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REPORT ON PREDICTIVE TESTING WITH
RESPECT TO THE CAPACITY OF CHEMICALS
TO INDUCE RESPIRATORY ALLERGY

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CONTENTS

MAILINGLIST	II
CONTENTS	III
SUMMARY	IV
SAMENVATTING	1
1. INTRODUCTION	2
2. IMMUNOLOGICAL HYPERSENSITIVITY REACTIONS	3
3. PREDICTIVE TESTING	9
3.1. Respiratory sensitizing tests	10
3.2. Skin sensitizing tests	12
3.2.1. Sensitization tests without adjuvant	12
3.2.2. Sensitization tests with adjuvant	12
3.2.3. Alternatives	13
4. CONCLUSIONS/RECOMMENDATIONS	15
5. REFERENCES	16

SUMMARY

At least 10% of the population of the western world is suffering from respiratory syndroms with characteristics of asthma or COPD (chronic obstructive pulmonary disease). The prevalence and severity of asthma like diseases is increasing and even in some countries mortality has risen. In the United Kingdom asthma is responsible for about 2000 deaths per year. In addition to inheritable components, it is known that exaggerated immune responses against inhaled compounds can lead to pulmonary diseases (respiratory allergy). More than half of the number of asthma cases are induced by type I hypersensitivity immune reactions (i.e. extrinsic asthma). The asthma cases that are not induced by these type I hypersensitivity reactions can be non-immunological mediated (i.e. intrinsic asthma) or induced by other subtypes of hypersensitivity reactions such as type III or IV hypersensitivity.

Predictive tests with respect to the capacity of chemicals to induce one or more types of respiratory hypersensitivity are necessary. Unfortunately there are almost no tests available in order to test the sensitizing capacity of compounds leading to respiratory allergy. The majority of the predictive tests are restricted to the capacity of chemicals to induce skin-type hypersensitivity reactions. The applicability of these tests for predictive testing regarding the capacity of compounds/chemicals to induce respiratory hypersensitivity is restricted.

SAMENVATTING

Minstens 10% van de westerse wereld bevolking lijdt aan een vorm van astma of CARA (chronische aspecifieke respiratoire aandoeningen). De incidentie en de ernst van deze respiratoire aandoeningen lijkt toe te nemen. Alleen in Engeland zijn deze aandoeningen al verantwoordelijk voor ongeveer 2000 doden per jaar. Het is bekend dat naast genetische (erfelijke) componenten ook immunologische overgevoeligheds reacties gericht tegen geïnhaleerde chemische stoffen een rol kunnen spelen bij deze luchtweg aandoeningen. Meer dan de helft van de astma gevallen wordt geïnduceerd door type I overgevoeligheid en zijn dus IgE afhankelijk (=allergische astma). De astma gevallen die niet door type I overgevoeligheid worden geïnduceerd zijn mogelijk niet immunologisch gemedieerd (=intrinsieke astma) of geïnduceerd door andere immunologische overgevoeligheds reacties (type III of IV).

Om bovengenoemde redenen zijn testen nodig om chemicaliën te onderzoeken met betrekking tot hun capaciteit om respiratoire overgevoeligheid te induceren. Deze testen zijn met betrekking tot de inductie van respiratoire overgevoeligheid echter niet voorhanden. Het merendeel van deze testen is bedoeld voor het screenen van de te testen chemicaliën op de mogelijke eigenschap om huidovergevoeligheid te induceren. De mogelijkheid om deze huidovergevoeligheds testen ook te gebruiken voor het testen op de potentie van deze stoffen om respiratoire overgevoeligheid te induceren is beperkt.

1. INTRODUCTION

The essential function of the immune system is defense against neoplastic cells and infectious agents such as parasites, viruses, fungi, and bacteria. The immune system is divided into two parts. The non-specific part is characterized as a physical/chemical barrier found in e.g. the skin and mucosal tissues of the respiratory and gastro-intestinal tracts. In addition to the cells of the physical/chemical barrier, such as epithelial and mucosal cells, also natural killer cells, macrophages and polymorphonuclear cells play an important role in the aspecific immune response. The specific part of the immune system can recognize, remember, and/or respond to unique antigens. Cells from the specific immune system are predominantly B- and T-lymphocytes.

The specific part of the immune response can be sub-divided into a humoral and a cellular immune system. In the humoral system B-lymphocytes and in the cellular immune system T-lymphocytes play a predominant role. B-lymphocytes, originating from the bone marrow, produce, after maturing into plasma cells, immunoglobulins that can bind antigens via the combining sites of their variable regions. Each matured B-lymphocyte (plasma cell) can produce antibodies with only one particular specificity. In general, antibody-binding serves to identify the antigen.

T-lymphocytes see antigen only as short stretches of amino acids, presented by molecules of the Major Histocompatibility Complex (MHC). MHC molecules are generally polymorphic between individuals. Within one individual or within one inbred strain of animals the MHC molecules are identical. A T-lymphocyte specific for e.g. ovalbumin will recognize ovalbumin only in the context of a certain MHC molecule. This phenomenon is called MHC restricted recognition. MHC and antigen are corecognized by the T cell receptor (TCR). The TCR is the antigen recognition molecule of T-lymphocytes. Antigen presentation takes place very quickly upon the entry of antigen into lymphoid tissue. Macrophages and Langerhans-dendritic cells will be responsible for the early recruitment and activation of especially CD4⁺ T-lymphocytes (i.e. T helper cells). Also B-lymphocytes with immunoglobulin molecules on their surface with specificity for the antigen can also participate by binding the antigen (proteins, high molecular weight compounds), processing it, and presenting it to the CD4⁺ T-lymphocyte. The precise mechanisms responsible for the presentation of low molecular weight compounds such as non-peptides are unknown at present. It is suggested that some of these

small molecular weight compounds can act as a hapten and bind to protein carriers leading to an immune response against this carrier/hapten complex, e.g. against a hapten bound to self-albumin or a hapten bound to the MHC itself.

In addition to an important role in cellular immunity, T-lymphocytes play also an important role in humoral immune responses: most humoral immune responses depend on helper T-cell activity. Isotype switch, an important phenomenon in type I hypersensitivity, also depends on activity of specific helper T-cells.

2. IMMUNOLOGICAL HYPERSENSITIVITY REACTIONS

Immune reactions usually form a host-defense system. But under certain circumstances immune reactions can lead to tissue injury. These *hyperimmune* reactions (hypersensitivity or allergy (Greek:allergy=altered reactivity)) can be induced by low molecular and high molecular weight compounds.

The major part of allergic diseases are induced by high molecular weight compounds, i.e. in majority proteins or (poly)saccharides having a size larger than 10 K daltons. In many instances the 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 induce specific IgE production or act as a hapten leading to T cell mediated immune responses. Most of these compounds possess electrophilic functionalities which enable the small molecular weight compounds (chemicals) to react upon contact with nucleophilic moieties such as sulfhydryl, hydroxyl, or amino groups on biologic molecules. Since it is generally accepted that covalent (or high affinity) binding of chemicals to macromolecules is essential to engender small molecules with immunogenicity, an electrophilic property of chemicals would be highly desirable for immunogenic activity. However an electrophilic functionality 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 contributing to the immunogenicity of small chemicals, is neither definitive nor sufficient for conferring respiratory sensitizing activity on chemicals.

Subtypes of allergy

The adverse reactions (allergy reactions) leading to tissue injury are mediated by different "specific" immune responses and are classified according to the immune mechanism that is responsible for this adverse immune reaction. Due to this classification allergy reactions are subdivided into 5 subtypes (1).

Type I, immediate hypersensitivity, is mediated by IgE and is pertinent to the pathogenesis of e.g. hay fever, atopic (allergic/extrinsic) asthma, and urticaria. This type of allergy is responsible for the major part of allergy. Nearly 10% of the western population suffers from Type I hypersensitivity reactions. The majority of these reactions are strongly localized anaphylactic reactions to extrinsic antigens (allergens) such as: grass pollen, birch pollen, cat dander, house dust mite (*Dermatophagoides pteronyssinus*), ragweed, wasp and bee sting compounds, penicillin etc. The antigens are mostly high molecular weight compounds.

After first contact with the specific antigen such as grass pollen antibody producing cells (B cells) will produce specific IgE and/or IgG4 both of which can bind to mast cells and basophils. Second contact with the allergen will trigger the whole cascade of the allergic reaction. Contact of the allergen with IgE armed inflammatory cells, such as mast cells and partly also basophils, will result in release of inflammatory mediators. In the bronchial tree, nasal mucosa, and conjunctiva this will induce asthmatic complaints. Even awareness of the

importance of sensitization to food allergens in the gut has increased. Contact of the food with IgE armed mast cells in the gut may induce local type I like reactions such as diarrhoea and vomiting or may allow the allergen to enter the body by causing alterations in gut permeability after mediator release. Allergen/antibody complexes may cause lesions at other places e.g. in the lung or skin.

Mast cells play a pivotal role in type I hypersensitivity reactions. Mast cells display high affinity receptors for the Fc part of IgE molecules. In addition to IgE also IgG4 can bind to specific receptors for this immunoglobulin on mast cell surfaces. However the majority of type I hypersensitivity is based upon the interaction between IgE and mast cells. In addition to mast cells basophils have also IgE receptors however the affinity of the IgE receptors on mast cells is much higher as compared to basophils.

The essential role of IgE in the pathogenesis of allergic diseases like atopic asthma has stimulated considerable study into the mechanisms by which IgE synthesis can be regulated. Using several animal models, like a model in which nude athymic mice were used, it is demonstrated that collaboration between T and B cells is essential for the induction of IgE synthesis by B cells (2). It is demonstrated that T helper cells stimulate and T suppressor cells inhibit IgE production by B cells (3,4). Recently it is found that T helper cells can be subdivided into two subtypes of T helper cells, i.e T helper 1 and T helper 2 cells (5,6). Especially T helper 2 cells play a crucial role in the induction of IgE synthesis (7).

Cross-linking of IgE by e.g. divalent hapten will trigger mediator release. Under normal circumstances release of mast cell mediators help to orchestrate the development of a defensive acute inflammatory reaction for example during parasitic (worm) infections. But under certain conditions as in atopic diseases, bronchoconstrictive and vasodilatory effects predominate and may become distinctly threatening.

Diagnosis of type I hypersensitivity is validated using the skin prick test or Prausnitz-Kustner test (passive cutaneous anaphylaxis). The allergen in the prick test is applied into the skin leading eventual to a skin weal and flare response. Radio-allergosorbent tests of patient sera can confirm the diagnosis hypersensitivity type I.

Type II hypersensitivity refers to cytotoxic antibodies produced by the host against his own tissues (auto-antibodies). This mechanism appears to be responsible for e.g. Goodpasture's syndrome. In this disease auto-antibodies (in majority IgG) against a basement membrane antigen are formed. Because this subtype hypersensitivity is in majority induced

by autoantibodies it is denoted as (auto)antibody-dependent cytotoxic hypersensitivity. Antibodies directed against e.g. membranes or cell surface antigens will bind to effector cells with Fc receptors. Activation of these "antigen" - antibody complexes will activate complement component C3 leading to cell lysis or membrane damage. Antibodies directed against cell surface antigens cause cell death not only by complement dependent lysis but also by adherence reactions leading to phagocytosis or through non-phagocytic extracellular killing by certain lymphoreticular cells (antibody-dependent cell-mediated cytotoxicity). Type II like hypersensitivity reactions may be responsible for e.g.: autoimmune haemolytic anaemia, Goodpasture's syndrome, haemolytic disease of newborns (HDNB), and Hashimoto's thyroiditis. Mismatched organ transplantation or blood transfusion may also induce type II like hypersensitivity responses leading to the killing of cells via immunoglobulin/complement dependent mechanisms.

Type III hypersensitivity involves tissue damages secondary to the formation of immune complexes. This reaction (Arthus reaction) is responsible for e.g. hypersensitivity pneumonitis or allergic alveolitis and possibly for late phase reactions (LAR) in asthma. This immune complex mediated hypersensitivity results from the deposition of immune complexes in blood vessel walls and tissues. Antigen-antibody binding can ultimately lead to the formation of insoluble complexes. These complexes may be responsible directly for local inflammatory reactions and platelet aggregation. If the antibody-antigen complex activates complement this will lead to the attraction of polymorphonuclear cells. These (aspecific) inflammatory cells can trigger a cascade leading to an intense inflammatory reaction. On the other hand activated complement components can stimulate mast cell degranulation as an anaphylatoxin or can activate IL-1 production by macrophages. The inflammatory reactions induced by type III like reactions are also called Arthus reactions. This reaction, an oedematous and inflammatory response, peaked at 3-8 hours after antigen provocation. The cells that are dominant in the inflammatory reaction are polymorphonuclear cells. Sometimes this is followed by infiltration of mononuclear cells, 24-48 hr after antigen provocation. It is hypothesized that type IV hypersensitivity mechanisms (see below) are involved in some forms of type III hypersensitivity, such as Pigeon Fancier's disease (a kind of extrinsic allergic alveolitis).

Diagnosis of type III hypersensitivity can be performed using the haemagglutination and precipitin tests (in vitro) or the arthus reaction (skin) or broncho-provocation (lungs) tests (in vivo).

Type IV hypersensitivity is exemplified by the well known delayed hypersensitivity cutaneous reaction to tuberculin. This type of hypersensitivity is likely to be responsible for granulomatous lung diseases, such as tuberculosis and sarcoidosis, and for inflammatory reactions found in some forms of chronic obstructive pulmonary diseases (COPD). Contact hypersensitivity, e.g. nickel allergy induced by earrings, is an example of a skin associated Type IV hypersensitivity reaction. Delayed type hypersensitivity (DTH) is a T cell dependent immune phenomenon manifested by an inflammatory reaction, at the site of antigen provocation (mostly the skin), that is maximal 1 to 2 days after antigen deposition. The DTH reaction was initially observed by Koch in the dermal reactivity of tuberculous individuals towards tuberculin (8). This is referred to as classic DTH. DTH reactions could in turn be subdivided into 4 subtypes (9). Tuberculin type hypersensitivity is denoted as the classical DTH type. This classical DTH reaction is induced by injection of protein antigens from e.g. the tubercle bacillus. The DTH response can be elicited months after priming (sensitization) and the reaction (erythema, induration) peaks 1 to 2 days after subsequent local interdermal antigen deposition. The local infiltrate consists of mononuclear cells. A second subtype of DTH is contact hypersensitivity. This subtype resembles the classic tuberculin subtype reaction, but it is predominantly an epidermal reaction, caused by contact-allergens. A third subtype reaction, the Jones-Mote hypersensitivity reaction, is characterized by a cutaneous infiltration of especially basophils. This response differs in several aspects from classic DTH responses. In contrast to the classical DTH reaction (tuberculin subtype) and the contact hypersensitivity subtype reaction serum can transfer the Jones-Mote reaction. It peaks somewhat earlier than the other subtypes, i.e. 24 hr after local antigen treatment. The last subtype of DTH is granulomatous hypersensitivity. In this form of DTH localized inflammatory responses are composed predominantly of mononuclear cells (especially macrophages). The infiltrating cells arise after chronic stimulation with persistent foreign materials or living agents (10). In contrast to the other subtypes the reaction time is at least a few weeks.

Type V, which is the last subtype of hypersensitivity is an autoimmune mediated response. In this subtype of hypersensitivity non-complement binding antibodies directed against certain cell surface components may actually stimulate rather than destroy the cell. This subtype of hypersensitivity can therefore be recognized as stimulatory hypersensitivity. An example is thyrotoxicosis, a disease in which autoantibodies (over)stimulate thyroid cell

receptors. In healthy persons only the thyroid stimulating hormone (TSH) is active in stimulating the thyroid receptor.

In summary Types I,II, III, and V depend on the interaction of antigen with antibodies and tend to be called immediate or intermediate type reactions. Type IV involves receptors bound to the lymphocyte surface and because of the longer time course this has been referred to as delayed type hypersensitivity (DTH). It is now clearly recognized that all the subtypes of hypersensitivity reactions as mentioned above can occur in the respiratory system leading to pulmonary disease.

At least 10% of the population of the western world is suffering from respiratory syndroms with characteristics of asthma or COPD (chronic obstructive pulmonary disease). The prevalence and severity of asthma like diseases is increasing and even in some countries mortality has risen (11-13). In the United Kingdom asthma is responsible for about 2000 deaths per year. In addition to inheritable components (14,15), it is known that exaggerated immune responses against inhaled compounds can lead to pulmonary diseases (respiratory allergy). More than half of the number of asthma cases are induced by type I hypersensitivity immune reactions (i.e. extrinsic asthma). The asthma cases that are not induced by these type I hypersensitivity reactions can be non-immunological mediated (i.e. intrinsic asthma) or induced by other subtypes of hypersensitivity reactions such as type III or IV hypersensitivity (16-21).

Complaints induced by pulmonary hypersensitivity can be subdivided (clinically) into early/immediate (EAR/IAR) and late onset reactions (LAR) (22,23). Inhalation of certain allergens may result in bronchoconstriction within several minutes after inhalation. In most cases this bronchoconstriction resolves within a few hours. This early airway narrowing is called the IAR. In some of the cases the airway narrowing persists and either does not return to baseline values or recurs after a few hours. This late airway narrowing (2-8 hr after inhalation) is called the LAR. In some cases isolated LAR occurs. The IAR is mostly characterized by smooth muscle contraction, vascular leakage, flush, hypotension, and mucus secretion whereas the LAR is mostly characterized by an inflammation consisting of eosinophils, neutrophils, fibrin deposition, and the occurrence of airway hyperresponsiveness. The LAR is often followed by a very late response (VLAR), i.e. 1-2 days after inhalation, that is characterized by an infiltrate of mononuclear cells and tissue destruction (23). Especially

the cellular phase of the inflammation is often associated with airway hyperresponsiveness, i.e. increased sensitivity for bronchoconstricting agents (24).

It is generally accepted that IAR to respiratory allergens are mediated by specific IgE antibodies. Direct evidence for this phenomenon has been obtained from studies employing high molecular weight compounds/allergens. With respect to low molecular weight compounds/chemicals, specific IgE has been identified in only a small percentage of affected individuals. This is in distinction to the situation noted with high molecular weight compounds where detection of specific IgE is a frequent occurrence. Caution is thereby warranted in acceptance of IgE as the sole mechanism of chemically induced respiratory sensitivity. A second major distinction between respiratory hypersensitivity induced by high molecular weight compounds in comparison to respiratory hypersensitivity induced by low molecular weight compounds is that most responses to low molecular weight compounds are manifested as LAR and not IAR. The mechanisms of LAR are uncertain. It has been hypothesised that LAR are analogous to type IV hypersensitivity reactions, and are mediated by CD4 positive T cells. Evidence for the involvement of T cells in late-phase respiratory responses to chemical sensitizers has been reported. Mononuclear cells were observed around bronchioli and other airways of mice sensitized to picrylchloride and challenged by intranasal exposure to the chemical. The histological changes were accompanied by physiologic changes, most notably airway hyperreactivity (25,26,27).

3. PREDICTIVE TESTING

Predictive tests for the evaluation of skin-sensitizing effects of chemicals exist since approximately 50 years (28). In 1981 and 1984 guidelines for the testing of sensitizing effects were published by respectively the OECD and the EC (29,30). In these guidelines 3 protocols in which adjuvants were not used, and 4 protocols in which Freund's Complete Adjuvant (FCA) was used, were included. The most recent guidelines for the testing of sensitizing effects were accepted in 1992 (OECD guidelines) (31). In addition to these protocols a few more tests are available for skin-sensitizing effects of chemicals are available (32,33).

In contrast, tests for the screening of sensitizing effects of compounds leading to respiratory allergy are less 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 compound can really induce respiratory hypersensitivity is doubtful.

3.1. Respiratory sensitizing tests

Methods for identifying respiratory sensitizing chemicals include:

1. In vitro methods such as determination of reactivity of suspect chemicals with proteins.
2. In vivo methods such as : a] Injection of a chemical to animals with subsequent determination of increase in total IgE, or specific antibody (IgE and/or IgG). b] Inhalation exposure of animals followed by determination of immediate (IAR) or late (LAR) onset reactions (lung function measurements), antibody production, pulmonary histopathology, and airway hyperreactivity (in vitro and in vivo).

In vitro methods: There are some in vitro methods available for the prediction of sensitizing properties of inhaled chemicals. Wass et al. (34) used high-pressure liquid chromatography techniques in order to monitor reactivity of a suspect chemical toward a lysine-containing peptide (i.e.binding). Evidence of binding was obtained with some well known respiratory sensitizers (chemicals). However, positive results were also obtained with reactive chemicals which have not been classified as respiratory sensitizers. It is unclear how this test would distinguish contact (type IV) from respiratory sensitizers (type I, IV and others?) as both groups of compounds are known to be chemically reactive.

Another in vitro approach is incorporated in a combined in vitro/in vivo procedure to evaluate the potential of low molecular weight compounds for causing respiratory hypersensitivity (35). This combined test is composed of four levels of testing. The first phase in this procedure is an examination of the structure of the chemical to assess its likely ability to react covalently with proteins. This part of this procedure includes especially literature studies to obtain information on reported immunogenic effects in man and animal of the chemical or structurally related chemicals. During the second phase an in vitro test of the chemical's ability to react with protein is performed. If the suspected compound can bind proteins the third level of experiments will be performed. In these studies the ability of the chemical to

invoke immunogenic changes in vivo were assessed. The chemical is injected subcutaneously into guinea pigs. These animals were injected two times a week during 4 weeks followed by a further injection one week later. Seven days after the last injection the titers of specific and non-specific antibodies is determined using the ELISA technique (IgG1 and IgE). A positive ELISA result is taken as evidence that the compound can modify the immune system leading to hypersensitivity (allergy). The passive cutaneous anaphylaxis test determines the titer of hypersensitivity. These findings are used as one indication of the respiratory potency of the compound. Additionally chemical-protein conjugates were administered intratracheally in guinea pigs in order to assess IAR. Diaphragmatic contractions were monitored during 10 minutes after intratracheal provocation in order to determine the possible IAR. Finally in the fourth level of screening the chemical is administered by inhalation which is the relevant route. Changes in respiratory rate and diaphragma contractions were monitored. Only IAR are monitored in this protocol.

It is necessary to test this approach further in order to determine the ability of this combined in vitro/in vivo approach to distinguish skin from pulmonary sensitizers.

In vivo methods: Different animal models are available in order to test sensitizing capacity of compounds. The majority of these animal models are restricted to high molecular weight compounds (proteins etc.). Few have been applied to test/use low molecular weight compounds. The guinea pig model developed by Karol et al. has proven to be valuable for chemical allergens (low molecular weight) (36). The mouse model developed by Garsen et al. demonstrated that low molecular weight compounds can induce respiratory syndromes (25-27). In addition to providing information as to the sensitization capability of chemicals, the animal models offer the opportunity to identify distinguishing features (i.e. patterns of antibody and cytokine production) between responses to high molecular weight vs. low molecular weight compounds.

Parameters that are determined in animal models after inhalatory exposure to chemicals are: changes in breathing (rate), pulmonary pressure, cyanosis, inflammation, airway reactivity, IAR and or LAR, Ig titers, vascular leakage.

3.2. Skin sensitizing tests

3.2.1. Sensitization tests without adjuvant

Bühler test: This guinea pig "skin" test was developed to screen strong and moderate sensitizers prior to testing in humans (37,38). The sensitivity of this test seems to be somewhat more sensitive than the Draize test. An advantage of the Bühler test is that the route of application is similar to that in man. A disadvantage of this test is that very low incidences of positive guinea pigs were obtained with well known sensitizers. Due to this disadvantage several false negatives were found. The test is improved using the occlusive application device, the Hill Top Chamber (39).

Draize test: This "skin" test is less sensitive than the Bühler test. Modifications of this test, such as increase in test-chemical concentration, or repeated induction procedures were made. However, these procedures failed to increase the test-sensitivity substantially. An advantage of this test is that this test is developed almost 50 years ago (28,40,41).

Open epidermal test: Open epidermal application of chemicals can be used for induction and challenge tests. This is the only test in which different concentrations for induction and challenge can be tested. It is possible to define the minimal sensitization and elicitation concentration of the chemical (42).

Whether these tests are useful for predictive screening with respect to the capacity of chemicals to induce respiratory allergy is doubtful.

3.2.2. Sensitization tests with adjuvant

Maximization test: In this "skin" test the sensitivity of the guinea pig test is increased by: 1. the injection of FCA during the sensitization phase, 2. using the maximal tolerated concentrations, and 3. irritation of the application site prior to the induction phase (43). False negatives in the Draize test are positive in this test. Modifications for non-injectable chemicals are available (44).

Split adjuvant test: A combination of adjuvant injections and open dermal application of the test-chemical (45).

FCA test: Similar as the open epidermal test. The induction consists now of 5 compound injections in adjuvant and epidermal open challenge (42).

Optimization test: This "skin" test is limited used probably due to the longer duration of the test because separate intradermal and epidermal challenge applications are regularly used. This test makes it possible to get information on the effects on intact and diseased skin (46).

Whether these tests are useful for predictive screening with respect to the capacity of chemicals to induce respiratory allergy is doubtful.

3.2.3. Alternatives

Tests using the mouse: The latest predictive *in vivo* protocols established use the mouse instead of the guinea pig. Three additional screening methods were accepted by the OECD in 1992.

1. Animals fed with higher amounts of vitamine A leading to enhanced responses in mice (47).
2. Combination of adjuvant injections and epidermal application of the chemical during induction and epidermal treatment of the ear for challenge. The ear thickness increment is the parameter. This test is not sensitive enough to screen moderate and weak sensitizers (48).
3. Treatment of mice on the ear during three days with the compound followed by measurement of the proliferation of the local lymph node cells (local lymph node assay) (49).

These protocols were in majority aimed at the the screening of potential skin-sensitizers and not respiratory sensitizers.

Tests based on cytokine profiles (mouse): In 1986, the existence of two CD4⁺ T helper (Th) cell subsets was discovered in mice, and they were designated Th1 and Th2 (5). 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 cell surface marker. They produce, however, defined patterns of cytokines that lead to strikingly different T cell functions (6,7). Roughly speaking, Th2 cells are more 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 humans (50).

Dearman et al. have shown that chemicals differ with respect to the types of hypersensitivity they preferentially induce (51-53). Importantly, the site of hypersensitivity (skin vs. respiratory system) proved important to the type of hypersensitivity (Th1 vs. Th2). However, this is not true in all cases. Garssen et al. (25-27) have shown that skin sensitization with the low molecular weight compound picrylchloride can induce respiratory hypersensitivity with features of type IV hypersensitivity. They have demonstrated that this respiratory hypersensitivity response was induced by T cell dependent mechanisms and that IgE was not involved. This suggests that Th1 cells can play an important role in the induction of pulmonary hypersensitivity by small molecular weight compounds such as picrylchloride. In man specific IgE can be detected in only a few patients suffering from TDI (toluene-di-isocyanate, a small molecular weight compound)-induced respiratory allergy. This does not mean that the immune system has no pivotal role in this disease. It can be hypothesized that TDI can induce respiratory allergy via Th1 mediated immune mechanisms such as type IV hypersensitivity in addition to Th2 mediated responses. Recently it is demonstrated that TDI can induce similar effects in the pulmonary system as was described in the picrylchloride model (Scheerens et al, unpublished).

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 profiles inform about the type of allergy induced (Th1 or Th2 mediated) (51-53). Compounds that induce Th1 mediated reactions are thought to be skin-sensitizers whereas compounds that induce Th2 mediated reactions are thought to be inducers of respiratory allergy. A disadvantage of this test is that Th1 mediated responses can occur in the respiratory system and not only in the skin.

4. CONCLUSIONS/RECOMMENDATIONS

Allergic diseases are, in addition to cardiovascular diseases and neoplastic diseases, the major death causes. Allergic diseases may affect more than 10% of the population and yet remains underdiagnosed. It's prevalence is increasing, even in some countries mortality due to these diseases has risen. Most studies are restricted to the induction of allergy by high molecular weight compounds, such as pollen and house mites, etc. Mechanisms responsible for the induction of allergy by small molecular weight compounds are less well known. Increasing knowledge of the mechanisms leading to allergic diseases induced by high and small molecular weight compounds can provide insight into the pathogenesis of allergic diseases and improve possibilities for predictive testing, prevention, and therapy.

The majority of the tests available are aimed at screening of sensitizing capacity of the compound on the skin. The results of these studies can be used for the prediction of sensitizing capacity with respect to respiratory allergy only partially.

The statement that compounds that can induce Th1 responses are inducers of only skin-allergy (type IV allergy) and that compounds that can induce Th1 and Th2 responses are inducers of skin and respiratory allergy is dangerous. It is true that compounds that can induce Th2 mediated immune responses can induce allergic reactions in the skin as well as in the respiratory tract. However compounds that can induce Th 1 mediated responses can induce respiratory allergic reactions in some cases. For this reason current tests are shortcoming with respect to screening of potency of the compound to induce respiratory hypersensitivity. New tests have to be developed and validated. The test developed by Garssen et al (25-27) may be one of these.

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