

Position Paper: Allergen standardization and skin tests

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Introduction

During the annual meeting in Glasgow, July 1990, the Executive Committee of the European Academy of Allergology and Clinical immunology (EAACI) decided to transform the Subcommittee on Skin Tests to the Subcommittee on Allergen Standardization and Skin Tests. Furthermore, the Business meeting of this Subcommittee decided to produce a revised version of the 1989 *Position Paper on skin tests used in type I allergy skin testing* and include in the new version a chapter on "Allergen standardization by *in vitro* and *in vivo* methods", replacing the chapter on "Biologic standardization". The new chapter includes a general description of factors which affect the potency and composition of allergen extracts and a second part focusing on standardization of allergenic extracts used for skin testing. The subcommittees on immunotherapy, provocation tests, and *in vitro* tests are responsible for preparing position papers on the Academy's view on standardization of allergens for immunotherapy, *in vivo* provocation tests, and *in vitro* tests, respectively. When outlining Chapter 4, we have been in contact with these subcommittees.

This position paper does not cover skin tests used for the study of immune complex diseases and delayed-type hypersensitivity.

The different chapters of the position paper on *Allergen standardization and skin tests* have been worked out by members of the Steering Committee of the Subcommittee on Allergen Standardization and Skin Tests. Draft chapters were circulated to all members and other interested parties and were discussed during a special session at the annual meeting in Zurich in May 1991. The final version was discussed and approved by the subcommittee and then by the EAACI Executive Committee during the Congress held in Paris in May 1992.

During recent years, methods and goals for allergen standardization have been the subject of intense discussions within the Academy, and among authorities and industries, and, recently, the EC regulatory authority has approved directives and guidelines for registration of allergenic preparations in Europe. These directives and guidelines will soon be implemented by member countries.

There is an international system for allergen standardization and a number of WHO/IUIS International Standards (IS) labeled in arbitrary international Units (IU). The Academy stresses the importance of assessing the potency of allergenic preparations, intended for all types of use, in IU, giving the method for calibration of potency against these IS, in order to allow comparison of different materials used in scientific investigations and to give an idea of the difference in potency between materials prepared by different manufacturers.

The Academy also promotes the use of biologically relevant units suitable for labeling commercially available extracts used for diagnosis, since such units allow better and more reliable diagnosis of sensitization and atopic disease.

A number of company-specific units have been launched: some of these units are equivalent to units prescribed by authorities; that is, the Nordic Biological Unit (BU/ml) and the US Allergy Unit (AU/ml). Other units are less well documented. For scientific purposes, it is essential that well-standardized extracts or pure allergenic components of such extracts be used.

Methods of skin testing vary among regions and also among medical specialties. At present, the skin prick test method using a lancet with 1-mm tip is the method of choice for routine diagnosis, especially among pediatricians. In contrast to results obtained by laboratory methods, skin test results are often presented without giving any details of the potency of the allergen preparation used, the method of testing, or reproducibility in the hands of the investigator. This fact reduces the value of most published studies using skin tests. It is our hope that the recommendations in this position paper on the performance, evaluation, and use of skin tests in diagnosis, standardization, and epidemiologic studies, will be followed by European allergologists and, especially, by editors of scientific journals.

Finally, we hope that this edition of the position paper will stimulate scientific work within the field.

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Abbreviations

AU, allergy unit; BS, biologic standardization; BU, biologic unit; CGRP, calcitonin gene-related peptide; ECF(ECA), eosinophil chemotactic factor (activity); ECP, eosinophil cationic protein; ELISA, enzyme-linked immunosorbent assay; EPR, immediate- or early-phase reaction(s); FDA, Food and Drug Administration, Bethesda, MD, USA; HETE, hydroxyeicosatetraenoic acid; histamine HO, histamine dihydrochloride; I-IRF, histamine-releasing factor(s); ICT, intracutaneous skin test(s) (intradermal skin test(s)); IHR, in-house reference preparation; IL, interleukin(s); IMP, intermediate product; ISP, international standard preparation(s); IT, immunotherapy; LPR, cutaneous late-phase reaction(s); MBP, major basic protein; MC, mast cell(s); OBRR, Office of Biologics Research and Review (FDA); PAF, platelet-activating factor (PAF acether); 51-5, short-term sensitizing antibodies; SPT; skin prick (puncture) test(s); ST, skin test(s); VIP, vasoactive intestinal peptide.

1. Pathophysiology of skin tests

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Studies of the mechanisms by which allergens and nonspecific agents induce skin reactions began over 50 years ago when Lewis and Grant described the “triple response”, but in recent years there has been a major improvement in our understanding of the pathophysiology of skin tests. The IgE-mediated allergic reaction of the skin results immediately in a dermal response which is marked by a wheal and flare reaction, and which is dependent on both chemical and neurogenic mediators (immediate reaction). This is often, but not always, followed by a late-phase reaction (LPR) developing over the next 3—5 h, peaking at 6—12 h, and resolving in approximately 24 h. The LPR appears as an ill-defined edematous reaction which is related temporally to an influx of inflammatory cells.

1.1. The type I hypersensitivity reaction

The ability to mount an IgE-mediated response to allergens is a prerequisite for the development of positive allergen skin tests. Some investigators have suggested that IgG antibodies have anaphylactic properties; others have questioned the existence of IgG short-term sensitizing antibodies. Other factors influencing skin reactivity to an allergen include the amount of allergen injected; the number, degree of sensitization, and releasability of cutaneous mast cells; and the reactivity of the skin to mediators released from the mast cells, particularly histamine.

Skin tests can also be performed with vaso active agents or mast cell degranulating agents. Histamine induces only a wheal and flare reaction, whereas kinins, PAF-acether, and mast cell secretagogues elicit both an immediate and a late-phase reaction.

1.2. Histology of the normal skin

T lymphocytes are scattered in the skin, whereas there are no B cells. T cells are present mainly around the superficial dermal vascular plexus, and occasionally in the epidermis and interstitial dermis. The main antigen-presenting cells (APC) in the skin are the Langerhans cells in the epidermis and the macrophages in the dermis. There are 5000—12000 mast cells per mm³, evenly distributed and frequently observed in close anatomic association with blood vessels and nerves, suggesting that they may be involved in the neuronal control of skin blood flow (4). These mast cells are predominantly of the formalin-sensitive type and contain tryptase and chymase (25). Only very few granulocytes are present in the normal skin, usually around superficial venules.

Epidermal changes associated with aging include flattening of the underside of the epidermis, reduction in the number of Langerhans cells and melanocytes, and decline in the number of melanosomes synthesized. Dermal changes include reductions in the collagenous and elastic fibers, fibroblasts, mast cells, and macrophages (22). These differences may explain age-dependent variation in skin reactivity to allergen and vasoactive amines.

1.3. Pathology of type I hypersensitivity reactions

After allergen injection, histopathologic studies of the skin show an influx of fluid within 5 mm, followed by the appearance of inflammatory cells, mainly neutrophils (10 mm after allergen injection) and eosinophils (later onset). Dunsky and Zweiman have developed a skin chamber technique to study the skin response to allergen: in this technique the epidermis is denuded by inducing a skin blister, after which the dermis is covered with a chamber so that mediators and cells can be recovered.

1.3.1. Immediate reaction

The immediate reaction is dependent on mast cells that are rapidly degranulated after allergen challenge (10) or injection of morphine. Mast cells in the skin apparently differ from those in the lung and intestine in being activated for histamine release by endogenous neuropeptides and morphine (15). Histamine release begins about 5 mm after allergen

injection and peaks at 30 mm. Tryptase is also released with a similar time course (27).

1.3.2. Late-phase reaction (LPR)

The LPR occurring after allergen challenge is due to IgE antibodies. Biopsies of the LPR reveal a mixed cellular infiltrate, predominantly made up of mononuclear cells but also including eosinophils, basophils, and neutrophils, and an extensive deposition of fibrin (7, 26). The same cellular pattern, however, can also be found after an immediate wheal and flare reaction that does not lead to a macroscopic LPR. Eosinophils are found to be activated, and the size of the LPR is correlated with the number of activated eosinophils (9). There is also extracellular deposition of eosinophil major basic protein (MBP), eosinophil-derived neurotoxin (EDN), and neutrophil granule proteins (elastase) in the skin during LPR (19). Activated T lymphocytes are also present, and there is a strong correlation between the numbers of CD4⁺ cells and activated eosinophils (9). There is indirect evidence that basophils cause the persistent release of histamine during continuous antigen administration in the skin, since such release is unaccompanied by release of tryptase or POD, (released from mast cells but not basophils), does not occur after codeine challenge (which activates mast cells but not basophils), and is inhibited by steroids (which inhibit the accumulation and release of histamine from basophils but not mast cells) (1).

Several mediators have been recovered from skin blister fluids after allergen challenge during the immediate and the late-phase reaction (11, 23, 28, 31). These include histamine (5 mm to 4 h), tryptase, kallikrein, thromboxane B, POD, (release starting 30 mm after challenge; peak at 6 h after challenge), LTC₄ (peak 2–4 h after challenge), and minimal concentrations of LTB₄ and PAF-acether (peak 5–6 h after challenge). Uncharacterized histamine-releasing factors have also been detected in skin blister fluids (29).

1.4. Injection into the skin of inflammatory mediators released during type I hypersensitivity reactions

Histamine mimics the allergen-induced wheal and flare reaction when injected into the skin by prick tests or intradermal reactions. However, injection of histamine never produces an LPR, in contradistinction to allergens or mast cell secretagogues. Histamine is the major, but not exclusive, mediator of the wheal and flare reaction. In contrast, histamine accounts for a limited portion of the LPR (11).

Sulfidopeptide leukotrienes, injected intradermally, induce a burning, erythematous wheal and flare reaction persisting for up to 4 h, with a characteristic central pallor supposedly caused by arteriolar constriction. Histologically, the wheal and flare reaction is associated with dermal edema and marked dilation of venules and capillaries.

LTB₄ injected intradermally in human skin has been shown to induce a transient wheal and flare reaction followed by an LPR whose histopathology demonstrates a polymorphonuclear leukocyte influx and some fibrin deposition.

POD, induces an asymptomatic wheal and flare reaction resolving within 2 h. Histologically, POD, injection causes dermal edema and vessel dilation, with a subsequent neutrophil infiltration beginning 2 h after challenge and lasting for a further 4 h. Topical application of 12-HEIL (12-hydroxy-5,8,10, 14-eicosatetraenoic acid) produces erythema and accumulation of neutrophils and monocytes in human skin. Blood flow is increased at 6 and

24 h (30). Cyclooxygenase products, such as prostaglandins and thromboxanes, probably contribute to IgE dependent skin reactions, both as modulators of mediator release and as vasoactive mediators (11).

Platelet-activating factor (PAF) administered intradermally has both acute and delayed effects, with a wheal and flare reaction that resolves within 30—60 min and that is followed by an LPR persisting for several hours (21). The wheal and flare reaction is due to the direct or indirect vasoactive properties of the autacoid. However, since PAF-acether is rapidly metabolized *in vivo* by fluid-phase and cell-associated phospholipases, its delayed effects can be explained only by its potent chemotactic activity, especially affecting eosinophils, or its effects on the release of IL-1 and tumor-necrosis factor. By a skin chamber technique, it was found that the prolonged exposure to this mediator induced the migration of neutrophils and basophils. Henocq & Vargaftig(14) showed that PAF-acether was able to attract eosinophils but only in allergic subjects. Moreover, PAF-acether is the most potent eosinophilotactic mediator, so its release induces an influx of various cells which can participate in cutaneous late-phase inflammation. The vasoactive and chemotactic effects of PAF appear to act locally on a small area, producing a highly focused inflammatory response (16).

A number of preformed mediators have been shown to be capable of activating the coagulation, fibrinolytic, and bradykinin pathways. The intradermal injection of a bradykinin analog leads to a late-phase reaction characterized by a cell infiltrate similar to that induced by allergen (18), whereas bradykinin itself causes vasodilation and vasopermeability but without inflammation.

1.5 Pharmacologic studies

Indirect evidence of the role of different mediators in allergic skin reactions can be obtained in studies of the modulation of reactions by pharmacologic or therapeutic agents. Concerning the immediate skin reaction, cromoglycolic acid does not inhibit codeine-induced histamine release, while antihistamines inhibit some of the components of the wheal and flare reaction but have no effect on the LPR. The LPR is blocked by prior administration of topical or systemic corticosteroids. Topical application of ETYA (eicosatetraenoic acid) can block both the cyclooxygenase and the lipoxygenase pathways of arachidonic acid metabolism and induce a small but significant reduction of the LPR. Cyclooxygenase inhibitors incompletely alter edema formation and dazoxiben, a selective inhibitor of thromboxane synthesis, increases the wheal and flare reaction and has some part in decreasing the LPR. Topical indomethacin, another cyclooxygenase inhibitor, decreases the flare without any effect on the wheal. PAF-antagonists decrease skin tests to PAF-acether and, to a lesser extent, those to allergens.

1.6. Cytokines

Cytokines are glycoproteins which are synthesized and secreted by various cells, bind to specific receptors, and regulate the activation, proliferation, and differentiation of immune as well as nonimmune cells. Keratinocytes are capable of secreting various immunomodulating cytokines or secretory regulatory peptides (IL-1 alpha, IL-1 beta, IL-6, IL-8, colony-stimulating factors, tumor-necrosis factor alpha, growth factors (transforming growth factor beta) and suppressor factors (epidermal cell-contractant interleukin-1)) involved in inflammation as well as in healing and repair processes. Many cells of the dermis are also capable of releasing cytokines: further studies are needed to clarify the role of different

cytokines in the cutaneous allergic reaction and to elucidate the lymphokine cascade *in vivo* (20).

Interleukin-1 promotes cell recruitment and influences allergic mediator release, and is released at sites of allergen-induced cutaneous reactions in human subjects 1 h and 10–12 h after allergen challenge (5). A histologic study of the LPR gained indirect evidence of cytokine secretion, as suggested by increased expression of HLA-DR on endothelial cells and *de novo* expression of the CD4 antigen on epidermal Langerhans cells (9). In a recent study using *in situ* hybridization, Hamid et al. (12) have observed that cells infiltrating the site of allergen-induced LPR transcribe mRNA for genes encoding for IL-3, IL-4, IL-5, and GM-CSF cluster, possibly representing the human equivalent of the murine Th2 cell phenotype.

In animals, recombinant (r) IL-1 alpha, IL-1 beta, TNF alpha, IL-1, -2, and IFN-gamma have been injected subcutaneously. Acute (6 h), resolving (48 h) inflammation was induced by the following cytokines in order of potency: rIL-1 alpha, rIL-1 beta, and rTNF alpha, whereas rIFN gamma had no effect (6). In human subjects, IFN-gamma injection induces a moderate, perivascular, lymphohistiocytic infiltrate and intense keratinocyte HLA-DR expression (24). However, IFN-gamma appears to suppress mast-cell-dependent skin reactions. Quantitative skin prick tests with allergen were done before and after 20 injections of either recombinant IFN-gamma (100 µg each) or placebo in 30 patients. IFN-gamma reduced skin wheal area significantly but had no effect on allergic bronchoconstriction (3).

Taken together, these studies appear to establish the importance of cytokines in the generation of the LPR, but further studies are required to clarify their role.

1.7. Neuromediators

The relationship between neurogenic and cellular inflammation in the generation of the cutaneous type I hypersensitivity reaction is beginning to be understood. Experimental support for the neurogenic hypothesis comes from studies which show that the indirect effect of histamine on the cutaneous microvasculature (in the peripheral flare around the injection site) is greatly diminished by prior application of a local anesthetic cream (13). The skin receives a rich supply of nerve fibers, many of them being the peripheral ramifications of primary sensory neurons involving axonal reflexes in the terminal arborizations of C-fibers containing substance P, neurokinin A, and calcitonin gene-related peptide (CGRP). The physiologic significance of the antidromic stimulation of small-diameter afferent nerve fibers localized in the skin is still incompletely known, but there is good evidence that some vascular effects of inflammation in the skin are neurogenic. Substance P releases histamine from skin mast cells and produces dose-related wheal and flare reactions in human skin. Neurokinin A induces a wheal but little or no flare and is less potent than substance P. CGRP induces both wheal and flare but is also less potent than substance P. In addition, CGRP induces a slow-onset, intense vasodilation in human skin which persists for several hours and is associated with leukocyte infiltration, a response which is not seen with substance P. The possibility that histamine and mast cells play a role in neurogenic inflammation in skin is still a matter of debate (8). However, two studies suggest that mast cell activation is not essential for the initial, vascular permeability phase of neurogenic inflammation in rodent skin (2, 17).

1.8. Schematic representation of type I hypersensitivity reactions

1.8.1. Immediate reaction

The wheal and flare reaction induced by an IgE mediated immune response is mainly due to the activation of mast cells releasing vasoactive agents, which cause both plasma extravasation and vasodilation. It is likely that cellular and neurogenic inflammation are linked, since histamine (more than other mediators) can trigger the release of substance P by axonal reflex, and substance P enhances the immediate reaction by causing the release of histamine from mast cells (positive feedback loop). The close proximity of the mast cells to vessels and nerves in the skin augments these processes.

1.8.2. Late-phase reaction

The exact mechanisms of this erythematous inflammatory reaction are less well characterized. Mast cells appear to be the trigger cells of the reaction, since the LPR almost never appears without a preceding immediate reaction. These cells release chemotactic mediators and possibly cytokines, attracting inflammatory cells to the site of the allergic reaction. It is also possible that the release of vasoactive mediators from mast cells increases vascular permeability, facilitating the access of inflammatory cells and the exposure of these cells to chemoattractant factors. It has been suggested that lymphocytes play a key role in the generation of late-phase inflammation, and they may be more important than mast cells in the generation of the LPR. Moreover, lymphocytes release histamine-releasing factors and other cytokines which can facilitate the release of mediators from mast cells and other cell types. Eosinophils are known to produce cytotoxic proteins such as MBP (major basic protein) and ECP (eosinophil cationic protein) that have been shown to be involved in the cutaneous LPR. They are likely to be key cells of the LPR. Macrophages and neutrophils release chemotactic factors, cytokines, oxygen free radicals, and enzymes that are involved in the inflammatory process. Neutrophils are the first cell type to appear in the inflammatory infiltrate after the immediate reaction. They have been shown to be in an activated state and are thus capable of initiating the cutaneous damage and attracting other cells, particularly eosinophils. Basophils are possibly involved: the late release of histamine is probably due to basophil activation, but the exact role of these cells remains to be elucidated.

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