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Problems in Allergen Standardization

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The fact that allergen extracts need standardization has been obvious since skin testing was introduced in 1873. The problem became more urgent with the introduction of desensitization in 1911. The first methods used were based on weight to volume ratios, and the Noon unit is a descriptive unit of this kind (1 Noon unit = the allergen extracted from 1 μg pollen). Noon units are simply a record of manufacturing procedure and, as such, cannot be verified by any assay of the extract. During the 1930s, it became clear that most allergens were proteins and the protein nitrogen unit (PNU) was developed by Robert Cooke and others (1 PNU = 0.01 μg protein).[1] At that time, the only available technique for testing potency was to compare skin testing results. For pollens, PNU has proved to be a very useful guide to the probable strength of an extract; however, it is not a measurement of allergen nor allergenic activity. Prolonged extraction of any pollen can increase protein without increasing allergen, whereas extraction of either ragweed pollen or mite culture with pyridine at high pH increases the protein yield while denaturing the allergens. Indeed, it was the realization that the extraction with pyridine denatured ragweed allergens that created much of the present impetus for allergen standardization.[2]

When standard extracts have been established nationally, they have generally not been accepted or used by other countries. For this reason, over the last 20 years, there has been an almost continuous effort to achieve international standards for allergens. A major effort has been made to define the exact conditions for skin testing so as to achieve accurate comparison not only between samples of one extract, but also between different allergens. This approach, supported strongly by Dr. Aas, in Norway, led to the successful development of the Hep, or histamine equivalent unit, in Scandinavia[3,4] and the rather similar unit that has recently been recommended in the United States.[5] An alternative to skin testing was made possible with the development of in vitro histamine release in 1967. However, it still requires patients and is technically demanding. The real problem with all biologic techniques is that they are too slow and are not suitable for routine use in commercial

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laboratories. As Norman and Lichtenstein put it in 1970, "Labeling extracts on the basis of biological activity requires the use of time-consuming biological methods in allergic volunteers, and simpler alternatives must be sought."[6]

The search for direct methods of measuring allergen in vitro started before the discovery of IgE or the purification of any major allergens. Using serum from rabbits immunized with allergen extract, it was possible to identify multiple proteins in the allergen extracts by immunodiffusion and to compare different samples of the same extract.[7] However, this did not give a direct guide to which antigens were allergens. Using inhibition of passive hemagglutination, the "strength" of allergen extracts could be compared with great sensitivity and considerable accuracy. However, because the hemagglutination was based on animal antisera, the assay was also measuring antigens that were not necessarily allergens. Two developments have changed the situation dramatically over the last 15 years. First, the discovery of IgE, which led to the availability of antisera to IgE and methods for measuring IgE antibody in vitro, and second, the purification of major allergens.[8–11] In fact, antigen E from ragweed pollen and the Group I protein from rye grass were purified before IgE was discovered. However, the proof that IgE antibodies against these major allergens could be measured in vitro added greatly to their acceptance. The ability to measure IgE antibody in vitro by the radioallergosorbent test (RAST) led directly to RAST inhibition assays, which are now the primary in vitro method for measuring the potency of an allergen extract. With the purification of major allergens, it has become possible to measure their concentration in an extract using rabbit antibodies. There are now about ten allergens that can be measured directly.

During the last 10 years, there have been several different initiatives toward establishing international standards. Many individuals have contributed, but Harold Baer, Bill Brighton, Kjell Aas, and Alain De Weck stand out in the earlier phase. Progress toward international standards since 1980 has depended on close collaboration between companies, regulatory agencies, scientists, and clinicians. Collaboration of this kind is not easy, as different groups often have very different reasons for wanting standardization and also a very different understanding of what is meant by a reference preparation. Over the last few years, much of the work has been organized from the Protein Laboratory, in Copenhagen, and much of the credit should go to Henning Lowenstein. In this reivew, we will outline the organization and objectives of allergen standardization, as well as the methods used, in order to illustrate the problems involved.

Coordination of Efforts Toward International Standardization

The International Union of Immunological Societies (IUIS) has established a committee on immunologic reference preparations and, associated with this, there is a subcommittee on allergens. In turn, this allergen subcommittee has a chairman, executive committee, and a steering committee. The steering committee has about 25 members, including representatives from the important companies producing allergens, the national regulatory agencies responsible for allergen preparations used for medical purposes, and scientists in-

terested in allergens/allergy. The objective of the steering committee has been to choose suitable allergens for standardization, to prepare allergen preparations, and to organize international collaborative trials so that reference preparations can be submitted to the WHO.[12] The procedure by which WHO assesses a candidate international reference preparation (IRP) is well defined.[13]

The steering committee has established a general policy as to how a candidate reference preparation should be prepared for a particular allergen. First, a small group of individuals are nominated to organize the studies on an allergen. Their first priority is to decide on the correct source materials for the allergen and a suitable source, ie, company, for obtaining or purchasing a sufficient quantity of allergen. If necessary, they may have to organize a preliminary trial to compare different sources and/or to establish the feasibility of various assay techniques. The next priority is to organize the manufacture of approximately 4000 glass-sealed ampules of lyophilized extract, together with small numbers (~200) of several other extracts of the same allergen. Each ampule will generally contain between 1 and 4 mg of protein, ie, the total quantity is 4–10 g of allergen protein. The samples are then sent coded to a series of laboratories in several different countries that have volunteered to the steering committee. Each laboratory is asked to assess the extracts using its own or a study RAST inhibition assay and to use any other technique that they consider useful in assessing allergen potency. Because of the importance of RAST inhibition, each of the studies so far has also provided ampules of lyophilized, pooled allergic serum suitable for RAST inhibition with that allergen.

The results of this international collaborative trial are then analyzed and written up by the group. The report is considered by each participating laboratory, and when agreed, it is submitted to the executive committee and then to WHO.[14–16] If the report is accepted by WHO, the preparation becomes a WHO international reference preparation (IRP). The ampules are stored frozen by a laboratory designated by the WHO and are made available to any laboratory that requests them. Even without setbacks, the procedure may take 2 or 3 years for each allergen.

Objectives

The objective of allergen standardization is for different laboratories and companies to be able to describe the strength of an allergen extract in units that have the same meaning. It would then be possible to use different extracts of the same allergen with confidence about their relative strength, ie, for skin testing, desensitization, or coupling to RAST particles. Equally important, it would also be possible for research workers to understand what strength of extract or quantity of an allergen was used by other laboratories.

The key requirement is to establish an international standard that will be generally accepted as *the standard* for each allergen. Before discussing the methods used to assess extracts, it is important to explain what is meant by a standard. The standard extract must be a typical extract, which is stored in a large number of very similar aliquots under conditions such that it will remain stable for a long time. In no sense does a standard have to be the best

extract or a model extract. The standard is a yardstick for making measurements of allergen extracts, not a blueprint for what allergen extracts should be. Furthermore, it is not absolutely necessary to decide in advance what it contains, as subsequent studies and improvements of methods may well modify any decision made. For an allergen standard, it is necessary that each glass-sealed ampule should contain sufficient allergen to be measured by any widely used technique of in vitro assay. In addition, it is obviously desirable that any major or minor constituent allergen proteins that are recognized for that allergen source are present. It would be nice to be able to measure the quantitative relationship between the different proteins in an extract, but this may have to wait until after the standard has been established.

Source Materials for Allergen Extracts

Good criteria are available for judging the quality of grass, weed, or tree pollens used for allergen extraction. In addition, the conditions of storage and extraction of pollens are well established. Thus, for the ragweed international reference preparation there was no argument about the source of pollen used. The Bureau of Biologicals, Washington, D.C., obtained ragweed pollen supplies over a 3-year period. The pollen was examined for purity by microscopy, defatted, and extracted in water for 22 hr at room temperature.[15] Similarly, there was very little problem choosing a timothy pollen source and deciding how to extract it.[16]

Obtaining agreement about an extract of *D. pteronyssinus* was far more difficult. In an earlier approach to standardization, Dr. Brighton, London, England, established a standard extract that was derived from isolated mite bodies, arguing that culture medium and debris should be excluded (NIBSC Code No. 77/622). However, the mites used had been grown in horse dander and horse proteins can be detected in that extract. Furthermore, the quantity of allergen in the ampules was rather low for many in vitro methods of assessment. By 1981, when the current allergen standardization steering committee was working, it was generally agreed that fecal material from the mites was at least as important as the mite bodies.[17–21] The available evidence suggests that extracts made from mite bodies probably contain all the relevant mite allergens; however, the extracts derived from bodies contain several proteins in increased proportions, some of which are irrelevant. The second problem with *D. pteronyssinus* extracts was that different companies were growing mites on different media. There are arguments for and against all the different media for production of clinical extracts. Clearly, it is undesirable to have nonmite proteins that are allergens present in the extract, eg, allergens from horse dander or from fish meal. Ideally, mites should be grown on a fully synthetic medium; to date, there are no published techniques for doing this. Some of the companies are using heat-denatured or semi-synthetic media to grow mites, but the details are commercial secrets. The classical technique for growing mites on human skin scales supplemented with *Sacharomyces cerevisiae* was developed by Spieksma and is still the most widely used.[17] Extracts from these cultures do not contain any nonmite allergens, but they do contain detectable human protein, approximately 0.3 μg human IgG/ml

(measured by radioimmunoassay). For an international standard, the growth medium may be relatively unimportant, as the extract is not intended to be used for treatment or diagnosis. To illustrate this point, it may be worth considering the international reference preparation for IgE; this is simply a human serum with a high level of total IgE. This serum has not been shown to be good (or even safe) for Prausnitz-Kustner (P-K) testing or for passive sensitization of basophils. In addition, very little is known about the other serum proteins or food proteins present in this serum. Nonetheless, the IgE WHO standard has been very successful as the reference point from which all standards used in the measurement of serum IgE are directly or indirectly derived.

There are problems with choosing source materials for many other allergens (Table 1). For bees, venom is now the logical choice because most clinics use venom. However, there are patients who appear to be allergic to proteins in the whole bodies, and there are allergists who still prefer whole body extracts. For cat allergen, there is good evidence that saliva is an important source of the major allergen, Cat 1.[22] However, most cat extracts are made from cat dander, and for practical reasons, it seems likely that the international reference preparation will be made in this way. For rats, mice, and guinea pigs, large quantities of protein are present in their urine, and some of these proteins appear to be the major allergens.[23] Furthermore, skin testing with urine from these animals gives more positive results than using serum or pelt extracts.[24] It is by no means accepted that urine is the correct source for a standard allergen preparation from these animals. In many ways, the key question to answer is what source is most widely accepted, or rather, will be most widely accepted, over the next 10 years.

Cross-Reacting Allergens

An interesting problem arises with closely related sources of allergens. With the grasses, it is clear that there is very close cross-reactivity among rye, timothy, orchard, sweet vernal, and several others. This cross-reactivity probably reflects the presence in each of a major allergen that fully cross-reacts with the group I protein in rye grass pollen.[9] It is perfectly possible to measure allergen in extracts of each of these using radioimmunoassay (RIA) for Rye I. Furthermore, skin testing with all these grasses gives remarkably similar results,[25] and many allergy clinics use grass pollen mix instead of skin testing with each separately. The decision was made to establish a reference preparation for timothy grass because it is very widely used. If, subsequently, it is decided to establish another grass standard (eg, rye), it will be difficult to distinguish that preparation from the timothy standard by assay of the major cross-reacting antigen or by RAST inhibition. Indeed, it would be very difficult to establish a pool of allergic sera from patients who were allergic to rye grass pollen distinct from timothy grass pollen (see below).

A very similar problem arises with the cross-reactivity that exists between *D. pteronyssinus* and *D. farinae*. It has been known for some years that most patients who show positive skin tests to one of these mites will also give positive reactions to the other mite. Furthermore, using extracts of each spe-

Table 1. Sources of Allergens and Purified Allergens in Relation to Standardization

Allergen	Source	Major allergen(s)	Units	Laboratories capable of measurements[a]
Plants				
Ragweed	Pollen	Ag.E, Ra3, Ra5	μg	>10
Rye grass	Pollen	Rye Group I, Group II	μg	~ 5
Timothy grass	Pollen	Ag25, Ag19	RP	6
Bermuda grass	Pollen	—	—	?
Fungi				
Alternaria	Spores culture	Alt I		
Cladosporium	Spores culture	Antigen 32		
Little animals				
D. pteronyssinus	Mite bodies, whole culture or fecal pellets	Ag P_1 (=Dp42)	μg	7
		DpX	2P	3
D. farinae	Mite bodies, whole culture or fecal pellets	Df 11 (=Df6)	RP	3
Cockroach	Whole culture or secretions		RP	1
Bees	Venom	Phospholipase A	μg	>10
Wasps or yellow jackets	Venom			
Big animals				
Cat	Pelt, dander or saliva	Cat 1	Units	3
Rat	Dander or urine	Rat urinary allergen	μg	2
Mouse	Dander or urine	—	—	1
Horse	Dander	—	RP	1 or 2
Penicillin				
Penicillium	Culture or synthesized	Penicilloyl hapten	μg or molarity	>10

RP, relative potency.
[a]Approximate number of laboratories capable of measuring major allergens.

cies on the solid phase of RAST to assay IgE antibodies in sera from mite allergic patients gives closely comparable results; assaying 58 sera from Hong Kong, the r value was 0.90, $P < 0.001$.[26] This suggests that IgE antibodies to one mite must cross-react with the other mite. However, comparing antigen P_1 with *D. farinae* extracts by immunodiffusion suggested that the cross-reactivity with this protein was poor.[27] Also, the RIA that we use for antigen P_1 is only partially inhibited by *D. farinae* extracts. On the other hand, there appeared to be other proteins in *D. farinae* extract that cross-react fully with

D. pteronyssinus proteins. These results suggested that the close correlation seen with RAST (or skin testing) was due to antibodies to proteins other than antigen P_1. However, this is probably not so, as results of RAST assay using *D. farinae* extracts shows a close correlation with IgE antibody to antigen P_1 in the same sera ($r = 0.91$, $P < 0.001$). As with the grass pollens, establishing a meaningful standard for *D. farinae* will require a group of patients or sera from patients who are allergic to this mite as distinct from *D. pteronyssinus*. Certainly, it would be unwise to attempt standardization of *D. farinae* until the relationship between the two mites has been better established.

In Vitro Assays Used in Allergen Standardization

Any assay performed on an allergen extract can, in theory, be matched in order to compare different extracts. However, many assays do not measure allergen proteins or allergenic activity, and in addition, there are special requirements for establishing an international standard. Assays must be simple enough and well enough defined to be used in many laboratories in several different countries. Secondly, the WHO requires that the assays can be expressed as "parallel line assays." This simply means that results for serial dilutions on a group of extracts will give parallel lines. Results in this form can then be more easily analyzed statistically.[13]

As can be seen in Table 2, many different methods were used to examine the candidate preparations for the first three allergens. In the following discussion, each technique will be considered relative to these three criteria:

1. Does it measure allergen?
2. Is it simple enough to be widely applicable?
3. Does it produce results that can be analyzed statistically?

RAST Inhibition

RAST inhibition is discussed first because it is the most widely used technique for in vitro allergen measurement, and because, in the opinion of the IUIS Steering Committee in 1981, it was the most useful technique for routine

Table 2. Numbers of Laboratories Carrying Out Different Analyses during International Collaborative Studies on Three Standards

	RAST inhibition	Major allergen assay	CIE/CRIE	IEF	Histamine release and skin tests	Countries
Ragweed $n = 12$	10	3	5/3	11[a]	1	5
D. pteronyssinus $n = 19$	12	6	7	6	2	11
Timothy $n = 14$	12	6[b]	8	11	6	10

[a]Ragweed IEF results gave "very similar binding patterns" for each of the extracts.
[b]Relative potency measurements of Ag25 and Ag19.

standardization. RAST inhibition was first used by Wide shortly after the introduction of RAST. The technique has been developed by many different groups, but particularly by Gleich and colleagues.[28] In order to carry out RAST inhibition, it is necessary to have:

1. A suitable allergen extract to link to the allergosorbent.
2. An activated solid phase, eg, microcrystalline particles or cellulose discs.
3. Sera or a serum pool from allergic patients.
4. Anti-IgE labeled with ^{125}I or an enzyme.

Allergen for the Solid Phase. In theory, the requirement for an allergen extract on the allergosorbent assumes that we know what a good extract is and how to measure it. In practice, using aqueous extracts has proved satisfactory and no major problems due to the extract on the allergosorbent have arisen during the preliminary studies or during the collaborative trials. When an extract is incubated with the activated particles, proteins in the extract bind covalently. The technique assumes that the allergens on the solid phase reflect the allergens in the extract. In most experiments with RAST or RAST inhibition, no serious assessment is made of the allergosorbent. If any assessment is made, it is simply assumed that any allergosorbent that will lead to optimal binding of anti-IgE using allergic serum is "good." Direct assessment of the binding of individual allergens in a mixture would require antisera directed against each allergen, which, for most allergen sources, are not available. A useful technique is to take the supernatant after binding to one batch of activated microfine cellulose and react it with a second batch. This procedure is repeated several times, and then each batch is tested in a RAST assay. If each batch gives parallel binding curves, then it is assumed that the binding in the first batch reflects all the proteins that can bind. Furthermore, using microfine cellulose, it is possible to combine the different batches.[15] The necessity of proving that an allergosorbent has allergen on it is illustrated by studies on *Cladosporium*. These studies demonstrated that a commercially available allergosorbent gave such low maximum binding of anti-IgE that most sera found to be positive with an experimental *Cladosporium* matrix would be judged to be negative.[29] Proteins may fail to bind either because of competition from other proteins, or low molecular weight material, or because the allergen inherently binds poorly.

Solid Phase for RAST. For practical reasons, many laboratories choose to use cellulose discs; however, the capacity of the discs for binding proteins is not very high, and the repeatability of the assays is less good than with particles. The particulate matrices, eg, Sepharose or microfine cellulose, have a much higher capacity for allergen and give more accurate results. The problem with these particles is that they are much slower to wash; furthermore, with RAST inhibition, high capacity is not a major benefit. In order to design a sensitive RAST inhibition assay, both the allergen on the allergosorbent and the serum should be limiting. If high capacity particles are used with excess allergen, it is possible to obtain high level binding of anti-IgE with an atypical serum that only has IgE antibody to a "minor" allergen. During the recent

collaborative trials, cellulose discs, acrylic beads, and microfine cellulose have been used successfully as solid phase.

Serum Pools for RAST Inhibition. Because individual patients become allergic to different proteins from a given allergen source, it is desirable to obtain sera from a group of patients (approximately 10).[14] Ideally, these patients will have allergic symptoms directly related to the relevant allergen source and no other sensitivity. In practice, sera are from patients whose dominant sensitivity, judged both by skin testing and symptoms, is to the relevant allergen. In addition, it is better to have sera from patients who have not received desensitization. Theoretically, desensitization might lead to sensitization to new allergens, but in practice, this has not been shown to be a problem. On the other hand, desensitization does increase IgG antibodies and may lead to the production of IgG antibodies to new allergens.[30,31] The presence of high levels of IgG antibody is likely to interfere with RAST assays when the allergen is limiting, and, as disucssed above, RAST inhibition is usually carried out with limited quantities of allergen.[28] Obviously, it is better to have sera with high levels of RAST binding, as the pool can be used for more tests. It could be argued that some sera from patients with low total IgE and low level of IgE antibody should be included because they may react to different allergens or determinants. In practice, sera with low levels of IgE antibody mixed in a pool would have no effect on the overall results.

For the international collaborative trial on *D. pteronyssinus*, a standard pool of allergic sera was established at NIBSC, London (Code No. 82/528). Details of the patients used to establish this pool and the antibodies in the sera are shown in Table 3. The important features are that most of the sera have high levels of RAST binding and IgE antibody to antigen P_1 as measured by ra-

Table 3. Sera Used for Standard Anti-*D. pteronyssinus* Pool in Collaborative Trial

Patient	Age/sex	Total IgE[a]	Anti-P_1[b] IgG	Anti-P_1[b] IgE	RAST inhibition[c] dilution	*D. pteronyssinus*	Other skin sensitivities
J.L.	22/M	500	1,100	117	1/17	+ + +	Cat + +
I.H.	25/M	2530	115	70	1/17	+ + +	Cat +
A.S.	15/F	4725	355	477	1/57	+ + +	Tree +/dog +
M.K.	28/M	1085	265	128	1/9	+ +	—
C.Sh.	45/M	250	325	65	1/5	+ + +	—
S.Bo.	20/M	309	90	81	1/4	+ + +	Dog + +/cat + +
S.Br.	18/M	2750	1,100	645	1/24	+ + +	Grass + + +/cat + +
S.Ba.	17/M	179	305	97	1/3	+ + +	Dog + +/grass + +/ *Cladosporium* + +
K.Sh.	21/F	850	2,325	242	1/14	+ +	—
P.J.	16/F	2910	430	358	1/48	+ + +	Grass pollen + + +/ Dog +/Cat +
Serum pool[d]		—	394	136	1/11	− − −	—

[a]International units.
[b]Units of binding activity (1 unit ~ 1 ng antibody protein).
[c]Dilution of serum giving 50% of maximum RAST binding to *D. pteronyssinus* discs.
[d]Serum pool was lyophylized in 0.9-ml aliquots (NIBSC Code No. 82/528) from Ford et al. 1985, ref. 41.

dioimmunoprecipitation, without very high levels of IgG antibody or total IgE. Another feature of these sera was that the patients had all been skin tested to a range of allergens and were shown to be more sensitive to *D. pteronyssinus* than to other allergens. Finally, *D. pteronyssinus* was the dominant mite found in dust from the houses of 9 of 10 of these patients. Several sera were rejected when making up this pool because of very high total IgE (>5000 IU/ml), because of another dominant sensitivity, eg, horse or cat, or because the patient lived in a house infested with *Euroglyphus maynei*. The serum pool used for the collaborative trial on ragweed was obtained from patients who were typically allergic to ragweed and had been sensitized in an area where ragweed is the dominant pollen at that time of year.[15] The sera used for the timothy grass pollen study were taken from patients who were sensitive to timothy extract but who had certainly been sensitized by exposure to a wide range of grass pollens. Patients in England are exposed to more rye grass pollen than timothy pollen, but show skin sensitivity to both.[25] In practice, it is most unlikely that this cross-reactivity will create any problems with use or assessment of the timothy IRP. Finding suitable sera for standardizing *D. farinae* may well prove difficult because, in most parts of Europe, *D. pteronyssinus* is dominant. There are areas in the south and east of the United States where *D. farinae* is dominant or codominant.[32,33] If *D. farinae* extracts are assessed or purified by reference to patients who are sensitized to *D. pteronyssinus*, only cross-reacting allergens will be identified and measured. The reference pools were meant to be used in the process of establishing an international standard and to help other laboratories to assess or establish their own assays. As the pools are stored lyophilized and frozen, they will also act as a useful reference standard for IgE antibody assays.

Radiolabeled Anti-IgE. For several years, anti-IgE has been a serious problem because the commercial supplies are expensive and the reagents are not easy to make. To specifically purify anti-IgE, you must either immunize with one myeloma and specifically purify on another one or immunize with Fc fragment of IgE and purify on Fc fragment or whole IgE. However, this problem is going to disappear rapidly as the monoclonal antibodies to IgE become more widely available. Some of the monoclonal anti-IgE preparations are proving to be excellent for RAST and RAST inhibition and will bring a new element of consistency to the assay.

Major Allergens and Assays for Major Allergens

Purification of allergens and establishment of the importance of a given allergen is a slow process. Once a protein has been purified and proven to be an important allergen, it is relatively easy to develop assays to measure it. These assays are of two types, those dependent on precipitation in gel, eg, rocket immunoelectrophoresis or radial immunodiffusion, or alternatively, immunoassays using radioactive or enzyme-linked markers. This diversity of assays is illustrated by the variety of techniques used to measure antigen P_1 in the collaborative study to establish the *D. pteronyssinus* standard (Table 4). Despite this wide variety of assays, the results for the relative potencies of

Table 4. Assay of Antigen P_1 in Coded *D. pteronyssinus* Extracts by Six Laboratories

Lab	Method[b]	Relative potency of extracts					Antigen P_1 (μg)	
		A[a]	B	C[a]	D	E	A	C
1	RIA	1.0	0.05	1.02	2.1	5.1	10	11
2	RIE	1.0	0.04	1.2	1.3	4.4	26	31
3	MoAb assay	1.0	0.03	0.94	1.4	4.1	—	—
4	Add[c] RIE	1.0	0.02	1.1	1.0	4.1	25	27
	Add SRID	1.0	0.06	1.13	1.0	5.7	19	22
5	SRID	1.0	0.10	1.1	1.1	4.1	15	15
	P_1 RAST	1.0	0.05	1.05	1.6	2.5	—	—
6	RIA	1.0	0.06	1.16	2.0	6.3	—	—
	Geometric mean	—	0.05	1.08	1.4	4.4		

[a]A and C were duplicates of the proposed reference preparation.
[b]RIA, fluid phase inhibition radioimmunoassay; RIE, rocket immunoelectrophoresis; MoAb assay, solid phase monoclonal antibody assay; SRID, single radial immunodiffusion; P_1 RAST, RAST inhibition assay specific for P_1.
[c]Add, addition.
Data from Ford et al.[14]

the extracts as assessed by antigen P_1 content were very close. There seems to be no reason to regard any of these assays as better than the others. On the other hand, there may well be practical advantages of one assay over another. Immunodiffusion-based assays require larger quantities of antigen and antiserum, and it would be difficult to prepare sufficient quantities of pure antigen or monospecific antisera to last for a long time. Radioimmunoassays (RIA) require very little rabbit antiserum; 1 ml of a potent antiserum can be sufficient for >100,000 tests. Similarly, very little allergen is required, and 1 mg should be sufficient for ~100,000 tests. However, RIA requires radiolabeling, and many laboratories find the techniques difficult. The alternative is to use an immunoassay based on a monoclonal antibody.[34] There are many possible techniques using monoclonal antibodies, and one alternative is shown in Fig. 1. Clearly, it is essential to establish that the monoclonal antibody is directed against an important allergen. This can be done either by comparison with CRIE or by screening with purified allergen. It would be difficult to establish that a monoclonal was directed against a major allergen without having first established which proteins were important. In practice, this requires purifying the relevant allergen. Once the specificity is established, it is relatively easy to design assays that do not require a supply of purified allergen. The control curves for these assays need only use an extract of *known* allergen content. Similarly, if allergen is required on a solid phase, whole extract can be used. In the near future, it is most likely that major allergen assays will be: (1) based on monoclonal antibodies, (2) much simpler to carry out than present assays, and (3) the most widely used assays for comparing or standardizing extracts. It is important to recognize that if the potency of allergen extracts was measured routinely by assays of several different defined components, the same international reference preparations could be used.

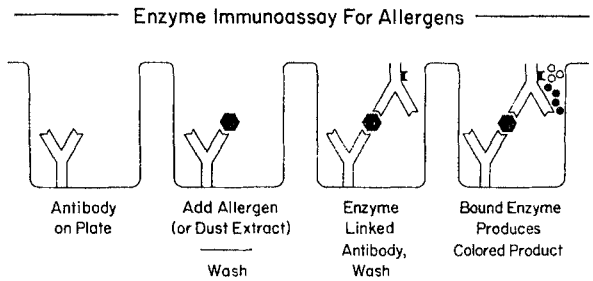


Figure 1. Outline of a possible allergen assay using monoclonal antibodies. The antibody bound to the plate can be monoclonal or conventional. The second antibody can be either specifically purified or monoclonal. The perioxidase label can be on the second antibody or on a third antibody (not shown), eg, rabbit anti-mouse Ig. A very similar assay can be carried out in which the second antibody is human IgE and the third antibody is radiolabeled goat anti-IgE.

Crossed-Immunoelectrophoresis (CIE) and Crossed-Radioimmunoelectrophoresis (CRIE)

Quantitative CIE can be used as a method of measuring the quantity of a defined protein in an extract and was used to measure timothy and *D. pteronyssinus* allergens (Table 2). CIE or Laurell electrophoresis has been more widely used as a technique for visualizing multiple proteins in an extract. Using human allergic sera and radiolabeled anti-IgE, CRIE is used to identify which of the multiple proteins seen in an extract using CIE are allergens.[35,36] During 1983, some friction developed within the IUIS Steering Committee between the allergen companies and those who wished to move allergen standardization forward rapidly; there were many reasons for this. However, there was a specific problem in relation to CIE/CRIE because the identification of individual allergens requires highly immune rabbit antisera. It is definitely unsatisfactory to have standardization dependent on an arbitrary (possibly nonrepeatable) reagent, such as hyperimmune rabbit antiserum. Using different antisera, it is often difficult to be sure which precipitates are equivalent. The problems with CRIE have recently been discussed by Lind and Lowenstein (1983)[37]:

1. The system for CRIE is dependent on a rabbit antiserum that produces a precipitate with the important allergens but does not block all the determinants to which IgE antibody is directed[37].
2. The interpretation of autoradiographs of CRIE is subjective and can be confused by nonspecific binding of allergens in precipitates.

At present, CIE and CRIE represent excellent techniques for assessing or comparing different extracts in a single laboratory, but it is difficult to see how they could be used by companies on a routine basis. Within a reference laboratory, it may be possible to use CRIE to define a range of typical values for minor as well as major allergens. However, these cannot be a *requirement* for commercial extracts, as it would be very difficult for a company to measure these values for themselves. The allergen companies have resisted any de-

scription of a standard that involves measurements they cannot easily duplicate. This in part reflects a tendency to regard allergen standards as model extracts rather than as a yardstick for measuring the strength of allergens. It is obviously important to distinguish between the tests that can be used routinely to measure the strength of allergen extracts and those tests, including CRIE, that are an important part of describing a standard extract but may not be suitable for routine assessment.

Isoelectric Focusing

Isoelectric focusing (IEF) of allergen extracts is a common method of assessment. For many pollens, the patterns are characteristic.[38,39] Indeed, IEF is the easiest technique to confirm the origin of a given pollen extract and may even be *sufficiently* sensitive to suggest that a given batch of pollen was not pure. However, the technique simply identifies a series of protein bands separated by their isoelectric points (pI), and gives no indication as to which of these proteins are allergens. Even if the patterns are scanned with a densitometer, the results cannot be regarded as quantitative.[39] The use of IEF is made more difficult by the presence of allergens that cross-react immunologically but have different isoelectric points. This is true of antigen P_1, for antigen 32 from *Cladosporium*, and to a lesser degree for many other allergens.[10,11,29] In addition, the group I protein of rye grass pollen cross-reacts almost completely with a protein in a variety of other grasses, but the cross-reacting proteins vary in isolectric point.[9] In conclusion, although IEF may be a useful technique for a company to use to compare different batches, it cannot be used in quantitative comparison with a standard or for statistical analysis. In the report of the collaborative study on timothy, the authors concluded, "We are not able to say whether absence of a band means that an important allergen is missing or whether it means that the extract is without a superfluous inactive protein."[16]

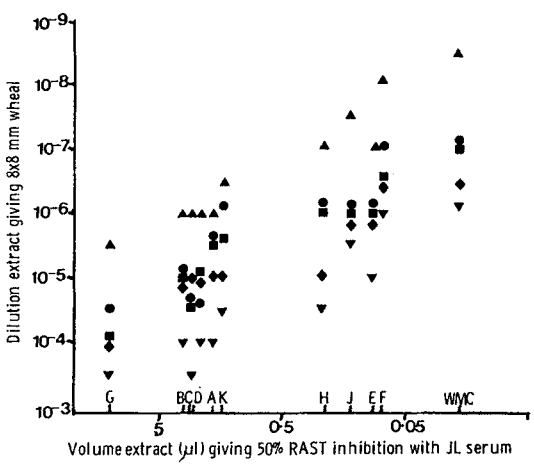
Skin Testing

Skin testing has been widely used for comparing the potency of allergen extracts. Carried out carefully, this can give quantitative and reproducible results that correlate well with other measurements of allergen potency. Early studies, using a constant dilution of extract, established that the size of wheal and flare was unreliable. All quantitative techniques now involve some form of end-point titration. End-point titration can be carried out either with prick or intradermal techniques. The end-point can be defined either by extrapolating to that dilution of extract that will give a wheal size of 8 × 8 mm (for example) or by carrying out a series of determinations close to the end-point and plotting the results. In a recent study, the size or erythema following intradermal tests gave more consistent results than wheal size.[5] However, erythema can only be measured on white skin and is completely unreliable on patients who have had atopic dermatitis. Most authors measure mean wheal size, ie, two diameters of the wheal at right angles, and express results as the dilution giving a particular end-point.[3,9,28,40]

Unlike the in vitro assays skin testing can provide a guide to the correct dose of allergen suitable for routine skin testing and, perhaps, to the dose for starting desensitization. Many desensitization regimens use a starting dose that is related to the threshold skin testing dose. Taking advantage of this, Turkeltaub et al.[5] have recommended that the concentration of an extract should be given in allergy units that are directly related to the quantity of allergen that will produce an end-point skin reaction. The Hep unit reflects that quantity of an allergen that will give the same skin reaction as a fixed quantity of histamine.[3] In both these systems, it is implied that the skin tests are carried out on typical highly allergic individuals. This involves major assumptions about the mean sensitivity of patients and implies that there is some consistent level of skin sensitivity at which patients will observe symptoms. This is not general experience, as symptomatic patients have been observed who have skin sensitivity to relevant major allergen over the range 10^{-7} μg/ml down to 10^{-3} μg/ml. Furthermore, it is likely that sensitivity to a particular allergen in different countries is not consistent. The real problem with skin testing is that it is time consuming, painful, and has a definite, though very small, risk to the patient. For many companies, simply obtaining a group of suitable skin-test-sensitive patients would be difficult.

In the preliminary study to choose a suitable candidate for the *D. pteronyssinus* IRP, ten coded extracts were assessed on five patients and three controls by end-point intradermal skin testing. The results showed a highly significant correlation with the results of RAST inhibition (Fig. 2) and antigen P_1 assay (Fig. 3).[41] These results are in keeping with many others in showing that either RAST inhibition or major allergen assay can satisfactorily replace

Figure 2. Comparison of RAST inhibition and quantitative skin tests. Ten coded extracts provided in lyophilized ampules were used for skin testing on five *D. pteronyssinus* allergic patients. The results are expressed as the dilution of extract necessary to produce an 8 × 8 mm wheal when 0.05 ml was injected intradermally. The extracts were also assessed by RAST inhibition using a crude *D. pteronyssinus* extract coupled by the discs (Bencard Ltd.) and a single serum (J.L.). Statistical analysis showed a highly signficant correlation between the two ($P < 0.001$), and the correlation was not improved by trying to fit lines of different slope to each patient.



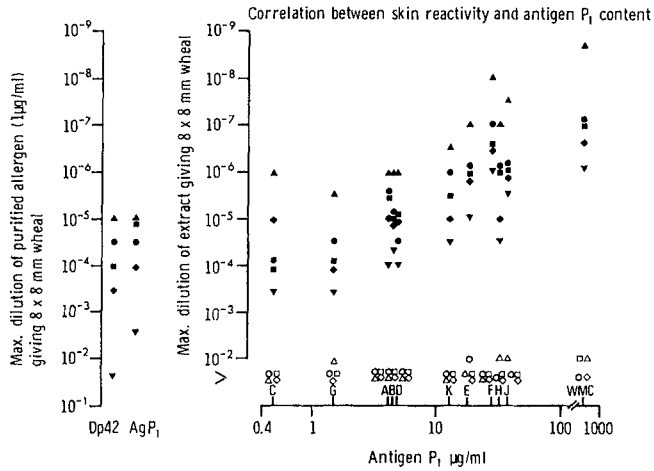


Figure 3. Correlation between antigen P_1 content and skin reactivity. Antigen P_1 content was measured by radioimmunoassay in the same ten extracts as described in Fig. 2. Skin-test results are the same as those in Fig. 2, but, in addition, results on four nonallergic patients are shown. Also shown are skin test results with purified allergens antigen P_1 and Dp42 in the same five allergic patients. For the patients, the P_1 content and skin tests showed a highly significant correlation ($P < 0.001$) (from ref 41).

skin testing. Clearly, the purification and assessment of a major allergen has always required skin testing. Similarly, it may be wise to carry out skin testing with candidate international reference preparations. On the other hand, there seems to be no reason to use skin testing either routinely or as part of the assessment of individual extracts by each company.

Histamine Release

Histamine release in vitro avoids any possible hazard to the patient, but otherwise has many of the same problems as skin testing. In particular, the requirement for a group of suitably allergic individuals who will consent to give blood regularly is a problem. The technical details are also difficult if the procedure is to give accurate quantitative results. During the collaborative studies on the first three IRPs, results from several laboratories using histamine release experiments generally did not lend themselves to statistical comparison. Therefore, the limited use of histamine release during these studies must reflect a general feeling that the procedure is too complicated for routine use in comparing different extracts.

Comparison of Potency from One Allergen to Another

Many traditional methods of standardizing allergens have been designed to give consistency to preparations of each allergen and also to serve as a direct guide to the relative strength of extracts of different allergens. Despite the inaccuracy of PNU, many allergists use PNU as an overall guide to the correct skin testing dose of extracts. Thus, intradermal skin testing is carried out with

10 PNU/ml or 100 PNU/ml, whereas desensitization is increased from 10 PNU to 10,000 PNU per injection. The fact the PNU values can act as a useful guide may also reflect the fact that the major pollen allergens often represent a significant proportion, ie, 5%–20%, of the extractable protein.[8,10,11] However, the success of PNU also reflects the fact that variation in allergen potency as great as tenfold has been tolerated in clinical practice.

In order to compare the allergen potencies between extracts of different pollens, it would be necessary to define patient groups and their sera as "equally" allergic to different allergens. Ideally, one could match patients as having equal symptom scores for a particular pollen count (eg, 150 grains/m^3) for two different pollens. This would be possible for comparing patients with hay fever caused by grass, ragweed, or birch pollen but it would be almost impossible for any other allergen. A poor man's guide would be to compare RAST binding by sera from different groups of patients. However, RAST binding makes assumptions about the quality of extracts bound to the discs or particles, and the argument rapidly becomes circular. Finally, it may be possible to compare different allergens according to their content of major allergen proteins; a ragweed extract containing 10 μg antigen E/ml would be declared equally "potent" to a rye grass extract containing 10 μg of the Group I protein or to a *D. pteronyssinus* extract containing 10 μg antigen P_1/ml, or to bee venom containing 10 μg phospholipase A/ml. This approach has great attractions, but can only be applied to allergens where measurement is relatively widely available. In addition, there is a danger that these measurements will be overinterpreted. Intradermal skin tests with *D. pteronyssinus* extract containing 10 μg antigen P_1 are often positive at a dilution of 1 part in 10 million; by contrast, venom skin tests are often carried out with 1:1,000 or 1:10,000 dilutions, and most venom allergic patients would be negative if tested with a 10^{-6} dilution of venom. Many of the companies do not wish to label their extracts with units that imply that a particular dose is correct for skin testing or treatment. At present, reaching agreement about the relative potencies of extracts of different allergens as part of establishing international standards would be extremely difficult.

Units

The choice of units has always posed problems in allergen standardization. We have already discussed the possiblity of using "clinically useful" units and have decided that they are neither practicable nor desirable for an international standard. At present, it is equally impracticable to use absolute units for all the allergens present in a given extract because this could only be approached for ragweed and bee venom. A ragweed reference preparation could possibly be defined as containing 10 μg antigen E, 1 μg Ra3, and 0.5 μg Ra5 per ampule. The policy of the IUIS Steering Committee has been to allot arbitrary units to each reference preparation. Defining arbitrary units is usually judged by practical considerations. Most workers prefer to work in whole numbers, where units are designed so that the smallest quantity commonly used will still contain a whole number of units. In practice, both PNU and Noon units are arranged so that the quantities used for skin testing or

starting desensitization are in whole numbers. The proposed international reference preparations have been alloted 100,000 units per ampule for timothy, ragweed and *D. pteronyssinus*. The unitage for *D. pteronyssinus* ignores the fact that this preparation contains less protein and probably less allergen, and the units are not designed to be directly comparable.

It is very important to realize that the assessment of a reference preparation can change after it has been established. The WHO standard serum used for IgM, IgG, and IgA (one serum) was first established using international units. Subsequently, it became possible to agree the absolute values, and the same serum is now considered to have an exact content of each isotype in micrograms. The standard serum for IgE was first established with international units. Subsequently, it was generally agreed that the IgE international unit equals 2.4 ng, but most authors have continued to use international units for IgE.[42] There is nothing to stop an investigator who is studying the series of reference preparations from publishing that his tests in vivo or in vitro indicate that their relative potency in relation to typical patients in a particular area was such that 1 U of *D. pteronyssinus* was equivalent to 2.5 U of ragweed and to 3.5 U of timothy. Similarly, the international reference preparations can be related to the Scandinavian standards, which have known strength in Hep units, or to United States standard extracts, which have known strength in allergen units. It is possible that in the future there will be general agreement about biologic units of potency that are comparable for each allergen. At that time, the same reference preparations could be used and their content would be redefined; it would not be necessary to establish new international reference preparations.

In conclusion, the ampules of freeze-dried extract have been alotted an arbitrary number of units. These units do not indicate the quantity necessary for treatment or for giving a positive skin test. Certainly, the units will in no sense indicate what dose of allergen is, or is not, "safe." The units will allow any investigator to compare his own extract (or substandard) to the international reference preparation and state, for example, "using RAST inhibition, the extract contained 6000 U of *D. pteronyssinus* allergen/ml." The reference preparation could also be used as a standard for assays of major allergen content. Thus, "the assay for antigen E (or antigen P_1) was standardized by use of a control extract substandardized from the IRP for ragweed (or *D. pteronyssinus*); the IRP has been shown to contain 60 μg antigen E (15 μg antigen P_1) per ampule." If subsequent investigations or improved techniques showed that the correct values for the standard were different, previous results could be reinterpreted. However, there would be no need to replace the standard. Once established, there are very good reasons for keeping a reference preparation for many years because experience with its use should progressively increase the value of having an international reference point.

Conclusions

It is perfectly possible to take a large volume of allergen extract, check that it gives positive skin tests in ten patients, lyophilize it, and then store it in ampules and declare that it is a standard preparation. However, before it can

be internationally accepted, it is necessary to convince an expert committee of the WHO that the potency of the preparation can be measured by several laboratories in several different countries and that the form in which it is stored is stable. There is ample evidence for a variety of allergens that, when they are stored lyophilized in glass ampules and frozen, they remain stable for many years, probably >10. The stability studies so far support this conclusion. Although, in theory, any method can be used to measure the potency, there is a strong prejudice in favor of in vitro techniques. Thus, there is a requirement for in vitro assays that actually measure allergen. Two types of techniques stand out in this respect—RAST inhibition and direct measurement of major allergen concentration.

Over the last 3 years, the allergen standardization subcommittee of IUIS has moved rapidly, working on allergen standards for 15 different allergens. In 1983–1984, three allergen preparations will be presented to the WHO and probably accepted. The candidate preparations have been assessed by a wide range of techniques, including skin testing, histamine release in vitro, Laurell electrophoresis, IEF, and CRIE. However, in each case, the primary data that have been analyzed statistically have come from RAST inhibition and assays for the quantity of a defined major allergen.

Each of the reference preparations has been allotted a number of arbitrary units. The units used do not have implications about the optimal dose for skin testing or desensitization. Equally, the units for the different allergens do not have a defined relationship to the others. That is, at present, the proposed international units for ragweed, timothy and *D. pteronyssinus* extracts are not related to each other.

In our opinion, the success of the present efforts to establish international standards has depended on the availability of suitable in vitro assays and the decision not to use biologic units. In addition, the present studies have benefited from past experience. Many of the most difficult problems in establishing standards and organizing collaborative trials have come from the actual mechanics of shipping extracts from one country to another. In previous attempts, extracts have spent days unfrozen at the wrong airport, radioactive reagents have proved unusable, and lyophilized extracts have been found to have so little activity that it was effectively impossible to measure their activity. Each of the three candidate IRPs that have been established has proved to be potent, easily measured by established techniques, and, over the first year, entirely stable.

There are many ways in which these standard extracts will be used. At a simple level they will act as the reference point for national or local standard preparations that can be described either in international units or in terms of major allergen content. Subsequent studies may lead to a different system of units that allows comparison between different extracts. For scientific studies on allergy, the availability of international standards will dramatically improve the quantitative aspects. Thus, all assays of allergen levels and all skin test reagents can be related to the international standard either in international units, as absolute measurements of major allergen, or as relative potency. In the past, establishing international standards, eg, for IgE and insulin, has had a very beneficial effect both in terms of understanding and acceptance

of measurements. There seems little doubt that the establishment of a series of international reference preparations for allergens will, in time, lead to improved national standardization and yield great benefits for both clinical allergy and the scientific acceptance of research in allergy.

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