

Allergen standardization

During the last 10 years, there has been rapid progress in the standardization of allergen extracts. However, standardization has taken several different forms and has produced considerable confusion about what the units mean and how they should be used in practice. This is due not only to the large number of different terms and units but also reflects genuine difference of opinion about the objectives of standardization. Two contrasting positions have emerged: (1) that the units used should help to predict the biologic response and that, because patients respond differently to the multiple proteins in an extract, standardization must involve an evaluation of the response of allergic patients to the extract, that is, by skin tests, and (2) the alternative view is that, because patients' responses are individual, any evaluation based on skin tests or the RAST depends on the selection of the patients tested and thus cannot be the basis for any long-term standardization. This view considers that standardization should aim to make quantitative measurements of defined constituents of an extract and that the unitage should not imply any prediction of the *in vivo* response.

These two positions inevitably lead in opposite directions. Historically, the earliest calls for standardization of allergen extracts followed shortly after the introduction of desensitization for hay fever, and the first unit was named after Noon himself. This unitage was based on the weight of pollen extracted and not of the allergen content of the extract. Similarly, definition of extracts by the ratio of the weight of raw material to the volume of fluid used to extract does not measure allergen content. However, there is no confusion about the meaning of Noon units or weight to volume units. The first unit that attempted to evaluate the quantity of protein in the extract was the PNU. The technique for PNU measures all protein in the solution and makes no distinction between biologically active allergens and other proteins. When techniques for measuring allergens, for example, antigen E (now *Amb a* I) and Rye I (now *Lol p* I), evolved, it became obvious that protein content was not necessarily related to allergen content.¹ These differences

Abbreviations used

PNU:	Protein nitrogen unit
BU:	Biological unit
FDA:	Food and Drug Administration
AU:	Allergy unit
MAB:	Monoclonal antibody
WHO:	World Health Organization
IS:	International standard
IU:	International unit

were particularly obvious when special conditions for extraction were used, such as alkaline pyridine. Pyridine, at pH 13, produces high levels of protein, and for some pollens (e.g., grass) the extracts are very potent; however, with other allergens (e.g., ragweed) the proteins are extensively denatured, leading to an extract with high PNU but very low potency. Results such as these spurred the creation of an allergen standardization subcommittee of the International Union of Immunological Societies. This subcommittee and, particularly, Drs. Aas, Baer, Brighton, Lowenstein, Norman, and de Weck, have worked toward a standardization system that evaluates the "allergenic" activity or potency of an extract.

There are three widely used methods to estimate the potency of an extract: (1) use the extract to skin test a series of patients, (2) measure the allergens *in vitro* by their ability to inhibit IgE antibodies, that is RAST inhibition, and (3) measure specific allergens in the extract.

Skin testing as part of national or international standardization was initially regarded as impractical but has now been established in two different forms:

1. The Nordic system based on testing the quantity (or dilution) of the extract that produces a wheal that is the same size as the wheal produced by a given concentration of histamine. These results were originally expressed as histamine equivalent potency (or HEP units) but are now expressed as BUs that have been adopted by Scandinavian countries and are widely used in Europe.
2. The system of AUs established by The Center for Biologics Evaluation and Research of the FDA is based on a different method of skin testing. The system proposed by Turkeltaub² used intradermal testing with threefold dilutions of an extract and evaluates the flare rather than the wheal size. Each extract is evaluated in individuals who are "highly

From the Division of Allergy and Clinical Immunology, Department of Medicine, University of Virginia, Charlottesville, Va.

Reprint requests: Thomas A. E. Platts-Mills, MD, Division of Allergy and Clinical Immunology, Department of Medicine, University of Virginia, Box 225, Charlottesville, VA 22908.

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TABLE I. Units used for allergen extracts

Units	Basis of the unit	Where used
Noon units	Allergen extracted from 1 µg of pollen	Obsolete
Wt/vol	Weight in grams per volume in milliliters	Worldwide
PNU*	1 PNU equals 0.01 µg of protein nitrogen	Worldwide
AU†	Skin testing to end point	USA
BU†	Skin testing relative to histamine	Nordic countries and other parts of Europe
IU‡	In vitro assays relative to WHO ISs	Worldwide

The SQ (standardized quality) unit is a special unit derived from BU which is used by ALK in Europe.

*Protein nitrogen is measured in micrograms by Kjeldahl technique or Ninhydrin assay.

†Notice. Although the unitage on an extract is established by skin testing a group of allergic individuals, the consistency of subsequent batches from the same manufacturer is maintained by RAST inhibition relative to a national standard.

‡IUs are not currently used for labeling extracts.

allergic" to the relevant extract, as defined by clinical history and or a sum of erythema diameter of >50 mm on prick testing. For each individual, the intradermal end point is expressed as the number of threefold dilutions that produce a summed erythema diameter of 50 mm. The mean value for 15 individuals, which ranges from 7 to 15, is used to define AUs for the extract. An extract with a mean dilution from 9 to 11 is allotted 1000 AU; from 11 to 13, 10,000 AU; and from 13 to 15, 100,000 AU. It should be noted that an extract, which, for example, elicits a result of 12.9, will be allotted 10,000 AU, whereas an extract that elicits a result of 13.1 will be allotted 100,000 AU. For both BUs and AUs, the objective is that units for one extract should be equivalent to units for other extracts derived from the same or another source.

An important problem with any system based on skin testing is patient selection. Each system uses a combination of history and skin testing to define patients for the tests. For patients with seasonal hay fever

caused by a pollen that has a distinct season and is common in a particular area, it is relatively easy to identify highly allergic patients and to confirm by skin reactivity. For house dust allergens, fungi, and many less common pollens, it is difficult to define typical symptoms, and therefore, skin testing becomes the major criterion of who is highly allergic. This leads to a situation that can easily become circular, that is, that an extract is used for skin testing to define who should be used to test the potency of the extract!

Shortly after RAST was introduced to measure IgE antibodies,³ the test was modified to test the potency of extracts. This system allows for direct comparison of two or more extracts in parallel and can produce accurate quantitative results.⁴ At the present time, RAST inhibition is widely used to evaluate extracts and compare these extracts to national standards. Indeed, all batches of standardized extracts currently marketed in the United States are routinely evaluated by allergen manufacturers using RAST inhibition. In general, companies find RAST inhibition a reliable in-house system for comparing the potency of one batch of extract with previous batches of the same extract. However, there is no way of comparing the RAST-inhibition potency of one extract (e.g., grass pollen) with the potency of an extract from a different source (e.g., cat dander). The main problems with RAST inhibition are that the results depend on the extract used for the solid phase and that the serum pools depend on the donors and are therefore difficult to reproduce. In addition, the assay uses considerable quantities of both serum and extract; therefore, even pools of 2 L of serum or more will not last for more than a few years in routine use. It is important to realize that new batches of standardized extracts that are labeled in AUs are compared with previous batches and FDA national standards by RAST inhibition (not by skin testing). Perhaps the biggest stumbling block for RAST inhibition internationally is that it is not possible to define what is being measured apart from saying the "potency" of the extract. Thus, at the present time, although RAST inhibition is widely used, there is no system of unitage that is based on this assay.

Most international and national biologic standards are based on measurements of specific proteins. The standards for immunoglobulin are a good example. These standards are lyophilized serum samples that contain a known quantity of IgG, IgA, IgM, or IgE. Initially, the measurements were based on arbitrary international units but have been changed to absolute units, that is, micrograms or milligrams, as the weight

estimates have been confirmed. There is no convincing way to measure any of the common allergens except by immunoassay (i.e., by use of an antibody specific for that protein). The only exception is that the hyaluronidase activity of venom can be used to evaluate the strength of some venoms. An essential requirement for allergen measurements is that the protein being measured has been purified and demonstrated to be an important allergen. Many allergens have not been purified, and an increasing number are well characterized, that is, by physicochemical criteria, amino acid sequence data, production of MABs and even, in some cases, cloning of the genes. Most, but not all, of these proteins are highly immunogenic in animals (as well as man), and it is relatively easy to develop polyclonal antisera or MAB. From the theoretical point of view, there is no real difference between an assay for *Fel d I* with polyclonal antiserum in radial immunodiffusion or with MAB in a two-site immunometric assay. The advantages of MAB are practical ones, (1) that specificity is constant, (2) that production is essentially unlimited, and (3) that the two-site ELISA assays are simple, rapid, and cost-effective.^{5, 6}

To use assay of a single allergen to standardize an extract, that allergen must be either an allergen that is an important quantitative component of all extracts from that source and to which most, that is, $\geq 80\%$ of allergic individuals respond, or an allergen that is present in all extracts in a consistent relationship to other components of the extract. Measurement of a single major allergen has worked well for ragweed extracts measuring *Amb a I* in micrograms and for cat extracts measuring *Fel d I* with FDA units (1 FDA unit of *Fel d I*, 4 μg). Problems arise if an extract contains several important allergens that are present in different proportions in extracts produced from different material or by different manufacturing techniques. With pollens, the source material is relatively consistent and can be checked for purity; however, different extraction procedures or processing may alter the ratio of different constituents. More difficult problems arise with dust mite, animal dander, or cockroach in which the source materials used may be dramatically different. For example, extracts made from whole bodies of mites have relatively more of the group II allergens. Currently, there are extracts of dust mites available in the United States that have ratios of group I: group II mite allergen ranging from 3:1 to 1:10. Furthermore, the group II allergens are much more stable than the group I allergens; thus, poor conditions of storage can lead to a selective loss of group I.⁷ Therefore, although

TABLE II. WHO ISs

ISs	Code No.	IUs
Timothy-pollen extract	NIBSC 82/520	100,000
Short ragweed-pollen extract	NIBSC 84/581	100,000
<i>D. pteronyssinus</i> extract	NIBSC 82/518	100,000
Birch-pollen extract	NIBSC 84/522	100,000
Dog-hair/dander extract	NIBSC 84/685	100,000
International reference reagent		
Bermuda grass-pollen extract	ATCC	

NIBSC, National Institute of Biological Standards and Control; ATCC, American Type Culture Center.

ISs under development: rye grass-pollen, cat, fur, *Alternaria*, and *D. farinae*.

*Reference to use of these standards should indicate the source and the code number; standards can be purchased from National Institutes of Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, England.

establishing a system of unitage based on specific measurements of major allergens is clearly the best long-term solution, it requires considerable understanding of the allergen source being evaluated.

Since 1980, ISs and international reference preparations (IRP) have been established for a series of different allergens (Table I). The process for establishing a standard is based on in vitro testing (including RAST inhibition and measurement of major allergens, as well as crossed immunoelectrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and isoelectric focusing) and has been outlined by Norman⁸ and Reed et al.⁹ For a standard to be accepted by the WHO, it must be tested in a series of laboratories in different countries. The choice of allergens, preparation of standards, and testing has been organized by the Allergy Standardization Subcommittee of the International Union of Immunological Societies. Reports of international collaborative trials are evaluated by the WHO expert committee.^{10, 12} The standards are a series (2000 to 4000) of glass-sealed ampules, each of which contains an aliquot of freeze-dried allergen extract. The WHO standards were designed to be used as a yard stick for in vitro evaluation of extracts from the same source material. It was not intended that ISs should be used for skin testing or immunotherapy; indeed, the WHO

specifically advises against any *in vivo* use of the standards. Thus, the whole thrust of the campaign to produce ISs is to establish *in vitro* systems that can be repeated consistently in different parts of the world. The expert committee of WHO has recently gone further in recommending that future standards should be based on *in vitro* measurements of specific proteins with the use of absolute units if possible.

ISs are stored at -70°C in a WHO-approved reference laboratory (e.g., National Institute of Biological Standards and Control in England or the American Type Culture Collection in the United States) (Table II). Each standard comes with a brief description of the source of the contents. For most of the standards, a report has been published that describes the preparation in detail.¹⁰⁻¹² The standards have a code number and have been allotted a number of IUs, usually 100,000 IU per ampule. It is appropriate to refer to either the total potency of an extract in IUs or to the concentration of a specific component in IUs. Alternatively, an IS can be used to standardize an assay of a specific allergen with absolute units. In this case, it would be normal practice to establish an in-house or laboratory standard that is compared to the IS. Measurements of mite allergen in the environment are routinely performed using as the positive control a standard extract that has been compared to the IS. In this case, the results are provided in micrograms based on the known content of the *Dermatophagoides pteronyssinus* IS that is 12.5 μg of *Der p* I. The advantages of this approach are well illustrated by the proposed threshold values for dust mite exposure as a risk factor for asthma¹³ and the recent article¹⁴ of a 10-year prospective study on the effects of exposure to mite allergen in early childhood.

CONCLUSIONS

The present confusion over standardization of allergen extracts reflects the fact that an ideal system has not been developed. The slow rate of progress has been a disappointment to many people involved with the efforts to establish standards. However, most extracts of the commonly used allergens are now carefully monitored by the companies, both by consistent manufacturing technique and by RAST inhibition. Indeed, the main benefits of international efforts to establish standardization may be in this increased awareness of the factors that influence potency and consequent improved consistency of extracts.^{8, 9} The systems of standardized unitage currently in use for commercially available extracts are both based on skin testing. The United States (isolationist as ever) uses

AUs, whereas the Nordic countries and other parts of Europe use BUs. At the present time, the two systems cannot be directly compared and are unlikely to be used in parallel in any country. The advantages of extracts labeled in standardized units are that they are generally very consistent from batch to batch and are of high overall potency. It is not safe to assume that an extract labeled as 10,000 AU of *D. pteronyssinus* (for example) from one company, is the same as an extract labeled 10,000 AU of *D. pteronyssinus* from another company. Indeed, we have observed major differences in content and ratio of allergens in extracts with the same AU. When different extracts are compared (e.g., grass pollen and cat) the AU or BU establishes a guide to the expected potency of extracts, which is a significant improvement over PNU or weight per volume. However, the two systems cannot be directly compared. It remains true that patients demonstrate enormous variation in their sensitivity to inhalant allergens, and any claim that unitage predicts the response *in vivo* should be treated with caution.

In the long run, the ideal system is to define the important allergen or allergens in an extract and measure them with absolute units standardized relative to an IS. Although this is obviously impractical for a large number of less common allergens, this goal is being achieved for the most important sources. Assays for individual allergens are increasingly available and simple, whereas WHO standards have been established or are in progress for ~8 allergens. In the future, it appears likely that major allergen measurements will progressively replace other systems of unitage. However, this will only occur as evidence for the reliability of measurements for each source is established.

Thomas A. E. Platts-Mills, MD

Martin D. Chapman, MD

Division of Allergy and Clinical Immunology

Department of Medicine

University of Virginia

Box 225

Charlottesville, VA 22908

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