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Assessment of allergic potential of chemicals for respiratory allergy

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SUMMARY

At least 10% of the population of the western world is suffering from respiratory syndromes with characteristics of asthma or COPD (chronic obstructive pulmonary disease). In addition to inheritable components, it is well known that exaggerated immune responses against inhaled compounds can lead to COPD. More than 50% of the number of asthma cases are induced by type I hypersensitivity immune reactions (i.e. allergic or extrinsic asthma). The asthma cases that are not induced by these type I hypersensitivity reactions can be non-immunologically mediated (i.e. intrinsic asthma) or induced by other types of hypersensitivity reactions such as type III or IV hypersensitivity. Predictive tests with respect to the potency of chemicals to induce one or more types of respiratory hypersensitivity are necessary in order to estimate the risk for exposure to these chemicals. The majority of the predictive tests are restricted to tests which are aimed to test the capacity of chemicals to induce skin-type hypersensitivity (type IV) reactions. The applicability of these tests for predictive testing regarding the potency of chemicals to induce respiratory hypersensitivity is restricted. In the present report a step by step approach is proposed for the identification and characterization of small molecular weight compounds as potential allergens, pertaining to the skin and the respiratory tract.

SAMENVATTING

Geschat wordt dat ongeveer 10% van de westerse wereld regelmatig CARA (Chronische Aspecifieke Respiratoire Aandoeningen waaronder astma) achtige klachten heeft. Naast een erfelijke component is bekend dat immunologische overgevoeligheids reacties tegen ingeademde stoffen (allergenen) ook een belangrijke rol spelen bij respiratoire aandoeningen zoals CARA. Bij meer dan de helft van het aantal astma patiënten spelen type I overgevoeligheids reacties een belangrijke rol (allergische astma). Astma dat niet door type I reacties wordt geïnduceerd kan niet-immunologisch geïnduceerd zijn (intrinsieke astma, niet-allergische astma) of geïnduceerd zijn door andere typen van immunologische overgevoeligheids reakties. Voorbeelden van dergelijke typen overgevoeligheids reacties zijn type III en IV overgevoeligheid. Om het risico van blootstelling aan chemische stoffen te schatten zijn voorspellende testen noodzakelijk. Deze testen moeten dan aangeven of een bepaalde klein moleculaire verbinding één of meerdere typen van overgevoeligheid kan induceren. De meerderheid van de beschikbare testen zijn beperkt tot testen voor de inductie van huidovergevoeligheid. De toepasbaarheid van dit type (huid)testen met betrekking tot voorspellende inductie van luchtweg overgevoeligheid is beperkt. In dit rapport wordt een getrapt systeem voorgesteld voor de identificatie en karakterisatie van klein-moleculaire verbindingen als potentieel allergeen ten aanzien van de huid of de ademhalingswegen.

1. INTRODUCTION

The function of the immune system is defense against neoplastic cells and infectious agents such as parasites, viruses, fungi, and bacteria. But under certain circumstances immune reactions can lead to tissue injury. These *hyper*immune reactions (hypersensitivity or allergy (Greek: allergy = altered reactivity)) can be induced by low molecular weight and high molecular weight compounds. The majority of allergies are induced by high molecular weight compounds, i.e. proteins or (poly)saccharides having a size larger than 10K daltons. In many instances such compounds possess biological activity, being proteolytic or hydrolytic enzymes. However, also small molecular weight compounds can induce allergic reactions. These small molecular compounds that can induce allergy-like reactions reveal some common features. Although there is less known about the precise mechanisms of allergy induced by small molecular weight compounds it is demonstrated that some of these compounds can act as a hapten and induce specific IgE production or lead to T cell mediated immune responses. Most of these compounds possess electrophilic functionalities which enable the small molecular weight compound to react upon contact with nucleophilic moieties such as sulfhydryl, hydroxyl, or amino groups on biological molecules. Since it is generally accepted that covalent (or high affinity) binding of chemicals to macromolecules is essential to engender small molecules with immunogenecity, an electrophilic property of chemicals would be highly predisposing for immunogenic activity. However, an electrophilic property of chemicals is not sufficient to endow a chemical with respiratory sensitivity activity. For example the aromatic diisocyanate biphenyl methane 4,4'-diisocyanate is recognized to be a respiratory chemical allergen whereas the unsaturated analog (dicyclohexyl methane-4,4'-diisocyanate) is not a respiratory sensitizer, although both compounds contain a pair of electrophilic isocyanate groups. It has been shown that the unsaturated analog is a potent contact sensitizer in humans even though normal use of the chemical results in exposure of workers to aerosols containing this chemical. Similarly, animal studies have shown the propensity of this chemical to cause dermal, rather than pulmonary sensitivity irrespective of the route of contact with the chemical. Inhalation of the unsaturated analog resulted in contact allergy and not respiratory allergy in guinea pigs. Humoral responses were absent in these animals whereas humoral responses occur in animals exposed to the saturated form of this chemical. Besides contact allergens, many non-allergens, also have electrophilic functionalities. Accordingly, this property, although exceedingly important in contribution to the immunogenicity of small chemicals, is neither definitive nor sufficient for conferring respiratory sensitizing activity of chemicals.

In an earlier report (Garssen and Van Loveren, 1994), predictive test were evaluated with respect to the usefulness of these test for the screening of sensitizing effects of chemicals leading to respiratory allergy. Predictive tests for the evaluation of skin-sensitizing effects of chemicals exist since approximately 50 years. Guidelines for the testing of sensitizing effects were published by respectively the OECD and the EC. The most recent guidelines for testing of sensitizing effects were accepted in 1992 by the OECD. In addition to the protocols mentioned in these guidelines a few more test are available for skin-sensitizing effects of chemicals. In contrast, tests for the screening of sensitizing effects of chemicals leading to respiratory allergy are less well available and accepted. Tests for skin-sensitizing effects of compounds are mostly used for the detection of potency of chemicals to induce respiratory allergy. Whether these tests indicate that a certain chemical can really induce respiratory hypersensitivity is highly doubtful. In the present report a step by step approach is proposed for the identification and characterization of small molecular weight compounds as potential allergens, pertaining to the skin and the respiratory tract.

2. SENSITISATION TESTS

2.1. Skin-sensitisation tests

Animal tests

In the majority of these tests guinea pigs are used as experimental animals. Examples of these tests are: the Draize test, the Freund's Complete adjuvant test, the optimization test, the maximization test, the split adjuvant test, the Buehler test and the open epidermal test (see Garssen and Van Loveren, 1994). For more than 40 years the guinea pig has been the animal of choice for predictive sensitisation tests (skin) and it is still the only animal species accepted by the authorities. The advantage of this species being that allergic reactions in guinea pigs resemble allergic reactions in human beings. A disadvantage of guinea pig models is that the immune system of the guinea pig has not been studied extensively. In contrast the immune system of the mouse has been investigated very extensively. For this reason new tests were developed using the mouse. Many of these mouse tests include a topical induction treatment on the mouse abdomen/thorax and a challenge application on the ear. In all these mouse models objective parameters for the measurement of the allergic potential are used, most commonly by assessing an increase in ear thickness or wet ear weight. An alternative to measuring ear thickness is the assessment of the cellular infiltrate using radioactive labeled cells. In addition, cell proliferation in draining lymph nodes can be measured, using tritiated thymidine. Most of the experiments on cellular mechanisms and methods in delayed type hypersensitivity have used strong allergens such as oxazolone, DNFB or isocyanates. Experience with weak allergens in predictive mouse protocols has been addressed in a few methods. Mice maintained on a diet supplemented with vitamin A acetate develop easier allergic reactions to weak allergens than animals not maintained on this diet (Maisey et al., 1986).

One such mouse test was recently refined by Gad et al. (1986). This test , i.e. the MEST test (mouse ear swelling test) is promising. The test has been validated using 50 test compounds. In the MEST test mice are sensitized by a comparatively rigorous regimen (the intradermal injection of test material in adjuvant followed by the daily application of the test material alone, for 4 consecutive days, to tape-stripped skin) and challenged on one ear with the test compound and on the contralateral ear with vehicle alone. Sensitization potential is evaluated by consideration of both the degree of oedema induced and the percentage of mice displaying a reaction. Vitamin A may be used instead of an adjuvant. Another promising test system is developed by Kimber et al. (1986). This test monitors the primary T cell response in the draining lymph node following topical application to the mouse ear. Mice are treated daily, for 3 consecutive days, on the dorsum of both ears with the test material or with an

equal volume of vehicle alone. Proliferative activity in draining lymph nodes is evaluated 5 days following the initiation of exposure. This method has the advantages of a short duration, an objective measurement of proliferation and minimal animal treatment. In contrast to the MEST and guinea pig assays, activity here is measured as a function of events induced during the induction, rather than elicitation, phase of contact sensitization. The initiation of "skin" sensitization is associated with, and dependent upon, the stimulation of T cell proliferative responses in lymph nodes draining the site of exposure. It is this response that is measured in the local lymph node assay (Kimber et al., 1986). Currently, chemicals are classified as possessing sensitization potential if with one test concentration a stimulation index, relative to vehicle-treated controls, of 3 or greater is induced.

The MEST and local lymph node assay offer significant advantages compared with the available guinea pig test methods. Important among these is the fact that in both cases there is an objective read-out.

Recently new techniques were developed based on the detection of cytokines in draining lymph nodes (Dearman et al., 1991 and 1992). In 1986, the existence of two CD4+ T helper (Th) cell subsets was discovered in mice, and they were designated Th1 and Th2. Their identification has greatly improved understanding the regulation of immune effector functions, not in the least on Type I and Type IV hypersensitivity responses. These Th subsets can not be differentiated on the basis of a cellular surface marker. They produce, however, defined patterns of cytokines that lead to strikingly different T cell functions. Roughly speaking, Th2 cells are efficient B cell helpers, especially in the production of IgE and IgG1, whereas Th1 mediate type IV hypersensitivity (e.g. delayed type hypersensitivity, DTH). In addition, they crossregulate by producing mutually inhibitory cytokines. These two types of Th cell subsets were originally identified in the mouse. Recently, it has become clear that they also exist in rats and humans.

For tests based on cytokine profiles (Th1 vs. Th2 mediated responses) mice were sensitized as mentioned above. Local lymph node cells were cultured and the cytokine profiles were analyzed. The cytokine profile informs about Th1 or Th2 mediated immune responses. Several authors (see Garssen and van Loveren, 1994) suggest that compounds that induce Th1 mediated reactions are active skin sensitizers whereas compounds that induce Th2 mediated reactions are active respiratory and skin sensitizers. The Th2 mediated immune reactions are immediate type hypersensitivity reactions that can occur in the skin and in the airways. The notion that Th1 mediated reactions are restricted to the skin is true with respect to the majority of compounds tested, however there are compounds that can induce Th1 mediated responses with the capacity to induce respiratory allergy.

In addition to these animal models there is much interest for the development of in vitro test systems in order to minimize the use of guinea pigs and mice. There are two reasons for further following this avenue: The first one is directed towards a fuller understanding of the relationship between the chemical structure and physicochemical properties and skin sensitizing activity. In this context, parameters that appear to be of particular importance are protein reactivity and lipophilicity associated with the capacity to penetrate into the viable epidermis. As such this information is relevant for the initial identification of the hazard. The second reason is of mechanistic nature. For instance, dendritic cells can be tested for their ability to stimulate chemical allergen-specific primary proliferative responses by naive T cells isolated from non-sensitized mice. Another approach makes use of the fact that the skin is an immunologically active tissue and that epidermal cells (both keratinocytes and Langerhans cells) produce cytokines constitutively or in response to external signals. At least some of the cytokines are required for the optimal initiation of skin-sensitization and it has been proposed that their induction or increased expression may provide useful molecular markers of contact sensitizing activity. If it can be shown that contact allergens provoke characteristic patterns of epidermal cytokine production then an in vitro method, using cell cultures or skin explants, may be possible. Yet, it should be borne in mind that some Th1 type immune resonses may also be involved in induction of respiratory allergy.

2.2. Respiratory-sensitisation tests

Sensitization of the respiratory tract is considered usually to be effected by IgE antibodies. Following primary exposure to the allergen a specific IgE antibody response is provoked. After subsequent inhalation of the same compound vasodilation and bronchoconstriction will occur within one hour (type I hypersensitivity, direct type hypersensitivity). In the majority of asthma patients type I hypersensitivity is a major feature. However, certain chemicals, especially low molecular weight compounds can induce respiratory allergy with characteristics of type IV hypersensitivity. In these cases inflammatory reactions can be found that have similarities with delayed type hypersensitivity (type IV) skin reactions and type III hypersensitivity reactions.

There are as yet no well defined or widely applied methods for the detection of chemicals that have the potential to cause sensitization of the respiratory system.

Animal tests

Guinea pigs models (Karol et al. 1993): Guinea pigs sensitized to free or protein-bound chemical allergens (by inhalation), such as toluene diisocyanate, will exhibit symptoms of pulmonary hypersensitivity following subsequent inhalation challenge with the same

compound. In such models hypersensitivity reactions are measured usually as a function of challenge-induced changes in respiratory rate or alterations in other breathing parameters such as tidal volume. Recently it has been found that significant changes in breathing patterns can be provoked in dermally sensitized guinea pigs by inhalation challenge with the free chemical. In this approach it is not necessarily to use hapten-protein conjugates. In a model developed by Garssen et al. (1991) similar findings were obtained using TNP as a hapten. A tiered approach to hazard assessment in guinea pigs has been proposed by Sarlo and Clark (1992). This comprises sequential analyses of physicochemical similarities with known allergens, the potential to associate covalently with protein, the ability to stimulate antibody responses and finally activity in a model of respiratory hypersensitivity in which animals sensitized by subcutaneous injection are challenged by intratracheal instillation.

Some authors (Karol et al., 1993; Sarlo et al., 1992) suggest that the mouse IgE test is sufficient to screen chemicals on their capacity to induce respiratory allergy. These authors observed that following topical exposure, only those chemicals known to cause occupational respiratory hypersensitivity in humans will provoke in mice a substantial increase in serum IgE titres. In contrast, chemicals that lack this potential for sensitization of the airways, but which nevertheless induce contact hypersensitivity, fail to stimulate similar changes in serum IgE. Representative results from mouse IgE tests performed with toluene diisocyanate (TDI), a human respiratory allergen, and 2,4-dinitrochlorobenzene, a potent contact sensitizer that fails to cause respiratory sensitization indicates that this test is useful. However, recently it is demonstrated that dinitrochlorobenzene (DNCB), dinitrofluorobenzene (DNFB) and picrylchloride (PCl), all potent contact sensitizers, can induce respiratory disorders in mice without detectable specific IgE. In some of these cases inflammatory reactions can be found that have similarities with delayed type hypersensitivity. Thus also without detectable IgE (Th2 mediated response) respiratory allergy may occur (e.g. Th1 mediated).

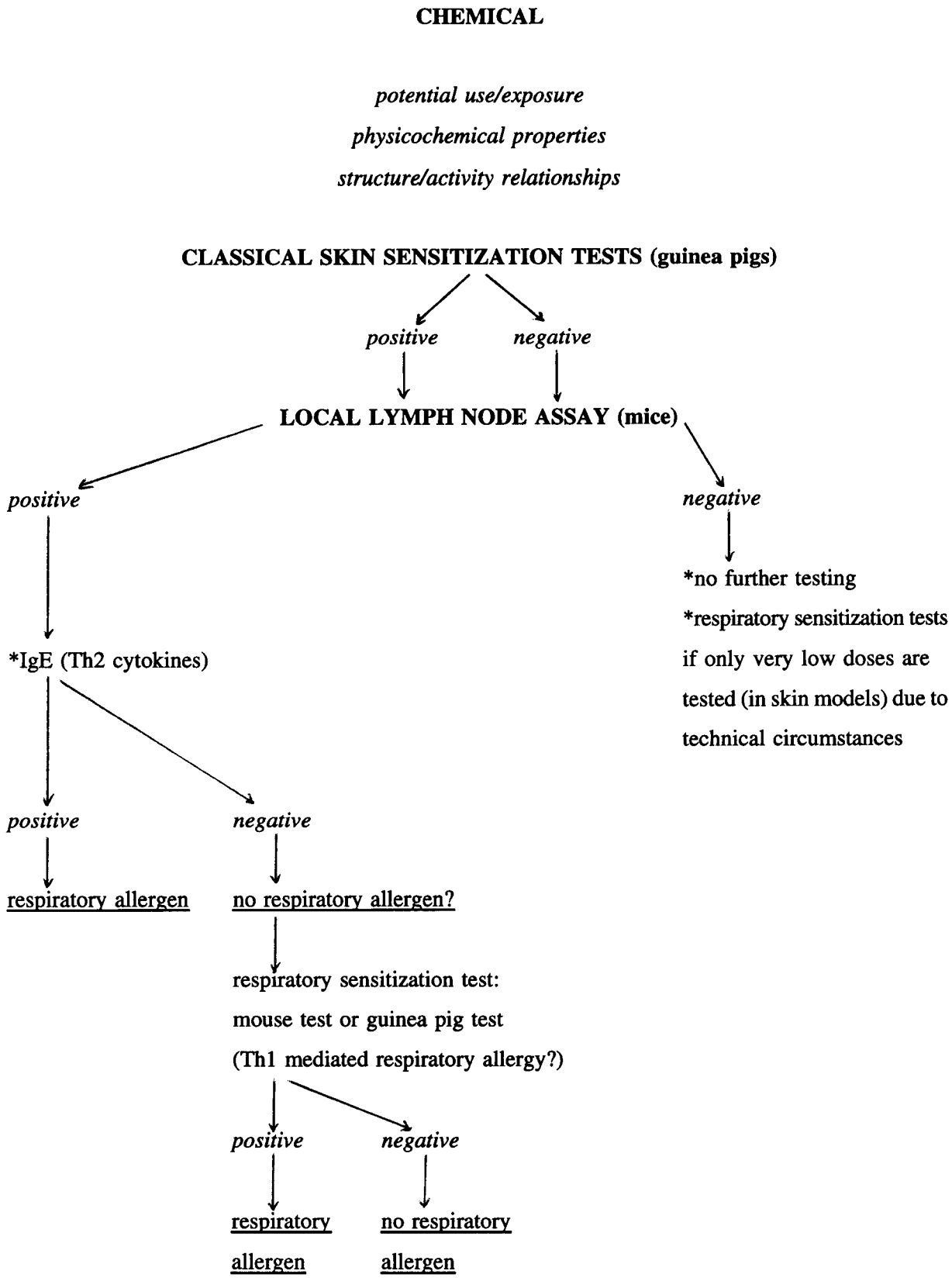
With respect to the sensitization capacity of chemicals animals can be exposed to compound on the skin or via the airways. Both routes will give enough information regarding the sensitizing capacity of the compound. However, if data dealing with respiratory allergic reactions are needed the animals have to be exposed to the compound via the airways after they have been sensitized (via the airways or the skin) in order to analyze whether the effector phase of the allergic reaction (i.e. airway hyperresponsiveness, pulmonar edema, pulmonary inflammation etc.) can occur in the pulmonary system.

3. RECOMMENDATIONS AND DISCUSSION

There are as yet no widely accepted and validated methods for the prospective evaluation of respiratory sensitizing potency or activity. In the present report a step by step approach is proposed for the identification and characterization of small molecular weight compounds as potential allergens, pertaining to the skin and the respiratory tract. This approach, schematized in Fig. 1, incorporates existing and newer developed test. The first step in hazard identification should include an examination of the physicochemical properties of the test chemical, particularly in the context of structure-activity relationships. With regard to identification of the respiratory hazard, important considerations include the volatility of the chemical and the likelihood that aerosols or vapours will be present during manufacture or use. Certain classes of chemicals may signal particular concern (e.g. isocyanates).

It appears that only those chemicals which exhibit at least some potential to cause skin sensitization in experimental animals are in principle able to induce respiratory allergy. Therefore an important step in screening of chemicals regarding their respiratory allergic potency is the performance of well conducted standard predictive tests for skin sensitization. The majority of these tests are restricted to the detection of delayed type hypersensitivity. Therefore additional tests are necessary. For this purpose the auricular lymph node assay (local lymph node assay) is proposed because this test can indicate whether sensitization occurs or not, indepedent upon the type of allergy that may be induced. Chemicals which lack the potential to cause sensitization, as determined by the local lymph node assay, can be classified also as lacking the ability to induce respiratory allergy and it is recommended that no further testing will usually be necessary. If, however, it has only been possible to perform the test at low concentrations for technical reasons, such as poor solubility of the test compound, and the results were negative, then respiratory sensitization testing may still be necessary. For the identification of the respiratory sensitization hazard of chemicals the methods described in section 2.2. can be used.

Figure 1. SCHEME FOR ASSESSMENT OF RESPIRATORY SENSITISATION



If the chemical can induce the production of Th2 cytokines (and IgE production) exposure to that chemical may lead to respiratory allergy. If it is indicated that no IgE titres are produced (no Th2 cytokine pattern in local lymph node assay) respiratory allergy will likely not be induced. However, there are some indications that certain low molecular weight compounds can induce respiratory hypersensitivity with features that are characteristic for asthma other than specific IgE titres such as: airway hyperresponsiveness, pulmonary oedema and pulmonary inflammation. Examples of compounds that can induce these phenomena without the induction of IgE production are: picrylchloride, di-nitro-chlorobenzene (DNCB), di-nitro-fluorobenzene (DNFB), trimelitic-anhydride (TMA), toluene-di-isocyanate (TDI), nickle, chromium salts, gold and colophony (see Garssen, 1991). With respect to compounds like these cytokine patterns that do not indicate Th2 responses may still have the potency to induce respiratory hypersensitivity, in these cases Th1 mediated immune responses may play a crucial role.

The models developed by Garssen et al. (1989 and 1991) and Stein-Streilein et al. (1983) may be useful for testing the capacity of low molecular weight compounds to induce IgE independent pulmonary hypersensitivity after exposure via the respiratory or skin route. In these models mice are used that are sensitized with low molecular weight compounds via the skin or via the respiratory tract. One week after skin or pulmonary sensitization the animals are challenged with the compound on the skin (ear) or intranasally. The read out systems are ear thickness measurements or wet ear weights if the animals were ear-challenged (i.e. contact hypersensitivity). If the animals are challenged intranasally the read out systems are: tracheal reactivity to cholinergic stimuli and serotonin (ex vivo/in vitro); pulmonary inflammation; pulmonary oedema and lung function (in vivo: pulmonary resistance and dynamic compliance) (Garssen et al. (1989 and 1991), Enander et al. (1983)).

Future refining of allergenicity tests may include the application of animal models/strains that have skewed their immune responses to either Th1 or Th2 responses.

References

Dearman RJ, Kimber I. Differential stimulation of immune function by respiratory and contact chemical allergens. Immunology 1991; 72: 563-570.

Dearman RJ, Mitchell JA, Basketter DA, Kimber I. Differential ability of occupational chemical contact and respiratory allergens to cause immediated and delayed dermal hypersensitivity reactions in mice. Int. Arch. Allergy Appl. Immunol. 1992; 97: 315-321.

Enander I, Ahlstedt S, Nygren H, Bjorksten B. Sensitizing capacity of derivates of picrylchloride after exposure of mice in the skin and in the lung. Int. Arch. Allergy Appl. Imm. 1983; 72: 59-66.

Gad SC, Dunn BJ, Dobbs DW, Reilly C, Walsh RD. Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). Toxicol. Appl. Pharmacol. 1986; 84: 93.

Garssen, J. Respiratory hyperresponsiveness induced by cellular immunity. PhD thesis, Utrecht, 1991.

Garssen J, Van Loveren H. Report on predictive testing with respect to the capacity of chemicals to induce respiratory allergy. RIVM report 312610002, 1994.

Garssen J, Nijkamp FP, Van Der Vliet H, Van Loveren H. T cell mediated induction of airway hyperreactivity in mice. Am. Rev. Resp. Dis. 1991; 144: 931-938.

Garssen J, Nijkamp FP, Wagenaar SjSc, Zwart A, Askenase PW, Van Loveren H. Regulation of delayed type hypersensitivity like responses in the mouse lung, determined with histological procedures: Serotonin, T cell-suppressor inducer factor and high antigen dose tolerance regulate the magnitude of T cell dependent inflammatory reactions. Immunology 1989; 68: 51-58.

Karol MH, Griffiths-Johnson DA, Skoner DP. Chemically induced pulmonary hypersensitivity, airway hyperreactivity and asthma. In: "Toxicology of the lung", Ed. Gardner et al. 2nd. edition, Raven Press, N.Y. 1993; 417-433.

Kimber I, Mitchell JA, Griffin AC. Development of a murine local lymph node assay for the detection of sensitization potential. Fd. Chem. Tox. 1986; 24: 1

Maisey J, Miller K. Assessment of the ability of mice fed on vitamin A supplemented diet to respond to variety of potential contact sensitizers. Contact Dermatitis 1986; 15: 17.

Sarlo K, Clark ED. A tier approach for evaluating the respiratory allergenicity of low molecular weight chemicals. Fund. Appl. Toxicol. 1992; 85: 55-58.

Stein Streilein J. Allergic contact dermatitis induced by intratracheal administration of hapten. J. Immunol. 1983; 131: 1748-1753.