

Studies on *Dermatophagoides pteronyssinus* allergens: Measurement of the relative potencies of *D. pteronyssinus* purified extracts by in vitro and in vivo methods

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*To standardize the *Dermatophagoides pteronyssinus* extracts used in clinical allergy practice, the relative potencies of several purified extracts were estimated by radioallergosorbent test (RAST) inhibition, histamine release, and intracutaneous titration testing, and the results were compared. The potencies of nine independently prepared *D. pteronyssinus* extracts were assayed with respect to an extract adopted as a reference. In vivo the cutaneous wheal surface after intradermal injections was measured on a population of *D. pteronyssinus*-sensitive subjects. In vitro RAST inhibition was performed with the reference extract as allergosorbent and with a pool of human IgE-rich sera from patients sensitized to *D. pteronyssinus* but untreated. For histamine release human basophils from patients allergic to *D. pteronyssinus* were used. In the three methods dose-response curves were plotted for the reference extract and the other extracts. A comparison of the measurement of in vitro and in vivo potencies is reported. Similar results were obtained with the three methods: all the extracts but one were as potent as the reference preparation. However, for routine purposes RAST inhibition appears to be the most convenient and reliable procedure.*

The preparation of standardized allergenic extracts in clinical and experimental allergy requires the drafting of well-defined procedures for all the steps in the production of these extracts. Hence for a given allergen the characteristics of the source material, the extraction procedure, and the final tests for identity, innocuity, toxicity, and activity must be established precisely. Allergen standardization with respect to biologic activity is a real approach to this problem.

Earlier studies were mainly concerned with measurement of the activities of different commercial extracts, particularly pollen extracts.

The methods utilized were skin tests in humans,¹⁻³

radioallergosorbent test (RAST)-based assay,⁴⁻⁶ and histamine release experiments.⁷ Good correlation was observed between the values obtained with the bioassay and those obtained with in vitro methods, either RAST inhibition⁸⁻¹⁰ or histamine release.¹¹

The aim of this study was to determine whether the different procedures available for measuring allergen potencies also give comparable results for a nonpollen allergen.

Because *Dermatophagoides pteronyssinus* is being increasingly recognized as a major allergen of house dust hypersensitivity,¹² we chose different batches of the mite extract for this purpose.

We investigated the activity of nine *D. pteronyssinus* extracts by three methods: intracutaneous titration testing, RAST inhibition, and histamine-release experiments.

Generally these three methods have been examined separately or compared in pairs. In this work we compare the results of potency measurement data based on the interaction of allergen components of *D. pteronyssinus* with IgE from the serum or fixed to mem-

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branes of blood basophils or of skin mastocytes. Moreover, rather than comparing potencies of extracts from various manufacturers, we compare the potencies of different batches of allergen extract produced in the same laboratory. The eight batches tested were obtained from eight individual mite cultures not simultaneously grown, extracted and purified by identical procedures, and characterized by isoelectric focusing. The ninth batch, made up of a mixture of the several batches, was taken as a reference, and the data are presented relative to this reference extract. The different procedures are discussed with regard to their reproducibility and adequacy in routine tests.

MATERIAL AND METHODS

D. pteronyssinus extracts

Cultures. The house dust mite, *D. pteronyssinus*, was grown and identified according to the method of Voorhorst et al.¹³ The culture medium was composed of 50% human skin scales and 50% dried yeast, *S. cerevisiae*. Culture was kept at 25° C with a humidity in equilibrium with saturated NH₄Cl. The culture recipients were shaken gently every 3 days. After a growth of 4 mo, the culture (containing about 7,000 living mites/gm of culture) was kept frozen until the day of extraction.

Experimental extracts. The eight extracts were obtained from eight individual cultures of *D. pteronyssinus*. Ten grams of whole culture (*D. pteronyssinus* plus medium) were ground with carborundum (3 gm) and were extracted in 230 ml borate buffer at pH 8.5. After centrifuging the supernatant liquid was neutralized at pH 6.5 and subjected to acetone precipitation at increasing concentrations. The fraction that precipitated between 50% and 80% acetone was isolated, washed, and dried. It constituted the experimental fraction and was stored at 4° C.

The culture medium alone was extracted by a protocol identical to that performed for the *D. pteronyssinus* extract.

Reference extract. The reference extract was a mixture of samples of the different batches of *D. pteronyssinus*. A potency of 1 was assigned to this extract.

Isoelectric focusing

Analytical isoelectric focusing. Analytical isoelectric focusing was performed using an LKB Multiphor apparatus and commercially available flatbed plates of polyacrylamide gel (PAG plates LKB of pH range 3.5 to 9.5) according to the manufacturer's instructions. The concentration of each allergen extract was 50 mg/ml phosphate-buffered saline (PBS) at pH 7.2. Samples of 10 μ l were applied to the gel surface. The PAG plate was focused at 22 w for 1.5 hr. The gel was then stained for protein identification (coomassie brilliant blue).

Preparative isoelectric focusing. Preparative isoelectric focusing was realized on an aliquot of 500 mg *D. pteronyssinus* reference extract. This aliquot was focused following the LKB procedure between pH 3.5 to 9.5. Immediately after the experiment a print was made, and the gel prepara-

tion was sectioned in 30 separate zones with a fractionating grid.

RAST inhibition

Reference serum. Sera rich in IgE specific for *D. pteronyssinus* were pooled from nine untreated *D. pteronyssinus*-sensitive patients as described earlier.¹⁴

Reference immunosorbent. The reference disks were prepared by coupling the reference extract of *D. pteronyssinus* to CNBr-activated paper disks.⁴ Unreacted sites on the disks were blocked with epichlorohydrine-polymerized amine (EPA).¹⁵

Test procedure. The assay developed by Yman⁶ was used with several modifications: 100- μ l aliquots containing 6 increasing quantities of the reference extract or of the other extracts to be tested were preincubated for 3 hr with 100 μ l of the reference serum diluted 1:10. In a control experiment the reference serum was incubated with 100 μ l PBS. Following this a reference paper disk was added to each mixture and incubated 3 hr at room temperature. After three washings in PBS, 50 μ l ¹²⁵I-labeled anti-IgE (Pharmacia Diagnostics, France) was added. After 18-hr incubation and three additional washings, the radioactivity bound to the paper disks was counted in a gamma counter. The counts observed for the uninhibited reaction constitute the 0% inhibition value.

All the experiments were conducted at least in duplicate. A dose-response curve was determined by plotting the percent RAST inhibition versus the varying quantities of each of the extracts investigated. The quantity of each extract required to obtain 50% inhibition was graphically determined and represented by C₅₀.

Histamine release

As described by May et al.,¹⁶ leucocytes were isolated from 30 ml venous blood from patients sensitive to *D. pteronyssinus*. Threefold dilutions were made for the different batches of *D. pteronyssinus* to be tested. Of each solution 0.1 ml was mixed with 0.9 ml cell suspension containing 1 to 2.10⁶ cells per milliliter. Triplicate incubations were performed, with constant agitation at 37° C for 40 min. The tubes were centrifuged at 300 $\times$ g for 8 min at 4° C, except for the incubations intended for the determination of total histamine content. Of the supernatant (or of the cell suspension) 0.85 ml was mixed with 0.1 ml 4 N perchloric acid, and after centrifuging the histamine content was quantified by the fluorometric method¹⁷ automatized by Siraganian.¹⁸

The histamine values refer to histamine base. Percentage histamine release was calculated by the formula 100 (e - b)/(c - b), where e is mean of fluorometric readings for experimental supernatant, b is mean of blanks, and c is mean of total histamine.

Intracutaneous titration testing

Patients were selected from the Allergy Service at the Hospices Civils of Strasbourg. All were skin tested prior to this study and were found to be skin reactive to *D.*

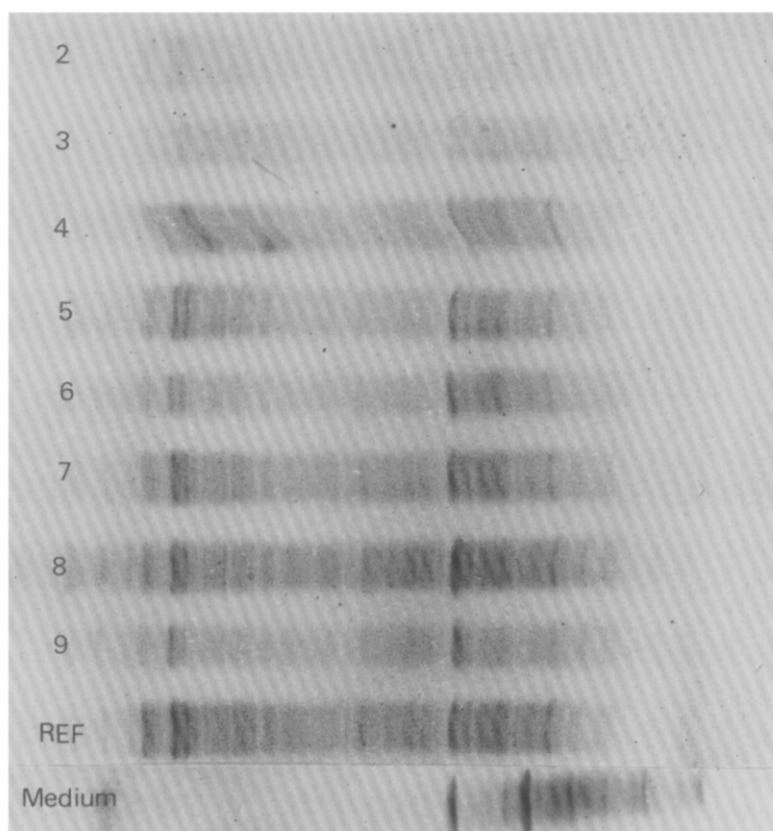


PLATE I. Characterization of nine batches of *D. pteronyssinus* extracts and of the culture medium extract by isoelectric focusing.

pteronyssinus reference extract at 10 $\mu\text{g/ml}$. For patients with a hyperreactivity to this dilution (diameter of the wheal ≥ 35 mm and pseudopods) a second skin test at 1 $\mu\text{g/ml}$ was performed. If no decrease of the reaction was observed the patient was excluded as not appropriate for a quantitative study of the extract potency.

All the patients selected had a high serum level of *D. pteronyssinus* specific IgE. Good correlation between skin test positivity and clinical history had been observed in all cases. Antihistaminics were withheld from all patients.

Three allergen batches were tested in skin reactions. For each of these batches a group of 48 patients was selected. The allergen concentrations used to determine the dose-response curve were 0.62 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$; 0.04 ml of each solution was injected intradermally in the external part of the arm of 16 patients (anatomically corresponding sites). After 20 min the wheal of the reaction was outlined with a ballpoint pen, and the markings were transferred by means of tape. The wheal figures were reproduced on homogeneous paper, cut out, and weighed.* The weight of each paper is proportional to the wheal area and reflects the intensity of the skin test reaction.

For each allergen concentration the mean value of the skin test reaction was calculated, and a log-log diagram

shows the intensity of skin test response versus allergen concentration.

From these cutaneous titration data the potency values of the three batches tested were calculated by the method recommended for quantitative bioassays by the European Pharmacopoeia.¹⁹

RESULTS

Isoelectric focusing

Plate I shows the isoelectric focusing pattern of the reference extract of the different batches of *D. pteronyssinus* and of an extract made from the culture medium alone.

The method enables individualization of about 30 components in each mite extract, focusing principally in the pH range 8 to 4. The culture medium extract presents a different pattern, showing only about 10 components grouped in the pH range 5.5 to 3.5.

Preliminary data* from the preparative isoelectric focusing of the *D. pteronyssinus* extract gave positive prick-test results in 10 *D. pteronyssinus*-sensitive patients with most of the fractions eluted from the gel.

*Brighton WD: Personal communication.

*Benezra C: Personal communication.

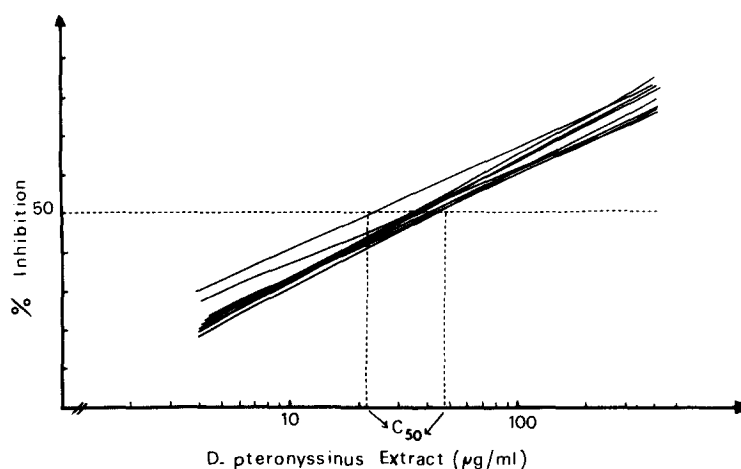


FIG. 1. RAST inhibition titration curves for eight individual experiments with reference extract.

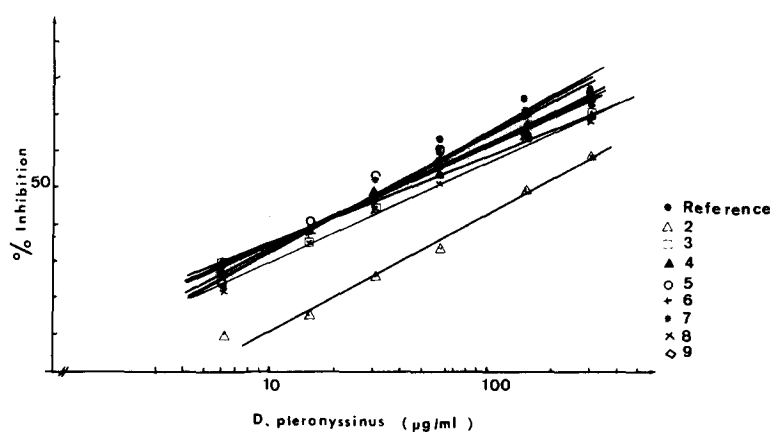


FIG. 2. RAST inhibition titration curves for nine batches.

These fractions were used at 100 $\mu\text{g/ml}$. Moreover these samples gave negative skin test results in six control patients not sensitive to *D. pteronyssinus*.

RAST inhibition

The precision of the RAST inhibition method was evaluated in eight identical experiments with the reference extract. The dose-response curves are shown in Fig. 1. Plotted on a log-log scale the inhibition curves are linear from 30% to 70% inhibition. The slight differences in the shape of the slopes are found to be not significant by analysis of covariance ($F_{32}^7 = 0.81$). The coefficient of variation for the C_{50} value is 18%, a result comparable to that of Yman.⁶

Fig. 2 and Table I give the results of the C_{50} values for the reference extract and for each of the eight tested batches. Each result is the mean value of at least two independent inhibition experiments.

The ratios of the C_{50} value of the reference extract to the C_{50} of the other extracts are also shown in Table

I. Statistical comparison by variance analysis reveals a difference between the nine extracts ($F_{15}^8 = 63$, $p < 0.001$). The difference between the potency C_{50} values, expressed in $\mu\text{g/ml}$, of the reference extract and one particular batch is significant ($p < 0.05$) when it is at least 15.4. Only batch 2 is found to differ significantly and has the lowest biologic activity. Earlier work¹⁴ showed that the culture medium extract did not have any inhibiting activity in this kind of experiment.

Cutaneous titration

The precision of our technique was estimated by verifying the homogeneity of the results of 39 initial wheal areas. The coefficient of variation (SD%) was found to be $\pm 11.5\%$.

Fig. 3 presents the cutaneous titration curves on a log-log scale for the reference extract and for batches 2 and 7. A linear relationship between concentration of *D. pteronyssinus* injected and average "wheal

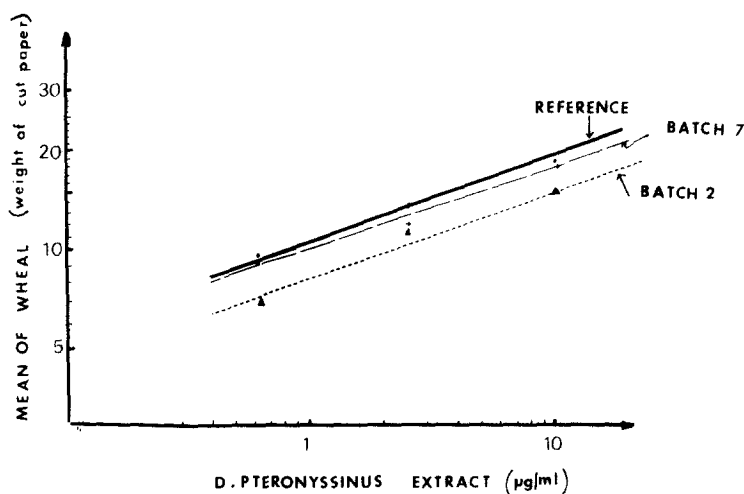


FIG. 3. Cutaneous titration curves.

TABLE I. Potency measurements of purified batches of *D. pteronyssinus* extracts by RAST inhibition

Batch	Mean C ₅₀ (µg/ml)	C ₅₀ reference
		C ₅₀ batch
Reference	41	1
2	153	0.26
3	44.1	0.92
4	45	0.91
5	34.5	1.18
6	33	1.24
7	33	1.24
8	52.5	0.78
9	37.5	1.09

weight" is observed. For the reference extract a linear regression line is obtained ($r = 0.74$, $p < 0.001$), and the regression is also significant for batches 2 and 7 ($p < 0.001$).

The lack of parallelism and the deviation from linearity for the three dose-response curves are not significant ($p > 0.05$); hence the three extracts are qualitatively equivalent.²⁰

No potency difference is observed between extract 7 and the reference extract (NS, $p > 0.05$). On the contrary the potency values of extract 2 and the reference are different ($p < 0.01$). The potency results for extracts 2 and 7 are given in Table II, as compared with the reference extract with an assumed potency of 1.

The skin reactivity of the culture medium extract was examined in 15 patients sensitive to *D. pteronyssinus* (intradermal injection). With this extract used at 10 µg/ml all the patients had negative skin test re-

TABLE II. Measure of *D. pteronyssinus* potency by cutaneous titration testing (ratio to reference extract)

Batch	Relative potency (95% fiducial limits)	Statistical test (F test)
2	0.45 (0.29-0.64)	F = 15 $p < 0.01$
7	0.75 (0.62-0.84)	F = 1.4 $p > 0.05$ (NS)

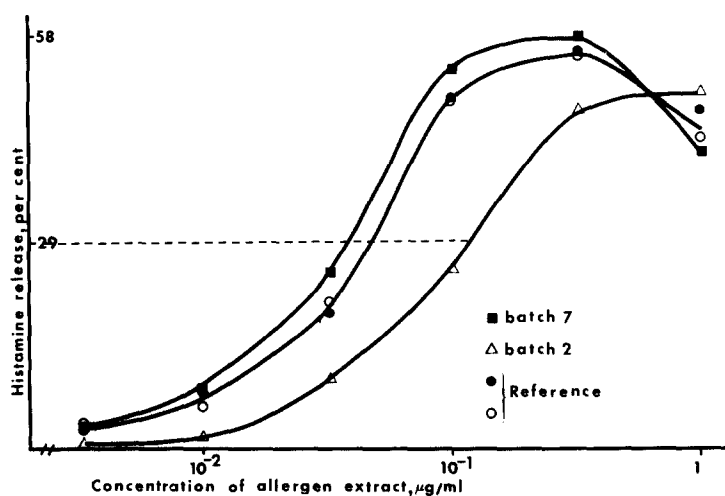
sults. Furthermore, earlier work²¹ showed that inhalation provocation tests on 64 patients with cutaneous and bronchial sensitivity to *D. pteronyssinus* remained negative with the culture medium extract. Thus the culture medium extract alone has no in vivo allergenic activity.

Potency measurements by histamine release

A typical experiment showing the histamine release curves with different batches of *D. pteronyssinus* is presented in Fig. 4. The reference extract curve was repeated twice by making two independent dilution series, and a good reproducibility was observed.

Within the same experiment (basophils from the same donor) the allergen batches tested induce the same maximum histamine release value; this flat portion of the curve cannot serve for the determination of the potency differences between batches. Nevertheless the steeper ascending part of the profiles, which clearly separate the activities of the batches, affords the possibility of measuring accurately the potencies of the different extracts tested.

In the experiment presented in Fig. 4 a concentration of 0.05 µg/ml of the reference extract induced the release of half (D_{50}) of the maximum histamine release value observed. The relative potencies for

FIG. 4. Histamine-release titration curves for *D. pteronyssinus* extracts.TABLE III. Potency values of *D. pteronyssinus* extracts by histamine release titration

Batch	D ₅₀ reference extract	Number of experiments
	D ₅₀ batch (mean)	
Reference	1	
2	0.55	4
7	1.16	2
8	0.78	2
9	1.00	2

TABLE IV. Relative potency of *D. pteronyssinus* extracts by three methods

Method Batch	Intracutaneous titration	Histamine release	RAST inhibition
Reference	1	1	1
7	0.75	1.16	1.24
8	—	0.78	0.78
9	—	1	1.09
2	0.45	0.55	0.26

all batches examined by histamine-release titration curves are reported in Table III. These values represent the ratio between D₅₀ of the reference extract included in each experiment and D₅₀ of each batch. For each assay Student's test was calculated to determine whether the differences observed between the means of the data of one batch and the reference are significant. Only extract 2 is significantly different from the reference extract ($p < 0.05$).

The histamine-release results observed with the culture medium extract are below the level of significance.

DISCUSSION

In this study intracutaneous titration testing, the RAST inhibition method, and the histamine-release procedure from human basophils were utilized to investigate the potencies of different batches of *D. pteronyssinus* extracts. No activity was observed with the culture medium extract within each of the three techniques. We therefore conclude that most of the components of mite extract are specific of *D. pteronyssinus*. Table IV summarizes the data obtained with different batches of the mite extract.

On one hand the comparison of batches 2 and 7 with the reference extract shows that the three methods yield similar results. Statistical analysis of these data show that batch 2 differs significantly from the reference extract, whereas batch 7 is not significantly different from the reference extract.

There are several possible explanations for the discrepancy in the biologic activity of batch 2. The homogeneous isoelectric focusing patterns (Plate I) exclude significant qualitative differences between batch 2 and the other batches. But the mite population may have been slightly different and hence the quantity of source material not absolutely identical. Moreover, carborundum extraction and acetone precipitations are procedures that cannot be performed in a fully repetitive way at each preparation.

On the other hand the comparison of the potency values obtained for batches 2, 7, 8, and 9 relative to the reference extract by the RAST inhibition and by histamine-release techniques shows that the same rank order is observed within these two methods. Thus both "in vitro" titration procedures give equivalent results.

These conclusions confirm the prior studies, al-

though the techniques are sometimes different. Gleich et al.⁸ and Yunginger et al.¹⁰ compared intradermal skin test end-point titration and RAST-based technique; Pepys et al.⁹ used skin-prick testing and RAST-based allergen assay, and Baer et al.¹¹ compared direct skin test and in vitro histamine release on human leucocytes.

The histamine-release test, if performed on the cells of one donor, has the disadvantage of selecting only the allergen components to which the basophils possess specific IgE on their membranes. But the convenience of this system is the screening for biologic activity at the cell level, a situation comparable to the skin test. However, this method can be used only as a research tool to supplement and possibly confirm routine testing, because of the scarcity of sensitive blood rich enough in basophils to permit this kind of experiment.

The RAST-inhibition method, as a radioimmunoassay, is specific, reproducible, and convenient. It is relevant as a guide for routine quantification of the activity of allergenic extracts. The value of the RAST-inhibition method in the standardization of allergens is to provide reference systems that can easily be stored and checked. The reference serum must be a pool derived from a large number of patients sensitive to the allergen under study and thus exhibiting IgE specificity for all components of the extract. The RAST-inhibition test prevails over the direct RAST test because of the uniformity of the experiments based on identical reference disks. The latter should contain all the natural allergenic components giving rise to the formation of IgE in humans. However, the RAST-inhibition method does depend on the availability of a specific anti-IgE standard.

For skin testing we report quantitative bioassays. The skin test titration procedure in our study required a large body of data obtained in a broad population study to eliminate individual reactivity. For each allergen batch 48 intradermal tests (on anatomically corresponding sites) were performed on 48 different individuals sensitive to *D. pteronyssinus*. Hence a very significant mean of intensity of skin test reactivity is obtained. We believe that this technique should express totally representative biologic activity. Skin testing offers the advantage of being close to clinical reality and should always be included in the study of the diagnostic value of the extracts. The cutaneous titration method is very time consuming for the physician and may create discomfort for the patients. Thus this in vivo method cannot be taken into consideration as a routine test for monitoring the activity of different allergen batches. For this reason we only examined three batches by this technique.

Once their equivalence with the bioassay is established, in vitro methods should replace skin testing for the determination of the potency of the extracts on a large and repetitive scale. We believe that RAST inhibition, as used in the present work, is a most satisfactory technique to evaluate the potency of allergen extracts.

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REFERENCES

1. Becker EL: Quantitative studies in skin testing. I. The assay of ragweed extracts by means of scratch test utilizing an "all or none" response. *J ALLERGY* **19**:108, 1948.
2. Swain HH, Becker EL: Quantitative studies in skin testing. V. The whealing reactions of histamine and ragweed pollen extract. *J ALLERGY* **23**:441, 1952.
3. Aas K, Belin L: Standardization of diagnostic work in allergy. *Acta Allergol* **27**:439, 1972.
4. Ceska M, Eriksson R, Varga JM: Radioimmunosorbent assay of allergens. *J ALLERGY CLIN IMMUNOL* **49**:1, 1972.
5. Foucard T, Johansson SGO, Bennich H, Berg T: In vitro estimation of allergens by a radioimmune antiglobulin using human IgE antibodies. *Int Arch Allergy* **43**:360, 1972.
6. Yman L, Ponterius G, Brand TR: RAST based allergen assay methods, in Karger S, editor: *Developments in biological standardization* 29, Basel, 1975, International Association of Biological Standardization Co., p. 151.
7. Lichtenstein LM, King TP, Osler AG: In vitro assay of allergenic properties of ragweed pollen antigens. *J ALLERGY* **38**:174, 1966.
8. Gleich GJ, Larson JB, Jones RT, Baer H: Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test. *J ALLERGY CLIN IMMUNOL* **53**:158, 1974.
9. Pepys J, Tangen O, Perkins FT, Tate H, Brighton WD: Examination of cocksfoot pollen (*Dactylis glomerata*) extracts by skin prick test in man, in Karger S, editor: *Developments in biological standardization* 29, Basel, 1975, International Association of Biological Standardization Co., p. 284.
10. Yunginger JM, Jones RT, Gleich GJ: Studies on alternaria allergen. II. Measurement of the relative potency of commercial alternaria extracts by the direct RAST and by RAST inhibition. *J ALLERGY CLIN IMMUNOL* **58**:405, 1976.
11. Baer H, Godfrey H, Maloney CJ, Norman PS, Lichtenstein LM: The potency and antigen E content of commercially prepared ragweed extracts. *J ALLERGY* **45**:347, 1970.
12. Pauli G, Bessot JC, Guisard G, Roegel E, Oudet P: Importance clinique des acariens chez les asthmatiques allergiques à la poussière domestique. *Rev Fr Allerg* **12**:141, 1972.
13. Voorhorst R, Spieksma FTh, Varekamp H: House dust atopy and the house dust mite, Leiden, 1969, Stafleu's Scientific Publishing Co., pp. 120-138.
14. Le Mao J: Evaluation de l'activité allergénique de préparations de *Dermatophagoides pteronyssinus* par dosage radio immunologique des antigènes extraits. *Ann Immunol (Inst Pasteur Paris)* **129C**:63, 1978.
15. Aalberse RC: Immunoglobulin E, allergens and their interaction. University of Amsterdam, 1974, Ph.D. thesis.
16. May CD, Lyman M, Alberto R, Cheng J: Procedures for immunochemical study of histamine release from leucocytes with small volumes of blood. *J ALLERGY* **46**:12, 1970.

17. Shore PA: Analysis of biogenic amines and their related enzymes, in Glick, editor: *Methods of biochemical analysis*. New York, 1971, Interscience, p. 89.
18. Siraganian RP: An automated continuous flow system for the extraction and fluorimetric analysis of histamine. *Anal Biochem* 57:383, 1974.
19. *Pharmacopée Européenne*, vol. II. Published under the direction of the Council of Europe, Ste Ruffine, France, 1971, Imp. Maisonneuve S.A.
20. Philippe J: *Les Méthodes statistiques en pharmacie et en chimie: Applications à la recherche, à la production et au contrôle*. Paris, 1967, Masson, pp. 263-265.
21. Pauli G, Bessot JC, Arujo-Fontaine A, Oudet P: L'asthme aux acariens. *Rev Fr Mal Resp* 5-6:711, 1973.

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