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Predictive testing for contact allergy

Comparison of some guinea pig and mouse protocols including dose-response designs

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Det som skrivs utan möda
läses i regel utan glädje.

Ur Första Klokboken av Gunnel & Kjell Swärd

To my family,
Magnus, Joakim and Fredrik

List of Original Papers

This thesis is based on the following papers, which will be referred to by their Roman numerals in the text:

I Montelius J, Wahlkvist H, Boman A, Fernström P, Gråbergs L, Wahlberg J E. Experience with the local lymph node assay: Inability to discriminate between allergens and irritants. Acta Derm Venereol (Stockh) 1994; 74: 22-27.

II Montelius J, Wahlkvist H, Boman A, Wahlberg J E. Murine local lymph node assay for predictive testing of allergenicity: Two irritants caused significant proliferation. Acta Derm Venereol (Stockh) 1998; 78: 433-437.

III Wahlkvist H, Boman A, Lidén C. Dose-response studies of contact allergens using 3 guinea pig models. Contact Dermatitis 1999; 41: 198-206.

IV Wahlkvist H, Boman A. Application of a dose-response protocol on the mouse ear swelling test (MEST) for contact allergy. Submitted.

V Wahlkvist H, Boman A, Montelius J, Wahlberg J E. Sensitizing potential in mice, guinea pig and man of the preservative Euxyl K 400 and its ingredient methyldibromo glutaronitrile. Contact Dermatitis 1999; 41: 330-338.

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Abbreviations

AO	Arachis oil
AAO	Acetone/arachis oil (4:1)
Ac:AO2	Acetone/arachis oil (1:2)
AOO	Acetone/olive oil (4:1)
CCET	Cumulative contact enhancement test
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNCB	2,4-dinitrochlorobenzene
DNFB	2,4-dinitrofluorobenzene
EC_3	Estimated concentration for a stimulation index of 3
EC_{50}	Estimated concentration sensitizing 50% of the animals
FCA	Freund's complete adjuvant
FCAT	Freund's complete adjuvant test
GPMT	Guinea pig maximization test
HC	Hydroxycitronellal
$K_2Cr_2O_7$	Potassium dichromate
LLNA	Local lymph node assay
MDBGN	Methyldibromo glutaronitrile
MEK	Methyl ethyl ketone
MEST	Mouse ear swelling test
OECD	Organization for Economic Co-operation and Development
OO	Olive oil
PE	Phenoxyethanol
p-PDA	*para*-phenylenediamine
QSAR	Quantitative structure-activity relationship
SDS	Sodium dodecyl sulfate
SI	Stimulation index
VAA	Vitamin A acetate

Contents

1. Introduction

Contact allergy (delayed hypersensitivity) may develop as a result of skin exposure to low molecular weight chemical substances (haptens) and can lead to allergic contact dermatitis. To minimize or avoid exposure to contact allergens in the environment, it is necessary to identify relevant allergens; thus knowledge about the sensitizing potential of chemicals is essential. To be able to make valid risk assessments, reliable predictive tests for contact allergy are of paramount importance.

1.1 Contact allergy

1.1.1 Contact dermatitis

Allergic and *irritant* contact dermatitis (eczema) are common occupational skin disorders, which are frequent causes of occupational illness. The most prevalent locations of occupational contact dermatitis are on the hands, arms and face (1). A combination of facts concerning occupational disorders reported to the Occupational Injury Information System (ISA) in Sweden during 1990-1991 shows occupations with an increased incidence of skin disorders (Figure 1) (2). However, contact dermatitis can also be due to non-occupational exposure to allergens and irritants in products (e.g. cosmetics, detergents, jewellery and skin care products) and is frequently seen in the general population (1, 3).

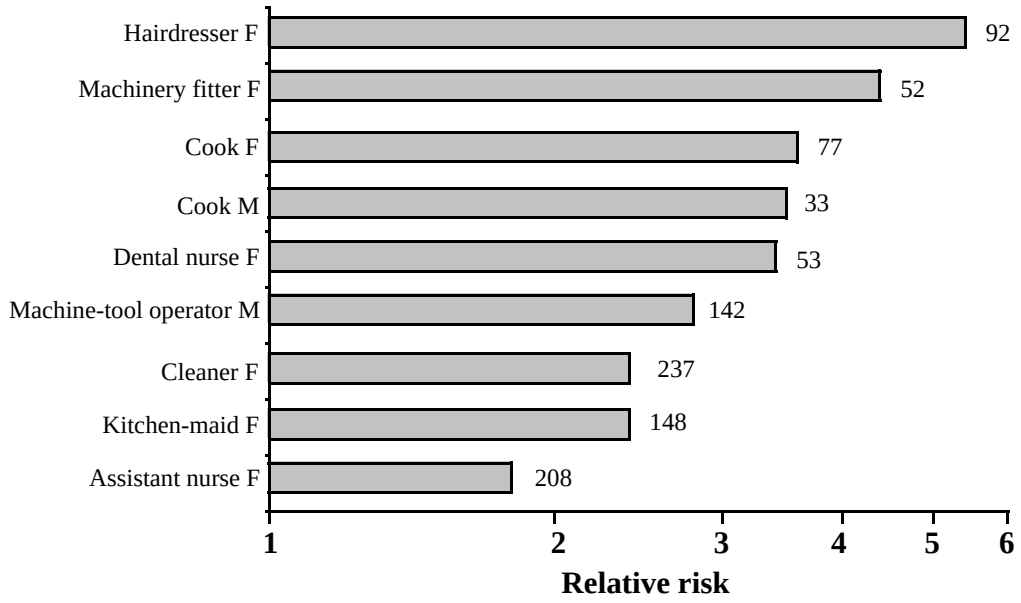


Figure 1. Occupations with an increased incidence of skin disorders, which were reported to the Occupational Injury Information System (ISA) in Sweden during 1990-1991 (2). Number of cases from each occupation is shown in front of the columns. F = female; M = male

Irritant contact dermatitis is considered to be more prevalent than allergic contact dermatitis (1). It is an unspecific inflammatory reaction in the skin, which is due to irritating substances. It is often more pronounced if the skin barrier is compromised due to frequent contact with water. The constitution of the skin in each individual is also of importance and a person with dry skin, i.e. an atopic individual, has an increased risk of developing irritant contact dermatitis (4-5). However, irritant contact dermatitis leads to an impaired skin barrier function and may increase the risk of developing an allergic contact dermatitis.

Allergic contact dermatitis is a specific inflammatory reaction due to sensitivity to a chemical (contact allergen). Individuals differ in their susceptibility to contact sensitization (6). Potent contact allergens may sensitize at the first exposure, while moderate or weaker contact allergens need several exposures to sensitize. Some contact allergens sensitize only after years of exposure. A contact allergy is diagnosed by patch testing, where standard allergens (i.e. the standard series (1, 7)) and perhaps also products from the work and leisure environment are applied to the skin of the patient's back under occlusion for 48 h (1, 7). The test reactions are examined after 72-96 h and the grade of reactivity is assessed according to international recommendations (7). The 10 contact allergens that elicited most positive reactions among occupationally exposed patients in Stockholm, Sweden during 1993-1998 are presented in Table 1. These findings were in agreement with those in a German multicenter study performed between 1990 and 1995 (10).

Table 1. Standard patch test results between 1993 and 1998 from the Department of Occupational and Environmental Dermatology, Stockholm County Council, Stockholm and data from sensitization experiments with guinea pigs.

Allergen	Humans[a]		Guinea pigs
	Number of positive	% positive	n positive/n exposed
Nickel sulfate	239	22.0	11/20[b]
Fragrance mix	91	8.1	-
Cobalt chloride, 0.5% pet.	84	7.6	43/50[b]
Potassium dichromate	77	6.9	10/10[b]
Colophony	61	5.5	13/20[b]
Balsam of Peru	49	4.4	positive[c]
Formaldehyde	36	3.2	17/19[b]
Thiuram mix	35	3.1	-
Kathon CG	28	2.5	7/20[b]
Germall II, 2% aq.	28	2.5	8/19[b]

a) 1100 patients were tested with each substance.

b) Experimental sensitization results from a list presented by Wahlberg and Boman in 1985 (8).

c) Experimental sensitization result from Klecak, 1985 (9).

- = no data available.

Millions of chemicals exist today and about 3700 of these have been described as contact allergens (11-12). Every year thousands of new substances are synthesized

and introduced on the market (12). If the components of a product show contact allergenic potential there is a risk of sensitization in the exposed population. It is therefore important to be able to use simple and reliable methods for predictive testing of contact allergens in chemicals and products before they are introduced on the market or when they are suspected as a cause of allergic contact dermatitis.

1.1.2 Contact allergen - antigen formation

The chemicals (contact allergens) suspected to give allergic contact dermatitis are usually low molecular weight chemicals (haptens), MW below 700 (13-14). When haptens come in contact with the skin they must first penetrate the skin barrier and bind to skin constituents, i.e. soluble or cell-bound host proteins, to form a complete non-self-antigen, which in turn can elicit an immune reaction (14). The sensitizing capacity of contact allergens depends on their ability to form these hapten-protein complexes. The sensitizer acts as an electrophile and the protein acts as a nucleophile in most of these reactions, with the nucleophilic function in the side groups (-NH_2, -SH, -S-, -N-, -NH and -OH) of the amino acids. Some haptens may instead easily form free radicals, which also bind to proteins in a free radical mechanism. The bonds formed between contact allergens and proteins are mostly of covalent nature, but metals form coordination bonds with proteins (15-16).

1.1.3 The skin - the Langerhans cell

The skin is the interface between man and his environment. The skin has two layers - the outer epidermis, which is epithelial and firmly attached to the underlying dermis of connective tissue. Beneath is the subcutis, i.e. loose connective tissue which usually contains an abundance of fat. In the epidermis there are four different cell types: keratinocytes, melanocytes, Merkel cells and the Langerhans cells. The Langerhans cell is a dendritic cell and there are approximately 800 Langerhans cells per square mm in human skin (17). Langerhans cells has also been studied in guinea pigs and mice (18-19). They are the prime antigen-presenting cells in the epidermis, and bear the important class-II major histocompatibility complex (MHC) antigens, which are the ubiquitous keys that make the immune system start to react when foreign substances penetrate the skin (17, 20). The Langerhans cells are the first line of defence of the immune system in human skin as well as in that of guinea pigs and mice.

1.1.4 Cells in the immune system

In the immune system, there are unspecific cells which react to all foreign materials (antigens) giving an innate (non-adaptive) response, but there are also cells which are activated by recognition of specific antigens, and which furthermore are able to create a memory of these specific antigens, giving an adaptive response. These two cell types are derived from a common stem cell, which differentiates into two main lineages, one for the myeloid cells (unspecific cells) and one for the lymphoid cells (specific cells). A dendritic cell lineage is also believed to develop from the stem cell. In the myeloid lineage the myeloid progenitor differentiates into, e.g., macrophages, granulocytes and mast cells. The lymphoid progenitor in the lymphoid lineage differentiates into, e.g., natural killer cells (NK-cells), B-cells

(which produces antibodies) and T-cells (including memory cells). The dendritic cell lineage includes the antigen presenting cells (APCs), e.g. Langerhans cells (21).

1.1.5 Contact hypersensitivity

Allergic contact dermatitis is the clinical manifestation of a type-IV (delayed) hypersensitivity reaction, which is a cell-mediated immunologic reaction. This hypersensitivity is an unfavorable side effect of a well-functioning immune system. The immune system defends us against infections and malignant cells, but in allergic contact dermatitis it has reacted to environmental chemicals. The contact allergic reaction has two phases: sensitization and elicitation (Figure 2). The sensitization phase takes at least one week from exposure in experimental animals. During sensitization the antigen (hapten-protein complex) is carried by Langerhans cells to the paracortical area of the draining lymph nodes where the antigen is presented to helper/inducer T cells (CD4+). About 5-7 days later specific memory T cells (CD4+) have been developed and circulate in the body. At a future contact with the hapten the memory T cells will recognize the antigen on the Langerhans cells and become activated to release cytokines and induce a cascade of inflammatory reactions. This is the elicitation phase. After 24-48 h, an inflammation has developed (an eczema) in the skin at the site of exposure (14, 20).

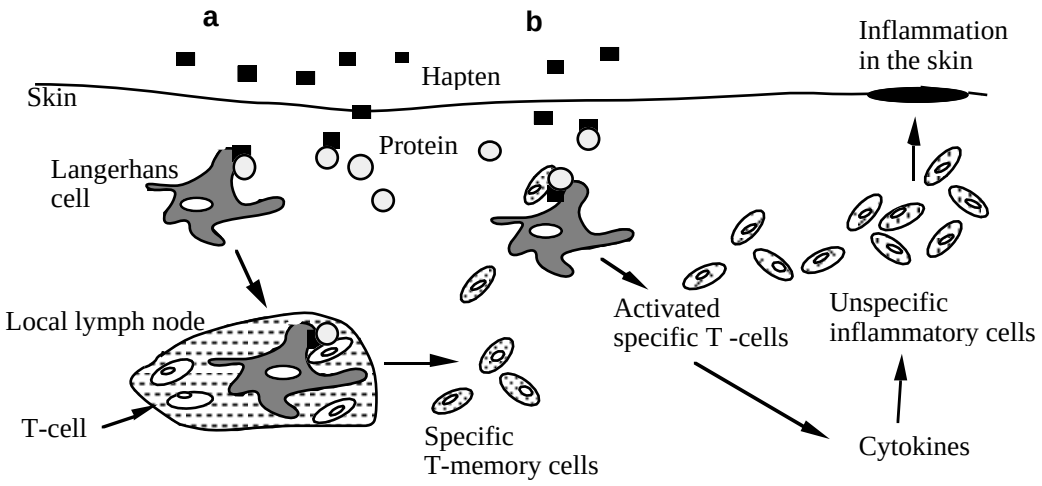


Figure 2. Schematic illustration of a delayed hypersensitivity reaction. Sensitization (a) and elicitation (b) in allergic contact dermatitis. a) The formed hapten-protein complex (antigen) is carried by the Langerhans cells to the draining lymph nodes where the antigen is presented to helper/inducer T cells, which results in development of specific memory T-cells. b) At a future contact with the hapten the memory T-cells will recognize this hapten-protein complex, which activates specific T-cells to proliferate and produce cytokines. Unspecific inflammatory cells will also be attracted to the site of exposure and an inflammation develops.

1.2 Predictive test methods

1.2.1 Human predictive test methods

Several predictive tests for the allergenic potential of chemicals have been in use for many years, with humans and experimental animals as subjects. Human test methods were mainly developed during the years 1944-1980 (Table 2). The preferred methods are the modified Draize procedure of Marzulli and Maibach developed in 1973-1974 (27) and the modified maximization technique of Kligman and Epstein developed in 1975 (28). One disadvantage of these tests is that large numbers of volunteers are needed if an experiment is to give reliable results. Another is that it is ethically less justifiable to perform these test on humans than on experimental animals due to the risk that the humans become sensitized for the rest of their lives and may develop eczema to the tested chemicals after future exposures. However, these tests do eliminate the need to extrapolate results from animals to humans.

Table 2. Human predictive test methods for contact allergens in chronological order.

Test methods	No. subj.	Skin site	No. patches	Duration of exposure	Rest (no. days)	Challenge	References
Repeated insult	100 ♂ 100 ♀	Arm, back	10	24h	10-14	Repeat patch	1944, (22) Draize
Prophetic	200	Arm, back	1	24-96h	10-14	48h	1944, (23) Schwartz, Peck
Repeated insult	200	-	10-15	24h	14-21	48h	1953, (24) Shelanski
Schwartz	200	Arm, thigh, back	1	72h	7-10	72h	1960, (25) Schwartz
Maximization	25	Forearm	5	24h	10	48h	1966, (26) Kligman
Modified Draize	200	Arm	10	48h	14	72h	1974, (27) Marzulli, Maibach
Modified Maximization	25	-	7	24h	10	48h	1975, (28) Kligman, Epstein
HRIPT[a]	80-120	-	9	24h-48h	14-17	24h	1980, (29) Stotts

[a)]HRIPT = Human repeat insult patch test.

1.2.2 Animal predictive test methods

The guinea pig is considered the most suitable experimental animal in predictive testing for contact allergy and it has been used for decades. However, the methods are quite costly and time-consuming. In addition it is sometimes hard to discriminate between allergic reactions and irritant reactions. Inspection and palpation are used for scoring, and are considered subjective. About 20 guinea pig protocols (Table 3) have been described and some have been compared and evaluated (36, 43-46). The methods use one of three different administration procedures at induction - intradermal, topical or both in combination - for sensitization. The results from guinea pig tests correlate well with the sensitizing properties of chemicals in man (9, 33, 44-45, 47-48) and are generally accepted by regulatory authorities (49). In the first international guidelines set forth in 1959 the Draize test was included (30) to screen out potent sensitizers. Since then several predictive guinea pig test methods have been developed.

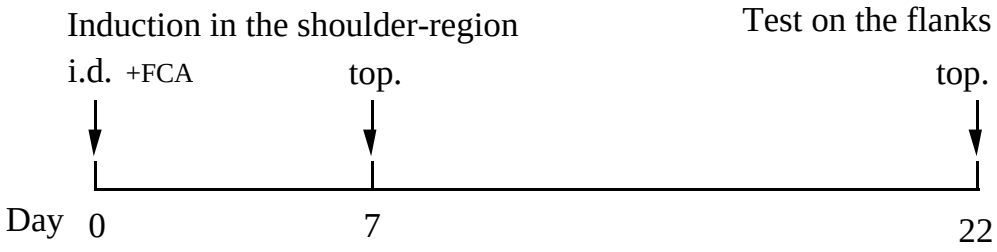
Table 3. Predictive test methods for contact allergens in guinea pigs in chronological order.

Method	References
Draize test	1959, Draize (30)
Buehler test	1965, Buehler (31)
Ear flank test	1967, Stevens (32)
Guinea pig maximization test	1969, Magnusson and Kligman (33)
Split adjuvant technique	1972, Maguire and Chase (34)
Optimization test	1975, Maurer et al. (35)
Freund's complete adjuvant test	1977, Klecak et al. (36)
Open epicutaneous test	1977, Klecak et al. (36)
TINA test	1977, Ziegler (37)
Modified guinea pig maximization test	1981, Sato et al. (38)
Single injection adjuvant test	1981, Goodwin et al. (39)
Cumulative contact enhancement test	1982, Tsuchiya et al. (40)
Epicutaneous maximization test	1983, Guillot et al. (41)
Guinea pig allergy test	1985, Doussou and Sicard (42)

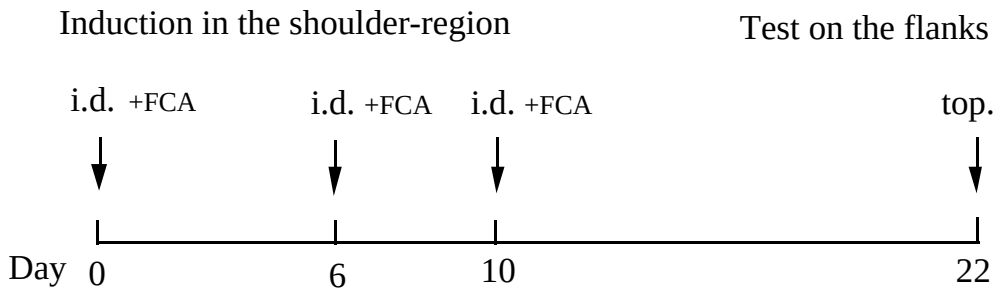
The Buehler test was developed in 1965 (31, 50-51); this test uses three short topical exposures (6 h) of the test chemical in the shoulder region at sensitization of the animals (induction), and patches are applied to elicit the sensitization (challenge tests) on the flanks. Magnusson and Kligman published an extensive study in 1970 (33, 47) where they investigated variables in the standard procedure for sensitization experiments in guinea pigs. They performed several different tests with focus on the experimental animal, the influence of some pharmaceuticals, ambient conditions, induction of hypersensitivity, potentiation of hypersensitivity by adjuvant, and elicitation of hypersensitivity and in the end developed a guinea pig maximization test (GPMT). The contact allergens were classified on a five grade scale (47). The GPMT method includes both intradermal administration with the addition of Freund's complete adjuvant (FCA) (52) and occlusive topical application (48 h) of the test substance in the shoulder region at induction, and challenge tests on the flanks (Figure 3a). In 1977 Klecak developed the Freund’s complete adjuvant test (FCAT) (9, 36), which has three intradermal administrations of the test

substance with FCA in the shoulder region at induction, and challenge tests on the flanks (Figure 3b). Tsuchiya et al. developed in 1982 the cumulative contact enhancement test (CCET) (40). The CCET method has four occlusive topical applications (24 h) of the test substance and intradermal administration of FCA in the shoulder region at induction, and challenge tests on the flanks (Figure 3c). The most commonly used predictive guinea pig methods in our laboratory are the GPMT, the CCET and the FCAT depending on which route of exposure is deemed most suitable for a particular study.

a) **Guinea Pig Maximization Test (GPMT)**



b) **Freunds complete adjuvant test (FCAT)**



c) **Cumulative Contact Enhancement Test (CCET)**

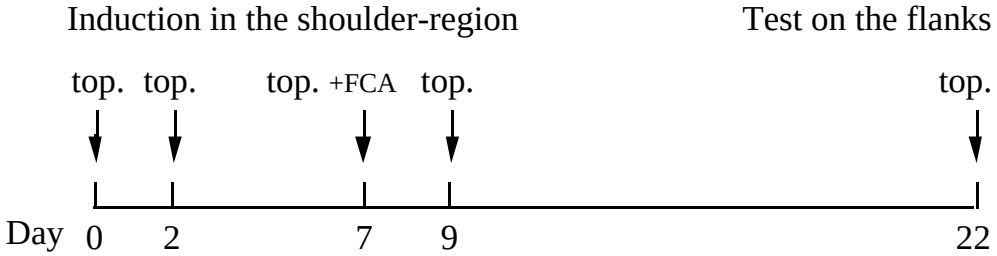


Fig 3. Time-schedules for three predictive guinea pig test methods a) The guinea pig maximization test (GPMT), b) The Freund's complete adjuvant test (FCAT) and c) The cumulative contact enhancement test (CCET). i.d. = intradermal; top. = topical

Two predictive guinea pig test methods are recommended in the current OECD guidelines (53). The GPMT with the use of FCA is the most extensively used predictive guinea pig method in Europe, whereas the Buehler test (without FCA) is the method of choice in the USA (45, 54). The use of FCA to enhance the non-specific immune response to contact allergens is controversial. Some investigators believe that the use of FCA in predictive test methods will lead to an overestimation of the allergenic potential of the chemical tested and that false positive reactions sometimes have been provoked due to induced hyper-irritability, referred as "angry back" or "excited skin syndrome" (55-59). GPMT has been shown in comparative studies to be more sensitive (43-45, 60) and to better correlate with results in human subjects (44-45) than the Buehler test. There is, however, a report which tries to explain the lower sensitivity of the Buehler test by variations of the test procedure (54).

To further improve the power of animal predictive tests for contact allergens, a multi-dose-response induction protocol for the GPMT has been developed (61-64). It increases the amount of information on the sensitizing capacity of a test substance that can be obtained from each experimental study. The protocol is combined with a statistical computer program, i.e. a logistic regression analysis, which uses all available test data in the analysis of the results (65). The program uses this logistic regression analysis to present curves fitted to the test results from the animal sensitization experiments and calculates significance of the dose-response relationship, the threshold concentration at sensitization and the estimated concentration sensitizing 50% of the animals (EC_{50}).

Table 4. Predictive test methods for contact allergens in mice in chronological order.

Method	References
Popliteal lymph node assay (PLNA)	1981, Gleichmann (66)
'VVA mouse' assay	1986, Maisey et al. (67)
Mouse ear swelling test (MEST)	1986, Gad et al. (68)
Local lymph node assay (LLNA) in vitro	1986, Kimber et al. (69)
Mouse ear sensitization assay	1988, Descotes (70)
Local lymph node assay (LLNA) in situ	1989, Kimber et al. (71)
Mouse ear swelling assay (MESA)	1991, Thorne et al. (72-73)
Sensitive mouse lymph node assay (SLNA)	1993, Ikarashi et al. (74)
Modified - Mouse ear swelling test (MEST)	1994, Gad (75)

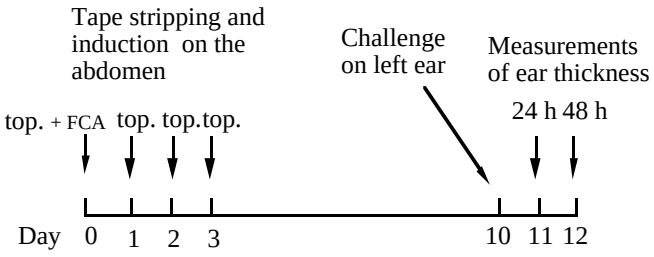
In recent years predictive test methods in mice have also become available (Table 4). Two predictive mouse tests, the murine local lymph node assay (LLNA) and the mouse ear swelling test (MEST), are recommended in the current OECD guidelines (53). The mouse was originally introduced for investigations of delayed contact sensitivity by Asherson and Ptak in 1968 and they used a mouse assay with MEST-like methodology (76). In 1986, Gad et al. were the first to standardize the MEST to be used for prediction of the skin sensitizing potential of environmental chemicals (68) by performing investigations of several parameters before a final test design

was established. The method includes intradermal administrations of FCA, tape stripping, and has four topical applications of test substance on the abdomen at induction. It has a challenge test on the ears, which measures the elicitation reaction as degree of edema (Figure 4a). In addition to the protocol recommended (68), there are other protocols using MEST methodology, which have been used in various studies (paper IV, 77). Variations in the protocols using MEST methodology have been described in several papers: variations include such as the site and number of applications for the induction procedure, different enhancement techniques, and the timing of challenge. Some variations in the protocols have recently been reviewed (paper IV, 78). Some modifications have led to development of new test methods (70, 72-73). The alterations are probably introduced as a result of the difficulty of classifying moderate or weak contact allergen as sensitizer using the recommended MEST protocol (79). Maisey and Miller used in 1986 the 'VAA mouse' assay (67, 80), which is similar to the recommended MEST protocol (68). Their protocol included a prolonged induction regime with six topical treatments and vitamin A acetate (VAA) supplemented diet and it was later referred to as the vitamin A enhanced ear swelling assay (VAESA) (81). It was demonstrated that VAA supplemented diet could increase the sensitivity of mouse assays using MEST methodology (67, 82). Several investigators have thereafter included VAA supplemented diet in their protocols (72-73, 83-84). A modification of the MEST protocol was done by Gad in 1994, where vitamin A acetate supplemented diet also was introduced (Figure 4b) (75, 85).

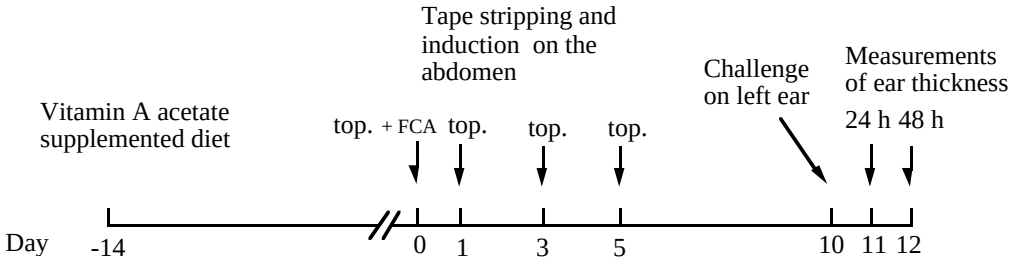
Kimber et al. developed the LLNA in 1986 (69). The method has three topical applications on the ears at induction, but no challenge test (Figure 4c). The method was first performed *in vitro* (69, 86), but was later modified to be carried out *in situ* (71, 87). It estimates the proliferative activity in the local lymph nodes by [^{3}H]thymidine incorporation, which is stated to correlate with the severity of the elicitation reaction induced by the test substance (88). The sensitivity of the method has later been enhanced through a few modifications (89-90). Some have tried to use different induction procedures in the hope of enhancing the sensitivity of the method (74, 77, 91-94) and one modification has led to the development of a new test method (74). Several studies in the last years have presented endpoints other then [^{3}H]thymidine incorporation to assess the sensitizing potential, e.g. use of an isotope with a shorter half-life (95), counting cells by a microscopic observation (93, 96-98), counting the proliferating cell nuclear antigen or a phenotypic detemination of subpopulations of cells by a flow-cytometric analysis (97-107) or detecting various cytokines by using an enzyme-linked immunosorbent assay (ELISA) or a reverse transcriptase-polymerase chain reaction (RT-PCR) (96-98, 100, 106-112). In some studies, alternative species have been used in the local lymph node assay, e.g. hamsters (113), guinea pigs (114-115) and rats (115-117). Some recent papers describe the use of more than one test method for the prediction of the sensitizing potential (98, 110, 118-121). A 'new' integrated model has also been proposed, which includes measurements of ear edema and flow cytometric analysis of the cells from the auricular lymph nodes whereafter the results are assessed by using a differentiation index (122). There has been a proposal for a

scheme for the ranking of the sensitizing potential of a substance based on data from several animal test methods (123).

a) **Mouse ear swelling test (MEST)**



b) **Mouse ear swelling test (MEST)**



c) **Local Lymph Node Assay (LLNA)**

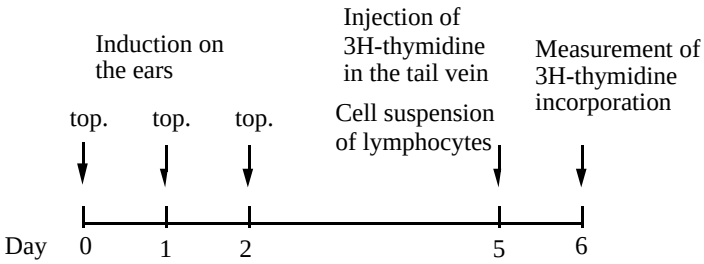


Figure 4. Time-schedule for predictive mouse test methods. a) The mouse ear swelling test (MEST). b) A modified version of the mouse ear swelling test (MEST). c) The local lymph node assay (LLNA). top. = topical

1.2.3 OECD-guidelines

The OECD Guideline for Testing of Chemicals 406 gives recommendations concerning skin sensitization and was last updated in 1992 (53). It recommends two predictive guinea pig tests -the guinea pig maximization test (GPMT) (8, 33, 47) and the Buehler test (31, 50-51), and two predictive mouse tests - the murine local lymph node assay (LLNA) (71, 87, 90) and the mouse ear swelling test (MEST) (68, 75, 85). The mouse models are currently suggested for preliminary screening of chemicals; in the case of a positive result the chemical may be classified as a

potential sensitizer, whereas if a negative result is obtained, a guinea pig test is recommended.

1.2.4 Update of prospective *in vitro* predictive test methods and some alternatives

Great effort is being made to reduce the use of experimental animals by refining and in the end replacing the experimental animal predictive test methods (124), and the development of *in vitro* test methods is currently in progress. Monolayer cultures in media (125-129) and three dimensional skin equivalents with an air-liquid interface (SKIN and EpiDerm) (130) have been developed and evaluated as potential *in vitro* systems to predict the contact sensitization potential of chemicals. However, the evaluated *in vitro* systems need further improvement (130) and at the moment no reliable predictive *in vitro* test method is available.

Quantitative structure-activity relationship (QSAR) studies (131-136) is a new approach to predicting the potential of contact allergens. However, only limited data are available concerning some groups of chemicals and there is probably no general QSAR that is valid for all chemicals. DEREK (Deductive Estimation of Risk from Existing Knowledge) is one expert system (computer program), which predicts potential skin sensitizers by identification of structural alerts (137). However, the risk of inducing contact allergy is related not only to the inherent allergenic potential (sensitization capacity) of a chemical. An important factor is penetration into the skin, which depends on the physico-chemical properties of the substance, concentration, skin barrier function and time of exposure (8). At present there is no available alternative predictive test method, which could replace the predictive animal test methods.

1.3 Risk assessment

Risk assessment is a procedure to define the adverse health effects resulting from the exposure of individuals or populations to hazardous chemicals. It comprises hazard identification, dose-response assessment, exposure assessment and risk characterization (estimation) (138-139). The European Union (EU) has developed criteria for classification of skin sensitizers on the basis of the properties of the chemicals. The basis for classification includes: 1) practical experience showing the substance or preparation to be capable of inducing sensitization by skin contact in a substantial number of persons 2) or positive results from an appropriate animal test. Compounds inducing at least 30% positive animals in an adjuvant test or 15% in a non-adjuvant test are classified as skin sensitizers. If a substance is classified as skin sensitizing the associated phrase R43 (‘May cause sensitization by skin contact’) must appear on the label of its package (140).

There is a Nordic proposal for a classification system for chemical allergens (141) causing skin allergy. All available data should be individually validated in accordance with the classification of carcinogens adopted by the International Agency for Research on Cancer (IARC) and the aggregate sum of knowledge regarding the substances should be used for classification. This system has five classification groups:

I -the substance causes allergic contact dermatitis in humans to a significant degree,

IIA - the substance probably causes allergic contact dermatitis in humans to a significant degree,

IIB - the substance possibly causes allergic contact dermatitis in humans to a significant degree,

III - the available data do not permit classification of the substance, and

IV - the substance is not a significant contact allergen and cannot cause allergic contact dermatitis in a significant number of persons.

Significant contact allergens are those classified in groups I, IIA and IIB (1, 141).

According to a 1996 report from a WHO Working Group, concerning criteria for classification of skin sensitizing substances in the working and general environments, substances may be classified into four different classes (139):

I - significant contact allergen,

II - probably a significant contact allergen,

III - not classifiable, and

IV - not a significant contact allergen.

2. Aims of the thesis

The purpose of this thesis is to evaluate some predictive test methods for contact allergens. It is done to provide information such that the test methods giving the clinically most relevant results should be used in risk assessment of chemical products and in research.

The specific aims of the present study are:

- To evaluate the LLNA, a predictive test for contact allergens in mice.
- To evaluate a slightly modified version of a multi-dose-response induction protocol applied on three predictive guinea pig tests for contact allergens: the GPMT, the CCET and the FCAT.
- To evaluate the application of an adjusted multi-dose-response induction protocol on a predictive test in mice, i.e. a modified MEST, for contact allergens.
- To compare the sensitizing potential of a preservative by using three predictive guinea pig tests for contact allergens - the GPMT, the CCET using a slightly modified multi-dose-response induction protocol, and the LLNA - and also to make comparisons with patch test results in dermatitis patients.
- To compare the estimates of the sensitizing potential of the substances obtained with the various predictive tests, to relate these estimates to human data from the literature, and thereafter compare the predictive test methods used.

3. Materials and methods

3.1 Animals

The experiments were carried out on outbred female Dunkin Hartley guinea pigs (average weight 300-350g) (AB Sahlins Försöksdjursfarm, Malmö, Sweden) [III, V], inbred female CBA/Ca strain mice (7-10 weeks) (B&K Universal AB, Sollentuna, Sweden) [I-II, V], and inbred female Balb/c strain mice (3-4 weeks) (B&K Universal AB, Sollentuna, Sweden and Charles River Sverige AB, Uppsala, Sweden) [IV]. The guinea pigs were housed in groups of three [III, V] and the mice in groups of four [I-II, V] or eight [IV] in Macrolon® cages on hardwood chip bedding under controlled environmental conditions. Pelleted standard diet (B&K Universal AB, Sollentuna, Sweden) [I-II, V] or pelleted standard diet (B&K Universal AB, Sollentuna, Sweden) supplemented with 280 IU/g of vitamin A acetate (AnalyCen Nordic AB, Lidköping, Sweden) [IV] were given to the mice and SDS pellets (AB Sahlins Försöksdjursfarm, Malmö, Sweden) [III, V] was given to the guinea pigs. Pellets and water were *ad libitum*. The guinea pigs were allowed to acclimatize for at least 7 days and the mice for 5 days prior to first exposure. The hair of the guinea pigs was removed with an electric clipper and shaver at induction and challenge exposure sites. The guinea pigs were numbered individually and randomly distributed to the cages. The mice [IV] were marked with Indian ink individually.

The studies were approved by the local ethical committee.

3.2 Chemicals

The *allergens* used were: Ethyl-*para*-aminobenzoate (benzocaine), *trans*-cinnamic aldehyde, 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrofluorobenzene (DNFB) [I]; Euxyl K 400 [V]; hydroxycitronellal (HC) [I and III-IV]; 2-mercaptobenzothiazole [I]; methyldibromo glutaronitrile (MDBGN) (1,2-dibromo-2,4-dicyanobutane) [IV-V]; 4-ethoxy-methylene-2-phenyl-2-oxazolin-5-one (oxazolone) [I, IV]; *para*-phenylenediamine (p-PDA) [I] and potassium dichromate ($K_2Cr_2O_7$) [III-IV].

The *irritants* used were: Chloroform/methanol [I]; 2-hydroxybenzoic acid methyl ester (methyl salicylate) [I-II]; nonanoic acid [II]; oxalic acid [I, IV]; sodium dodecyl sulphate (SDS) and Triton X-100 [I].

The *vehicles* used were: Acetone:olive oil (4:1) (AOO) [I, IV]; arachis oil (AO) [III, V]; acetone:arachis oil (1:2) (Ac:AO2) [V]; acetone:arachis oil (4:1) (AAO) [IV]; dimethylformamide (DMF) [I-II, IV-V]; dimethylsulfoxide (DMSO) [IV]; methyl ethyl ketone (MEK) [I-II]; olive oil (OO) [III, V]; phenoxyethanol (PE) [V] and physiological saline [III].

3.3 Predictive test methods using mice [I-II, IV-V]

3.3.1 Local lymph node assay (LLNA) [I-II, V]

3.3.1.1 The assay

The LLNA (Figure 5) was carried out in studies I-II, V as recommended (90). Mice in groups of four were treated with three topical applications of 25 µl of test substance at one of three different concentrations (Tables I-II, paper I and Table I-II, paper II) on the dorsum of both ears (days 0, 1 and 2). Control mice (n=4) were treated in the same way with the vehicle alone or were untreated. At day 5, all mice were injected intravenously through the tail vein with 20 µCi [^{3}H]thymidine in 250 µl of phosphate-buffered saline (PBS). After 5 h the mice were sacrificed and the draining auricular lymph nodes were excised, pooled for each group and the average lymph node weight was determined. A single-cell suspension of lymph node cells was prepared, washed, precipitated and the incorporated [^{3}H]thymidine was determined by β-scintillation counting.

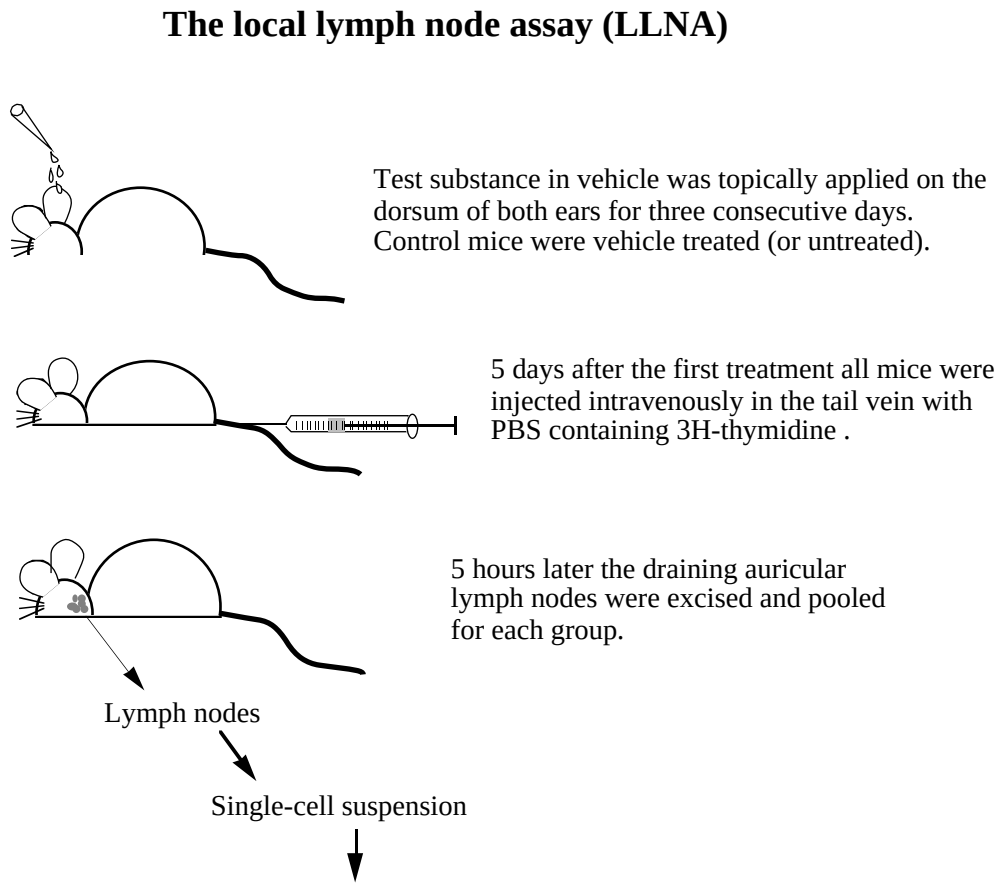


Figure 5. The protocol for the local lymph node assay (LLNA) (90).

3.3.1.2 Calculation of results and the criteria for classification

Results were expressed as mean disintegrations per minute/lymph node (dpm/node) for each experimental group. A stimulation index (SI), i.e. test group value/control

group value, was calculated for each concentration of each substance tested. According to the method (90), a chemical is classified as a sensitizer if two criteria are fulfilled: 1) at least one concentration of the test chemical must induce a SI of a threefold or greater value than that of the vehicle control; and 2) the results must not be incompatible with a biological dose-response. The relative potency may be ranked as a function of the concentration required to induce a stimulation index of 3, and this concentration is expressed as an EC_3 value (estimated concentration for SI=3) (142-143).

3.3.2 A modified mouse ear swelling test (MEST) using a multi-dose-response induction protocol [IV]

3.3.2.1 Irritancy threshold studies and calculation of the relative increase of ear thickness

The mice were given VAA supplemented diet prior to pretest and maintained on it thereafter. Each test substance or the vehicle alone was topically applied on the dorsum of both ears on ten mice. Ear thickness measurements were performed on all ears using a spring-loaded micrometer (Oditest, H C Kröplin, GMBH, Schlüchtern, FRG). The measurements were carried out just prior to application of the test substance (day 0) and were repeated 24 h (day 1) and 48 h (day 2) after application of test substance. The relative increase in ear thickness in percent for each ear was calculated using the following formula:

$$\text{Relative increase in ear thickness in \%} = \frac{B - A}{A} \times 100$$

In this equation, B = the mean ear thickness at post-challenge (24 h or 48 h) and A = the mean ear thickness prior to challenge (0 h). Based on these calculations, the concentration that gave a mild irritation and the highest non-irritating concentration (< 10 % increase in ear thickness) were chosen as the highest concentration for induction and for challenge, respectively.

The modified mouse ear swelling test (MEST)

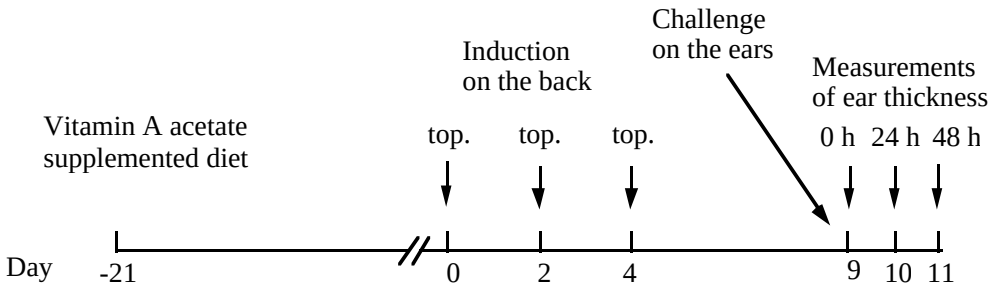


Figure 6. Time-schedule for the modified mouse ear swelling test (MEST).
top. = topical

3.3.2.2 The modified mouse ear swelling test (MEST)

In paper IV the MEST was performed essentially as described originally (68), but with some modifications (Figures 6-7): The mice were given VAA supplemented diet prior to test and maintained on it thereafter. A multi-dose-response induction protocol (62) was followed. The mice were divided into five groups with eight animals in each. One of the four concentrations of each test substance, or the vehicle alone (the control group) was applied in each respective group (Table 9). The *induction* was carried out by a total of three topical applications of 100 µl of test substance in vehicle or the vehicle alone every second day (days 0, 2, 4) on the clipped and shaven back of the mice (application site: 2x3 cm). After five days (day 9), the mice were *challenged* topically with 25 µl of the chosen concentration of test substance in vehicle on the dorsum of both ears. Ear thickness measurements were performed on days 9 to11 as described in section 3.3.2.1. The highest relative increase in ear thickness in percent on either ear of each mouse was used for further analysis.

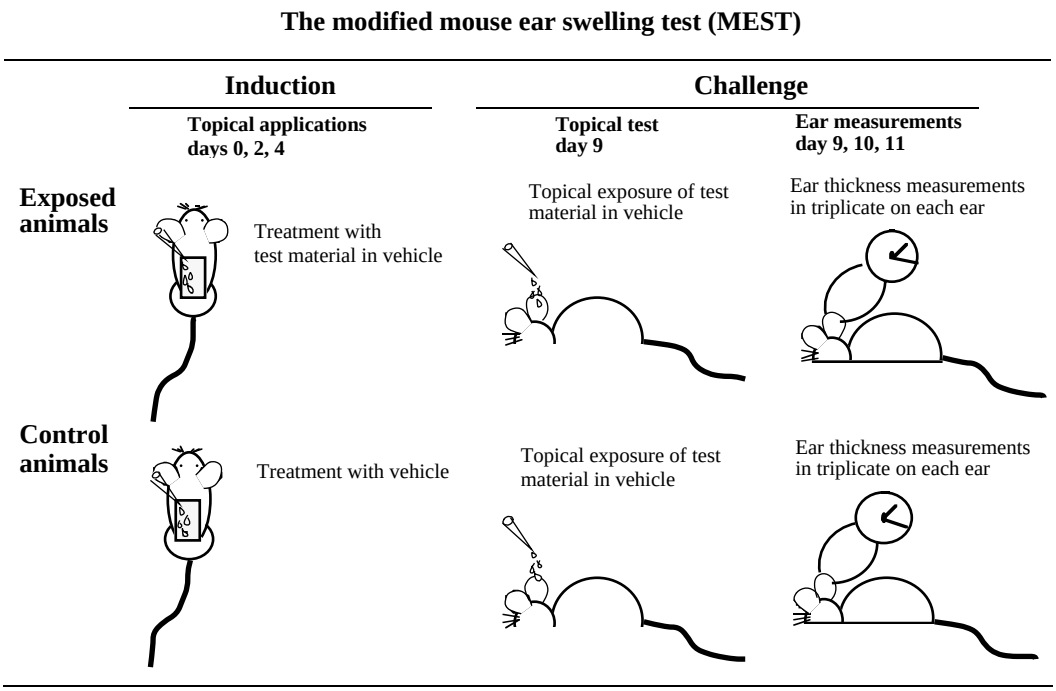


Figure 7. The protocol for the modified ear swelling test (MEST), which is a modification of the original MEST protocol (68).

3.3.2.3 Interpretation of results

To interpret the calculated relative increase in ear thickness, by performing statistical dose-response analysis, several hypothetical 'positive' sensitization response criteria (here called: sensitization criteria) were set, whereas Gad et al. (68) defined a 'positive' response as a 20 % relative increase in ear thickness. Hypothetical sensitization of each mouse was judged individually and to be classed as sensitized, at least one ear of each mouse should have a relative increase in ear thickness of at least 5 %, 10 %, 15 % or 20 % depending on the different sensitization criteria used. The number of sensitized mice was counted at each sensitization criterion. This gave lower sensitization rates in the induction groups when a higher sensitization criterion is used. The relative number of sensitized mice in each

induction group, giving series of sensitization rates at each sensitization criterion for a test substance, was statistically analyzed (62, 65) (section 3.5.2). When no significant dose-response relationship was obtained for a test substance, the sensitization rates of the mice in the different induction groups were given at the sensitization criterion of 10%, as this was chosen in the pretest to be the non-irritant concentration for the test substances and the vehicles. The sensitizing capacity of a test substance was assessed by calculating some parameters using the statistical analysis program (62, 65). To be able to compare the sensitivity of this modified MEST with the original protocol (68), the relative ear thickness in percent (termed percent ear swelling in the original protocol (68)) from a test substance was calculated for the different induction groups by using the following formula:

$$\text{Relative ear thickness in \%} = \frac{D}{C} \times 100$$

In this equation, D = the sum of the mean ear thickness from all ear measurements (all B) in an induction group at post-challenge (24 h or 48 h) and C = the sum of the mean ear thickness from all ear measurements (all A) prior to challenge (0 h).

3.4 Predictive test methods using guinea pigs [III, V]

3.4.1 General procedure for the guinea pig test methods [III, V]

3.4.1.1 Irritancy threshold studies
In pre-tests the test substances were applied topically on the flanks for 24 h using closed patch test on 3-6 animals and injected (intradermally) on 3-6 animals. The concentration that gave minimal irritation and the highest non-irritating concentration were selected to be used as the highest concentration for topical induction and for challenge, respectively. The highest tolerable concentration for intradermal induction was selected on the basis of the pre-test or on previous experience.

3.4.1.2 Induction
The induction was performed in paper V according to the methodology description presented in section 3.4.3. The concentrations of the substance used are presented in Table 12 [V].

3.4.1.3 Challenge and re-challenge
On day 22, patch testing was performed on the flanks for 24 h using Aluminium Finn Chambers 8 mm Ø (Epitest Ltd Oy, Tussula, Finland) on Scanpor® tape (Norgesplaster AS, Norway) and acrylastic bandage (Beiersdorf, FRG). Usually, seven different challenge concentrations were used, in addition to one vehicle control. The concentrations were randomly distributed to avoid bias from differences in anatomical location. Test reactions were read blindly at 48 h and 72 h after application. The minimum criterion for a positive reaction was a confluent erythema (++) (144). The concentrations used at challenge and re-challenge are summarized in Table 5 [III], Table 12 in the result section [V] and Table 2 in paper V.

Table 5. Concentration ranges for allergens used at induction and challenge.

Series	Animal group	Allergen	Method	Induction conc. (%)		Challenge conc. (%) top.
				id	top.	
I	1 - 6	$K_2Cr_2O_7$	GPMT	0.003 - 0.3	0.01; 1	0.01 - 0.1
II	7 - 12	$K_2Cr_2O_7$	GPMT	0.0003 - 0.03	0.01; 1	0.01 - 0.1
III	13 - 18	$K_2Cr_2O_7$	CCET	-	0.01 - 1	0.0003 - 0.3
IV	19 - 24	$K_2Cr_2O_7$	CCET	-	0.0003 - 0.03	0.003 - 0.3
V	25 - 30	$K_2Cr_2O_7$	FCAT	0.0001 - 0.01	-	0.0003 - 0.3
VI	31 - 36	HC	GPMT	0.03 - 3	1; 100	0.01 - 10
VII	37 - 42	HC	CCET	-	1 - 100	0.01 - 10
VIII	43 - 48	HC	FCAT	0.03 - 3	-	0.01 - 10

Concentrations in % (w/w) were increased by a factor of 3 giving a dose range of 10, 100 or 1000. Potassium dichromate ($K_2Cr_2O_7$) in saline at induction and challenge. Hydroxycitronellal (HC) in arachis oil at induction and in olive oil at challenge. Intradermal concentrations (id) and topical concentrations (top.)
GPMT = Guinea pig maximization test. CCET = Cumulative contact enhancement test. FCAT = Freund's complete adjuvant test.

3.4.2 Specific procedures for the guinea pig test methods using a multi-dose-response induction protocol [III, V]

3.4.2.1 Induction

A modification of the multi-dose-response induction protocol (62) was used in paper III and V. In each of the experiments the animals were divided into 6 groups (generally with 8 animals in each) with 5 induction concentrations and one sham treated control group. The topical exposure in the GPMT was applied at two concentrations – one higher and one lower – alternating between the experimental groups in each series, starting with the lower topical concentration in the group with the highest intradermal concentration applied (62). The concentrations used, are summarized in Table 5 [III], Figure 17 in the result section [V] and Table 2 in paper V. The inductions were done according to the three methods as described in 3.4.3-3.4.5.

3.4.3 Guinea pig maximization test (GPMT) [III, V]

The GPMT method (Figure 8) was carried out in accordance with the original protocol (8, 33, 47) [V] and as discussed in section 3.4.1, or with the modifications mentioned in section 3.4.2 [III]. The first exposure at induction (day 0) was made by three pairs of intradermal injections of 0.1 ml in the shoulder region on each animal; emulsion of FCA/vehicle (or FCA/water) (1:1), test substance in vehicle and test substance in emulsion of FCA/vehicle (or FCA/water) (1:1). One week later (day 7) a second exposure of 0.2 ml of test substance in the vehicle was applied topically to the same area (each application: 2x4 cm) for 24 h using an occlusive dressing with filter paper (Whatman, England) on Blenderm® tape (3M) and acrylastic bandage. Challenge was performed as described in section 3.4.1.3.

Guinea Pig Maximization Test (GPMT)

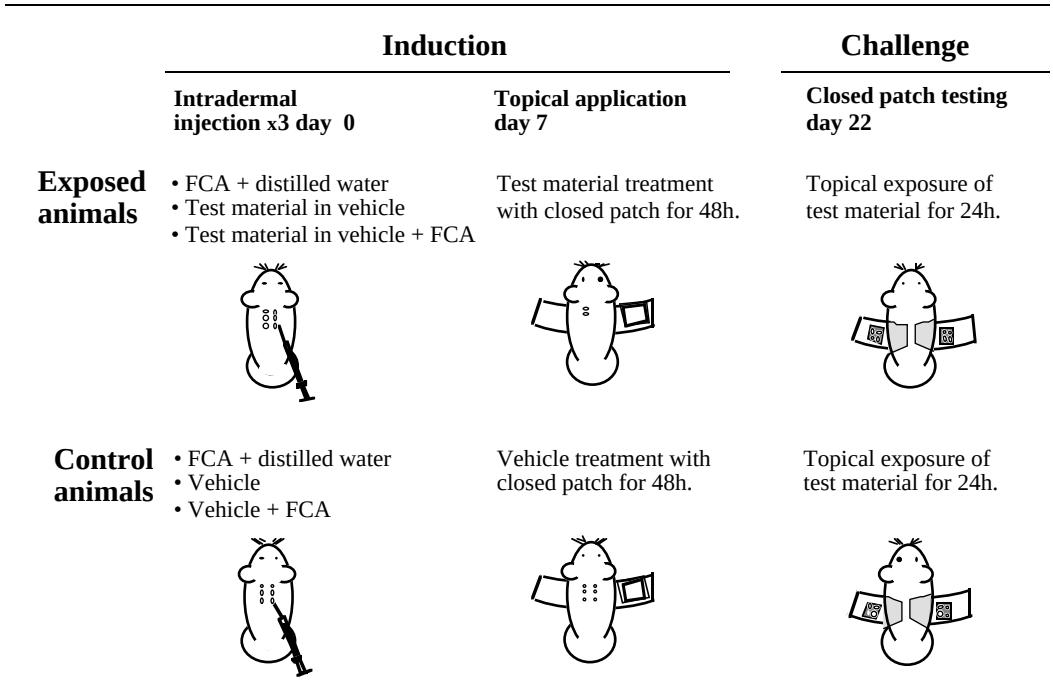


Figure 8. The protocol for the guinea pig maximization test (GPMT) (8, 33, 47) with sham-treated control animals.

3.4.4 Cumulative contact enhancement test (CCET) [III, V]

The original description of CCET (Figure 9) (40) was followed, including the modifications mentioned in section 3.4.2 [III, V]. Induction was carried out by four topical applications (days 0, 2, 7 and 9) of 0.2 ml test substance in the shoulder region (each application: 2x4 cm) for 24 h using an occlusive dressing as described in the GPMT method and with two intradermal injections of 0.1 ml FCA (day 7) in the same region. Challenge was performed as described in section 3.4.1.3.

Cumulative Contact Enhancement Test (CCET)

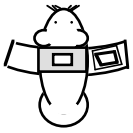
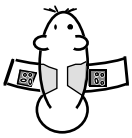
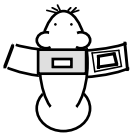
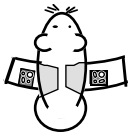
	Induction		**Challenge**
	Topical application days 0, 2, 7, 9	**Intradermal injection day 7**	**Closed patch testing day 22**
Exposed animals	Test material treatment with closed patch for 24h.	FCA i.d. x 2	Topical exposure of test material for 24h.
			
Control animals	Vehicle treatment with closed patch for 24h.	FCA i.d. x 2	Topical exposure of test material for 24h.
			

Figure 9. The protocol for the cumulative contact enhancement test (CCET) (40) with occluded challenge and sham-treated control animals.

3.4.5 Freund's complete adjuvant test (FCAT) [III]

The FCAT (Figure 10) was performed as described originally (9, 36), with the modifications mentioned in section 3.4.2 [III]. Induction was carried out by giving three intradermal injections (day 0, 6, 10) of 0.1 ml of test substance in FCA/vehicle (or FCA/water) emulsion (1:1). Challenge was performed as described in section 3.4.1.3.

Freund's Complete Adjuvant Test (FCAT)

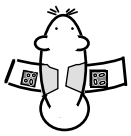
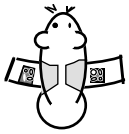
	Induction	**Challenge**
	Intradermal injection days 0, 6, 10	**Closed patch testing day 22**
Exposed animals	Test material in vehicle + FCA	Topical exposure of test material for 24h.
		
Control animals	Vehicle + FCA	Topical exposure of test material for 24h.
		

Figure 10. The protocol for the Freund's complete adjuvant test (FCAT) (9, 36) with sham-treated control animals.

3.5 Statistical analysis [III-V]

3.5.1 Fisher's exact test [V]

The results from the GPMT study in paper V were analyzed using Fisher's exact test (145-146).

3.5.2 Logistic regression analysis [III-V]

The statistical PC computer program "Program for multi-dose-response analysis", i.e. a logistic regression analysis (65), included in the multi-dose-response induction protocol developed for the GPMT (62) was used in papers III-V. This computer program calculates and adapts the best fitted monotone or non-monotone model curve to the observed sensitization rates in the experiments for the tested substances, i.e. contact allergens. It also calculates chi-square (χ^2) for goodness of fit to the curve and dose-response relationship. The series of sensitization rates

giving the best fit to the curve and a statistically significant dose-response was usually chosen for further analysis.

The threshold concentration at sensitization was chosen empirically to be the concentration giving at least the lowest possible sensitization rate of 0.125 (one animal out of eight), and this sensitization rate was used in the analysis. The maximal sensitization rate was decided to be the highest number of sensitized animals obtained in any experiment group at the chosen induction and challenge series [III, V] or sensitization criterion [IV]. The estimated concentration sensitizing 50% of the animals (EC_{50}) was calculated by the program. Since the program failed to calculate the threshold concentration and EC_{50} for the non-monotone dose-response curves, these curves were cut at the point where they started to decrease [III, V]. This procedure resulted in monotone curves with fewer induction concentrations, from which it was possible to calculate the parameters mentioned. The obtained monotone dose-response curves, which were similar to the non-monotone dose-response curves given, were used for the calculations in paper IV.

3.6 Patch testing in patients [V]

Patients referred to and examined at the Department of Occupational and Environmental Dermatology in Stockholm, were tested with a standard series and with products and materials from their work environment. The concentrations of the substances used are shown in the results section (Table 14). Finn chambers® (Epitest Ltd Oy, Tussula, Finland) on Scanpor® tape (Norgesplaster AS, Norway) were used and the exposure time was 48 h (7). The readings took place on 2 occasions- on Day 3 (24 h after removal of the patches) and on Day 5-7. The test reactions were recorded according to the ICDRG (147).

4. Results

4.1 Prediction of sensitizers using the LLNA [I-II]

4.1.1 The outcome with eight allergens and six irritants [I-II]

The ability of the predictive test method, LLNA, to discriminate between allergens and irritants was investigated by testing 14 chemicals. In paper I, seven of eight allergens and all five irritants tested were classified as sensitizers using the LLNA (Figure 11). The contact allergens known to be moderate to potent sensitizers in guinea pigs (DNFB, oxazolone, DNCB, t-cinnamic aldehyde, 2-mercaptobenzothiazole, p-PDA) all gave clear positive results and showed a dose-response relationship (Table I in paper I). The SI-values of the less potent contact allergens HC and benzocaine, were, respectively, slightly above (SI=3.4) and slightly below (SI=2.9) the limit (SI=3) for being classified as allergens in the LLNA. The five irritants tested (SDS, oxalic acid, methyl salicylate, the non-ionic surfactant Triton X-100 and a mixture of chloroform/methanol (2:1)) gave SI-values above or equal to the limit (SI=3) and also showed a clear dose-response (Table II in paper I).

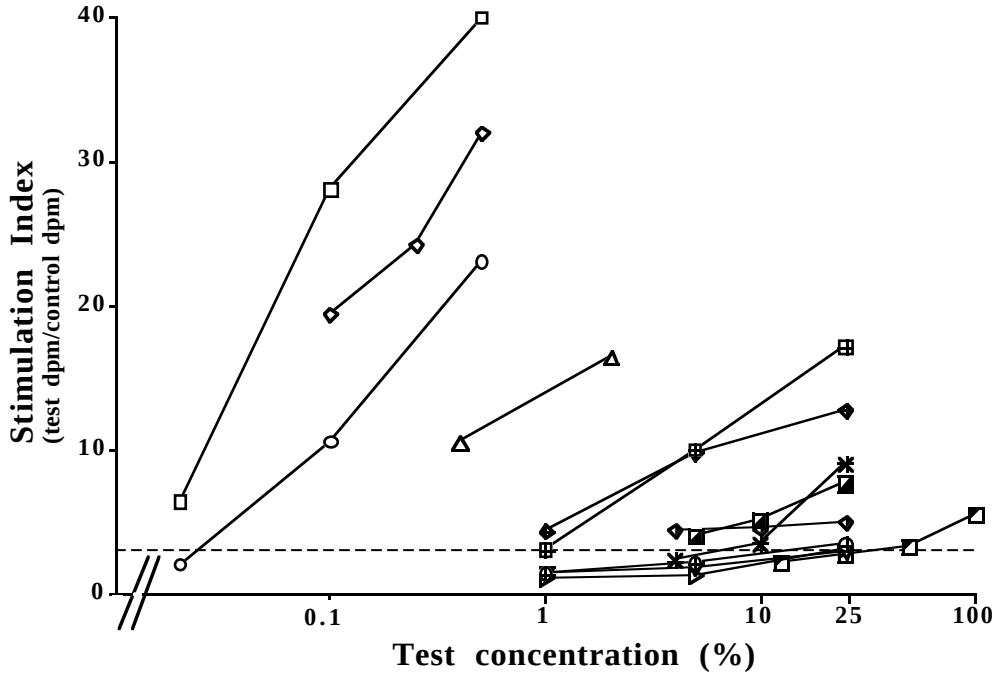


Figure 11. Summary of test results using the LLNA of eight allergens: DNFB (□); oxazolone (◇); DNCB (○); t-cinnamic aldehyde (⌖); 2-mercaptobenzothiazole (⊞); p-PDA (△); HC (⊕); benzocaine (▽, almost totally obscured by HC) and five irritants: SDS (◪); oxalic acid (◆); methyl salicylate (▷); Triton X-100 (✳); nonanoic acid (◩); chloroform/methanol (2:1) (not shown). The dotted horizontal line (- - - - - - -) shows three times the control value (SI=3). Data from Tables I and II in paper I, and from Table II in paper II.

In paper II, one additional irritant (nonanoic acid) (Figure 11) was tested and one irritant (methyl salicylate) was re-tested in two different vehicles (Figure 12) in the LLNA. Both irritants caused a dose-dependent increase in cell-proliferation and according to the method, were classified as contact allergens when tested at higher concentrations. The use of DMF or MEK as vehicles had only marginal effects on the results (Figure 12 and Table I in paper II) and the proliferation activity in lymph nodes of mice treated with either neat vehicle was just slightly increased compared to that in naive mice (Table I in paper II).

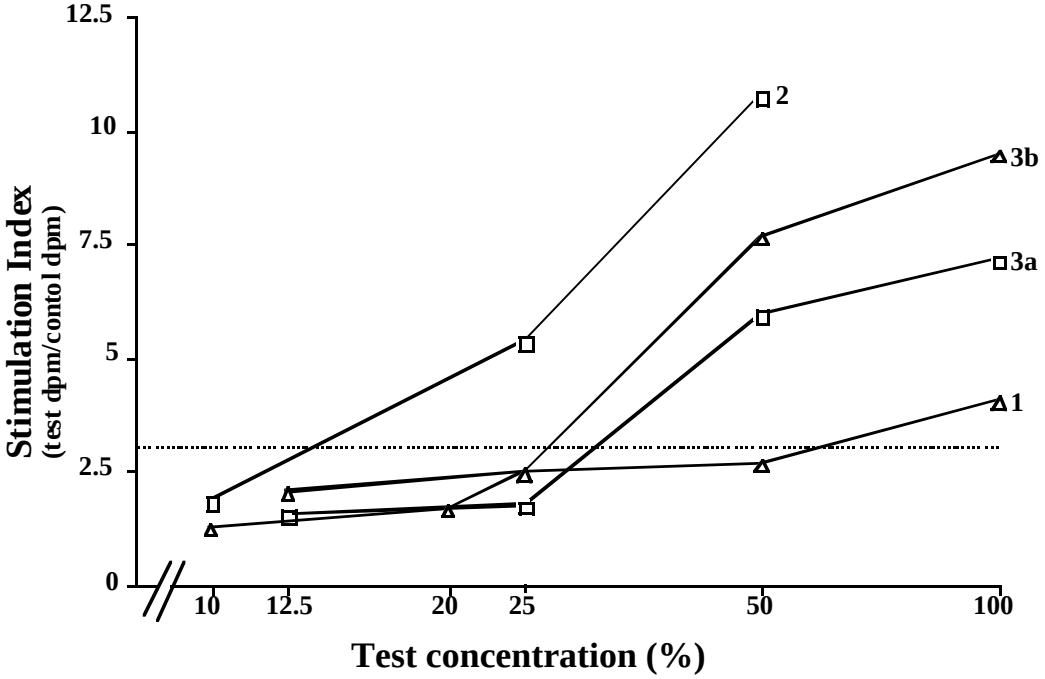


Figure 12. Test results obtained using the LLNA with methyl salicylate in the vehicles MEK (□) and DMF (△).The experiments (1, 2, and 3a and 3b) were done on three occasions and 3a and 3b were performed on the same occasions. The dotted horizontal line (-------) shows three times the control value (SI=3). Data from Table I in paper II.

4.1.2 SDS induced proliferation [I]

SDS was tested according to different schedules (3, 4, 5 or 6 days), in two experiments, to investigate the time course of the proliferation induced in the LLNA. A maximal or almost maximal proliferation was induced as early as 3 days after the first application of SDS with an SI-value around 5 to 6, which was maintained or slightly elevated at day 6 (Figure 13).

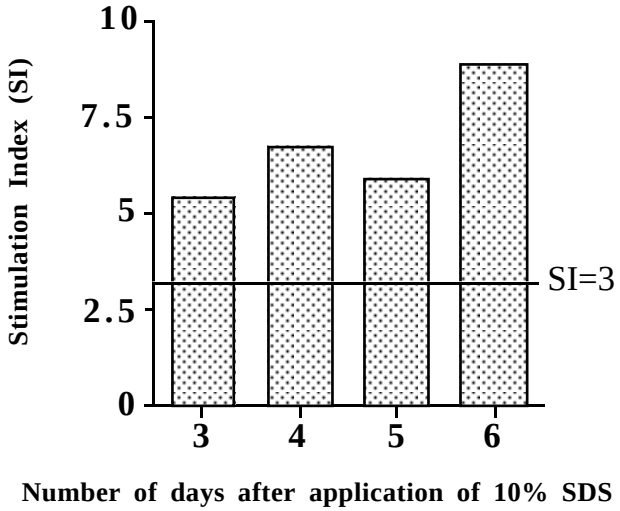


Figure 13. Stimulation index of 10% SDS using the LLNA - 3, 4, 5 and 6 days after the first of three daily applications. The horizontal line (_____) shows three times the control value (SI=3). Data from Table III in paper I.

4.1.3 The addition of SDS to the test samples [I]

The effect of addition of SDS, an irritant, to the vehicle when testing two well known allergens in LLNA was investigated. The proliferative activity of 10% SDS in combination with different concentrations of the allergens HC and t-cinnamic aldehyde was studied and compared with the proliferation caused by the same concentrations of the allergens without SDS. There was a parallel shift upwards of the dose-response curve for HC when 10% SDS was applied (Figure 14a). The proliferative activity induced by SDS is added to the proliferation induced by the allergen. The shift upwards of the dose-response curve of t-cinnamic aldehyde, when 10% SDS had been applied, was slightly different (Figure 14b). The proliferative activity induced by the combination of allergen and SDS was larger than the sum of the proliferation they induced separately.

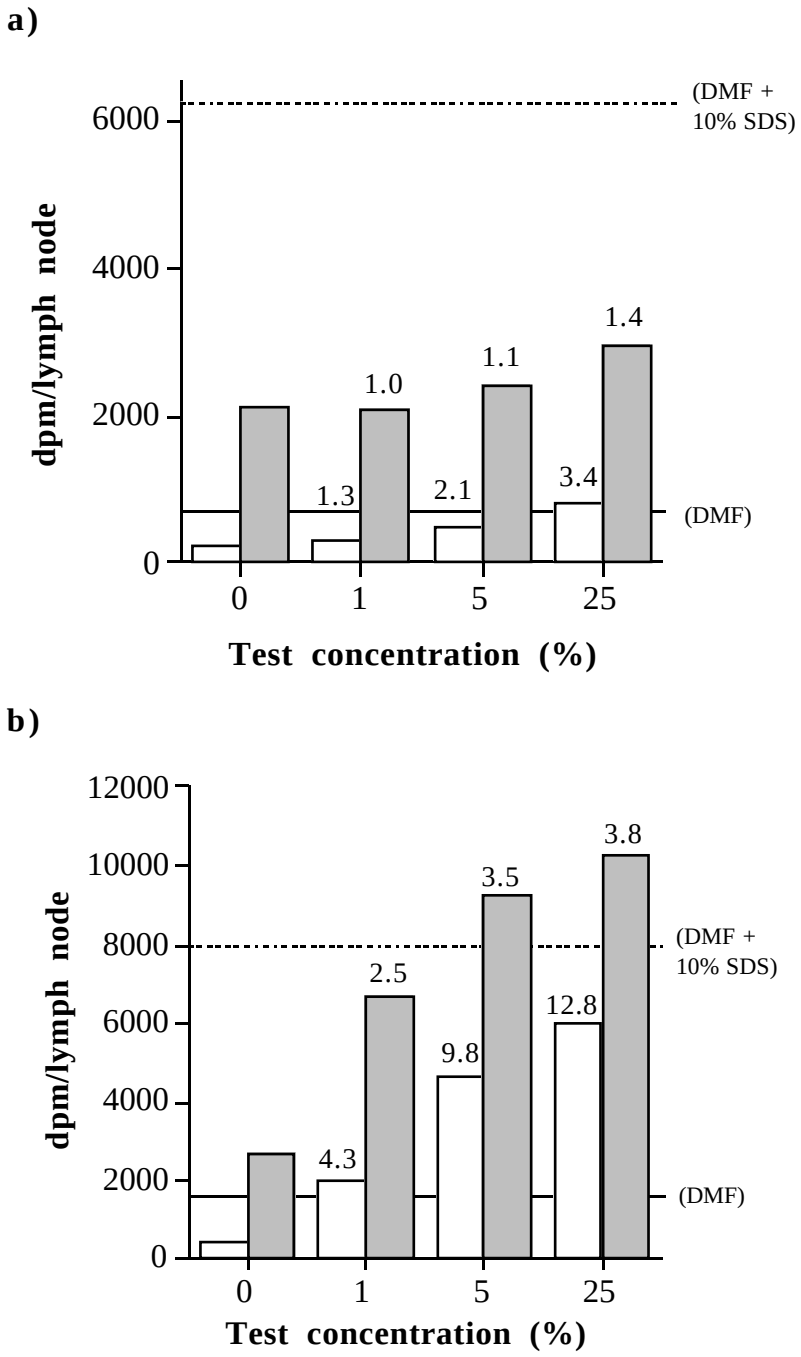


Figure 14. The proliferation activity in the lymph nodes using the LLNA after treatment with an allergen and after combined treatment with an irritant and an allergen. a) HC (□) and HC with 10% SDS (▨). b) t-cinnamaldehyde (□) and t-cinnamaldehyde with 10% SDS (▨). The horizontal lines (——) and (– – – –) show three times the control value with DMF and DMF + 10% SDS, respectively (SI=3). Numbers on bars indicate SI values.

4.1.4 EC_3 values for two irritants [II]

The application of LLNA to rank the relative skin-sensitizing potential of chemicals was investigated. The relative potency is ranked as a function of the concentrations required to give SI=3. This concentration is expressed as EC_3 (estimated concentration for SI=3). The EC_3 for the two irritants, methyl salicylate and

nonanoic acid, were read from the graph in Figure 1 in paper II and compared with the EC_3-values for five allergens and one irritant from the literature (Table 6). Methyl salicylate and nonanoic acid were only slightly less potent inducers of proliferation in the LLNA than the allergens eugenol, penicillin G, hexyl cinnamic aldehyde, and HC.

Table 6. Relative activity of induced proliferation in local lymph node assay (LLNA) for some allergens and irritants.

Substance	EC_3[a]		References
	(%)	(M)	
DNCB	0.0765	0.00383	(148)
Eugenol	5.8 - 14.5	0.353 - 0.883	(142)
HC	20	1.28	(149)
Hexyl cinnamic aldehyde	6.85 - 9.63	0.317 - 0.445	(150)
Methyl salicylate	15 - 65	0.99 - 4.27	(II)
Nonanoic acid	35	2.21	(II)
Penicillin G	20	0.561	(151)
SDS	1.5 - 17.1	0.052 - 0.593	(142)

a) EC_3 value is defined as the concentration of the test material required to elicit a stimulation index of three in the LLNA (142-143) and is given in % (w/v) and in mol/dm^3 (M). HC = hydroxycitronellal. SDS = sodium dodecyl sulfate.

4.2 Prediction of sensitizing capacities using a multi-dose-response induction protocol in the GPMT, the CCET and the FCAT [III]

4.2.1 Sensitization experiments with the model contact allergens

The application of the slightly modified multi-dose-response induction protocol on three guinea pig models - GPMT, CCET and FCAT - was investigated. The model contact allergens, $K_2Cr_2O_7$ and HC, resulted in a clear sensitization of the exposed animals when this protocol was applied. However, in the initial experiments testing $K_2Cr_2O_7$ in the GPMT and the CCET (nos. I and III, Table 5), almost a maximal sensitization was attained in all exposed induction groups, groups 13-18 (data not shown). This made it impossible to study dose-response relationships. Therefore, the induction concentrations were lowered in the two following experiments with the GPMT and the CCET and resulted in different numbers of positive reactions in each experimental series. Re-challenge was done with $K_2Cr_2O_7$ in the GPMT (no. II, Table 5) and the CCET (no. III, Table 5) at 2 weeks and at 5 weeks after the first challenge, respectively, and confirmed the sensitization results at the first challenge (data not shown). A variable degree of dose-response was seen in all tests both for the challenge concentration gradient and in the induction concentration gradient as demonstrated with HC in CCET in Table II in paper III.

4.2.2 Data assessment

The logistic regression analysis computer program in the protocol was used to calculate which series of results from each sensitization experiment could give the best fit to a dose-response curve. Those results are summarized in Table 7 and were used for further dose-response analysis.

Table 7 Results from sensitization experiments using the multi-dose-response induction protocol in the GPMT, CCET and FCAT.

	Challenge conc. for $K_2Cr_2O_7$						Challenge conc. for HC					
	GPMT: 0.03%		CCET: 0.1%		FCAT: 0.1%		GPMT: 3%		CCET: 3%		FCAT: 10%	
Animals	Group	No. pos/tested	Group	No. pos/tested	Group	No. pos/tested	Group	No. pos/tested	Group	No. pos/tested	Group	No. pos/tested
Exposed	7	3/4	19	5/9	25	7/8	31	2/8	37	5/8	43	6/8
	8	4/5	20	7/9	26	6/8	32	4/8	38	7/8	44	4/8
	9	3/5	21	3/9	27	5/8	33	3/8	39	7/7	45	3/8
	10	2/5	22	1/8	28	3/8	34	5/8	40	6/8	46	1/8
	11	0/5	23	1/8	29	3/8	35	2/8	41	3/8	47	0/8
Control	12	2/5	24	0/8	30	1/8	36	0/8	42	2/8	48	0/8

The data shown were those fitting best to one of the model curves and they were used for logistic regression analysis (Table 8, Figures 15a-b).
The number of positive animals/number of tested animals at challenge for each induction group is given. Further details are presented in Table 5. Allergens: potassium dichromate ($K_2Cr_2O_7$) and hydroxycitronellal (HC). GPMT = Guinea pig maximization test. CCET = Cumulative contact enhancement test. FCAT = Freund's complete adjuvant test.

The best fitted dose-response curves for each of the methods - GPMT, CCET, FCAT - are shown for $K_2Cr_2O_7$ in Figure 15a and for HC in Figure 15b. The fitted curves from the results with $K_2Cr_2O_7$ were non-monotone for the GPMT and the CCET and monotone for the FCAT. The fitted curves from the results with HC were non-monotone for all three methods. A significant dose-response relationship was found for the curves fitted from the CCET and FCAT for both model allergens. However, no dose-response relationship was seen for the curves fitted from the GPMT with the model allergens tested, due to divergent elicitation results.

The calculations of different parameters by the analysis computer program are summarized in Table 8. The χ^2 values for goodness of fit and dose-response relationship, the maximal sensitization rate, the EC_{50} and the threshold concentration for these six curves are given for a constant challenge concentration. There was no clear pattern in the sensitivity of the three methods used. The FCAT was the most sensitive test for $K_2Cr_2O_7$ and gave the highest response rate and the lowest EC_{50} and threshold concentration. For HC the highest sensitization rate was seen in the CCET, but the lowest EC_{50} and threshold concentration were seen in the GPMT. None of these test methods could be recommended in preference to the others.

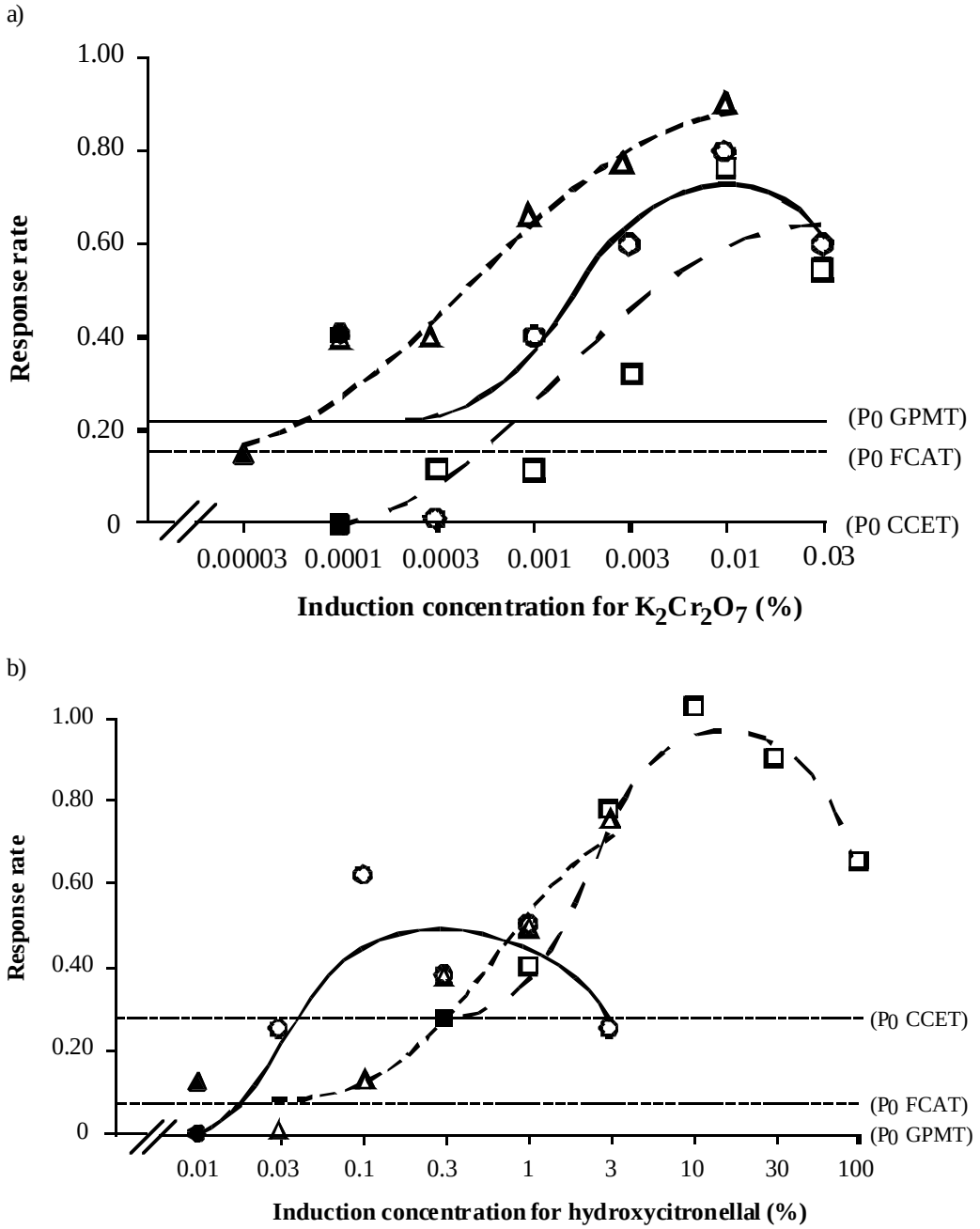


Figure 15. Dose-response curves at constant challenge concentrations using the multi-dose-response induction protocol on the guinea pig maximization test (GPMT) (○), the cumulative contact enhancement test (CCET) (□), and the Freund's complete adjuvant test (FCAT) (△) for a) Potassium dichromate ($K_2Cr_2O_7$) and b) Hydroxycitronellal (HC). Challenge reactions read at 72 h. Data from Tables 5 and 7. The $\log_{10}$ for the vehicle control group, i.e. concentration 0% (filled symbols), is not defined, but in the graph assigned a value by the computer program at the onset of each curve by a factor of three from the lowest induction concentration used in each experiment. (Figures reproduced with the permission from the publisher).

Table 8. Results from sensitization experiments in guinea pigs using the multi-dose-response induction protocol.

Allergen	Method[a]	Ch. conc.[b]	Statistical sign.[c]	Max. rate[d] (pos./exp.)	EC_{50}[e]		TC[f]	
					%	molal	%	molal
$K_2Cr_2O_7$	GPMT[g]	0.03	ns	4/5	$2.5x10^{-3}$	$8.5x10^{-5}$	$4.4x10^{-6}$	$1.5x10^{-7}$
	CCET	0.1	**	7/9	$4.2x10^{-3}$	$1.4x10^{-4}$	$6.6x10^{-4}$	$2.2x10^{-5}$
	FCAT	0.1	**	7/8	$4.5x10^{-4}$	$1.5x10^{-5}$	1.1x10-7	$3.7x10^{-9}$
HC	GPMT[g]	3	ns	5/8	$6.8x10^{-2}$	$4.0x10^{-3}$	$1.6x10^{-2}$	$9.3x10^{-4}$
	CCET	3	**	7/7	1.8	$1.1x10^{-1}$	0.31	$1.8x10^{-2}$
	FCAT	10	**	6/8	0.89	$5.2x10^{-2}$	$7.2x10^{-2}$	$4.2x10^{-3}$

Test results from the 72 h reading are used for the analysis; data from Tables 5 and 7. Allergens: potassium dichromate ($K_2Cr_2O_7$) and hydroxycitronellal (HC).

[a)]All methods followed the multi-dose-response induction protocol with a constant challenge concentration.

[b)]Challenge concentration for the curve chosen.

[c)]All curves had a goodness of fit to the curve, i.e., χ^2_{FIT}, which was not significant (ns) and all curves were accepted (Table 4 in paper III). The dose-response relationship, i.e. $\chi^2_{D\text{-}R}$, was calculated; ** $p<0.01$ or not significant (ns).

[d)]Maximal sensitization rate. The maximal number of animals with a positive reaction/number of animals exposed.

[e)]Estimated concentration. Calculated induction concentration sensitizing 50% of the animals, given in % (w/w) and molal (mol/kg).

[f)]Threshold concentration. Lowest induction concentration that can sensitize, given in % (w/w) and molal (mol/kg). Chosen empirically to be at the least the animal response rate 1/8=0.125.

[g)]Calculated values using results from the GPMT method were based only on the id administration. GPMT = Guinea pig maximization test. CCET = Cumulative contact enhancement test. FCAT = Freund's complete adjuvant test.

4.3 Sensitization results using a multi-dose-response induction protocol in a modified MEST [IV]

4.3.1 Sensitization experiments with four allergens and one irritant

The application of an adjusted multi-dose-response induction protocol on a modified MEST was evaluated with four chemicals previously identified as contact allergens: oxazolone, $K_2Cr_2O_7$, MDBGN and HC, and the irritant oxalic acid. Different sensitization criteria were used to obtain a variable degree of dose-response relationship with the induction concentration gradients used for the substances tested (data not shown), as is exemplified with the strong contact allergen oxazolone (positive control) in Table 1 in paper IV. The relative ear thickness after application of the test substances was calculated for the different induction groups at the sensitization criteria chosen by the statistical analysis (section 4.3.2) (Table 9). A clear increase in the relative ear thickness was seen with oxazolone (up to 30 %) and $K_2Cr_2O_7$ (up to 11 %), while no or possibly slight increases were shown for the other substances tested. Data from literature on the relative ear thickness and sensitization rates were compared with our results for some of the substances tested (Table 10).

Table 9. Results from the modified mouse ear swelling test (MEST) with the test substances using the multi-dose-response induction protocol. The relative ear thicknesses for the respective induction groups are also given.

Substance	Challenge conc.[a] (w/v %)	Induction conc.[a] (w/v %)	Sens. criterion[b]	Measuring time	Sens. rate (pos/exp)	Relative ear thickness(%)
Oxazolone	0.3	AOO	15 %	48 h	0/8	104
	in AAO	0.0003			2/8	108
		0.003			5/8	115
		0.03			7/8	119
		0.3			8/8	130
$K_2Cr_2O_7$	0.3	DMSO	10 %	24 h	1/8	104
	in DMSO	0.03			4/8	107
		0.1			5/8	108
		0.3			7/8	111
		1.0			5/6	110
MDBGN	3	DMF	5 %	48 h	2/6	103
	in DMF	0.3			2/8	102
		1.0			5/7	105
		3.0			6/7	103
HC	10	DMF	10%	24 h	0/8	103
	in DMF	3			0/8	104
		10			1/8	105
		30			1/8	103
		100			2/8	105
Oxalic acid	0.3	DMF	10%	48 h	0/8	99
	in DMF	0.1			1/7	103
		0.3			1/7	103
		1.0			0/7	100

The sensitization rates were further used in the dose-response analysis (Table 11 and Figure 16a-c).
Induction and challenge: Oxazolone was applied topically in acetone/olive oil, 4:1 (AOO) at induction and in acetone/arachis oil, 4:1 (AAO) at challenge. Potassium dichromate ($K_2Cr_2O_7$) was applied topically in dimethylsulfoxide (DMSO). Methyldibromo glutaronitrile (MDBGN), hydroxycitronellal (HC), and oxalic acid were applied topically in dimethylformamide (DMF).
[a)]HC was applied topically; concentrations are given in v/v %.
[b)]Series of sensitization rates were chosen on the basis of hypothetical sensitization criteria in percent. Usually the series that gave the best fit to a model curve and a statistically significant dose-response relationship was chosen. If no statistical significance was obtained the results were presented at a 10% (no irritation in pretest) sensitization criterion.

Table 10. Results obtained with the contact allergens tested in the modified mouse ear swelling test (MEST) using a multi-dose-response induction protocol. For comparison, literature data from mouse assays using MEST methodology are also presented.

Substance	Ind. conc.[a] (%)	No. appl.[b]	Ch. conc.[c] (%)	Vehicles[d]	Relative ear thickness (%)	Sens. rate (%)	Statistical sign.[e]	References
Oxazolone	0.3	3	0.3	AOO, AAO	130	100	***	paper IV
Oxazolone	5.0	4	0.1	Ac	134	100	nd	1986 (68)
Oxazolone	3.0	3	0.1	Ac	140	nd	**	1988 (70)
Oxazolone	0.1	4	0.1	Ac	161	100	**	1993 (152)
$K_2Cr_2O_7$	1.0	3	0.3	DMSO	111	88	**	paper IV
$K_2Cr_2O_7$	2.0	4	2.0	25% EtOH	114	40	nd	1986 (68)
$K_2Cr_2O_7$	2.0	3	2.0	EtOH	109	nd	*	1988 (70)
MDBGN	3.0	3	3.0	DMF	105	86	*	paper IV
HC	100	3	10	DMF	105	25	ns	paper IV
HC	30	6	20	AOT	nd	nd	***	1986 (67)
HC	25	6	20	MEK	nd	nd	***	1991 (81)

Contact allergens: 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), potassium dichromate ($K_2Cr_2O_7$), methyldibromo glutaronitrile (MDBGN), and hydroxycitronellal (HC).
Vehicles: Acetone (Ac), acetone:arachis oil, 4:1 (AAO), acetone:olive oil, 4:1 (AOO), acetone:oil:Tween 80, 4:3:1 (AOT), dimethylsulfoxide (DMSO), dimethylformamide (DMF), ethanol (EtOH), methyl ethyl ketone (MEK).
[a)]The induction concentration (highest) used.
[b)]Number of topical treatments applied.
[c)]The challenge concentration used.
[d)]The vehicles used at induction and challenge.
[e)]Statistical significance of the test results with the statistical analysis used in each respective study.
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and not significant (ns).
nd = not determined.

4.3.2 Data assessment

The resulting sensitization rates in the induction groups for all substances tested at the various sensitization criteria are shown i Table 9. These data represent in general the best fit to the model curves using the logistic regression analysis computer program and the sensitzation rates were used further in the analysis of the results.

This protocol could detect the moderate to strong contact allergens, i.e. oxazolone and $K_2Cr_2O_7$. and the weak contact allergen MDBGN as sensitizers. A significant dose-response relationship was obtained for the fitted curves for these contact allergens (Table 11). The sensitization rates for oxazolone at a 15 % sensitization criterion gave a good fit to a non-monotone curve (Table I in paper IV, Tables 9, 11 and Figure 16a). A slightly better fit of the sensitization rates for oxazolone to a monotone curve at 10 % sensitization criterion with a statistically significant dose-response relationship was also obtained. However, this curve was not chosen, since several of the sensitization rates were at maximum giving a smaller spread of the sensitization rates in the interval of induction concentrations tested (data not shown). The sensitization rates obtained for $K_2Cr_2O_7$ gave a fitted non-monotone curve at the 10 % sensitization criterion (Tables 9, 11 and Figure 16b). No curve

could be fitted to the sensitization rates for MDBGN at a 10 % sensitization criterion due to the low increases in the relative ear thickness (Table 9). The sensitization rates for MDBGN at a 5 % sensitization criterion were used in the analysis and gave an acceptable fitted monotone curve (Tables 9, 11 and Figure 16c).

Table 11. Sensitizing potential of five chemicals in the modified mouse ear swelling test (MEST) using a multi-dose-response induction protocol.

Substance	Sensitization criterion[a]	Statistical significance[b]	Max. rate[c] (pos./exp.)	EC_{50}[d]		TC[e]	
				(%)	(M)	(%)	(M)
Oxazolone	15 %	***	8/8	0.002	$9.2x10^{-5}$	$1.0x10^{-4}$	$4.6x10^{-6}$
$K_2Cr_2O_7$	10 %	**	7/8	0.03	$6.8x10^{-4}$	$8.4x10^{-7}$	$2.9x10^{-3}$
MDBGN	5 %	*	6/7	0.7	$2.6x10^{-2}$	0.04	$1.5x10^{-3}$
HC	10 %	ns	2/8	nd	nd	nd	nd
Oxalic acid	10 %	ns	1/7	nd	nd	nd	nd

Data from Table 9. Dose-response curves are shown in Figures 16a-c. Allergens: oxazolone, potassium dichromate ($K_2Cr_2O_7$), methyldibromo glutaronitrile (MDBGN), and hydroxycitronellal (HC). Irritant: oxalic acid.

[a)] Series of sensitization rates were chosen on the basis of hypothetical sensitization criteria in percent. Usually the series that gave the best fit to a model curve and a statistically significant dose-response relationship was chosen. If no statistical significance was obtained the results were presented at a 10 % (no irritation in pretest) sensitization criterion.

[b)] All presented curves had a goodness of fit to the curve, i.e. χ^2_{FIT}, which was not significant (ns) and the curves were all accepted (Table III in paper IV). The dose-response relationship, i.e. $\chi^2_{D\text{-}R}$, was calculated; *** $p<0.001$, ** $p<0.01$, * $p<0.5$ and not significant (ns).

[c)] Maximal sensitization rate. The maximal number of animals with a positive reaction/number of animals exposed.

[d)] Calculated induction concentration sensitizing 50 % of the animals, given in percent (w/v) and M (mol/L). For substances not giving a statistically significant dose-response calculations were not done (nd).

[e)] Lowest induction concentration that can sensitize, given in percent (w/w) and M (mol/L). Chosen empirically to be at the least the animal response rate of 1/8=0.125. For substances not giving a statistically significant dose-response calculations were not done (nd).

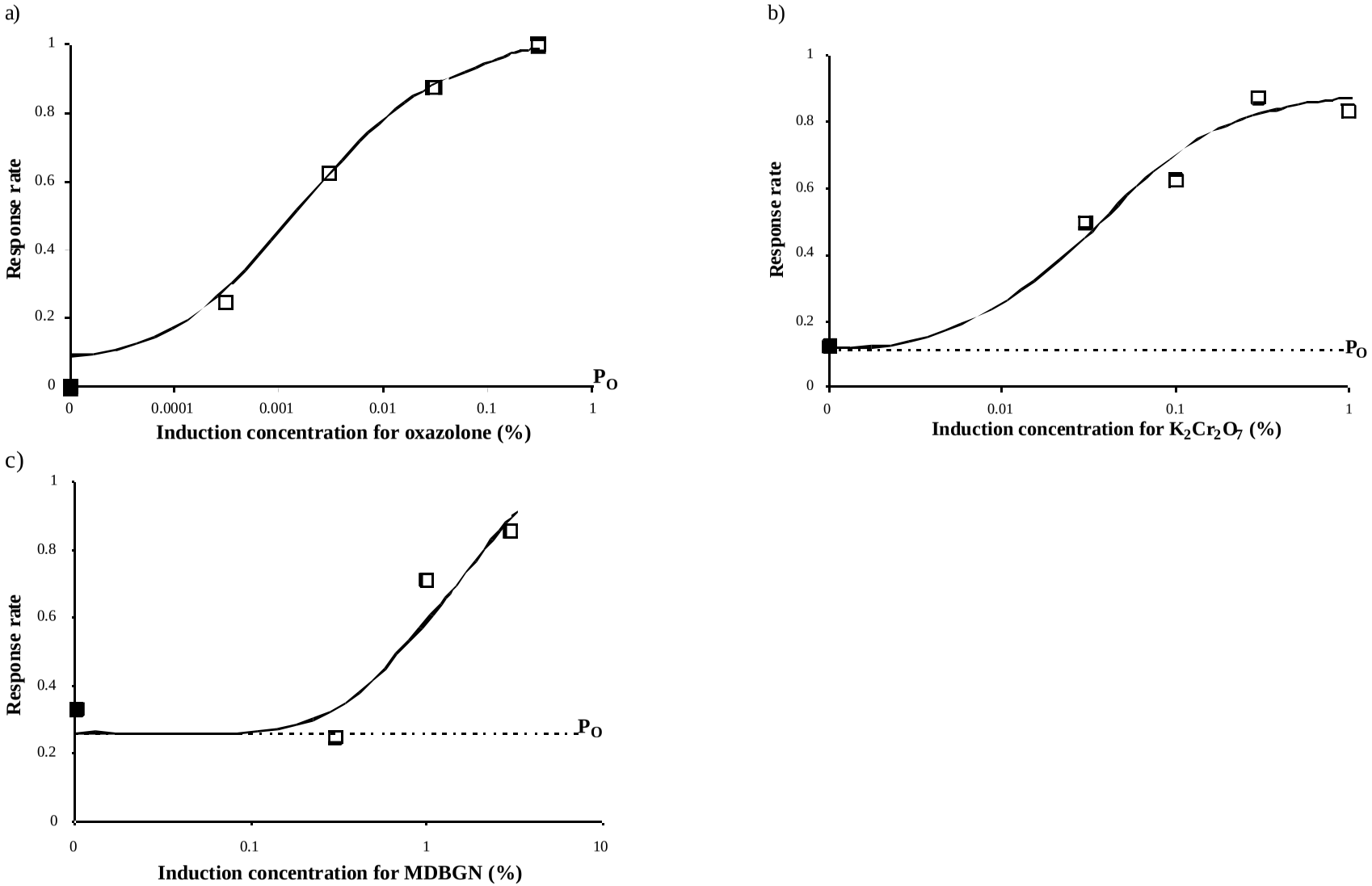


Figure 16. The dose-response curves obtained by using the multi-dose-response-induction protocol in the modified ear swelling test (MEST). a) Oxazolone at a 15 % sensitization criterion. b) Potassium dichromate ($K_2Cr_2O_7$) at a 10 % sensitization criterion. c) Methyldibromo glutaronitrile (MDBGN) at a 5 % sensitization criterion. Results from the 24 h measurement were used for the analysis. Data from table 9. The $\log_{10}$ for the vehicle control, i.e. concentration 0% (filled symbols), is not defined, but in the graph assigned a value by the computer program at the onset of each curve. P_0 = baseline for statistical calculation.

The sensitizing capacities of the contact allergens - oxazolone, $K_2Cr_2O_7$ and MDBGN - are shown in Table 3, where the values for threshold concentration for sensitization, EC_{50} and maximal sensitization rate are given. However, no statistically significant dose-response relationship could be confirmed for the weak contact allergen, HC. A 'negative' response was obtained with the irritant, oxalic acid (negative control), at the concentrations tested (Table 9, 11).

4.4 The allergenicity of a preservative according to different test methods (V)

The allergenicity of Euxyl K 400 and one of its ingredients MDBGN was investigated using three different predictive test methods, the GPMT, the CCET using a slightly modified multi-dose-response induction protocol, and the LLNA. The ability of these three test methods to detect the allergenicity of the Euxyl K 400 and/or MDBGN varied.

4.4.1 Sensitization experiment with GPMT

Sensitization with MDBGN gave a few positive reactions at the challenge concentration 1% MDBGN in OO and a single positive reaction was also seen with the challenge concentration 1% MDBGN in PE (Table 12). However, no statistically significant sensitization was induced by MDBGN in OO or in PE at any concentration tested. The challenge concentration 3% MDBGN in OO, which gave minimal irritation in the irritancy threshold study, was also included in the test. However, some of the control animals reacted to this concentration (data not shown).

Table 12. Results from sensitization studies in guinea pigs according to the guinea pig maximization test (GPMT) using methyldibromoglutaronitrile (MDBGN) for induction[a].

	Challenge material[b]						
	MDBGN (% in OO)				MDBGN (% in PE)[c]		
Guinea pigs	1	0.3	0.1	OO	1	0.3	PE
Exposed (n=15)	3	1	0	0	1	0	1
Control (n=15)	0	0	1	1	0	0	0
p exposed/control	ns	ns	ns	ns	ns	ns	ns

The number of animals with positive reactions at 72 h after test application is given.
[a)]*Induction*: MDBGN 0.1% in arachis oil intradermally and MDBGN 10% in arachis oil topically (w/w).
[b)]*Challenge material*: MDBGN in olive oil (OO) or MDBGN in phenoxyethanol (PE) (w/w).
[c)]Euxyl K 400 (20% MDBGN:80% PE) was diluted with additional PE to obtain the concentrations of MDBGN used.
ns: not significant

4.4.2 Sensitization experiment with CCET using a multi-dose-response induction protocol

Various dose-response relationships were seen among the guinea pigs sensitized with MDBGN in the different induction groups at the challenge concentrations used (Table 2 in paper V). The induction series and challenge series chosen for further analysis were the series with numbers of positive test reactions (sensitization rates) giving the best fit to the model curves. Curves with a significant dose-response were obtained for both the induction series at a constant challenge concentration of 3% (Figure 17 and Table 13) and the challenge series at a constant induction concentration of 3 % (Figure 1b in paper V and Table 13). The dose-response curve was non-monotone for the induction series (Figure 17) and monotone for the challenge series (Figure 1b in paper V). The sensitizing potential of MDBGN is presented in Table 13, as are the maximal sensitization rate, the EC_{50} and the threshold concentration for sensitization for these curves.

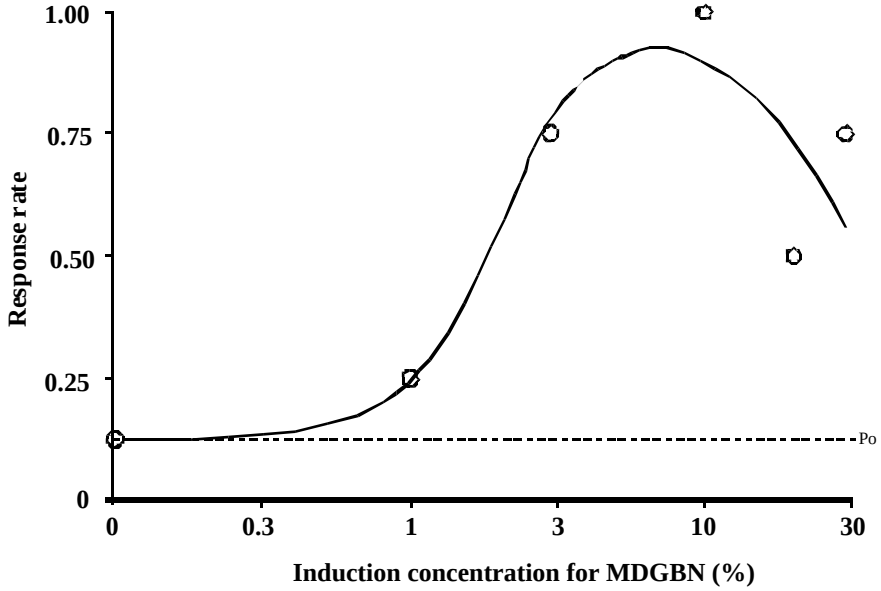


Figure 17. Dose-response curve obtained for methyldibromo glutaronitrile (MDBGN) at a constant challenge concentration of 3 % using the multi-dose-response-induction protocol in the cumulative contact enhancement test (CCET). Challenge reactions read at 72 h. Data from Table 2 in paper V. The $\log_{10}$ for the vehicle control group (concentration 0%) is not defined, but in the graph assigned a value by the computer program at the onset of the curve. P_0 = baseline for statistical calculations. (Figure reproduced with the permission from the publisher).

Table 13. Sensitizing capacity of methyldibromoglutaronitrile (MDBGN) in guinea pigs according to the cumulative contact enhancement test (CCET) using a dose-response protocol.

Constant conc.[a] used for	Conc.[b] (%)	Statistical sign.[c]	Max. rate[d] (pos./exp.)	EC_{50}[e]		TC[f]	
				%	molal	%	molal
Challenge	3	**	8/8	1.9	$7.2x10^{-2}$	0.3	$1.2x10^{-2}$
Induction	3	***	6/8	0.7	$2.8x10^{-2}$	0.02	$8.6x10^{-4}$

Data from Table 2 in paper V.

[a)]The dose-response protocol was followed; either the challenge concentration or the induction concentration was constant.

[b)]Challenge or induction concentration for the curve chosen.

[c)]All the curves had a goodness of fit to the curve, i.e., χ^2_{FIT}, which was not significant (ns) and all the curves were accepted. The dose-response relationship was calculated, χ^2_{D-R}, ** $p<0.01$ and *** $p<0.001$.

[d)]Maximal sensitization rate. The maximal number of animals with a positive reaction/number of animals exposed.

[e)]Calculated induction concentration sensitizing 50% of the animals, given in % (w/w) and molal (mol/kg).

[f)]Lowest induction concentration that can sensitize, given in % (w/w) and molal (mol/kg). Chosen empirically to be at the least the animal response rate 1/8=0.125.

4.4.3 Sensitization experiment with LLNA

The proliferation induced in the lymph nodes by Euxyl K 400 and MDBGN showed a dose-response relationship (Figure 18). Both Euxyl K 400 and MDBGN diluted in DMF or PE gave SI-values above three at the test concentration 5% and they were classified as sensitizers (Figure 18, Table 4 in paper V). The resulting lymph node weights and the [^{3}H]thymidine incorporation are also shown in Table 4 in paper V.

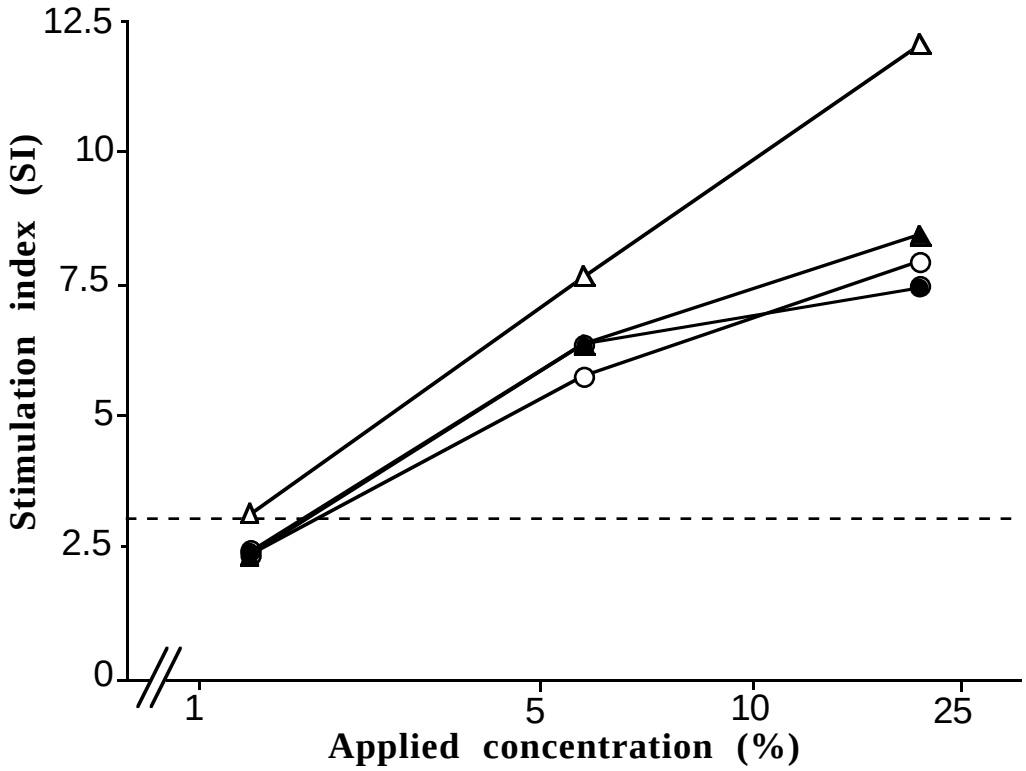


Figure 18. Proliferation induced by methyldibromo glutaronitrile (MDBGN) in phenoxyethanol (PE) (experiment 1 =▲, experiment 2a =△) and in dimethylformamide (DMF) (experiment 2b =●, experiment 3 =○), in sensitization experiments using the LLNA. Experiments 2a and 2b were performed on the same occasion. For test with 20% MDBGN in PE, Euxyl K 400 was used. The dotted (**- - - - - - -**) horizontal line shows three times the vehicle-treated control value (SI=3). Data from Table 4 in paper V. (Figure reproduced with the permission from the publisher).

4.4.4 Patch testing of patients

The frequency and causes of positive patch test reactions to Euxyl K 400 and MDBGN in patients with work-related contact dermatitis was also investigated to make comparisons possible with the experimental data. The preservative Euxyl K 400 was included in the standard series 1991-1996 and one of its ingredients MDBGN in 1996-1998. The patch testing of 1770 patients – referred due to work-related contact dermatitis - resulted in nine with relevant positive reactions (Table 14). Seven patients were positive to Euxyl K 400 and two patients were positive to MDBGN. The majority of those had been *occupationally* exposed (V), by using soaps, shampoos, creams, lotions or cleansing agents. One case was related to an adhesive and one to a cutting fluid containing MDBGN. There were also nine unexplained positive reactions to Euxyl K 400. The total frequency of positive patch test reactions varied between 0.9 and 1.8 % (Table 14) and more detailed data are presented in Table 5 in paper V.

Table 14. Patch test results with Euxyl K 400 and methyldibromo glutaronitrile (MDBGN) when included in the standard series at an occupational dermatology clinic.

Test preparation	Conc. (% in pet.)	Test period	Total pos.		Relevance
			n	%	n
Euxyl K 400	0.1	Dec-91 - Sept-92	3	1.4	1
Euxyl K 400	0.5	Aug-93 - Aug-95	5	0.9	3
Euxyl K 400	1.0	Sept-92 - Aug-93 Aug-95 -April-96	8	1.8	3
MDBGN	0.3	April-96 -Dec-98	7	1.3	2

4.5 The estimated sensitizing potentials of the tested substances and human data from literature [I-V]

The sensitizing potentials of all the substances studied in this thesis were estimated and used for comparison of the five predictive test methods used. The estimated sensitizing potentials were based on the degree of sensitization among the experimental animals in each test method, and the concentrations used were also taken into consideration (Table 15). Published patch test results from dermatitis patients are also presented for comparison (Table 15).

Table 15. Summary of the substances tested in the predictive animal test methods used in this thesis. The degree of the sensitization response (+++ = marked, ++ = medium, + = low and - = negative) in each test method used was estimated for each test substance, taking into account the concentration range over which the substance was tested within a test method.
The allergens and the irritants are presented in alphabetic order.

Substances	Predictive animal test methods					Patch test results[a] (references)
	GPMT	CCET	FCAT	LLNA	MEST	
Allergens[b]:						
Benzocaine	nt	nt	nt	(-)	nt	1.7 % (10)
t-CN	nt	nt	nt	+(+)	nt	10.2 % (153)
DNCB	nt	nt	nt	+++	nt	positive (154)
DNFB	nt	nt	nt	+++	nt	positive (155)
HC	++	+++	++(+)	+	-	10.2 % (153)
$K_2Cr_2O_7$	++(+)	++	+++	nt	+	4.6 % (10)
2-MBT	nt	nt	nt	++	nt	0.9 % (10)
MDBGN	-	+++	nt	+(+)	(+)	1.7 % (10)
Oxazolone	nt	nt	nt	+++	+++	nt
p-PDA	nt	nt	nt	++	nt	5.0 % (10)
Irritants[c]:						
Chloroform /MeOH	nt	nt	nt	+	nt	negative (156)
MS	nt	nt	+	nt	-	negative (156)
NNA	nt	nt	nt	+	nt	negative (157)
Oxalic acid	nt	nt	nt	+	-	negative (156)
SDS	nt	nt	nt	+	nt	negative (158)
Triton X-100	nt	nt	nt	+	nt	negative (159)

[a)] Patch testing was performed in dermatitis patients.
[b)] *Contact allergens:* Benzocaine, T-cinnamic aldehyde (t-CN), 2,4-dinitroclorobenzene (DNCB), 2,4-dinitrofluorobenzene (DNFB), hydroxycitronellal (HC), potassium dichromate ($K_2Cr_2O_7$), 2-mercaptobenzothiazole (2-MBT), methyldibromo glutaronitrile (MDBGN), 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (Oxazolone), p-phenylenediamine (p-PDA).
[c)] *Irritants:* Chloroform/MeOH, methyl salicylate (MS), nonanoic acid (NNA), Oxalic acid, sodium dodecyl sulfate (SDS), Triton X-100.
Animal test methods: the guinea pig maximization test (GPMT), the cumulative contact enhancement test (CCET), the Freund's complete adjuvant test (FCAT), the local lymph node assay (LLNA), the mouse ear swelling test (MEST)
not tested = nt

5. General discussion

In this thesis predictive test methods for contact allergy were investigated. A slightly modified multi-dose response induction protocol was applied to three guinea pig predictive test methods - the GPMT, the CCET and the FCAT - and evaluated with two model allergens. The application of a multi-dose response induction protocol on a modified predictive test method in mice – the MEST - was evaluated with some allergens and one irritant. A predictive test method in mice - the LLNA - was evaluated with both allergens and irritants. The ability of three predictive test methods – the GPMT, the CCET using a slightly modified multi-dose response induction protocol, and the LLNA - to detect the allergenicity of a preservative was studied and the results were compared with results from patch tests in dermatitis patients. The predictive test methods were also compared with each other based on the results from the substances studied.

5.1 Evaluation of a slightly modified multi-dose-response induction protocol on three guinea pig models [III]

A multi-dose-response induction protocol has been developed and evaluated for the GPMT to improve the power of predictive tests for contact allergens (62). It is combined with a statistical computer program, i.e. logistic regression analysis, which uses all available test results in the analysis of the results (65). Some studies done with this protocol can be found in the literature (61, 63). The protocol has also been evaluated in some other extended dose-response studies using the GPMT (64, 160). This protocol, slightly modified with an increased number of animals in each experimental group and an increased number of challenge concentrations, was applied to three predictive guinea pig methods - the GPMT (33), the CCET (40) and the FCAT (36) - using two model contact allergens (paper III). These methods have different induction procedures; the GPMT uses both topical and intradermal administration, the CCET uses topical administration only and the FCAT uses intradermal administration only. The multi-dose-response induction protocol (62) was easily applied on the CCET and the FCAT since they have only one induction route, where the test substance can be administered in an increasing concentration gradient. Fitted curves with a significant dose-response for the model allergens were obtained. However, the GPMT has two induction routes - intradermal and topical - and when the multi-dose-response induction protocol was used (62), the intradermal administration was given in an increasing concentration gradient and the topical administration was given at two concentrations (one higher and one lower) which were alternated between the induction groups (62). The topical administration influenced the results in the GPMT with the model allergens and gave inconsistent results (Table 7). No dose-response could be seen in those fitted curves (Figure 15a-b and Table 8). Our interpretation is that the respective influences of intradermal and topical exposure vary with the chemical under study, an interpretation which is also discussed elsewhere (61). Adjustment of the induction procedure for the GPMT so that it can handle two increasing concentration gradients is desirable. This is indicated in a previously published report (161).

The logistic regression analysis PC computer program (65) was used to obtain the best fitted *dose-response curves* for each of the experiment series. The fitted curve with $K_2Cr_2O_7$ was monotone for the FCAT. Monotone dose-response curves have been seen in earlier published studies using GPMT with cinnamic aldehyde (62-63), mercaptobenzothiazole (62), and methyl methacrylate (64). Non-monotone fitted curves were obtained with $K_2Cr_2O_7$ for the GPMT and the CCET and with HC for all three sensitization methods used (paper III). Non-monotone dose-response curves have been reported in studies using GPMT with formaldehyde (61-64), mercaptobenzothiazole (62-63) and nickel sulphate (161). The dose-response relationship was significant for the curves fitted from the CCET and FCAT, but no dose-response relationship was seen for the curves fitted from the GPMT for the two model allergens tested. This is in contrast to previously published studies with this multi-dose-response induction protocol using the GPMT (61-64, 161). However, it is consistent with one other independent study using the GPMT to examine the effect of Tween® 80 (162).

Occasional reactions due to irritation were seen at challenge test sites in the control animals as well as in the vehicle controls (paper III), and are a matter of concern (55-59, 163-165). The number of animals was increased in each group, a confluent erythema was chosen as the criterion for a positive reaction and the readings from 72 h were chosen to minimize the influence of irritation in data assessment. Nonetheless, the base levels (P_0) were slightly influenced due to these irritation reactions in a few curves (Figures 15a-b). Re-challenge was done with $K_2Cr_2O_7$ in the GPMT and CCET, and confirmed the results obtained at the first challenge (data not shown). The value of re-challenge has earlier been discussed and it was concluded that the GPMT can benefit from this procedure to verify a sensitization or if positive reactions have been observed in the sham-treated controls at the first challenge (58, 164, 166-167). However, new controls should be added.

One important advantage of this multi-dose-response induction protocol (62), compared to standard single dose administration (33, 36, 40), is that it minimizes the risk of using an *induction concentration* that is too high and giving a result in the down slope portion of a non-monotone dose-response curves. Multiple concentrations have been used in many earlier studies at induction (168-169), at challenge (170-172) or at both (9, 173-176) and a dose-response relationship is generally seen. Visualization of those results is usually done graphically (161, 175), but without the aid of a logistic regression analysis computer program much information is lost. However, when following the original recommendations for induction concentrations in the dose-response induction protocol (62), the initial experiments with $K_2Cr_2O_7$ in the GPMT and the CCET (nos. I and III, Table 5) gave an almost maximal sensitization at all the induction concentrations used (data not shown). This was also shown for Kathon CG, another potent sensitizer, using the same multi-dose-response induction protocol (62-63). The induction concentrations were then lowered in the following two experiments with the GPMT and CCET and resulted in various numbers of positive reactions in each experimental series (Table 7). The modification using a *challenge concentration* gradient, instead of only one test concentration according to the protocol (62),

minimizes the risk of obtaining a weak elicitation reaction or no reaction at all in sensitized animals.

Modifying the protocol by increasing the number of animals in each experimental group (6 groups with 8 animals in each) partly counteracts the original objective of better use of the same number of animals (6 groups with 5 animals in each compared to 2 groups with 15 animals in each), but as it makes the result more reliable and minimizes the influence of occasional false positive reactions, it is an alteration worth considering (145). Obtaining information on suspected contact allergens tested in sensitization studies, i.e. a threshold concentration for sensitization, a maximal sensitization rate and an EC_{50}, should be of great value for risk assessment of allergens.

It can be concluded that the dose-response protocol was easily applied to the CCET and FCAT, which have only one exposure route, and where the logistic models could be fitted to the data. However, for the GPMT with two exposure routes, the topical doses at induction interacted with the logistic model. The protocol would benefit from further developments: A linear increase should be included for the topical application as for the intradermal application at induction in the GPMT. The induction doses should - when necessary - be lowered. Further challenge concentrations should be included. The ability of the computer program to calculate threshold concentration and EC_{50} for non-monotone curves should be extended.

5.2 Evaluation of a multi-dose-response induction protocol applied on a modified mouse ear swelling test [IV]

The MEST has been standardized and evaluated in order to be used for *prediction of the skin sensitizing potential* of environmental chemicals (68). It has some advantages compared to the guinea pig test methods, concerning speed, labor-intensiveness and cost, and the use of an objective end point. The assays using MEST methodology have been further evaluated (67, 70, 73, 79, 81, 83, 152, 177-178), and compared with guinea pig test results (80, 179) and human test results (180). However, doubts have been raised about the method's ability to detect weak and moderate contact sensitizers when the original MEST protocol is used (79), and some modifications in the MEST methodology have been introduced over the years. Some studies have been able to detect weak to moderate contact allergens by including smaller modifications together with vitamin A acetate (VAA) supplemented diet (73, 83), whereas others, despite their efforts to include modifications to obtain a more sensitive test method, did not manage to detect all the weak and moderate sensitizers tested (70, 152, 177).

The MEST performed in this study (Figure 6-7) was *a modification of the original MEST protocol* (Figure 4a) (68). VAA supplemented diet was used to enhance the sensitivity of the MEST, as it has been shown in several studies to increase the sensitivity of the predictive test method (72-73, 82-84). The induction was performed on the back, which in some studies has been shown to induce a higher sensitization rate of the animals (unpublished result, 78, 84) than when the original protocol is followed. The number of topical applications was reduced to three as done in other MEST studies (78, 118-119, 122, 181). Challenge was

performed at day 9 (5 days after last application at induction). This interval of 5 days from induction to challenge was used in the modified MEST protocol by Gad (Figure 4b) (75), and also in some other studies (72-73, 78, 182). In the present study the challenge was performed by application of test substance on both ears as also described earlier (67, 81-83, 118), instead of using only one ear for the test substance and the other ear for the vehicle control. The reason for this was that the mice may redistribute the substances applied on the different ears through their natural grooming behavior and therefore the application of the vehicle control was omitted. The sensitization criterion for the test substances was set to be at a higher level than the increases in ear thickness induced by irritation. The modifications are discussed in more detail in paper IV.

A multi-dose-response induction protocol developed for the GPMT (62) was slightly adjusted for the application to this modified MEST protocol. Following the dose-response protocol and the earlier evaluation of the protocol (section 5.1) the test animals were divided into five groups with eight animals in each. Relative increases of ear thickness were calculated for each animal, since the statistical analysis, i.e. the logistic regression analysis, has to assess the data on a relative basis in each individual animal. A sensitization criterion of at least a relative increase in ear thickness of 20 % as in the original MEST (68) was considered to be too great an increase, since the individual variation of ear thickness was not taken into account. Hypothetical sensitization criteria were introduced and a sensitization was considered to have occurred if at least one ear on one or more animal, had at minimal a relative increase in thickness corresponding to the statistically chosen sensitization criterion. The advantage of this statistical program is that it makes it possible to analyze different series of sensitization data and to determine which series gives the best fit to the available model curves (62).

Four chemicals, previously identified as contact allergens, and one irritant were used for the evaluation in the present study and some sensitization data with *dose-response relationships* were obtained (data not shown). Dose-response relationships for contact allergens are well known and have been demonstrated in various studies using both guinea pig (section 5.1) (161, 170, 172, 175) and mouse (section 5.2-5.3) (72-73, 180, 182-183) predictive test methods. However, an additional advantage of this logistic regression analysis is that it uses all the obtained experimental data from the different induction groups to calculate the significance of the dose-response. In several studies of the sensitizing properties of potent sensitizers, a non-monotone dose-response curve was seen (72-73, 180, 182-183). In those studies, the data were analyzed with linear regression analysis, but the application of the present logistic regression analysis would most likely have given additional information. In the *dose-response analysis* of our data, different sensitization criteria had to be used. It was shown that with a stronger sensitizer a higher sensitization criterion could be used. A monotone dose-response curve was obtained for the weak contact allergen MDBGN at a 5 % sensitization criterion (Figure 16c). This sensitization criterion was lower than the allowed 10 % increase in ear thickness due to irritation from the challenge application. However, this 5 % sensitization criterion gave a statistically accepted curve with a statistically significant dose-response relationship, which could not be obtained with a 10 %

sensitization criterion. The non-monotone dose-response curves (section 5.1) obtained for the strong and moderate contact allergens, i.e. oxazolone (the positive control) (Figure 16a) and $K_2Cr_2O_7$ (Figure 16b), were quite similar to the monotone curve. For these two last contact allergens, a dose-response curve could be obtained at a 10 % sensitization criterion, however, a better curve was found with the 15 % sensitization criterion for oxazolone. The irritant reactions (the 'sensitized' mice) in the control groups for $K_2Cr_2O_7$ and MDBGN were also included in the calculations of the dose-response curves and because of them the statistical program made small adjustments of the baseline (P_0) in the data assessment (Table 9 and Figures 16b-c). The obtained dose-response curves were used for further calculations of the sensitizing capacity for the tested substances (Table 11), e.g. the estimated concentration sensitizing 50 % of the animals (EC_{50}). This parameter can be compared to some earlier studies using "the estimated dose to cause 50 % sensitization" (SD_{50}), which, however, has only been interpolated from plots (72-73, 180, 182-183).

The sensitizing potential of three of the tested contact allergens (Table 11) shows that the highest sensitization rate and lowest EC_{50} are seen for oxazolone (a common strong experimental contact allergen in animals) and followed by the moderate contact allergen $K_2Cr_2O_7$ and the weak contact allergen MDBGN. The observations for the last mentioned allergens are in accordance with human data (10). However, the threshold sensitization concentration for $K_2Cr_2O_7$ was, in this study, lower than the one for oxazolone. The weak contact allergen, HC was not detected as a sensitizer using this modified MEST (Table 11) in contrast to human data (10). HC gave only a few sensitization reactions and a minimal relative increase in ear thickness (Table 9). The irritant (negative control), i.e. oxalic acid was correctly shown to give a 'negative' response using the modified MEST protocol (Table 11). Only occasional irritant reactions were seen and no relative increase in ear thickness in the induction groups was detected (Table 9).

During the *interpretation of the results* from earlier studies, different methods or no statistical methods at all have been used. This makes comparisons of our test results to the test results from other studies difficult to perform. Relative ear thickness for each test substance was for this reason calculated for the induction groups using measurements of all exposed ears (Table 9). Some of our results of the contact allergens tested were compared with literature data (Table 10), which showed a relatively good correlation between our test results and other test results. It is likely, however, that HC has to be applied more than three times topically at induction and that a higher challenge concentration had to be used than the one used in this study (10 %), since a sensitization was obtained after six topical applications with a challenge of 20 % (67, 81).

It can be concluded that this modified MEST using the dose-response protocol could detect the tested moderate to strong contact allergens as sensitizers, but not one of the two weak contact allergens. The irritant (negative control) gave a 'negative' response.

5.3 Evaluation of the local lymph node assay [I-II]

The LLNA has been developed to be used as a *predictive test method* for contact allergens (90). It offers many advantages over guinea pig methods regarding speed, labor-intensiveness and cost, and gives an objective end result not affected by the color of the chemical tested (71). It has been used in several studies for prediction of the allergenic potential of substances (152, 184-185). The method has been extensively evaluated in inter- and intralaboratory studies (89, 186-190), with human maximization test results (191), and in comparative studies with guinea pig methods (89, 186-187, 192-193). It has been shown to identify moderate to strong allergens and some weak allergens (86, 193-194). However, the ability of this assay to discriminate between weak allergens and irritants has been questioned and an increasing number of substances, considered to be non-sensitizers, have been shown to induce cell-proliferation in the LLNA (paper I, 81, 149, 185, 194-195). Nonetheless, during a meeting in the USA for the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in 1998, the peer review panel recommended the LLNA as a stand-alone alternative for predictive testing of contact allergens (196). However, eight modifications must be included in the protocol, e.g. the mice should be assessed individually, statistical analysis should be performed and the classification for a sensitizer should include a SI ≥ 3, a statistical significance and dose-response information (196).

Use of the LLNA for predictive testing showed in the present study that the method was reliable for predictive screening of six moderate to strong contact *allergens* (SI = 12.8 - 39.9) (Figure 11). This was in agreement with previously published results based on the LLNA (86, 91, 186-187, 190, 192, 194), which tested chemicals classified as moderate to strong allergens in guinea pigs (8, 33, 187, 192). However, the method failed to discriminate between weak to moderate contact allergens (SI = 3.4 - 17.1) and irritants (SI = 5.0 - 10.7). HC and benzocaine, classified as moderate sensitizers in guinea pig tests (8, 33, 192), gave low SI-values as also presented in an earlier published report (192). Benzocaine was not classified as a contact allergen at 25% test concentration (SI < 3). However, later studies with benzocaine, using DMF as vehicle instead of AOO, occasionally gave SI-values high enough for its classification as a sensitizer (197).

A number of *irritants*, without previously known sensitizing properties, were tested in the present study: chloroform/methanol (2:1) (198), methyl salicylate (96, 188, 199-200), nonanoic acid (157, 201-202), oxalic acid (203), SDS (33) and Triton X-100 (204). All these gave a clear proliferation in at least one of the concentrations used and could thus be classified as contact allergens. Comparable results have been reported with the irritants: nonanoic acid, methyl salicylate and SDS (81, 142). In contrast, in some other reports, irritants do not induce a marked proliferative response (95, 165, 186, 192, 194, 205-206). In the case of SDS, this discrepancy could be due to variations in SDS preparations from different manufacturers (207). However, 10% SDS from two different suppliers (paper I) has been tested on eight additional occasions and gave at all times SI-values above the limit (SI=3) (paper I). The dose-response seen for the irritants tested was also seen in a study where different concentrations of SDS were tested (142, 208).

However, the proliferative activity observed after application of Triton X-100 may be due to allergenicity of ethoxylated non-ionic surfactants (166). Other substances regarded as non-sensitizers have been reported to induce marked cell proliferation in the LLNA: mineral oil (195), benzalkonium chloride, salicylic acid (194), copper chloride (206) and in addition, in later studies, heavy and light mineral oil, monoolein, squalene, squalane, pristane, Arlacel A, peanut oil, olive oil and Freund's complete and incomplete adjuvant (paper II, 149, 209).

Presenting substances tested in LLNA with *EC_3 values*, has been suggested as a better way of ranking substances according to their proliferation activity, as EC_3 values indicate a substance's relative skin-sensitizing potential (90, 142-143, 149, 205, 210). However, the calculated EC_3 for methyl salicylate and nonanoic acid in paper II, was at about the same level as four of the five allergens compared in Table 6. Thus, the EC_3 could not be used to distinguish allergens from the two irritants tested.

Methyl salicylate has previously been reported to elicit positive results in the LLNA (81), coincident with our results in paper I and II. These findings are in contrast to other studies claiming methyl salicylate to be negative in the LLNA (95, 186, 192, 194, 205-206). However, most of these other studies used AOO as vehicle (95, 204-206). These dissimilarities in results of the proliferation assay for methyl salicylate have been proposed to be due to the vehicle used, i.e. DMF (95). Methyl salicylate was tested with the two different vehicles DMF and MEK in the LLNA, and nearly the same level of proliferation was obtained (Figure 12 and paper II). It is more interesting to note that the test concentrations used in most of the studies with negative outcome for methyl salicylate were low (25% or less) (95, 186, 188, 192, 205), which in Figure I in paper II only occasionally gave positive results. However, in a recently published paper 100 % methyl salicylate was tested in the LLNA with a negative result (165).

The choice of *vehicle* could, though, be of interest when the proliferation activity is studied in the LLNA. In some studies, oxazolone (180) and DNCB (211) have been tested with various vehicles and different results were obtained (ref). Benzocaine gave a low SI-value in the study in paper I, probably because of incomplete absorption due to evaporation of acetone in the vehicle AOO (Table I in paper I and 197). AOO treatment alone induces highly variable proliferative responses and AOO is therefore not a suitable vehicle (149, 212), and OO possesses an inherent capacity to induce high proliferation in the LLNA (149). Nevertheless, another published report states that AOO is a reasonable first-choice vehicle even though presenting similar results (212) as in our earlier report (149). When the vehicle-treated control group values vary on different test occasions this may result in unspecific changes in SI-values for the test substance. In this study, variations in the vehicle treated or the untreated control group were seen in the range of 100-500 dpm per lymph node (Tables I-III in paper I and Tables I-II, paper II). Some other vehicles, e.g DMF, DMSO or Azone®, are known to increase skin absorption, which also may influence the result (213).

It has been proposed that adding 10% SDS, an irritant, to all vehicles used could reduce the *background noise* sometimes induced by irritation (90). This cannot be recommended since some allergens may act synergistically with the irritant, as

shown with t-cinnamic aldehyde (Figure 14b), while others may give an additive response, as shown with HC (Figure 14a) (paper I). This synergistic effect seems not to be due to increased absorption (208), though it could be due to the strong irritating properties of t-cinnamic aldehyde (paper I).

The *time-pattern* of the effects of 10% SDS (33) on proliferation was tested in LLNA (paper I) due to the classification of 10% SDS as a sensitizer. It has been suggested that the proliferative activity seen for irritants could be due to 'environmental' antigens or non-antigenic-specific lymphocyte proliferation (89-90) The proliferation reached a level that could classify SDS as a sensitizer 3 days after the first application. This fast kinetic profile accords well with proliferative responses induced by potent allergens (88) and with the time course of Langerhans cell accumulation in draining lymph nodes after SDS treatment (208). SDS treatment of the skin in humans also induces a large increase in the amounts of various inflammatory cytokines and cells, including Langerhans cells in the draining lymph (214-217).

It can be concluded that the LLNA was reliable for predicting moderate to strong contact allergens. However, the method failed to discriminate between weak to moderate contact allergens and irritants.

5.4 Comparison of results from three predictive animal test methods and patch test in dermatitis patients: the allergenicity of a preservative [V]

The preservative Euxyl K 400 has two ingredients, methyldibromo glutaronitrile (MDBGN) (20%) and 2-phenoxyethanol (PE) (80%) (218). MDBGN is considered to be the principal allergen, whereas PE is a rare sensitizer (219-220). The preservative, i.e. Euxyl K 400, has given a number of sensitized dermatitis patients since the introduction on the market and in a German multicenter study carried out between 1990-1995, MDBGN is number 15 on the toplist when common allergens from the standard series were tested (10). There are several case reports and multicenter studies on contact allergy to Euxyl K 400 and its two ingredients in the literature (218, 221-230), and the use of MDBGN in products is monitored in Sweden (231-232) (V). The attempts to define the allergenicity of Euxyl K 400 experimentally have earlier given conflicting results. Seven human repeat insult patch tests (HRIPT) and eleven studies using the guinea pig maximization test (GPMT) with MDBGN were reported negative (233). Both ingredients in one GPMT study (234) and MDBGN in a modified Freund's complete adjuvant test (235) gave some sensitization reactions. In two optimization tests performed, 1/20 and 13/20 of the guinea pigs reacted to PE given intradermally, but not to topical challenge (0/20) (236).

An investigation of the allergenicity of MDBGN and Euxyl K 400 using three different animal models for predictive testing: the LLNA in mice, and the GPMT and the CCET using a dose-response protocol in guinea pigs. The results were compared with the frequency and causes of positive patch test reactions to Euxyl K 400 and MDBGN in patients with work-related contact dermatitis. The choice of the experimental methods used for the present investigation was based on their different

induction routes. The GPMT (33) has a combination of one intradermal administration and one topical application at induction, while the LLNA (92) and the CCET (40) have multiple topical applications only, three and four times, respectively. The GPMT and the LLNA are two of the four predictive test methods recommended in the OECD guidelines (53). However, it is also stated that there may sometime be circumstances where other predictive test methods may be used (53).

The patch testing of 1770 patients - referred due to work-related contact dermatitis - with Euxyl K 400 or MDBGN resulted in 9 with relevant positive reactions (Table 5). The majority had been exposed to soaps, shampoos, cleansing agents, creams and lotions, i.e. the same type of exposure as in non-occupational cases (218-222, 226-228, 237-238). Two predictive animal test methods, i.e. the CCET with the application of a multi-dose-response induction protocol and the LLNA confirmed human data. The fitted dose-response curve obtained of results from *the CCET* was non-monotone (section 5.1) for the induction series (Figure 17) and monotone for the challenge series (Figure 1b in paper V). Single positive reactions were seen at some challenge concentrations in both the exposed and vehicle treated animals (Table 2 in paper V), a phenomenon which was further discussed in paper V and also in section 5.1. This slightly influenced the base level (P_0) in the data assessment (Figure 17 and Figure 1b in paper V). The sensitizing capacity of MDBGN was presented as EC_{50}, the threshold concentration for sensitization and the maximal sensitization rate (Table 13). *The LLNA* classified Euxyl K 400 and MDBGN diluted in DMF or PE as contact allergens with SI-values above 3 at the test concentration 5% (Table 4 in paper V and Figure 18). The results with MDBGN in the two different vehicles, DMF and PE, were similar, indicating that PE did not have any strong contact allergenic or irritating properties in the LLNA. Euxyl K 400 can also be classified as sensitizing since it was the product used when testing 20% MDBGN in PE (Table 4 in paper V and Figure 18). Some positive reactions to MDBGN were found in *the GPMT* (Table 12) at the highest test concentration (1%) in both vehicles, but no statistically significant difference compared to the controls was found. This result was in agreement with earlier test results from GPMT studies (233-234).

It is reasonable to believe that the outcome in the GPMT method was due to too few topical treatments at induction, i.e. that the total dose applied topically was too low, for this particular test substance. It was probably not due to the topically applied concentration being too low, since the results from the CCET study gave a maximal sensitization rate at the induction concentration 10% (Table 2 in paper V and Figure 17), the same concentration used for the single topical application in the GPMT (Table 12). The various vehicles used at induction could not explain the variation in reactivity, since acetone most likely had evaporated at the time of application. The *divergent results* from the various animal predictive test methods indicate that multiple topical applications of MDBGN, as in the CCET method and in the LLNA, are required to obtain sensitization. The need for repeated topical applications could be due to MDBGN being extremely labile in biological systems in the presence of sulfhydrylgroups (239-240), a fact which is further discussed in paper V. Divergent results when using the GPMT, the CCET and the LLNA have

also been obtained in an investigation on the allergenic properties of sulphanilic acid (241). However, in that study the GPMT showed that sulphanilic acid had an allergen potential, whereas the CCET and the LLNA failed to detect the substance as a sensitizer. In the present study it also seems likely, as discussed in section 5.1, that the influence of intradermal and topical exposure on sensitization varies with the chemical tested.

It can be concluded that the choice of test method could be significant for the possibility to detect a sensitizer and for these particular substances a predictive test method with multiple topical applications had to be used.

5.5 Comparison of the animal predictive test methods used

Comparisons between various predictive animal test methods are difficult to perform, since they have various prerequisites such as, e.g., different sensitization and challenge procedures, different applications sites, different immunological enhancement techniques, and also the use of different animal species. There are several inbred mouse strains available; however, the choice of strain for a test method could make the assay less sensitive to one or more of the substances under study. The guinea pigs are outbred, giving a genetic heterogeneity, which can be viewed as a small model for the individual genetic diversity in humans. However, it is also possible to have a mix of guinea pigs, which may have a lower susceptibility to one or more of the substances under study. In addition, the results are presented differently for the various test methods. One predictive test method presents its results by including the degree of the 'positive' sensitization reactions in the prediction of the sensitizing potentials, i.e. the LLNA (90), while the other test methods discussed in this thesis, i.e. the MEST (68), the GPMT (33), the CCET (40), and the FCAT (36) may present both the degree of the elicitation reaction and the relative number of sensitized animals. However, a comparison was made of the five predictive test methods used with all the substances tested in this thesis, and the results are summarized in table 15. Literature data on patch test results in dermatitis patients were also included in this table for the tested contact allergens (Table 15). The degree of sensitization response in each test method was estimated for each test substance, and the concentrations used were taken into consideration. Table 15 shows that four contact allergens, i.e. oxazolone, $K_2Cr_2O_7$, MDBGN, and HC, and one irritant, i.e. oxalic acid, have been tested with at least two predictive test methods in this thesis.

Oxazolone is a strong experimental contact allergen, which was used as a positive control in the test methods (Table 15). This substance showed a strong sensitization in both the LLNA (Table I in paper I and Figure 11) and the MEST (Tables 9 and Figure 16a). *Oxalic acid* (203) obtained a 'negative' response in the MEST (Table 9). However, in the LLNA this irritant was classified as a sensitizer as well as the other tested irritants (Table II in paper I and Figure 11), used as 'negative' controls in the methods (Table 15).

The moderate contact allergen $K_2Cr_2O_7$ (Table 15) gave a higher sensitization rate with stronger reactions in the three guinea pig test methods (Table 7 and Figure 15a) than in the MEST (Table 9 and Figure 16b). The FCAT showed the highest sensitization rate for $K_2Cr_2O_7$ (Tables 7 and Figure 15a), followed by the GPMT

and then the CCET. In an earlier experimental study, the GPMT gave also a high sensitization rate for this substance (8). In the mouse methods a variable degree of sensitization has been presented. The MEST (68, 70) only showed weak sensitization responses to $K_2Cr_2O_7$, but with the LLNA some have obtained a low response (92, 115, 185, 242), while other have obtained a moderate response (120, 191-192, 205, 243). The optimization test gave a very high sensitization rate with an intradermal challenge, but only a moderate sensitization with a topical challenge (236). $K_2Cr_2O_7$ is included in the standard series used for patch testing in dermatitis patients (Table 15).

Divergent sensitization results were obtained with the weak contact allergen *MDBGN* in the two guinea pig test method tested (Table 15). In the CCET (Table 2 in paper V and Figure 17) MDBGN showed a strong sensitization, while the sensitization results from the GPMT (Table 12) were not statistically significant. Similar results were seen in several earlier studies using the GPMT (233-234). Some weak reactions were observed when using a modified FCAT (235), however, the test results could be questioned (paper V). The LLNA showed stronger sensitization to MDBGN than the MEST did, while compared to the guinea pig test methods, both mice test methods showed a low sensitization (Table 15). The sensitization result from the LLNA (Table 4 in paper V and Figure 11) was stronger than the results from the MEST (Table 9), since the sensitization criterion for the MEST had to be lowered to 5 % to obtain a 'positive' response. However, the interpretation of the result was somewhat difficult when the LLNA was used, since the results with MDBGN were in the same SI-range as some well-known irritants, e.g. SDS (paper I-II). However, MDBGN seems to require several topical applications to obtain a 'positive' sensitization. This substance is included in the standard series used for patch testing in dermatitis patients (Table 15).

The weak contact allergen *HC* was tested in the three guinea pig test methods and in both the LLNA and the MEST (Table 15). However, the level of sensitization did not agree in mice and in guinea pigs. In all three guinea pig test methods HC showed a stronger sensitization than in the mouse test methods (Table 15). The strongest response to HC was seen in the CCET (Table 7 and Figure 15b), whereas in the LLNA, HC was barely classified as a sensitizer (Table I in paper I and Figure 11) and in the MEST, HC obtained a 'negative' response (Table 9). In earlier published studies with HC tested in the GPMT according to the original protocol (33) the substance was classified as a low to moderate allergen (8). However, in the MEST, a weak statistically significant response has been shown (67, 81). HC has also been classified by others as a weak sensitizer in the LLNA (191-192). The discrepancies could be due to differences in exposure concentrations. This substance is included in the fragrance mix in the standard series used for patch testing in dermatitis patients (Table 15).

The predictive animal test methods that correctly could predict the sensitizing potential for the weak and moderate contact allergens tested were the CCET and the FCAT (Table 15).

5.6 Concluding remarks and recommendations

Results from the animal predictive test methods are widely used in risk assessment of chemicals at advisory and regulatory institutes and authorities. Chemical and pharmaceutical production companies are very dependent on a proper risk assessment before their products are released on the market. To perform a proper risk assessment of a test substance (section 1.3), several different investigations must be done and one of these is a sensitization study of a test substance with a predictive animal test method. This study is supposed to give an answer as to whether the substance has an inherent potential to sensitize. Therefore, it is important that the test method gives a reliable test result for the prospected use of that substance. Sometimes it is also interesting to know how strong the sensitizing potential is for a particular substance compared with other substances, that could be used for the same purpose in a product. However, it is also always of great importance to know the extent of exposure of humans. If the exposure of humans to one substance has become too extensive, and an increasing sensitization frequency to that substance has been noted at patch testing, another substance could take its place in a product, if a lower degree of sensitization has been shown for that second substance.

Even though LLNA and MEST have some advantages compared to the guinea pig test methods concerning speed, labor-intensiveness and cost, and the use of an objective end point, the methods are not at present capable of replacing the predictive guinea pig test methods. The LLNA is useful for detection of moderate to strong contact allergens (SI = 12.8 - 39.9), but it does not adequately discriminate between weak to moderate contact allergens (SI = 3.4 - 17.1) and irritants (SI = 5.0 - 10.7). The LLNA could falsely classify substances with exclusively irritating properties to be allergens or, alternatively, overestimate the allergenicity of chemicals with both allergenic and irritating properties. The MEST is judged to be less capable of detecting potential contact allergens than the LLNA, but on the other hand no false ‘positive’ reactivity with the irritant tested was seen. Both the LLNA and MEST gave lower sensitization responses with the weak and moderate contact allergens tested than the guinea pig test methods did. Neither the LLNA nor the MEST could be recommended as the only predictive test method for contact allergens. However, they could be used for screening of sensitizers. If a SI<12 is obtained for a substance using the LLNA or if no statistical significant dose-response relationship is obtained using a multi-dose-response induction protocol on the modified MEST, a retest with a guinea pig test is recommended. It is preferable is to use a multi-dose-response induction protocol in the CCET or the FCAT, since they gave reliable results with the substances tested and are easy to perform. It should also be noted that one substantial benefit of using predictive guinea pig test methods is the possibility to study cross-reactivity (244). This cannot be done in the LLNA, due to its design. It is possible, however, to perform cross- reactivity studies in the MEST, but only with one test substance on each mouse.

It is recommended that the investigator, whose aim it is to study the sensitizing potential of a substance should choose the induction procedure that is most relevant for the prospected use. The investigator should be aware of the possibility of using other available standardized predictive animal test methods. A test method with a

particular induction route may be more suitable for testing a substance than one of the recommended methods. The investigator should also be observant of the varying ability of the predictive test methods to detect the sensitizing potential of a substance. Dose-response designs of predictive test methods increase the amount of information obtained from each sensitization study, but are not generally included in all animal test methods today. The investigator should consider including dose-response designs in the protocols used when the sensitizing potential of a substance is investigated, since there is a risk of missing a sensitization due to a too low or a too high induction (or challenge) concentration if only one induction (or challenge) concentration is used. Calculation of EC_{50} values improves the possibility of proper ranking of contact allergens and augments the information used in risk assessment.

6. Conclusions

- A variety of predictive test methods for the investigation of potential contact allergens are available.

- Dose-response designs of predictive test methods increase the amount of information obtained from each sensitization study and should be considered for inclusion in the protocols when the sensitizing potential of a substance is investigated.

- Application of a multi-dose-response induction protocol to guinea pig test methods with only one induction route, i.e., the CCET and the FCAT, makes them a good choice of methods for predictive testing. However, for a guinea pig test method with two induction routes, i.e. the GPMT, the topical doses at induction interacted with the logistic model.

- It is possible to use the multi-dose-response induction protocol applied on the modified MEST for screening of sensitizers. However, if no statistically significant dose-response relationship is obtained, a retest with a guinea pig test method should be performed.

- It is possible to use the LLNA for screening of sensitizers. However, if a stimulation index (SI) < 12 is obtained, a retest with a guinea pig test method should be performed.

- Guinea pig test methods gave in general a higher sensitization rate than the mouse test methods for the substances tested.

- The guinea pig test method giving the highest sensitization rate varied with the particular substance tested in this thesis.

- The MEST was judged to be less capable of detecting potential contact allergens than the LLNA, but on the other hand no false ‘positive’ reactivity with the irritant tested was seen.

- The choice of test method could be significant for the possibility to detect a sensitizer, since the predictive test methods have various capacities to detect the sensitizing potential of a substance. The test method should have the induction procedure that is most relevant for the prospected use of the substance being tested.

7. Summary

Wahlkvist H. *Predictive testing for contact allergy: Comparison of some guinea pig and mouse protocols including dose-response designs.* Arbete och Hälsa 1999: 21.

Contact allergy (delayed hypersensitivity) may develop as a result of skin exposure to contact allergens (haptens) and can lead to allergic contact dermatitis. The purpose of this study was to evaluate some predictive animal test methods for contact allergens. It was done with the aim that the test methods giving the clinically most relevant results should be used in risk assessment of chemicals and in research.

A slightly modified multi-dose-response induction protocol was evaluated with two model contact allergens when applied to three guinea pig predictive test methods. The protocol was easily applied to the cumulative contact enhancement test (CCET) and the Freund's complete adjuvant test (FCAT), which have only one induction route. However, for the guinea pig maximization test (GPMT) with two induction routes, the topical doses at induction interacted with the logistic model. The protocol would benefit from further development and some modifications are suggested. Calculations of the estimated concentration sensitizing 50 % of the animals (EC_{50}) improves the possibility for proper ranking of contact allergens and augments the information used in risk assessment. The calculated EC_{50}-values for the model allergens were: 0.00045 % in the FCAT, 0.0025 % in the GPMT and 0.0042 % in the CCET for potassium dichromate ($K_2Cr_2O_7$), and 0.068 % in the GPMT, 0.89 % in the FCAT and 1.8 % in the CCET for hydroxycitronellal (HC).

A multi-dose-response induction protocol was applied on a modified mouse ear swelling test (MEST) and evaluated with four contact allergens and one irritant. This protocol could detect the moderate to strong contact allergens as sensitizers, but not one (HC) of the two weak contact allergens. The irritant (negative control) gave a 'negative' response. The EC_{50}-values calculated for the three detected allergens were 0.002 % for oxazolone, 0.03 % for $K_2Cr_2O_7$ and 0.7 % for methyldibromo glutaronitrile (MDBGN).

The murine local lymph node assay (LLNA) is a predictive test method, but its ability to discriminate between allergens and irritants has been questioned. Eight contact allergens and six irritants were investigated in the evaluation of the LLNA. The moderate to strong allergens gave clearly 'positive' results (stimulation index (SI) = 12.8 – 39.9), but one weak allergen (benzocaine) was not classified as a sensitizer (SI<3). The irritants tested, i.e. chloroform/methanol, methylsalicylate, nonanoic acid, oxalic acid, sodium dodecyl sulfate (SDS), Triton X-100, however, gave also 'positive' results (SI = 5.0 – 10.7), not distinguishable from the results with weak and moderate contact allergens (SI = 3.4 – 17.1). The addition of 10% SDS could not be used to reduce the induced proliferation due to irritation from the test chemicals, nor could an alternative choice of vehicle.

The allergenicity of a preservative, i.e. Euxyl K 400, and one of its ingredients, MDBGN, was investigated in three different animal predictive test methods, and patch testing in dermatitis patients was performed for comparison. The CCET using

a multi-dose-response induction protocol (EC_{50} = 1.9 % for MDBGN) and the LLNA (SI = 7.4 - 7.9 for MDBGN and 8.4 - 12.0 for Euxyl K 400) confirmed the sensitization potential of the substance based on dermatitis patients patch test results (total frequency varied between 0.9 - 1.8 %). However, the results from the GPMT were not statistically significant.

In conclusion, even though the LLNA and the MEST have some advantages compared to the guinea pig test methods concerning speed, labor-intensiveness and cost, and the use of an objective end point, the methods are at present not capable of replacing the predictive guinea pig test methods. Both the LLNA and MEST gave a lower sensitization rate with the weak and moderate contact allergens tested than the guinea pig test methods did. The MEST is judged to be less capable of detecting potential contact allergens than the LLNA, but on the other hand no false 'positive' reactivity with the irritant tested was seen. Dose-response designs of predictive test methods increase the amount of information obtained from each sensitization study and should be considered for inclusion in the protocols used when the sensitizing potential of a substance is investigated.

Investigators are advised to select the predictive test method with the induction procedure that is most relevant for the prospected use of the substance being tested. A test method with a particular induction route may be more suitable for testing a substance than one of the recommended methods, so there is also a possibility to use other available standardized predictive animal test methods. However, that predictive test methods have a varying capacity to detect the sensitizing potential of a substance is evident.

Key words: contact allergens, contact allergy, cumulative contact enhancement test, dose-response, evaluation, Freund's complete adjuvant test, guinea pig maximization test, local lymph node assay, mouse ear swelling test, patch test, predictive testing, statistical analysis.

8. Sammanfattning (summary in Swedish)

Wahlkvist H. *Predictive testing for contact allergy: Comparison of some guinea pig and mouse protocols including dose-response designs.* Arbete och Hälsa 1999: 21.

Kontaktallergi (fördröjd överkänslighet) kan utvecklas vid hudexponering för kontaktallergen (hapten) och kan orsaka allergiskt kontakteksem. Syftet med studien var att utvärdera några djurmetoder för prediktiv testning av kontaktallergen. Tyngdpunkt lades vid att den testmetod som ger de kliniskt mest relevanta resultaten bör användas vid riskvärdering av kemikalier och inom forskning.

Ett delvis modifierat protokoll för induktion med multi-dos-respons utvärderades med två modellkontaktallergen som applicerades på tre prediktiva testmetoder på marsvin. Protokollet kunde utan svårigheter tillämpas på ”the cumulative contact enhancement test” (CCET) och ”the Freund´s complete adjuvant test” (FCAT), vilka har endast en exponeringsväg vid induktion. Men i ”the guinea pig mazimization test” (GPMT) som använder två exponeringsvägar, interagerade den topikala dosen i induktionen med den logistiska modellen. Protokollet skulle kunna förbättras ytterligare genom några modifieringar som föreslogs. Beräkning av den koncentration som sensibiliserar 50 % av djuren (EC_{50}) förbättrar möjligheten att rangordna kontaktallergen och ökar mängden information för riskvärdering. De beräknade EC_{50}-värdena för modellallergenen blev 0,00045 % med FCAT, 0,0025 % med GPMT och 0,0042 % med CCET för kaliumdikromat ($K_2Cr_2O_7$), och 0,068 % med GPMT, 0,89 % med FCAT och 1,8 % med CCET för hydroxycitronellal (HC).

Ett induktionsprotokoll för multi-dos-respons applicerades på ett modifierat ”mouse ear swelling test” (MEST) och utvärderades med fyra kontaktallergen och en irritant. Protokollet kunde identifiera de testade moderata till starka kontaktallergenen som sensibiliserande, men inte det ena (HC) av två svaga kontaktallergen. Irritanten (negativ kontroll) gav ett ’negativt’ svar. De beräknade EC_{50}-värdena för de tre ämnen som identifierades som allergen var 0,002 % för oxazolon, 0,03 % för $K_2Cr_2O_7$ och 0,7 % för metyldibromoglutaronitril (MDBGN).

”The murine local lymph node assay” (LLNA) är en prediktiv testmetod, vars förmåga att skilja mellan allergen och irritanter har ifrågasatts. Åtta kontaktallergen och sex irritanter användes i utvärderingen av LLNA. De moderata till starka allergenen gav tydliga ’positiva’ resultat (stimuleringsindex (SI) = 12,8 – 39,9), men ett svagt allergen (bensokain) blev inte klassificerat som sensibiliserande (SI<3). Irritanterna som testades, kloroform/metanol, metylsalicylat, nonansyra, oxalsyra, natriumdodecylsulfat (SDS), Triton X-100, gav också ’positivt’ resultat (SI = 5,0 – 10,7), som inte kunde särskiljas från resultaten med svaga och moderata allergen (SI = 3,4 – 17,1). Tillägg av 10 % SDS minskade inte proliferationen som orsakats av irritation från testkemikalierna, inte heller användandet av annan vehikel gjorde detta.

Den allergiframkallande potentialen hos ett konserveringsmedel, Euxyl K 400 och en av dess ingredienser, MDBGN, undersöktes med tre olika prediktiva testmetoder

på djur, och resultaten jämfördes med resultat från lapptestning på eksempatienter. CCET med ett induktionsprotokoll för multi-dos-respons (EC_{50} = 1,9 % för MDBGN) och LLNA (SI =7,4 – 7,9 för MDBGN och 8,4 – 12,0 för Euxyl K 400) bekräftade substansernas allergiframkallande förmåga, liksom lapptestresultat hos eksempatienter giorde (total frekvens positiv varierade mellan 0,9 % och 1,8 %). Resultaten från GPMT var inte statistiskt signifikanta.

Slutsatsen är att, även om LLNA och MEST har några fördelar jämfört med marsvinsmetoderna beträffande tidsåtgång, arbetsinsats och kostnad, och användande av objektiv mätmetod, kan metoderna inte ännu ersätta marsvinsmetoderna. Både LLNA och MEST gav lägre sensibiliseringsfrekvens med de testade svaga och moderata kontaktallergenen än marsvinsmetoderna. MEST bedöms ha sämre förmåga att upptäcka potentiella kontaktallergen än LLNA, men å andra sidan iakttogs inte någon falsk positiv reaktivitet med den testade irritanten. Dos-responsdesign hos prediktiva testmetoder ökar mängden information från varje sensibiliseringsstudie och bör övervägas att inkluderas i protokoll för undersökning av den sensibiliserande potentialen hos substanser.

Rekommendationen blir att en prediktiv testmetod med en induktionsväg som är mest relevant för den framtida användningen av den testade substansen bör användas. En testmetod med en viss induktionsväg kan vara mer lämpad för testning av substansen än någon av de rekommenderade metoderna, så det är också möjligt att använda andra tillgängliga standardiserade prediktiva testmetoder utförda på djur. Men, det är uppenbart att de prediktiva testmetoderna har olika förmåga att identifiera den sensibiliserande potentialen hos substanser.

Nyckelord: cumulative contact enhancement test, dos-respons, utvärdering, Freund’s complete adjuvant test, guinea pig maximization test, kontaktallergen, kontaktallergi, lapptestning, local lymph node assay, mouse ear swelling test, prediktiv testning, statistisk analys

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