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RISK ASSESSMENT OF CONTACT ALLERGENS: A FEASIBILITY STUDY

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SAMENVATTING

Huid sensibilisatie, allergische contact dermatitis, is weliswaar geen levensbedreigende aandoening, maar toch kan deze aandoening het functioneren van getroffenen ernstig beperken.

Daarom wordt bij de toelating van stoffen, waaronder chemicaliën, huishoudprodukten en kosmetica, inzicht in de sensibiliserende eigenschap van de stoffen geëist.

In het algemeen wordt deze sensibiliserende eigenschap in diermodellen onderzocht. Door de opzet van deze diermodellen is slechts inzicht in de intrinsieke sensibiliserende eigenschap mogelijk.

Voor een goede risicoschatting is echter meer inzicht in de kwantitatieve aspecten van het sensibiliserend karakter van een stof nodig. In deze studie is onderzocht welke beperkingen de huidige diermodellen voor een dergelijke kwantitatieve risicoschatting hebben. Vervolgens worden aanpassingen aan de testprotocollen voorgesteld, alsmede additioneel onderzoek met behulp van *in vitro* modellen. Tenslotte wordt een test strategie voorgesteld.

SUMMARY

Skin sensitization, allergic contact dermatitis, is not a life-threatening condition but it can be very impairing. For that reason contemporary legislation prescribes the assessment of the skin sensitizing properties of substances like chemicals, house-hold and personal-care products.

In general skin sensitization is assessed by animal test models. However the commonly used animal models (guinea pig and mouse models) are limited to hazard identification, i.e. the assessment of the intrinsic sensitizing capacity.

Every day practice shows the need for quantitative risk assessment. For that reason in this study currently used animal tests are analyzed for their limitations to quantitative risk assessment. Recommendations are put forward to adapt the test protocols and additional *in vitro*-methods are suggested to quantify the immune response.

Furthermore a test strategy is suggested.

1. INTRODUCTION

Skin sensitization is also known as contact allergy and allergic contact dermatitis. Contact allergy is a persistent condition, once sensitized to a substance (e.g. chemicals, house-hold and personal-care products) an individual is at risk of dermatitis whenever exposed to the same substance. Although allergic contact dermatitis is not a life-threatening condition, it can be very impairing. Therefore, contemporary legislation prescribes the assessment of the skin sensitizing properties of substances.

Though, sensitization test procedures using human volunteers are developed ethical reasons (the risk to induce sensitization in healthy volunteers and the use of positive control agents) and logistic problems (a large number of non-homogenous volunteers is necessary to obtain a sufficient sensitivity) do hamper these test methods to be used in the assessment of contact allergy.

International guidelines (1, 2, 3) recommend a limited number of standardized predictive animal tests. In general the guinea pig is the laboratory animal of choice. Nevertheless, mouse models have been developed (4,5,6).

The first useful test was developed by Draize, Woodard and Calvery in 1944 (7), which was based on the immunological observations described by Landsteiner and co-workers (8-12). In the last three decades variations on the basic procedures and several new procedures have been introduced. (13-19). Besides the variation in administration of the testsubstance to guinea pigs, i.e. intradermal, topical, combination of both ways and occlusiveness, the use of Freund's complete adjuvant to enhance the immunological response are the major differences in the test procedures.

These tests are used to classify substances (e.g. in the European Union under Annex VI of the Dangerous Substances Directive), however, there is no international agreement on the prevalence of test methods or on the interpretation of the results. Furthermore, the relevance to human risk assessment is unclear. Additionally, the sensitivity of each tests is still difficult to assess, except that in general methods using adjuvant are superior to non-adjuvant methods in detecting weak contact sensitizers. For that reason the usefulness of the commonly used animal tests is limited to hazard identification and therefore cannot be used for quantitative human risk assessment.

Every day practice, e.g. the occurrence of contact allergy for cosmetics, shows the need for quantitative risk assessment. Although the development of contact allergy depends on several features like the degree of exposure to a substance (concentration, time, circumstances) and genetic susceptibility, the intrinsic sensitizing capabilities (weak, moderate or strong sensitizer) are also important. The aim of this study is to delineate the shortcomings in the experimental design of the predictive animal tests for a quantitative risk assessment and to indicate possible adaptations.

2. BASIC PRINCIPLES OF SKIN SENSITIZATION

The immunologic adverse reactions determined in these animal models are classified as a type IV hypersensitivity reaction (20). This delayed type hypersensitivity is a T-cell mediated skin response which take 24-48 hours after antigen contact to reach maximum intensity. The delayed type hypersensitivity reactions can be subdivided into four subtypes, the tuberculin type, the contact hypersensitive type, the Jones-Mote type and the granulomatous type. Regarding the scope of this report only the principle immunological reactions of the contact hypersensitive type will be summarized. Detailed information on the other subtypes can be found elsewhere (21,21).

Two main phases can be distinguished in the process of skin sensitization these are the induction and the elicitation phase. Sensitization is developed in the induction phase. Some important events in the induction phase are: skin penetration of the allergen; binding of allergens to so-called Langerhans cells; T-cell recognition of allergen-modified Langerhans cells; proliferation of specific T-cells; dissemination of primed effector and memory T-cells.

In the elicitation phase memory T-cells recognise the allergen by re-exposure through the Langerhans cells and will become activated. This will lead to the production and release of lymphokines, such as interleukin-2 (IL-2), IL-3 IL-4 and interferon-γ. These mediators will attract more lymphocytes and macrophages. The latter population becomes activated and non-selectively aggressive. Local tissue destruction and mononuclear cell infiltration leads gradually to an eczematous, local inflammatory reaction.

Chemicals tested for skin sensitization are in general of low molecular weight, i.e. 1000 dalton or less. To induce an allergic response, i.e. to act as an antigen, these haptens have to conjugate to skin proteins. In the induction phase the hapten-protein complex binds to so-called class II Major Histocompatibility Complex (MHC) molecules on the Langerhans cellsurface. The Langerhans cells are the only cells in normal skin which are capable of presenting antigens to T-cells. It is thought that the hapten-protein complex is internalized by the Langerhans cell and is processed into immunogenic antigen fragments. These fragments are expressed in combination with MHC class II molecules at the cell surface membrane. Hereafter the Langerhans cells migrate to the draining lymph nodes. Mediators like IL-1β and tumour necrosis factor α (TNF-α) play an pivotal role in this

process (22, 23). In the paracortex of the draining lymph nodes clustering of the antigen-carrying Langerhans cells and antigen specific T-cells occur. The antigen/MHC class II complex is recognized by the T-cell receptor (TCR). The occupance of TCR results in a cascade of intracellular signals ultimately leading to induced synthesis or activation of nuclear factors that then augment transcription of lymphokine genes, especially IL-2. This partly autocrine cascade is supported by a.o. IL-1β derived form Langerhans cells. Within a few days the generation and expansion of antigen specific memory cells occurs. These memory cells are released into the circulation and may enter the skin. Re-exposure of the antigen to memory cells will start the elicitation phase.

Keratinocytes are capable to produce and secrete various mediators. Most of these mediators are only produced after noxious stimuli. Application of contact allergens leads to an upregulation in transcription of i.e. IL-6, GM-CSF (granulocyte macrophage/colony stimulating factor) and TNF-α. These mediators affect Langerhans cell phenotype and function. It is suggested that induction of contact allergy does not only require the allergen presentation by Langerhans cells, but also the hapten induced alteration of cytokine production by keratinocytes.

3. PREDICTIVE SKIN SENSITIZATION TESTS

3.1. Guinea pig predictive tests

Nowadays both guinea pigs and mice are used as experimental animals in prospective test. However, from all the methods included in the OECD and EU guidelines just two guinea pig methods are used most frequently for regulatory purposes. In Europe the Guinea Pig Maximisation Test (GPMT) is preferred, in the USA the Bühler test is used more frequently. In table 1 an overview of the main aspect of the most commonly used test procedures is given. A detailed description of the test procedures can be found in several reviews (24-26). The guinea pig methods can be grouped either by the way of application, i.e. epicutaneous, intradermal, or by the use of Freund's complete adjuvant (FCA). FCA stimulates in a non-specific manner the immune systems of animals. Therefore the FCA methods tend to be more sensitive than non-adjuvant methods.

In principle the approach in the various guinea pig methods is the same. During the induction phase the guinea pigs are exposed to the testsubstance. After a rest period of 10 to 14 days the animals are challenged by a single re-exposure to the same testsubstance. Sensitization is determined by inspecting the skin reactions to the challenge exposure or by comparing the skin reactions following induction and challenge in individual animals. The wide variety in procedures is caused by the intention of the originators to optimise the induction phase of the test method. Intradermal application of the test substances is chosen to ensure that the presentation to immunocompetent cells in the skin is not hampered by skin permeability problems. Other investigators chose epidermal application because this way of application mirrors the possible human exposure. Occlusion of the application site will facilitate the skin penetration of the test substance. The epidermal tests are believed to be more suitable for testing formulations.

FCA is used to enhance the immunological response, so that substances with a low sensitization potency will sensitize sufficient animals in a small experimental group. However, the use of FCA has some drawbacks. The adjuvant itself induces small necrotic reactions. In combination with a high reactive test substance these necrotic areas can merge into large necrotic lesions. Besides the impact on the welfare of the animals such lesions can also impair sensitization. Thus underestimate the sensitizing potential of strong sensitizers possibly through the induction of tolerance. Furthermore, there is a debate

whether adjuvant treated control animals are more susceptible for irritative compounds than non-treated animals.

As is shown in table 2 the various guinea pig methods results in a large variation in the incidences of sensitization for several compounds. These discrepancies do not only result from differences in test method but may also result form differences in induction dose and the used vehicles . In routine testing the choice of the vehicle is usually rather arbitrarily. However, the rate of skin permeability depends largely on the characteristics of the vehicle and together with factors as temperature and pH the vehicle affects the release of the allergen. (27-29).

It is shown that higher concentrations and higher dosages increase the sensitization incidence (17, 27, 30). A thresholds exist for both the induction and the elicitation dose. White et al. (31) showed that sensitization is depended on the dose per unit area of the skin, thus with intradermal concentration. Moreover, the immunological response is also depended on the number of stimulatory Langerhans cells which reach the draining lymph node (32, 33). In general less attention is paid to the dose-response relation in the induction phase. In the Draize test and in the Optimization test fixed induction concentrations are used. Most other methods use induction concentrations which induce mild to moderate skin irritation in the guinea pig. Only the Open Epicutaneous Test (OET) (25) and a modified GPMT by Andersen (34) use several induction concentrations. The dose-response relationship between induction doses and the sensitization frequencies showed to be non-linear (34).

A dose-response relationship was also shown for the elicitation phase in the GPMT and Bühler test (35). The shapes of the log dose-response curves for a strong and weaker sensitizer, dinitrochlorobenzene and *p*-phenylenediamine, respectively, were similar in that the initial portions of the curves were linear, followed by a more shallow curve as the response approached zero.

Not only variations in the sensitization incidence can be introduced in the induction phase, but also in the elicitation phase. Although the macroscopic reaction pattern in sensitized guinea pig skin is limited to the formation of edema and erythema, evaluation of the various grades in erythema and edema is highly subjective. There is a need for quantitative evaluation. Only in the Optimization test (16) objective assessment, by measuring the diameter of the erythema and the skinfold thickness, is performed. Andersen

& Staberg (36) showed that measurements of skin blood flow by laser-doppler flowmetry and guinea pig skin thickness can be useful as supplements to the visual scoring.

The usefulness of histopathological evaluation of the application site, especially after challenging with dyes, is still under debate. It has been difficult to discriminate reliably between irritation reactions and allergic reactions, therefore identification of a sensitization response is only possible when the inflammatory response in the treated animal is notably more marked than in the control animals (37, 38).

Regardless the observed variations the Guinea Pig Maximisation test, the Freund's Complete Adjuvant test, the Optimization test, the Split Adjuvant test, the Bühler test, the Draize test and the Open Epicutaneous test are all allowed to be used for classification and labelling of sensitizing substances. The only restriction is that substances are considered to be positive when ≥30% of the test animals, in an adjuvant method, show positive responses in the absence of reactions in the control animals. In a non-adjuvant test method a response of ≥15% positive animals leads to classification as a sensitizing substance.

3.2. Mouse predictive tests

As part of basic immunological research the immune system of the mouse is studied more extensively than that of the guinea pig. From that knowledge predictive mouse assays were developed. In 1992 the OECD accepted three screenings methods: the mouse ear swelling test (MEST) according to Gad et al. (5), the noninvasive mouse ear swelling assay (MESA) according to Thorne et al. (39, 40) and the mouse local lymph node assay (LLNA) according to Kimber et al. (6, 41).

The induction procedure in the MEST is very rigorous. Besides the intradermal injections of FCA, the test substance is applied repeatedly to the abdomen which is stripped by adhesive tape. Sensitization is measured by the increase of the ear thickness. The responses of known sensitizing chemicals were modest. It is indicated that the test is not sensitive enough to detect moderate and weak sensitizers. The MESA is a modification of the MEST. In the MESA induction is performed at irritative concentrations of the test substance. The immunological response is not enhanced by the use of FCA. Supplementation of vitamin A in the diet enhanced the immunological response, so weak sensitizers cold be detected. Due to the use of an objective parameter dose-response relations can be studied in the MEST and MESA.

In contradiction to the other methods the LLNA determines only the induction capability of test substance. The main event studied in this assay is the proliferative response of T-cells in lymph nodes draining the exposure site. Mice are treated on the dorsum of the ear for three consecutive days. The proliferative activity in the draining lymph nodes is determine by the *in situ* incorporation of ^{3}H-thymidine. Comparative studies with guinea pig methods and inter-laboratory validation studies show that the LLNA provide a reliable method to identify chemicals which have a significant potential to induce skin sensitization (42-44).

3.3. Alternative methods

3.3.1 In vitro tests

Skin sensitization is a multicomponent reaction involving several organ systems. For that reason the possibilities to develop a practical *in vitro* model is limited. However, the various parts of the immunological response can be assessed in *in vitro* models. There are several models to estimate skin penetration, ranging from animal and human skin samples to perfused pig ears (45-47). Induction phenomena can be studied in isolated mouse and human epidermal Langerhans cell cultures (48, 49) or in *ex vivo* models as the local lymph node assay as developed by Kimber and colleagues (50). The release of relevant mediators can also be studied human and animal skin explants (51).

The abovementioned methods are, in general, still in the developmental stage. Although, positive results are shown with known sensitizers relevance to hazard identification and quantitative risk estimation is unclear.

3.2.2 Quantitative structure-activity relationships

Several expert systems and mathematical models are developed to assess contact allergy. (52-54) These models combine physico-chemical characteristics, like chemical structures, chemical reactivity, skin permeability, and clinical human and animal data to identify structural alerts for skin sensitization. The initial results show that these approaches are promising. However, the knowledge of all the necessary model descriptors is limited. Therefore the usefulness of these models to identify contact allergens is restricted, although the model can be applied within a group of related chemicals.

4. RISK ASSESSMENT

Three main features are important in the risk assessment of contact allergens for humans. These are the likely human exposure to the substance, the human susceptibility and the allergic potency of the substance. Each of these features include several pitfalls which make a reliable estimation difficult. The exposure assessment includes both the intended use of the substance and any reasonable foreseeable misuse. Furthermore, data on the frequency and duration of the activities of the employee and consumers must be known. Because induction to a sensitizer is probably related to the exposure time and exposure concentration. Untill now no clear relationship is postulated. Furthermore, this relation might differ from compound to compound. Additionally, information is needed on skin penetration and other physico-chemical characteristics. Genetic constitution is an important element in the assessment of the human susceptibility. Furthermore, susceptibility is enhanced in already sensitized individuals. The amount of allergen required to induce sensitization in a non-sensitized person is much greater than that required to elicit a response in a previously sensitized person (55). As is shown in the abovementioned paragraph the estimation of the sensitizing potency of substance by predictive animal test is difficult. Except the OET none of the commonly used animal test establish dose-response relationships. Due to the complex test procedure, longer duration and greater numbers of animals, the inter-laboratory experience with this test is limited. Although the evaluation of the response in the GPMT is ranked on a semi-quantitative scale, the relevance of this classification is unclear. Several publications have assessed the skin sensitization potential in the guinea pig of small groups of human contact sensitizers (56, 57). These studies show that, with very few exceptions, substances that have sensitized man can also sensitize guinea pigs. It is still questionable whether the reverse statement is also true. Although negative maximization data are used to classify substances as non-sensitizers.(29). Up to now only the intrinsic skin sensitizing potency of a substance can be assessed in the predictive animal tests.

Regarding the limitation of the animal tests Robinson and co-workers (58, 59) suggested the following risk assessment strategy for new product ingredients. The first step is an analytical characterization and literature survey to determine structural alerts suggestive of skin sensitization. Subsequently a Bühler test is performed to determine inherent skin

sensitization potential. If the substance is positive an additional guinea pig testing is needed to provide dose response data to ensure that the risk of inducing contact allergy in human volunteers is judged to be minimal. Eventually the risk is assessed in approximately 80-120 volunteers by a human repeat insult patch test (HRIPT). Whenever possible, the data are compared with results from appropriate 'benchmark' standards, chemicals and products with known skin sensitizing potentials under similar employee and consumer exposures. Although the abovementioned strategy looks promising, some serious drawbacks are incorporated. The procedure is time consuming and laborious. Furthermore, the procedure is expensive due to the performance of the HRIPT. In order to assess the responsiveness of the volunteers a positive control should be included. However, such approach is unethical. Another, although minor drawback is the absence of internationally agreed test protocols. Regardless the drawbacks human data might be very usefull to assess the risk to the population of already marketed compounds.

A quantitative prospective risk assessment can be performed when commonly used guinea pig methods are adapted in such a way that quantative information on induction potential and dose-response relationships can be estimated.

5. CONCLUSIONS/RECOMMENDATIONS

More than a half century research on skin sensitization results in a broad scale of predictive animal test. Due to their design, evoking a response in a limited time period, the majority of the predictive animal tests will only discriminate reliable between sensitizers and non-sensitizers, thus may serve in the hazard identification of substances. However, every day practice shows the need for a more quantitative risk assessment.
With the aid of currently developed alternative methods, physico-chemical data (electrophility, partion coefficients, structural alerts) and minor adaptation of most used guinea pig methods, i.e. the GPMT and the Bühler test, adequate dose response relationships can be obtained for performing a quantitative risk assessment.
The following research strategy is suggested:

- primarily skin penetration capacity is investigated e.g. in *in vitro* models. These experiments might be supported by mathematical models which may estimating the ultimate exposure of the test compound to the immune system and may be useful by *in vitro-in vivo* and interspecies extrapolation (60).
- secondly induction is quantified by the dose-response relation of cytokine profiles and production and/or Langerhans cell migration. These phenomenons can be studied in both human and animal tissue explants (61, 62).
- thirdly based on the knowledge obtained in the *in vitro* models, animals are sensitized and a dose response relationship is estimated in the elicitation phase in order to optimize the determination of the sensitization respons.

Initial validation of this strategy can be performed with known sensitizers. Suitable "weak" sensitizers are e.g. triethanolamine, dicyanodiamide and diethylene glycodimethacrylate. Kathon GC, coumarine and hydroxycitronellal are model compounds in the class "moderate sensitizing. Neomycinesulphate and 1-chloro-2,4,-dinitrobenzene are regarded as "strong" sensitizers.

Table 1. Commonly used tests methods for skin sensitization.

test	induction	no. applications	challenge
FCA guinea pig tests			
guinea pig maximisation test (Magnusson & Kligmann)	a: intradermal/intradermal in adjuvant/adjuvant; b: epidermal (occlusive)	a: 2 b: 1	epidermal (occlusive)
Freund's complete adjuvant test	intradermal in adjuvant	5	epidermal (open)
Optimization test	a: intradermal; b: intradermal in adjuvant	a: 4 b: 6	a: intradermal b: epidermal (occlusive)
Split Adjuvant Test	a: epidermal (occlusive); b: adjuvant	a: 2 b: 1	epidermal (occlusive)
non-FCA guinea pig tests			
Draize test	intradermal	10	intradermal
Bühler test	epidermal (occlusive)	3	epidermal (occlusive)
Open epicutaneous test	epidermal (open)	21	epidermal (open)
mouse tests			
Mouse ear swelling test (MEST)	a: intradermal adjuvant; b: epidermal, abraded skin (open)	a: 2 b: 3	epidermal
Local lymph node assay (LLNA)	epidermal	3	-

Table 2. Comparative results of sensitization assays in guinea pigs.

	GPMT	SAT	OT	FCAT	Draize	Bühler	OET
dihydrocoumarin	100	100	100	100	100	100	88
p-phenylenediamine	80-100	100	5-65	-	0-100	100	-
formaldehyde	100	0-70	10-100	30	10-70	30-60	0-38
penicillin G	90-100	80-100	33-100	67-100	35	-	0-50
benzocaine	60-85	40-60	0-90	40-50	0	20	38-63
cinnamic aldehyde	60-100	100	100	-	10-20	0-50	100

The percentages of sensitized guinea pig is shown. Data are obtained from the literature.

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