



CONTACT HYPERSENSITIVITY: ANIMAL TESTS

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Abstract—After reviewing general aspects and the historical development of predictive animal skin sensitization tests, the recommended tests of the OECD and EC are discussed. Background information is given on the changes that have occurred during the last 2 yr. Although many factors are known to influence induction and elicitation of contact allergy in animals, the discussion is focused on the choice of induction concentration and the vehicle. Differences in general screening strategies between Europe and the USA are considered. The paper concludes with a discussion about the importance of in-house validation of the methods used for proper prediction of the sensitizing potential of chemicals and with remarks on predictive *in vitro* tests.

Introduction

Protocols of predictive contact allergy tests can be divided generally into three main phases: the induction phase, which represents the first contact with the chemical and during which the antigen is presented to lymphocytes in the draining lymph nodes, the rest period without treatment during which sensitization develops, and the challenge or provocation phase during which skin reactions are elicited after subsequent exposure to the chemical. Various routes of application, such as intradermal, epidermal open or epidermal occlusive, have been used in predictive animal tests during the last 50 years.

The protocol of Draize (Draize *et al.*, 1994) was the first method that was included in a guideline (Draize, 1959). The test was designed to screen out potent sensitizers. Negative results with known human sensitizers such as benzocaine, neomycin or nickel sulfate (Magnusson and Kligman, 1969) was the main reason for developing other protocols.

A considerable change in the development of tests came with the introduction of Freund's complete adjuvant (FCA) injections during the induction phase. FCA enhances the immunological response of the animals and functions as a depot for the distribution of the allergen (Waksman, 1979). With the introduction of FCA, it proved possible to detect not only potent but also moderate and weak contact allergens.

Methods in guidelines

Guidelines for the testing of sensitizing effects were published in 1981 by the OECD and in 1984 by

the EEC. Until recently both guidelines included seven methods, four protocols that require adjuvant in the induction phase, and three tests that do not (Table 1).

The OECD guideline of 1981 listed all seven methods without indicating any preference. The EEC favoured the use of the maximization test described by Magnusson and Kligman (1969). Both guidelines described the test in tabular form and many modifications of the original protocol were possible.

Working groups of the Federal Health Office in Germany, of the European Industrial Ecology Toxicology Centre (ECETOC, 1990) and of the OECD subsequently discussed changes to the guidelines and aimed to reduce the number of recommended tests. From surveys in Europe and the USA it was apparent that the maximization test is the most frequently used test in Europe, but that the Buehler test was the most common in the USA. As a consequence of these surveys, these two tests are now the primary accepted methods in the new guidelines of the OECD (1992) and EEC (1992). The EEC still favours the maximization method as the first recommended test.

Besides the reduction in the number of recommended tests, the following major changes were included in the new guidelines.

- (1) It is recommended that tests are performed according to the original literature.
- (2) It is recommended that moderate and/or weak allergens are used for validation of the method used.
- (3) Other protocols can be used in special cases.
- (4) The OECD accepts the mouse methods, the mouse ear swelling test (MEST) (Gad *et al.*, 1986) and the local lymph node assay (LLNA) (Kimber and Weisenberger, 1989) as screening tests and all positive results obtained in one of

Abbreviations: FCA = Freund's complete adjuvant; LLNA = local lymph node assay; MEST = mouse ear swelling test.

Table 1. Methods in OECD and EEC guidelines

Reference	Route of treatment for	
	Induction	Challenge
<i>Non-adjuvant test</i>		
Draize (1959)	id	id
Buehler (1965)	EOC	EOC
Klecak <i>et al.</i> (1977)	EO	EO
<i>Adjuvant test</i>		
Magnusson and Kligman (1969)	id + FCA and EOC	EOC
Maguire (1973)	id + FCA and EOC	EOC
Klecak <i>et al.</i> (1977)	id + FCA	EO
Maurer <i>et al.</i> (1980)	id and id + FCA	id and EOC

id = intradermal injection EOC = epidermal occlusive application EO =
epidermal open application FCA = Freund's complete adjuvant injection

the mouse tests need not be confirmed in a guinea pig test. Negative results in mouse tests, in contrast, have to be confirmed using a guinea pig method.

Influence of induction concentration on sensitization

Based on experience in humans of the high sensitivity of allergic individuals, it was thought that the elicitation of allergic reactions was not dose dependent. It is clear now that the induction and the elicitation phases of sensitization are in fact dose dependent. The *in vivo* experience in dose-response relationships was confirmed in *in vitro* lymphocyte transformation tests (Li and Aoyama, 1992). Magnusson and Kligman (1969 and 1970) recommended quite high concentrations for the intradermal and epidermal applications. The selected concentrations should even have a slight irritant effect during induction and the challenge concentration should be just under the threshold for irritation. In combination with the adjuvant injections, the selection of high doses may lead to strong irritant effects. Further research demonstrated that such combination effects may diminish the response rate and not maximize it. Schäfer *et al.* (1978) showed that the optimal concentrations for the testing of para-substituted benzenes are in the range of 1–2% and that higher concentrations have an inhibitory effect. Similar results were published by Andersen (1987) for formaldehyde and by Rohold *et al.* (1991) for nickel sulfate.

Choice of vehicles

The choice of vehicle for intradermal, epidermal open and epidermal occlusive application of test compounds is important, and several publications have shown that the vehicle influences the outcome of sensitization tests (Magnusson and Kligman, 1970;

Wahlberg, 1973). It is clear that with the vehicle best adapted to the physicochemical properties of the test compound, the penetration of the compound is influenced and the incidence of positive animals is higher than with a standard vehicle for all test compounds. However, our conclusion, based on studies with water-soluble and water-insoluble compounds, was that the choice of vehicle will not have such a strong influence on the outcome of the test (Maurer, 1983). It is clear that besides the choice of the vehicle, the type of patch test material and the volume of solution or cream applied influences reactivity.

Testing strategies

Surveys on the most frequently used sensitization methods also demonstrated differences in testing strategies between Europe and USA. The intention of Buehler was to screen out strong and moderate sensitizers in the animal tests and to test negative or weakly positive compounds in humans as a second step (Buehler, 1965). Magnusson and Kligman (1969), in contrast, wanted to test compounds in the most sensitive test and to omit testing in humans. The testing strategy should also be dependent on the type of chemicals examined. Industrial compounds may be tested only for labelling purposes or transportation classification. It is commonly accepted that certain classes of industrial chemicals are skin sensitizers. On the other hand, it is unacceptable that ingredients of cosmetics or pharmaceutical preparations are sensitizers. In the case of individuals with skin disorders the difference of sensitivity of intact and diseased skin must be considered.

Based on the actual recommended methods in the OECD guidelines, different step-like strategies can be included in the development process of a chemical (Table 2).

Table 2. Testing strategies

Step	Compound	Strategy 1	Strategy 2	Strategy 3
1	Raw chemical	MEST/LLNA	MEST/LLNA	MEST/LLNA
2	Raw chemical	Buehler test	GPMT	GPMT
3	Formulation	Buchler test	Buchler test	Modified GPMT

MEST = mouse ear swelling test LLNA = local lymph node assay GPMT = guinea
pig maximization test

Step 1. Screening of raw materials at an early stage of development in the mouse MEST or LLNA.

Step 2. Testing of raw materials in a guinea pig test for labelling purposes or for reasons of safety of handling within the production units.

Step 3. Testing of final formulations to get additional information on the sensitizing potential at the actual use concentration and on the influence of additional compounds in the formulation.

In the first screen it is possible to detect strong and moderate sensitizers in tests of short duration, but the threshold of sensitivity of these tests is presently unknown. However, the inclusion of these tests in the OECD guideline, with the acceptance of positive results for labelling purposes, was a major step forward. Guinea pig tests in the second step will still be important for further development of new chemicals, although testing requirements will be dependent on the intended use of the compounds. The maximization test is a very sensitive method and will detect sensitizers with strong or weak potentials. Results from the Buehler test give the impression that the method is less sensitive than the maximization protocol. The sensitivity of the Buehler test is much more dependent on correct performance (Robinson *et al.*, 1989).

Additional testing of the final formulation in a third step would have some advantages. It would be possible to detect the effects of the actual concentration of the allergen in comparison with those of the concentration used in Step 2 and to assess the influences of other ingredients. The best comparison between the tests of Steps 2 and 3 is only possible if the same test protocol has been used or the same sensitivity of the test is achieved. In cases in which the maximization protocol is used in the second step it would be advisable for Step 3 to use a modified version with only epidermal occlusive application of the test compound during the induction phase instead of intradermal injections. This modified protocol has been validated and showed the same sensitivity as the original version (Maurer and Hess, 1989).

Validation of test methods

The sensitivity of the guinea pig strain used must be checked at regular intervals with known skin sensitizers. Strong contact allergens have been used in recent years. During the discussions in the working groups of ECETOC, OECD and EEC, it was agreed generally that the use of moderate or weak sensitizers would be more efficient and would demonstrate better the sensitivity of the strain and the quality of the test performance. The recommended compounds from the different groups are not identical and some strong contact allergens are still included in the EEC recommendations (Table 3).

The reference compounds recommended by the OECD have been tested recently in two laboratories

Table 3. Recommended reference compounds for in-house validation

Organization	Recommended reference compounds
ECETOC (1990)	Benzocaine, neomycin sulfate, hydroxycitronellal
EEC (1992)	<i>p</i> -Phenylenediamine, 2,4-dinitrochlorobenzol, potassium dichromate, neomycin sulfate, nickel sulfate
OECD (1992)	Hexyl cinnamic aldehyde, mercaptobenzothiazole, benzocaine

(Basketter *et al.*, 1993). The compounds were tested in the maximization and Buehler tests as well as in the LLNA. The interlaboratory variation in the selection of vehicles and test concentrations is well documented. The results obtained demonstrate that the classification of the sensitizing potential may differ from one laboratory to another and shows how important it is to perform in-house validation with reference compounds of different potentials. The study demonstrates also the importance of the quality of the performance of the sensitization test and its implications for the classification and labelling of compounds. It is stated, "Thus, a laboratory which runs a more stringent version of a given assay may achieve a positive classification for a weakly sensitizing chemical which another laboratory may fail to detect, while both laboratories nevertheless meet the minimum standards" (Basketter *et al.*, 1993).

Classification of sensitizing potentials

Magnusson and Kligman (1969) classified compounds into five classes of skin sensitizing potency. Even in the case of a negative result in a guinea pig maximization test, the compound still is classified as weak sensitizer. The classification of Magnusson and Kligman was based on the clinical experience that allergic reactions may be observed in single cases to compounds that are negative in a guinea pig test.

Guinea pig tests can only predict the sensitizing potential of a compound; they cannot predict whether a positive compound will act as a weak or a strong sensitizer in humans under conditions of use, because many factors other than the potency of the compound alone are involved in humans. Nickel sulfate, for example, is a weak to moderate sensitizer in guinea pigs, but is one of the most frequent causes of allergic contact dermatitis in humans. The main reason is its wide distribution and the high frequency of exposure. Dinitrochlorobenzene, the classical reference compound with a very strong sensitizing potential, has induced only a few cases of allergy in humans because industrial use is very limited.

The real sensitizing risk of a chemical can only be evaluated on the basis of good epidemiological data. In a recent publication (Knudsen *et al.*, 1993), criteria for the validation of published data for guinea pigs and humans are discussed and a classification of significant allergens is proposed. A group of the

Federal Health Office in Germany is also working on the classification of allergens on the basis of animal and human data. Good progress has been achieved in a realistic classification of allergens on the basis of guinea pig and human data, but much work has still to be done.

In vitro possibilities

As already mentioned, the acceptance of the mouse tests, MEST and LLNA, is a good step towards refining the concepts of contact allergy testing. Further refinement of the mouse test protocols will also increase the sensitivity of the methods (Mori, 1992).

On the other hand, we are far away from a predictive and validated *in vitro* method. Much effort has been put into *in vitro* studies with lymph node cells from animals sensitized *in vivo* (von Blomberg *et al.*, 1990), on the binding capacity of allergens with proteins (Philp, 1965; Weibel and Hansen, 1989) and in culturing and exposing Langerhan's cells to allergens *in vitro*. An interesting approach was based on the combination of binding capacity and the induction of cell proliferation (Bos, 1984).

Information from *in vitro* tests has been available for many years. However, none of these tests has reached the stage of interlaboratory validation or acceptance by registration authorities.

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