

# A collaborative study on the first international standard of *Dermatophagoides pteronyssinus* (house dust mite) extract

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*A collaborative study was carried out to assess the suitability of a preparation to serve as the International Standard for Dermatophagoides pteronyssinus (house dust mite) extract. The proposed international standard of D. pteronyssinus, two additional freeze-dried extracts, and a commercially available skin testing solution were tested in the study. Nineteen laboratories in 11 different countries participated. The assay methods used included RAST inhibition, crossed immunoelectrophoresis/crossed radioimmunoelectrophoresis, isoelectric focusing, quantitative skin testing, and various other methods for assessing total allergenic activity. In addition, six laboratories measured the quantity of antigen P<sub>1</sub>, and three laboratories measured antigen DpX in each of the preparations. On the basis of the results from this study, the World Health Organization established the preparation as the International Standard for D. pteronyssinus extract with an assigned unitage of 100,000 IU per ampule. The units refer both to the total allergenic activity of the ampule and to that of the individual allergens, such as P<sub>1</sub> and DpX. (J ALLERGY CLIN IMMUNOL 75:676-86, 1985.)*

The need for standardization of allergen extracts used in diagnosis and treatment of allergic disease has

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## Abbreviations used

|        |                                                         |
|--------|---------------------------------------------------------|
| CIE:   | Crossed immunoelectrophoresis                           |
| CRIE:  | Crossed radioimmunoelectrophoresis                      |
| GCV:   | Geometric coefficient of variation                      |
| GM:    | Geometric mean                                          |
| HCCT:  | Human complement consumption test                       |
| HR:    | Histamine release                                       |
| IEF:   | Isoelectric focusing                                    |
| IS:    | International standard                                  |
| IUIS:  | International Union of Immunological Societies          |
| NIBSC: | National Institute for Biological Standards and Control |
| QST:   | Quantitative skin test                                  |
| RIA:   | Radioimmunoassay                                        |
| RIE:   | Rocket immunoelectrophoresis                            |
| RMDT:  | Rat mastocyte degranulation test                        |
| SRID:  | Single radial immunodiffusion                           |
| WHO:   | World Health Organization                               |

long been realized by practicing allergologists, manufacturers, research scientists, and national control authorities. The Allergen Standardization Subcommittee of the IUIS met in Geneva, Switzerland, in February 1981 to plan a program of allergen stan-

dardization and decided to produce IS for 15 different allergens.\* The allergens were selected on the basis of frequency of use and their relative importance as judged by responses to a questionnaire to manufacturers and control authorities. Work was begun immediately on producing an IS for each of the three allergen extracts heading the priority list. These were *Phleum pratense* (timothy grass) pollen, *Ambrosia elatior* (short ragweed) pollen, and *Dermatophagoides pteronyssinus* (house dust mite).

The working group for dust mite extract (A. F., H. L., and T. P. M.) prepared a specification for the *D. pteronyssinus* IS that was circulated to manufacturers interested in producing this extract. The NIBSC in London, England, acted as the coordinating laboratory. Eight extracts were submitted and, together with an extract already available freeze-dried at NIBSC, were coded and distributed to the laboratories of the working group. These studies were conducted in order to identify a suitable extract for the IS. Techniques for assessing the potency of *D. pteronyssinus* mite extracts were also investigated. This work was described in a preliminary report to the subcommittee and is published elsewhere.<sup>1</sup> One of the eight extracts was chosen as the candidate IS because (1) it appeared to be suitable in terms of potency and allergen content, (2) it contained no allergenic contaminants from the culture medium, (3) complete details of its manufacture were available, and (4) sufficient quantity was available in the same form as had been used for the preliminary study. Subsequently, the proposed IS was tested according to WHO guidelines<sup>2</sup> in an international collaborative study in parallel with three other coded extracts. The report† of this study was submitted to the WHO Expert Committee on Biological Standards in July 1984, and this article describes that study in an abridged form.

## MATERIAL AND METHODS

### Coded extracts

Participants received five extracts of *D. pteronyssinus* coded A to E. These included coded duplicates of the proposed IS.

*The proposed IS.* This extract was coded A and C. The *D. pteronyssinus* mites had been cultivated on human dander and yeast according to the method of Voorhorst and

Spieksma,<sup>3</sup> checked for identity by Dr. Spieksma,<sup>1</sup> and the cultures were extracted in an aqueous buffer. After centrifugation, the supernatant was filtered for sterility, dialyzed against 0.005 mol/L of ammonium bicarbonate, again filtered, and supplied to NIBSC freeze-dried in large vials. It was reconstituted to 1 mg/ml with distilled water, homogenized by stirring and filtration (Unipore polycarbonate membranes, Bio-Rad Laboratories, Richmond, Calif.) down to a pore size of 0.20  $\mu$ m, distributed into glass ampules in 1 ml volumes, freeze-dried, and sealed under nitrogen.<sup>4</sup>

Initially, a "trial fill" of 500 ampules (NIBSC code 82/568) was prepared in order to establish conditions for freeze-drying and to investigate the stability of the preparation. Subsequently, the "main fill" of 4000 ampules (NIBSC code 82/518) was prepared. The variation in weight from ampule to ampule was found to be not more than 0.29%.

The protein content of the proposed IS measured by the Lowry et al.<sup>5</sup> method was approximately 300  $\mu$ g per ampule. Moisture content by a modified method of Büchler et al.<sup>6</sup> was 0.25% and by a modified method of Baker,<sup>7</sup> and commercial quality assurance methods were found to be satisfactory. The residual oxygen per ampule was below the limits of detection (2%) as examined by Laser Raman spectroscopy. The extract was tested for sterility and was found to be sterile. The extract was also tested for the presence of hepatitis B antigen by RIA and was found to be negative.

*Extract B.* This was the existing freeze-dried extract of *D. pteronyssinus* held at NIBSC (code 77/622). It had been prepared by extraction of clean mites separated from a culture grown on horse dander. A 0.1% w/v extract was made in phosphate buffered saline and had been freeze-dried from 0.8 ml volumes at NIBSC.

*Extract D.* This was an extract made from a *D. pteronyssinus* whole mite culture commissioned for the collaborative study. A similar extract made by the same manufacturer from a different batch of mites was examined in the preliminary study and was found to fulfill the criteria for the IS. The details of manufacture of this extract were not disclosed, but it was known to have been dialyzed against 1 mmol/L of ammonium bicarbonate and freeze-dried. The material was reconstituted in distilled water to 1 mg/ml and freeze-dried in 500 ampules at NIBSC.

*Extract E.* This was a commercially available skin prick testing preparation supplied by a third manufacturer. The extract was made from a culture of *D. pteronyssinus* mites grown on nonectodermal mammalian protein of neither horse nor human origin. The raw material used for making this extract contained mite feces, whole mite bodies, exoskeletons, eggs, and nymphs. It was extracted with 50% aqueous glycerol containing 6% w/v of sodium chloride and 0.5% w/v of phenol, dialyzed, and presented as a liquid in ampules (not freeze-dried).

### Human serum pool P

Serum from 10 patients with clinical sensitivity to *D. pteronyssinus* house dust mite was collected at the Clinical

\*Geneva meeting documents. Geneva, February 1981. Available in Europe from H. Løwenstein, The Protein Laboratory, Sigurdsgade 34, DK-2200 Copenhagen N, Denmark; outside Europe from R. Goldstein, NIAID, National Institutes of Health, Westwood 755, Bethesda, MD 20205 U. S. A.

†Unpublished working document WHO/BS/83. 1417. Available from WHO, 1211 Geneva 27, Switzerland.

**TABLE I.** Summary of methods used in collaborative study

| Method                   | No. of laboratories | No. of assays | Notes on techniques (references)                                 |
|--------------------------|---------------------|---------------|------------------------------------------------------------------|
| RAST inhibition          | 13                  | 44            | Paper disc <sup>*8</sup>                                         |
| CIE/CRIE                 | 7                   | 16            | Rabbit antisera and human allergic pool <sup>†10,11,14-16</sup>  |
| Specific allergen assays | 6                   | 16            | RIA, RIE, SRID, quantitative CIE <sup>10,20</sup>                |
| IEF                      | 5                   | 11            | — <sup>‡21,22</sup>                                              |
| QST                      | 3                   | 5             | Intradermal and prick techniques <sup>§23,24</sup>               |
| HR                       | 3                   | 3             | — <sup>25,26</sup>                                               |
| ELISA inhibition         | 2                   | 4             | Inhibition of microplate ELISA of Phadezym RAST <sup>27,28</sup> |
| Direct RAST              | 2                   | 3             | Paper discs <sup>9</sup>                                         |
| RMDT                     | 1                   | 4             | — <sup>29</sup>                                                  |
| HCCT                     | 1                   | 5             | — <sup>30,31</sup>                                               |

\*Laboratory 2 used two separate serum pools; the provided serum pool P and an in-house pool. Laboratory 7 used polystyrene beads coupled to antibodies against trinitrophenyl (TNP) and TNP-conjugated mite extract to create the solid phase. Nine laboratories used <sup>125</sup>I-labeled anti-IgE from Pharmacia, whereas four laboratories used their own preparations of <sup>125</sup>I-labeled anti-IgE

†Six laboratories used the ammonium sulphate precipitated fraction of rabbit hyperimmune sera to mite extract from ALK at concentrations between 2 µl and 25 µl per square centimeter. Laboratory 10 used two antisera, one against whole culture and one against pure *D. pteronyssinus* mite bodies. One laboratory used the human pool serum pool P for CIE, whereas three laboratories used their own pools.

‡Four laboratories used PAG plates (pH 3.5 to 9.5) (LKB, Bromma, Sweden), whereas two prepared their own plates. Two further laboratories attempted IEF but found that the contents of the ampules were not sufficient for successful banding.

§Intradermal skin testing to end point was carried out on three patients by laboratory 1, and quantitative prick testing on 32 patients by laboratory 9 and on nine patients by laboratory 14, respectively.

Research Center, Harrow, Middlesex, England. The sera were individually tested by RAST, and all were found to be strongly positive. In addition, each serum was tested for the absence of hepatitis B antigen by RIA. The details of the clinical criteria used in selecting the patients and the results of the assays are presented in the article describing the preliminary studies.<sup>1</sup> The sera were pooled, filtered, ampuled in 0.9 ml volumes, freeze-dried, and sealed into glass ampules under nitrogen at NIBSC (code 82/528). This serum pool was prepared as a reference reagent for use in the collaborative study and thereafter.

## Participants

Nineteen laboratories in 11 countries participated in the study and included manufacturers, national control laboratories, and research laboratories (Appendix). Throughout this article the laboratories are identified by a code number that does not correspond to the order in which they are listed in the Appendix.

## Design of study

Participants were requested to assay the coded extracts by RAST inhibition, HR, CIE/CRIE, IEF, and/or any other methods that they routinely used. The organizers stated that it would be preferable if participants could use local serum pools and their own versions of each method, but ampules of serum pool P and IUIS method sheets were available on request. Participants were requested to carry out at least three assays of RAST inhibition, two each of other techniques, and to include all five extracts in each assay. Finally, they were requested to return the raw data together with their own interpretations of the results.

## Assay methods

The methods used in the study are summarised in Table I in the order of the number of laboratories with each method used together with relevant references.

The techniques used for RAST inhibition and direct RAST were versions of published methods.<sup>8,9</sup> Similarly, the methods of CIE/CRIE used for composition and that of quantitative immunoelectrophoresis<sup>10,11</sup> were generally well recognized.

The quantities of specific allergens, antigen P<sub>1</sub> and antigen DpX, were measured by a variety of methods including RIA, RIE, quantitative immunoelectrophoresis, SRID, and by techniques with the use of monoclonal antibodies.<sup>10-20</sup> One laboratory used purified P<sub>1</sub> on RAST discs to create a P<sub>1</sub>-specific RAST-inhibition assay.<sup>18</sup>

Additionally, IEF<sup>21,22</sup> was used for examination of the composition of the extracts.

Biologic activity was assessed by QST<sup>\*23,24</sup> and HR.<sup>25,26</sup> Other, less well recognized methods such as ELISA inhibition,<sup>27,28</sup> RMDT,<sup>29</sup> and HCCT<sup>30,31</sup> were also used to verify activity.

## Stability studies

The effects of freeze-drying and storage at temperatures up to 45° C on the proposed IS (82/518) and the trial fill

\*Mehta V, Wainscott G, Tomenson B, Hordle D: The Graeco-Latin square skin prick testing methods. Unpublished IUIS document, available in Europe from H. Løwenstein, The Protein Laboratory, Sigurdsgade 34, DK-2200 Copenhagen N, Denmark; outside Europe from R. Goldstein, NIAID, National Institutes of Health, Westwood 755, Bethesda, MD 20205 U. S. A.

**TABLE II.** Geometric means of potency ratios in terms of extract A by RAST inhibition for individual laboratories

| Laboratory   | No. of assays | Potency ratio |            |           |           |
|--------------|---------------|---------------|------------|-----------|-----------|
|              |               | C = A         | B          | D         | E*        |
| 1            | 3             | 0.88 (11)     | 0.82 (24)  | 5.5 (18)  | 28 (290)  |
| 2p           | 3             | 1.61 (35)     | 5.50 (12)  | 43.0 (21) | 27 (95)   |
| 2h           | 3             | 1.09 (21)     | 0.40 (25)  | 4.2 (28)  | 11 (35)   |
| 3            | 3             | 0.94 (37)     | 0.33 (18)  | 9.4 (22)  | 15 (15)   |
| 4            | 3             | 1.19 (27)     | 0.76 (43)  | 6.4 (33)  | 6.8 (50)  |
| 5            | 3             | 0.82 (76)     | 0.36 (51)  | 4.9 (120) | 14 (230)  |
| 7            | 3             | 1.11 (10)     | 0.062 (22) | 2.4 (20)  | 4.3 (6)   |
| 9            | 3             | 1.87 (13)     | 0.23 (55)  | 7.3 (85)  | —         |
| 11           | 3             | 1.13 (30)     | 2.80 (34)  | 31.0 (22) | 15 (36)   |
| 13           | 5             | 0.64 (87)     | 0.38 (120) | 2.0 (94)  | 1.7 (130) |
| 15           | 3             | 1.30 (58)     | 0.97 (58)  | 9.9 (37)  | 12 (17)   |
| 16           | 3             | 0.68 (38)     | 1.01 (31)  | 13.0 (54) | 19 (69)   |
| 17           | 3             | 1.06 (11)     | 0.43 (58)  | 9.6 (4)   | 11 (51)   |
| 18w          | 2             | 1.03 (9)      | 0.89 (3)   | 6.5 (15)  | 21 (0)    |
| 18b          | 1             | 1.02 (—)      | 4.50 (—)   | 18.0 (—)  | 17 (—)    |
| Overall GM†  |               | 1.05          | 0.70       | 8.7       | 12.0      |
| Overall GCV† |               | 33%           | 220%       | 120%      | 110%      |

The figures in the parentheses are the geometric coefficients of variation of the potency estimates in percent.

p = assays performed by use of serum pool P; h = assays performed by use of in-house serum pool; w = assays performed by use of whole mite culture extract on discs; b = assays performed by use of clean mite body extract on discs.

\*Potency of 1 ml of extract E relative to the ampule contents of extract A.

†The overall GM and GCV are calculated as the GM and GCV of the mean estimates of the laboratories.

(82/568) were studied at NIBSC by use of RAST inhibition.

### Statistical analysis

Assay data from RAST inhibition and ELISA inhibition were analyzed as parallel line assays,<sup>32</sup> relating, respectively, logit of the percentage inhibition and log absorbance to log dose. The significance of the deviations of these relationships from linearity and from parallelism was assessed by analysis of variance. Any assay demonstrating significant deviation ( $p < 0.01$ ) of the result was noted, but all assays were included in the subsequent analyses. All the raw data were also plotted to aid in the evaluation of the above analyses. For each assay potencies of the extracts were expressed relative to extract A (the proposed IS).

For the other quantitative assay methods, the potency estimates used in the analyses were those supplied by the participants or calculated from the activities listed for the extracts.

Potency estimates were combined by taking the geometric mean, and the reproducibility of the potency estimates was calculated as the GCV.<sup>33</sup>

### RESULTS

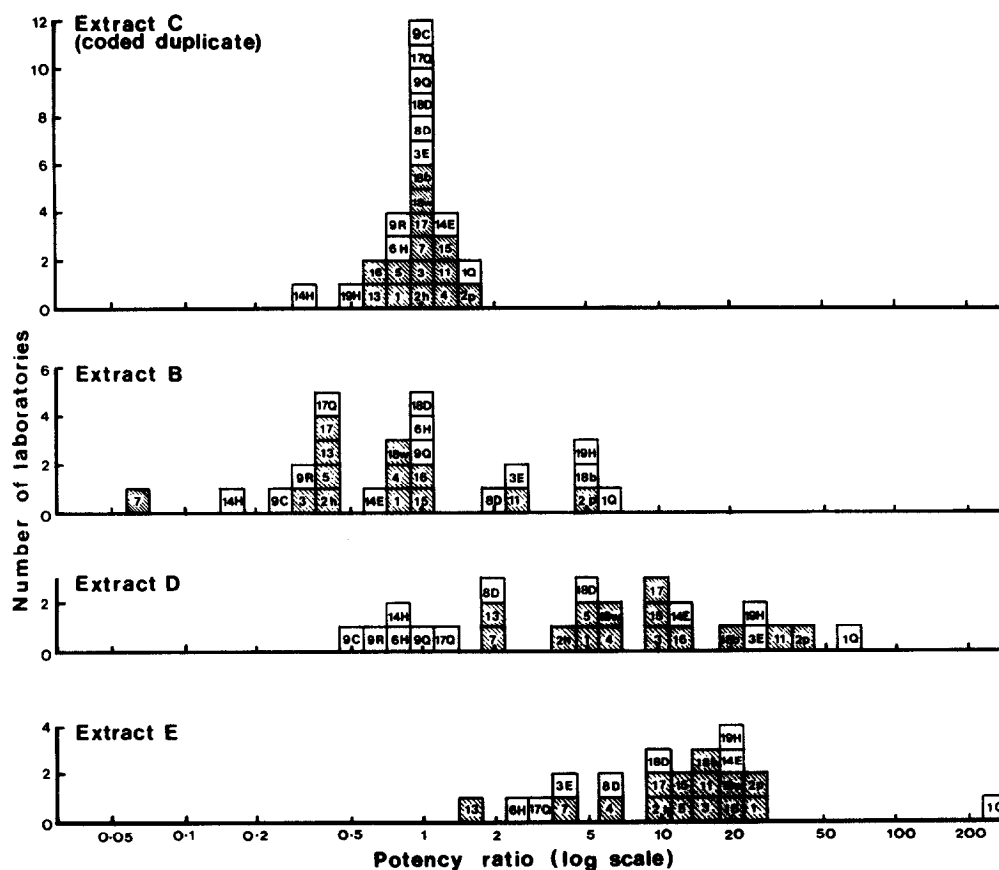
Eighteen of the 19 participating laboratories submitted results in the collaborative study. One laboratory did not submit results because of technical prob-

lems. All methods used except CRIE and IEF provided quantitative data.

### RAST inhibition

RAST inhibition was the predominant method used for estimates of total allergenic potency with 13 laboratories returning results on 44 assays. Data from five laboratories demonstrated satisfactory parallel line assays, but in the other laboratories significant deviations from parallelism of the log dose-response relationships were found in some assays. Most of the deviations from parallelism were due to extract B that demonstrated lines with slopes that consistently differed from those of the other preparations. When data for extract B were excluded, satisfactory parallel line assays were obtained in 11 of the 13 laboratories.

For each preparation the geometric means of the potency estimates obtained by individual laboratories were calculated (Table II). The reproducibility of the potency estimates within laboratories expressed as the GCV of the estimates is also illustrated in Table II. The frequency distributions of the mean potency estimates of the laboratories are illustrated in Fig. 1. The overall mean potency estimate for extract C rel-



Characteristic CIE patterns were obtained by all laboratories. For the proposed IS (extracts A and C), up to 15 precipitates were observed, and four of these could be demonstrated to bind IgE by use of CRIE (Fig. 3). Two further precipitates not clearly observed by use of Coomassie brilliant blue stained CIEs were visible on CRIE x-ray films. Antigen P<sub>1</sub> (= Ag 42 = Dpt12) could be observed in all the ex-

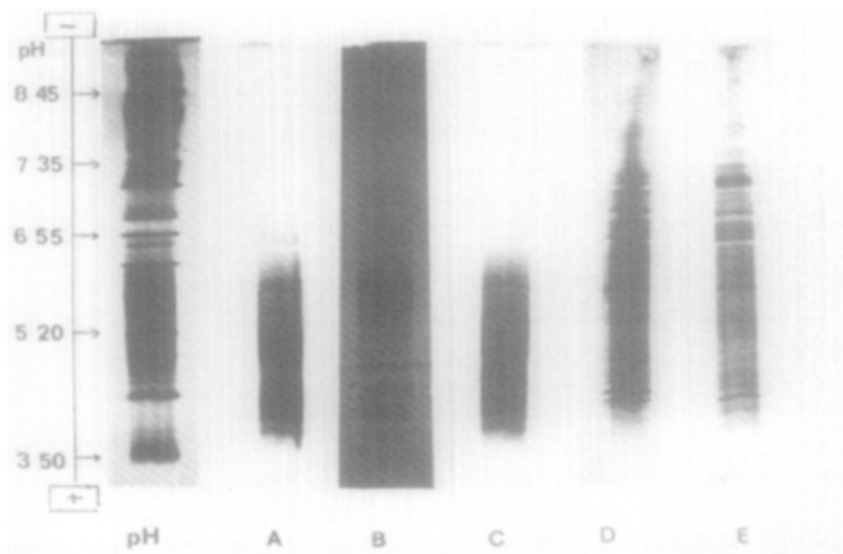


**FIG. 2.** Characteristic *D. pteronyssinus* IEF extracts A to E by laboratory 5 with pH gradient. Samples A, B, C, and D were dissolved, respectively, in 300  $\mu$ l, 200  $\mu$ l, 300  $\mu$ l, and 500  $\mu$ l of distilled water. Sample E was desalted on Sephadex G-25, lyophilized, and dissolved to one fifth of its original volume. For each sample 15  $\mu$ l was applied for IEF. This was performed in 5% polyacrylamide gels containing Pharmalyte (Pharmacia, Uppsala, Sweden), pH range 3 to 10. Samples were focused for 1.5 to 2 hr by use of a maximum of 30 W and 2000 V. Gels were fixed and stained according to the method of Merril et al.<sup>21</sup>

tracts as a closed precipitin arc. Since extracts A to D were freeze-dried, they could be applied at a higher concentration than the fluid extract E. Applying large volumes (E) caused some distortion of the precipitin patterns. Estimates of antigen  $P_1$  were possible on CIE by comparing the areas under the arcs for different extracts, and similar estimates were possible for antigen DpX on CRIE.

### Specific allergen assays

Six laboratories measured antigen  $P_1$  by use of different assay methods and antisera raised in rabbits to their own purified preparations of  $P_1$ . The mean estimates of potency of the laboratories relative to extract A are presented in Table III. In general there was close agreement between laboratories for extracts C, D, and E, but extract B demonstrated more variation. Extract B also had much lower potency estimates than the other extracts. When the results for antigen  $P_1$  were expressed as microgram equivalents per ampule, more variation between laboratories was observed (range 10 to 31  $\mu$ g) for the proposed IS. This variation probably reflects differences in the internal standards for this purified antigen used by the four laboratories. By use of the reported values for the proposed IS, antigen  $P_1$  represented between 1% to 3% of the dry weight and between 3% to 10% of the protein present.

Three laboratories expressed results as a relative

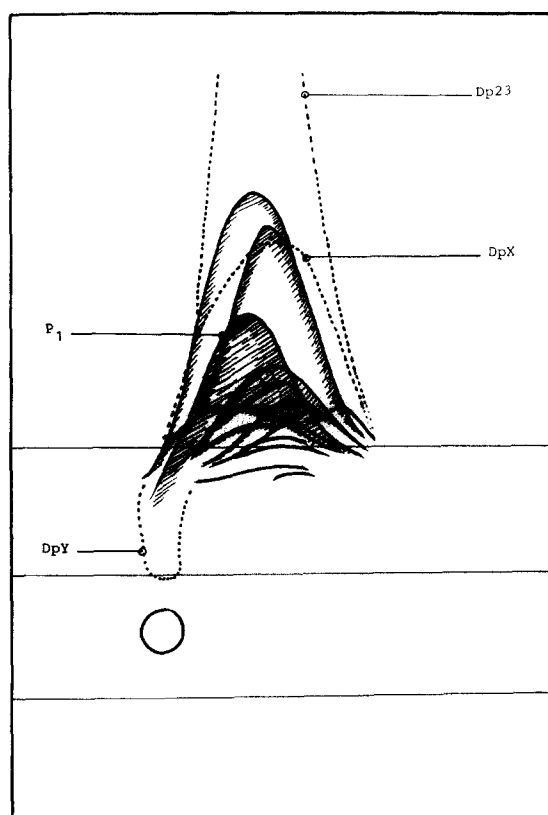


**FIG. 3A.** Identification of the components of the proposed IS by CIE and CRIE. A drawing of the precipitates observed on CIE and CRIE. The dotted lines denote precipitates observed only on CRIE.

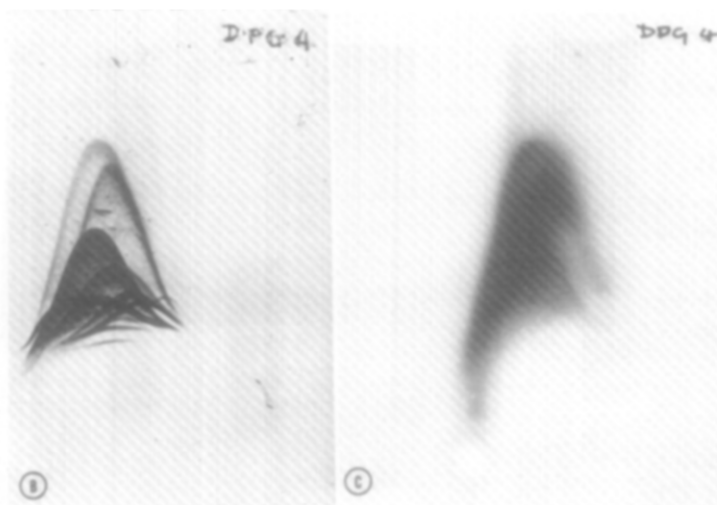


**FIG. 3. B-C.** B, Appearance of CIE slide. C, Appearance of CRIE autoradiograph. CIE and CRIE were performed as follows: The contents of one ampule of extract A were dissolved in 50  $\mu$ l distilled water and 5  $\mu$ l was applied. Agarose (type HSA, Litex; Glostrup, Denmark) at 1% (w/v) of solution in barbital buffer (0.073 mol/L Tris, 0.024 mol/L of barbital, and 0.0006 mol/L of calcium lactate, pH 8.6) was poured at 1.5 mm thick. First dimension was run at 10 V per centimeter for 25 min at 4° C. Second dimension was run in a 1.3 mm thick gel including the ammonium sulphate-precipitated fraction of rabbit hyperimmune serum to *D. pteronyssinus* mite culture extract (ALK, Copenhagen, Denmark). This immune serum was present at 10  $\mu$ l per square centimeter, and the gel was run at 1.5 V per centimeter for 18 hr. The plate was pressed and washed successively in saline solution and distilled water, cold-air dried, and incubated with *D. pteronyssinus* serum pool P diluted 1:15 for 24 hr. After washing, the plate was incubated with radioiodine-labeled anti-IgE (Pharmacia, Uppsala, Sweden), approximately 0.25  $\mu$ Ci for 24 hr. After washing, the plate was hot-air dried and autoradiographed for 7 days on Kodak XS-1 x-ray film and stored at -70° C in a cassette with an intensifier screen. The plate was stained in Coomassie brilliant blue. According to the IUIS nomenclature proposal for isolated and/or well characterized allergens, the new names will be: P<sub>1</sub>, Ag 42, Dpt 12 = Der.p.I; DpX = Der.p.II; Dp 23 = Der.p.III.

potency for antigen DpX (Table IV). These results were very consistent for A and C but demonstrated considerable interlaboratory variation for the other extracts. Values for DpX were not demonstrated in micrograms because of the lack of a purified preparation of this allergen.

### Stability studies

When RAST inhibition was used, the log dose-response curves for the freeze-dried extracts and liquid material stored at -150° C were comparable. The relative potency of the reconstituted freeze-dried material relative to the frozen material for the proposed IS was 65% (95% confidence interval 33% to 127%) and for the trial fill was 130% (95% confidence interval 80% to 240%). Thus it appeared that no adverse effects on the potency of the materials had occurred during freeze-drying.

In accelerated degradation studies there was no significant loss in potency nor change in the slopes of the dose-response curves of the samples stored at either +37° C or +45° C after a period of 7 to 9 months

storage, although the estimates of potency had wide confidence limits (Table V). Further studies are being performed to assess the stability of the freeze-dried IS.

### DISCUSSION

The most widely used assay method was RAST inhibition that, for practical reasons, is the easiest technique for measuring total allergenic activity. In individual assays the proposed IS was found to elicit dose-response curves comparable with those for extracts D and E that are similar to commercial extracts. Potency estimates from other quantitative assays for total allergenic activity, such as histamine release and skin testing, were in reasonable agreement with those from RAST inhibition.

During these studies it became clear that extract B was different from the others. In some assays it was less potent, in RAST inhibition it sometimes elicited log dose-response lines with slopes different from those of the other extracts, and it demonstrated atypically low antigen P<sub>1</sub> values. This extract was origi-

**TABLE IIIA.** Estimates of antigen P<sub>1</sub> (= Ag 42 = Dpt 12) in the extracts by six laboratories in terms of P<sub>1</sub> content of extract A

| Assay method               | Lab code no. | No. of assays | P <sub>1</sub> content in terms of extract A |      |     |     |
|----------------------------|--------------|---------------|----------------------------------------------|------|-----|-----|
|                            |              |               | B                                            | C    | D   | E*  |
| RIA                        | 1            | 1             | 0.05                                         | 1.02 | 2.1 | 5.1 |
| RIE                        | 4            | 2             | 0.04                                         | 1.20 | 1.3 | 4.4 |
| P <sub>1</sub> -McAb assay | 7            | 3             | 0.03                                         | 0.94 | 1.4 | 4.1 |
| Addition RIE               | 10           | 3             | 0.02                                         | 1.10 | 1.0 | 4.1 |
| Addition SRID              | 10           | 3             | 0.06                                         | 1.13 | 1.0 | 5.7 |
| SRID                       | 11           | 4             | 0.10                                         | 1.10 | 1.1 | 4.1 |
| P <sub>1</sub> RAST        | 11           | 1             | 0.05                                         | 1.05 | 1.6 | 2.5 |
| RIA                        | 17           | 2             | 0.06                                         | 1.16 | 2.0 | 6.3 |
|                            | GM           |               | 0.05                                         | 1.08 | 1.4 | 4.4 |
|                            | GCV          |               | 63%                                          | 8%   | 34% | 32% |

McAb = monoclonal antibody.

\*Relative potency of 1 ml of E compared with one ampule of A.

**TABLE IIIB.** Estimates of antigen P<sub>1</sub> (= Ag 42 = Dpt 12) in the extracts by six laboratories in micrograms per ampule of P<sub>1</sub>

| Assay method  | Lab code no. | No. of assays | µg/ampule of P <sub>1</sub> |      |    |    |     |
|---------------|--------------|---------------|-----------------------------|------|----|----|-----|
|               |              |               | A                           | B    | C  | D  | E*  |
| RIA           | 1            | 1             | 10                          | 0.56 | 11 | 22 | 53  |
| RIE           | 4            | 2             | 26                          | 1.00 | 31 | 33 | 111 |
| Addition RIE  | 10           | 3             | 25                          | 0.6  | 27 | 27 | 99  |
| Addition SRID | 10           | 3             | 19                          | 1.2  | 22 | 23 | 110 |
| SRID          | 11           | 4             | 15                          | 1.4  | 15 | 16 | 60  |

\*Calculated as microgram per milliliter.

**TABLE IV.** Estimates from three laboratories of relative concentration of DpX in the extracts

| Assay method   | Lab code no. | No. of assays | DpX content in terms of extract A |      |      |      |
|----------------|--------------|---------------|-----------------------------------|------|------|------|
|                |              |               | B                                 | C    | D    | E    |
| RIE            | 4            | 2             | 1.01                              | 1.17 | 2.5  | 520* |
| Addition RIE   | 10           | 3             | 1.2                               | 1.03 | 1.9  | 8.5  |
| Addition SRID  | 10           | 3             | 2.4                               | 1.14 | 4.0  | 11   |
| Dpx-McAb assay | 7            | 3             | 3.5                               | 1.14 | 16   | 40   |
|                |              | GM†           | 1.8                               | 1.12 | 4.1  | 16   |
|                |              | GCV           | 80%                               | 6%   | 160% | 130% |

McAb = monoclonal antibody.

\*Doubt about relative concentrations used in this assay, therefore omitted from GM.

†GM and GCV of estimates from each method.

nally prepared in 1976 from isolated *D. pteronyssinus* mites that had been grown on horse dander. The details of the procedure used to isolate those mites are not available, and it is therefore difficult to speculate on the reasons why this extract appears to be different. One possibility is that extract B does not include fecal

particles as do those extracts made from whole cultures. There is good evidence that a whole culture extract incorporating fecal particles more clearly resembles the allergens to which patients are naturally exposed.<sup>34</sup> However, all other materials, including the proposed IS, were derived from whole cultures and

**TABLE V.** Potencies of the trial fill (82/568) and of the proposed IS (82/518) accelerated degradation samples each in terms of the material stored at  $-20^{\circ}\text{C}$  obtained by RAST inhibition

| Extract     | NIBSC code no. | Period stored (mo) | Potency ratio          |                        |
|-------------|----------------|--------------------|------------------------|------------------------|
|             |                |                    | + $37^{\circ}\text{C}$ | + $45^{\circ}\text{C}$ |
| Trial fill  | 82/568         | 6                  | 0.83 (0.50 to 1.37)*   | 0.63 (0.38 to 1.03)    |
|             |                | 9                  | 0.67 (0.38 to 1.18)    | 0.63 (0.36 to 1.10)    |
| Proposed IS | 82/518         | 4                  | 1.25 (0.65 to 2.42)    | 1.41 (0.73 to 2.72)    |
|             |                | 7                  | 0.87 (0.38 to 1.97)    | 1.14 (0.50 to 2.61)    |

\*Ninety-five percent confidence interval.

appeared to behave in a manner similar to each other in RAST inhibition.

By use of IEF, the extracts demonstrated differences that were partially due to differences in the quantity of extract applied and partially due to staining techniques. In several laboratories, extract D demonstrated more cathodic bands than the other extracts. However, no laboratory attempted to demonstrate which of the bands represented important allergens so that we cannot state whether the additional bands indicate different allergens or a high concentration of positively charged proteins, possibly from the culture medium. It is well established from previous work that antigen  $P_1$  can focus at pH ranges from 4.5 to 7.1.<sup>13</sup> In addition, it has been found that antigen DpX in the whole extract focuses in the anodic region with variable isoelectric points.<sup>15</sup> Since variations in experimental conditions in separate laboratories elicit different IEF patterns, it is very difficult at present to make meaningful comparisons of *D. pteronyssinus* mite extracts; therefore this technique cannot be recommended for standardization.

The use of CIE and CRIE demonstrated that at least 17 distinct antigens were present in the proposed IS and that up to six of these antigens bound to IgE. It was also clear that the relative proportion of the different antigens can vary in extracts derived from various sources. Thus, the total allergenic activity of an extract represents a contribution from several separate allergens. The distribution of allergens in the IS is in no way a blueprint of what an extract should contain either with regard to total potency or relative concentrations of the allergens. The results of the study have demonstrated that the IS can be used as a yardstick to measure antigens  $P_1$  and DpX, and our results suggest that the proposed IS will prove suitable for measuring these and other allergens as techniques become available. For the moment it is suggested that the proposed IS be used as a yardstick for measuring either total allergenic potency by assays such as RAST in-

hibition or antigens  $P_1$  and DpX activities by any of the techniques that are available. It is important when units are reported in terms of the IS that it is made clear what methods are being used.

On the basis of this collaborative study, the WHO Expert Committee on Biological Standards met in 1984 and established the preparation coded 82/518 as the international standard for *D. pteronyssinus* extract with assigned units of 100,000 IU per ampule.<sup>35</sup> This unitage refers both to the total activity of the ampule and to that of the individual allergens. This standard is available from NIBSC, London, England.

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## REFERENCES

1. Ford AW, Rawle FC, Lind P, Spieksma FTM, Løwenstein H, Platts-Mills TAE: Standardization of *Dermatophagoides pteronyssinus*: assessment of potency and allergen content in ten coded extracts. *Int Arch Allergy Appl Immunol* 76:58, 1985
2. WHO Expert Committee on Biological Standardization. Guidelines for the preparation and establishment of reference materials and reference reagents for biological substances. WHO Tech Rep Ser 626:101, 1978
3. Voorhorst R, Spieksma FTM: Fünfzehn Jahre hausstaubmilbenforschung in Leiden. *Allergologie* 1:93, 1978
4. Campbell PJ: International Biological Standards and Reference Preparations. II. Procedures used for the production of biological standards and reference preparations. *J Biol Stand* 2:259, 1974
5. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265, 1951
6. Büchler W, Meier J, Bersier P: Bestimmung von kleinen Wassermengen in lyophilisierten. *Z Anal Chem* 237:104, 1968
7. Baker PRW: The microdetermination of residual moisture in freeze-dried biological materials. *J Hyg* 53:426, 1955
8. Yman L, Ponterius G, Brandt R: RAST-based allergen assay methods. *Dev Biol Stand* 29:151, 1975
9. Ceska M, Eriksson R, Varga JM: Radioimmunosorbent assay of allergens. *J ALLERGY CLIN IMMUNOL* 49:1, 1972
10. Løwenstein H: Quantitative immunoelectrophoretic methods

- as a tool for the analysis and isolation of allergens. *Prog Allergy* 25:1, 1978
11. Lind P, Løwenstein H: Identification of allergens in *Dermatophagoides pteronyssinus* mite body extract by crossed radioimmuno-electrophoresis with two different rabbit antibody pools. *Scand J Immunol* 17:263, 1983
  12. Chapman MD, Platts-Mills TAE: Purification and characterization of the major allergen from *D. pteronyssinus* antigen P<sub>1</sub>. *J Immunol* 125:587, 1980
  13. Chapman MD: Purification and characterization of the major allergen from the house dust mite *D. pteronyssinus* antigen P<sub>1</sub>. London, 1981 (Ph.D thesis)
  14. Lind P: Standardization of mite extracts: qualitative and quantitative investigations of three kinds of preparations with CRIE and RAST. *Allergy* 35:227, 1980
  15. Lind P: Characterization of a partially purified allergen preparation of *D. pteronyssinus* and two isolated major allergens. XIV Scandinavian Congress of Allergology. Göteborg, Sweden, 1981 (abst)
  16. Lind P, Weeke B, Løwenstein H: A reference allergen preparation of the house dust mite, *D. pteronyssinus*, produced from whole mite culture—a part of the DAS 76 study. Comparison with allergen preparations from other raw material. *Allergy* 39:259, 1984
  17. Stewart GA: Isolation and characterization of the allergen Dpt 12 from *Dermatophagoides pteronyssinus* by chromatofocusing. *Int Arch Allergy Appl Immunol* 69:224, 1982
  18. Aalberse R, Lind P: (in preparation)
  19. Read SM, Northcote DH: Minimization of variation in the response to different proteins of the Coomassie blue G dye binding assay for protein. *Anal Biochem* 116:53, 1981
  20. Mancini G, Carbonara AO, Heremans JF: Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2:235, 1965
  21. Merril CR, Goldman D, Sedman SA, Ebert MH: Ultrasensitive stain for proteins in polyacrylamide gels. *Science* 211:1437, 1981
  22. Blakesley RW, Boezi JA: A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G 250. *Anal Biochem* 82:580, 1977
  23. Norman PS: Skin testing. In Rose NR, Friedman H, editors: *Manual of clinical immunology*, ed 2. Washington, D. C., 1980, American Society of Microbiology, pp 586-9
  24. Aas K: Allergenextrakte—allgemeine betrachtungen allergenextrakte. In Aas K, Brede, editors: *Allergen-extraete*. Symposium im Georg-Speyer-Haus zu Frankfurt-am-Main, 1978, pp 38-48
  25. Mesheryakova SA: Fluorometric method of histamine assay in blood and tissue. *Lab Delo* 2:103, 1971
  26. Siraganian RP, Hook WA: In Rose NR, Friedman H, editors: *Manual of clinical immunology*, ed 2. Washington D. C., 1980 American Society of Microbiology, p 808
  27. Voller A, Bidwell D, Bartlett A: Microplate enzyme immunoassays for the immunodiagnosis of sinus infection. In Rose NR, Friedman H, editors: *Manual of clinical immunology*, ed 1. Washington, D. C., 1976, American Society of Microbiology, pp 506-12
  28. Kauffman HF, van der Heide S, Beaumont F, de Monchy JGR, de Vries K: The allergenic and antigenic properties of spore extracts of *Aspergillus fumigatus*: a comparative study of spore extracts with mycelium and culture filtrates extracts. *J ALLERGY CLIN IMMUNOL* 73:567, 1984
  29. Schwartz J, Vardinon N: In vitro prevention of direct mast cell disruption by specific antibody. *Int Arch Allergy* 30:67, 1983
  30. Beerens L, Guikers CLH: Value of fluid phase complement consumption as a means of in vitro allergen standardization. *Der Biol Stand* 29:235, 1975
  31. Petrunov B: Inactivation of the human complement by atopic allergens as a method of their standardization. Tenth European Congress of Allergology and Clinical Immunology. Prague, 1977. *Allergol Immunopathol* 5:319, 1979
  32. Finney DJ: Statistical method in biological assay, ed 3. London and High Wycombe, 1978, Charles Griffin & Co, Ltd, pp 69-104
  33. Kirkwood TLB: Geometric means and measures of dispersion. *Biometrics* 35:908, 1979
  34. Tovey ER, Chapman MD, Wells CW, Platts-Mills TAE: The distribution of dust mite allergen in the houses of patients with asthma. *Am Rev Respir Dis* 124:630, 1981
  35. Expert Committee on Biological Standards. WHO Tech Rep Ser (in press)

## Appendix

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## Allergens of the house dust mite *Dermatophagoides farinae*—Immunochemical studies of four allergenic fractions

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*Analysis of the allergenic components of the house dust mite Dermatophagoides farinae was performed. Four different types of proteins reactive with human anti-mite IgE antibodies were identified as four distinct radioprecipitins in radioimmunoelectrophoresis. These were designated as mite allergen reactive with human IgE antibodies (Me) Me 1, Me 2, Me 3, and Me 4 according to their electrophoretic mobility from the slower to the faster. Me 1 and Me 2 were the first and the second prevailing allergens, respectively, in 133 atopic patients subjected to the study. Me 1, on purification by gel filtration and anion exchange chromatography, elicited a single radioprecipitin by radioimmunoelectrophoresis. Me 1 also elicited a single band in sodium dodecyl sulphate-polyacrylamide gel electrophoresis and a single peak in high-performance liquid chromatography. Both methods elicited identical values of 17,000 daltons for the molecular weight of Me 1. The isoelectric point of Me 1 was suggested to be 8.0. Me 1 elicited higher skin reactions compared to that obtained by the crude mite extract when it was assessed in seven atopic patients by serial titration intradermal skin testing. (J ALLERGY CLIN IMMUNOL 75:686-92, 1985.)*

The mite was suggested many years ago to be one of the major causative allergens found in house dust that is well known for its allergenicity in atopic asthma and other atopic diseases.<sup>1, 2</sup> More recently, the skin reaction to the extract of the pyroglyphoid mite of the *Dermatophagoides* sp has been reported to correlate well with that induced by house dust in atopic indi-

viduals,<sup>3-5</sup> whereas others have found correlations in RAST and in provocation tests.<sup>6, 7</sup> We also previously reached the conclusion that the house dust mite was the most common source of allergen found in house dust in Japan<sup>8-11</sup> as it was for a large portion of Europe<sup>12-15</sup> and the west coast of the United States.<sup>16</sup>

We have attempted to purify and characterize the allergenic components of the house dust mite *Dermatophagoides farinae* by use of a variety of immunochemical methods and have reported that the most allergenic fraction was distributed in the range of 20,000 to 30,000 daltons in molecular weight and that the major protein component was accompanied by a small amount of polysaccharide.<sup>17, 18</sup> We have made further attempts in this study to identify the component most allergenic to human atopic individuals by use of RIEP. This method has the advantage of directly iden-

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