

SKIN TESTING IN ALLERGEN STANDARDIZATION AND RESEARCH

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As described in the article by Tripathi and Patterson elsewhere in this issue, Charles Blackley performed the first skin test in 1865, reporting his findings in 1873.¹⁹ Since then, numerous methods for skin testing have been described and more or less validated. Those methods currently perceived to be most important are discussed.

The purpose of skin testing is to demonstrate the presence of allergen-specific immunoglobulin E (IgE) antibodies on skin mast cells. Inoculation of minimal amounts—a few picoliters in the case of skin prick testing or 0.05 to 0.1 mL in the case of intradermal skin testing—of allergen in the skin induces an immediate reaction in sensitized individuals. If the allergen dose is high in relation to the patient's sensitivity, there also is a late reaction. The immediate reaction consists of a central wheal with surrounding erythema appearing within the first half hour; it depends on interaction of IgE on mast cells and soluble allergen in the interstitial fluid. One allergen molecule binds to two IgE molecules, inducing mast-cell activation and the release of preformed mediators (such as histamine) and mediators formed by the mast-cell membrane (e.g., leukotrienes and prostaglandins). Interleukins (soluble messenger substances such as IL-4 and IL-5) are released, attracting other cells such as T cells, neutrophils, and eosinophils. These cells are involved in the late-phase reaction.

Skin testing primarily is performed to diagnose allergic diseases or,

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more correctly, the IgE-mediated allergic part of these multifactorial diseases. This article concentrates on the use of skin tests for allergen standardization and research.

SKIN TEST METHODS

Intracutaneous Skin Test—Intradermal Skin Test

Intradermal test procedures are well established. Research procedures have been defined by the US Food and Drug Administration (FDA), Office of Biologics,^{105, 106} and The Netherlands Society of Allergology.¹

FDA Procedure

The intradermal method as used in the United States also has been discussed by Turkeltaub.¹⁰⁵ The many variants in the literature are summarized below.

Materials. Materials include a small 0.5-mL hypodermic syringe graded in 0.01- to 0.05-mL (10–50 μ L) increments with a short beveled 27-gauge needle, test solution, translucent tape, fine-tipped marker or ballpoint pen, and record sheet.

Skin Test. The needle is inserted into the superficial layer of the skin almost parallel to the skin surface, bevel down. An amount of 0.05 mL should be injected to increase reproducibility.¹⁶ Normally, the same size of bleb is induced, as leakage can occur and the volumes are fairly small. It was reported that the best precision was obtained by injecting a defined volume of test solution.⁸⁶ Dreborg et al found, however, that injecting an exact volume using a micrometer dosing device was inferior to creating visually similar bleb sizes.⁵¹

Site of Testing. Intradermal skin tests can be performed on arms or on the back.

Test Solutions. Standardization should be performed using scientific methods for defining the allergenic composition and documenting the concentration of major allergens. The methods normally used for this purpose are not equivalent in performance. Special care should be taken when using homogeneous reagents, such as recombinant allergens and monoclonal antibodies. Natural allergen sources contain a number of proteins, each of which is a mixture of isoallergens that each have unique epitope structures. Thus, a recombinant standard should be selected carefully to fully represent the natural mixture of isoallergens. Likewise, monoclonal antibodies do not bind to all proteins with allergenic properties (neither to all epitopes on naturally occurring pure allergens or all epitopes on a single isoallergen) and not with equal affinity to all isoallergens. Their assay performance should be compared with that of polyclonal antibodies raised by repeated immunization

using purified natural allergens comprising all isoallergens of one protein/allergen from the allergen source material.⁴

Standardized allergen extracts/preparations should be in an aqueous solution with an added stabilizer (e.g., human serum albumin [HSA] 0.03%). The use of glycerol as a stabilizer is not recommended because of its skin-irritating properties. When glycerol is used to stabilize allergens, the control solution should be diluted in parallel with the allergen solutions in glycerol, so the same concentration for both the negative control and allergen solutions is used at least down to 0.5% glycerol.¹⁰⁵

The diluent should be used as a negative control, and histamine should be used as a positive control. In Europe, histamine dihydrochloride 0.1 or 0.01 mg/mL (0.54 or 0.054 mmol/L) (i.e., 0.06- or 0.006-mg histamine base/mL [dose 3 and 0.3 µg]) is used. In the United States, histamine base 0.1 µg is the recommended intracutaneous dose. Other reference substances have no advantage, and there is less documentation available for these substances.

Location of Tests. For standardization purposes, the FDA recommends use of the arms. Specific sites are indicated in the FDA's written protocol.

Recording Results. After 15 minutes, the contour of the erythema is encircled without crossing any part of the red skin. The drawing should be transferred by pressing translucent adhesive tape against the drawing and then affixing it to a record sheet.

Reading. The longest diameter and the midpoint orthogonal diameters of the erythema are measured and added, producing the sum (Σ) of erythema diameters (Fig. 1).

The erythema is larger, increases more rapidly, and is more diffuse than the wheal contour, and the slope of the dose-response relationship using a linear/log model is steeper than that of the wheal (Fig. 2).

Calculation. In standardization, the extract concentration producing Σ of erythema diameters equal to 50 mm is determined for each patient. In diagnostic testing, some investigators use lower cutoffs, but Turkeltaub¹⁰³ has documented the diagnostic efficacy when the Σ erythema diameters are equal to 50 mm with a ragweed extract containing 10^{-5} µg/mL or less, Amb e 1, the major allergen of short ragweed.

Skin Prick Test—Skin Puncture Test

The modified skin prick test method was devised by Jack Pepys in the 1970s.⁶² This method is no longer recommended in research because it is less reproducible^{6, 14, 26, 27, 58, 71, 72, 106} than the Østerballe method (Allergy Pricker),⁷⁹ which uses a lancet with a 1-mm tip and shoulders that prevent further penetration. According to the original publication, the lancet should be jabbed through a drop of test solution; however, the reproducibility is then bad. A modified method has been described,^{31, 34} and its reproducibility documented.^{31, 33, 41, 45, 50, 53}

Material. Materials include steel lancets with 1-mm tip and shoul-

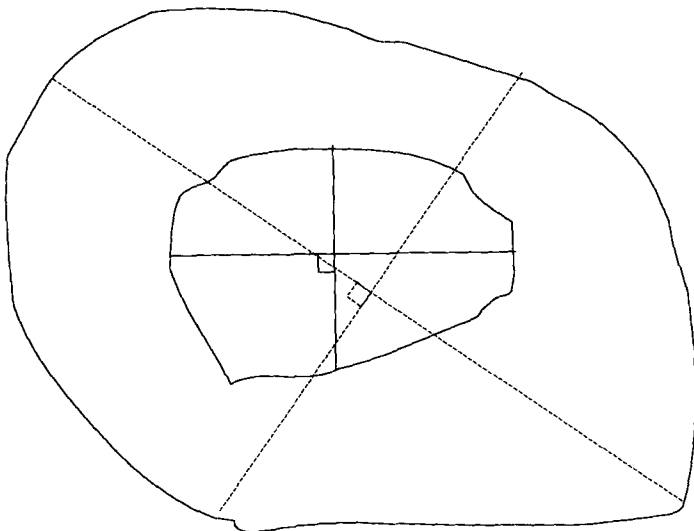


Figure 1. The wheal and the erythema. The longest and midpoint orthogonal diameters are measured. According to the US Food and Drug Administration method, the erythema diameters are added, and the Σ of erythema diameters are used for calculations, whereas according to the Nordic council, the mean of the wheal diameters should be used.

ders, test solutions, translucent tape, fine marker or ballpoint pens, and record sheets.

Skin Test. Two drops of the same test solution are applied to the skin surface at a distance of at least 3 cm from other tests. The tip of the lancet is pressed at 90° through the drop for 1 second, applying the same pressure with the volar aspect of the finger tip every time (Fig. 3). The two tests with the same solution should be allocated to mirrored sites on the skin.

Site of Testing. The most common location for testing is the volar aspect of the forearms. No statistical difference in reactivity has been observed between the proximal and distal area of the forearm, but Taudorf found a higher reactivity of the left forearm that might be a result of the number of statistical tests performed.⁹⁹ There is a statistical difference in the wheal size between the back (larger) and the forearms.⁷⁰ Test sites should be at least 2 cm apart.⁷⁰

When many tests have been performed, some authors have used the costal part of the back.^{41, 43, 45, 50, 53} There is a circadian rhythm with maximum wheal sizes at noon,⁸⁸ and there is also a seasonal variation of skin sensitivity⁷⁷ such as the variation in conjunctival,⁶⁸ nasal sensitivity,^{65, 107} and in specific immunoglobulin E (IgE) levels^{69, 87} to seasonal allergens.

Test Solutions. Test solutions are standardized allergen extracts/preparations in aqueous solution with a stabilizer added, such as HSA

(0.03%). Glycerol 50% often is used as a stabilizer, and it is recommended in the United States. Regarding standardization, the FDA method was described previously, and for Europe there is a position paper by the European Academy of Allergology and Clinical Immunology (EAACI).^{2,3}

The diluent should be used as the negative control and histamine as the positive control. In Europe histamine dihydrochloride 10 mg/mL (54.3 mmol/L) is recommended by authorities⁷⁵ and the EAACI.^{2,3} In the United States, histamine base 1 to 6 mg/mL is recommended by different manufacturers, but there is at present no general agreement. Other reference substances, such as opiates, have no advantage and lack basic documentation.

Recording Results. After 15 minutes, the contours of the wheal should be drawn on the surrounding erythema, close to the wheal edge, without crossing any part of the wheal. The drawing is then transferred to a record sheet by means of a translucent adhesive tape by pressing the tape against the drawing and then affixing the tape to the record sheet.

Reading. Most clinicians use the wheal size for evaluation of skin prick tests. It is important that the wheal is accompanied by erythema, although most often the size of the erythema is not used for evaluation.

There are basically three methods for evaluation whealing reactions:

1. To visually compare the area of the allergen-induced whealing reaction to that of histamine (positive control) as proposed by Aas and Belin in 1974⁵. This methodology is semiquantitative and not very useful because small differences in size mean large differences in sensitivity.
2. To measure the longest and the midpoint orthogonal diameter with a transparent ruler, calculate the mean diameter ($(D_1 + D_2)/2$) (Fig. 1) and use it as an expression of skin reactivity.
3. To calculate the area of the wheal by means of planimetry or by scanning the wheal area using any scanner. The best scanning method is by a computer program supplied by UCB Institute of Allergy using a handheld scanner.^{84, 85} The area is calculated directly in the computer program and can be used for statistical analysis.

For routine use, the mean diameter method is most convenient, whereas in research trials the area as calculated by the scanning method should be used. The semi-quantitative +++ method is not recommended for research, as the precision of the skin prick test method is poor anyway,^{41, 45, 53} and the histamine response varies with the degree of allergy/skin reactivity.⁹⁵ This semiquantitative method is not useful for estimating differences in sensitivity, because small differences in wheal size mean large differences in sensitivity.^{41, 50} There are two indications for using histamine as a positive reference: (1) histamine should be used to detect nonreported antihistamine consumption, and (2) histamine can be used, like in bronchial provocation testing, for evaluation of the reactivity/hyper-reactivity of the organ (i.e., the skin).⁴⁰

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Figure 2. *See legend on opposite page*

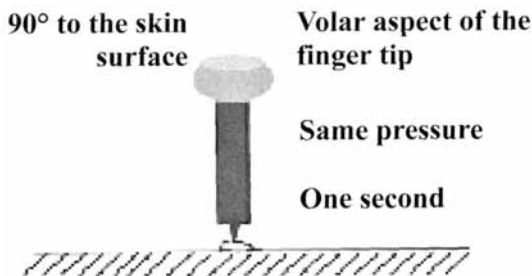


Figure 3. Method for skin prick testing.

Documentation of the Skin Prick Test Method

Dose Response. In the early 1980s, Björkstén et al¹⁸ documented the dose response of allergen. They found the slope to be 0.19 using the model ($\log \text{conc} = a + b \cdot \log \text{diameter}$). This finding has later been confirmed in many patients and centers.^{18, 41, 43, 45, 49, 50, 53} The dose response of skin prick testing is best fitted to a log/log model, which means that the slope is straight within reasonable limits (i.e., from the lower cut off limit up to at least 500 mg/mL of histamine dihydrochloride and 1 mg of major allergen/mL and that the coefficient of variation [CV] is similar within this range, using log transformed data). The individual dose response varies between allergens and individuals.^{41, 45}

Using the area, the slope is twofold steeper for mathematical reasons, 0.3875 ± 0.02 .⁴¹ For practical reasons, the slopes 0.4 and 0.2 can be used, using the area and the diameter for calculations, respectively.

The Precision of the Skin Prick Test. The precision varies considerably between centers. Dreborg et al^{18, 41, 45} found the CV to vary between 10% and as much as 145% in some centers. In most centers involved in these trials in Europe, the CV was between 30% and 60% using the area and between 15% and 30% using the mean diameter. It was shown that the CV did not differ between wheal sizes using log transformed data,^{41, 45, 50} indicating that the dose-response relationship was best fitted to the log/log model.

Other methods have not been documented to the same extent.

Figure 2. A, Dose-response relationship for the erythema sum of diameters. There is always a negative result. For example, an erythema with diameters less than the initial bleb (set to 0 mm) means that the slope crosses the x-axis at one of the three-fold concentrations, has no continuous scale, but discrete variables. B, Parallel line bioassay of two allergenic extracts from similar source materials. (Adapted from Turkeltaub PC, Rastogi SC, Baer H, et al: A standardized quantitative skin-test assay of allergen potency and stability: Studies on the allergen dose-response curve and effect of wheal, erythema, and patient selection on assay results. *J Allergy Clin Immunol* 70:343-352, 1982; with permission.)

Rather, as mentioned, a number of test methods/devices have been shown to give less precise results.^{6, 14, 26, 27, 58, 71, 72, 106}

Cutoff of the Skin Prick Test Method. The cutoff of a method (i.e., the level above the background, indicating a true positive test) is always critical. In skin prick testing this means a response that significantly differs from the response to testing with the diluent or with no solution at all. Data lower than the cutoff may not be used in scientific trials or in routine skin testing, as the response obtained does not differ from zero.

In skin prick testing there are few trials on this subject. The European Position paper on this subject^{2, 3} prescribes 3 mm as the cutoff point for several reasons: (1) when reading the result, it is very subjective to read results less than 3 mm in diameter, and (2) there are no systematic investigations of the background reactivity. The problem is that such a background level is valid only in patients without any dermatographism. Therefore, it is important to always test with a negative control solution to define the background to separate allergens; (3) only one study has examined results in clinically nonsensitive patients. Eigenmann et al⁵⁷ tested those persons who were positive and negative in double-blind placebo controlled food challenges. The clinical cutoff (i.e., the 95 percentile of the wheal size in those negative to the challenge) differed between 3 mm in diameter (wheat) to 5 mm (cow's milk). Because they used nonstandardized allergens, their results can never be repeated and their results cannot be used with the next batch from the same manufacturer; (4) the dose response of allergen and histamine is flat. An increase of the diameter of the wheal by a factor of 1.5 means a 10-fold increase in sensitivity. If it would be possible to evaluate such responses, there would be 10-fold increases in sensitivity from 1.4 mm in diameter to 2 mm and from 2 to 3 mm in diameter, and so forth.

Interpretation in Relation to Disease

Sensitivity and Specificity of the Skin Prick Test

It is valuable to give the sensitivity and specificity (positive and negative predictive values)⁶⁰ of diagnostic tests such as the skin prick test. These tests never give a 100% correct answer to the question of whether the patient is clinically sensitive to the allergen in question (i.e., having symptoms upon exposure) but only information on whether he or she is sensitized. Testing only determines whether the patient is sensitized according to the method used. Obtaining information on whether the patient has symptoms from contact with the allergen tested is most valuable to the clinician. It is most important for meaningful calculations to determine a suitable gold standard, sensitization, or properly defined clinical disease.^{46, 78, 83, 94} This is difficult, however, and there are few published studies.

In evaluating results of diagnostic studies, the 95% confidence inter-

val gives important information. The majority of data on sensitivity and specificity given on the diagnostic value of allergy tests are of little value, as no relevant gold standard was used, and the confidence intervals were not given.

Likelihood Ratios

The positive and negative likelihood ratios,²⁵ with the 95% confidence limits, make the results still more meaningful²²; however, there so far are no such data published regarding skin prick testing used in allergy diagnosis.

ALLERGEN STANDARDIZATION

There is one US method for in vivo allergen standardization of allergenic preparations^{102, 104} and one Nordic method⁷⁵ used with different names by European manufacturers.³

US Method for Allergen Standardization

To assure the safety of skin testing and specific immunotherapy (allergen vaccination), the FDA requires standardization of some allergenic extracts according to a method developed within the agency.¹⁰²

Material. Materials include 1- to 0.5-mL syringe graded in increments of 0.05 mL or less, a thin (27-gauge) short beveled hypodermic needle, and serial threefold dilutions of allergenic extract.

Skin Test. A 0.05-mL amount of extract is injected into the dermis, the bevel facing upwards. The extract should cause a bleb about 3 mm in diameter.

Site of Testing. The injection should be performed on sites indicated on a scheme supplied with a detailed protocol by FDA, using the lower and upper arm.

Test Solutions. Standardized allergen extracts/preparations in 50% glycerol solution are diluted threefold with buffered saline with HSA. Skin testing starts with dilution 15. If this concentration gives a positive response, then lower concentrations are tested. If there is a negative response, three higher concentrations are tested at a time.

Location of Tests. Tests do not need to be performed in duplicate, as the wheal response has a much better precision⁵¹ (i.e., the CV is less than 10%). The erythema, not the wheal, is used for evaluation.²

Patients Included. Twenty adult individuals, highly sensitive to the allergen to be tested, are included.

Recording Results. After 15 minutes, the contour of the erythema should be drawn on the surrounding pale skin, either on a tape or on the skin, and then transferred to a record sheet by means of the tape.¹⁰²

Reading. The longest and the orthogonal diameters of the erythema

are measured, and the sum (Σ) of erythema diameters is used for calculations.

Evaluation. The highest concentration giving no reaction (i.e., no erythema outside the wheal area) is denoted zero sum of erythema diameters. The concentration of extract producing Σ of erythema diameters that are equal to 50 mm is calculated per patient¹⁰² (see Fig. 2A). The dilution causing Σ of erythema diameters equal to 50 mm in the median-sensitive patient denotes the unit. Extracts causing this reaction with dilution 14 (13–15) are given the potency 100,000 AU/mL, those extracts inducing such a reaction with dilution 12 (11–13) are given the potency 10,000 AU/mL, and so on. Because the end of the curve is fixed to zero, the slope is steep, and the determination is precise.

Remarks. The main objective of the method is to assure that highly sensitive patients do not have adverse reactions to intracutaneous testing or immunotherapy. Therefore, highly sensitive patients are included. The drawback is that for some allergens it is difficult to gather highly sensitive patients within a reasonable time frame, the intracutaneous method is painful, the normal distribution of patient reactivity is not precise at the ends, and there will be great variations between individuals selected (Fig. 4). There is so far no documentation of the reproducibility of the method using the same material (i.e., the same material from the same manufacturer) in different hospitals, clinics, or different regions, documenting the repeatability of the unit.

Nordic Council Method for Biologic Standardization of Allergenic Preparations

In Europe the philosophy has been different. The first authority that developed a method for biologic standardization with the aim to equilibrate the biologic activity between allergen extracts prepared from different allergen source materials was the Nordic Council on Medicines.⁷⁴ The document has since been modified,⁷⁵ and the method chosen was based on research of the group of Dreborg.*

There are also rules from the European Union (EU) in a directive²⁴ and in the European Pharmacopoeia.⁶¹ These documents are partly similar although not identical. Because the EU and pharmacopoeia procedures have not been documented, the method according to the Nordic Guidelines⁷⁵ are to be discussed.

Material. Materials include a lancet with 1-mm tip and shoulders, test solutions, translucent tape, fine marker or ballpoint pen, and record sheet.

Patients. At least 20 consecutive adults (15–50 years of age) with clinical sensitivity to the allergen in question, attending a department for allergy, clinically allergic to the allergen in question are included.

Skin Test. A pretest is performed on the volar aspect of the forearm

*References 32, 33, 41, 43, 45, 49, 50, 53, 75

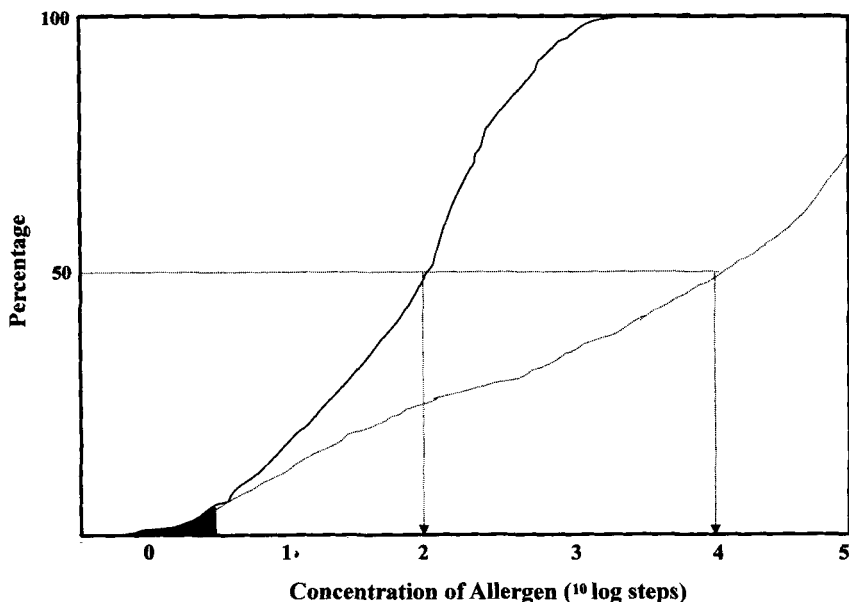


Figure 4. Distribution of CH_{10} in a general population without any selection (*dotted line*) spreading over six potencies of 10 and in a sample from a specialist clinic with a four potencies of 10 difference in sensitivity (*solid line*). The median sensitive patients defines the unit, 10,000 biologic units (BU)/mL or 10 histamine equivalents in skin prick testing (HEP). The triangle on the x axis illustrates the patients selected according to the US Food and Drug Administration method for biological standardization. (Data from Dreborg S: Diagnosis in vivo and in vitro. In Mygind N, Naclerio R (eds): Allergic and Non-Allergic Rhinitis: Clinical Aspects. Copenhagen, Munksgaard, 1992, pp 82–94; and Dreborg S, Basomba A, Belin L, et al: Biological equilibration of allergen preparations: Methodological aspects and reproducibility. Clin Allergy 17:537–550, 1987.)

with the three lowest of six 10-fold dilutions of allergen and histamine dihydrochloride 1 mg/mL, all in duplicate. The concentration inducing a wheal of approximately the same size as that of histamine dihydrochloride 1 mg/mL and two 10-fold higher concentrations, histamine dihydrochloride 10 and 1 mg/mL, are used in the final test on the back.

Four drops of each test solution are applied to the skin surface in separate locations at least 3 to 4 cm in distance from each other. The tip of the lancet is pressed at 90° through each drop for 1 second, applying the same pressure with the volar aspect of the finger tip every time (Fig. 5). The four tests with the same solution should be allocated to mirrored sites on the skin of the thoracic part of the back according to a scheme included in the method.^{33, 48, 75} The same lancet can be used for the four tests with the same solution.

Site of Testing. The site of testing is the thoracic part of the back. No statistical difference in reactivity has been observed between the

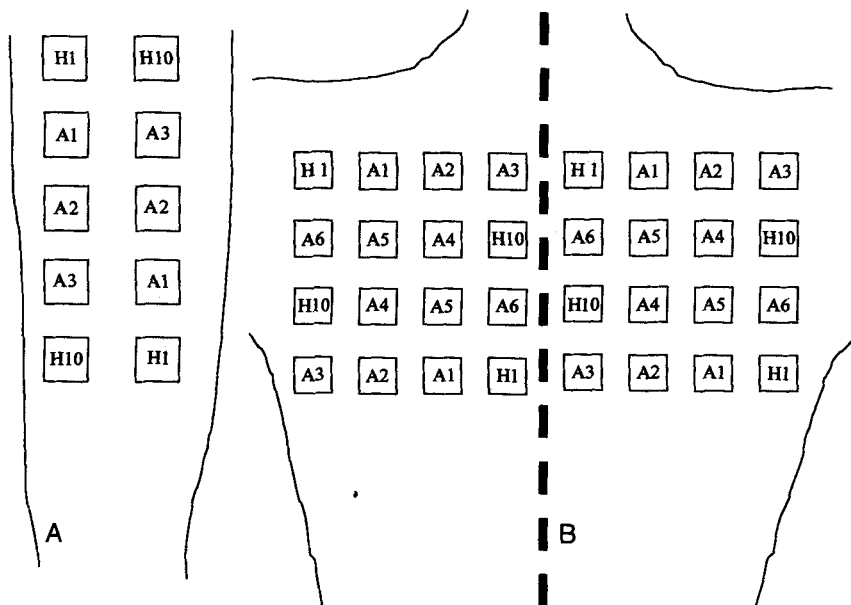


Figure 5. A, The allocation of test sites on one forearm for duplicate tests with three ten-fold dilutions (6, 5, and 4) of the same extract, histamine dihydrochloride 1 and 10 mg/mL, for selection of test solutions for the final test on the back. B, Allocation of test sites testing histamine 1 and 10 mg/mL and two different allergens (A1-3 and A4-6). Quadruplicate tests are performed with each solution. (Data from Dreborg S, Einarsson R, Longbottom J: The chemistry and standardization of allergens. In Weir DM (ed): Handbook of Experimental Immunology, vol 10. London, Blackwell Scientific, 1986, pp 1-28 and Nordic Council on Medicines: Registration of Allergen Preparations: Nordic Guidelines, ed 2, publication no 7. Uppsala, Nordic Council on Medicines, 1989, p 1.)

proximal and distal area of the thoracic part of the back (personal observation).

Test Solutions. Test solutions include standardized allergen extracts/preparations that are freeze-dried and reconstituted with albumin diluent (i.e., buffered saline with 0.03 % HSA. The highest concentration used should contain about 10 mg dry weight (dw), corresponding roughly to 4 mg of protein and up to 1 mg of major allergen. The potency of a 10-mg dw/mL allergen solution varies between species and method for manufacturing. There should be a high concentration of allergens available inducing a positive response in all patients with clinical symptoms induced by the allergen in question. Six 10-fold dilutions are prepared in albumin diluent (HSA 0.03 % in buffer solution). Histamine dihydrochloride 10 mg/mL (54.3 mmol/L)¹⁰⁰ and 1 mg/mL also are tested in quadruplicate.⁷⁵ Two related allergens (e.g., *Dermaphagoides pteronyssinus* and *D. farinae*) or two grasses can be tested in parallel in areas in which both allergens are prevalent so that patients are exposed to both of them (see Fig. 5).

No negative test is prescribed, but tests with diluent should be performed above and below the test area.

Registration. After 15 minutes, the contours of the wheals should be drawn on the surrounding erythema, close to the wheal edge, without crossing any part of the wheal. The drawing should be transferred by means of a translucent adhesive tape by pressing the tape against the drawing and then affixing the tape to a record sheet.

Reading. The wheal area should be used for calculations. The area of the wheal is calculated by means of planimetry or scanning the wheal as described previously.^{84, 85}

Calculations. Using the median wheal area or median of mean diameters for each concentration, the slope of the dose-response relationship according to the log/log model is calculated. The concentration Ch_{10} (i.e., the concentration eliciting an allergen wheal area of the same size as that of histamine dihydrochloride in the same patient) is calculated using the model Ch_{10} equal to the (response to allergen/response to histamine).^{1/b} The median Ch_{10} among the sample of patients denotes the unit 10,000 biologic unit (BU)/mL (also called 10 HEP, histamine equivalent in skin prick testing).

Remarks. The method has been documented regarding the following: amount of material in dw needed to elicit a 10,000-BU/mL activity for a number of common allergens,^{42, 45, 49, 53} reproducibility of the unit between different regions of Europe (Scandinavia, UK, Germany, Spain),^{42, 45, 49, 53} and slope and precision of the allergen dose-response relationship.^{41, 45, 50} By increasing the number of patients included, the confidence limits are reduced and a more precise estimate can be obtained.³⁷ The method also can be used to estimate the difference in sensitivity between populations by including random samples from different regions.

The mode of the distribution of threshold concentrations has been documented in conjunctival⁵² and bronchial provocation tests³⁵ performed with different allergens in hundreds of patients per allergen before the start of immunotherapy trials (Fig. 6) and in histamine release experiments performed in some of these patients.⁵²

The major allergen content of the standardized freeze-dried extracts used for biologic standardization was investigated for its major allergen content. It varied within a factor of 2 (i.e., from 0.6–2 ng of major allergen per BU.⁴⁷ The amount of major allergen/BU varied less between precise than does the standard method of RAST inhibition of FDA for determination of the relative potency of allergenic extracts from the same allergen source material in relation to the FDA standard of the same species according to the limits prescribed by the FDA (i.e., 50–200% of the labeled potency of the standard).

The distribution of reactivity (Ch_{10}) of patients included in biologic standardization trials is Gaussian, spreading over four potencies of 10 (i.e., the concentration eliciting a Ch_{10} response differs from 100 times less than the unit to 100 times higher concentration) in these materials (see Fig. 4).^{33, 41, 45} When the general population is included, the difference

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Figure 6. A, Distribution of threshold concentrations for conjunctival provocation test with birch (*open bars*) and timothy (*solid bars*) pollen allergen extracts in children and adults before the start of allergen vaccination. The mode was at 1000 to 3200 ng major allergen/mL. B, Threshold concentration causing asthma in bronchial challenge. *D. farinae* (*open bars*), *D. pteronyssinus* (*shaded bars*), and *C. herbarum* (*solid bars*). All patients tested with *D. farinae* were adults, whereas more than half of those tested with *D. pteronyssinus* (47 children, 27 adults) and *C. herbarum* (32 children, 31 adults) were school children. The mode of distribution is similar to that in conjunctival tests and skin prick tests. (*Adapted from Dreborg S: Bronchial provocation tests with biologically standardised allergenic preparations. In Melillo G, Norman PS, Marone G [eds]: Clinical Immunology, vol 2. Philadelphia, BC Decker, 1990, pp 185–191; with permission and Dreborg S, Rosendahl A, Einarsson R: The activity of biologically equilibrated allergen preparations in conjunctival provocation test and leukocyte histamine release. In Dreborg S: The Skin Prick Test: Methodological studies and clinical applications. Linköping University Medical Dissertation No. 239, Linköping, 1987; with permission.*)

in sensitivity increases to six potencies of 10 (1 million times difference) (i.e., clinically sensitive individuals react with a wheal of the same size as that of histamine 10 mg/mL in the same patient to 100 times less material, but less sensitive individuals get such a reaction to 10,000 times more material than needed in the median patient in the sample used for biologic standardization)³⁶ (Fig. 4).

The drawback of the method is that the patient selection prescribed can only be used for common inhalant allergens^{11, 37, 47} in patients with a clear-cut clinical history of sensitivity to just that allergen.⁵³ Other solutions must be found for some pollen allergens of minor importance (e.g., plantain, which flowers in Europe during the grass pollen season and seldom causes problems without a dominating grass pollen allergy).⁵³ Other allergens include food allergens, occupational allergens, allergens to mice, guinea pigs, and rats to which humans are exposed occasionally or for short periods at home or in the workplace, and uncommon allergens. Because it has been documented that the biologic activity of allergenic extracts of common inhalant allergens correlates with the major allergen content,⁴⁷ it has been proposed to use the major allergen content as the basis for reporting the biologic activity of experimental allergen extracts, extracts of uncommon allergens, and extracts to which humans are exposed in another way than that of common inhalant allergens (e.g., food allergens).^{11, 33, 47}

As mentioned, several manufacturers use techniques for standardization of their allergenic extracts similar to the method prescribed in the Nordic Guidelines³ but using company-specific "fantasy units" and without reporting their exact procedures and results. In the United States, the authorities prescribe exactly which documentation should be presented by manufacturers before selling standardized extracts, but marketing nonstandardized extracts is still allowed. In Europe, on the other hand, there is a directive from the EU²⁴ giving less precise instructions than those of the Nordic Guidelines.⁷⁵ In some countries, especially in northwestern Europe, only standardized allergen extracts from one source or closely related sources such as related grasses are on the market ready to use. In other countries (e.g., France) and as in the United States, most injections of allergen extracts are given as individually prescribed formulations, often as mixtures of nonstandardized extracts prepared from different source materials without documented stability and probably partly degraded, as they contain known and unknown enzymes capable of degrading allergens effectively. The standardization concepts described will only play a role in the long run. On the other hand, the present use of naturally occurring allergenic source materials for production of extracts, standardized regarding composition and potency, with gradually increasing open declaration of the major allergen content, may be an intelligent way to gradually make customers used to potency declaration by weight. Then new specific epitope/recombinant therapy that is standardized by weight or molecular content will be more easily accepted.

DETERMINATION OF RELATIVE POTENCY OF ALLERGENIC EXTRACTS

US Method by Intracutaneous Testing

This method is based on the paper by Turkeltaub et al in 1982 that discusses the dose response of intradermal skin testing¹⁰⁶ (see Fig 2B). There are, however, reports on general reactions in connection with such intradermal tests, probably because many tests are performed in the same sensitive person at the same time.²⁸

This method also is recommended in Europe for determination of relative potency of allergens^{2, 3} in case in vivo tests are needed, because the dose response of the erythema is steeper than that of the whealing reaction at least up until 60 to 70 mm Σ of erythema diameters, which probably makes the method more precise than methods based on skin prick testing.

Skin Prick Testing for Estimation of Relative Potency

As mentioned previously, because the intracutaneous method is more precise for estimation of relative potency because of the steep dose response of the erythema induced by intracutaneous injection of allergen, this method also is recommended in Europe.^{2, 3}

By using the same protocol as for biologic standardization, which uses quadruplicate tests with each of three 10-fold dilutions of a standard and an allergen extract to be compared with that standard and then evaluated by parallel line bioassay, the skin prick method also can be used.^{2, 44} Using the median slope of allergens (i.e., 0.4), two or more extracts can be compared using quadruplicate tests with one concentration of each of the extracts³² and calculating the concentration that induces a 3-mm wheal or any wheal size, assuming that the slopes are parallel.

This method assumes that the median slope 0.4 (0.3875) is valid for all allergens and cannot be used in case of investigation of new preparations (e.g., recombinant allergens) that may have other properties. Testing over a wider range of concentrations in a number of patients with known in vitro sensitivity to the compound or its epitopes would be recommended.

ALLERGY RESEARCH

Skin tests are used within many fields of allergy research. Only the most important are mentioned in this article.

Epidemiology of Atopic Sensitization

Skin tests, especially skin prick tests, often are used in epidemiologic trials for documentation of allergic sensitization. If the skin prick test is used in such trials, some rules must be followed:

1. Only standardized allergens with known and documented composition and potency (i.e., in meaningful units such as micrograms of major allergen as estimated by a defined method) can be used. Preferably, the material should be comparable with International Standards (when available), and a sample should be kept freeze-dried for comparison *in vivo* and *in vitro* with subsequent batches or materials used in other trials using extracts produced from similar allergen source materials.
2. Only methods with documented precision or methods that are documented in the department performing the tests should be used. The best way to document precision in centers participating in the study is to ask the testing personnel to perform a number of tests, at least four in each individual, in a sample of individuals (greater than 50) to allow detection of minor differences in technique reflected by the CV. Either a defined allergen or histamine solution should be used. No difference in size should be detected. If such comparison is performed before and after the trial, differences in technique of any importance can be excluded. The technique of the technician should be documented at intervals by 20 tests in at least five people using histamine solution, and the result should be kept on file for future documentation. All tests should be in duplicate, and the mean/median histamine wheal size should be calculated. The range should be no more than ± 1 mm at the 6-mm mean diameter level.⁴⁰
3. Because there are clear methodologic problems with the skin prick test, it is recommended that allergen-specific IgE against one allergen important in the region is measured among a random sample of participating individuals from all centers. Then, only well-documented methods for IgE determinations may be used, not changing composition of allergens and methods for coupling without notice, to allow comparison over time and with results from similar trials.

There are few if any epidemiologic trials that fulfil the above given criteria. The author believes that the best trial is probably the International Study of Asthma and Allergy in Children (ISAAC) study protocol, phase two, that prescribes in-house proficiency testing, but it is impossible to gather all investigators and assistants for comparison of the technique.

One example of the problems involved in published studies is the European study on asthma which used the Phazet needle (dry allergen on an 'Allergy Pricker').¹⁵ This device was convenient, but the potency was defined only before the start of production,¹⁵ and varied between

batches.³⁹ Thus, data obtained in such trials can never be repeated and are of little value to researchers in the future and also for comparison with results from other trials.

Patient Characterization

Skin tests often are used for characterization of patients included in clinical trials (e.g., studies on pharmaceuticals). The same strict rules should be applied as for epidemiologic studies or else the information has no relevance.

Study of the Allergic Reaction

To study the mechanisms involved in the allergic reaction and the effect of chemicals, drugs, and mediators, several techniques are available.

Intradermal Skin Testing

Intradermal skin testing was used to study the late-phase reaction of the type I immediate skin reaction.^{29, 92, 93} Since then, modern techniques have been used to study this reaction, as in the studies by Steven Durham's group using *in situ* hybridization to study the interleukin response during the late-phase skin reaction and changes in this response during immunotherapy (allergy vaccination).^{55, 56, 108}

Temperature

The temperature of skin test lesions has been studied by two groups.^{12, 96} In 1980 Stuttgen et al,⁹⁶ using AGA Thermovision, found increased heat development in skin test areas suppressed by antihistamines. They also found that the heat radiation continued long after the disappearance of the erythema. Bagnato et al¹² studied the increase in temperature of the skin during immediate skin reactions using an infrared thermography camera. They compared the increase in temperature with the mean wheal diameter. These parameters correlated well ($r = 0.94$; $P < 0.001$). The CV of the temperature measurements were low (4.6%).

Ultrasound

Ultrasound has been used to study the thickness and volume of the whealing response to histamine.⁸⁹ Serup et al found that the thickness was correlated to the wheal diameter up to a diameter of 5 mm ($r = 0.64$; $P < 0.001$), after which wheals only expanded tangentially. Wheal thickness as measured by ultrasound was considered a potentially important parameter in experimental studies of inflammatory skin reactions.

Blood Flow by Laser Doppler Flow and Laser Doppler Imaging

Serup et al⁹⁰ used the laser doppler flow technique in the 1980s to study the whealing response to histamine. They measured the superficial blood flow in the center of wheals and 5, 10, 15, and 20 mm from the center (i.e., at distinct points), not the whole wheal and erythema. In the wheal center, the blood flow was increased but neither different concentrations of histamine nor the control reaction could be differentiated. Five and 10 mm from the center, however, there was a significant difference between the different solutions of histamine tested as well as the control reactions. The best differentiation was obtained 5 and 10 minutes after skin prick testing.

Laser doppler imaging (LDI) allows computer-aided recording of results and monitoring over 200×200 mm (i.e., several skin reactions at the same time). This method has been used by several groups mainly to study patch test results but also to study skin tests for immediate hypersensitivity.^{7, 8, 23, 97} Andersson et al⁸ point out the possibility of combining LDI with microdialysis to study skin reactivity. The author's group studied the time course of skin prick testing with histamine dihydrochloride, 10 mg/mL (M. Brinck, unpublished data). The maximum size of the histamine wheal response by scanning was after 18 minutes and not after 10 minutes as documented by Voorhorst et al using the intracutaneous test.¹⁰⁹

Evaporation

Evaporation of humidity can be measured and has been used in the study of skin tests in humans and combined with LDI⁵⁹ to get further information on the reactions.

Microdialysis

Microdialysis is another new method that can be used to study allergic reactions,^{9, 76, 80, 81} the effect of antiallergic drugs,^{9, 23, 82} and the effect of mediators on the skin.²¹

In principle, a fine dialysis tube is inserted into the skin. The tube can be perfused by a buffer solution. Then mediators released at the site of skin testing challenges by allergens bio-active amines such as histamine, prostaglandins, or leukotrienes. Samples for mediator analyses can be obtained in the dialysis tube and measured quantitatively in the buffer solution.

Petersen et al⁸² found that at the site of prick testing with allergen, histamine is released only in the center of the wheal, but not under the periphery of the whealing response or under the erythema. Other findings have shown that salmeterol and salbutamol have similar but weak anti-inflammatory properties.⁸²

Skin Chamber

In this technique, a blister (a small fluid-filled bleb) is induced by applying a negative pressure to a small area of the skin, the top of the bleb is removed by a pair of fine scissors, and a chamber filled with buffer solution is attached on top of the small skin lesion. The cavity of the chamber is filled with buffer and the material to be studied (e.g., allergen). The cells are counted or mediators such as histamine released into the supernatant are measured.⁵⁴

Using this method some information has been obtained regarding cells and mediators released by the allergen-IgE interaction; however, the allergen concentrations used were high at 10,000 PNU/mL. If standardized and stable extracts are used, the potency is comparable with at least 100,000 ng of major allergen/mL to be compared with the recommended dose for intracutaneous testing in Holland (i.e., 30 BU/mL^{96, 97} of major allergen/or about 30 ng/mL).⁶⁶ The doses used in skin chamber experiments were too high and not physiologic. Results obtained with this methodology should be interpreted with caution. Many of the results obtained with this methodology have been summarized.³

Biopsies

Biopsies were performed several decades ago to document the allergic inflammation of the late phase of the IgE-mediated immediate reaction.^{29, 92, 93} In recent years biopsies of the late reactions have been performed to study the immunologic events especially in relation to allergy vaccination, finding (e.g., by *in situ* hybridization) a shift from a T_{H2} toward a T_{H1} interleukin pattern.^{55, 56, 108}

Evaluation of the Activity of Antiallergic Drugs

Skin testing has been performed for many years as a method to evaluate the antiallergic properties of antiallergic drugs, such as antihistamines.^{13, 20, 66, 67} The method has not been used in an optimal way because the lin/log model for evaluation has been used.²⁰ Using this model, it seems that the reduction is less at low response levels (Fig. 7).⁶⁷ This fact may mislead those who have to make decisions on optimal therapeutic doses of antihistamines. So far, side effects, especially drowsiness, have been the main dose-limiting factor, but with less sedating preparations it seems reasonable in some cases to increase the dose to obtain better results.

Evaluation of the Action of Mediators of Inflammation

Intradermal testing with allergens has been used to document the influx of cells,^{29, 92, 93} mediators released,^{63, 64, 91, 98} and the action of mediators^{10, 17, 30, 101, 110, 111} when used for skin testing. New techniques with

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Figure 7. The slope plotted lin ($\circ$) log and log/log ($\bullet$) using the same data. A 70% decrease of the histamine wheal, which is commonly reported in antihistamine evaluation trials, means a reduction to 2 mm diameter. It is difficult to quantitate such reductions regardless of the evaluation system used. When considering, however, that a reduction from 2 mm (67% reduction) to 1.4 mm diameter (78% reduction) means a tenfold difference, no significant difference has been found at the level of 67% and 78% reduction in most trials published so far (*Modified from Dreborg S: Diagnosis in vivo and in vitro. In Mygind N, Naclerio R (eds): Allergic and Non-Allergic Rhinitis: Clinical Aspects. Copenhagen, Munksgaard, 1992, pp 82–94; with permission.*)

analysis of interleukins, receptors, and messenger RNA (mRNA) can enhance the knowledge about allergic mechanisms.

SUMMARY

Skin tests are valuable tools in allergen research and allergen standardization. To be useful tools, the methods used must be supervised properly. When skin tests are used in scientific trials and the results are reported, the materials and methods must be defined clearly, the precision given, and the methods to guarantee similarity in technique between centers must be supplied. The skin is more easily inspected by the eye, and reactions can be more easily supervised than in the lung or gut. Some techniques such as microdialysis and LDI can more easily be applied to the skin than to inner organs.

Despite the differences between these organs, the skin can be used for initial work on new concepts for allergic reactions. The role of cell-mediated reactions in atopic dermatitis also could be applied to rhinitis and asthma because similar dendritic cells and other cells involved are also present in the other organs mentioned.

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