

What I wrote in 1962 (3) still applies: "Advances in the understanding of the pathogenesis of the atopic diseases will only come when standardized methods using "pure" antigens and antibodies are available". Progress seems slow but progress is taking place, particularly in the Scandinavian countries.

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Analysis and Standardization of Allergen Extracts Intended for Therapeutic Use

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Over the past decade, many papers and international meetings have been devoted to establishing methods and requirements for analysis and standardization of allergen extracts. In fact, manufacturers and control authorities are in demand to produce procedures enabling precise and viable quantitation of allergenic activity as well as analysis of extracts. Such an approach would allow physicians to prescribe uniformly labelled extracts of known quality and guarantee therapeutic procedures.

Minimum requirements have also to be established for drafting monographs for Pharmacopoeia.

It has been stated (3) that potencies determined by skin testing or by RAST inhibition of commercial extracts of short ragweed pollen showed about a 1000-fold difference as to their real activity and one can conclude

that allergenic extracts, actually on the market, often do not satisfy the general requirements of drugs. At present, it is even not advisable to substitute an extract of one manufacturer with that of another without again carrying out skin testing to ensure safety and therapeutic activity, because none of the quantitation systems actually used indicates a precise expression of the biological activity in standardized units, a condition leading to much confusion.

The concentrations of extracts are also for the time being expressed in different units such as the Noon unit, the total nitrogen unit, the protein nitrogen unit or weight by volume (5).

The flaw in these methods of standardization of allergenic extracts is that correlation of biological activity

of impure preparations with chemical analysis (or other terms) such as protein content or quantity of raw material (extract) is not evident.

Several propositions have been made during recent years to overcome the lack of standardization of allergenic extracts, with respect to biological activity. The methods proposed have not yet been generally accepted, but certainly satisfy several criteria (1). Appropriate methods of standardization of allergen extracts by Skin Prick Testing (SPT), Radio-Allergosorbent-Tests (RAST) and Quantitative Immunoelectrophoretic methods (QIE) have been studied in several laboratories and detailed recommendations and technical survey given (1,2,4).

Studies were also undertaken in our laboratories during the last few years to characterize and standardize the biological activity of our extracts, especially with regard to house dust and pollen preparations.

Large quantities of extracts have been produced and purified and their activity has been established by means of skin testing in reference to an internal standard (5). In this way, it should be easy to correlate this standard either to an international reference preparation and express the biological activity in generally accepted units, or to those obtained by prick tests with a histamine equivalent as proposed by Aas et al. (1). Of course, difficulties will arise in countries where on ethical grounds, standardization can not be required by tests performed in humans. An alternative method of biological standardization has been proposed by means of RAST-based techniques and the order of correlation coefficients compared to skin test titration has been found to be highly significant in several studies.

It has, nevertheless, to be considered that while skin tests are very easy to carry out and allow activity to be checked at any moment, RAST-tests do not express biological activity and have to be carried out under carefully controlled conditions in specialized laboratories using materials of high quality such as discs, pooled sera of selected patients, labelled anti-IgE.

The analysis of allergen extracts was carried out in our laboratories by several biological methods: the *in vivo* method of skin testing and *in vitro* methods of double immunodiffusion, immunoelectrophoresis, isoelectrofocusing and crossed immunoelectrophoresis. Incubation of immunoprecipitates with specific IgE from patients and subsequent reaction with ^{125}I -anti-IgE and autoradiography permitted to identify specific allergens. Serum pools made up from a large number of untreated allergic patients classified as class 5 or 4 by Phadebas RAST were used.

Studies were mostly performed with whole and purified timothy pollen extracts. Antibodies were raised in rabbits by injection of whole timothy pollen extract. Difficulties were encountered to get sera reacting with a large number of antigenic components. Immunization was carried out by several procedures and mostly over periods exceeding 1 year. Only bleedings of selected animals showing numerous precipitating antibodies were

pooled and used.

Extraction and purification of allergens were carried out under carefully determined conditions so as to avoid degradation of antigens into low molecular weight fractions. All extracts were immediately frozen or lyophilized and kept at low temperature. Activity of fractionated timothy pollen allergens, determined by skin testing, revealed a molecular weight distribution between about 20 000 and 44 000 and two allergen containing peaks of about 26 500 and 41 000.

Enzyme activity of whole extract and fractions of purified timothy pollen has been determined by means of the Api-Zym System (API System—La Balme les Grottes — 38390 Montalieu Vercieu-France). The following enzymes were present in crude extract: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, acid phosphatase, phosphamidase, α -galactosidase, β -glucosaminidase and α -mannosidase.

All of the isolated allergenic fractions of a purified extract were devoid of enzymic activity. On the contrary, activity of some enzymes present in crude material was also present in fractions of molecular weights exceeding 65 000 daltons and having no *in vivo* allergenic activity.

Double immunodiffusion and immunoelectrophoresis using rabbit anti-timothy serum revealed the presence of at least 20 antigens in timothy pollen extract, many of which were also present in extracts of cocksfoot, rye grass and cultivated rye pollens. Analysis by crossed immunoelectrophoresis also revealed the extreme antigenic heterogeneity of grass pollen extracts.

Radio-immunotechniques enabled to identify major and minor antigens in timothy extract capable of specifically binding IgE of pooled sera from hay fever patients.

Allergens detected by means of a rabbit anti-timothy pollen serum and subsequent incubation with pooled sera from patients and labelled anti-IgE were also present in extracts of the three other grass pollens, although in different proportions.

These results indicate the presence of a large number of identical antigens in the grass pollen extracts, of which only a very few can be classified as major allergens.

Extracts of pollen were analyzed by isoelectrofocusing (6) and a high number of components were also found to be present. Immunological identity of separated bands are, however, difficult to characterize by this method. One has to bear in mind that identical proteins differing slightly in molecular composition could result in the separation of several bands and are, for instance, present in crystalline diphtheria toxin and highly purified serum albumin.

Similar studies are also underway to analyze extracts of house dust and *Dermatophagoides pteronyssinus*. Analysis by biological methods and isoelectrofocusing also revealed the presence of a large number of allergens.

It can be concluded that allergenic extracts contain a large number of antigens of which some are major

allergens capable of producing high levels of specific IgE in man. It seems obvious that these allergens have to be isolated before being used for therapeutic purposes, thus eliminating the injection of minor allergens to atopic individuals and the risk of subsequent sensitization.

The potency of allergen extracts has to be expressed in units of biological activity determined by appropriate methods in reference to a generally accepted standard.

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A Practical Approach to Allergen Standardization

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The quality of all allergen preparations depends upon optimal raw materials. The selection of these materials must be based on a combination of RAST methods and quantitative immunoelectrophoresis. Any purification steps subjected to the extract should be monitored by the same methods. The purpose of this communication is to describe a practical approach to the standardization of stable, freeze-dried reference preparations and the tests necessary to ensure only small variations in the subsequent batches.

Allergen preparations to be used in a practical way for diagnostic and therapeutic purposes must be given units which reflect the allergenic activity observed *in vivo*. Histamine Equivalents in Prick testing (HEP) obtained by skin prick tests (SPT) of the allergen solution in parallel with a 1 mg/ml (5.45 mmol/l) histamine solution, have been suggested by Aas and coworkers (1). In Fig. 1 an allergen preparation (B) which has been biologically standardized by this approach, is compared with two theoretical preparations (A and C) standardized by *in vitro* methods only. The possible wheal diameters of SPT obtained in a number of patients display a great variation when testing any of the three preparations. The reactions of

preparations A and C are, however, in one case too small and in the other too great. The biologically standardized preparation B exhibits on the other hand a median reaction equivalent to the mean histamine reaction.

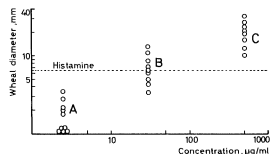


Fig. 1. Possible wheal diameters of SPT obtained in a number of patients when tested with three theoretical allergen preparations, plotted as a function of the corresponding concentrations. The preparations A and C were standardized by *in vitro* methods only, while preparation B was biologically standardized.

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