

A new method of allergen standardization

Passive cutaneous anaphylaxis inhibition in a mouse-to-rat system in comparison with other methods

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Various methods have been employed for standardization of potency of allergen extracts. We used passive cutaneous anaphylaxis (PCA) inhibition in a mouse-to-rat system as a new means of standardization. Several allergen extracts, including mite, short ragweed, house dust, Aspergillus, Candida, and Japanese cedar, were examined. Mouse IgE antibodies were produced after two or three injections of antigen and alum. The potency measured by PCA inhibition in the mouse-to-rat system was compared with that of protein nitrogen (PN) content, end point of prick test in humans, and 50% radioallergosorbent test (RAST) inhibition. Our studies suggest that the mouse-to-rat system could be used to determine allergenic potency and to standardize allergen extracts.

Various methods have been employed for the quantitative measurement of potency of allergens. These include weight by volume, pollen (or Noon) units,¹ total nitrogen content,² protein nitrogen units (PNU), and protein nitrogen (PN) content.³ These values are by no means a direct measurement of allergenic potency, nor do they necessarily correlate with allergenic potency.^{4, 5} The titer of the Prausnitz-Küstner (P-K) test and the end point of the direct skin test are also used for allergen standardization, but these tests cause discomfort to the patients. Recently, the release of histamine^{6, 7} and the RAST inhibition⁸⁻¹⁴ have been proposed. The 50% RAST inhibition method is especially recommended by the International Committee of Allergen Standardization¹⁵ as a better method for measurement of the potency of allergen extracts. On the other hand, mice are widely

used as suitable animal models of IgE antibody production.¹⁶⁻¹⁸ Consequently, we have tried to see whether or not this animal can be used for the measurement of the potency of allergen extracts. In this paper we report the possible usefulness of the inhibition test of passive cutaneous anaphylaxis (PCA) in the mouse-to-rat system in comparison with PN content, the end point of the skin test in humans, and 50% RAST inhibition for the standardization of allergen.

MATERIALS AND METHODS

Allergens

Two different extracts of house dust mite (*Dermatophagoides farinae*), two of short ragweed (*Ambrosia elatior*), three of house dust, three of *Aspergillus fumigatus*, three of *Candida albicans*, and three of Japanese cedar (*Cryptomeria japonica*) were obtained from Torii Pharmaceutical Company (Tokyo, Japan). Some of them were extracted with different methods; those extracted with an identical method were extracted on different occasions. PN content of these allergens was determined by analyzing the precipitate obtained with phosphotungstic acid by the method of Stull, Cooke, and Tennant.³ The extracts were stored undiluted at 4° C until used. They were diluted with saline for the skin test and PCA inhibition, and with RAST incubation buffer (phosphate buffer, 0.05 M; NaCl, 0.9%; bovine serum albumin, 0.3%; NaN₃, 0.05%; polysorbate 20 [Tween 20] 0.1%; pH, 7.4) for the RAST.

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Skin test in humans

Six to 17 allergic patients (17 subjects with mite, 10 with short ragweed, 13 with house dust, 6 with *Aspergillus*, 12 with *Candida*, and 9 with Japanese cedar) were tested by the prick method with a twofold serial dilution of the appropriate allergen extracts to assay the in vivo potency of the extracts on human skin. These patients were suffering from either asthma or allergic rhinitis and had been skin-tested previously with positive results. Some of them were tested with several allergens. Bronchodilators and antihistamines were discontinued for more than 12 hr before the test. The prick test was performed on the back or forearm and the positive reaction was read when erythema was more than 10 mm × 10 mm in 15 min. The end point of the skin test was determined as the maximum dilution with positive reaction. The patients used for skin testing were not always those used for supplying serum for the RAST.

50% RAST inhibition

Antisera. The sera were obtained from several patients who were highly allergic to the allergen. Equal volumes of the sera were mixed and stored at -20°C until used. When the sera were ready to be used, they were diluted 1:10 or 1:50 with RAST incubation buffer.

Solid-phase antigen. Cellulose paper discs (Toyo Roshi Kaisha Ltd., Tokyo) were coupled to the allergen according to the method of Ceska, Eriksson, and Varga.¹⁹ About 20 mg of lyophilized antigen in 10 ml of phosphate-buffered saline (PBS) was used for coupling to 100 to 200 paper discs. The allergen-coupled paper discs were stored in RAST incubation buffer at 4°C .

Inhibitors. A serial twofold dilution of allergenic extract was made and 100 μl of each dilution was used as an inhibitor.

Radiolabeled anti-IgE. Anti-IgE was kindly offered by Dr. Johanson (Uppsala, Sweden). Using the method of Klinman and Taylor,²⁰ 50 μl of anti-IgE was labeled with ^{125}I , then diluted, divided into several tubes, and stored at -20°C until used. Labeled anti-IgE was diluted to about 100,000 cpm per 50 μl for RAST.

RAST inhibition was performed according to the method of Gleich and co-workers,⁸⁻¹¹ with some modifications (Fig. 1). The potency of allergenic extract in 50% RAST inhibition test was expressed as follows. The isotope count of the samples was compared to that of the control samples with no inhibitor. The percentage of inhibition was calculated and plotted on semilog graph paper. The amount of allergen extract necessary for 50% RAST inhibition was determined from the curve, and the reciprocal was considered as the potency of the allergen extract. RAST inhibition test was performed more than three times for each allergen.

PCA inhibition test in mouse-to-rat system: Reagin production in mice

At least 5 BALB/C mice, 8 wk of age, were used for immunization. Optimal immunizing doses had been determined in the preliminary experiments as mite, 10 μg ; short ragweed, 10 μg ; house dust, 25 to 100 μg ; *Aspergillus*, 10 to 50 μg ; and *Candida*, 50 to 100 μg . Aluminum hydroxide

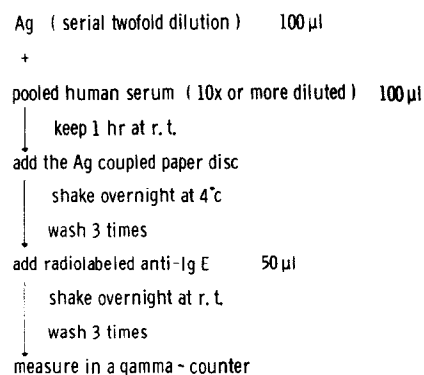


FIG. 1. Procedure of 50% RAST inhibition test.

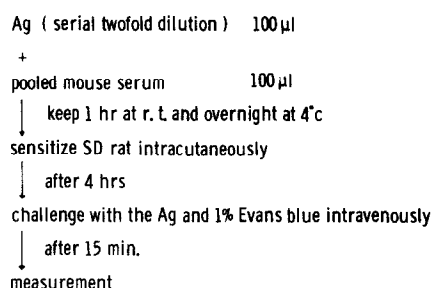


FIG. 2. Procedure of PCA inhibition test in mouse-to-rat system.

gel (alum), 1.8 mg, was used as adjuvant. Male Sprague-Dawley (S-D) rats were used for skin reactions.

From 10 to 100 μg of each antigen with 1.8 mg of alum was injected intraperitoneally into a BALB/C mouse as a primary dose. Four to six weeks later, the same dose of each antigen with alum was injected in the same route as a booster immunization. House dust antigen was injected three times to produce sufficient reagin. Serum was then obtained from the retroorbital venous plexus weekly thereafter, pooled, and stored at -20°C . These reaginic titers to all antigens, with the exception of Japanese cedar, to which reaginic antibody could not be produced by any means, were more than 1:160 on S-D rats. Each serum was diluted 50 to 500 times for PCA inhibition.

The PCA inhibition procedure is shown in Fig. 2. The concentrations of challenging antigens were as follows: mite, 4 mg/ml; house dust, 10 mg/ml; *Aspergillus* and *Candida*, 10 mg/ml; and short ragweed, 10 mg/ml. The end point titration of the allergen extract to inhibit the PCA reaction was considered as the potency of the allergen in PCA inhibition test. The PCA inhibition test was carried out in duplicate on the back of each rat and performed more than three times for each allergen.

RESULTS PN content

The PN contents of original extracts are shown in Table I. The numbers in parentheses show the ratio of the potency in PN content.

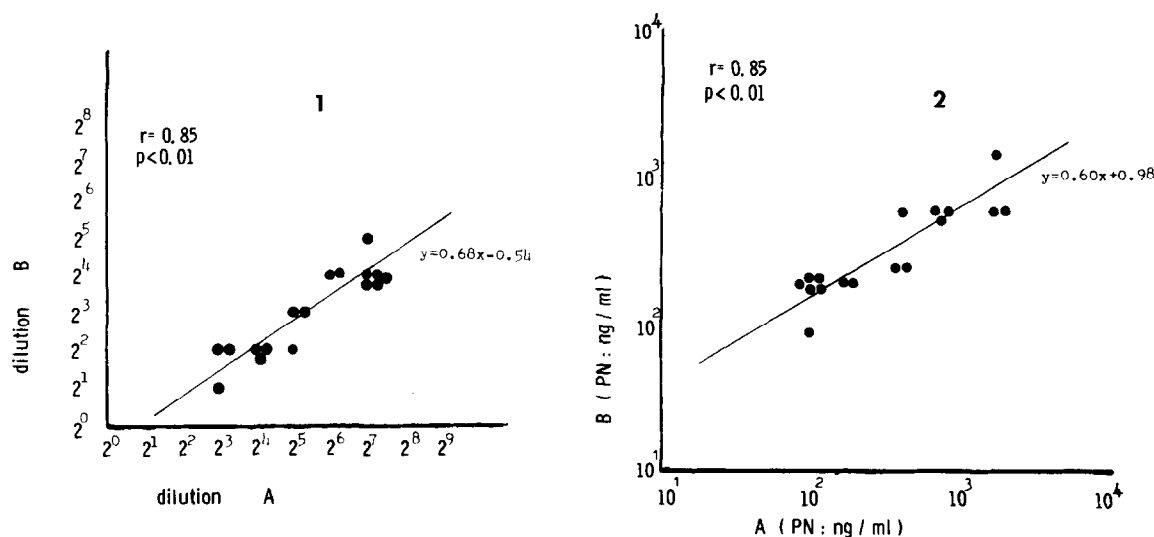


FIG. 3. Correlation of the end point of the prick test in humans with two different mite extracts. Fig. 3, 1 shows results by dilution. Fig. 3, 2 shows results by PN content. X- and y-axes are plotted by indices of 2 (Fig. 3, 1) and 10 (Fig. 3, 2). Each point represents one subject.

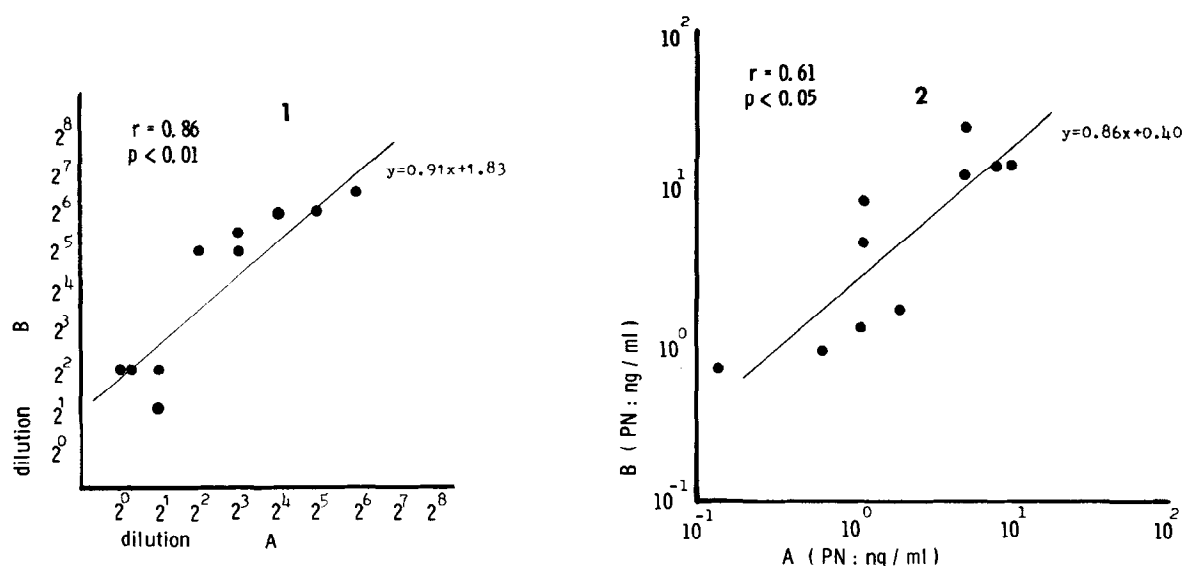


FIG. 4. Correlation of the end point of the prick test in humans with two different short ragweed extracts. Fig. 4, 1 shows results by dilution. Fig. 4, 2 shows results by PN content. X- and y-axes are plotted by indices of 2 (Fig. 4, 1) and 10 (Fig. 4, 2). Each point represents one subject.

Skin test in humans

The skin test in humans was performed by the prick method. Fig. 3 represents one example of the skin test made to determine the relationship between the potency of two different extracts of mite antigens. Fig. 3, 1 is by dilution, and Fig. 3, 2 is by PN content. The potency of A was 4.7 times stronger than that of B. The PN content of the end point was almost the same for both A and B. The correlation coefficient was 0.85 ($p < 0.01$) in both figures. Fig. 4 represents the relationship between the two different short rag-

weed extracts. The potency of B was about three times stronger than that of A ($r = 0.86$, $p < 0.01$). Table I shows the end point and PN content of the skin test in humans.

50% RAST inhibition

Fig. 5 shows one example of the several tests of the RAST inhibition curve of different mite extracts. The volume of mite extract (Fig. 5-1) and the PN content (Fig. 5-2) in 100 μ l of inhibitor is plotted logarithmically on the horizontal axis. The inhibition

TABLE I. Comparison of allergenic potency assayed by PN content, prick test in humans, 50% RAST inhibition, and PCA inhibition in mouse-to-rat system

						PCA inhibition in mouse-to-rat system
Antigen	PN (μg/ml)	Prick test in humans		50% RAST inhibition		End point
		End point	PN (μg/ml)	Volume of antigen (μl/100 μl)	PN (ng/100 μl)	
<i>Mite</i>						
A	13.58 (1.0)*	2 ^{5.3} † (1.0)*	0.35‡	1.4§ (1.0)*	19	2 ^{4.0} † (1.0)*
B	2.72 (0.2)	2 ^{3.1} (0.2)	0.32	7.0 (0.2)	19	2 ^{1.0} (0.1)
<i>Short ragweed</i>						
A	9.46 (1.0)	2 ^{2.3} (1.0)	1.92	20.5 (1.0)	194	2 ^{5.0} (1.0)
B	59.62 (6.3)	2 ^{3.9} (3.0)	3.99	3.9 (5.3)	233	2 ^{6.5} (2.8)
<i>House dust</i>						
A	3.01 (1.0)	2 ^{2.4} (1.0)	0.11	8.0 (1.0)	24	2 ^{4.0} (1.0)
B	45.34 (15.1)	2 ^{2.6} (1.1)	0.75	9.0 (0.9)	400	2 ^{5.1} (2.1)
C	11.76 (3.9)	2 ^{2.5} (1.1)	0.21	12.0 (0.7)	141	2 ^{4.8} (1.7)
<i>Aspergillus</i>						
A	72.69 (1.0)	2 ^{4.0} (1.0)	4.54	0.5 (1.0)	36	2 ^{9.2} (1.0)
B	15.48 (0.2)	2 ^{2.8} (0.4)	2.22	1.5 (0.3)	23	2 ^{6.7} (0.2)
C	192.77 (2.7)	2 ^{5.0} (2.0)	6.02	0.17 (2.9)	33	2 ^{10.2} (2.0)
<i>Candida</i>						
A	136.14 (1.0)	2 ^{4.4} (1.0)	6.45	0.08 (1.0)	11	2 ^{8.0} (1.0)
B	272.89 (2.0)	2 ^{5.9} (2.8)	4.57	0.07 (1.1)	19	2 ^{10.0} (4.0)
C	23.04 (0.2)	2 ^{2.7} (0.3)	3.55	0.6 (0.1)	14	2 ^{7.0} (0.5)
<i>Japanese cedar</i>						
A	1.46 (1.0)	2 ^{1.8} (1.0)	0.42	2.7 (1.0)	4	ND
B	6.67 (4.6)	2 ^{1.2} (0.7)	2.90	3.2 (0.8)	21	ND
C	2.84 (1.9)	2 ^{0.6} (0.4)	1.87	13.0 (0.2)	37	ND

*Numbers in parentheses show the ratio of potency in each method.
†Mean value (arithmetic) of the end point; maximum dilution producing positive reaction is considered the end point.
‡PN contents of the end point of the prick test in humans.
§Volume of antigen in 100 μl of inhibitor which shows the 50% RAST inhibition.
||PN contents in 100 μl of inhibitor showing the 50% RAST inhibition.

curve shows nearly a linear pattern. The volume of mite extract to show 50% inhibition was 1.4 μl of A and 7.0 μl of B. The potency expressed as the reciprocal is 0.71 of A and 0.14 of B, providing the ratio of potency 1.0:0.2. The PN content was about 19 $\mu\text{g}/100 \mu\text{l}$. Another example of the inhibition curve by *Aspergillus* extracts is shown in Fig. 6. Table I summarizes the data obtained. (The 50% RAST inhibition is indicated by PN content and volume of antigen in 100 μl of inhibitor.)

PCA inhibition test in the mouse-to-rat system

PCA inhibition tests were performed several times for each allergen. Fig. 7 shows one example of short ragweed antigens. Since the reactivity of the skin was different in each rat, the arithmetic mean of the end point is shown in Table I.

DISCUSSION

In this study we compared several methods of allergen standardization, which included PN content of allergens, prick test in humans, 50% RAST inhibition, and PCA inhibition using the mouse-to-rat system. The requirement for the standardization of allergens is primarily for the clinical field where allergens are used for skin test and provocation test to detect the causative allergens, and used also for desensitization therapy. We believe the skin test in humans to be the most suitable method for allergen standardization. However, this method has difficulties, such as availability of subjects, discomfort to patients, and possibility of anaphylaxis. Other methods have been tried by many investigators. Among them, 50% inhibition of RAST has been recommended as a suitable technique. However, the RAST method does not directly reflect the biological potency of allergens.

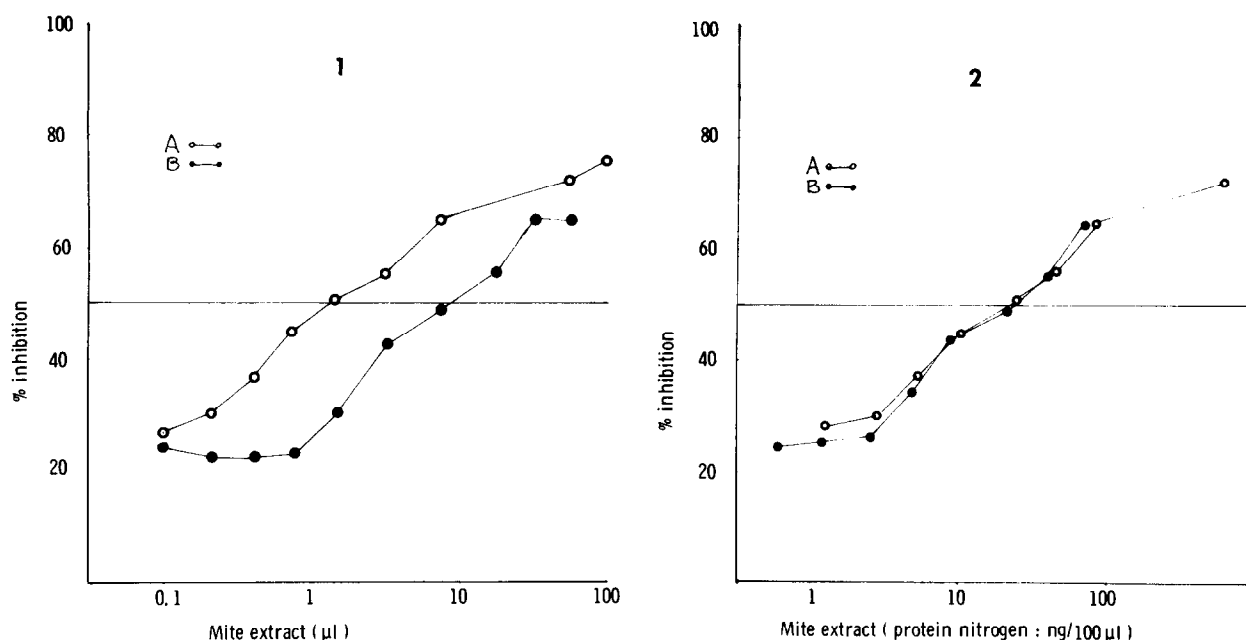


FIG. 5. Inhibition of mite RAST by mite extracts. X-axis is plotted by volume (Fig. 5, 1) and PN content (Fig. 5, 2) of the inhibitor added. Y-axis is plotted by % inhibition.

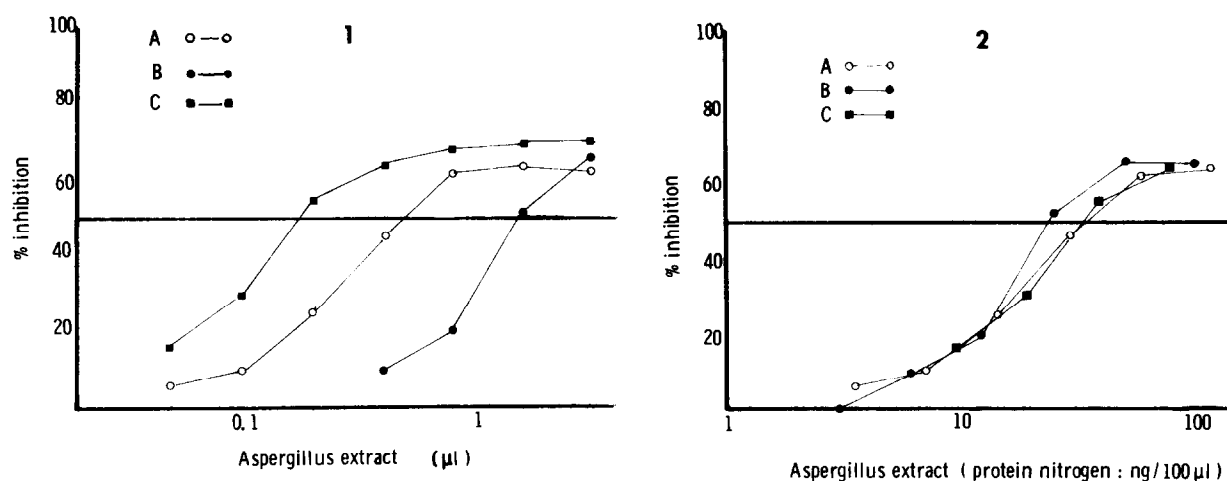


FIG. 6. Inhibition of *Aspergillus* RAST by *Aspergillus* extracts. X-axis is plotted by volume (Fig. 6, 1) and PN contents (Fig. 6, 2) of the inhibitor added. Y-axis is plotted by % inhibition.

Consequently, we have studied the mouse-to-rat system as an assay of the biological potency of allergens.

Many studies have shown that PN content does not necessarily represent the potency of allergens to elicit skin test results in humans. In this study, however, PN content did correlate with the skin test to some extent, with the exception of house dust and Japanese cedar. A relatively good correlation was found, probably because the antigens used in this study were obtained from the same company and the sources of allergens were similar although the process of extraction was different. Considering this fact, PN content

does have some utility for standardization of the potency of allergen extracts.

The 50% RAST inhibition test is the method most recommended at the moment. In our study, however, the correlation between the 50% RAST inhibition test and the end point of the skin test in humans was not as high as was expected. The crude extracts we used probably had multiple antigenic determinants; therefore, each patient did not necessarily produce reaginic antibody against the same determinant. The pooled sera was obtained from a limited number of patients; therefore, our pool might not cover all allergenic de-



FIG. 7. An example of PCA inhibition test in rat using mouse reagin.

terminants equally. Another reason for the poor correlation might be the existence of IgG antibody against the same determinant.

The PCA inhibition test in a mouse-to-rat system was used for standardization of the allergen extracts for the first time and we were able to obtain a similar correlation with the human skin test in comparison with 50% RAST inhibition (by Wilcoxon's paired rank test). The end points were considered to be good parameters of the allergenic potency. Therefore, it was thought that mouse reagin could be used as a substitute for human reagin to determine allergenic potency. Moreover, mice are good reagin producers,¹⁶⁻¹⁸ and the reagin titer persists for more than several months, making this a more convenient method for study. At first, we tried a P-K skin test on rats, using mouse reagin to obtain the end point.²¹ The process was as follows: One-tenth milliliter of 50- to 250-fold saline dilution of each serum was injected into the rat intracutaneously. Several hours later, P-K sites were injected with twofold serial dilutions of antigen extracts. The maximum dilution eliciting positive skin reaction was considered to be the end point. This correlates comparatively well with the end point of the skin test in humans. However, the end



FIG. 8. An example of P-K type skin test using mouse reagin.

point was rather difficult to determine (Fig. 8). Consequently, we next tried PCA inhibition instead of the P-K type skin test. The end point was then quite easy to determine (Fig. 7), but some problems remained in using this method. The BALB/C mice produced a high titer of the IgE antibodies against mite, short ragweed, *Candida*, *Asperigillus* after two injections, and house dust after three injections, but this strain of mouse did not produce IgE antibodies against Japanese cedar antigen. Neither DBA nor C3H mice produced IgE antibodies against Japanese cedar. Therefore, we could not perform a PCA inhibition test with Japanese cedar antigen.

According to recent reports,^{22, 23} pretreatment with low dosage of irradiation or cyclophosphamide could increase the production of IgE antibodies in mice by suppressing the function of suppressor T cells. Using these pretreatments, nonresponder mice might be made to produce the IgE antibodies against an antigen such as Japanese cedar. Such an attempt is now in progress.

The most important unsolved problem is whether or not the antigenic specificity of IgE antibody is the same in humans as in mice. To answer this problem, we tried the inhibition of RAST and human skin reac-

tion with mouse IgE antibodies, and the inhibition of PCA in a mouse-to-rat system with human IgE antibodies, but we could not obtain sufficient and reliable evidence. Even though the specificity was different in man and mouse, this method could be used for the standardization of allergen extracts if the potency of allergen by PCA inhibition correlates significantly well with the potency of skin test in humans; however, more data are needed to make a broad generalization.

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