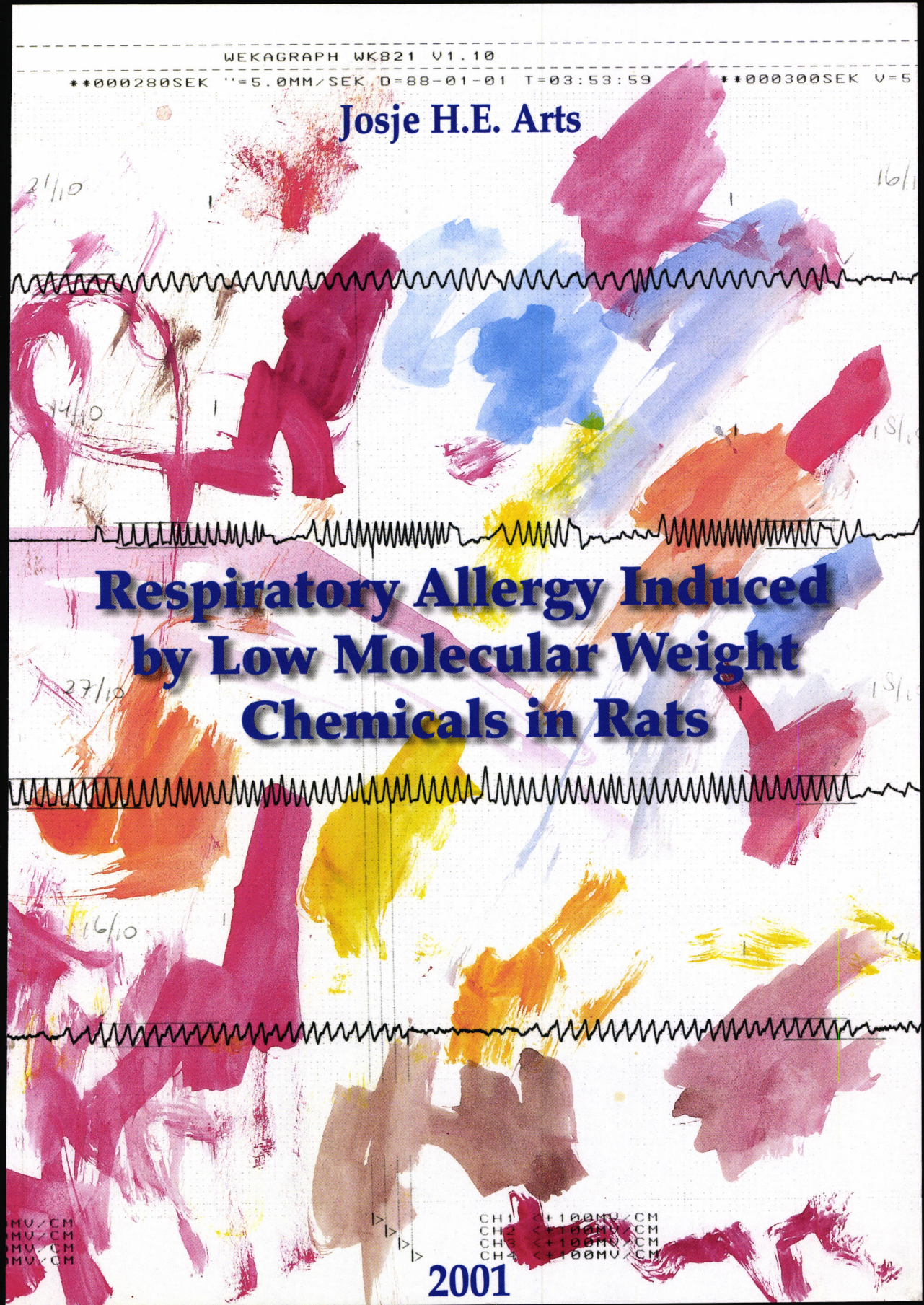
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Josje H.E. Arts
Respiratory Allergy Induced by Low Molecular Weight Chemicals in Rats
2001

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Luchtwegallergie geïnduceerd door klein-moleculaire stoffen in de rat
(met een samenvatting in het Nederlands)

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Promotores:

Prof. Dr. V.J. Feron (Universiteit Utrecht)

Prof. Dr. W. Seinen (Universiteit Utrecht)

Co-promotores:

Dr. C.F. Kuper (TNO Nutrition and Food Research, Zeist)

Dr. M.A. Bloksma (Universiteit Utrecht)

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Chapter 1

General introduction

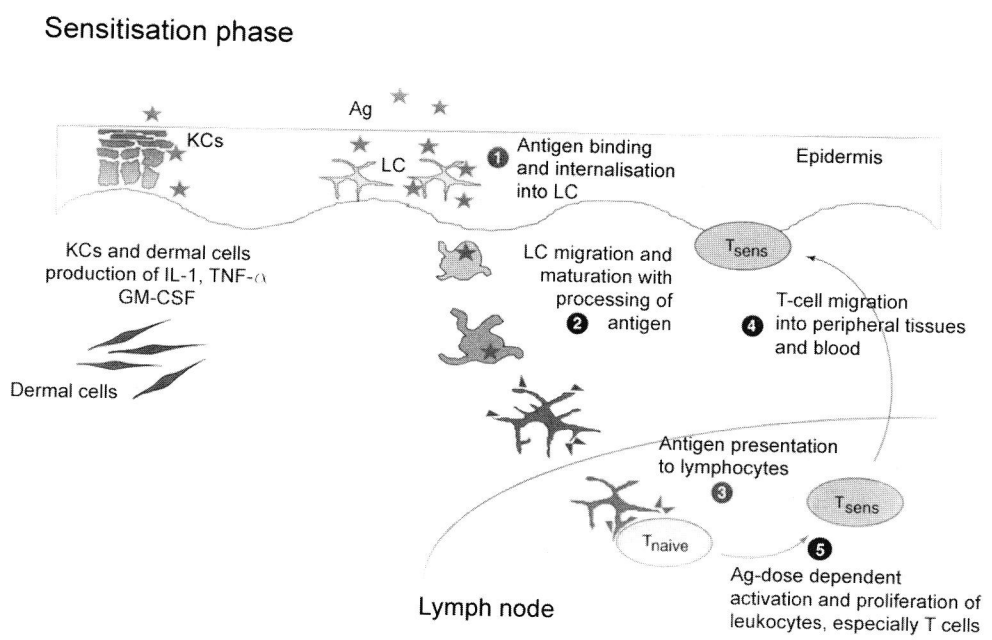
Introduction

Allergy is a diverse family of diseases caused by untoward immune reactions that may ultimately lead to tissue inflammation and organ dysfunction. The immune reactions are caused by exogenous (environmental, occupational or food-related) substances. These are called antigens, or allergens to refer to the allergic reactions they provoke. Allergy comprises a two-phase process: (1) a generally symptom-free sensitisation phase and (2) a symptomatic effector phase. After initial encounters with a particular allergen a primary immune response is mounted resulting in a state of heightened responsiveness to this allergen (induction or sensitisation). Upon subsequent exposures (challenges), a more vigorous and accelerated secondary immune response is provoked, that can result in clinically manifest adverse health effects (Figure 1).

Two important types of allergic diseases at the workplace caused by exposure to exogenous substances are allergic contact dermatitis and asthma. Asthma is the most frequent diagnosis of acute occupational respiratory disease in the UK (Meredith et al., 1991; Ross et al., 1995). In the Netherlands, it has been estimated that 500-2,000 workers may develop occupational asthma each year (Heederik et al., 1999a). Occupational asthma resulting from respiratory sensitisation can be life threatening (Fabbri et al., 1988; Ehrlich, 1994), and the majority of patients do not completely recover even when removed from exposure for several years (Saetta et al., 1992; 1995; Chan-Yeung and Malo, 1995; O'Neill, 1995).

The number of known respiratory allergens is increasing and includes both high molecular weight substances (usually proteins, >5000 Daltons), and low molecular weight (LMW) chemicals (<<5000 Daltons) which acquire allergenic potential after binding to a body protein (Cullen et al., 1990; Chan-Yeung and Malo, 1995). Well-known examples of respiratory sensitising LMW chemicals are diisocyanates, reactive dyes and acid anhydrides (Briatico-Vangosa et al., 1994).

Given the serious health problems of respiratory allergy together with the continuous introduction of new chemicals into workplaces, early identification of chemical respiratory sensitisers is very important. This requires reliable test methods. At the moment prediction of respiratory sensitisation potential from analysis of structure alone is unreliable while the few animal models available are not widely applied or fully validated.



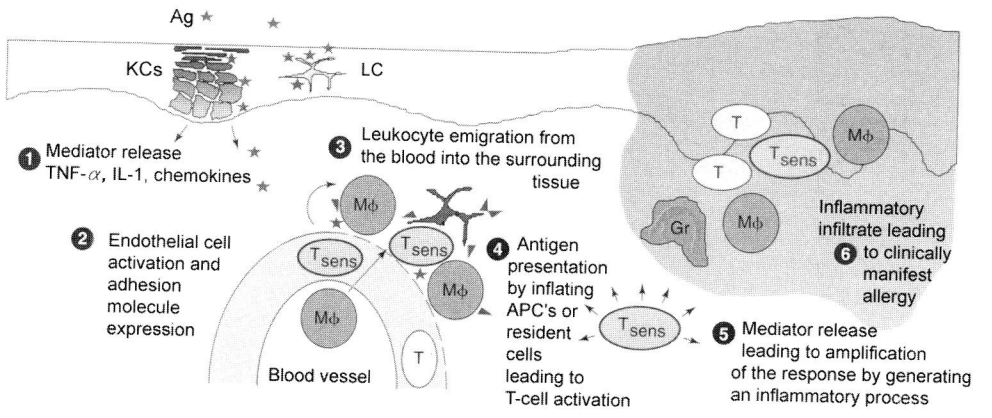


Figure 1 – A hypothetical scheme to describe the sensitisation phase and the challenge phase in allergy, exemplified by allergic contact dermatitis. Modified from Grabbe and Schwarz (1998). Abbreviations: Ag, antigen; APC, antigen presenting cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; Gr, granulocyte; IL-1, interleukin-1; KC, keratinocyte; LC, Langerhans cell; Mφ, monocyte/

Occupational allergic airway diseases – Definitions, classification, diagnosis, prevalence and risk factors

There are a large number of LMW chemicals that are able to cause occupational allergy in susceptible individuals. Many of these chemicals can cause contact allergy. Chemicals that are known to cause respiratory allergy are far fewer in number, and the number of chemicals that is known to cause both contact and respiratory allergy in humans is even less (Hardy and Devine, 1979; Davies, 1984; Dearman et al., 1994). Due to the lack of validated (animal) models, substances capable of inducing respiratory allergy are usually only identified by recognition of allergic symptoms in typical subpopulations, notably exposed workers (case reports) (Grammer et al., 1989; Chan-Yeung and Malo, 1995). All published cases of respiratory allergy to LMW chemicals have been derived from occupational and not from consumer exposure (Briatico-Vangosa et al., 1994).

In the following subsections, an overview is given on work-related allergic airway diseases, mainly focusing on occupational asthma.

Definitions of occupational allergic airway diseases

Work-related respiratory diseases which are the result of a specific immunological reaction to a substance from the environment may involve (a) the upper respiratory tract giving rise to rhinitis, (b) the conducting airways giving rise to allergic asthma, and (c) the peripheral gas exchanging parts of the lungs giving rise to e.g. hypersensitivity pneumonitis (Kazantzis, 1994).

- *Allergic rhinitis* is an immediate type (type I) allergy which is characterised by periods of nasal discharge, sneezing, and congestion that persists on average at least 0.5 to 1 hour per day (Mygind and Weeke, 1983), and is often accompanied by sinusitis and/or conjunctivitis (watery or prickly eyes). Upon prolonged exposure, chronic inflammation of the nasal mucous membrane may develop.
- *Allergic (extrinsic) asthma* is generally defined as an immediate type (type I) respiratory disease characterised by periods of acute airway obstruction, conducting airways inflammation, and increased sensitivity of the airways to a variety of stimuli also named bronchial hyperreactivity (Sheffer, 1991a). These acute attacks are interspersed with complaint-free periods, occurring either spontaneously, after medication, or after discontinuation of exposure. Upon encountering a respiratory allergen, allergic individuals may either

respond acutely, have late-onset symptoms, or both. The early reaction upon recognition of the allergen results from direct effects of mediators released, whereas the late reaction is the consequence of an inflammatory reaction of the bronchial mucosa.

Examples of substances that can induce occupational asthma are given in Table 1. Although most high molecular weight substances that induce asthma do so by a type I mechanism, it has become clear that some LMW chemicals can do so by other ways. This will be discussed in more detail in following sections.

- *Hypersensitivity pneumonitis, also named extrinsic allergic alveolitis*, differs from asthma in that it is an inflammatory disease of the respiratory bronchioles or alveoli characterised by a reduction in the functional lung capacity rather than an obstruction of the airflow as in asthma. Unlike asthma, symptoms like fever, sweating, cough, and dyspnoea generally appear 4-8 h after allergen challenge. If exposure to the sensitising chemical continues, the lung can become scarred (fibrosing alveolitis) leading to severe breathlessness (O'Neill, 1995). Presently, hypersensitivity pneumonitis is considered to be the consequence of a cell-mediated (type IV) hypersensitivity reaction as well as an immune complex-mediated (type III) allergic reaction. The type III reaction is characterised by phagocyte and complement activation, especially in the early stages of the reaction (Salvaggio, 1987; Regal, 1997; Terr, 1997). Hypersensitivity pneumonitis due to exposure to organic dust is often known under names related to occupation: farmers' lung, mushroom workers' lung, and malt workers' lung. Examples of substances, including LMW chemicals, found to induce hypersensitivity pneumonitis at the workplace are presented in Table 1. Interestingly, all LMW chemicals known to induce hypersensitivity pneumonitis are also known to induce allergic asthma.

Problems in defining occupational asthma

Over the past years, asthma at the workplace has proved to be a difficult and controversial subject for regulatory authorities. Adoption of a clear definition has been hampered by a poor understanding of its mechanisms and confusion about terminology. Although several substances that induce occupational asthma do so by an allergic IgE-mediated, type I mechanism, it has become clear that some LMW chemicals can induce occupational asthma by other allergic, non-IgE-mediated mechanisms (Maestrelli et al, 1997).

Table 1 – Substances known to induce allergic asthma and/or hypersensitivity pneumonitis at the workplace

	Allergic asthma	Hypersensitivity pneumonitis
High molecular weight chemicals	Animal dander and/or urinary proteins Latex Flour Enzymes	Organic dust from animal, vegetable or microbial origin
Low molecular weight chemicals	Diisocyanates - toluene diisocyanate (TDI) - diphenylmethane diisocyanate (MDI) - hexamethylene diisocyanate (HDI) Acid anhydrides - trimellitic anhydride (TMA) - phthalic anhydride - maleic anhydride - himic anhydride - tetrachlorophthalic anhydride - tetrahydrophthalic anhydride - hexahydrophthalic anhydride - methyltetrahydrophthalic anhydride Metal compounds - platinum - cobalt - beryllium - nickel - chromium Plicatic acid (Western Red Cedar) Reactive dyes Amines - ethylene diamine - hexamethylene tetramine - chloramine-T	Diisocyanates - toluene diisocyanate - diphenylmethane diisocyanate - hexamethylene diisocyanate Acid anhydrides - trimellitic anhydride - tetrachlorophthalic anhydride Metal compounds - beryllium

References: Alanko et al., 1978; Barker et al., 1998; Baur et al., 1995; Bernstein et al., 1984; Bernstein, 1996; Brisman, 1994; Butcher et al., 1993; Chan-Yeung and Malo, 1993b; Cullinan and Newman Taylor, 1997; Drexler et al., 1994; Durham et al., 1987; Evans et al., 1986; Freiman and Hardy, 1970; Grammer et al., 1992; Halpin et al., 1994; Heederik et al., 1999b; Herzog et al., 1991; Hollander et al., 1997; Hostynek, 1997; Houba et al., 1998; Jones Williams and Wallach, 1989; Kusaka, 1993; Liss et al., 1984; 1993; 1997; Malo et al., 1983; Marabini et al., 1993; Merget et al., 1994; Moller et al., 1985; Nielsen et al., 1988; Nieuwenhuijsen et al., 1999; Topping et al., 1986; Turjanmaa et al., 1996; Venables, 1989; Williams, 1989; Yokota et al., 1997, Zeiss, 1991; Zeiss et al., 1980; Zeiss et al., 1992; Zeiss and Patterson, 1993.

Epidemiological studies further suggest that airborne irritants have a profound influence on allergic responses, most probably because of the induction of airway inflammation. These irritants may also provoke allergy-like symptoms in susceptible individuals (Samet, 1995). Due to the similarity in clinical symptoms, it is difficult to distinguish immune-mediated respiratory allergy caused by LMW chemicals from irritant-induced reactions.

As a consequence, no clear distinction is made between respiratory sensitisation (mechanism) and disease (symptoms). This is also reflected in the definition of occupational asthma used by the Subcommittee on Occupational Allergy of the European Academy of Allergology and Clinical Immunology. This definition is primarily focused on clinical symptoms, i.e. "occupational asthma is a disease characterised by variable airflow limitation, bronchial hyperresponsiveness or both, due to conditions in a particular workplace, which is reflected in periods of dyspnoea especially at night and in the early morning" (Anonymous, 1992). The poor understanding of the mechanisms involved and the interdisciplinary character of the subject has resulted in a lack of consensus about terminology with different classifications of occupational asthma as a logical consequence (see next section).

Classifications of occupational asthma and inducers of the disease

In contrast to the Committee above (Anonymous, 1992), Chan-Yeung and Malo (1995) describe two categories of asthma at the workplace (Table 2a).

Table 2a – Categories of asthma at the workplace according to Chan-Yeung and Malo (1995)

Category 1	Category 2
Occupational asthma a. asthma with latency b. asthma without latency	Work-aggravated asthma

Category 1 is *occupational asthma*, subdivided into asthma with and without latency. *Asthma with latency* develops after a period of exposure that may vary from a few weeks to several years. It includes all cases of immune-mediated asthma, and agents that cause this type of asthma are subdivided by the authors into those that induce IgE-dependent and those that induce IgE-independent reactions. Chemicals, that can induce asthma with latency, include high as well as low molecular weight

chemicals. *Asthma without latency* (irritant-induced asthma) results from exposure to high irritating concentrations of gases, fumes and other chemicals such as uranium hexafluoride, isocyanates, ammonia, chlorine or hydrazine, on one or several occasions. High concentrations of these substances most probably cause toxic and irritation effects on the respiratory tract resulting in airway obstruction. This type of airway obstruction has also been named 'Reactive Airways Dysfunction Syndrome' (RADS; Brooks et al., 1985; 1993; Bernstein et al., 1993; Newman Taylor and Pickering, 1994). It has been estimated that at least one in 20 and possibly one in every six cases of occupational asthma was induced by exposure to irritants (Tarlo and Broder, 1989). Category 2 according to Chan-Yeung and Malo (1995) is *Work-aggravated asthma*, i.e. pre-existing or concurrent asthma that is aggravated by irritants or physical stimuli in the workplace. Examples of stimuli that do not cause specific immune reactions but can provoke asthma-attacks in asthmatic individuals are physical exercise, temperature changes, SO2, and diesel exhaust gases (Chan-Yeung et al., 1993; Samet, 1995; Venables and Chan-Yeung, 1997).

Yet another classification of asthma at the workplace is being used by the Nordic Committee on Building Regulations (NKB, 1993; Table 2b).

Table 2b – Categories of asthma at the workplace according to NKB (1993)

Category 1	Category 2	Category 3
Allergy	Non-specific hyperresponsiveness	Specific chemical hypersensitivity

Occupational airway diseases in this classification are based on three principal forms of hypersensitivity, which may all lead to the same type of symptoms in the airways: (1) *Allergy*, defined as "a specific immunological response to protein components, mediated by specific IgE antibodies", (2) *Non-specific hyperresponsiveness*, defined as "an altered function in the cells and organs with excessive reactions to different kinds of stimuli, in which inflammation of the mucous membranes in the airways plays a central role", and (3) *Specific chemical hypersensitivity*, which "comprises specific hypersensitivity to single reactive chemicals of low molecular weight (often lower than 300 Daltons) and for which no specific immunological reaction has to be demonstrated".

With respect to substances inducing occupational asthma, the current EC-labelling criteria for dangerous substances (CEC, 1967; amended several times and adapted to technical progress for the 28th time recently), which are used to classify substances amongst others on their potential to induce respiratory allergy, include all chemicals that can induce asthma (-like) attacks. As stated: "The condition will have the clinical character of an allergic reaction. However, immunological mechanisms do not have to be demonstrated". In this guideline, it is further emphasised that "Substances that elicit symptoms of asthma by irritation only in people with bronchial hyper-reactivity should not be assigned R42" (risk phrase 42: respiratory sensitiser). In addition, the decision on classification needs to take into account "the size of the population exposed" and the "extent of exposure". Thus, a high-production-volume chemical found in large quantities in many workplaces throughout the world, might not warrant the R42 phrase if only a few cases of asthma with its use have been reported over the years (Evans, 1997). An example of such a compound could be glutaraldehyde. In contrast, three cases of asthma among a workforce of 20 in contact with a specific chemical might well indicate the need for classification as a respiratory sensitiser (Evans, 1997).

Diagnosis of occupational asthma

Notwithstanding the above-indicated differences in definition of occupational asthma and the classification of inducers of occupational asthma, ways to diagnose individuals suffering from work-related respiratory problems have been proposed. The diagnosis of occupational asthma can be based on a 5-step procedure (Table 3) including clinical history, physical examination, lung function and immunological tests, bronchial provocation tests and/or assessment of airway hyperresponsiveness.

Table 3 – Suggested 5-step procedure to diagnose occupational asthma (Anonymous, 1992)

Steps	Diagnostic procedure
1	History suggestive of occupational asthma
2	Confirmation of bronchial asthma with demonstration of reversibility of bronchial obstruction, of non-specific bronchial hyperreactivity and of increased diurnal variability of peak expiratory flow rates (PEFR)
3	Confirmation of work-related bronchoconstriction with serial measurements of PEFR and of non-specific bronchial reactivity
4	Confirmation of sensitisation to occupational agents with skin tests and/or *in vitro* tests to detect specific immunoglobulins
5	Confirmation of causal role of occupational agent with specific bronchial challenges

The degree of bronchial hyperresponsiveness can be assessed by challenging the individual with non-specific stimuli such as histamine or methacholine. Challenge with the suspected allergen can be used to confirm a diagnosis of occupational asthma in an individual worker and is in fact the only way to determine the causative agent.

The most commonly used lung function measurements are the peak expiratory flow rate (PEFR), forced expiratory volume in one second (FEV1), forced vital capacity (FVC) and maximum midex-piratory flow (MMEF). PEFR measures the maximum flow rate during a forced expiratory manoeuvre, FEV1 measures the volume of air expired in one second at maximum expiration, FVC measures the total volume of air expired as rapidly as possible and MMEF measures the slope of the line between 25% and 75% of FVC.

With respect to immunological tests, skin prick tests, radio-allergosorbent tests (RAST) or enzyme-linked immunosorbent assays (ELISA) can be used to detect the presence of specific antibodies. For instance, IgE is considered to play a role in respiratory allergy induced by protein aeroallergens and several LMW chemicals.

Prevalence of occupational asthma

It has been reported that at least 2% of all asthma cases may be of occupational origin (Chan-Yeung and Lam, 1986). With respect to high molecular weight chemicals, the prevalence of occupational asthma may range from approximately 4-10% among people exposed to laboratory animals to 44% among workers in small bakeries. With respect to LMW chemicals, it may range from 1 to more than 20% among people exposed to isocyanates and up to almost 30% among workers exposed to acid anhydrides (Wernfors et al., 1986; Sheffer, 1991b; Chan-Yeung and Malo, 1995; Heederik et al., 1999a). The reported prevalences, however, appear to depend strongly on the way of diagnosing.

Risk factors for occupational asthma

Risk factors for the development of occupational asthma reportedly are: (1) exposure to allergens, (2) genetic predisposition to atopic diseases, (3) aspecific bronchial hyperreactivity, and (4) exposure to volatile or particulate irritants, like cigarette smoke.

(1) With respect to exposure to allergens, the prevalence rate of sensitisation and/or respiratory symptoms upon exposure to proteinaceous allergens

seems to be related to exposure intensity (Hollander et al., 1997; Houba et al., 1998; Heederik et al., 1999b; Nieuwenhuijsen et al., 1999). Also, for a number of LMW chemicals a relationship between degree of exposure and the prevalence of respiratory allergy has been shown (Chan-Yeung and Malo, 1995).

- (2) Atopy is the genetic predisposition to produce increased levels of IgE after exposure to allergens. The prevalence of asthma induced by high molecular weight substances, i.e. type I (IgE-) mediated asthma, is higher in atopic than in non-atopic individuals (Chan-Yeung et al., 1993a; Beckett, 1994; Brooks, 1995; Chan-Yeung and Malo, 1995; Bernstein, 1997; Cullinan and Newman Taylor, 1997; Venables and Chan-Yeung, 1997). For LMW chemicals this is less clear since the prevalence of isocyanate- or plicatic acid-induced asthma is about the same in atopics as in non-atopics (Beckett, 1994; Bernstein, 1996; 1997; Cullinan and Newman Taylor, 1997).
- (3) Aspecific bronchial hyperreactivity has also been indicated as a risk factor. However, it is not clear whether aspecific bronchial hyperreactivity is an independent risk factor or a symptom of occupational asthma (Beckett 1994; Renstrom et al., 1995; Venables and Chan-Yeung, 1997; Xu et al., 1997).
- (4) Smoking seems to be a risk factor for sensitisation and development of occupational asthma upon exposure to platinum salts and acid anhydrides (Beckett, 1994; Chan-Yeung et al., 1995; Bernstein, 1997; Venables and Chan-Yeung, 1997). Smoking was rated even more important than atopy in predisposing to sensitisation to platinum salts (Chan-Yeung and Malo, 1995) but not to detergent enzymes or laboratory animals (Venables et al., 1988; Chan-Yeung and Malo, 1995; Bernstein, 1997; Kruize et al., 1997). Furthermore, the majority of non-atopic workers with diisocyanate- or plicatic acid-induced asthma had never smoked (Bernstein, 1997; Venables and Chan-Yeung, 1997).

Acid anhydrides as a cause of occupational allergic airway diseases

Acid anhydrides are LMW chemicals that have been associated with asthma, hypersensitivity pneumonitis, rhinitis, and, much less frequently, contact dermatitis (Zeiss et al., 1992; see Table 1 for a list of acid anhydrides that are known to cause occupational asthma and hypersensitivity pneumonitis). Since trimellitic anhydride (TMA) is an ideal compound to study respiratory allergy induced by LMW chemicals, a more detailed description of the acid anhydrides in general, and TMA

in particular (Fig. 2), is given below.

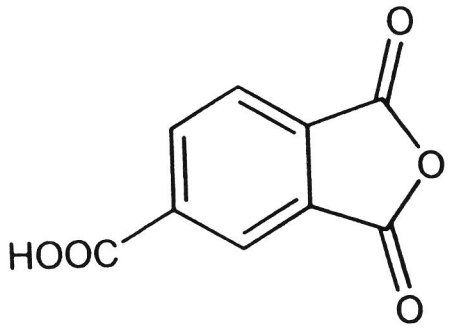


Figure 2 Molecular formula of TMA (1,2,4-benzenetricarboxylic acid, cyclic 1,2-anhydride; CAS Reg. No. 552-30-7)

Acid anhydrides are used in industry for more than 50 years as curing agents in the production of epoxy and alkyd resins, which have widespread applications in paints, plastics and adhesives. They are also used in the production of dyes, pesticides, pharmaceuticals, and isolators. The reactive O=C-O-C=O group not only makes these anhydrides appropriate as a basic material for the above indicated applications but this reactivity is most probably also responsible for the irritating and sensitising properties of the acid anhydrides. Several of these have been reported to cause occupational asthma and/or hypersensitivity pneumonitis (Table 1).

The binding of these compounds to self-proteins most probably is responsible for the development of new, allergenic epitopes. Based on the presence of specific IgE antibodies it is generally assumed that the asthmatic reaction upon exposure to the acid anhydrides develops through an antibody (IgE)-dependent mechanism (Venables, 1989; Fabbri et al., 1993). Using skin prick tests, ELISA or RAST, specific IgE was shown to be involved in 7-63% of the persons occupationally exposed to anhydrides (Liss et al., 1993; Drexler et al., 1994; Welinder et al., 1994; Baur et al., 1995; Yokota et al., 1997). A positive correlation was found between the presence of specific IgE and the extent of exposure (Moller et al., 1985; Zeiss et al., 1992). However, the relation between specific IgE and occurrence of asthma is not straightforward: 40-100% of persons with acid anhydride-induced asthma showed specific IgE, whereas specific IgE was found in 8-35% of occupationally exposed subjects not having asthma (Venables, 1989).

Data on the incidence of occupational asthma and rhinitis caused by acid anhydrides are limited. Kanerva and Vaheri (1993) reported that 2% of all cases of occupational allergic rhinitis in Finland between 1981 and 1987 were caused by exposure to acid anhydrides. In Germany (Strassburger et al., 1996), the UK (Gannon and Burge, 1993) and Finland (Karjalainen et al., 2000), 0.2%, 0.6%, and 0.7%, respectively, of all cases of occupational asthma were ascribed to these compounds. Exposure to hardening agents, resins and glues that contain acid anhydrides may also be (partly) responsible for the development of occupational asthma (5-8% of all cases; Meredith, 1993; Madan, 1996).
In studies of acid anhydride-exposed workers, the prevalence of occupational asthma varied between 2 and 28% (Moller et al., 1985; Wernfors et al., 1986; Nielsen et al., 1988; Venables, 1989; Grammer et al., 1992; Nielsen et al., 1992; Liss et al., 1993; Zeiss and Patterson, 1993; Chan-Yeung and Malo, 1994; Drexler et al., 1994). However, provocation tests to prove the diagnosis were usually not carried out.

With respect to TMA, besides IgE-mediated asthma and rhinitis, two other immunological and one non-immunological clinical syndromes have been reported to be caused by exposure to this compound (Table 4).

Table 4 – Immunological and non-immunological syndromes induced by TMA (Zeiss and Patterson, 1993)

Types	I/NI*	Time of first manifestation of symptoms	Concentrations	Way of exposure
1. IgE-mediated asthma and rhinitis	I	Months to years	Low	Dust/Fumes
2. IgG-mediated asthma (TMA-flu)	I	Months to years	Moderate	Dust/Fumes
3. Pulmonary haemorrhage-haemolytic anaemia	I	Weeks to months	High	Fumes/Spraying
4. RADS	NI	Single exposure	High	Dust/Fumes/ Spraying

* I = immunological; NI = non-immunological ; RADS = Reactive Airways Dysfunction Syndrome

The immunological syndromes are IgG-mediated asthma with systemic symptoms (TMA flu) and pulmonary haemorrhage-haemolytic anaemia. TMA flu has characteristics of hypersensitivity pneumonitis whereas pulmonary haemorrhage-

haemolytic anaemia results from antibody binding to circulating red blood cells and to pulmonary vascular cells. The development of these two immunological syndromes has been related to the degree and way of exposure. The non-immunological syndrome is Reactive Airways Dysfunction Syndrome (RADS), most probably caused by exposure to high TMA concentrations which resulted in toxic and irritating effects on the respiratory epithelium leading to airway obstruction (Zeiss and Patterson, 1993).

Testing for chemical-induced respiratory allergy: hazard identification

The current EC-labelling criteria for dangerous substances (CEC, 1967) are meant to classify substances - amongst others - on the basis of their potential to induce allergy and/or irritation. The reason to mention irritation here is that most (if not all) chemical allergens also have irritating properties. Furthermore, as indicated previously, it is difficult to distinguish immune-mediated respiratory allergy caused by LMW chemicals from irritant-induced airway reactions due to the similarity in clinical symptoms.

With respect to skin effects, a chemical is usually classified as a skin sensitiser (risk phrase R43) upon a positive skin sensitisation test such as the validated guinea pig maximization test (OECD 406) and/or as a skin irritant (R38) upon a positive result in a validated skin irritation test (OECD 404). In analogy, there are two risk phrases for respiratory effects, i.e. respiratory sensitisation (R42) and respiratory irritation (R37), but validated tests are lacking. Although a number of animal test protocols has been published to detect respiratory allergy (see for reviews Briatico-Vangosa et al., 1994; Pauluhn et al., 1999), none of these are widely applied or fully accepted. Also with respect to airway irritation testing, the test most often used, viz. the sensory irritation test (Alarie, 1973; ASTM, 1984), is not included into the current OECD guidelines.

A first - logical - step in hazard identification of LMW chemicals to induce respiratory allergy is examination of physical properties. 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, though, a next step to discriminate between respiratory allergens and inducers of other types of airway reactions could be to look for structure-activity relationships. Agius et al. (1991) hypothesised 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 presently insufficient information available to predict respiratory sensitisation potential from analysis of structure alone (Briatico-Vangosa et al., 1994).

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 (Kochman et al., 1990) are likely to be relevant. Chemicals known to cause occupational asthma such as trimellitic anhydride, isocyanates, and chloramine-T were found to bind easily to proteins *in vitro* (Patterson et al., 1978; Evans et al., 1986; Jin and Karol, 1988; Wass and Belin, 1990). However, a binding study *in vitro* cannot be considered representative of the potential of inducing an immunological response, let alone allergy, which is a complex pathophysiological event involving the interaction of many cell types and cytokines. *In vivo* animal models, therefore, seem more valuable to study the potential to cause respiratory allergy. Ideally, to investigate allergen-induced changes, animal models of allergic asthma should resemble the processes and/or reactions as observed in humans as closely as possible.

In vivo animal models to study the potential to cause respiratory allergy

Presently, there are two proposed ways – either of which not validated – to determine a chemical's potential to cause respiratory allergy: (1) measuring inhalation challenge-induced respiratory reactions of intradermally or subcutaneously sensitised guinea pigs (e.g. Karol, 1988) or (2) measuring total serum IgE concentrations of mice after topical exposure to immunogenic concentrations of the test material (IgE test; Dearman et al., 1992a).

(1) Guinea pig models

In guinea pigs, respiratory sensitisation to LMW chemicals has been achieved via single or repeated inhalation exposures, and via intradermal or subcutaneous application. LMW chemicals tested positive were amongst others p-tolyl isocyanate, hexyl isocyanate, toluene diisocyanate, diphenylmethane-4,4'-diisocyanate, trimeric hexamethylene diisocyanate, trimellitic anhydride, and phthalic anhydride (Karol et al., 1979; Karol, 1980; 1983; Cibulas et al., 1986; DeCeaurriz et al., 1987;

Botham et al., 1988; Karol and Thorne, 1988; Pauluhn and Eben, 1991; Sarlo and Clark, 1992; Sarlo et al., 1994; Pauluhn, 1997). Parameters used in these studies to show lung function changes were breathing frequency, tidal volume (TV), 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 (Thorne and Karol, 1988; Pauluhn and Eben, 1991; Pauluhn and Mohr, 1994; Pauluhn, 1997).
The guinea pig models that examine the potential of chemicals to induce respiratory allergy have the advantage of being a functional endpoint test; i.e. respiratory symptoms can be studied. In addition, like the human lungs, lungs of guinea pigs are a major shock organ for anaphylactic responses to allergens (Sarlo and Ritz, 1997). However, the tests are time-consuming, costly, and may require the use of hapten-protein conjugates which hampers comparison to the human situation. Furthermore, unlike in humans, in the majority of the studies, the onset of respiratory responses was immediate with reactions occurring either during or shortly after the challenge period. Only in very rare instances were delayed-onset or dual responses reported.

(2) Mouse model (IgE test)
This model is based on the finding that chemicals which have the potential to cause respiratory allergy in man, such as TMA, phthalic anhydride, toluene diisocyanate, diphenylmethane-4,4'-diisocyanate, and hexamethylene diisocyanate 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 (Dearman and Kimber, 1991; 1992; Dearman et al., 1991; 1992ab; Hilton et al., 1995). The mouse IgE test is cost-effective but is not a functional endpoint test, i.e. it is not known whether increases of total serum IgE are indicative of actual induction of respiratory allergic symptoms.

Aim of the study
The aim of the studies presented in this thesis was to develop an animal testing strategy in the rat that can be used for the detection of LMW respiratory allergens.

This test method has its basis in the tiered approach suggested by Briatico-Vangosa et al. (1994).

In Chapter 2, the Local Lymph Node Assay (LLNA), a test to measure sensitisation potential via dermal application, is described. In the LLNA, five rat strains were tested using two model compounds, viz. TMA and DNCB, known inducers of respiratory allergy and contact allergy, respectively. Strain-specific and compound-specific differences were investigated. Moreover, results were compared with those in the BALB/c mouse, since this species was originally used. Next, (Chapter 3) two genetically different rat strains were selected, notably the high IgE-responding Brown Norway (BN) and the low IgE-responding Wistar strain. These strains were tested for IgE responses, using the same compounds and methyl salicylate, a skin irritant devoid of sensitising properties. Chapter 4 deals with the question whether increases of total serum IgE levels can be associated with manifest functional and morphological changes of the airways after inhalation challenge. In order to obtain more detailed information on the rat respiratory allergy model, various parameters of inflammation and lung function, including non-specific airway hyperresponsiveness *in vivo* and *in vitro* were investigated (Chapter 5). Finally, it was examined whether allergen-induced lung function changes can be distinguished from irritant-induced lung function changes (Chapters 5 and 6, respectively). In Chapter 7 the results have been summarised and discussed.

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Chapter 2

Local lymph node activation in rats after dermal application of the sensitizers 2,4-dinitrochlorobenzene and trimellitic anhydride

Josje H.E. Arts, Sonja C.M. Dröge, Nanne Bloksma and C. Frieke Kuper

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Local Lymph Node Activation in Rats after Dermal Application of the Sensitizers 2,4-Dinitrochlorobenzene and Trimellitic Anhydride

J. H. E. ARTS*†, S. C. M. DRÖGE†, N. BLOKSMA‡ and C. F. KUPER†

†TNO Nutrition and Food Research Institute, Division of Toxicology, PO Box 360, 3700 AJ Zeist, The Netherlands and ‡Research Institute of Toxicology (RITOX, Utrecht University, The Netherlands

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Abstract—Five rat strains were compared for their performance in the local lymph node assay (LLNA), a promising test system for the identification of the skin-sensitizing potential of chemicals in the mouse. The contact sensitizer 2,4-dinitrochlorobenzene (DNCB) and the contact and respiratory sensitizer trimellitic anhydride (TMA) were used as model chemicals and responses in rats were compared with those in BALB/c mice. The chemicals were applied to the dorsum of both ears, once daily for three consecutive days; 2 days (mice) or 3 days (rats) thereafter, proliferating cells were labelled by ip injection of BrdU 2 hr before the animals were killed. Systemic effects were subsequently assessed by determination of spleen, liver and kidney weights, skin effects by determination of swelling and inflammatory cell infiltration of the ears, and immune effects by determination of weight and proliferative activity of the local lymph nodes (LLN). Following application (× 3) of DNCB or TMA, minor systemic effects were observed, as indicated by slightly elevated spleen and liver weights in a few rat strains and the mice. Skin effects, consisting of increased ear thickness and presence of mononuclear inflammatory cell infiltrates, were observed in all rat strains treated with DNCB or TMA. LLN weights had increased, as had the proliferative activity in these nodes. It was concluded that effects induced by DNCB and TMA in all five rat strains were comparable with those in mice.

INTRODUCTION

The study of contact sensitizing chemicals was initiated by Landsteiner and Jacobs (1935), who showed that intradermal injection of nitro- and chlorosubstituted benzenes into guinea pigs followed by epidermal challenge several days later led to specific contact dermatitis. The introduction of a scoring system to assess erythematous reactions (Draize *et al.*, 1944) resulted in the first test to measure the sensitizing potential of chemicals in guinea pigs. Several tests have now been developed, the occluded patch test (Buehler, 1965 and 1985) and the guinea pig maximization test (Magnusson and Kligman, 1969 and 1970) being the most widely accepted by regulatory agencies. The tests comprise a sensitizing phase followed by a challenge phase. Skin reactions after challenge with the chemical are assessed visually and the sensitizing potential is calculated from the number of animals exhibiting a positive response. Although the assays are well validated and play an important role in predicting the sensitizing potential of chemicals, they also have a major limitation: the visual assessment of erythema is subjective and may be obscured when coloured or irritant chemicals are examined (Kimber, 1989).

Ashershon and Ptak (1968) introduced the mouse as an experimental model to study contact sensitization and at present there are various murine test methods for the identication of contact allergens. These can be subdivided into two types: (1) endpoint tests measuring challenge-induced increases in ear thickness of sensitized animals [e.g. the mouse ear swelling test (MEST); Gad *et al.*, 1986] or (2) tests measuring sensitization-induced activation of the lymph nodes draining the site of application (Oort and Turk, 1965; Turk, 1967; Turk and Stone, 1963), currently termed the local lymph node assay (LLNA; Kimber *et al.*, 1986 and 1994). In the LLNA, proliferative activity, as measured by labelled thymidine incorporation, is studied in the auricular lymph nodes of mice after topical application of the chemical to both ears. The LLNA promises to be a good candidate to replace, at least in part, the guinea pig

*Author for correspondence.

Abbreviations: TMA = trimellitic anhydride; DNCB = 2,4-dinitrochlorobenzene; BN = Brown Norway; PVG = Piebald Virol Glaxo; SD = Sprague–Dawley; BrdU = bromodeoxyuridine; AOO = acetone + olive oil; LLN = local lymph nodes; LLNA = local lymph node assay; IgE = immunoglobulin E; PBS = phosphate buffered saline; SI = stimulation index.

models in the near future for recognition of sensitizing chemicals, although irritant non-sensitizing chemicals also might be positive in this assay (Basketter and Scholes, 1992; Edwards *et al.*, 1994; Montelius *et al.*, 1994; Robbins *et al.*, 1991).

Recently, Kashima *et al.* (1994) described a useful alternative lymph node cell proliferation assay in guinea pigs using a single 24-hr occlusive patch, an intradermal injection or a combination of these.

The present study was carried out to test whether the rat, being the commonly preferred species in protocol toxicity studies, is a useful experimental animal in the LLNA. The LLNA would gain in significance if it were valid in several animal species. Furthermore, risk assessment could be improved by comparing the concentration that causes sensitization with the concentration that causes toxicity in the same animal species. We therefore investigated LLN responses using bromodeoxyuridine (BrdU) incorporation after topical application of two model allergens, trimellitic anhydride (TMA) and 2,4-dinitrochlorobenzene (DNCB). TMA is known to induce immediate-type allergic reactions, which are associated with specific immunoglobulin E (IgE) antibody responses. DNCB is known to induce T-cell-mediated delayed-type hypersensitivity reactions (Dearman and Kimber, 1991). Five different rat strains were used to see whether strain differences were present and results were compared with responses in the BALB/c mouse.

MATERIALS AND METHODS

Animals and maintenance

Female, 10–11-wk-old Fischer 344 (F344), Brown Norway (BN), and Piebald Virol Glaxo (PVG) inbred rats, Wistar random-bred rats, Sprague–Dawley (SD) outbred rats and 8–9-wk-old BALB/c inbred mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) and acclimatized for at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received the Institute's grain-based open-formula diet and unfluoridated tap water *ad lib.* All animal procedures were approved by the TNO Commission of Animal Welfare.

Test materials

DNCB (purity at least 98%; Sigma, St Louis, MO, USA) and TMA (purity 97%; Aldrich, Brussels, Belgium) were dissolved in a 4:1 (v/v) solution of acetone (Merck, Darmstadt, Germany) and raffinated olive oil (Chempri BV, Raamsdonksveer, The Netherlands) (AOO).

Experimental design

Desired concentrations of the chemicals in 75 μl (rats) or 25 μl (mice) AOO were applied to the dorsum of both ears on days 0, 1 and 2. Controls received AOO or were left untreated. The rats were slightly anaesthetized with ether before each application, since they offered resistance to the treatment. Ears were inspected for hyperaemia before and after each application. Proliferating cells were labelled by ip injection of the thymidine analogue BrdU (150 mg/kg body weight; Sigma) on day 4 in the case of mice and (because of the assumed slower immune responsiveness of rats) on day 5 in the case of rats. 2 hr after BrdU treatment, animals were anaesthetized and bled to death through the abdominal aorta. Ear thickness was measured immediately thereafter with an electronic pawmeter (Eykman–Winkler Institute of Microbiology, Utrecht University, The Netherlands) and animals were observed grossly for abnormalities. Lymph nodes draining the skin of the ear LLN, superficial cervical lymph nodes, kidneys, liver and spleen were excised, freed from fat and weighed. Next, the shells of the ears were removed as completely as possible from the head, and the dorsal ear area was calculated with the aid of calibration paper.

Immunohistochemistry

For determination of mitotic activity, local lymph nodes were fixed in 70% ethanol, embedded in paraffin wax and 5-μm sections were prepared. After deparaffination, the sections were immersed in 0.3% H_2O_2 (v/v) in methanol for 15 min to inhibit endogenous peroxidase, hydrolysed in 1 N HCl (60 min, 37°C) and subsequently preincubated in 25% normal goat serum for 20 min to reduce background staining. Next, the sections were successively incubated with the monoclonal antibody anti-BrdU (Beckton Dickinson, San Jose, CA, USA) for 60 min, with a biotin-labelled rabbit-anti-mouse antibody (Dakopatts, Glostrup, Denmark), with a peroxidase-conjugated streptavidin (Dakopatts) and finally incubated for 10 min with the chromogen 3′3′-diaminobenzidine-tetrahydrochloride (Sigma), dissolved in phosphate buffered saline (PBS) at a concentration of 0.5 mg/ml (pH 7.4), and supplemented with 0.05% (v/v) H_2O_2. After each incubation step, sections were rinsed in PBS. The sections were slightly counterstained with haematoxylin.

Mitotic activity was determined in lymph node sections at five sites of the paracortex (a typical T-cell compartment), at three sites of the cortex (a B-cell compartment) and the medulla, without prior knowledge of the treatment. At each site, at least 25 cells of which the nuclei were located at the crossings of the horizontal and vertical lines of a 10×10 intraocular grid were examined at high power magnification ($\times 400$) to determine the mean percentage of BrdU-positive (BrdU+) cells per compartment and the average percentage of BrdU+ cells per section. As a measure of total proliferation, the average percentage of BrdU+ cells per section was multiplied by lymph node weight.

For determination of inflammatory cell infiltrates, cryostat sections of ears of four rat strains that were

snap-frozen on dry ice were incubated with the mouse monoclonal antibodies MRC OX19 (CD5; recognizing virtually all T cells; Pharmingen, San Diego, CA, USA) or ED1 [specific for the majority of monocytes and macrophages, Dijkstra *et al.* (1985); kindly provided by C. D. Dijkstra]. Sections were then incubated with peroxidase-conjugated rabbit anti-mouse Ig (RAMPO, Dakopatts) for 30 min and finally incubated with the chromogen 3′3′-diaminobenzidine-tetrahydrochloride and H_2O_2, as described above. Control sections were processed in the same way, except for the leucocyte-specific antibodies which were replaced by PBS. The presence and composition of inflammatory cell infiltrates were assessed on the basis of the number of OX19- and ED1-positive cells in the ear sections.

Statistics

Body and organ weights were analysed by one-way analysis of variance (ANOVA) followed by the two-sided Dunnett's multiple comparisons test. Ear thickness and data on proliferative activity were analysed by ANOVA followed by pairwise *t*-tests.

RESULTS

Strain-dependent systemic effects of 1% DNCB and 50% TMA

Three daily applications of DNCB or TMA did not affect body and kidney weights of BALB/c mice and all rat strains tested (data not shown). Relative spleen weights were significantly increased by DNCB in BALB/c mice and Fischer rats, and absolute and relative liver weights by TMA in SD and PVG rats only. The latter strain also showed a relative increase of liver weight in response to DNCB (Table 1).

Strain-dependent reactions of the ears to 1% DNCB and 50% TMA

Rats had on average 1.8 times larger ear areas and 2.5 times thicker ears than mice, while receiving a three times larger vehicle volume than mice.

Hyperaemia of the ears was not observed in mice nor in any of the rat strains after application of TMA, whereas DNCB induced hyperaemia in three of six BN rats after the second application only.

Ear thickness generally had increased in mice and all rat strains tested; significant increases were reached in BALB/c mice, Fischer, BN and SD rats in the case of DNCB, and in BALB/c mice, Fischer and PVG rats in the case of TMA (Table 2). No significant differences were observed between responses to DNCB and TMA in the same strain, although in BN rats the response to DNCB tended to be greater than that to TMA, whereas in BALB/c mice the reverse tendency was observed.

Immunohistochemical examination of the ears of four rat strains revealed the presence of inflammatory cell infiltrates after DNCB or TMA treatment, but not after AOO treatment. The incidence and composition of the infiltrates varied with the strain and treatment. DNCB induced infiltrates consisting of macrophages and T lymphocytes in three of three BN rats and one of three Wistar rats; infiltrates consisting of macrophages without significant T-cell numbers were seen in two of three PVG rats and in another Wistar rat. TMA induced infiltrates of macrophages and T lymphocytes in the ears of three of three Fischer, two of three Wistar, one of three BN and one of three PVG rats. In no instance were significant granulocyte numbers observed. The infiltrates were predominantly located in the epithelium, dermis and upper part of the subcutis and, when prominent, were accompanied by crusts and marked acanthosis.

Table 1. Strain-dependent effects of DNCB and TMA on absolute and relative spleen and liver weights

Organ	(weight)	Treatment	BALB/c mice	Rats				
				Fischer	Wistar	BN	SD	PVG
Spleen	(g)	AOO	0.097 ± 0.003	0.431 ± 0.016	0.418 ± 0.014	0.335 ± 0.019	0.539 ± 0.019	0.365 ± 0.010
		DNCB	0.110 ± 0.005	0.465 ± 0.013	0.452 ± 0.025	0.341 ± 0.015	0.578 ± 0.025	0.367 ± 0.013
		TMA	0.106 ± 0.005	0.455 ± 0.009	0.452 ± 0.031	0.435 ± 0.063	0.607 ± 0.021	0.377 ± 0.012
Spleen	(g/kg)	AOO	4.81 ± 0.09	2.51 ± 0.03	2.11 ± 0.07	2.11 ± 0.06	2.61 ± 0.09	2.42 ± 0.04
		DNCB	5.54 ± 0.24*	2.74 ± 0.08*	2.26 ± 0.08	2.18 ± 0.06	2.72 ± 0.11	2.40 ± 0.03
		TMA	5.28 ± 0.19	2.67 ± 0.04	2.32 ± 0.12	2.75 ± 0.43	2.86 ± 0.09	2.42 ± 0.07
Liver	(g)	AOO	0.954 ± 0.022	5.25 ± 0.18	7.15 ± 0.22	4.96 ± 0.21	6.27 ± 0.23	5.35 ± 0.15
		DNCB	0.931 ± 0.035	5.46 ± 0.22	7.35 ± 0.23	5.00 ± 0.18	6.82 ± 0.14	5.81 ± 0.09
		TMA	0.924 ± 0.106	5.29 ± 0.15	6.99 ± 0.35	5.24 ± 0.21	6.93 ± 0.12*	5.90 ± 0.20*
Liver	(g/kg)	AOO	47.4 ± 0.7	30.6 ± 0.4	36.1 ± 1.0	31.3 ± 0.5	30.3 ± 0.8	35.5 ± 0.8
		DNCB	46.8 ± 1.6	32.0 ± 0.9	36.9 ± 0.4	32.0 ± 0.5	32.2 ± 0.4	38.1 ± 0.6*
		TMA	45.9 ± 1.1	31.0 ± 0.6	36.0 ± 1.4	32.8 ± 0.6	32.7 ± 0.5*	37.9 ± 0.3*

Groups of six mice or rats received 1% DNCB or 50% TMA in AOO, or AOO alone, on the dorsum of both ears on days 0, 1 and 2, and an ip injection of BrdU on day 4 (mice) or day 5 (rats). Animals were autopsied 2 hr after BrdU treatment and organs were weighed.
Values are means ± SEM.
Asterisks indicate significant differences from corresponding controls (*$P < 0.05$; ANOVA followed by two-sided Dunnett's multiple comparisons test).

Table 2. Strain-dependent effects of DNCB and TMA on ear thickness

		Ear thickness				
		AOO	DNCB		TMA	
Species	Strain	(× 10E-2 mm)	(× 10E-2 mm)	(%)	(× 10E-2 mm)	(%)
Mouse	BALB/c	20.8 ± 0.4	23.9 ± 0.7*	15	28.0 ± 1.7*	35
Rat	Fischer	50.5 ± 1.5	56.7 ± 1.9*	12	57.2 ± 1.1*	13
	Wistar	54.5 ± 1.9	59.9 ± 2.3	10	58.7 ± 1.3	8
	BN	47.9 ± 0.9	61.0 ± 4.8*	27	55.4 ± 2.4	16
	SD	53.4 ± 1.6	63.8 ± 3.4*	19	61.4 ± 2.2	15
	PVG	52.4 ± 1.0	56.7 ± 0.9	8	58.2 ± 2.0*	11

Groups of six mice or rats received 1% DNCB or 50% TMA in AOO or AOO alone on the dorsum of both ears on days 0, 1 and 2, and an ip injection of BrdU on day 4 (mice) or day 5 (rats). Animals were autopsied 2 hr after BrdU treatment and ear thickness was measured.
Values are means ± SEM (absolute values) and (for DNCB- and TMA-treated groups) as percentage increase compared with AOO-treated controls.
Asterisks indicate significant differences from corresponding controls (*$P < 0.05$; ANOVA followed by pairwise t-tests).

Strain-dependent reactions of the local lymph nodes to 1% DNCB or 50% TMA

In rats, the lymph nodes that appeared enlarged after a 3-day application of DNCB or TMA were located dorsally to the lower poles of the submandibular glands, at the junction of the external jugular veins and the anterior and posterior facial veins. In most animals, only one node at each side was found, but occasionally two or even three nodes were observed, especially in BN rats. All nodes at the above-mentioned location were considered to be the LLN and were collected and weighed together.

In BALB/c mice as well as all rat strains tested, significantly increased mean LLN weights were observed relative to controls after TMA as well as DNCB application. The stimulation index (SI) was highest in mice and lowest in BN rats both for TMA and DNCB application; in the other rat strains the response varied slightly (Table 3). Untreated rats showed slightly higher LLN weights than did vehicle-treated controls (data not shown). The rather low stimulation index in BN rats may result from the high control weights in this strain.

Control LLN showed low percentages of proliferating cells, as judged by BrdU staining, in the different lymph node compartments of most strains. Higher percentages were found in the medulla of BALB/c mice and Fischer rats, and in both the medulla and cortex of BN rats (Fig. 1a). DNCB and

Table 3. Strain-dependent effects of DNCB and TMA on weight and proliferative activity in local lymph nodes (LLN)

Species	Strain	Treatment	LLN weight† (mg)	SI‡	BrdU+ cells†§ (%)	SI‡	BrdU+ cells/LLN†	SI‡
Mouse	BALB/c	AOO	4.0 ± 0.5	—	5.0 ± 2.1	—	0.2 ± 0.1	—
		DNCB	18.6 ± 0.7**	4.7	15.4 ± 2.4**	3.1	2.8 ± 0.4**	11.8
		TMA	18.7 ± 1.6**	4.7	16.0 ± 0.9**	3.2	3.2 ± 0.3***	13.3
Rat	Fischer	AOO	12.4 ± 1.2	—	3.5 ± 0.7	—	0.4 ± 0.1	—
		DNCB	51.9 ± 1.3**	4.2	19.6 ± 3.9***	5.6	10.2 ± 2.1	22.6
		TMA	35.7 ± 3.2**	2.9	18.5 ± 2.1***	5.3	6.5 ± 0.9**	14.5
	Wistar	AOO	19.0 ± 1.7	—	3.3 ± 0.8	—	0.6 ± 0.2	—
		DNCB	77.0 ± 5.6**	4.1	22.4 ± 2.8***	6.8	17.5 ± 2.8**	28.2
		TMA	59.1 ± 5.5**	3.1	22.6 ± 2.4***	6.9	13.7 ± 2.4**	22.0
	BN	AOO	29.6 ± 2.9	—	5.2 ± 1.0	—	1.6 ± 0.4	—
		DNCB	63.3 ± 4.1**	2.1	15.8 ± 3.2	3.0	10.0 ± 2.0	6.1
		TMA	65.6 ± 4.6**	2.2	15.8 ± 4.5	3.0	11.1 ± 3.7	6.8
	SD	AOO	20.7 ± 2.6	—	1.6 ± 0.6	—	0.3 ± 0.1	—
		DNCB	82.6 ± 4.1**	4.0	14.4 ± 3.4*	9.0	12.4 ± 3.4*	44.1
		TMA	79.0 ± 5.5**	3.8	14.0 ± 4.9	8.7	11.6 ± 3.9*	41.4
	PVG	AOO	13.6 ± 1.5	—	2.8 ± 0.5	—	0.4 ± 0.1	—
		DNCB	49.3 ± 2.5**	3.6	13.9 ± 2.3**	5.0	6.7 ± 1.0***	17.6
		TMA	38.7 ± 2.0**	2.9	20.6 ± 1.5***	7.4	8.1 ± 0.9***	21.2

Groups of five or six mice or rats received 1% DNCB or 50% TMA in AOO or AOO alone on the dorsum of both ears on days 0, 1 and 2, and an ip injection of BrdU on day 4 (mice) or day 5 (rats). LLN at both sites were removed 2 hr after BrdU treatment, weighed, and processed for BrdU staining to determine the percentage or total number of BrdU+ cells per LLN.
†Values are means ± SEM.
‡Stimulation index (mean ratio of values found in LLN of chemical- and AOO-treated animals, respectively).
§Mean of the average percentages found in paracortex, cortex and medulla.
Asterisks indicate significant differences from corresponding controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ANOVA followed by Dunnett's or t-test).

TMA increased proliferative activity in all lymph node compartments of all groups, but responses in the different compartments differed with the strain and compound used (Fig. 1b,c). In Fischer and Wistar rats, proliferative activity in the paracortical lymph node area was higher than in the cortical area after DNCB treatment, and vice versa after TMA application. In BN rats, the proliferative activity was more or less the same in cortex and paracortex after DNCB treatment, whereas a higher cortical reaction was seen after TMA application. In BALB/c mice in particular, and to a lesser extent in PVG and SD rats, the paracortical reaction was greater than that in the cortex, regardless of which compound had been applied.

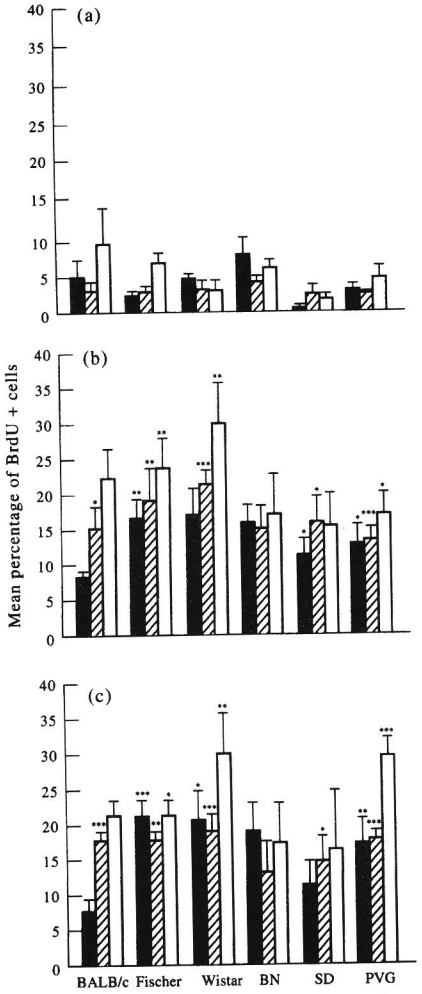

Fig. 1. Strain-dependent area-associated proliferative activity of local lymph nodes (LLN) after application of DNCB or TMA. Groups of five or six mice or rats received 1% DNCB (b) or 50% TMA (c) in AOO, or AOO alone (a), on the dorsum of both ears on days 0, 1 and 2, and an ip injection of BrdU on day 4 (mice) or day 5 (rats). LLN at both sites were removed 2 hr after BrdU treatment and processed for BrdU staining to determine the percentage of BrdU+ cells in three areas in the cortex (■), five areas in the paracortex (▨) and three areas in the medulla, (□). Asterisks indicate significant differences from corresponding controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ANOVA followed by t-test).

The mean percentage of BrdU+ cells per section was significantly increased by DNCB or TMA in BALB/c mice and in all rat strains apart from the BN strain (Table 3). The stimulation index was highest in SD rats and lowest in BN rats and BALB/c mice. Virtually the same ranking was seen when stimulation indices of the mean total proliferative activity per LLN were calculated.

Dose–response study

DNCB caused a dose-dependent increase of lymph node weight, being significant at the two highest concentrations tested in the three rat strains selected (Table 4). Linear dose–effect relationships were also seen when the average percentage of BrdU+ cells and the total number of BrdU+ cells/LLN were considered, but sensitivity of the strains differed with regard to the parameters. Fischer rats already showed significant increases of both parameters in response to 0.25% DNCB, whereas Wistar rats required 0.5% to do so. BN rats showed an increase of the average percentage of BrdU+ cells at 1% only, but showed a significant increase of the total number of BrdU+ cells/LLN already at a level of 0.5%. In the case of TMA, no clear dose–effect relationships could be noted. All doses tested caused significant increases of all parameters in all strains and effects appeared to be of similar magnitude or tended to have an optimum at the medium dose tested (Table 4).

DISCUSSION

The LLNA with DNCB and TMA as described in mice (Kimber *et al.*, 1994; Kimber and Weisenberger, 1989) was tested in five rat strains, using BrdU instead of tritiated thymidine as a label to assess induced prolferative activity, and LLN weights and ear thickness and inflammatory cell infiltrates as additional parameters. LLN weight appeared to be a very consistent parameter, since it was significantly increased by TMA and DNCB in all strains at all levels except for 0.25% DNCB (Tables 3 and 4). Induced proliferative activity per LLN, however, is possible the best parameter, since it yielded by far the highest SI for both compounds in all strains, and since weight increase may include non-specific effects, such as oedema.

In the murine LLNA, total proliferative activity per LLN, as measured with tritiated thymidine, is the parameter generally used and compounds inducing at least a threefold increase of this parameter relative to

vehicle-treated controls are considered to be sensitizers (Kimber *et al.*, 1994). Accordingly, our observations that DNCB and TMA increased total BrdU incorporation per LLN in all strains well above threefold, indicates that the rat may be a suitable test animal for recognition of these (and probably other) sensitizers and that the BrdU method gives reliable results. Thus, routine assessment of BrdU incorporation, for instance by ELISA or flow cytometry, may offer a rapid and suitable, non-radioactive alternative to the tritiated thymidine method. The rather laborious immunohistochemical assessment of BrdU incorporation, however, permits acquisition of more detailed information (Fig. 1). Notably, DNCB tended to induce more proliferation in the T-cell-dependent paracortical area than in the B-cell-dependent cortical area, whereas TMA tended to do the reverse, particularly in Fischer and Wistar rats. These observations agree very well with the type of allergy predominantly induced by these chemicals, namely T-cell-mediated delayed-type hypersensitivity by DNCB and IgE antibody-mediated allergy by TMA. A higher paracortical than cortical reaction was also found for DNCB (Kimber *et al.*, 1986) and for the typical contact sensitizer oxazolone (Kimber *et al.*, 1991).

Further, in our study, both chemicals caused clear proliferative responses in the medulla, a compartment that predominantly harbours activated B cells and antibody-forming plasma cells on lymph node activation. It suggests that both chemicals cause antibody formation, as formally demonstrated in the murine LLNA (Bloksma *et al.*, 1993).

According to Tilney (1971), rats rarely have auricular lymph nodes; instead, the ear was reported to be drained by the facial nodes, which in turn drain into the posterior cervical lymph nodes. In this study, most animals had one node at each side at the location of the facial nodes. However, in rats with more than one node at each side at that location, auricular lymph nodes may have been present. All lymph nodes at the facial lymph node location were enlarged after application of DNCB or TMA. Since the weight of the other lymph nodes in the neck region (superficial cervical lymph nodes) were unchanged (data not shown), it seems justifiable to conclude that application of either chemical on the ears resulted in drainage by the auricular/facial lymph nodes only.

The observed different responsiveness of the different rat strains to DNCB and TMA was not unexpected, since the genetic make-up is known to have a marked influence on immune responsiveness. BN rats generally showed the lowest responses, and SD and Wistar rats the highest. The latter two strains generally also showed the highest intrastrain variability, as apparent from the large SEM. Since SD and Wistar rats were the only random/outbred strains used in this study, the variability may be explained by genetic differences between members of these strains and emphasizes the influence of the genetic make-up. Consequently, the differences in sensitivity between

Table 4. Strain-dependent effects of various concentrations of DNCB and TMA on weight and proliferative activity in LLN

Rat strain	Treatment	LLN weight† (mg)	SI‡	BrdU+ cells†§ (%)	SI‡	BrdU+ cells/LLN†	SI‡
Fischer	AOO	11.6 ± 0.7	—	2.9 ± 0.3	—	0.3 ± 0.0	—
	0.25% DNCB	17.7 ± 1.4	1.5	12.3 ± 0.5***	4.3	2.2 ± 0.2**	6.8
	0.5% DNCB	29.0 ± 2.8**	2.5	12.9 ± 0.4***	4.5	3.7 ± 0.4**	11.6
	1% DNCB	49.3 ± 3.5**	4.2	14.8 ± 1.8***	5.1	7.4 ± 1.2*	23.1
	12.5% TMA	32.9 ± 3.4**	2.8	16.2 ± 0.9***	5.6	5.3 ± 0.7**	16.6
	25% TMA	41.4 ± 3.9**	3.5	15.3 ± 1.8***	5.3	6.4 ± 1.0*	20.1
	50% TMA	35.9 ± 2.7**	3.1	18.7 ± 2.7***	6.5	6.8 ± 1.1*	21.2
Wistar	AOO	17.7 ± 0.8	—	2.8 ± 0.6	—	0.5 ± 0.1	—
	0.25% DNCB	25.8 ± 3.3	1.4	7.0 ± 1.9	2.5	2.0 ± 0.6	4.1
	0.5% DNCB	40.1 ± 2.9**	2.3	10.8 ± 0.6***	3.9	4.3 ± 0.4**	9.0
	1% DNCB	63.8 ± 4.4**	3.6	13.0 ± 0.5***	4.7	8.3 ± 0.8**	17.3
	12.5% TMA	65.9 ± 3.0**	3.6	15.1 ± 1.4***	5.4	10.0 ± 1.3***	20.9
	25% TMA	74.0 ± 2.1**	4.1	16.7 ± 0.7***	6.0	12.4 ± 0.6***	25.7
	50% TMA	56.8 ± 4.8**	3.1	15.2 ± 1.7***	5.5	8.7 ± 1.4***	18.1
BN	AOO	27.5 ± 1.7	—	4.2 ± 1.2	—	1.1 ± 0.3	—
	0.25% DNCB	30.1 ± 0.8	1.1	6.1 ± 1.6	1.5	1.8 ± 0.5	1.7
	0.5% DNCB	41.4 ± 1.4**	1.5	9.9 ± 1.4	2.4	4.1 ± 0.6*	3.8
	1% DNCB	54.7 ± 4.1**	1.9	10.5 ± 2.7*	2.5	5.7 ± 1.6**	5.2
	12.5% TMA	57.5 ± 1.5**	2.0	15.7 ± 0.7***	3.7	9.0 ± 0.5***	8.4
	25% TMA	64.1 ± 3.5**	2.3	14.4 ± 1.2***	3.4	9.3 ± 1.0**	8.6
	50% TMA	60.7 ± 5.3**	2.1	15.3 ± 1.6***	3.7	9.2 ± 1.1**	8.5

Groups of rats received DNCB or TMA in AOO (five rats per group) or AOO alone (eight rats per group) on the dorsum of both ears on days 0, 1 and 2, and an ip injection of BrdU on day 5. LLN at both sites were removed 2 hr after BrdU treatment, weighed, and processed for BrdU staining to determine the percentage or total number of BrdU+ cells per LLN.

†,‡,§As in Table 3.

Asterisks indicate significant differences from corresponding controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ANOVA followed by Dunnett's or t-test).

the rat strains may give perspective in using strains representative for specific human populations.

Responses of the BALB/c mice, however, were remarkably similar to those of the rats. This suggests that the adaptations of the mouse LLNA protocol to the rat (i.e. a three times larger volume of the same concentration of sensitizer, and DNA labelling on day 5 instead of day 4) were well chosen, although a longer delay may be considered. This is concluded from the observations that the mice showed the highest relative increase of LLN weight, but almost the lowest increase of proliferative activity per LLN. It suggests that the peak of proliferation and thus the peak increase of cells had occurred prior to day 4, which is supported by data from the literature (Kimber *et al.*, 1986; Kimber and Weisenberger, 1989).

Dose–effect studies in three rat strains showed that DNCB, tested at concentrations of 0.25, 0.5 and 1%, caused a linear increase in LLN weight and proliferative activity, while TMA, tested at concentrations of 12.5, 25 and 50%, caused responses of approximately similar magnitude as those with 1% DNCB (Table 4), as was also found by Dearman and Kimber (1991) in the mouse. Further, the data show that the range of concentrations of TMA used was apparently too high, the medium concentration not infrequently tending to cause the highest responses. This phenomenon has been observed with other chemicals and has been attributed to down-regulation of the immune response as a consequence of over-vigorous stimulation (Anderson, 1990; Edwards *et al.*, 1994; Kimber and Weisenberger, 1991).

Ear thickness was consistently, but not always signficantly, increased by DNCB and TMA, and not clearly related to the presence of mononuclear cell infiltrates (T lymphocytes with or without macrophages), since these were only seen in a portion of the exposed rats. The infiltrates, however, are likely to point at local delayed-type hypersensitivity reactions to residues of the chemicals. This is not unexpected, since 1% DNCB and 50% TMA were reported to induce an equal state of sensitization for contact hypersensitivity (Dearman and Kimber, 1991).

It was concluded that effects induced by TMA and DNCB in all five rat strains were comparable with those in mice, suggesting that rats also may be used as test animals in the LLNA.

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Chapter 3

Local lymph node activation and IgE responses in Brown Norway and Wistar rats after dermal application of sensitizing and non-sensitizing chemicals

Josje H.E. Arts, Sonja C.M. Dröge, Steven Spanhaak, Nanne Bloksma, André H. Penninks and C. Frieke Kuper

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Local lymph node activation and IgE responses in Brown Norway and Wistar rats after dermal application of sensitizing and non-sensitizing chemicals

Josje H.E. Arts[a,*], Sonja C.M. Dröge[a], Steven Spanhaak[a], Nanne Bloksma[b], André H. Penninks[a], C. Frieke Kuper[a]

[a]*TNO Nutrition and Food Research Institute, Toxicology Division, P.O. Box 360, 3700 AJ Zeist, The Netherlands*
[b]*Research Institute of Toxicology (RITOX), Utrecht University, Utrecht, The Netherlands*

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Abstract

The local lymph node assay (LLNA) and the IgE test in the mouse are proposed models for predictive recognition of low molecular weight chemicals causing IgE-mediated allergic airway reactions in man. Since rats are commonly used in routine toxicity studies and a previous study (Arts et al. (1996) Food Chem. Toxicol. 34, 55–62) has shown that several rat strains were found appropriate for the LLNA, the suitability of the rat for the IgE test was examined in the present study. Serum IgE concentrations were examined following topical exposure of Brown Norway (BN) and Wistar rats to each of four chemicals with known diverse sensitization potential in humans: trimellitic anhydride (TMA), a dermal and respiratory sensitizer; dinitrochlorobenzene (DNCB), a dermal sensitizer with no or limited potential to cause respiratory allergy; formaldehyde (FA), a skin irritant and dermal sensitizer with equivocal evidence for respiratory sensitizing potential; methyl salicylate (MS), a skin irritant devoid of sensitizing properties. Of the four tested chemicals, only exposure to TMA resulted in a significant increase in serum IgE concentration and this response was only evoked in the high-IgE-responding BN rat. The latter two chemicals were also tested for lymph node activation, in casu the ear-draining lymph nodes. FA caused a dose-dependent activation of the draining lymph nodes whereas MS was inactive. The results as obtained with TMA, DNCB and MS in the rat are in agreement with human data. The results with FA though, indicate the need for further studies of chemicals that have both irritant and sensitizing properties at about similar concentrations or may act through non-IgE-mediated immune mechanisms.

Keywords: IgE; Lymph node activation; Rats; Dermal application; Sensitizers

Abbreviations: BN, Brown Norway; BrdU, bromodeoxyuridine; DNCB, 2,4-dinitrochlorobenzene; FA, formaldehyde; LLN, local lymph node; MS, methyl salicylate; TMA, trimellitic anhydride.

* Corresponding author.

1. Introduction

Various chemicals of industrial importance such as isocyanates, reactive dyes and organic acid anhydrides are known to cause respiratory allergy, generally becoming manifest as occupational asthma and/or rhinitis. These are usually the consequence of immediate type hypersensitivity reactions (Coombs and Gell, 1975) that are almost invariably mediated by allergen-specific IgE (Botham et al., 1988, 1989).

As yet there are no validated methods or internationally harmonized guidelines for the identification of respiratory sensitizing chemicals. Recently, a tiered approach was suggested (Briatico-Vangosa et al., 1994) to this end. The first step proposes an examination of physicochemical properties particularly in the context of structure–activity relationships (Sarlo and Clark, 1992). The second step suggests the performance of a standard predictive test for recognition of skin sensitizing activity, such as the guinea pig maximization test (Magnusson and Kligman, 1969) or the murine local lymph node assay (LLNA; Kimber et al., 1986; Kimber et al., 1994). The last step proposes the determination of the chemical's potential to cause respiratory sensitization by either of two ways: (1) measuring inhalation challenge-induced respiratory reactions of intradermally or subcutaneously sensitized guinea pigs (Karol, 1988) or (2) measuring the serum IgE concentrations of mice after topical exposure to immunogenic concentrations of the test material (Dearman et al., 1992).

The first method (Karol, 1988) has the advantage of being an endpoint test but is time-consuming, costly, and often requires the use of a hapten–protein conjugate. By contrast, the mouse IgE test is cost-effective but has the disadvantage that it is not yet known whether increases of total serum IgE are indicative of actual induction of specific IgE or respiratory sensitization.

Since rats are more commonly used in routine toxicity studies than mice, knowledge of the effects of a given chemical in the LLNA and the IgE test in the rat would facilitate comparison of its immunological and toxic properties. In a previous study using trimellitic anhydride (TMA) and 2,4-dinitrochlorobenzene (DNCB), several rat strains were found appropriate for the LLNA (Arts et al., 1996). In the present study, the suitability of the rat for the IgE test was studied. To this end, two rat strains were used, the low-IgE-responding Wistar strain and the high-IgE-responding Brown Norway (BN) strain (Diaz-Sanchez and Kemeny, 1991), and four different chemicals with diverse sensitization potential in man: DNCB, a dermal sensitizer (Kimber and Weisenberger, 1989); TMA, a dermal sensitizer at high concentrations and a respiratory sensitizer (Bernstein et al., 1983; Botham et al., 1989; Dearman and Kimber, 1991); formaldehyde (FA), a skin irritant, and a dermal sensitizer after prolonged exposure (Foussereau et al., 1982; Glass, 1961; Jordan et al., 1979; Maibach, 1983) and a supposed respiratory sensitizer (Hendrick et al., 1982; Nordman et al., 1985; Sheffer, 1991); methyl salicylate (MS), a skin irritant being negative in predictive human sensitization tests (Basketter et al., 1994). Further, FA and MS were also tested for lymph node activation, in casu the ear-draining lymph nodes, to enable comparison with results obtained with DNCB and TMA (Arts et al., 1996).

2. Materials and methods

2.1. Animals and maintenance

Female, 10- to 11-week-old random-bred Wistar and inbred BN rats were purchased from Charles River Wiga GmbH, Sulzfeld, Germany, and acclimatized for at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received the Institute's grain-based open-formula diet and unfluoridated tap water ad libitum. All animal procedures were approved by the TNO Commission of Animal Welfare.

2.2. Test materials

Trimellitic anhydride (TMA; purity 97%; Aldrich, Brussels, Belgium), 2,4-dinitrochlorobenzene (DNCB; purity at least 98%; Sigma, St.

Louis, MO, USA), formaldehyde (FA; commercial grade 37%; Boom, Meppel, the Netherlands) and methyl salicylate (MS; purity at least 99%; Sigma, St. Louis, MO, USA) were dissolved in 4:1 (v/v) acetone (Merck, Darmstadt, Germany) and raffinated olive oil (Chempri, Raamsdonksveer, the Netherlands) (AOO).

2.3. Local lymph node activation

Selected concentrations of the chemicals in 75 μl AOO were applied to the dorsum of each of both ears on days 0, 1, and 2. Controls received AOO. An intraperitoneal (i.p.) injection of 150 mg/kg body weight of the thymidine analog, bromodeoxyuridine (BrdU; Sigma, St. Louis, MO, USA), was given on day 5 to label proliferating cells. Two hours after the BrdU treatment, animals were anaesthetized and bled to death via the abdominal aorta. Ear-draining lymph nodes were collected and processed as described by Arts et al. (1996). In short, lymph nodes were fixed in 70% ethanol, embedded in paraffin wax and sectioned. Sections were successively incubated with a monoclonal anti-BrdU antibody, with a biotin-labelled rabbit-anti-mouse antibody, peroxidase-conjugated streptavidin, and finally with the chromogen, 3,3′-diaminobenzidine tetrahydrochloride. Mitotic activity was determined in lymph node sections at five sites of the paracortex and at three sites of the cortex and medulla, without prior knowledge of the treatment. In each lymph node, at each standardized site, 50 cells of which the nuclei were located at the crossings of the horizontal and vertical lines of a 10×10 intra-ocular grid were examined at high power magnification ($400 \times$) to determine the mean percentage of BrdU positive (BrdU+) cells per compartment and the weighed average percentage of BrdU+ cells per section. As a measure of total proliferation, the average percentage of BrdU+ cells per section was multiplied by lymph node weight.

2.4. Serum IgE responses

Animals received 150 μl of the selected concentrations of the chemicals on each flank (approximately 12 cm^2 each) which had been shaved with an electrical razor 1–4 days earlier. Seven days after the first application, all animals received 75 μl of the same chemical at 50% of the initial concentration on the dorsum of each of both ears. Controls received AOO only. The IgE test was performed with the highest concentration of FA and MS tested in the LLNA, and in the case of DNCB and TMA, at a concentration causing significant proliferation in the LLNA (Arts et al., 1996).

For determination of serum IgE concentrations, blood was sampled by orbital puncture (days 8 and 14) and from the abdominal aorta at autopsy (day 21). Serum samples were stored at -20°C until analysis. Serum IgE was measured in individual or pooled (of two rats each) sera by means of an ELISA in which volumes of 100 μl were used, incubations were performed at 37°C unless indicated otherwise, and wells were washed with demineralized water containing 2% Tween 20 after each incubation. Flat-bottomed wells of microtiter plates (Nunc, Roskilde, Denmark) were incubated with 5 μg/ml of streptavidin (Boehringer Mannheim GmbH, Germany) in 0.1 M sodium carbonate buffer (pH 9.6) overnight at 4°C. Wells were next incubated with a 1:500 dilution of biotin-conjugated monoclonal mouse-anti-rat IgE antibodies (Serotec, Oxford, UK) in PBS containing 1% BSA for 30 min. Then graded dilutions of test sera (up to 1:32) and a monoclonal rat IgE standard at concentrations ranging from 1 to 1000 ng/ml (Serotec, Oxford, UK) in PBS containing 1% BSA were added to duplicate wells and incubated for 45 min. This was followed by an incubation with a 1:1000 dilution of peroxidase-labeled monoclonal mouse-anti-rat IgE (Zymed, San Francisco CA, USA) in PBS containing 1% BSA for 30 min after which substrate (3,3′,5,5′-tetramethylbenzidine dihydrochloride dehydrate and hydrogen peroxide) was added. The colour reaction was terminated after 20 min at room temperature by the addition of 2 M sulphuric acid to measure substrate conversion as optical density at 450 nm using the BIO-RAD 3550 Microplate Reader (BIO-RAD, Richmond CA, USA). The concentration of IgE in test serum samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as μg IgE/ml serum.

2.5. Clinical signs, body and organ weights

All animals were observed at least once daily and weighed shortly before the first chemical application, in the case of the IgE test at weekly intervals thereafter, and at autopsy. At autopsy, kidneys, liver and spleen were collected and weighed.

2.6. Statistics

Body weights were analyzed by one-way analysis of covariance (COVAR) followed by the two-sided Dunnett's multiple comparison test. Organ weights were determined by ANOVA-LSD in the case of 4 rats/group and by ANOVA-DUNNETT in the case of 5 rats/group. Data on proliferative activity and serum IgE concentrations were analysed by ANOVA followed by pairwise t-tests.

3. Results

3.1. Local and systemic effects after application of the test chemicals

MS and FA at the various concentrations used caused no visible changes of the ears after each of the three daily applications; the third application of 10% FA resulted in restlessness in Wistar as well as BN rats. The treatments did not affect body, kidney, liver and spleen weights, except for 25% MS that significantly increased kidney weights in Wistar rats (about 14%; $P < 0.01$; data not shown).

Application of 1% DNCB or 25% MS on the shaved flank skin caused no skin reactions, but application of 10% FA or 50% TMA locally induced dermal encrustations, erythema or scaliness in most Wistar and BN rats. The dermal reactions were first seen 6 days after the flank application, generally disappeared 2 days later and reappeared about 5 days later in a few rats. Application of the chemicals did not affect body, kidney, liver and spleen weights.

3.2. Local lymph node activation

FA significantly increased LLN weights of both strains at the two highest (5 and 10%) concentrations; Wistar rats showed a clearly dose-dependent response, whereas the response of BN rats levelled off at 5% (Table 1). The proportion of proliferating cells, as measured by the percentage of BrdU + cells, increased slightly more than the LLN weight, although significant increases were only seen in BN rats treated with 10% FA. The latter strain showed a clear dose-dependent response, but the response of Wistar rats levelled off at 5% FA. The same dose-response relationship was found for the total proliferative activity per LLN (BrdU + cells/LLN). This parameter showed the most prominent increases which were significant in both strains at the two highest concentrations.

The FA-induced proliferation appeared largely confined to the paracortical area (Fig. 1a) and was statistically significant at concentrations of 5 and 10% in Wistar rats and at a concentration of 10% in BN rats. The proliferative activity in the cortical and medullar area was statistically indifferent amongst treated rats and controls, but proliferative activity in the B-cell harbouring cortex tended to increase in BN rats.

MS did not increase LLN weight and proliferation in both strains (Table 1, Fig. 1b). Rather, it tended to decrease these parameters.

3.3. Serum IgE responses

The mean concentration of serum IgE in vehicle-(AOO) treated rats was 0.10–0.15 μg/ml for Wistar rats and 0.5–1.7 μg/ml in BN rats (Tables 2 and 3), being consistent with data from other studies (Abadie and Prouvost-Danon, 1980; Diaz-Sanchez and Kemeny, 1991).

In BN rats, topical exposure to 50% TMA on day 0 and to 25% on day 7 resulted in significantly increased serum IgE levels on days 14 and 21 when compared to vehicle-treated controls (Table 2). In contrast, DNCB, FA and MS failed to change the serum IgE levels of BN.

To study the reproducibility and strain-dependence of the effects of DNCB and TMA, a second

Table 1
Strain-dependent effects of various concentrations of FA and MS on weight and proliferative activity in local lymph nodes

Strain	Treatment	LLN weight (mg)	SI[a]	BrdU+ cells[b] (%)	SI	BrdU+ cells/LLN	SI
Wistar	AOO	34.3 ± 2.8	–	4.7 ± 1.1	–	1.5 ± 0.3	–
	2.5% FA	39.5 ± 1.5	1.2	8.1 ± 1.4	1.7	3.2 ± 0.6	2.1
	5% FA	$46.4 \pm 4.3^{**}$	1.4	10.8 ± 0.7	2.3	$5.0 \pm 0.7^{*}$	3.3
	10% FA	$50.0 \pm 3.2^{***}$	1.5	10.1 ± 2.5	2.1	$5.0 \pm 1.2^{*}$	3.2
	5% MS	29.3 ± 0.9	0.9	4.1 ± 1.5	0.9	1.2 ± 0.4	0.8
	12.5% MS	27.6 ± 1.6	0.8	2.1 ± 0.4	0.4	0.6 ± 0.1	0.4
	25% MS	28.8 ± 1.5	0.8	4.2 ± 1.2	0.9	1.2 ± 0.4	0.8
BN	AOO	39.4 ± 1.0	–	5.7 ± 0.9	–	2.2 ± 0.3	–
	2.5% FA	45.0 ± 1.4	1.1	5.8 ± 1.4	1.0	2.7 ± 0.7	1.2
	5% FA	$56.2 \pm 2.9^{**}$	1.4	11.9 ± 3.0	2.1	$6.7 \pm 1.7^{*}$	3.0
	10% FA	$55.2 \pm 4.5^{**}$	1.4	$13.8 \pm 1.1^{*}$	2.4	$7.6 \pm 1.0^{*}$	3.4
	5% MS	36.6 ± 3.2	0.9	5.8 ± 2.0	1.0	2.2 ± 0.7	1.0
	12.5% MS	33.8 ± 5.0	0.9	6.0 ± 0.9	1.1	2.1 ± 0.6	1.0
	25% MS	34.8 ± 2.5	0.9	7.2 ± 3.4	1.3	2.7 ± 1.5	1.2

Groups of four rats received FA or MS in AOO at various concentrations (w/v) or AOO alone on the dorsum of both ears on days 0, 1 and 2, and an i.p. injection of BrdU on day 5. LLN at both sites were removed 2 h after BrdU treatment, weighed, and processed for BrdU staining to determine the percentage or total number of BrdU+ cells/LLN. Data have been expressed as means ± S.E.M. (standard error of the mean).

[a]Stimulation index, being the mean ratio of values found in LLN of chemical- and AOO-treated animals, respectively.

[b]Weighed mean of the average percentages found in the paracortex, cortex and medulla.

Statistics: ANOVA-LSD or ANOVA/t-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Linear trend analysis showed statistical significance in both the percentage of BrdU+ cells as well as in BrdU+ cells/LLN; $P<0.05$ (% BrdU+ cells in Wistar rats), $P<0.01$ (% BrdU+ cells in BN and BrdU+ cells/LLN in both Wistar and BN rats).

study was carried out. TMA induced significant increases in serum IgE concentrations of BN rats of the same order of magnitude as those observed in the first study (Tables 2 and 3), but failed to do so in Wistar rats (Table 3). DNCB appeared incapable of changing the IgE levels of both strains.

4. Discussion

The present study shows that FA increased LLN weights and proliferation in Wistar and BN rats whereas MS did not. The results of MS are consistent with the negative results obtained in mice with MS concentrations as high as 25% (Basketter et al., 1994) but at variance with those of Montelius et al. (1994) who found sensitizing activity in mice at concentrations of 25% but not at 10 or 5%. Since MS also appeared negative in predictive guinea pig (Kimber et al., 1991) and human (Basketter et al., 1994) sensitization tests, it was concluded that the LLNA test in the rat correctly identified MS as a non-sensitizer.

FA concentrations of 5 and 10% increased LLN proliferation slightly above 3-fold both in Wistar and BN rats. These results were in good agreement with those observed in mice, showing about 4-fold increases at concentrations of 2–10% (Kimber et al., 1991; Basketter et al., 1994). Based on the criterion (Kimber et al., 1994) that compounds inducing at least a 3-fold increase in draining lymph node proliferation in mice relative to vehicle-treated controls should be considered contact sensitizers, FA has to be classified a contact sensitizer on the basis of the rat LLNA results. FA, however, has irritating properties, the threshold for human skin being about 2% (WHO, 1989). Since chemical irritants devoid of sensitizing properties such as sodium dodecyl sulphate, oxalic acid, nonaic acid, chloroform/methanol (2:1) and the non-ionic surfactant triton X-100 appeared able of stimulating LLN proliferation 3- to 10-fold (Robbins et al., 1991; Basketter et al.,

1994; Montelius et al., 1994), irritant properties of FA might have substantially contributed to the LLN proliferation observed in this study. Therefore, FA probably is a weak sensitizer at most, which is in accordance with the observation that FA sensitization is rarely observed in dermatological practice, despite being classified as a strong contact sensitizer in guinea pigs (Cronin, 1982).

The moderate LLN proliferation induced by FA appeared to be confined to the T-cell-dependent, paracortical area, whereas the known sensitizing chemicals, TMA and DNCB, caused very vigorous proliferation in the paracortical as well as in the B-cell-dependent cortical areas of BN and Wistar LLN (Arts et al., 1996). TMA and DNCB also caused significant antibody formation in the murine LLNA (Bloksma et al., 1993). Lower doses of TMA and DNCB, however, induced less proliferation and less or no activation in the B-cell-dependent areas (Arts et al., 1996), and exponentially less antibody production (Vandebriel et al., 1996). Thus, the inability of even high concentrations of FA to cause significant proliferation in B-cell-dependent areas are probably in support of the conclusion above that it is at most a weak sensitizer.

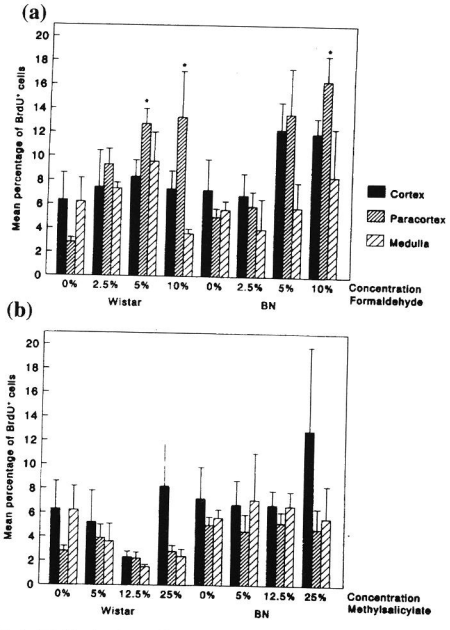

Fig. 1. (a) Strain-dependent area-associated proliferative activity of local lymph nodes after application of FA. Groups of four rats received 2.5, 5 or 10% FA in AOO or AOO alone on the dorsum of both ears on days 0, 1 and 2, and an i.p. injection of BrdU on day 5. LLN at both sites were removed 2 h after BrdU treatment and processed for BrdU staining to determine the percentage of BrdU + cells in three areas in the cortex, five areas in the paracortex and three areas in the medulla. Statistics: ANOVA/t-test; $*P < 0.05$. (b) Same as in (a) except for MS at concentrations of 5, 12.5 and 25%.

Results of the IgE test showed that TMA induced significant increases in total serum IgE levels in BN rats after two dermal applications, but that DNCB, FA and MS failed to do so. The positive result with TMA and the negative result with DNCB as noted in the rat, agree with findings in the mouse IgE test (Dearman and Kimber, 1991; Dearman et al., 1992; Hilton et al., 1995) and with data in man (Bernstein et al., 1983; Botham et al., 1989) and guinea pigs (Botham et al., 1989; Blaikie et al., 1995) that TMA, but not DNCB, is capable of causing respiratory sensitization. Moreover, the negative results with MS were expected since it is not a sensitizer, as also indicated by the rat LLNA. Thus, the rat IgE test appeared suitable for predicting the respiratory sensitization properties of these chemicals, although it should be noted that it is not yet known whether increases of total serum IgE are indicative of actual induction of specific IgE or respiratory sensitization.

Whether or not the negative result with FA in the rat IgE test is correct is open for discussion regarding the equivocal data obtained in studies with patients suspected to have respiratory problems as a consequence of FA sensitization. Firstly, FA-specific IgE antibodies could not be detected in such patients (Kramps et al., 1989; Salkie, 1991). Secondly, negative bronchial provocation tests (Frigas et al., 1984) as well as positive bronchial provocation and pulmonary function tests have been reported, albeit in a limited number of persons (Hendrick and Lane, 1977; Wallenstein et al., 1978; Hendrick et al., 1982; Nordman et al., 1985). Although the human data are ambiguous and incomplete, it is possible that FA is

Table 2
IgE concentrations in serum of BN rats after dermal application of DNCB, TMA, FA or MS

Treatment		IgE concentration in serum (μg/ml)		
Day 0	Day 7	Day 8	Day 14	Day 21
AOO (control)	AOO (control)	0.74 ± 0.15	0.53 ± 0.08	0.52 ± 0.08
DNCB (1%)	DNCB (0.5%)	0.89 ± 0.50	0.50 ± 0.16	0.35 ± 0.06
TMA (50%)	TMA (25%)	0.77 ± 0.16	$3.93 \pm 0.54^{**}$	$5.25 \pm 0.61^{**}$
FA (10%)	FA (5%)	0.43 ± 0.07	0.54 ± 0.23	0.30 ± 0.05
MS (25%)	MS (12.5%)	0.47 ± 0.06	0.73 ± 0.31	0.68 ± 0.26

Groups of five rats received DNCB, TMA, FA or MS in AOO at various concentrations (w/v) or AOO alone bilaterally on the shaved flanks followed by application of the same chemical at 50% of the initial concentration on the dorsum of both ears 7 days later. Blood was sampled for determination of IgE concentrations by orbital puncture on days 8 and 14 or from the abdominal aorta on day 21 at necropsy. Data have been expressed as means $\pm$ S.E.M. (standard error of the mean).
Statistics: ANOVA/t-test; $^{**}P<0.01$.

able of causing IgE-independent respiratory sensitization. If so, the IgE test is inappropriate to detect such compounds. It is also possible, however, that the highest applied concentration of FA (10%) was too low to elicit IgE production. This is suggested by the studies of Hilton et al. (1995) in the mouse IgE test, showing that induction of significant increases of serum IgE by chemical respiratory allergens required application concentrations over 3- to 30-fold that inducing a 3-fold increase of LLN proliferation. We, however, did not test FA concentrations exceeding 10% because of the overt skin reactions already at 10% FA. On the other hand, whether prolonged administration of lower concentrations of FA will ultimately result in increased IgE levels is the subject of further studies.

The fact that BN, but not Wistar rats, produced IgE in response to TMA exposure indicated that the chance to develop IgE-mediated respiratory allergy to TMA requires a particular genetic predisposition. Evidence for such predisposing factors has been obtained earlier in mouse and rat models of respiratory allergy to high molecular weight allergens (Xu et al., 1992; Saloga et al., 1993) and in humans with allergic asthma (Amelung et al., 1992; Meyers et al., 1994). By contrast, the genetic control appears less strict for proliferative responses in the ear-draining lymph nodes (Kimber and Weisenberger, 1989; Arts et

Table 3
Strain-dependent IgE concentrations in serum after dermal application of DNCB and TMA

Treatment		IgE concentration in serum (μg/ml)		
Day 0	Day 7	Day 8	Day 14	Day 21
Wistar				
AOO (control)	AOO (control)	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
DNCB (1%)	DNCB (0.5%)	0.11 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
TMA (50%)	TMA (25%)	0.11 ± 0.01	0.14 ± 0.01	$0.19 \pm 0.01^{\#}$
BN				
AOO (control)	AOO (control)	1.69 ± 0.22	1.34 ± 0.18	1.18 ± 0.15
DNCB (1%)	DNCB (0.5%)	1.11 ± 0.22	1.23 ± 0.17	1.42 ± 0.25
TMA (50%)	TMA (25%)	1.89 ± 0.23	$3.39 \pm 0.51^{*}$	$3.84 \pm 0.26^{**}$

Groups of seven rats received DNCB or TMA in AOO at various concentrations (w/v) or AOO alone bilaterally on the shaved flanks followed by application of the same chemical at 50% of the initial concentration on the dorsum of both ears 7 days later. Blood was sampled for determination of IgE concentrations by orbital puncture on days 8 and 14 or from the abdominal aorta on day 21 at necropsy. Data have been expressed as means $\pm$ S.E.M. (standard error of the mean).
Statistics: ANOVA/t-test; $^{*}P<0.05$; $^{**}P<0.01$ as compared to AOO controls, $^{\#}P<0.01$ as compared to DNCB-treated rats.

al., 1996) and those in the popliteal lymph nodes (Patriarca et al., 1994; Bloksma et al., 1995). This is not unexpected since proliferation is required for all types of sensitization while differentiation to IgE production requires fine-tuned additional mechanisms.

In summary, the results obtained with TMA, DNCB and MS in the rat are in agreement with human data. The results with FA though, indicate the need for further studies of chemicals that have both irritant and sensitizing properties at about similar concentrations or may act through non-IgE-mediated immune mechanisms.

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Chapter 4

Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals

Josje H.E. Arts, C. Frieke Kuper, Stan M. Spoor and Nanne Bloksma

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Airway Morphology and Function of Rats Following Dermal Sensitization and Respiratory Challenge with Low Molecular Weight Chemicals

Josje H. E. Arts,*[,1] C. Frieke Kuper,* Stan M. Spoor,* and Nanne Bloksma†

*Toxicology Division, TNO Nutrition and Food Research Institute, Zeist, The Netherlands; and †Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences and Faculty of Biology, Utrecht University, The Netherlands

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Local lymph node activation and increased total serum IgE levels are suggested to be predictive parameters of airway hypersensitivity caused by low molecular weight (LMW) chemicals. Whether increases of total serum IgE are indicative of actual induction of specific airway reactions (morphological and functional) after inhalation challenge was examined in the present study. In Brown Norway (BN) and Wistar rats, serum IgE concentrations were examined following topical exposure of chemicals with known diverse sensitization potential in humans: trimellitic anhydride (TMA), a dermal and respiratory sensitizer; dinitrochlorobenzene (DNCB), a dermal sensitizer with no known potential to cause respiratory allergy; and methyl salicylate, a skin irritant devoid of sensitizing properties. Functional and histopathological changes in the respiratory tract were examined after subsequent inhalatory challenge with these chemicals. Of the three tested chemicals, only topical exposure to TMA resulted in a significant increase in total serum IgE concentrations in the high-IgE-responding BN rat. Upon subsequent inhalatory challenge of these rats, TMA induced specific airway reactions which included a sharp decrease in respiratory rate during challenge, followed by an increase in breathing rate with a concomitant decrease in tidal volume 24 and 48 h after inhalatory challenge, and histopathological changes in the larynx and lungs of animals necropsied 48 h after challenge. Interestingly, despite low IgE levels, TMA induced histopathological changes in the larynx and lungs of Wistar rats too. Laryngeal changes were also observed in Wistar rats upon sensitization and challenge with DNCB. These data suggest that increased total serum IgE after topical sensitization is associated with immediate-type specific airway reactivity after inhalation challenge in BN rats and thus may be a valuable parameter in testing for respiratory sensitization potential of LMW compounds. Histopathological examination upon subsequent inhalation challenge of sensitized low-IgE-responders may provide information on other allergic inflammatory airway reactions. © 1998 Academic Press

Occupational asthma is an allergic respiratory disease that can be caused by LMW[2] chemicals such as isocyanates, reactive dyes, and acid anhydrides. Such chemicals are generally thought to react with self proteins upon dermal or respiratory exposure and so may induce sensitization. If specific IgE antibodies are induced, inhalation of the chemical will then mediate the disease-producing immediate type hypersensitivity reaction.

Despite insight into the mechanisms of the diseases, respiratory-sensitizing chemicals are usually only identified in practice, i.e., by recognition of allergic symptoms in a part of occupationally exposed populations. To prevent these occupational diseases, development of tests predicting the sensitizing properties of chemicals in man has gained increasing attention in the last decade, and various tests have been suggested. A tiered approach including three steps has been suggested by Briatico-Vangosa *et al.* (1994). The first step proposes an examination of physicochemical properties particularly in the context of structure–activity relationships (Sarlo and Clark, 1992). The second step suggests the performance of a standard predictive test for recognition of skin sensitizing activity, such as the guinea pig maximization test (Magnusson and Kligman, 1969) or the murine LLNA (Kimber *et al.*, 1986, 1994). The last step proposes the determination of the chemical's potential to cause respiratory sensitization by either of two ways: (1) measuring inhalation challenge-induced respiratory reactions of intradermally or subcutaneously sensitized guinea pigs (Karol, 1988) or (2) measuring the serum IgE concentrations of mice after topical exposure to immunogenic concentrations of the test material (IgE test; Dearman *et al.*, 1992). Regarding the LLNA, we have shown that various rat strains are suitable for recognizing the skin-sensitizing chemicals DNCB and TMA (Arts *et al.*, 1996). Further, BN, but not Wistar, rats appeared suitable for the IgE test. Notably, BN rats showed increased serum IgE levels upon dermal application of TMA that, unlike DNCB, are associated with respiratory sensitization in man

[1] To whom correspondence should be addressed at TNO Nutrition and Food Research Institute, Toxicology Division, P.O. Box 360, 3700 AJ Zeist, The Netherlands. Fax: + 31 30 69 602 64; E-mail: j.arts@voeding.tno.nl.

[2] Abbreviations used: BN, Brown Norway; DNCB, 2,4-dinitrochlorobenzene; *f*, frequency; LLNA, Local Lymph Node Assay; LMW, low molecular weight chemicals; MS, methyl salicylate; TMA, trimellitic anhydride

(Arts *et al.,* 1997). MS, an irritant devoid of sensitizing properties, gave negative results (Arts *et al.,* 1997).

Though a positive IgE test is considered to indicate that a chemical can cause respiratory sensitization, this has not been formally proven. We, therefore, investigated whether increases of total serum IgE levels after topical exposure are associated with sensitization of the respiratory tract and with specific functional and morphological changes of the airways after inhalation challenge at peak serum IgE levels. To this end, we examined the three aforementioned chemicals with diverse sensitization potential in man, namely TMA, a dermal and respiratory sensitizer (Bernstein *et al.,* 1983; Botham *et al.,* 1989; Dearman and Kimber, 1991); DNCB, a dermal sensitizer with no known potential to cause respiratory allergy (Kimber and Weisenberger, 1989); and MS, a skin irritant devoid of sensitizing properties (Basketter *et al.,* 1994), using two rat strains, the low-IgE-responding Wistar strain, and the high-IgE-responding BN strain (Diaz-Sanchez and Kemeny, 1991).

METHODS

Animals and maintenance. Female, 10- to 11-week-old, random-bred Wistar and inbred BN rats were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) and acclimatized for at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received the Institute's grain-based open-formula diet and unfluoridated tap water *ad libitum.* All animal procedures were approved by the TNO Commission of Animal Welfare.

Materials. TMA (purity 97%) was supplied by Aldrich (Brussels, Belgium). DNCB (purity at least 98%) and MS (purity at least 99%) were obtained from Sigma (St. Louis, MO). Acetone was supplied by Merck (Darmstadt, Germany) and raffinated olive oil by Chempri (Raamsdonksveer, The Netherlands).

Test scheme and groups. Unless specified otherwise, studies were conducted according to the following scheme:

day 0	7	20 or 21	21 or 22	23 or 24
↑	↑	↑	↑	↑
dermal sensitization	dermal sensitization	blood sampling (IgE)	respiratory challenge	necropsy

Sensitization procedure. Chemicals were applied at appropriate concentrations in a 4:1 (v/v) mixture of acetone and olive oil (AOO) as the vehicle (Arts *et al.,* 1997). Animals received 150 μl of the chemicals in AOO on each flank (approximately 12 cm^2 each) which had been shaved with an electrical razor at least 3 days earlier. Seven days after the first sensitization, they received 75 μl of the same chemical at 50% of the initial concentration on the dorsum of each of both ears. Controls received vehicle only.

Serum IgE responses. Individual serum samples were prepared from blood withdrawn by orbital puncture on Day 20 or 21 and stored at −20°C until analysis of IgE levels by means of an ELISA as described in Arts *et al.* (1997). In short, flat-bottomed wells of microtiter plates (Nunc, Roskilde, Denmark) were incubated with 5 μg/ml of streptavidin (Boehringer Mannheim GmbH, Germany) in 0.1 M sodium carbonate buffer (pH 9.6) and next incubated with a 1:500 dilution of biotin-conjugated monoclonal mouse-anti-rat IgE antibodies (Serotec, Oxford, UK). Then duplicate wells were incubated with graded dilutions of test sera and a monoclonal rat IgE standard (Serotec, Oxford, UK), and subsequently with a 1:1000 dilution of peroxidase-labeled monoclonal mouse-anti-rat IgE (Zymed, San Francisco CA) after which substrate (3,3′,5,5′-tetramethylbenzidine dihydrochloride dehydrate and hydrogen peroxide) was added. The color reaction was terminated by the addition of 2 M sulphuric acid to measure substrate conversion as optical density at 450 nm using the BIO-RAD 3550 Microplate Reader (BIO-RAD, Richmond CA). The concentration of IgE in test serum samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as micrograms IgE per milliliter serum.

Atmosphere generation and analysis. An all glass nebulizer designed at the Institute was used to generate liquid aerosols from freshly prepared solutions of the chemicals in acetone (Schaper and Brost, 1991). The acetone concentration was kept below 3000 ppm (7.4 g/m^3), which is considered to be far below a level inducing sensory irritation (Alarie, 1973; De Ceaurriz *et al.,* 1981; Schaper and Brost, 1991). Concentrations of TMA and DNCB were based on a study performed in guinea pigs (Blaikie *et al.,* 1995).

Atmospheric concentrations of TMA and DNCB were determined gravimetrically by filter sampling and those of MS by calculations based on the nominal concentration and the complete evaporation of the test solution. Particle size distributions of TMA and DNCB in the test atmospheres were determined using a 10-stage cascade impactor (Andersen, Atlanta, GA). Due to the small sampling air flow rate (2–5 L/min) and the large total volume required for analytical and particle size determinations (20–200 L/sample), samples were not collected during challenge exposure but prior to or after challenge. The mass median aerodynamic diameter of the TMA aerosols was 0.6 μm with geometric standard deviations between 1.8 and 2.9. DNCB particles were on average smaller than 0.4 μm.

Respiratory challenge and lung function measurements. Rats were restrained in Battelle tubes and placed individually into one of four plethysmographs that were connected to a central exposure chamber for nose-only exposure to the test atmosphere. Each plethysmograph was provided with a pressure transducer which sensed changes created by in- and expiration and transmitted amplified signals to a polygraph recorder, so allowing determination of respiratory frequency (f) and pattern.

Using this experimental set up, rats were subsequently exposed to fresh air for 20 to 40 min (prechallenge period), then to the test atmosphere for exactly 15 min (challenge period), and again to fresh air for 15 min (postchallenge period). Respiration was monitored each minute for 20 s, starting 6 min prior to the actual challenge. Thus means of frequencies were calculated from 6 prechallenge, 15 challenge, and 15 postchallenge values. A few groups were challenged twice with TMA at an interval of 24 h.

One day before challenge and by 1–2, 24, and 48 h after challenge, respiratory frequency and various other parameters were assessed using a plethysmograph with a separate head and body chamber and matched pressure transducers. For this purpose, unanaesthetized rats were placed into modified Battelle tubes with a water-wetted silicon diaphragm to give an air tight seal between head and neck at the one side and thorax and abdomen at the other. The tube was placed in the body chamber with the open end of the tube fitting into the front chamber. To prevent rebreathing, the air in the front chamber was constantly refreshed with an air flow of 1 L/min using a critical orifice; this flow was passed over a mesh wire resistance.

Breathing parameters (frequency, tidal volume, and minute volume) were determined by means of recording the pressure signal in the volume-calibrated body chamber during approximately 1 min. Lung mechanical properties were obtained by determination of the transfer impedance (Z_{tr}) as described in detail by Oostveen *et al.* (1992). Hereto, small (0.1 kPa) sinusoidal pressure fluctuations in the frequency range of 16–208 Hz (16-Hz harmonics) were applied around the thorax. These pressure fluctuations superimposed on those of the spontaneous breathing yielded small sinusoidal pressure changes which were measured in the head chamber (5 times for 4 s). The pressure signals were processed by Fast-Fourier Transformation, and if internal coherence was > 0.95 (Michaelson *et al.,* 1975), Z_{tr} for each frequency and means of $Re[Z_{tr}]$ (real part of Z_{tr}) and $Im[Z_{tr}]$ (imaginary part of Z_{tr}) as a function of frequency were calculated. Using an appropriate parameter estimating program (Oostveen *et al.,* 1992), the parameters were directly linked to the physiological variables airway resistance, compliance, and inertance.

Local effects, body and organ weights, and haematology. All animals were observed at least once daily and weighed shortly before the first chemical application, at weekly intervals thereafter, and at necropsy 48 h after (the last)

challenge. Animals were killed by exsanguination from the abdominal aorta under ether anaesthesia. Blood was collected for determination of total and differential white blood cell counts and the animals were examined for gross pathological changes. Liver, kidneys, spleen, and the lungs with trachea and larynx were removed and weighed.

Respiratory tract pathology. The nose, larynx, trachea, and lungs were preserved in neutral, phosphate-buffered 4% (v/v) formaldehyde (the lungs after inflation with the preservative) and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin. Sections of the nasal passages were cut at 6 levels, according to Young (1986) and Woutersen *et al.* (1994). Sections of the larynx were cut transversally at the base of the epiglottis, at the level of the ventral pouch and the arythenoid projections, and between the caudal part of the larynx and the first tracheal ring. The cranial part of the trachea was cut transversally, the caudal part longitudinally, together with the bifurcation and the two mainstem bronchi. Each lung lobe was cut at one median sagittal level. All tissues were examined microscopically.

Statistics. Body weights were analyzed by one-way analysis of covariance (COVAR) followed by the two-sided Dunnett's multiple comparison test. Organ weights and absolute haematology values were determined by ANOVA-DUNNETT. Relative haematology values were analyzed by Kruskal-Wallis nonparametric analysis of variance followed by the Mann/Whitney U test. Data on serum IgE concentrations were analyzed by ANOVA followed by pairwise t tests. The lung function parameters were analyzed by comparing the differences in challenge/prechallenge and postchallenge/prechallenge values of sensitized animals with those of unsensitized control animals using ANOVA followed by pairwise t tests.

TABLE 1
Lung Weights in BN and Wistar Rats after Dermal Sensitization and Subsequent Inhalation Challenge with TMA

Strain	Sensitization	Mean challenge concentration (mg/m^3)	Absolute weight (g)	Relative weight (g/kg)
BN	Vehicle	—	1.31 ± 0.02	7.69 ± 0.22
	Vehicle	52	1.33 ± 0.04	8.53 ± 0.36
	50/25%	52	2.11 ± 0.11**	13.39 ± 0.59**
	50/25%	31	2.31 ± 0.07**	14.50 ± 0.42**
	50/25%	16	2.08 ± 0.12**	12.88 ± 0.64**
BN	Vehicle	51/50	1.31 ± 0.04	8.15 ± 0.26
	50/25%	51/50	2.28 ± 0.13**	14.23 ± 0.71**
Wistar	–	–	1.03 ± 0.03	5.18 ± 0.17
	Vehicle	41	1.02 ± 0.02	4.80 ± 0.13
	50/25%	38/41	1.43 ± 0.06**	6.75 ± 0.32**

Note. Groups of 6 rats each received 50% TMA (w/v) bilaterally on the shaved flanks on Day 0 and 25% TMA (w/v) on the dorsum of both ears 7 days later. Controls received vehicle only. Animals were challenged once (Day 21) or twice (Days 21 and 22) by inhalation exposure to various TMA concentrations. Data have been expressed as means ± SEM. Statistics: ANOVA/Dunnetts test, $^{**}p < 0.01$. For comparison, absolute and relative lung weights are also indicated for vehicle-sensitized but nonchallenged BN rats ($n = 6$) and for untreated Wistar rats ($n = 5$).

RESULTS

Local Effects, Haematology, Body and Organ Weights

Flank application of MS caused no skin reactions, but TMA and DNCB caused local dermal encrustations, erythema, or scaliness in most rats. Reactions to TMA appeared 1 day after application and generally persisted for 3–5 days. In a few BN rats, they persisted a few days longer, whereas in a few Wistar rats they reappeared 1 day after ear application and lasted for up to 2–4 days. Flank skin reactions to DNCB lasted for up to 2 days after application in BN rats and for up to 5 days in Wistar rats.

Ear application of DNCB or MS caused no visible local reactions, but TMA induced scaliness of the ear skin of most BN rats and of one Wistar rat for up to 2–3 days. No differences in total and differential white blood cell numbers, including eosinophils, were observed between sensitized and control groups (data not shown). Dermal treatments did not affect body weights, but 2 days after an inhalatory exposure to DNCB, MS, or TMA slight body weight loss in several Wistar and BN rats was noted, irrespective of previous sensitization (data not shown). None of the chemicals affected absolute or relative kidney, liver, and spleen weights (data not shown), but TMA challenge consistently increased absolute and relative lung weights of the sensitized rats, especially of the BN strain; a concentration-response relationship was not observed. Absolute and relative lung weights of TMA-challenged control BN and Wistar rats were similar to those of nonchallenged or nontreated rats (Table 1).

Serum IgE Levels

Control Wistar rats had, as expected, low IgE serum levels (mean levels ranged from 0.00 to 0.01 μg/ml), and dermal treatment with TMA, DNCB, or MS did not significantly change these levels (data not shown). Mean IgE levels of vehicle-treated control BN rats varied from 0.35 to 0.71 μg/ml, with the exception of one control group in which 3 out of 12 outliers were found resulting in a mean value of 1.46 μg/ml (Table 2). Outliers ($n = 2$) were also found in a group of 46 age-matched, untreated BN rats showing a mean value of 0.52 ± 0.10 μg/ml. The levels in vehicle-treated rats were consistent with data from a study by Diaz-Sanchez and Kemeny (1991).

Dermal exposure to DNCB and MS did not affect the IgE levels, but TMA caused a highly significant increase in two separate experiments (Table 2).

Functional Respiratory Changes

Breathing frequencies and patterns of control BN and Wistar rats were comparable and appeared not changed by sensitization and/or challenge with all chemicals with the exception of TMA challenge of TMA-sensitized BN rats (Table 3). In the latter animals, inhalation challenge significantly decreased breathing frequencies within 1 or 2 min of exposure. This generally lasted throughout the challenge period and was the result of irregularly lengthened pauses between breaths. While these changes had returned to normal shortly after the challenge, breathing frequencies had increased by 1–2 h and especially 24 h after challenge as compared to the prechallenge

TABLE 2
IgE Concentrations in Serum of BN Rats after Dermal Application of TMA, DNCB, or MS

Sensitization	IgE concentration in serum (μg/ml)
Control	0.35 ± 0.06 (6)
TMA	3.43 ± 0.44 (12)***
Control	1.46 ± 0.48 (12)
TMA	4.55 ± 0.25 (24)***
Control	0.71 ± 0.23 (6)
DNCB	0.71 ± 0.11 (6)
Control	0.47 ± 0.11 (5)
MS	0.75 ± 0.16 (6)

Note. Groups of rats received 50% TMA, 1% DNCB, or 25% MS (w/v) bilaterally on the shaved flanks on Day 0 and 25% TMA, 0.5% DNCB, or 12.5% MS (w/v) on the dorsum of both ears 7 days later. Controls received vehicle only. Blood was sampled by orbital puncture on Day 20 or 21, and serum was analyzed for IgE concentrations. Data have been expressed as means ± SEM for the indicated number of rats (in parentheses). Statistics: ANOVA/*t* test, ***$p < 0.001$.

frequency (Fig. 1). A second TMA challenge at the latter time caused the same change of breathing pattern and the same relative decrease of breathing frequency as observed during the first challenge. Because of the high prechallenge breathing frequency, however, the second TMA challenge reduced the absolute frequency to control levels. This high frequency reappeared shortly after the second challenge, further increased by 1–2 and 24 h, and was still high at 48 h. As far as measured, the increased breathing frequencies were attended with reduced tidal volumes. Despite the increased frequency, it resulted in reduced minute volumes by 2 h after the first and second challenge, but normal minute volumes were obtained at 24 and 48 h after the second challenge (Fig. 1). In a separate experiment, challenge with different TMA concentrations showed the absence of a clear concentration dependence of the respiratory reactions in sensitized BN rats (Fig. 2). In this experiment, changes were generally similar to the preceding one.

Airway resistance, compliance, and inertance assessed by 1–2, 24, and 48 h after respiratory challenge with different TMA concentrations showed no significant changes (data not shown).

Histopathological Changes in the Respiratory Tract

Naive and singly TMA-challenged control BN rats had a normal histology of the nasal passages, larynx, trachea, and extrapulmonary bronchi (Table 4; Figs. 3A and 3B; data not shown). In sensitized BN rats, TMA challenge caused a concentration-dependent incidence in mixed inflammatory cell infiltrates, located ventrally and at the tip of the arythenoid projections. This type of laryngitis consisted of subepithelial aggregates of predominantly macrophage-like cells with varying numbers of granulocytes including some eosinophils and mononuclear inflammatory cells. The inflammation slightly protruded into the laryngeal lumen and was covered by a partly hyperplastic, partly ulcerated epithelium (Fig. 3C).

Lungs of naive BN rats contained low numbers of granulomas, while TMA challenge of the vehicle-treated rats increased the numbers of eosinophils inside or nearby the granulomas in half of the rats and induced more prominent goblet cell ex-

TABLE 3
Changes in Respiratory Frequency (*f*) in BN and Wistar Rats after Dermal Sensitization and Subsequent Inhalation Challenge with TMA, DNCB, or MS

Strain	Treatment		*f* (s^{-1})		
	Sensitization	Inhalation challenge	Before challenge	During challenge	After challenge
BN	Control	41 mg/m^3 TMA	2.08 ± 0.12	2.15 ± 0.24	2.50 ± 0.18
	TMA	38 mg/m^3 TMA	2.37 ± 0.20	1.64 ± 0.16*	2.34 ± 0.15
	Control	7.3 mg/m^3 DNCB	2.18 ± 0.08	2.06 ± 0.05	2.11 ± 0.08
	DNCB	7.5 mg/m^3 DNCB	2.01 ± 0.13	1.96 ± 0.12	1.90 ± 0.08
	Control @	18 mg/m^3 MS	2.03 ± 0.09	2.02 ± 0.10	2.01 ± 0.14
	MS	18 mg/m^3 MS	2.12 ± 0.05	1.95 ± 0.07	1.99 ± 0.09
Wistar	Control	41 mg/m^3 TMA	2.22 ± 0.07	2.81 ± 0.14	3.08 ± 0.20
	TMA	38 mg/m^3 TMA	1.91 ± 0.08	2.75 ± 0.28	2.90 ± 0.13
	Control	7.3 mg/m^3 DNCB	2.21 ± 0.16	2.08 ± 0.17	2.05 ± 0.15
	DNCB	7.5 mg/m^3 DNCB	2.16 ± 0.09	2.08 ± 0.10	2.10 ± 0.09
	Control @	18 mg/m^3 MS	2.26 ± 0.15	2.28 ± 0.19	2.25 ± 0.13
	MS	18 mg/m^3 MS	2.29 ± 0.17	2.28 ± 0.18	2.24 ± 0.19

Note. Groups of 6 rats each received 50% TMA, 1% DNCB, or 25% MS (w/v) bilaterally on the shaved flanks on Day 0 and 25% TMA, 0.5% DNCB, or 12.5% MS (w/v) on the dorsum of both ears 7 days later. Controls received vehicle only. On Day 21 or 22 animals were challenged by inhalation exposure to each of the compounds. Data have been expressed as means ± SEM for a 6-min prechallenge, a 15-min challenge, and a 15-min postchallenge period. @ = 5 instead of 6 rats per group; Statistics: ANOVA/*t* test on differences in challenge/prechallenge and postchallenge/prechallenge values between control and test groups, *$p < 0.05$.

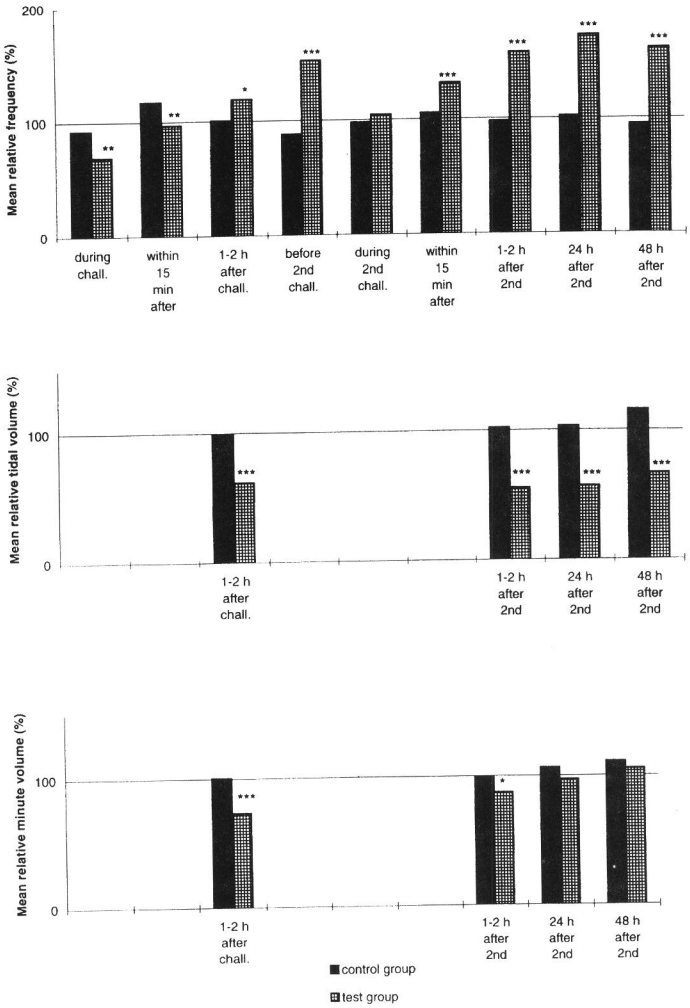

FIG. 1. Mean relative changes in respiratory frequency, tidal volume, and minute volume in groups of 6 BN rats after dermal sensitization with either vehicle (control group) or 50% TMA (test group) and two subsequent inhalation challenges with 51 and 50 mg/m^3 TMA. Statistics: ANOVA/t test on differences in challenge/prechallenge and postchallenge/prechallenge values between control and test group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

pression (goblet cell hyperplasia and hypertrophy) in one rat. TMA challenge of sensitized BN rats caused similar, but slightly more prominent, goblet cell expression and increased numbers of eosinophilic aggregates. Whereas none of these effects appeared clearly related to the inhaled TMA concentration, the distinct increase in the number and size of the granulomas appeared to be concentration-related, whereas the induction of haemorrhages in sensitized BN rats was only seen after challenge with the highest TMA concentration used (Table 4). The noncaseating granulomatous inflammation was observed in the interstitium and the lumina of the alveoli, the alveolar duct, and the terminal bronchioli, occasionally even

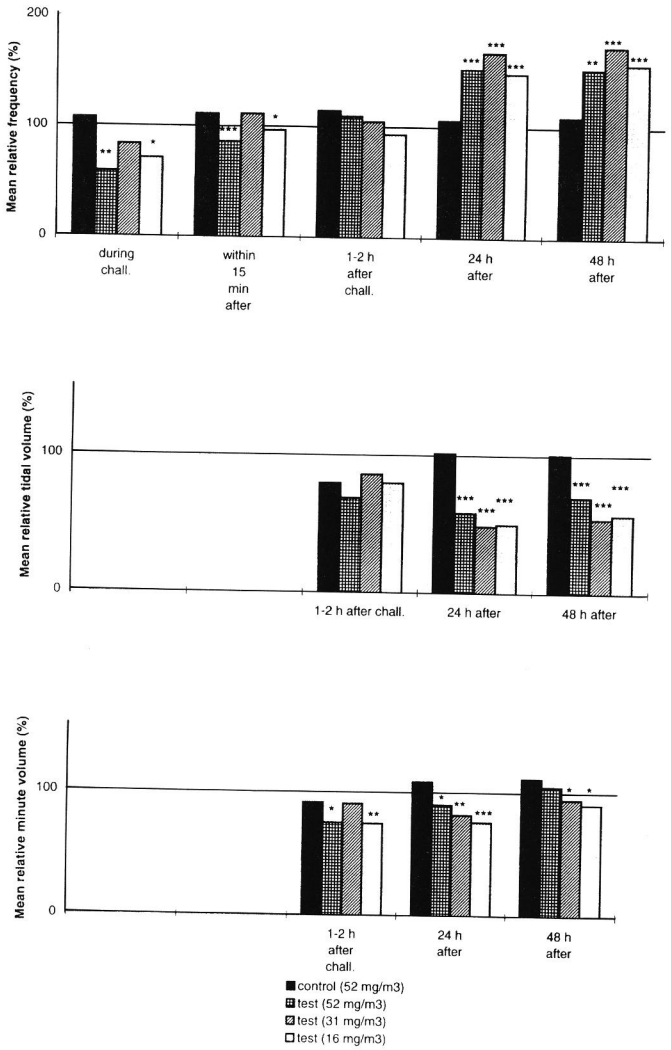

FIG. 2. Mean relative changes in respiratory frequency, tidal volume, and minute volume in groups of 6 BN rats after dermal sensitization with either vehicle (control group) or 50% TMA (test groups) and subsequent inhalation challenge with various concentrations of TMA. Statistics: ANOVA/*t* test on differences in challenge/prechallenge and postchallenge/prechallenge values between control and test groups. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

obliterating the lumina. In more progressed cases, the inflammation took up a considerable portion of the lung lobes. Two sequential challenges with TMA caused virtually the same changes in control and sensitized BN rats as seen after a single challenge, respectively (Table 4). These results were confirmed in the larynx and lungs in a separate study (data not shown).

Naive and TMA-challenged control Wistar rats showed no respiratory tract pathology, but half of the sensitized Wistar

TABLE 4

Number of BN and Wistar Rats Showing Respiratory Tract Changes after Dermal Sensitization and Subsequent Inhalation Challenge with TMA

Strain:	BN					BN		Wistar		
Sensitization:	–	Control	TMA	TMA	TMA	Control	TMA	–	Control	TMA
Mean challenge conc. (mg/m^3):	–	52	52	31	16	51/50	51/50	–	41	38/41
Larynx										
Marked focal mixed inflammatory cell infiltrate	0	0	6	5	2	0	6	0	0	3
Lungs										
Haemorrhages	0	0	6	0	0	0	6	0	0	5
Increased goblet cell expression										
Slight	0	1	4	1	1	0	3	0	0	0
Marked	0	0	0	2	0	0	3	0	0	0
Increased number of eosinophilic aggregates	0	3	4	6	5	2	3	0	0	0
Mean number of microgranulomas/lobe										
0–10	6	6	0	0	0	6	0	0	0	0
10–15	0	0	0	0	4	0	0	0	0	0
15–>20	0	0	4	6	2	0	5	0	0	0
Large confluent granulomas	0	0	2	0	0	0	1	0	0	0
Focal, predominantly lymphocytic infiltrates with few microgranulomas	0	0	0	0	0	0	0	0	0	6

Note. Groups of 6 rats each received 50% TMA (w/v) bilaterally on the shaved flanks on Day 0 and 25% TMA (w/v) on the dorsum of both ears 7 days later. Controls received vehicle only. Animals were challenged once (Day 21) or twice (Days 21 and 22) by inhalation to various TMA concentrations. Animals were killed, and airways were removed for examination 48 h after the (last) challenge. Results were compared with unsensitized and unchallenged rats. Nasal passages, trachea, and extrapulmonary bronchi did not exhibit treatment-related histopathological changes.

rats developed a similar focal laryngitis as seen in BN rats upon challenge with TMA. Likewise, TMA challenge of sensitized, but not control, Wistar rats caused pulmonary haemorrhages. It also caused focal perivascular and peribronchiolar inflammatory cell infiltrates, mainly around the smaller bronchioli (Table 4). The infiltrates largely consisted of mononuclear and neutrophilic polymorphonuclear cells. Further, small interstitial granulomas and accumulations of alveolar macrophages were observed. No histopathological changes were observed in nasal passages, trachea, and extrapulmonary bronchi.

DNCB caused no exposure-related histopathological changes in the respiratory tract of BN rats (Table 5). In contrast, DNCB-sensitized Wistar rats developed a ventrally located laryngitis characterized by a marked lymphocytic cell infiltrate after inhalation challenge (Fig. 3D). In addition, Wistar lungs had an atelectasis-like appearance, i.e., they had not fully expanded upon inflation with the fixative after DNCB inhalation, irrespective of sensitization. Nasal passages, trachea, and extrapulmonary bronchi did not exhibit treatment-related histopathological changes.

MS caused no histopathological changes of the respiratory tract of both rat strains (data not shown).

DISCUSSION

Two 1-week-spaced dermal applications of TMA elevated total serum IgE levels in BN rats 2 weeks after the last application, and inhalation challenge with TMA at that time evoked a significant change of airway function. Both effects were absent in identically exposed Wistar rats and in BN and Wistar rats after similar exposure to DNCB or MS. The absence of TMA-induced effects in low-IgE-responding Wistar rats was in accordance with findings in TMA-treated, low-IgE-responding Sprague–Dawley rats (Leach *et al.*, 1987). The airway response of sensitized BN rats to TMA was similar to that described in TMA-sensitized guinea pigs (Botham *et al.*, 1989; Blaikie *et al.*, 1995), namely an immediate decrease of the breathing frequency as a consequence of a lengthened pause between breaths and a rapid recovery after withdrawal of TMA. Although nonsensitizing irritants can evoke physiologically the same reactions (Briatico-Vangosa *et al.*, 1994), the observed respiratory reaction to TMA in BN rats is not likely to be the result of an irritant response because it required previous sensitization. The airway response is possibly related to increased IgE production since Wistar rats (this study), that fail to mount a significant IgE response upon TMA exposure, did not display airway reactions to TMA. Thus, regarding the capacity of TMA to increase nonspecific as well as specific IgE in the BN rat (Andius *et al.*, 1996; Vento *et al.*, 1996; Pullerits *et al.*, 1997), the immediate respiratory reaction to TMA may be the result of a classical type I hypersensitivity reaction (Coombs and Gell, 1975).

Bronchoconstriction is a hallmark symptom of allergic human asthma (Boushey *et al.*, 1980). The immediate TMA-induced bronchoconstriction in BN rats can be considered as severe because mean breathing frequency during the 15-min

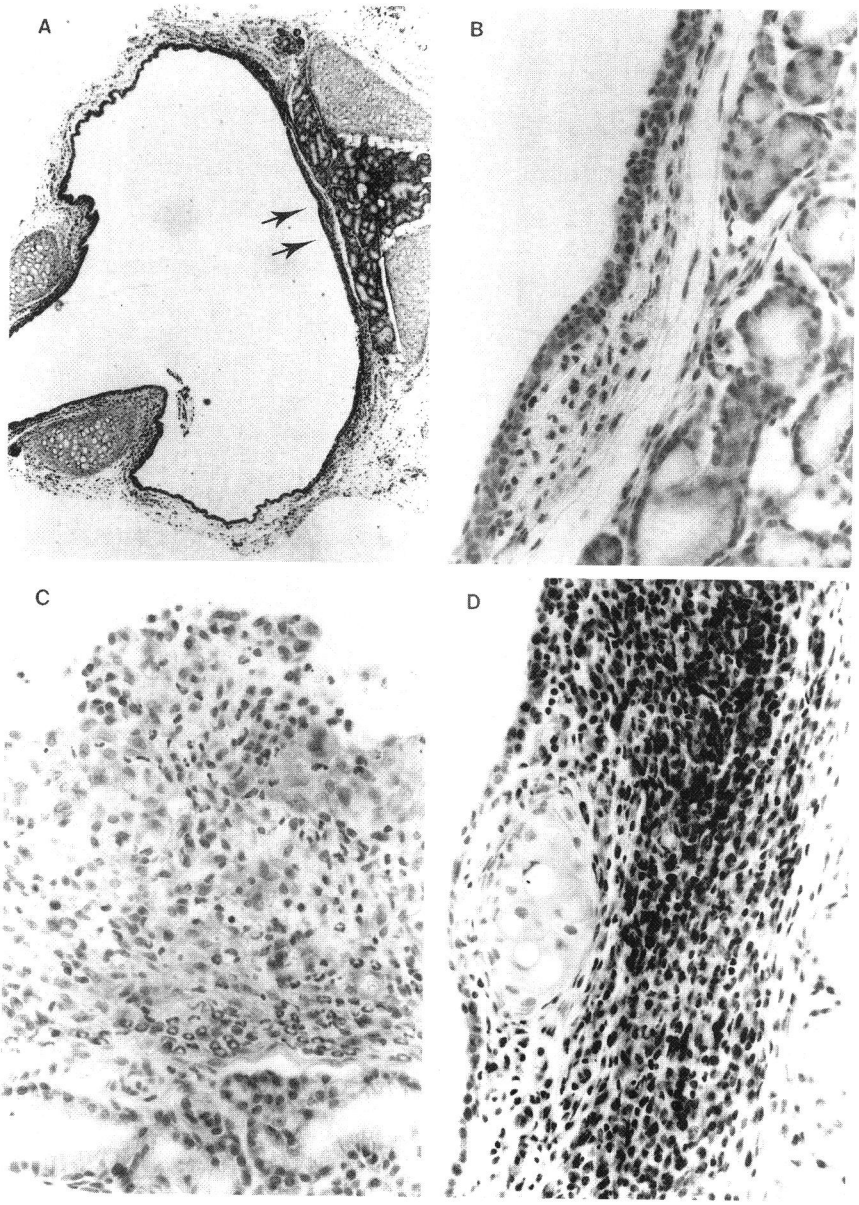

FIG. 3. (A) Larynx of an unsensitized, TMA-challenged BN rat. Arrows indicate the site depicted in detail in B (50×). (B) Detail of the larynx of an unsensitized TMA-challenged BN rat (400×). (C) Detail of the larynx of a TMA-sensitized and -challenged BN rat. Hypersensitivity laryngitis: a mixed inflammatory reaction with slight cell necrosis and eruption of inflammatory cells (400×). (D) Detail of the larynx of a DNCB-sensitized and -challenged Wistar rat. Infiltrate consists predominantly of lymphocytes (400×).

TABLE 5
Number of BN and Wistar Rats Showing Respiratory Tract Changes after Dermal Sensitization and Subsequent Inhalation Challenge with DNCB

Strain	BN			Wistar		
Sensitization	–	Control	DNCB	0	Control	DNCB
Mean challenge conc. (mg/m^3)	–	7.3	7.5	0	7.3	7.5
Larynx						
Marked focal mononuclear cell infiltrate	0	0	0	0	0	6
Lungs						
Mean number of microgranulomas/lobe (0–10)	6	6	6	0	0	0
Atelectasis-like appearance (see text)	0	0	0	0	6	6

Note. Groups of 6 rats each received 1% DNCB (w/v) bilaterally on the shaved flanks on Day 0 and 0.5% DNCB (w/v) on the dorsum of both ears 7 days later. Controls received vehicle only. Animals were challenged on Day 21 or 22 by inhalation exposure to DNCB. Animals were killed and airways were removed for examination 48 h after challenge. Results were compared with unsensitized and unchallenged rats. Nasal passages, trachea, and extrapulmonary bronchi did not exhibit treatment-related histopathological changes.

challenge period was generally reduced by 30% or more in most animals (Karol *et al.*, 1980, 1985; Botham *et al.*, 1989; Blaikie *et al.*, 1995). Like other animal models of allergic asthma (Botham *et al.*, 1989; Hayes *et al.*, 1992; Obata *et al.*, 1992; Blaikie *et al.*, 1995; Hessel *et al.*, 1995ab) the BN rats rapidly recovered from the immediate bronchoconstriction, since a normal breathing frequency was noted shortly after inhalation challenge. There was also no bronchoconstriction at 1–2 h, and at 24 and 48 h after challenge as judged by the normal or enhanced breathing frequency and the normal airway resistance at these times. However, the reduced tidal volumes at these times and the reduced minute volumes at 1–2 and 24 h after challenge were indicative of a rather persistent effect on lung function. In our study, using a fixed sensitization dose of TMA, the response to increasing inhalation concentrations of TMA was similar for all concentrations. Apparently, all concentrations were high enough to evoke a full-blown functional respiratory response. These results agree with those obtained in guinea pigs after intradermal sensitization with a fixed dose of TMA and several challenge concentrations (Blaikie *et al.*, 1995; Botham *et al.*, 1989).

Histopathology of the lungs of TMA-sensitized and -challenged BN rats showed features associated with increased IgE levels and allergic asthma, namely eosinophilic aggregates and goblet cell hyperplasia and hypertrophy (Aikawa *et al.*, 1992; Bentley *et al.*, 1993; Virchow *et al.*, 1994; Bernstein *et al.*, 1997). However, similar inflammatory reactions were seen in TMA-challenged control BN rats, though less markedly and less frequently, suggesting the involvement of a not yet understood, nonspecific, reaction to TMA peculiar to the BN strain, since TMA challenge did not evoke eosinophil and goblet cell reactions in Wistar rats.

Besides the allergic asthma-associated pathology in the BN lungs, sensitization-dependent granulomatous inflammation resembling hypersensitivity pneumonitis (extrinsic allergic alveolitis) in man were found. The granuloma development increased with increasing challenge concentrations, while haemorrhages were seen at the highest challenge concentration only. Such a concentration dependency has been noted in TMA-exposed humans as well (Zeiss and Patterson, 1993). Conversely, haemorrhages and granuloma development are also considered to represent acute and subacute features, respectively, of human hypersensitivity pneumonitis (Terr, 1997). This agrees with the observations that TMA-related granuloma development in humans requires chronic exposure for at least several months (Zeiss and Patterson, 1993). Therefore, the prominent granuloma formation in TMA-sensitized BN rats already 2 days after a single respiratory challenge was unexpected. It is suggestive of a predisposition in this rat strain which may be related to the presence of microgranulomas in the lungs of naieve BN rats, as observed in the present study and by others (Ohtsuka *et al.*, 1997). Such microgranulomas were absent from the lungs of naive and sensitized Wistar rats, while challenge upon sensitization induced a pathology consistent with mild acute hypersensitivity pneumonitis, i.e., haemorrhagic alveolitis and terminal bronchiolitis with a clear contribution of neutrophilic polymorphonuclear cells, besides mononuclear cells, and few interstitial microgranulomas. Thus the preexistent microgranulomas in BN lungs may accelerate development of hypersensitivity pneumonitis as induced by TMA. Presently, hypersensitivity pneumonitis is considered to be the consequence of a cell-mediated (Type IV; Coombs and Gell, 1975) hypersensitivity reaction as well as an immune complex-mediated (Type III) allergic reaction. The latter would cause phagocyte and complement activation, especially in the early stages of the disease (Salvaggio, 1987; Terr, 1997). Thus this study shows that TMA-sensitized BN rats concomitantly express two or three types of allergic reactions in the lung after respiratory challenge, although TMA-related allergic reactions in man are usually reported as separate syndromes (Zeiss and Patterson, 1993; Bernstein *et al.*, 1997). In rats, this is clearly dependent on the genetic makeup, since similarly exposed Wistar rats did not display functional and morphological evidence of Type I allergic asthma. The divergent TMA

pathology in BN and Wistar rats is not surprising, because the BN strain, unlike most other rat strains, is highly biased to the development of immune reactions mediated by so-called Th2 cells (Peszkowski *et al.,* 1994). These cells produce a set of cytokines that favor the production of IgE and other particular antibody isotypes and counteract the development of Th1 cells that mediate Type IV delayed hypersensitivity reactions.

This predominant Th2 profile in BN rats probably also explains the absence of laryngitis in DNCB-treated BN rats, because the laryngitis induced by this compound in Wistar rats was a typical Type IV hypersensitivity reaction as judged by the almost pure lymphocytic infiltrate. The laryngitis induced by TMA, however, differed from that induced by DNCB, because the inflammatory infiltrate predominantly consisted of cells that morphologically resemble macrophages.

Its morphology beared resemblance to a mixed Type III/IV reaction, as seen in hypersensitivity pneumonitis, and can be considered a hypersensitivity laryngitis. The TMA-induced laryngitis was observed both in BN and Wistar rats and was similar as to morphology and severity. The incidences of laryngitis were challenge concentration-dependently increased in BN rats, affecting all 6 at 50 mg/m^3 and 5 out of 6 at 31 mg/m^3, whereas 3 out of 6 Wistar rats exhibited laryngitis at a concentration of 38/41 mg/m^3. Together data indicate that the development and nature of allergic airway reactions in larynx as well as lungs upon exposure to sensitizing chemicals is defined by intrinsic properties of the chemical as well as the exposed individual, as earlier concluded for chemically induced allergic contact dermatitis (Peszkowski *et al.,* 1994).

Although the DNCB-induced laryngitis in Wistar rats confirmed the well-known capacity of DNCB to cause delayed hypersensitivity reactions, interstitial pneumonia was not observed. Such a pneumonia, however, was seen with nitrochlorobenzenes in Wistar rats and in BALB/c mice after intranasal challenge (Garssen *et al.,* 1989; Zwart *et al.,* 1994; Satoh *et al.,* 1995). Since the laryngitis in the challenged Wistar rats indicated appropriate sensitization, the most likely reason for the absence of interstitial pneumonia is that the DNCB aerosol did not penetrate sufficiently into the lower airways. Finally, MS did not induce any histopathological changes in both control and test animals, confirming that this chemical is devoid of sensitizing potential (Basketter *et al.,* 1994).

With regard to the suggested tiered approach for recognition of respiratory-sensitizing chemicals (Briatico-Vangosa *et al.,* 1994), our present and previous studies with TMA, DNCB, and MS (Arts *et al.,* 1996, 1997) indeed indicated that the LLNA correctly identified TMA and DNCB as sensitizing chemicals and that the IgE test correctly identified TMA as a potential inducer of allergic asthma, provided an appropriate rat strain is used. However, the IgE test does not predict a chemical's capacity to cause allergic airway reactions other than allergic asthma. Histopathological examination upon subsequent inhalation challenge of sensitized low-IgE-responders as performed in the present study, therefore, may provide additional information on other allergic inflammatory airway reactions.

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Chapter 5

Respiratory allergic reactions to trimellitic anhydride in Brown Norway rats

Josje H.E. Arts, Nanne Bloksma, Thea Leusink-Muis,

C. Frieke Kuper

Submitted

Abstract

Occupational exposure to low molecular weight (LMW) chemicals such as acid anhydrides can result in occupational asthma, an allergic disease characterised by episodic airway obstruction, airways inflammation and non-specific airways hyperresponsiveness (AHR).

Previously we showed that inhalation of the allergen trimellitic anhydride (TMA) by sensitised Brown Norway (BN) rats induced an early asthmatic response and an airway response at 24 and 48h, increased lung weights and marked airway inflammation. The present study investigated whether AHR characterised this model of occupational asthma and whether irritant and allergic effects of TMA on the airways could be discriminated.

BN rats were sensitised by dermal application of TMA and challenged by inhalation of a slightly irritating concentration of TMA. Controls were treated and/or challenged with vehicle. Three weeks after sensitisation, total IgE was measured with ELISA and specific IgE by passive cutaneous anaphylaxis assay. Lung function was measured before, during and after challenge by recording breathing pattern, frequency and tidal volume. AHR to methacholine was measured one day after challenge in a whole body plethysmograph or in isolated perfused tracheas. Bronchoalveolar lavage was also performed to measure total protein, lactate dehydrogenase, N-acetyl-glucosaminidase, and total and differential leukocyte numbers in the fluid. Larynx, trachea and lungs were examined histopathologically. TMA sensitisation and challenge induced all changes indicated above, elevated total and TMA-specific IgE levels, caused AHR *in vivo* and *in vitro*, and increased levels of biochemical parameters and numbers of eosinophils and neutrophils in lavage fluid. During TMA challenge of vehicle-treated rats a breathing pattern typical of irritation was noticed whereas in TMA-sensitised rats, besides a decrease in breathing frequency, a breathing pattern typical of sensitisation could be distinguished from the irritant pattern.

In conclusion, TMA challenge caused sensitisation-dependent asthma-like early changes of breathing pattern, that clearly could be distinguished from irritant-induced changes, and AHR 24h after challenge. Functional changes were accompanied by inflammatory changes characteristic of asthma and biochemical evidence of airway damage.

Introduction

Occupational exposure to low molecular weight (LMW) chemicals such as acid anhydrides, diisocyanates and reactive dyes can result in allergic respiratory diseases, occupational asthma being the most common disease. The disease is characterised by episodic airway obstruction, airways inflammation and non-specific airways hyperresponsiveness (AHR) to a variety of stimuli, including histaminergic and cholinergic agents (Sheffer, 1991; Anonymous 1992). Occupational asthma is a disorder of great health concern because of the high morbidity, the frequently poor reversibility upon withdrawal from exposure, and the sometimes life-threatening reactions (Briatico-Vangosa, 1994). Development of disease requires sensitisation, that is thought to be triggered by dermal or respiratory exposure to the sensitising chemical, binding of the chemical to self-proteins and subsequent presentation to chemical-specific lymphocytes. In susceptible individuals this may ultimately result in the production of specific IgE antibodies. Inhalation of the chemical allergen then evokes the disease-producing immediate type hypersensitivity reaction becoming manifest as tightness of the chest because of bronchoconstriction.

In animal models, mostly sensitised guinea pigs, several LMW allergens have produced respiratory changes during inhalation challenge consisting of increased or decreased breathing frequencies (Karol et al., 1980; Karol, 1988; Botham et al., 1988; 1989; Dearman et al., 1991; Pauluhn and Eben, 1991; Pauluhn and Mohr, 1994; Blaikie et al., 1995; Pauluhn et al., 1999a). Further, likewise in human (occupational) asthma, inhalation of LMW allergens by sensitised animals can cause non-specific AHR. This AHR is apparent by increased bronchoconstriction to, for instance, a cholinergic agonist (Scheerens et al., 1996; 1999). Both parameters, immunologically specific and non-specific bronchoconstriction, have been used in toxicology to identify LMW respiratory allergens.

Alterations in breathing, however, have also been found upon inhalation of irritating compounds, and distinction of irritant-induced alterations from specific allergen-induced alterations is not easy (Karol, 1991; Briatico-Vangosa et al., 1994; Pauluhn and Mohr, 1994; Pauluhn et al., 1999a). In addition, irritating compounds may induce inflammatory airway reactions that are not readily distinguishable from allergen-mediated airway inflammation. This is a problem since LMW allergens usually display irritant effects at higher concentrations.

Discrimination between LMW allergens and irritants is very important since a

generally valid occupational exposure limit may be assessed for LMW irritants whereas no such level can be assessed for LMW allergens. This is due to the large inter-individual variation in threshold for sensitisation and provocation of an allergic response (Briatico-Vangosa et al., 1994). Further, in screening it is important to know the irritating potential of LMW respiratory allergens.

Trimellitic anhydride (TMA) is a typical respiratory allergen, which is also known for its airway irritating effects. In a previous study, we have demonstrated the airway sensitising properties of TMA in the BN rat. Dermal sensitisation with TMA resulted in increased total serum IgE levels and subsequent inhalation challenge with non- or slightly irritating concentrations of TMA caused bronchoconstrictive reactions characterised by reduced breathing frequency during challenge, and local inflammation characteristic of asthma (Arts et al., 1998).

In the present study we examined whether allergen-specific bronchoconstriction and inflammation were attended by non-specific AHR *in vivo* and *in vitro*, and whether allergen- and irritant-induced alterations could be distinguished on the basis of breathing patterns during challenge.

Material and methods

Animals and maintenance

Female, 7-8-week-old, inbred Brown Norway (BN) rats were purchased from Charles River Deutschland (Sulzfeld, Germany; BN/CrlBR), IFFA CREDO (L'Arbresle Cedex, France; BN/OrlIco), and Harlan UK Ltd. (Blackthorn, UK; BN/SsNHsd). Wistar rats (Crl:[WI]WUBR), used for the PCA assay (see further) were supplied by Charles River Deutschland. The animals were acclimatised for at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received the Institute's grain-based open-formula diet and unfluoridated tap water ad libitum. All animal procedures were approved by the TNO Commission of Animal Welfare.

Materials

Trimellitic anhydride (TMA; purity 97%) was obtained from Aldrich (Brussels, Belgium), acetone from Merck (Darmstadt, Germany), raffinated olive oil from Chempri (Raamsdonksveer, The Netherlands), and methacholine chloride RBI from SanverTECH (Heerhugowaard, The Netherlands).

Test scheme and groups

Studies were generally conducted according to the following scheme:

Day	-1	0	7	20	21	22
	↑	↑	↑	↑	↑	↑
	pre-bleed (IgE)	dermal sensitisation	dermal sensitisation	blood sampling (IgE)	respiratory challenge and function	AHR, BAL, necropsy, blood sampling (IgE)

The test group was sensitised and challenged with TMA (+/+). Control groups were treated and challenged with vehicle and TMA (-/+), TMA and vehicle (+/-), and vehicle and vehicle (-/-), respectively.

Sensitisation procedure

TMA was applied at a concentration of 50% (w/v) in a 4:1 (v/v) mixture of acetone and olive oil (AOO) as the vehicle (Arts et al., 1997). Animals received 150 µl on each flank (approximately 12 cm^2 each) which had been shaved with an electrical razor 2-3 days earlier. Seven days after the first sensitisation, they received 75 µl of 25% TMA on the dorsum of each of both ears.

Serum IgE levels

Individual serum samples were prepared from blood withdrawn by orbital puncture and/or via the abdominal aorta at necropsy. The serum samples were stored at -20°C until analysis of total serum IgE levels by means of an ELISA (Arts et al., 1997) or the presence of TMA-specific IgE antibodies using the Passive Cutaneous Anaphylaxis (PCA) assay (Knippels et al., 1998).

In short, the ELISA test procedure was as follows: flat-bottomed wells of microtitre plates (Nunc, Roskilde, Denmark) were incubated with 5 µg/ml of streptavidin (Boehringer Mannheim GmbH, Germany) in 0.1 M sodium carbonate buffer (pH 9.6) and next incubated with a 1:500 dilution of biotin-conjugated monoclonal mouse-anti-rat IgE antibodies (Serotec, Oxford, UK). Then duplicate wells were incubated with graded dilutions of test sera and a monoclonal rat IgE standard (Serotec, Oxford, UK), and subsequently with a 1:1000 dilution of peroxidase-labeled monoclonal mouse-anti-rat IgE (Zymed, San Francisco CA, USA) after which substrate (3,3',5,5'-tetramethylbenzidine dihydrochloride dehydrate and hydrogen peroxide) was added. The colour reaction was terminated by the addition of 2 M sulphuric acid to measure substrate conversion as optical density at 450 nm using the BIO-RAD 3550 Microplate Reader (BIO-RAD, Richmond CA, USA). The concentration of IgE in test serum samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as µg IgE /ml serum.

In short, the PCA assay was carried out as follows: backs and flanks of naive (untreated) BN and Wistar rats were shaved and injected intradermally with 0.1 ml of the undiluted test sera, followed 72 h later by an intravenous injection of 1 ml of a 1:1 mixture of a solution of Evans blue (2% in sterile saline) and either a solution of TMA-HSA (5 mg/ml sterile saline; kindly donated by Dr. V. Warbrick, Astra-Zeneca, UK) or TMA-BSA (5 mg/ml; kindly donated by Dr. R. Pieters, RITOX, Utrecht University, The Netherlands). After 10 min and 120 min responses at the site of the serum injection were measured as the diameter of dye extravasation. A blue coloured spot of at least 5 mm in diameter was considered a positive response.

Atmosphere generation and analysis

An all glass nebulizer designed at the Institute was used to generate the test atmospheres from freshly prepared solutions of TMA in acetone (Schaper and Brost, 1991) or acetone alone. The acetone concentration was kept between 2000 and 5000 ppm (~ 5-12 g/m^3), which levels are considered to be far below the level inducing sensory irritation (Alarie, 1973; De Ceaurriz et al., 1981; Schaper and Brost, 1991). The concentration of TMA was based on a previous study performed in rats (Arts et al., 1998). Atmospheric concentrations of TMA were determined gravimetrically by filter sampling and those of acetone by calculations based on the nominal concentration and its complete evaporation. The particle size distribution of TMA in the test atmospheres was determined using a 11-stage (Institute's design) or a 10-stage Andersen cascade impactor (Andersen, Atlanta, USA). Due to the small sampling air flow rate (2-5 l/min) and the large total volume required for analytical and

particle size determinations (40-70 l/sample), samples were not collected during the challenge exposure but immediately prior to or after challenge. The mass median aerodynamic diameter (MMAD) of the aerosolised TMA particles was on average 1.0±0.2 µm with a mean geometric standard deviation (gsd) of 2.1±0.8.

Respiratory challenge and lung function measurements

For respiratory challenge and measurement of changes in respiration, rats were treated as described previously (Arts et al., 1998). Using this experimental set up, two TMA-sensitised and two vehicle-treated rats at a time, were first exposed to fresh air for 20 to 40 min (pre-challenge period) and then to the TMA or vehicle atmosphere for exactly 7 min (challenge period). Respiration (breathing frequency and pattern) was monitored semi-continuously by means of recording the pressure signal during approximately 20 sec at each measurement, i.e. a few min prior to the actual challenge, during challenge, and within one hour after challenge. Respiration was also occasionally monitored 3h, 6h and/or 24h after challenge.
To determine the degree of respiratory distress, the gross respiratory response (GRR) was assessed according to Ritz et al. (1993) based on the number of normal breaths between retractions (in our study indicated by the irregularly lengthened pauses) relatively to normal breathing. The minimum score according to this classification is 0.0 (no retractions), the maximum score 7.0 (anaphylactic shock).
Lung function was assessed using a two-chamber whole-body plethysmograph as described by Arts et al. (1998). Each animal was placed into this set-up at least 20 min before challenge, remained there until one hour after challenge, and was replaced for short periods of time 3h, 6h and/or 24 h after challenge. Breathing parameters (frequency, tidal volume and minute volume) and breathing pattern were determined semi-continuously at all time periods (including challenge) by means of recording the pressure signal in the volume-calibrated body chamber during approximately 20 sec at each measurement. Lung mechanical properties (airway resistance, compliance and inertance) as measured by the determination of the transfer impedance (Oostveen et al., 1992) were obtained at about the same time periods (except during challenge when the mesh wire resistance had to be removed).

In vivo non-specific airway responsiveness

In vivo non-specific airway responsiveness (AHR) to methacholine was evaluated one day after airway challenge in unrestrained animals using a ventilated bias flow whole-body plethysmograph (BUXCO Electronics, Sharon CT, USA) as described by Hamelmann et al. (1997). Bronchoconstriction is known to induce changes in breathing pattern and is reflected 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 (Hamelmann et al., 1997). Each animal was placed in a small wire mesh cage (20x11x9 cm), which in turn was placed into the animal chamber, to avoid disturbance of measurements due to the extensive mobility of the rats (Michielsen et al., 1999b). Measurements were started by recording of baseline Penh values during 3 min. Then aerosols of saline and 2-fold increasing concentrations of methacholine chloride in saline (0.2 – 50 mg/ml), produced by an ultrasonic nebulizer, were given for 3 min to record breathing for 3 min thereafter. Between aerosols normal air was provided for at least 15 min. In several rats the full range of concentrations of methacholine was tested, but rats showing severe respiratory distress or abnormal behaviour (see below) already at lower concentrations were not tested further to prevent further animal discomfort. The PC200 value (i.e. the concentration of methacholine corresponding to a 100% increase in Penh as compared to the Penh of aerosolised saline) was calculated to quantify airway reactivity. In addition, airway reactivity was assessed by inspection of the recorded breathing patterns (presence of apnoeic periods). Further, animals were observed for presence of

hunched posture, salivation, dark eyes and exophthalmus.

Local effects, body and organ weights, and necropsy
All animals were observed at least once daily and weighed shortly before the first chemical application, at weekly intervals thereafter and just prior to necropsy. At necropsy, animals were anaesthetised with ether, killed by exsanguination from the abdominal aorta and examined grossly for abnormalities. Liver, kidneys, spleen, and lungs with trachea and larynx were removed and weighed.

Airway histopathology
Neutral, phosphate-buffered 4% (v/v) formaldehyde was used to inflate and preserve the lungs and to preserve the larynx and trachea. Next these organs were embedded in paraffin wax. The larynx was sectioned transversally at three levels, i.e. at the base of the epiglottis, at the level of the ventral diverticulum and the arythenoid projections, and between the caudal part of the larynx and the first tracheal ring. The cranial part of the trachea was sectioned transversally, the caudal part longitudinally and included the bifurcation and the two mainstem bronchi. Lung lobes were cut at one sagittal level. Sections (5 µm) were stained with haematoxylin and eosin and examined microscopically under code.

Bronchoalveolar lavage
Animals were anaesthetised with Nembutal followed by exsanguination from the abdominal aorta. After binding of the left lobe, which was used for histopathological evaluation (see above), the right lung lobes were lavaged according to Hooftman et al. (1988) two times with a volume of 23 ml saline per kg bw. The total amount of retracted lavage fluid was weighed and retained on ice. The bronchoalveolar cells were isolated from the supernatant by centrifugation (250 g) during 5 minutes at 4°C and resuspended in 0.5 ml saline to assess total cell and differential cell numbers. Total cell numbers were counted using an automated haematology analyser (K-800, Sysmex, Toa, Kobe, Japan). The percentage of viable cells was determined using an acridine orange/ethidium bromide staining method in combination with fluorescence microscopic evaluation. For differential cell counts, cytospins were prepared and stained with May-Grunwald Giemsa. At least 400 cells were counted per animal to determine absolute numbers and percentages of macrophages/monocytes, lymphocytes, neutrophils and eosinophils. Supernatants were used for determination of total protein (Lowry et al., 1951), lactate dehydrogenase (LDH) and N-acetyl glucosaminidase (NAG), using an automatic analyser (Hitachi 911, Hitachi Instruments Division, Japan).

Isometric *in vitro* airway responsiveness
Animals received a lethal dose of Euthesate (0.6 kg/bw ip). When involuntary spasms had disappeared, the thorax was opened and the animals were exsanguinated by cardiac puncture. Then tracheas were dissected in toto, freed of connective tissue and blood vessels, and perfused in an organ bath containing Krebs-bicarbonate solution (in mM: NaCl, 118.1; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.2; NaHCO3, 25.0; KH2PO4, 1.2; glucose, 8.3) according to the method of Pavlovic et al. (1989) and modified by Garssen et al. (1990). Two hooks were inserted through opposite sides of the tracheal wall so to include the intact smooth muscles. One hook was attached to a fixed point in the organ bath, the other hook was connected to an isometric transducer (Harvard Bioscience, Kent, UK). The tracheal tension was set at an optimal counter weight of 2000 mgf. The inside of the trachea was perfused (2 ml/min) with the same Krebs solution by means of a peristaltic pump. The Krebs solution was kept at 37°C and continuously gassed with 5% CO2 in O2. Every 15 min the buffer was refreshed on both sides until a stable tone was reached (usually within 60 min). After equilibration, contractile

reactivity, expressed as changes in mg force, was measured by sequential application of increasing concentrations of methacholine chloride (10^{-8} to 10^{-3} M) via the luminal Krebs buffer. After these measurements, tracheas were processed for histopathological evaluation (see above). The same was done with larynges and lungs immediately after isolation of the tracheas.

Statistics

Body weights were analysed by one-way analysis of covariance (COVAR) followed by the two-sided Dunnett's multiple comparison test. Organ weights, absolute cell counts and biochemical parameters in BAL fluid were determined by ANOVA-DUNNETT. Relative cell numbers in BAL fluid were analysed by Kruskal-Wallis non-parametric analysis of variance followed by the Mann/Whitney U-test. Data on total serum IgE concentrations were analysed by ANOVA followed by pairwise t-tests. The lung function parameters were analysed by comparing the differences in challenge/pre-challenge and post-challenge/pre-challenge values between test and control groups using ANOVA followed by pairwise t-tests. *In vivo* airway hyperreactivity data, i.e. PC200 values and visual scores of control and test rats, were analysed by the non-parametric Pearson Chi-squared test. The dose-response curves of the *in vitro* trachea hyperresponsiveness of control and test rats were fit according to the equation:

$$y = d + \frac{ax^c}{b + x^c}$$

which was the best among several alternatives. Next, the parameters a, b, c, and d were statistically analysed between test and control group rats using the two-sample t-test.

Results

Local effects, body and organ weights

Flank application of TMA caused local dermal erythema, scaliness and/or encrustations in 50% of the BN rats two to three days after application. Ear application of TMA caused no grossly visible local reactions. TMA did not change the weights of body, kidney, liver, and spleen in both strains as compared to vehicle (-/-) treatment (data not shown). Likewise, TMA treatment did not change lung weights in both strains at all times, with the exception of the (+/+) BN rats 24h after TMA challenge. These animals showed an increase in absolute and relative lung weight (Table 1).

Serum IgE levels

Pre-treatment total serum IgE levels of 44 of the 46 BN rats measured ranged from 0.08 to 1.41 µg/ml (overall mean 0.39 ± 0.29 µg/ml). The two remaining animals showed very high total IgE levels (i.e. 2.8 and 3.7 µg/ml) and were left out for statistical analysis. This high total IgE levels may point to the presence of pre-existing inflammation (Germann et al., 1998), indicating that pre-bleed of animals may be needed to meaningfully evaluate the results.

Table 1 – Lung weights of BN rats after dermal sensitisation and subsequent inhalation challenge with TMA

Treatment	Time of necropsy after challenge	Lung weight	
		Absolute (g)	Relative (g/kg)
-/+	5 hours	1.37 ± 0.09	8.59 ± 0.46
+/+		1.36 ± 0.04	8.39 ± 0.44
-/-	24 hours	1.27 ± 0.04	8.47 ± 0.35
+/-		1.14 ± 0.02	7.79 ± 0.14
-/+		1.27 ± 0.05	8.32 ± 0.43
+/+		1.71 ± 0.07 * *	11.46 ± 0.42 * *
-/-	14 days	1.45 ± 0.03	8.45 ± 0.31
+/-		1.30 ± 0.06	7.13 ± 0.22 * *
-/+		1.32 ± 0.07	7.58 ± 0.26
+/+		1.48 ± 0.05	7.47 ± 0.51

Groups of 4 or 6 rats received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. Animals were necropsied 5h, 24h or 14 days after challenge with either TMA (49-53 mg/m^3) or vehicle (acetone). Treatment: vehicle-treated and –challenged rats (-/-), TMA-sensitised and vehicle-challenged rats (+/-), vehicle-treated and TMA-challenged rats (-/+), and TMA-sensitised and –challenged rats (+/+). Data have been expressed as means ± SEM. Statistics: ANOVA/t-test, ** $p<0.01$

In (-/- and -/+) control rats, the total IgE levels remained low (viz. mean levels of 0.43-0.49 µg/ml), whereas TMA sensitisation (+/- and +/+ groups) caused a highly significant increase in total IgE levels in two separate experiments (Table 2; Study I and II). Highly significantly increased total serum IgE levels when compared to controls were also observed in two other studies (Table 2; Study III and IV).

In order to confirm the presence of TMA-specific IgE antibodies, several representative sera were also tested in a PCA assay (Table 3). All sera tested from unsensitised (-/- or -/+) control BN rats were negative in the PCA assay, whereas all

Table 2 – Total serum IgE levels in BN rats after sensitisation with either vehicle or TMA

Study	Treatment	IgE levels (µg/ml)		
		before sensitisation	before challenge	24h after challenge
I	-/- or -/+	0.38 ± 0.07	0.49 ± 0.07	0.43 ± 0.08
	+/- or +/+	0.29 ± 0.03	5.54 ± 0.47 * * *	4.51 ± 0.36 * * *
II	-/+	0.39 ± 0.10	0.48 ± 0.13	nd
	+/+	0.78 ± 0.20	4.05 ± 0.08 * *	nd
III	-/- or -/+	nd	nd	0.99 ± 0.09
	+/- or +/+	nd	nd	3.94 ± 0.43 * * *
IV	-/- or -/+	nd	0.94 ± 0.17	nd
	+/- or +/+	nd	4.33 ± 0.43 * * *	nd

Groups of 15-16 rats (studies I and III) or 6 rats (studies II and IV) received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. Blood was sampled by orbital puncture before the first sensitisation, the day before challenge, or from the abdominal aorta just before necropsy 24h after challenge, and serum was analysed for total IgE concentrations. Treatment: vehicle groups (vehicle-treated and –challenged (-/-) rats and vehicle-treated and TMA-challenged (-/+) rats), and TMA sensitised groups (TMA-sensitised and vehicle-challenged (+/-) rats and TMA-sensitised and –challenged (+/+) rats. nd = not determined. Data have been expressed as means ± SEM. Statistics: ANOVA/t-test, ** $p<0.01$, *** $p<0.001$

sera tested from sensitised (+/- or +/+) BN rats showed positive PCA results, i.e. presence of TMA-specific IgE antibodies, using both naive BN and Wistar rats as recipients. There was, however, no clear association between specific and total IgE (Table 3).

Table 3 – Comparison of PCA and ELISA results of representative sera from BN rats after sensitisation with either vehicle or TMA

Treatment	BN *		Wistar *	Total serum IgE (µg/ml)
	TMA-HSA spot Ø (mm)	TMA-BSA spot Ø (mm)	TMA-BSA spot Ø (mm)	
Vehicle	0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0		0.94 0.75 0.85 1.11 0.52 0.59 1.29 0.40
TMA	15 13 15 17 19 16 17 14	16 16 13 15 14 15 12 15		6.18 2.36 4.52 5.00 6.14 2.80 3.68 7.17
TMA			12 14 10 11	4.39 5.43 2.58 4.84

BN rats received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. Blood was sampled from the abdominal aorta 2 weeks after the last sensitisation, and serum was analysed for total serum IgE levels (ELISA) and presence of TMA-specific IgE antibodies (PCA) using either TMA-HSA or TMA-BSA and using either naive BN or Wistar rats (*) as recipients.

Respiratory changes

Breathing frequencies of vehicle-treated (-/-) or TMA-sensitised (+/-) control BN rats were comparable and appeared unchanged by challenge with acetone. Neither did breathing frequency change in vehicle-treated, TMA-challenged (-/+) control BN rats. In contrast, TMA challenge of sensitised (+/+) BN rats decreased mean breathing frequencies to 50% of the original rate starting within 1 or 2 minutes of exposure (Table 4; Figure 1a).

Table 4 - Changes in breathing frequency of BN rats after dermal sensitisation and subsequent inhalation challenge with TMA or vehicle

Study	Treatment	Breathing frequency (s^{-1})		
		before challenge	during challenge	after challenge
I	-/-	2.68 ± 0.04	2.39 ± 0.13	nm
	+/-	2.86 ± 0.29	2.37 ± 0.23	nm
	-/+	2.87 ± 0.13	2.81 ± 0.30	nm
	+/+	3.04 ± 0.11	1.52 ± 0.08 * * *	nm
II	-/+	2.67 ± 0.18	3.10 ± 0.22	3.06 ± 0.12
	+/+	2.69 ± 0.14	2.05 ± 0.06 * *	2.80 ± 0.11
III	-/-	3.03 ± 0.21	2.70 ± 0.19	3.11 ± 0.21
	+/-	2.96 ± 0.08	2.38 ± 0.16	2.72 ± 0.07
	-/+	3.47 ± 0.18	3.10 ± 0.18	3.62 ± 0.13
	+/+	3.33 ± 0.22	1.93 ± 0.14 * *	3.13 ± 0.22
IV	-/-	2.79 ± 0.11	2.54 ± 0.09	2.60 ± 0.12
	+/-	2.77 ± 0.14	2.56 ± 0.10	2.50 ± 0.12
	-/+	2.93 ± 0.18	2.65 ± 0.18	2.74 ± 0.15
	+/+	2.77 ± 0.23	1.57 ± 0.06 * * *	2.33 ± 0.13

Groups of 6 or 9 rats each received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. On day 21 animals were challenged by inhalation exposure to either TMA (45-54 mg/m^3) or vehicle (acetone). Breathing frequency was measured each minute for about 20 sec, starting a few min prior to the actual challenge (means of 3-12 pre-challenge values). During challenge, respiration was either monitored each min for about 20 sec or continuously (means of 7-22 challenge values). Respiration was also measured within one hour after challenge (14-30 post-challenge values). Treatment: vehicle-treated and –challenged rats (-/-), TMA-sensitised and vehicle-challenged rats (+/-), vehicle-treated and TMA-challenged rats (-/+), and TMA-sensitised and –challenged rats (+/+). Data have been expressed as means ± SEM, nm=not measured. Statistics: ANOVA/t-test on differences in challenge/pre-challenge and post-challenge/pre-challenge values between the groups using the (-/-) group (studies I, III, IV) or the (-/+) group (study II) as control group; ** $p<0.01$, *** $p<0.001$

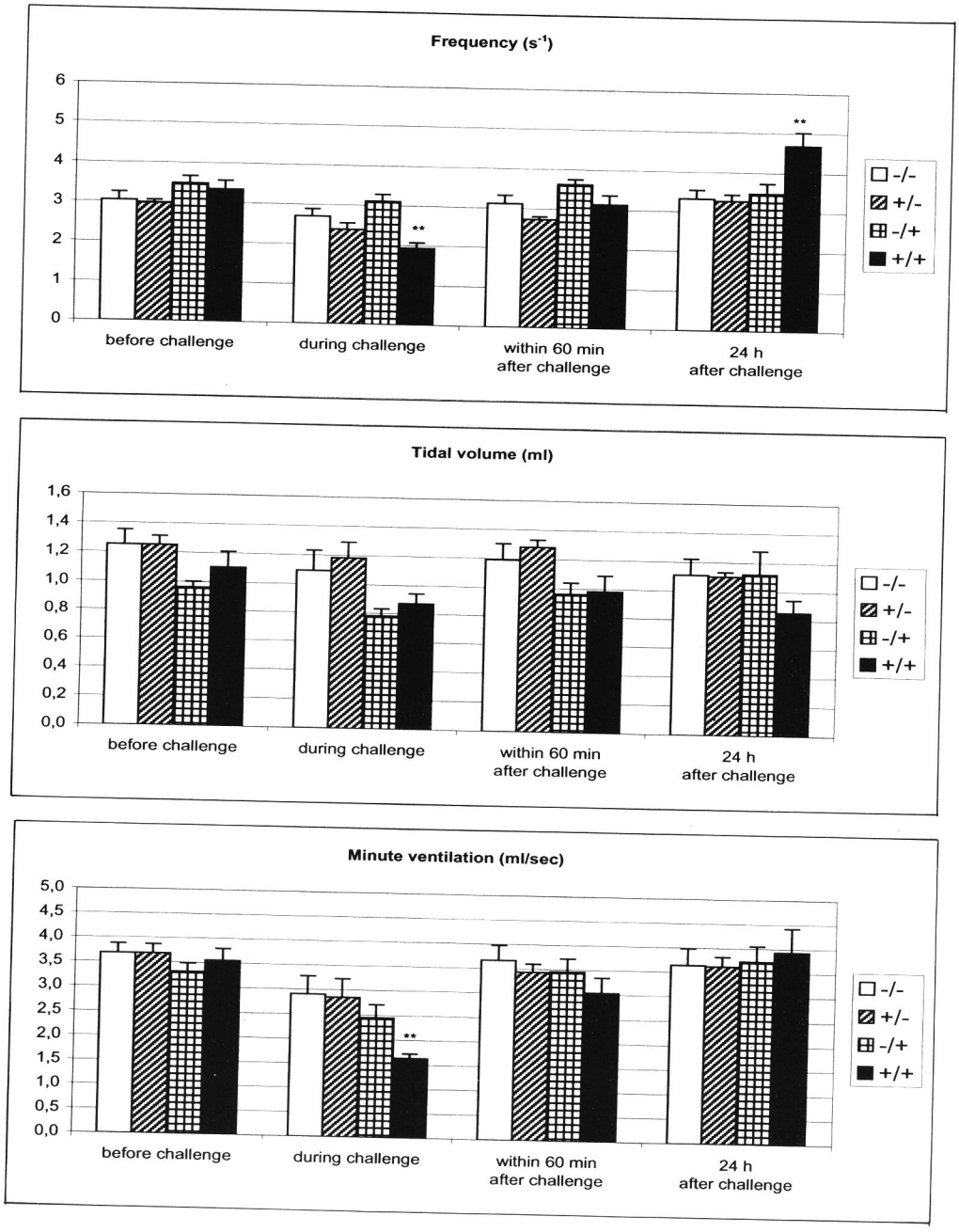

Figure 1
Mean relative changes in respiratory frequency, tidal volume and minute ventilation in groups of 6 BN rats after dermal sensitisation on the shaved flanks (day 0) with 50% TMA (w/v) followed by 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. On day 21 animals were challenged by inhalation exposure to either 49 mg/m^3 TMA or vehicle (acetone). Treatment: vehicle-treated and –challenged rats (-/-), TMA-sensitised and vehicle-challenged rats (+/-), vehicle-treated and TMA-challenged rats (-/+), and TMA-sensitised and –challenged rats (+/+). Statistics: ANOVA/t-test on differences in challenge/pre-challenge and post-challenge/pre-challenge values using the (-/-) group as the control group; ** $p < 0.01$

Breathing patterns of control (-/- and +/-) BN rats during acetone challenge appeared normal with a few slight irregularities. Breathing pattern of a few vehicle-treated, TMA-challenged (-/+) controls had changed. The pattern had a spiked form instead of a waveform, and there was a slight increase in the pause between breaths at the end of expiration (at peak expiratory pressure). Further, the time of inspiration and expiration appeared to be slightly decreased (Figure 2a).

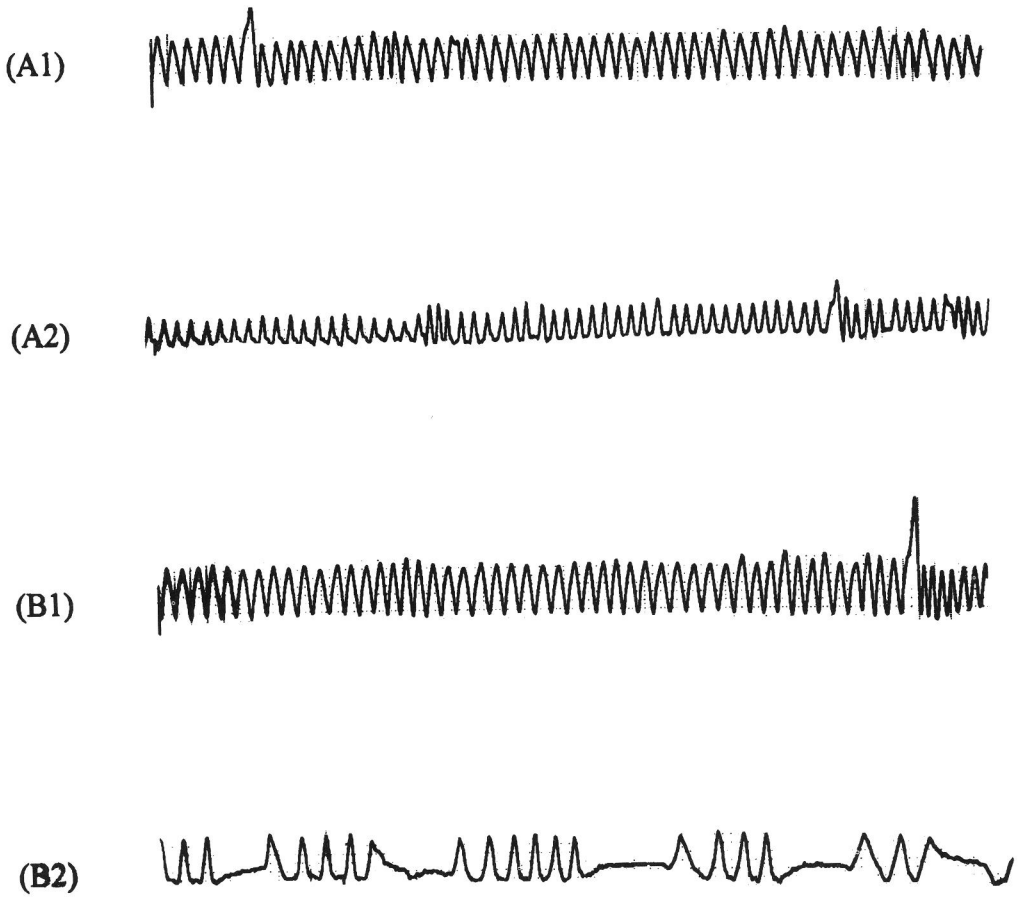

Figure 2
Representative breathing patterns of BN rats before and during challenge exposure to 45 mg/m^3 TMA. (A1) non-sensitised rat, pre-exposure; (A2) non-sensitised rat, during challenge; (B1) sensitised rat, pre-exposure; (B2) sensitised rat, during challenge

In contrast, increases in inspiratory and expiratory time and irregularly lengthened pauses (apnoeas) between a varying number of breaths were seen in all TMA-sensitised and –challenged (+/+) rats (Figure 2b). In one out of four experiments challenge of (+/+) rats reduced the breathing rate less drastically, although the breathing pattern resembled that of the corresponding groups.

Respiratory distress in TMA-sensitised and –challenged (+/+) BN rats was moderate regarding the obtained gross respiratory response (GRR) score (Ritz et al., 1993) of 4.0±0.6, corresponding to a mean of 6-15 respirations between each pause. Rats of the control groups (-/-, +/- and -/+) had no retractions and had, therefore, a GRR score of 0.0.

Recovery of BN rats after TMA challenge differed. In the vehicle (-/+) group, changes in breathing pattern had almost completely returned to normal immediately after challenge, whereas in TMA-sensitised (+/+) BN rats divergent recovery times were noted. A few animals resumed a normal breathing pattern about 10 min after challenge, whereas in some other animals the breathing pattern was still slightly abnormal by 1h after challenge.

Tidal volume and minute ventilation did not change during and after challenge of animals of the three control groups (-/-, +/-, and -/+) when compared to pre-challenge, whereas the minute ventilation was significantly reduced during TMA challenge of the sensitised (+/+) BN rats. This decrease was mainly caused by a decrease in breathing frequency, because tidal volume was hardly reduced (Figure 1b). The breathing parameters appeared normal at 3h and 6h after challenge (data not shown), but at 24h, although the minute ventilation had not changed, the tidal volume decreased and breathing frequency increased (Figure 1c). Airway resistance, compliance and inertance assessed within one hour after TMA challenge, and 3h, and 6h or 24h thereafter did not show significant changes as compared to pre-challenge values in all groups (data not shown).

In vivo non-specific airway responsiveness

TMA-challenge of sensitised (+/+) BN rats resulted in cholinergic hyperresponsiveness 24h after challenge as indicated by the on average about 4 times lower concentration of methacholine needed to double the Penh value as compared to the (-/+) control group (Figure 3). In the latter group, however, two animals also showed increased AHR at low methacholine concentrations, whereas one animal of the sensitised group showed AHR at a relative high methacholine concentration.

The concentrations causing clear signs of respiratory difficulty as indicated by apnoeic periods and/or visual distress were even more indicative of hyperresponsiveness, since on average 6 times less methacholine was needed to provoke these effects in the sensitised (+/+) group as compared to the unsensitised (-/+) control group (Figure 3).

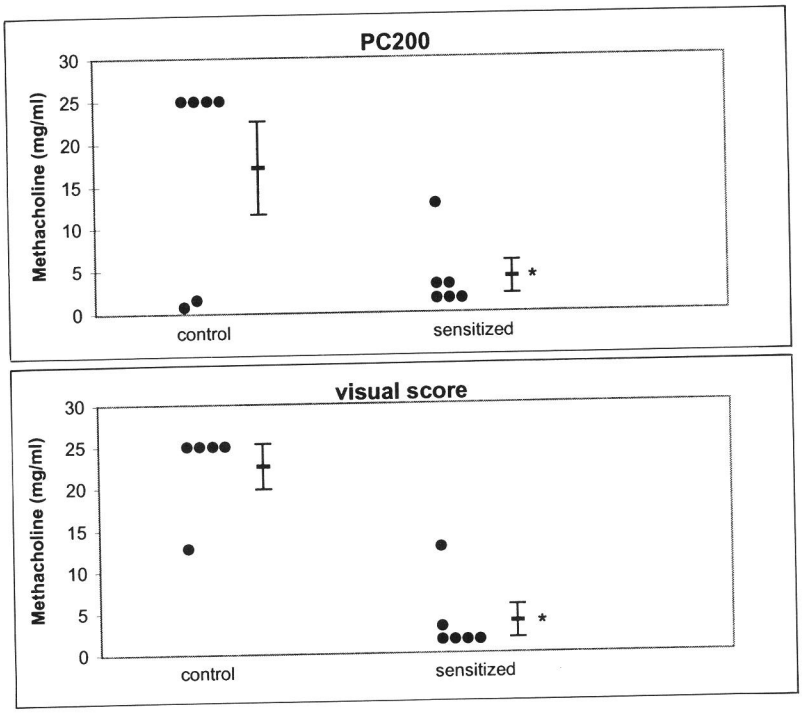

Figure 3
Non-specific hyperresponsiveness (AHR) in vivo. A group of 6 BN rats received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. The control group of 6 rats received vehicle (AOO) only. Both groups were challenged on day 21 by inhalation exposure to 46 mg/m^3 TMA. Animals were exposed to increasing concentrations of methacholine. Airways hyperresponsiveness was expressed as the concentration of methacholine that induced an increase of 100% of aerosolised saline Penh values (PC200), and as the concentration of methacholine that induced visible signs of respiratory distress and/or other symptoms (hunched posture, salivation, dark coloured eyes, and exophthalmus (visual score). Each circle represents one rat; horizontal and vertical bars represent groups mean and SEM, respectively. One control animal was accidentally not visually examined. Statistics: Pearson Chi-squared test; * $p<0.05$

Isometric *in vitro* airway responsiveness

Perfusion of increasing concentrations of methacholine through the tracheas of control (-/+) BN rats resulted in a concentration-dependent contraction of tracheal smooth muscles to an average maximum (Emax) of 900 mgf. The tracheas of TMA-sensitised and -challenged (+/+) rats showed significantly increased reactivity to methacholine as compared to controls, reaching a significantly increased mean Emax value of 1450 mgf, i.e. an increase of 60% (Figure 4). EC50 values were comparable for both groups, i.e. $1.9*10^{-6}$ and $2.5*10^{-6}$ M for the (-/+) control and TMA-sensitised (+/+) group, respectively.

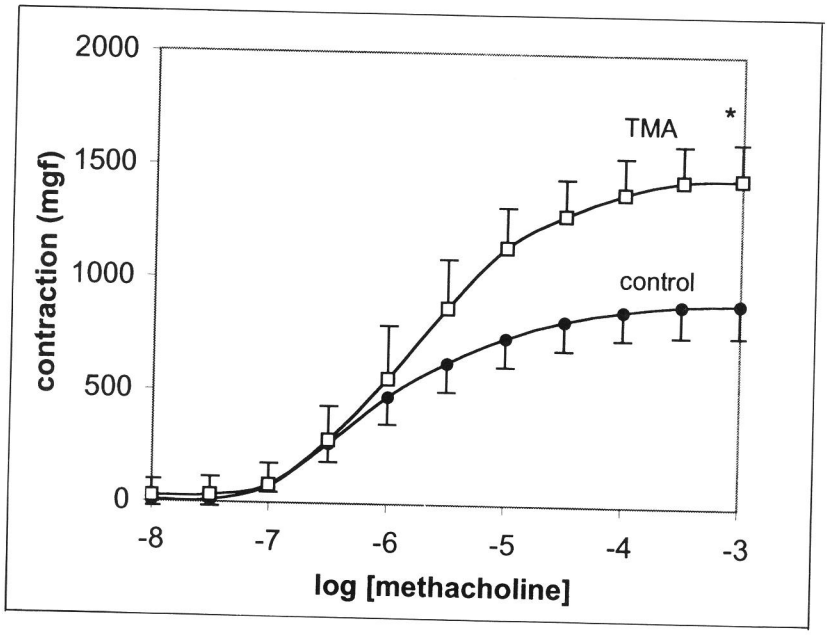

Figure 4
Non-specific airway hyperresponsiveness (AHR) *in vitro*. A group of 5 BN rats received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. The control group of 5 rats received vehicle (AOO) only. Both groups were challenged on day 21 by inhalation exposure to 54 mg/m^3 TMA. Animals were killed 24h after challenge and tracheas were removed for measurement of tracheal responsiveness to cumulative concentrations of methacholine (ranging from 10^{-8} to 10^{-3} M) in a perfused organ bath set up. Results are expressed as means ± SEM. Statistics: two-sample t-test on the best fit of the two dose-response curves; * $p<0.05$

Bronchoalveolar lavage

Total and differential cell counts and levels of total protein, LDH and NAG in BAL were similar in the three (-/-, +/- and -/+) BN control groups. In contrast, highly increased numbers of eosinophils, neutrophils, and high levels of total protein, LDH and NAG were found upon TMA-challenge of sensitised (+/+) BN rats while total cell number and the number of lymphocytes tended to increase (Figures 5 and 6).

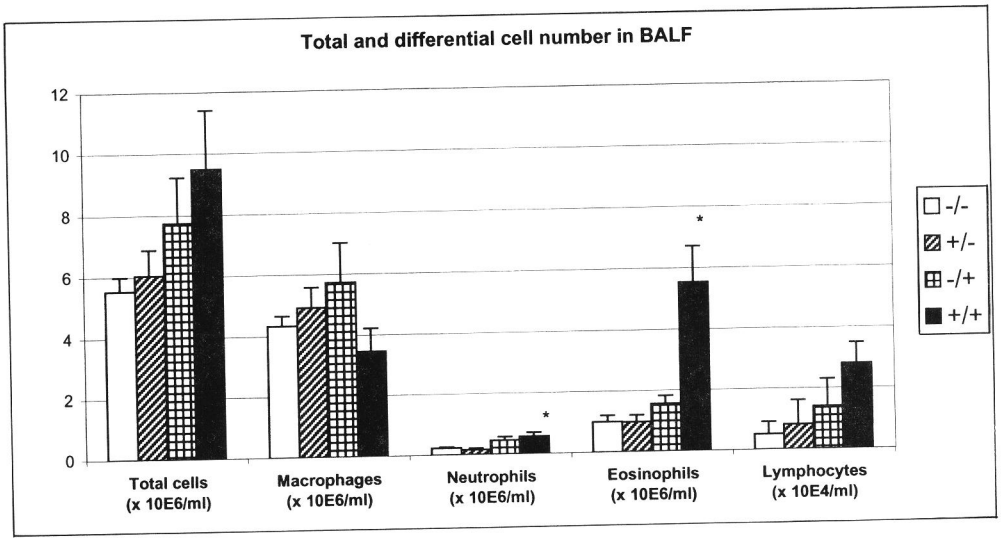

Figure 5
Total cell number and total numbers of alveolar macrophages/monocytes, neutrophils, eosinophils and lymphocytes in BAL fluid (BALF) of BN rats after dermal sensitisation and subsequent inhalation challenge with TMA or vehicle. Groups of 6 rats each received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. On day 21 animals were challenged by inhalation exposure to either 45 mg/m^3 TMA or vehicle (acetone). One day later the animals were sacrificed and their lungs were lavaged. Treatment: vehicle-treated and –challenged rats (-/-), TMA-sensitised and vehicle-challenged rats (+/-), vehicle-treated and TMA-challenged rats (-/+), and TMA-sensitised and –challenged rats (+/+). Data have been expressed as means ± SEM. Statistics: ANOVA/Dunnett's test, * $p<0.05$

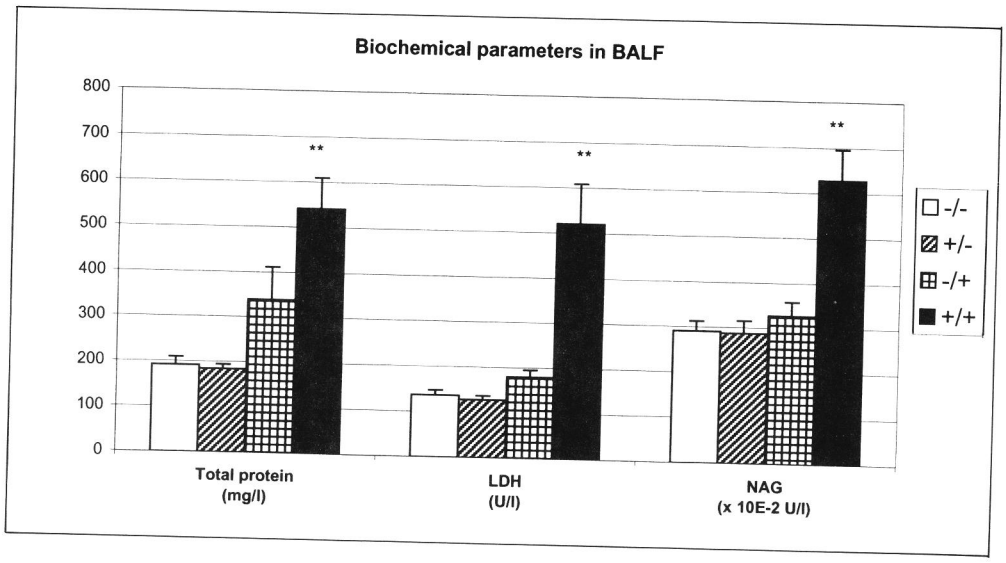

Figure 6
Biochemical parameters of airway damage (total protein, lactate dehydrogenase (LDH) and N-acetyl-glucosaminidase (NAG)) in BAL fluid (BALF) of BN rats after dermal sensitisation and subsequent inhalation challenge with TMA or vehicle. Groups of 6 rats each received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. On day 21 animals were challenged by inhalation exposure to either 45 mg/m^3 TMA or vehicle (acetone). One day later the animals were sacrificed and their lungs were lavaged. Treatment: vehicle-treated and –challenged rats (-/-), TMA-sensitised and vehicle-challenged rats (+/-), vehicle-treated and TMA-challenged rats (-/+), and TMA-sensitised and –challenged rats (+/+). Data have been expressed as means ± SEM. Statistics: ANOVA/Dunnett's test, ** $p<0.01$

Histopathological airway changes

Control (-/-, +/-, and -/+) BN rats had a normal histology of the larynx with the exception of one animal of the -/+ group that showed a very slight laryngitis consisting of some granulocytes and lymphocytes scattered in the lamina propria. TMA-sensitised and –challenged (+/+) BN rats, in contrast, showed moderate or severe laryngitis consisting of subepithelial aggregates of predominantly macrophages with varying numbers of granulocytes including some eosinophils and lymphocytes/monocytes (Table 5).

Table 5 – Histopathology of the larynx and lungs of BN rats necropsied 24h after TMA challenge

	Treatment			
	-/-	+/-	-/+	+/+
LARYNX				
Number of animals with laryngitis Very slight Moderate Severe	0 0 0	0 0 0	1 0 0	0 2 4
LUNGS				
Number of animals with increased eosinophilia Slight Moderate	2 0	1 0	0 1	3 3
Number of animals with goblet cell hyperplasia Slight Moderate	0 2	1 0	1 1	2 4
Number of animals with haemorrhages# 1 haemorrhage 2 haemorrhages >2 haemorrhages	2 2 0	2 0 0	0 2 0	0 0 6
Number of inflamed vessel transsections/ Number of uninflamed vessel transsections	0.9 ± 0.1	1.3 ± 0.4	0.8 ± 0.3	2.4 ± 0.4
Number of inflamed bronchioli transsections/ Number of uninflamed bronchioli transsections	1.9 ± 0.6	1.8 ± 0.4	0.4 ± 0.1	1.5 ± 0.3
Degree of granulomatous inflammation	1.6 ± 0.3	2.2 ± 0.2	2.3 ± 0.2	1.8 ± 0.3

Groups of 6 BN rats each received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. Animals were challenged on day 21 by inhalation to either 45 mg/m^3 TMA or vehicle (acetone). Animals were killed and airways were removed for examination 24h after challenge. Regarding the degree of pulmonary granulomatous inflammation, the sections were graded as follows: (0) no inflammation, (1) slight inflammation with scattered microgranulomas covering <5% of the lung section, (2) moderate inflammation with scattered microgranulomas covering >5% to <30% of the lung section, (3) severe inflammation with scattered microgranulomas covering >30% to <50% of the lung section. Treatment: vehicle-treated and –challenged rats (-/-), TMA-sensitised and vehicle-challenged rats (+/-), vehicle-treated and TMA-challenged rats (-/+), and TMA-sensitised and –challenged rats (+/+). Data have been expressed either as the number of animals showing an effect or as means ± SEM; # examined in main lung lobe.

The inflammation slightly protruded into the pharyngeal lumen. No treatment-related changes were observed in the trachea. Pulmonary inflammation was observed in animals of all groups. However, animals of the +/+ group showed a higher incidence of eosinophilic aggregates, more prominent goblet cell expression and a higher incidence and degree of haemorrhages when compared to the other three groups. In addition, the ratio of inflamed vessel transsections and uninflamed vessel transsections tended to be slightly higher in sensitised and challenged (+/+) animals when compared to the other groups (Table 5).

Discussion

In the present study we examined parameters of inflammation and lung function in high IgE-responding BN rats with the aim to investigate (1) whether respiratory challenge of TMA-sensitised animals with TMA induced non-specific airway hyperresponsiveness (AHR), a characteristic feature of asthma, and (2) whether allergen- and irritant-induced alterations by TMA could be distinguished.

Likewise in our previous studies (Arts et al., 1998), two one-week-spaced dermal applications of TMA elevated total serum IgE levels in BN rats by two weeks after the last application and inhalation challenge at that time resulted in an immediate reduction of the breathing frequency in sensitised (+/+) rats, but not in unsensitised (-/+) controls. In addition, airway reactions, increased lung weights and histopathological airway changes were observed 24h after TMA challenge.

Since two additional control groups included in the present study, i.e. vehicle-treated and -challenged (-/-) rats and TMA-sensitised but vehicle-challenged (+/-) rats, also did not display the above-mentioned changes in lung function and pathology, it can be concluded that prior sensitisation is required for the changes induced by TMA inhalation. Moreover, since sensitisation elevated levels of TMA-specific IgE, the immediate airway response to TMA challenge is probably mediated by specific IgE antibodies. Thus the response in BN rats resembles the early asthmatic response of IgE-mediated occupational asthma (Zeiss and Patterson, 1993). The resemblance of the BN model to occupational asthma is further indicated by amongst others the appearance of cholinergic hyperresponsiveness after TMA challenge of sensitised, but not controls.

In the case of occupational asthma induced by LMW allergens, however, it is mandatory to verify whether effects are not merely due to irritant properties of the chemical allergen. Respiratory challenge of control (-/+) BN rats with about 50 mg/m^3 caused short pauses between single, spike-formed breaths with slight decreases in the time of inspiration and

expiration. This breathing pattern was considered to be typical of pulmonary irritation (Schaper and Brost, 1991), and in view of the generated particle size, i.e. a mean MMAD value of 1 µm, lungs could indeed have been reached. However, the absence of significant changes in breathing frequency indicates that 50 mg/m^3 of TMA is only slightly irritating to the airways of vehicle-treated BN rats (Arts et al., 2001). In contrast, the breathing pattern of the sensitised (+/+) animals at the same challenge concentration was characterised by an increase in inspiratory and expiratory time and irregularly lengthened pauses (apnoeic periods) between a varying number of breaths, resulting in a decreased breathing frequency (Figure 2). Thus, although challenge with 50 mg/m^3 of TMA caused slight irritation, breathing patterns between the two groups (-/+ and +/+) BN rats differed to such an extent that sensitisation-induced changes could be clearly distinguished from irritation-induced ones.

Increases in numbers of eosinophils, neutrophils, and lymphocytes, as well as levels of total protein, LDH and NAG in bronchoalveolar lavage fluid upon TMA challenge also required prior sensitisation. However, the slight increases in some of the parameters upon TMA challenge of vehicle-treated control rats, may point at an additive role of irritation, known to increase most of these parameters as well (Pauluhn et al., 1999b; Swiercz et al., 2000).

The observed allergic asthma-associated pathology in TMA-sensitised and –challenged (+/+) BN rats, namely eosinophilic aggregates and goblet cell hyperplasia, confirmed the functional evidence of the disease, though some changes were also seen in the control groups albeit at a lower degree and incidence. Besides allergic asthma-associated pathology, a characteristic laryngitis was seen, a less frequently reported feature of occupational respiratory allergy (O'Hollaren, 1995; Sala et al., 1996). In addition, sensitisation-dependent pulmonary haemorrhages were observed 24h after TMA challenge as also noted earlier in a similar study 48h after challenge. These haemorrhages have been attributed to a combined type III/IV allergic reaction (Arts et al., 1998). A difference with the previous study, however, is that lungs also showed moderate granulomatous inflammation irrespective of treatment, except for a slightly higher number of perivascular infiltrates in the +/+ rats. Inflammatory abnormalities in lungs of naive BN rats have been observed earlier by us and others (Arts et al., 1998; Germann et al., 1998; Michielsen et al., 1999ab) and attempts to identify an infectious agent have failed (Germann et al., 1998). 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. Notably, TMA-sensitised BN rats showed very consistent airway responses to TMA, i.e. in all sensitised rats of present and previous studies, decreases in breathing frequency were observed during

challenge with TMA. They probably respond in a more consistent way than sensitised guinea pigs that have been shown to react to TMA with increased or decreased breathing rates (Botham et al., 1989; Blaikie et al., 1995). This might be related to the abundance of bronchial smooth musculature and the switch of breathing pattern from nasal to oronasal breathing in the latter species when exposed to irritant concentrations (Dodd et al., 1986; Pauluhn et al., 1999a). Because of the uniformity of the response in BN rats, challenge-induced decreases in mean breathing frequency of groups can be compared and statistically evaluated. This is to be preferred to categorising challenge-induced responses in normal, moderate or severe as common practice with guinea pigs. In contrast, BN rats seem less appropriate for AHR measurements using the BUXCO equipment. During the course of the measurements, several BN rats developed a flat breathing pattern during TMA challenge and/or showed reactions already to saline. From these results, it could be questioned whether the BN rat is suitable to study this aspect of occupational asthma.

In identically treated low IgE-responding Wistar rats, increased total serum IgE levels and specific IgE antibodies, and specific and non-specific in vivo bronchoconstriction reactions were absent (data not shown). The absence of functional airway changes was in agreement with previous findings (Arts et al., 1998) and with findings in TMA-treated, low-IgE responding Sprague-Dawley rats (Leach et al., 1987).

Since TMA sensitisation of BN rats increased levels of total and TMA-specific IgE antibodies and TMA challenge caused respiratory reactions in TMA-sensitised BN rats only, it is supposed that the measurement of total serum IgE in high IgE-responding animals provides an appropriate means to assess respiratory sensitisation potential of LMW chemicals.

Overall, the responses of humans to inhaled TMA have been reproduced in the BN rat, i.e. immediate bronchoconstrictive reactions during challenge, a persistent effect on lung function, AHR, BAL eosinophilia, and airway inflammation. Moreover, allergic and irritant responses to TMA could be clearly distinguished on the basis of breathing patterns during exposure.

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Chapter 6

Respiratory irritation by trimellitic anhydride in Brown Norway and Wistar rats

Josje H.E. Arts, Martijn W. de Koning, Nanne Bloksma and C. Frieke Kuper

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RESPIRATORY IRRITATION BY TRIMELLITIC ANHYDRIDE IN BROWN NORWAY AND WISTAR RATS

Josje H. E. Arts

TNO Nutrition and Food Research, Zeist, The Netherlands

Martijn W. de Koning

TNO Nutrition and Food Research, Zeist, and Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

Nanne Bloksma

Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, and Faculty of Biology, Utrecht University, Utrecht, The Netherlands

C. Frieke Kuper

TNO Nutrition and Food Research, Zeist, The Netherlands

Several acid anhydrides are known for their sensitizing and irritative properties. Since both irritation and respiratory allergy can cause changes of lung function, proper testing of allergen-dependent effects on the respiratory tract requires knowledge of the respiratory irritant effects. To study the latter effects, groups of female Brown Norway (BN) and Wistar rats were exposed for 30 min to a range of concentrations (10 to 300 mg/m^3) of the well-known respiratory allergen trimellitic anhydride (TMA). Breathing pattern and frequency were monitored before, during, and after exposure. Animals were necropsied and lung weights were determined 1 day after exposure. In BN rats, changes in breathing pattern were seen at levels of 29 mg/m^3 and higher and decreases in frequency at 60 mg/m^3 and higher, whereas in Wistar rats changes in both pattern and frequency (increases followed by decreases) were seen at levels of 34 mg/m^3 and higher. Changes in breathing pattern consisted of a spiked form instead of a wave form of the respiratory cycle, with a pause between breaths at the end of expiration. The length of the pause increased with increasing concentrations of TMA while the duration of the respiratory cycle decreased slightly, implying that breathing frequency was mainly determined by the magnitude of the increase in pause. These reversible changes in breathing pattern and frequency were considered to be suggestive of lower airway irritation, rather than upper airway irritation. No concentration-related changes in lung weights were observed. The highest level at which no acute airway irritation as based on both breathing pattern and frequency was observed in both rat strains was 14 mg/m^3.

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Address correspondence to Josje H. E. Arts, MSc, TNO Nutrition and Food Research, Department of Target Organ Toxicology, PO Box 360, 3700 AJ Zeist, The Netherlands. E-mail: j.arts@voeding.tno.nl

Acid anhydrides are low-molecular-weight (LMW) chemicals that have been used in industry for more than 50 years as curing agents in the production of epoxy and alkyd resins and in the manufacture of the plasticizer dioctyl phthalate. Epoxy and alkyd resins have widespread applications in paints, plastics, and adhesives. Other applications are production of dyes, pesticides, pharmaceuticals, isolators, and thermostable polyvinyl chloride (PVC). The functional RCOOCOR′ group endows acid anhydrides with the reactivity needed for these applications, but this reactivity is most probably also responsible for the irritative and sensitizing properties of these LMW chemicals (Bernstein et al., 1984; Topping et al., 1986; Durham et al., 1987; Nielsen et al., 1988; Grammer et al., 1992; Zeiss et al., 1992; Liss et al., 1993; Drexler et al., 1994; Yokota et al., 1997; Barker et al., 1998). Trimellitic anhydride (TMA) is a model compound among the acid anhydrides. This compound, besides inducing immunoglobulin E (IgE)-mediated asthma and rhinitis, is known to cause two other immune-mediated syndromes and a nonimmune syndrome. The latter syndrome is clinically characterized by airflow obstruction as a result of toxic and irritative effects on the airway epithelium and is designated as reactive airways dysfunction syndrome (RADS; Zeiss & Patterson, 1993; Bernstein et al., 1993; Brooks & Bernstein, 1993). Since airflow obstruction is a key symptom of IgE-mediated asthma and since irritant concentrations of chemical allergens can cause such functional airway reactions in unsensitized animals (Karol, 1991; Briatico-Vangosa et al., 1994; Pauluhn & Mohr, 1994), a lung function parameter able to distinguish toxic and immune-mediated effects is desired for the assessment and control of health risks.

Since sensitized rats of the high IgE-responding Brown Norway (BN) and low IgE-responding Wistar strain have shown different allergic airway reactions upon challenge with TMA (Arts et al., 1998), the purpose of the present study was to evaluate the acute airway irritating effects of TMA in naive rats of these two strains.

METHODS

Animals and Maintenance

Female, 7- to 8-wk-old, inbred Brown Norway (BN/SsNHsd) rats were purchased from Harlan UK Ltd. (Blackthorn, UK), and random-bred Wistar rats (Crl:[WI]WUBR) of the same age were purchased from Charles River Deutschland (Sulzfeld, Germany). The animals were acclimatized for at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received the institute's grain-based open-formula diet and unfluoridated tap water ad libitum. All animal procedures were approved by the TNO Commission of Animal Welfare.

Materials

Trimellitic anhydride (TMA; purity 97%) was obtained from Aldrich (Brussels, Belgium). TMA solutions were prepared shortly before use by dissolving it in pesticide-grade acetone (Merck, Darmstadt, Germany).

Atmosphere Generation and Analysis

An all-glass nebulizer designed at the institute was used to generate the test atmospheres from the solutions of TMA in acetone (Schaper & Brost, 1991) or acetone alone. The acetone concentration in air was kept between 3000 and 5000 ppm (~7–12 g/m^3), levels that are considered to be far below a level inducing sensory irritation (Alarie, 1973; De Ceaurriz et al., 1981; Schaper & Brost, 1991). Atmospheric concentrations of TMA were determined gravimetrically by filter sampling and those of acetone by calculations based on the nominal concentration and the complete evaporation. The particle size distribution of TMA in the test atmospheres was determined using a 10-stage Andersen cascade impactor (Andersen, Atlanta, GA). Due to the low sampling airflow rate (2–5 L/min) and the large total volume required for analytical and particle size determinations (up to 500 L/sample), samples were not collected during exposure but prior to or after exposure. The mass median aerodynamic diameter (MMAD) of the TMA aerosol was between 0.5 and 2.2 μm (Table 1).

Study Conduct

Animals were weighed shortly before exposure and randomly assigned to a group (n = 4) receiving a given concentration of TMA. The rats were individually restrained in Battelle tubes, and each tube was then placed into one of four plethysmographs that were connected to a central exposure chamber for nose-only exposure to the test atmosphere. Each plethysmograph was provided with a pressure transducer that sensed changes created by inspiration and expiration and transmitted amplified signals to a polygraph recorder, allowing determination of respiratory frequency and pattern during challenge. Using this experimental setup, 4 rats at a time were subsequently exposed to fresh air for 30 min (preexposure period), to the appropriate TMA concentration or vehicle atmosphere for 30 min (exposure period), and again to fresh air for 30 min (recovery period). Respiration was monitored for 20-s periods in the preexposure period (at 1-min intervals

TABLE 1. Particle size distribution in TMA aerosols

Concentration (mg/m^3)	MMAD (μm)	GSD
10	0.50	2.0
30	0.90	2.8
	1.00	2.6
50	0.90	1.9
100	2.00	1.9
	1.70	2.0
200	2.00	1.9
	2.20	1.7

Note. MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation, $(84\%/16\%)^{1/2}$.

starting 6 min prior to the TMA exposure), during the exposure period (at 1-min intervals during the first 5 min and then at 2-min intervals), and during the recovery period (at 1-min intervals during the first 10 min and then at 3-min intervals). Respiratory frequency was expressed as the number of breaths per second.

At necropsy, the day after exposure, the animals were weighed, anesthetized, killed by exsanguination from the abdominal aorta, and examined grossly for pathological changes. Lungs with trachea and larynx were removed and absolute and relative (relative to body weight) lung weights were determined.

Data Handling and Statistics

For each animal, the maximum changes in breathing frequency during exposure—that is, at those points where the plateau value was reached in the time–response curve—were used to calculate the mean value. Mean pre- and postexposure (recovery) values were obtained by averaging the data obtained. Thus means of frequencies were calculated from 6 preexposure (PRE), 6–17 exposure (EXP), and 13 recovery (REC) values. To reduce variability, each animal was used as its own control. Hence, concentration effects were evaluated by a repeated-measures two-way analysis of variance (ANOVA). Significant effects were subsequently evaluated post hoc by one-way analysis of variance (ANOVA) of the relative changes, that is, (PRE – EXP)/(PRE) and (PRE – REC)/(PRE), followed by the Dunnett test to evaluate individual contrasts. The RD50 value, the statistically derived concentration at which animals are breathing at 50% of their original frequency, was assessed according to Alarie (1973). In addition, the RD50 value was calculated according to a method described by Bos et al. (1992), based on the reaction dynamics at the receptor sites and assuming that the maximal response is 100%, using the equation:

$$\log \mathrm{RD50} = \log C + \log [(100 - R)/R]$$

where C is the concentration (mg/m^3) and R the response (%). Lung weights were analyzed by one-way ANOVA followed by the Dunnett test.

RESULTS

Respiratory Changes

Control BN rats exposed to the vehicle (acetone) did not show changes in breathing frequency when compared to exposure to normal air. BN rats exposed to 14 or 29 mg/m^3 of TMA showed normal breathing frequency as well. At 60 mg/m^3, 2 out of 4 animals had a decrease in breathing frequency of 24 and 29%, respectively, whereas 1 other rat breathed at a slightly increased rate. The mean breathing frequency of the group, however, was not different from that of the controls. At TMA levels of 100 mg/m^3

and higher, significant reductions in breathing frequency were observed in all animals (Figure 1). The changes in frequency started rapidly upon exposure to TMA, and plateau values were reached within 30 min of exposure.

Changes in breathing pattern of the BN rats were observed at TMA concentrations of 29 mg/m^3 and higher. They were characterized by (1) a slight decrease in the duration of inspiration and expiration and (2) a spiked instead of a wave form of the respiratory cycle with, at the end of expiration (at peak expiratory pressure), a pause between breaths (Figure 2a). The length of these pauses increased with increasing exposure concentrations of TMA. Like the changes in frequency, changes in breathing pattern started rapidly upon exposure to TMA.

Breathing frequency and/or pattern almost completely returned to normal immediately after exposure at levels of 100 mg/m^3 and below, whereas at levels of 100 mg/m^3 and higher the whole recovery period was required to attain normal breathing, resulting in a significantly reduced frequency relative to preexposure values at these concentration levels (data not shown).

The RD50 value (Alarie, 1973) for BN rats was 260 mg/m^3, with 95% confidence limits of 185 and 441 mg/m^3 and a correlation coefficient of 0.905. Calculation of the RD50 value according to Bos et al. (1992) resulted in a slightly higher mean RD50 value (±SD) of 312 (±109) mg/m^3.

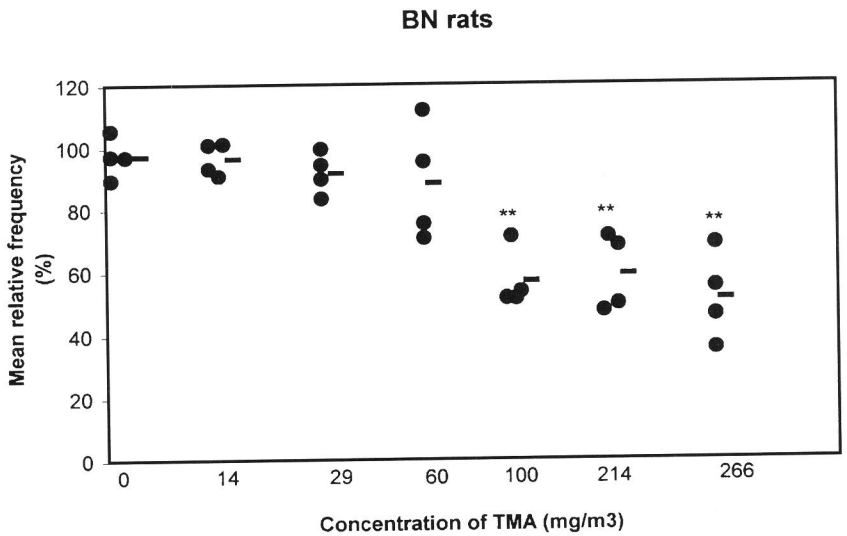

FIGURE 1. Mean relative changes in breathing frequency in groups of four BN rats during inhalation exposure to either vehicle or one of various concentrations of TMA. Dots indicate individual data expressed relative to preexposure frequency (set at 100%); horizontal bars indicate group means. Statistics: repeated-measures two-way ANOVA followed by one-way ANOVA/Dunnett on (PRE – EXP)/(PRE) values between vehicle and test groups; double asterisk indicates significance at $p < .01$.

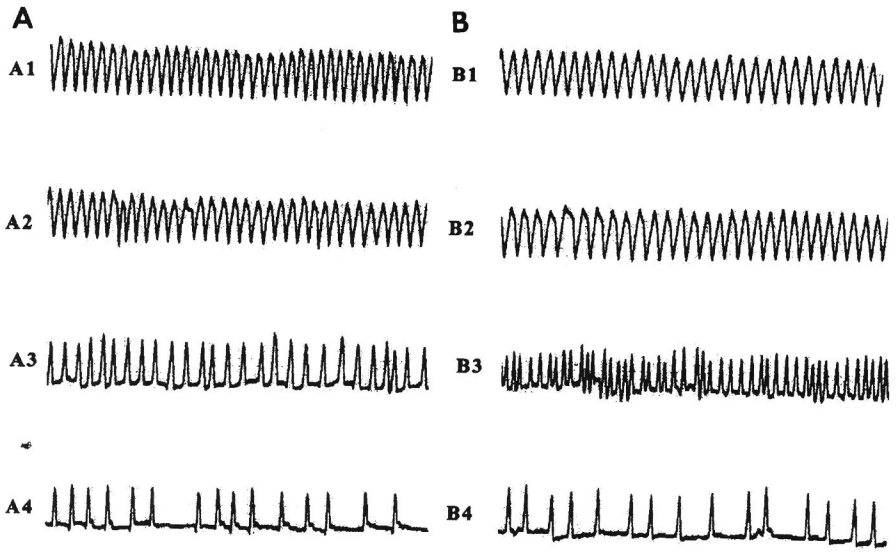

FIGURE 2. Representative breathing patterns of (A) BN and (B) Wistar rats before and during a 30-min inhalation exposure to vehicle (acetone) or TMA: (A1) preexposure, (A2) acetone exposure, (A3) 29 mg/m^3 TMA, and (A4) 214 mg/m^3 TMA; (B1) preexposure, (B2) acetone exposure, (B3) 34 mg/m^3 TMA, and (B4) 215 mg/m^3 TMA.

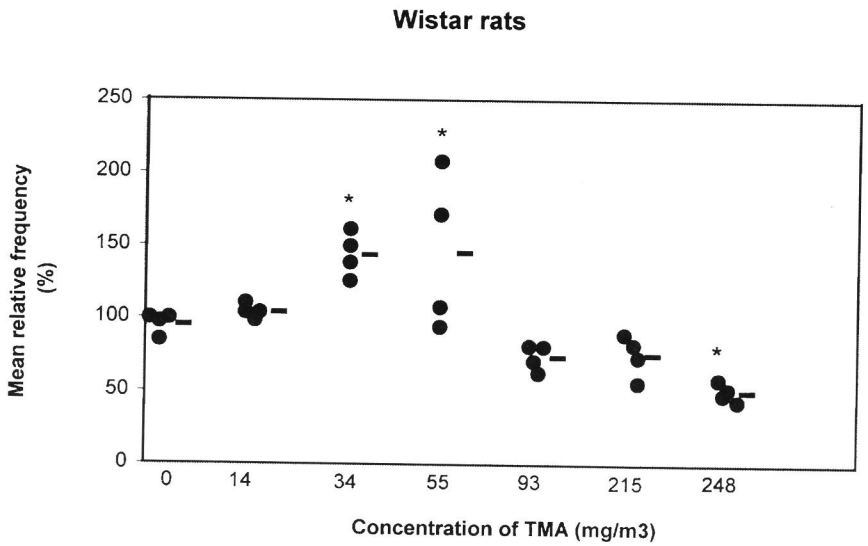

FIGURE 3. Mean relative changes in breathing frequency in groups of four Wistar rats during inhalation exposure to either vehicle or one of various concentrations of TMA. See further description in Figure 1. Statistics: repeated-measures two-way ANOVA followed by one-way ANOVA/Dunnett on (PRE – EXP)/(PRE) values between vehicle and test groups; asterisk indicates significance at $p < .05$.

Like BN rats, Wistar rats showed no changes of breathing frequencies during exposure to acetone or 14 mg/m^3 TMA. Exposure to 34 or 55 mg/m^3, however, resulted in a significant increase in breathing frequency. In contrast, exposure to 93 mg/m^3 and higher reduced breathing frequency in all groups, although statistical significance was reached at the highest level only (Figure 3).

The breathing pattern of Wistar rats exposed to 14 mg/m^3 was considered to be normal, whereas the changes in breathing pattern at levels of 34 mg/m^3 and higher were comparable to those in BN rats (Figure 2b), that is, a spiked form with a slightly decreased inspiration and expiration time and a concentration-dependent increased pause between breaths.

Similarly, the changes in breathing pattern and frequency started rapidly upon exposure to TMA, and reached plateaus within 30 min of exposure. Mean postexposure breathing frequency was still higher at levels of 34 and 55 mg/m^3. Breathing frequency and pattern almost completely returned to normal shortly after exposure to 93 mg/m^3, whereas this required the full 30-min recovery period at levels of 215 and 248 mg/m^3 (data not shown).

Since TMA caused no linear changes in the breathing frequency of Wistar rats, RD50 values could not be calculated. However, breathing frequency was reduced to about 50% at a concentration level of 248 mg/m^3, which was comparable to the concentration resulting in a 50% decrease in BN rats.

Gross Pathology and Lung Weight

Gross observation of the lungs of BN rats, controls included, showed the presence of small hyaline areas and a few petechiae. TMA exposure did not increase the incidence and severity of these lesions. No macroscopic lung changes were observed in Wistar rats. No concentration-related changes in absolute and relative lung weights of BN and Wistar rats were observed (data not shown).

DISCUSSION

This study aimed to characterize the acute airway-irritating effects of the respiratory allergen TMA in naive rats in order to study whether respiratory irritant effects of TMA could be distinguished from immune-mediated reactions. A further question was whether BN and Wistar rats would display different respiratory irritant reactions to TMA, because the two strains showed quite different respiratory responses when challenged after sensitization (Arts et al., 1998).

Both in naive BN and Wistar rats, TMA aerosols induced reversible changes in breathing pattern and frequency. The wave-form breathing pattern became spiked, respiratory-cycle timing decreased slightly, and the pause between breaths lengthened. Since the increase in pause was concentration-dependent, the breathing frequency was mainly deter-

mined by the magnitude of the increase in pause (Schaper & Brost, 1991). Such changes are typical of pulmonary irritation, that is, stimulation of C-fiber vagal nerve endings that are located in the bronchial epithelium and alveolar walls (Alarie, 1981). Many airborne chemical irritants have been shown to elicit this type of stimulation, which typically results in a concentration-dependent lengthening of the pause after expiration and consequently a decreased breathing frequency. The finding of this typical pattern in naive rats exposed only once to a particular concentration of TMA indicates that TMA causes pulmonary irritation in two different rat strains at roughly the same concentrations. It also confirms that TMA aerosol particles with an MMAD between 0.5 and 2.2 μm, as obtained in this study, were small enough to reach the bronchi and/or alveoli. Further, although deposition of TMA aerosol particles in the nose and throat also must have occurred, signs of sensory irritation, namely, lengthening of the expiration time as a consequence of stimulation of the local trigeminal nerve endings (Alarie, 1973), were absent. Actually, the TMA-induced respiratory changes observed in this study were the same as seen in mice in which irritation of the higher airways was prevented by exposure to TMA via a trachea cannule (Schaper & Brost, 1991).

In addition to the absence of typical signs of sensory irritation, signs of allergic respiratory reactions were absent. The latter are characterized by a normal wave-form breathing pattern with irregularly lengthened pauses (apneas) between a varying number of breaths, also resulting in a decreased breathing frequency (Arts et al., 1998). While the absence of allergic respiratory reactions was expected because of the use of naive animals in this study, data on breathing pattern indicate that TMA-induced irritant effects were clearly distinguishable from TMA-induced allergic responses. If this is also true for other respiratory allergens, it may provide additional information for the distinction between irritant-induced reactions and IgE-mediated allergic reactions.

Although TMA caused respiratory changes characteristic of pulmonary irritation, it lacks many properties of classical pulmonary irritants. Notably, TMA effects on breathing frequency were prompt and recovery was fast, whereas classical pulmonary irritants gradually decrease the frequency to a nadir several hours later, whereafter recovery is generally poor (Weyel et al., 1982; Weyel & Schaffer, 1985; Ferguson et al., 1986). Moreover, irritating concentrations of TMA did not affect lung weight the day after exposure, while classical irritants increase lung weights 18–24 h after exposure by inducing edema (Weyel et al., 1982; Weyel & Schaffer, 1985; Ferguson et al., 1986). The presence of small hyaline areas and a few petechiae as observed macroscopically on the lungs of BN rats was not considered to be treatment related. Inflammatory abnormalities in lungs of healthy BN rats have been earlier observed by us and other groups, and attempts to identify an infectious agent have failed (Germann et al., 1998).

Although both BN and Wistar rats decreased breathing frequency in response to TMA concentrations of about 100 mg/m^3 and higher, Wistar, but not BN, rats increased breathing frequency upon exposure to TMA concentrations between 30 and 60 mg/m^3. Such a biphasic irritant response was observed earlier in Swiss Webster mice at slightly different concentrations (Schaper & Brost, 1991). The observed increase in frequency was caused by a decreased respiratory cycle time and shortened pauses between breaths. Like the decrease in frequency, it is characteristic of pulmonary irritation. Although these data seem to indicate that Wistar rats are more susceptible to the irritant effects of TMA than BN rats are, changes in breathing pattern were seen at roughly the same concentrations.

In summary, TMA induced reversible airway reactions that were indicative of pulmonary irritation and consisted of clear changes in both breathing frequency and pattern when compared to effects of control (vehicle) exposure. The TMA-induced changes in breathing frequency were seen at higher levels than the levels that were needed to provoke respiratory reactions in sensitized animals in our previous studies. Furthermore, the changes in breathing patterns caused by irritant concentrations of TMA were clearly distinguishable from those elicited by the allergic reaction (Arts et al., 1998).

With respect to the current threshold limit value (TLV ceiling) of 0.04 mg/m^3 (ACGIH, 1999), the level at which no acute airway irritation was observed in rats in the present study, 14 mg/m^3, suggests a reasonable margin of safety in unsensitized humans. However, this may be different in sensitized subjects, since at concentrations as low as 0.04 mg/m^3 pulmonary hemorrhages have been observed in sensitized rats (Leach et al., 1987).

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Chapter 7

Summary and general discussion

All occupational respiratory LMW allergens known to date have been identified by recognition of allergic symptoms in exposed workers. The serious health problems of respiratory allergy together with the continuous introduction of new chemicals into workplaces emphasise the importance of a predictive testing strategy for LMW chemicals. Predictive tests should be (a) reliable, with few false-positive or false-negative results, (b) able to distinguish between allergen- and irritant-induced reactions, which is considered necessary for proper risk assessment, and (c) cost-effective. At present, there are no widely applied or fully validated (animal) models to identify respiratory LMW allergens. The objective of the studies described in this thesis was aimed to develop a testing strategy in the rat based on existing methods in the mouse, namely the Local Lymph Node Assay (LLNA) and the IgE test. The IgE test protocol was extended with an inhalatory challenge to study respiratory tract responses. The rat appeared to be a suitable animal model to study responses to allergenic LMW chemicals such as airway obstruction, non-specific airway hyperresponsiveness (AHR), and airway inflammation that are considered characteristic features of occupational asthma. Furthermore, the use of rats enables serial blood sampling for assessing IgE kinetics, and allows interpretation of allergy test results 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.

Local Lymph Node Assay

The murine Local Lymph Node Assay (LLNA; Dearman et al., 1992ab), which has recently been accepted as a stand-alone test (OECD 429; OECD 2000), investigates sensitisation potential by measuring cell proliferation in the lymph nodes draining the route of application, that is the ear. Irritating compounds were not found to induce cell proliferation (Dearman et al., 1992ab), although some studies suggest this distinction between irritants and allergens was not always evident (Robbins et al., 1991; Basketter and Scholes, 1992; Edwards et al., 1994; Montelius et al., 1994). Although intended to be a test to assess skin sensitisation potential, all tested human LMW respiratory sensitisers (with the exception of metal compounds) were also positive in the LLNA, using the BALB/c mouse (Dearman et al., 1992ab). Therefore, this assay was used as a starting point. All five rat strains tested (notably Wistar, SD, Fischer 344, PVG and BN) appeared suitable for identifying TMA and DNCB as sensitising chemicals (Chapter 2). In addition, Wistar and BN rats appeared suitable for identifying formaldehyde as a sensitising chemical, while methyl salicylate, an

irritant devoid of sensitising properties, gave negative results (Chapter 3). The fact that not only BALB/c mice but also five different rat strains tested positive suggests that the LLNA is a robust test. The BN rat appeared to be the least sensitive of the five rat strains tested (Chapter 2), that is, responses in this strain were clearly weaker than in the other strains. Therefore, results of the LLNA in BN rats should be considered with caution.

As expected, both the skin allergen (DNCB) and the respiratory allergen (TMA) tested positive in this assay, supporting the notion that the LLNA identifies the sensitisation potential of both skin and respiratory allergens. However, several respiratory allergens (such as TMA, TDI and MDI) have only occasionally been reported to cause skin allergy in humans (Hardy and Devine, 1979; Dearman et al., 1994). The reverse is also true; there are very few reports of respiratory sensitisation following exposure to skin allergens such as HMDI and IPDI (Davies, 1984). A likely explanation for the difference between skin and respiratory allergens could be a difference in the way of antigen presentation between skin and respiratory tract. Indeed some studies suggest variable effects of these two types of allergens on Langerhans cells (Aiba and Katz, 1991; Cumberbatch et al., 1992). For correct hazard identification, therefore, further studies to elucidate the underlying mechanisms are desirable.

IgE test

It is well established that respiratory allergy to proteins in humans 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, not at least because specific IgE antibodies could not be demonstrated in a large number of symptomatic individuals sensitised to certain diisocyanates (Karol et al., 1978; 1979ab; 1980; Butcher et al., 1980; Karol and Alarie, 1980; White et al., 1980; Karol, 1980; 1981; Baur and Fruhmann, 1981; Danks et al., 1981; Buthcher et al., 1983; Baur et al., 1984; Butcher and Salvaggio, 1986; Cartier et al., 1989; Baur et al., 1994; Karol et al., 1994; Kimber and Dearman, 1997; Beckett, 2000), as well as in a number of persons with acid anhydrides-induced asthma (Venables, 1989).

Several factors may be involved in the failure to demonstrate specific IgE antibodies in sensitised, symptomatic individuals: too much time between the last exposure and measurement (Karol, 1980), difficulties in preparing relevant chemical-protein

conjugates necessary for the measurement (Butcher et al., 1976; Paggiaro et al., 1983; Sarlo and Ritz, 1997), the ability of diisocyanates to cross-link IgG, IgA and IgE, leading to mediator release and immune complex formation that could result in both immediate and late responses in the absence of any identifiable IgE-antibody response (Zeiss, 1991), and/or production of sub-classes of IgG rather than IgE that have been shown to mediate early-onset reactions (Karol and Graham, 1997). It is, therefore, possible that immediate respiratory hypersensitivity reactions to chemical allergens operate independently of IgE antibodies.

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. Determination of IgE, whether or not mechanistically involved, could, therefore, provide an appropriate means to assess respiratory sensitisation potential. Indeed, respiratory, but not skin allergens, generally have been shown to increase total serum IgE levels or produce specific IgE anti-hapten antibodies in high IgE-responding BALB/c mice (Dearman and Kimber, 1991a; 1992; Dearman et al., 1991; 1992ab; Hilton et al., 1995; Potter and Wederbrand, 1995).

Using two rat strains, it was found that the high IgE-responding BN strain showed increased total serum IgE levels upon exposure to TMA but not upon application of DNCB, formaldehyde or methyl salicylate. The low IgE-responding Wistar strain failed to show increased total serum IgE levels with these chemicals at any of the time points measured (Chapter 3). Since increases of total IgE do not necessarily have to be accompanied by increases of allergen-specific IgE, TMA-specific IgE antibodies were also measured using the PCA assay. All sera tested from TMA-sensitised BN rats clearly showed presence of TMA-specific IgE antibodies, whereas all sera tested from non-sensitised BN rats were negative in this assay (Chapter 5). Sera of TMA-sensitised as well as non-sensitised Wistar rats were also negative (data not shown). The capacity of TMA to increase non-specific as well as specific IgE in the BN rat was also found by others (Andius et al., 1996; Vento et al., 1996; Pullerits et al., 1997).

From these results it is concluded that the measurement of total serum IgE in high IgE-responding animals would provide an appropriate means to assess respiratory sensitisation potential of LMW chemicals.

Inhalatory challenge

To study the relevance of elevated IgE levels, it was investigated using TMA, DNCB and methyl salicylate, whether increases of total serum IgE after topical exposure were associated with sensitisation of the respiratory tract and with specific functional and inflammatory changes of the airways after inhalation challenge.

Functional and inflammatory changes

The results of the studies with TMA, DNCB and methyl salicylate in BN and Wistar rats showed that increased total serum IgE after topical application is indeed associated with immediate-type specific airway reactivity upon inhalation challenge and AHR *in vivo* and *in vitro*. Functional changes during TMA challenge and increased airway hyperresponsiveness following methacholine exposure 24h later were observed in TMA-sensitised BN rats only (Chapters 4 and 5). Further, inflammation characterised by eosinophilic infiltration around bronchioli and blood vessels, goblet cell hyperplasia and hypertrophy, and increases in BAL eosinophils were typically observed in TMA-sensitised and –challenged BN rats, which suggests that these inflammatory characteristics are also associated with specific IgE. There was, however, no association with blood eosinophilia.

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 (Chapters 4 and 5).

Wistar rats did not display morphological evidence of type I allergic asthma but showed pulmonary changes consistent with mild acute hypersensitivity pneumonitis, with a clear contribution of lymphocytic infiltrates. These rats, moreover, demonstrated a mixed type III/IV laryngitis (Chapter 4). The presence of hypersensitivity pneumonitis-like inflammation in Wistar rats was in accordance with findings in TMA-exposed, low-IgE responding Sprague Dawley rats (Leach et al., 1987; Zeiss et al., 1988; 1989; 1992).

In contrast, sensitisation and challenge with the typical skin allergen DNCB resulted in laryngitis in Wistar rats only (Chapter 4). As judged by the almost pure lymphocytic infiltrate, this laryngitis was caused by a type IV reaction. 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 (Botham et al., 1989; Garssen et al., 1989; 1991; 1993; 1994; Garcia et al., 1992; Buckley and Nijkamp, 1994; Zwart et al., 1994; Satoh et al., 1995). Since the BN strain is highly biased as to the development of immune reactions mediated by Th2 cells, whereas Wistar rats are predisposed to Th1 responses (Peszkowski et al., 1994), the divergent pathology in these two strains is not surprising.

Although not characterised by episodic airway obstruction as in occupational asthma, occupational hypersensitivity pneumonitis and allergic laryngitis are also of particular interest as work-related respiratory allergic diseases. Hypersensitivity pneumonitis is well known because of its inflammatory character which, upon continuing exposure, may lead to severe breathlessness and absence from work (O'Neill, 1995). 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 (O'Hollaren, 1995; Sala et al., 1996).

Dose-response relationships

With respect to functional changes in BN rats following sensitisation and challenge with TMA initially no concentration-response relationship was obtained (Chapter 4). However, unpublished studies using lower challenge concentrations have shown a concentration below which adverse effects did not occur (manuscript in preparation). These results indicate that challenges should be performed at a certain minimum exposure level to allow investigation of the reactions described above.

With respect to morphological changes in BN rats, the severity of the granulomatous inflammation was concentration-dependent whereas haemorrhages were seen at the highest challenge concentration only (Chapter 4). Such a concentration-dependency has also been found in TMA-exposed humans (Zeiss and Patterson, 1993; Bernstein et al., 1997).

The relevance of sensitisation via skin for respiratory allergy

The dermal route appeared to be very effective for airway sensitisation with TMA and DNCB (Chapters 4 and 5). The efficacy of topical application for sensitisation with LMW chemicals in both rats (present studies) and mice (Hamelmann et al., 1999; Scheerens et al., 1999) suggests that skin exposure can be a significant risk factor in respiratory allergy in man. 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 (Karol, 1986; Nemery and Lenaerts, 1993; HSE, 2000). 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 (Liu et al., 2000).
Sensitisation of the respiratory tract via the skin has been chosen because it avoids inflammation of the airways prior to challenge which could complicate the interpretation of the response upon challenge (Holt and Sedgwick, 1987; Briatico-Vangosa et al., 1994; Kimber et al., 1996; Pauluhn et al., 1999). 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 (Karol, 1980; Botham et al., 1988; Pauluhn and Eben, 1991; Sarlo et al., 1994; Pauluhn, 1997). This may indicate that development of a specific immunological unresponsiveness or tolerance had occurred, as was shown by Sedgwick and Holt (1985), Holt and Sedgwick (1987), and Dearman and Botham (1990). 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 (Pauluhn and Eben; 1991).

Differences between respiratory irritant- and allergen-induced airway reactions
Irritants can influence the occurrence, severity, duration and type of allergic reactions in man (Venables and Chan-Yeung, 1997) or can cause asthma-like reactions and inflammation of the airways in the absence of prior exposure (Chan-Yeung and Malo, 1995). In addition, it has been observed in animals that changes in breathing parameters may also be caused by the irritant properties of the allergen, e.g. some compounds may cause a decrease in breathing frequency due to sensory irritation, whereas others may cause an increase due to pulmonary irritation (Karol, 1991).
By comparing TMA-sensitised with non-sensitised rats, breathing variables specific for either allergy or irritation were examined. 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 (Chapter 6). Since the onset of the response to TMA was rapid instead of slow, the recovery complete and fast rather than poor and delayed, and lung weights were not

affected, TMA was considered not to be a classical pulmonary irritant but a chemical capable of stimulating C-fibre nerve endings located in the alveolar walls and bronchial epithelium (Alarie, 1973; 1981; Schaper and Brost, 1991). 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 (Chapters 4 and 5). Several studies have reported that challenge concentrations exceeding the irritant threshold concentration are required to elicit functional allergic airway responses (Tao et al., 1991; Obata et al., 1992; Pauluhn and Mohr, 1994). However, in the case of TMA, challenge concentrations below the irritant threshold appeared capable to cause an early asthmatic reaction (unpublished results).

Overall, it was concluded that (1) based on examination of breathing pattern and frequency, it is very well possible to discriminate between TMA-induced irritant- and allergic reactions, and (2) since these reactions could be induced at the same challenge concentration(s), reactions in sensitised animals should always be compared with those in non-sensitised controls to assess the type of airway reaction involved.

Hazard and risk assessment

In the context of hazard identification, as indicated previously, it is supposed that the measurement of total serum IgE in high IgE-responding rats provides an appropriate means to assess respiratory sensitisation potential of LMW chemicals. An additional inhalation challenge of TMA-sensitised BN rats may provide the opportunity to identify concentrations below which adverse effects are unlikely to occur. In many instances, the exposure concentration to set off asthmatic symptoms is lower than the concentration necessary to sensitise a worker and much lower than the concentration necessary to provoke an irritation reaction (Sandler, 2000). Although toxicologists are still miles away from recommending health-based occupational exposure levels for respiratory allergens based on animal studies, the presence of a no-observed-adverse-effect level in sensitised rats suggests that assessment of safe exposure levels is feasible. Further, the correct distinction between respiratory allergens and non-sensitising airway irritants may facilitate effective risk assessment and management, i.e. 'real respiratory allergens', which may be life threatening already at very low concentrations, may be distinguished from the large bulk of airway irritants, the effects of which are usually reversible

following short time exposure.

Inhalation challenge of TMA-sensitised high IgE-responding BN rats not only induced type I allergy-associated asthma-like functional and morphological pulmonary changes, but also type III/IV allergic inflammatory reactions in larynx and lungs. These latter changes were also observed in low IgE-responding Wistar rats. Assuming that the reactions in this low IgE-responding Wistar strain may be representative of reactions in non-atopic subjects, this would imply that these subjects also can become sensitised and display airway inflammation. Thus, excluding atopic subjects from employment in jobs where exposure to respiratory allergens cannot be avoided would not help to prevent occupational allergic airway diseases.

Moreover, in view of the delayed type (type IV) mediated reactions observed in the larynx of Wistar rats after sensitisation and challenge with the skin allergen DNCB, it is reasonable to suppose that chemicals preferentially inducing skin sensitisation may also represent a risk of respiratory allergy in the case of inhalation exposure. The very low vapour pressure and/or a relatively large particle size of typical skin allergens such as DNCB, DNFB and picryl chloride, however, precludes the generation of airborne concentrations that can induce allergic reactions. Examination of the ability of a chemical to attain such concentrations by evaporation or its use (i.e. spraying, nebulising, atomising etc.) should, therefore, be a starting point to decide whether or not testing for respiratory sensitisation is necessary.

Finally, the efficacy of skin application for sensitisation to LMW chemicals in both rats and mice suggests that dermal exposure can be a significant risk factor in respiratory allergy in man.

Summary and conclusions

Based on our studies, the following tiered approach is proposed for the identification of respiratory LMW allergens (see Figure 1):

Stage 0: Examination of the risk of inhalatory exposure on the basis of physical characteristics and/or use of the chemical.

Stage 1: When inhalation exposure is likely, assessment of sensitising potential by the LLNA in BN rats. In case of a negative result, the substance is considered a non-sensitising chemical provided that skin absorption has taken place. In case of a positive result, further testing may be considered unnecessary when, for classification purposes, one is interested in sensitising potential only.

TIERED APPROACH FOR TESTING OF LMW RESPIRATORY ALLERGENS

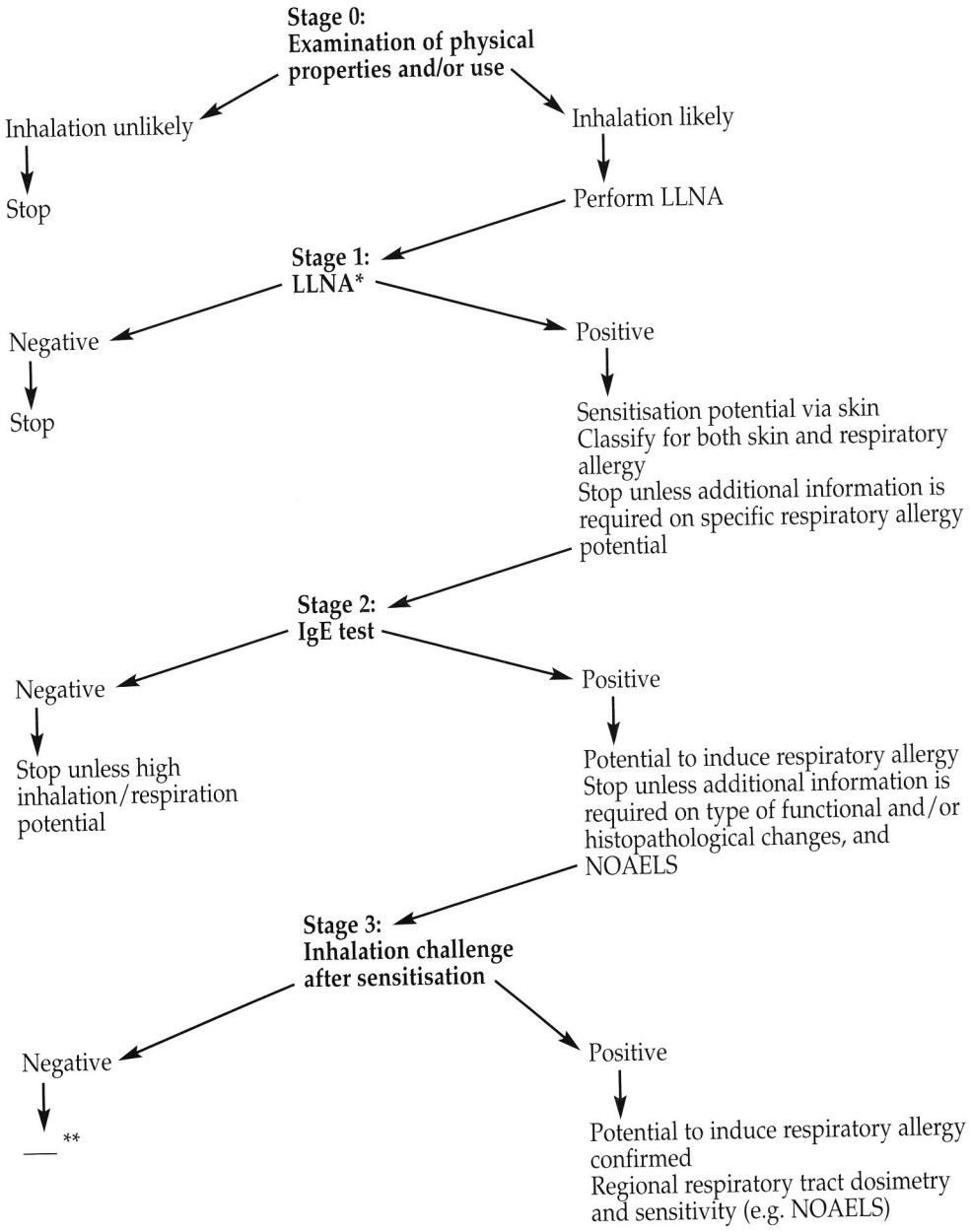

Figure 1 – Tiered approach for detecting LMW respiratory allergens (except metal compounds); the possibility of a LMW chemical to sensitise VIA the respiratory tract is not tested; * Guinea Pig Maximization Test (GPMT) (or other accepted alternatives) also possible; ** Negative with respect to functional changes; morphological changes may still occur (see text); NOAEL = no-observed-adverse-effect level

As such the chemical should be classified for both skin and respiratory allergy.

Stage 2: In case further studies into the positive result in stage 1 are required, assessment of the capacity to increase total serum IgE in BN rats. Such a test is also recommended for existing chemicals which have already been identified as skin sensitisers and for which inhalation exposure is likely to occur.

Stage 3: In case of a positive result in stage 2, assessment of functional and morphological airway changes in the sensitised BN rat upon an inhalative provocation at the time that total serum IgE levels are high. Concentration levels below which adverse effects are unlikely to occur can be established. Further, histopathological examination should confirm type I allergy and/or provide information on other types of inflammatory airway reactions.

Overall, using this tiered approach, the responses of humans to inhaled TMA have been reproduced in the high IgE-responding BN rat and this approach has been found to clearly distinguish a respiratory allergen (TMA) from a skin allergen (DNCB), and from a non-sensitiser (methyl salicylate). With TMA, positive results were obtained in both the LLNA and IgE-test, and inhalation challenge indeed resulted in asthma-like functional and morphological reactions, and non-specific airway hyperreactivity in sensitised BN rats only. In sensitised low IgE-responding Wistar rats, reactions were limited to non IgE-associated morphological airway changes. Moreover, immune-mediated functional airway reactions in BN rats induced by TMA could be distinguished from irritation reactions induced by the same compound. With DNCB, it has been shown that LMW chemicals capable of inducing skin sensitisation may also represent a risk of respiratory allergy when inhalation exposure is likely to occur.

Testing of other (respiratory) sensitisers will have to prove the usefulness of the tiered approach in the rat as described in this thesis.

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Abbreviations

AHR	airway hyperresponsiveness
AOO	acetone – olive oil
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
BN rat	Brown Norway rat
BrdU	bromodeoxyuridine
DNCB	2,4-dinitrochlorobenzene
DNFB	2,4-dinitrofluorobenzene
ELISA	enzyme-linked immunosorbent assay
f	frequency
FA	formaldehyde
GPMT	guinea pig maximisation test
GRR	gross respiratory response
gsd	geometric standard deviation
HMDI	dicyclohexylmethane-4,4'-diisocyanate
HSA	human serum albumin
IgA / E / G	immunoglobulin A / E / G
IPDI	isophorone diisocyanate
LDH	lactate dehydrogenase
LLNA	local lymph node assay
LMW	low molecular weight
MDI	diphenylmethane-4,4'-diisocyanate
MMAD	mass median aerodynamic diameter
MS	methyl salicylate
NAG	N-acetyl-B-D-glucosaminidase
NOAEL	no-observed-adverse-effect level
PBS	phosphate buffered saline
PC200	provocative concentration of an aspecific stimulus (e.g. methacholine) required to cause a 100% increase in Penh as compared to the Penh of aerosolised saline
PCA	passive cutaneous anaphylaxis

Penh	enhanced pause, a dimensionless empirically established value; increases in Penh reflect changes in breathing pattern that are considered to represent bronchoconstriction
PVG rat	Piebald Virol Glaxo rat
R 37	'irritating to respiratory system' (risk phrase)
R 38	'irritating to skin' (risk phrase)
R 42	'may cause sensitisation by inhalation' (risk phrase)
R 43	'may cause sensitisation by skin contact' (risk phrase)
RADS	reactive airways dysfunction syndrome
RAST	radio-allergosorbent test
RD50	statistically derived concentration at which animals are breathing at 50% of the original frequency
SD rat	Sprague Dawley rat
SI	stimulation index
TDI	toluene diisocyanate
Th1 cell	T helper 1 cell
Th2 cell	T helper 2 cell
TLV	threshold limit value
TMA	trimellitic anhydride
TV	tidal volume

Samenvatting

Samenvatting

Allergie is een verzamelnaam voor een groep van ziekten die veroorzaakt wordt door ongewenste immuunreacties tegen lichaamsvreemde stoffen die uiteindelijk kunnen leiden tot weefselontstekingen en verminderd functioneren van het lichaam. Deze stoffen, die allergenen worden genoemd, komen voor in het voedsel, de ingeademde lucht of kunnen via de huid worden opgenomen. Allergie ontstaat door een bifasisch proces. In de eerste fase wordt na herhaalde blootstelling aan een allergeen een overgevoeligheid voor het betreffende allergeen opgebouwd (sensibilisatie). In de tweede fase ontstaat na verdere blootstellingen (challenges of provocaties) een versnelde en sterkere immuunreactie, die uiteindelijk kan leiden tot klinische symptomen.

Op de werkplek komt zowel huidallergie als luchtwegallergie voor. In **Hoofdstuk 1** wordt een overzicht gegeven van de literatuur over luchtwegallergie.

Luchtwegallergie, en dan met name astma, is de meest gestelde diagnose van luchtwegaandoeningen op de werkplek in het Verenigd Koninkrijk. Er wordt geschat dat in Nederland elk jaar 500 tot 2000 werknemers beroepsastma ontwikkelen. De meerderheid van de patiënten met beroepastma herstelt niet volledig, zelfs niet ettelijke jaren na de laatste blootstelling; beroepsastma kan zelfs fataal zijn.

Het aantal bekende luchtwegallergenen neemt toe en omvat zowel groot-moleculaire stoffen (meestal eiwitten; > 5000 Daltons) als klein-moleculaire stoffen (<< 5000 Daltons). Laatstgenoemde moeten eerst binden aan een lichaamseiwit voordat ze allergene activiteit vertonen. Bekende voorbeelden van klein-moleculaire luchtwegallergenen zijn diisocyanaten, bepaalde kleurstoffen (in het Engels 'reactive dyes' genoemd) en zure anhydriden. Deze stoffen worden veel in de industrie gebruikt.

Als gevolg van de continue introductie van nieuwe chemicaliën op de werkplek en de ernstige gezondheidseffecten van luchtwegallergie is een vroege opsporing van luchtwegallergenen uitermate belangrijk. Het is niet goed mogelijk om alleen op basis van de structuurformule te voorspellen of een stof luchtwegallergie kan veroorzaken. De meeste luchtwegallergenen zijn meestal pas geïdentificeerd na (h)erkenning van luchtwegklachten bij blootgestelde werknemers. Om niet langer werknemers als proefdieren te laten fungeren, zijn er geschikte testmethoden nodig. De ontwikkeling van testmethoden voor de identificatie van luchtwegallergenen

wordt bemoeilijkt door het ontbreken van een goede definitie van beroepsastma. Dit wordt veroorzaakt door een slecht begrip van werkingsmechanismen en verwarring over terminologie. Uit epidemiologisch onderzoek is gebleken dat irriterende, niet allergene, klein-moleculaire stoffen een allergische luchtwegreactie sterk kunnen beïnvloeden, zeer waarschijnlijk als gevolg van het induceren van ontstekingsreacties in de luchtwegen. Daarnaast kunnen irriterende stoffen luchtwegklachten veroorzaken die lijken op luchtwegallergie, waardoor het vaak moeilijk is om luchtweg-irritatie te onderscheiden van luchtweg-allergie. Als gevolg hiervan wordt in de huidige regelgeving geen duidelijk onderscheid gemaakt tussen verschillende mechanismen die tot dezelfde klinische symptomen leiden.

Het doel van de studies beschreven in deze dissertatie was om een teststrategie te ontwikkelen die gebruikt kan worden voor de identificatie van klein-moleculaire luchtwegallergenen. Als uitgangspunt voor de teststrategie dienden twee beschreven modellen in de muis. Voor deze teststrategie werd de rat gekozen omdat herhaalde bloedafname (om het verloop van de antilichaamspiegels te kunnen volgen) beter mogelijk is bij de rat dan bij de muis. Ook zijn de resultaten van de allergietesten beter interpreteerbaar in de context van overig uitgevoerd toxiciteitsonderzoek, gezien het bijna exclusieve gebruik van de rat bij dit soort onderzoek.

In **Hoofdstuk** 2 is het onderzoek beschreven dat werd uitgevoerd met behulp van de *Local Lymph Node Assay* (LLNA), een test om na te gaan of een stof sensibiliseert nadat deze is aangebracht op de huid. De LLNA is oorspronkelijk een test in de muis en werd aangepast voor de rat. Vijf rattenstammen werden getest waarbij gebruik werd gemaakt van twee modelstoffen, namelijk trimellietzuuranhydride (TMA) en 2,4-dinitrochloorbenzeen (DNCB). Deze stoffen zijn bekende veroorzakers van respectievelijk luchtwegallergie en huidallergie. Verschillen in reactie tussen stammen en tussen de stoffen werden nader onderzocht. Bovendien werden de resultaten vergeleken met die verkregen bij de muis. In alle vijf rattenstammen (Wistar, Sprague Dawley, Fischer 344, PVG en Brown Norway) en de BALB/c muis werden TMA en DNCB correct als sensibiliserende stoffen geïdentificeerd. De BN-rat leek het minst gevoelig, dat wil zeggen, de relatieve respons in deze stam was duidelijk minder in vergelijking met die bij de andere stammen. Dit betekent dat bij marginale resultaten met de BN rat hogere doseringen moeten worden getest of dat een andere stam moet worden gekozen. Het feit echter dat niet alleen de muis maar

ook alle vijf rattenstammen positieve resultaten in de LLNA lieten zien suggereert dat de LLNA een robuuste test is.
Omdat zowel een huidallergeen (DNCB) als een luchtwegallergeen (TMA) positief was in de LLNA, is deze test niet geschikt voor het maken van onderscheid tussen huid- en luchtwegallergenen. Daarvoor is verder onderzoek nodig.

Een verhoogde immuunglobuline E (IgE)-spiegel na huidapplicatie van het allergeen wordt geassocieerd met het vermogen van een stof om luchtwegallergie te induceren. Twee rattenstammen werden geselecteerd, één stam die een goede IgE-respons heeft, de 'high IgE-responding' Brown Norway (BN) rat, en één stam die dat niet heeft, de 'low IgE-responding' Wistar-rat (**Hoofdstuk 3**). Deze twee stammen werden, wederom gebruikmakend van TMA en DNCB, getest op hun vermogen om (niet-specifieke) IgE-spiegels in het serum te verhogen. Daarnaast werden methylsalicylaat, een huidirriterende stof zonder allergene eigenschappen en formaldehyde, een huidallergeen en twijfelachtig luchtwegallergeen, getest. De twee laatstgenoemde stoffen werden eerst in de LLNA getest bij beide rattenstammen. Ook voor formaldehyde en methylsalicylzuur werkte de LLNA goed: formaldehyde gaf een (licht) positieve reactie te zien en methylsalicylaat niet.
De 'high IgE-responding' BN-rat vertoonde verhoogde IgE-spiegels in het serum na huidapplicatie van TMA maar niet na huidapplicatie van DNCB, formaldehyde of methylsalicylaat. De 'low IgE-responding' Wistar-rat vertoonde geen enkele maal verhoogde IgE-spiegels. Op basis van deze resultaten werd geconcludeerd dat het meten van het totaal-IgE-gehalte in 'high IgE-responders' een geschikte manier is om (sterke) luchtwegallergene activiteit van stoffen vast te stellen.

In de studies beschreven in **Hoofdstuk 4** werd onderzocht of toegenomen totaal-IgE-gehalten in serum na het opbrengen van een stof op de huid geassocieerd konden worden met functionele en histopathologische veranderingen van de luchtwegen na een inhalatoire blootstelling (provocatie of challenge). De resultaten van de studies met TMA, DNCB en methylsalicylaat bij zowel BN- als Wistar-ratten toonden aan dat verhoogde totaal-IgE-gehalten inderdaad geassocieerd waren met genoemde veranderingen. Als gevolg van inhalatoire blootstelling aan TMA van met TMA-gesensibiliseerde BN-ratten bestonden de functionele veranderingen uit vertraagde ademfrequentie en verandering van adempatroon tijdens blootstelling en een toegenomen ademfrequentie en verlaagd teugvolume 48 uur na blootstelling.

Tevens waren de longgewichten verhoogd. De morfologische veranderingen bestonden uit ontstekingshaarden in de luchtwegen gekenmerkt door infiltratie van witte bloedcellen karakteristiek voor astma (eosinofielen) rond de kleine bronchiën en bloedvaten, en toename in aantal (hyperplasie) en vergroting (hypertrofie) van slijmbekercellen. Naast deze met allergisch astma-geassocieerde pathologie, veroorzaakte TMA-provocatie bloedingen en ontstekingen in de longen die wezen op een andere allergische luchtwegaandoening (extrinsieke allergische alveolitis; EAA). In het strottenhoofd (larynx) werd voorts een karakteristieke ontsteking (laryngitis) aangetroffen. Blootstelling aan TMA van met TMA-gesensibiliseerde Wistar-ratten, daarentegen, resulteerde niet in functionele reacties. Ook werd 48 uur na blootstelling geen allergisch astma-geassocieerde pathologie gezien maar werden wel ontstekingen gelijkend op EAA in de longen en een karakteristieke ontsteking van de larynx (laryngitis) geconstateerd. Bovendien resulteerde sensibilisatie met en inhalatoire blootstelling aan het typische huidallergeen DNCB ook in laryngitis, alleen bij Wistar-ratten. Het verschil in luchtwegpathologie was goed verklaarbaar op basis van het verschil in genetische aanleg van de twee gebruikte rattenstammen. Hoewel EAA en allergische laryngitis niet worden gekenmerkt door luchtwegvernauwing door contractie van gladde spieren zoals bij astma, zijn deze twee ziekten toch van belang als allergische beroepsziekten. EAA en laryngitis kunnen, zij het op andere wijze, uiteindelijk ook leiden tot ernstige benauwdheid.
Aannemende dat de Wistar-rat model staat voor niet gevoelige (niet-atopische) personen, betekent dit dat deze personen ook gesensibiliseerd kunnen worden en ontstekingen in de luchtwegen kunnen vertonen. Dit houdt tevens in dat beroepsallergie niet kan worden voorkomen door gevoelige (atopische) personen de toegang te ontzeggen tot werkplekken waar blootstelling aan luchtwegallergenen kan plaatsvinden. Bovendien kan worden geconcludeerd dat inademing van typische huidallergenen een risico vormt voor luchtwegallergie.

Uit bovengenoemd onderzoek is eveneens gebleken dat het opbrengen van de stof op de huid erg effectief kan zijn om ook de luchtwegen te sensibiliseren. Dit suggereert dat blootstelling van de huid een belangrijke risicofactor is voor het ontstaan van luchtwegallergie bij de mens. De reden waarom voor het hier beschreven onderzoek werd gekozen voor het sensibiliseren van de luchtwegen via de huid in plaats van de luchtwegen was tweeledig: (1) op deze wijze werd voorkomen dat aspecifieke ontstekingsreacties in de luchtwegen ontstaan die de

interpretatie van de provocatie-reactie zouden kunnen bemoeilijken en (2) uit de literatuur bleek dat herhaalde inhalatoire blootstelling tolerantie kan doen ontstaan, dat wil zeggen dat dieren niet meer reageren op het allergeen, ook niet bij hoge concentraties van dit allergeen.

Om het BN-rat luchtweg-allergiemodel nader te karakteriseren werden verschillende longfunctie- en luchtwegontstekingsparameters onderzocht (**Hoofdstuk 5**), inclusief aspecifieke luchtweghyperreactiviteit, hetgeen een kenmerk is van allergisch astma. Inhalatoire blootstelling aan TMA van met TMA-gesensibiliseerde BN-ratten resulteerde 24 uur na provocatie in toegenomen aspecifieke luchtweghyperreactiviteit zowel *in vivo* als *in vitro*. Tevens bleken op hetzelfde tijdstip de aantallen eosinofielen en neutrofielen en de hoeveelheden eiwit, lactaatdehydrogenase en N-acetyl-glucosaminidase in longspoelsel te zijn verhoogd. Met name de biochemische parameters zijn indicatief voor luchtweg-schade.
Naast een verhoging van het totaal-IgE-gehalte werd ook TMA-specifiek IgE aangetoond in de gesensibiliseerde BN-rat.

Ten slotte werd onderzocht of de door allergeen-geïnduceerde veranderingen in de luchtwegen onderscheiden konden worden van de door irritatie-geïnduceerde luchtwegveranderingen (respectievelijk **Hoofdstuk 5 en 6**). TMA-provocatie van met TMA- gesensibiliseerde BN-ratten induceerde een voor astma kenmerkend adempatroon dat goed onderscheiden kon worden van door irritatie-veroorzaakte veranderingen in controle dieren. Eerst-genoemde dieren vertoonden langere in- en uitademingstijden. Ook traden er pauzes van onregelmatige duur op tussen een variabel aantal ademhalingen (zie figuur 2B in Hoofdstuk 5). Dit leidde tot een verlaagde ademfrequentie. Het door irritatie veroorzaakte ademhalingspatroon van niet-gesensibiliseerde ratten bestond uit een spitse in plaats van een golvende vorm en uit een geringe toename van de duur van de pauze tussen elke ademhaling aan het eind van de uitademing (zie figuur 2A in Hoofdstuk 5 en figuur 2A en 2B in Hoofdstuk 6). De duur van de pauze nam toe met de TMA-concentratie. Daardoor nam ook de frequentie af bij hoge concentraties TMA. Wistar-ratten vertoonden hetzelfde adempatroon ongeacht voorafgaande sensibilisatie.
In dit onderzoek (**Hoofdstuk 6**) kon tevens de concentratie worden vastgesteld waarbij geen acute luchtwegirritatie meer kon worden waargenomen.

Op basis van deze resultaten werd geconcludeerd dat het zeer wel mogelijk is om door TMA-geïnduceerde allergische luchtwegreacties te onderscheiden van irritatiereacties. Het is daarom aan te bevelen dat luchtwegreacties van gesensibiliseerde dieren altijd worden vergeleken met die van niet-gesensibiliseerde dieren.

In **Hoofdstuk 7** zijn de resultaten samengevat en bediscussieerd.
De volgende teststrategie ter onderkenning van luchtwegallergenen werd voorgesteld (zie figuur 1, Hoofdstuk 7):
Stap 0: Beoordeling van de kans op inademing van een stof op basis van fysische karakteristieken en/of gebruik.
Stap 1: Indien inademing mogelijk is, vaststellen van het vermogen van de stof tot sensibilisatie in de LLNA. In geval deze test negatief is wordt de stof als niet sensibiliserend beschouwd, mits voldoende duidelijk is dat de stof daadwerkelijk door de huid is opgenomen. Indien deze test positief uitvalt en er geen behoefte is aan verdere classificatie, is meer onderzoek niet nodig. Zo'n stof dient dan als huid- en luchtwegallergeen te worden geclassificeerd.
Stap 2: Om een positief resultaat verkregen in stap 1 nader te karakteriseren, nagaan of de stof het totaal-IgE-gehalte verhoogt in een 'high IgE-responding' dier zoals de BN-rat (IgE-test). Deze test wordt ook aanbevolen voor stoffen die reeds eerder als huidallergeen geclassificeerd waren maar die ook ingeademd zouden kunnen worden.
Stap 3: In geval van een positieve IgE-test, nagaan of functionele en histopathologische veranderingen tijdens en na inhalatoire blootstelling in de gesensibiliseerde BN-rat optreden. Indien veranderingen worden waargenomen kunnen concentraties worden vastgesteld waarbij geen effecten meer optreden. Bovendien kan op basis van de aard van de histopathologische veranderingen worden vastgesteld of het om allergisch astma gaat of om andere typen luchtwegallergie.
Met deze teststrategie was het mogelijk om een luchtwegallergeen (TMA) te onderscheiden van een typisch huidallergeen (DNCB) en een irriterende, niet allergene stof (methylsalicylaat). Door het testen van andere (luchtweg)allergenen zal moeten worden vastgesteld in hoeverre de ontwikkelde teststrategie algemeen bruikbaar is.

Dankwoord

Tja, het dankwoord. Het makkelijkste zou natuurlijk zijn om iedereen die aan het tot stand komen van dit proefschrift heeft geholpen bij deze te bedanken. Maar dan blijft de rest van de pagina zo leeg. Omdat tevens het dankwoord een van de meest gelezen passages in een proefschrift is, en de meesten het vaak toch wel leuk vinden hun naam te zien, waag ik een poging, met daarbij meteen een verzoek om vergeving indien ik toch onverhoopt iemand ben vergeten.
Een proefschrift is een werk van velen en niet alleen van de schrijver dezes. En zonder begeleiding, zowel vakkundig als emotioneel, is het bijna onmogelijk. Ik wil daarom graag als eerste mijn promotoren, Prof. Dr. Vic Feron en Prof. Dr. Willem Seinen, bedanken voor het in mij gestelde vertrouwen. Ondanks mijn drukke baan en twee jonge kinderen, hebben ze mij toch altijd een hart onder de riem kunnen steken bij elk stapje op het te volgen pad. Beste Vic, ik heb zelden iemand ontmoet die altijd zo enthousiast was bij alles wat met Toxicologie te maken had, en jouw opmerking op elk ingeleverd manuscript 'Ziet er prima uit' en (of maar) 'Zie een paar tekstuele veranderingen' of ' Zie een paar suggesties' gaven de burger moed. Beste Willem, ook jou dank ik hartelijk voor alle nuttige opmerkingen. En wat 'auto body shop workers' nu precies doen, moeten we nog maar eens bespreken.
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De start van het promotieonderzoek was ergens in 1993 en werd versneld door de komst van Drs. Sonja Dröge. Sonja, ook al hebben we elkaar lang niet gezien c.q. gesproken, door jouw inzet en bijdrage hadden we een vliegende start, hetgeen meteen leidde tot de eerste twee publicaties. Vele jaren later (1999) werd Sonja opgevolgd door Martijn de Koning, die wat langer bleef dan afgesproken. Maar ook jouw bijdrage zal leiden tot twee publicaties. Een ervan is al opgenomen in dit proefschrift (Hoofdstuk 6), de andere is in de maak (dosis-respons relaties). Beiden, erg bedankt voor jullie hulp.
Onderzoek doen naar luchtwegallergie zonder inhalatoire blootstellingen is als een vis happend naar lucht op het droge. En werkzaam op het terrein van de inhalatie-toxicologie was het niet meer dan logisch om het onderzoek in die richting uit te breiden c.q. te buigen. Per slot van rekening hebben weinig onderzoeksinstituten mogelijkheden op dit gebied. Ik ben daarom veel dank verschuldigd aan leden en ex-leden van 'ons Inhalatieteam': Stan Spoor, Frank Hendriksma, Gerard Roverts, Evert Duistermaat en last but not least mijn kamergenoot Hans Muijser (ook al zit er nu een glaswand tussen), vanwege zijn algemene adviezen en raad omtrent de te gebruiken statistiek plus al het extra werk dat anders langer zou zijn blijven liggen. Van nieuwkomer Carolien Mommers hoop ik dat we nog veel gaan samenwerken

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Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 6 december 1960 te Nijmegen. Na het voltooien van de middelbare schoolopleiding (VWO – Gymnasium B) aan het Elshofcollege in dezelfde plaats begon zij in september 1979 haar studie aan de toenmalige Landbouw Hogeschool te Wageningen (nu Wageningen Universiteit en Researchcentrum), richting Humane Voeding. In de kandidaatsfase werd haar belangstelling voor de toxicologie gewekt hetgeen resulteerde in een verzwaard hoofdvak Toxicologie in de doctoraalfase. Tijdens de 6-maanden stageperiode werd onderzoek op het gebied van de Immunotoxicologie verricht aan de Universiteit Utrecht (vakgroep Veterinaire Farmacologie, Farmacie en Toxicologie). De overige twee doctoraalvakken bestonden uit Voedingsleer/Celbiologie en Pedagogiek/ Algemene Didactiek. Na haar afstuderen (september 1986) is zij in november van dat jaar bij de werkgroep Inhalatietoxicologie van TNO Voeding in dienst getreden, eerst als study director daarna tevens als Product Manager Inhalatietoxicologie. In 1994 werd zij geregistreerd als toxicologisch onderzoeker (SMBWO). Het promotie-onderzoek werd uitgevoerd bij TNO Voeding in de periode 1993-2000 onder begeleiding van Dr. C.F. Kuper, Dr. M.A. Bloksma, Prof. Dr. V.J. Feron en Prof. Dr. W. Seinen.

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Stellingen

1. Het beschikbaar hebben van (dier)modellen die stoffen kunnen identificeren die luchtwegallergie veroorzaken zal, net zoals voor huidallergie, hopelijk leiden tot een classificatie op basis van het werkingsmechanisme en niet op symptomen (dit proefschrift).

2. Het verschil tussen IgE-gemedieerde en niet IgE-gemedieerde reacties hangt zowel van het individu als het allergeen af (o.a. dit proefschrift).

3. *Antibody measurements are only as good as the chemical-protein conjugate used in serological tests*. Sarlo and Ritz (1997) In: I. Kimber and R.J. Dearman (eds.) Toxicology of chemical respiratory hypersensitivity, Taylor & Francis Ltd., London, UK, 107-120

4. Het restrictieve gebruik van de term contactallergie voor cel-gemedieerde allergische reacties in de huid is onjuist aangezien ook voor het krijgen van luchtwegallergie contact met het allergeen nodig is.

5. Of de huid kan dienen als route voor sensibilisatie van de luchtwegen wordt nog steeds betwijfeld. Het omgekeerde echter niet: de skin prick test wordt algemeen geaccepteerd als een methode voor het vaststellen van luchtwegallergie.

6. Irritant-induced asthma verhoudt zich tot allergisch astma als hyperplasie tot een tumor.

7. Het is voor risk assessment noodzakelijk dat sensibiliserende stoffen ingedeeld worden op 'potency' en niet alleen op 'potential'.

8. Met betrekking tot overgevoeligheid voor voedingsmiddelen wordt de term allergie niet alleen in de volksmond meestal onjuist gebruikt, ook diverse wetenschappelijke disciplines maken vaak een ratatouille van overgevoeligheid, allergie, pseudo-allergie en intolerantie.

9. De keuze voor kinderen is evenals de keuze voor het niet willen krijgen van kinderen een egocentrische keuze.

10. Straten in woonwijken worden tegenwoordig voorzien van allerlei snelheidslimiterende obstakels behalve die straten waar lijnbussen rijden waardoor deze als racebaan, ook door die bussen, worden benut.

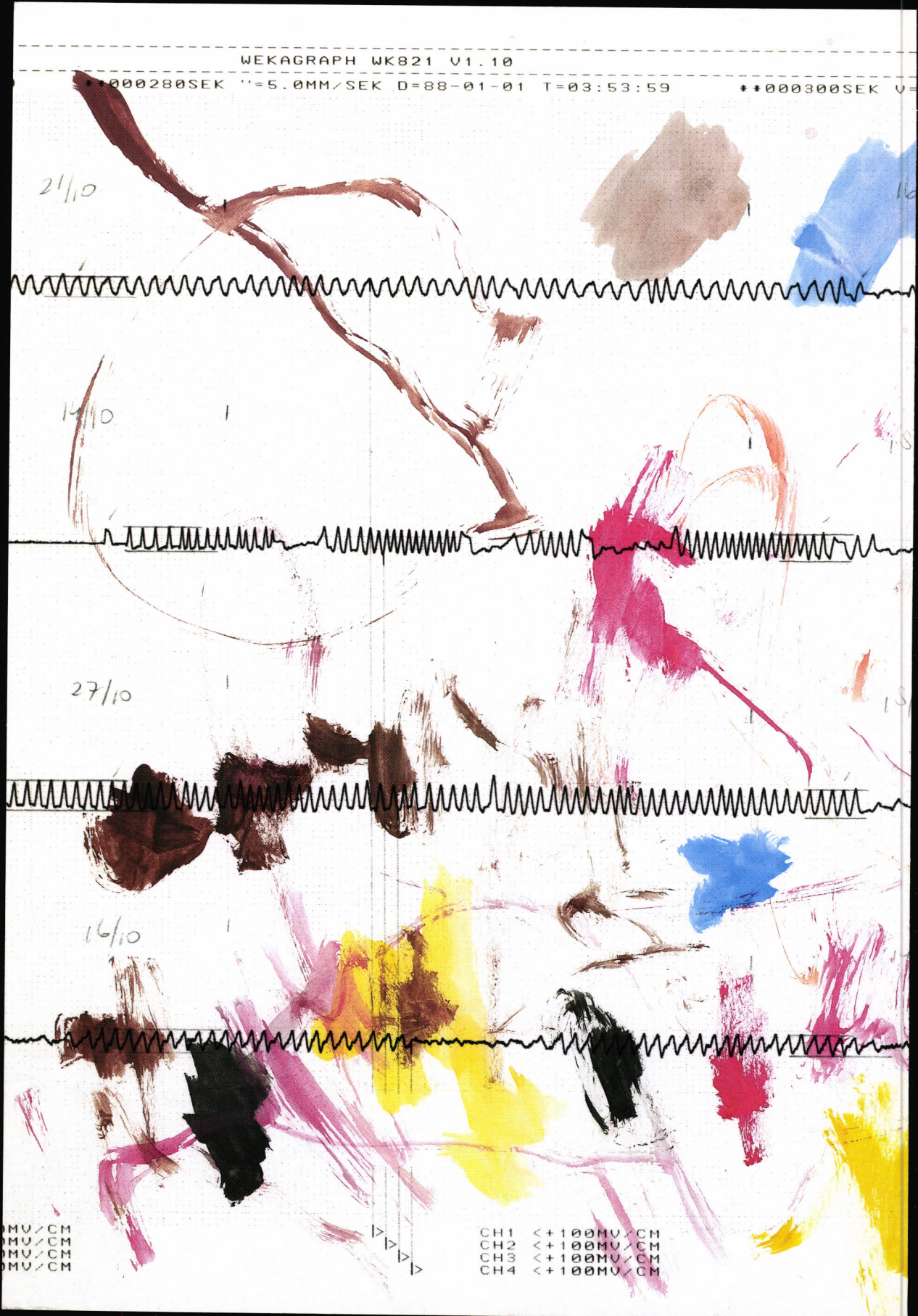
11. Zogenaamde ruimtewagens bieden ruim zicht behalve aan degenen die er achter rijden.

12. Onbekend maakt onbemind gaat zeker op voor de cricketsport.

13. Het is makkelijker een tuinarchitect om de tuin te leiden dan hem het hof te maken.

14. Het aantal typefouten in een verstuurde e-mail is recht evenredig met de vermeende mate van drukte van de verzender.

Stellingen behorende bij het proefschrift
Respiratory allergy induced by low molecular weight chemicals in rats
Josje H.E. Arts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