

ALLERGY TO INSECT STINGS

IV. Diagnosis by Radioallergosorbent Test (R.A.S.T.)¹

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Radioallergosorbent tests (RAST(s)) have been developed and assessed for the diagnosis of insect hypersensitivity by using a purified allergen from honeybee venom, phospholipase A₂, and crude yellow jacket venom. Sera from 193 patients positive both by history and skin test to one of these insects were compared with various groups of control sera. Eighty percent of sera from skin test-positive patients were RAST positive; positive RAST were found in 16% of sera tested from skin test-negative patients. A highly positive RAST correlates well with a positive skin test and clinical sensitivity, but serum IgE is not measurable in many patients with mast cell or basophil bound antibody. Since biologically important reactions of antigen with IgE require that the antibody be cell bound, skin testing would be preferred to RAST if one were limited to a single test for the diagnosis of insect allergy.

It has been known for almost 60 years that "reagins" in the blood of atopic individuals are associated with specific allergen sensitivities. This was demonstrated by Prauznitz and Küstner (1), who injected serum from allergic patients into normal skin, allowed 24 to 48 hr for tissue "fixation", and challenged the prepared skin site with antigen. This was the first demonstration that a humoral substance was responsible for allergic disease. The P-K test, named for these investigators, has been used for several decades to detect the presence of reaginic antibodies. Skin testing of allergic patients with small quantities of the suspected allergens is a more direct way to detect IgE antibodies associated with allergic diseases (2). Recent work has shown that careful skin testing with extracts known to contain relevant allergens yields good correlations between the severity of clinical symptoms and the quantity of allergen required to elicit a positive skin response (3). A newer diagnostic technique, *in vitro* histamine release from human basophil

leukocytes after antigenic challenge, has also been shown to correlate significantly with clinical symptoms and with direct skin tests (4). Histamine release is more easily quantitated than are skin tests and, with the advent of new automated procedures, little blood is required for assessing sensitivity to multiple allergens. The equipment and expertise, however, are available in few laboratories, thus limiting the diagnostic potential of this assay.

In 1967, Wide *et al.* (5) introduced the radioallergosorbent test (RAST(s))³ as a method to measure the small quantities of IgE antibodies present in serum. The RAST is widely accepted as the serologic method of choice for measurement of allergen-specific IgE antibodies. It has been used to determine the presence of specific IgE antibodies directed against various allergens, including those from such diverse sources as ragweed, birch, and codfish, as well as to follow the changes in IgE antibody levels resulting from natural exposure to allergens and from immunotherapy (6-9). The results of the RAST, although semi-quantitative, have been shown to correlate with skin test results and with bronchoprovocation studies with several allergens (10, 11).

In insect allergy, diagnosis, except by history, has been almost impossible due to the use of inappropriate reagents, whole body extracts. Recently, venoms from the Hymenoptera have been shown to be diagnostically useful both *in vitro* by histamine release and *in vivo* by skin testing (12, 13). The Hymenoptera venoms are currently available in few centers and testing requires the physical presence of the patient. In contrast, sera can be analyzed by RAST with little inconvenience or danger to the patient. Since accurate diagnosis in this potentially life-threatening disease is of primary importance, development and assessment of the potential of the RAST in this disease state seemed necessary.

We have developed RAST specific for IgE antibodies to one of the major allergens in honey bee venom, phospholipase A₂ (Phos A), and to yellow jacket venom (14). Primary attention was given to these allergens since, in our area, more than 90% of patients with insect hypersensitivity react to the venom of one of these insects. Patients were considered hypersensitive to insect stings if, coupled with a history of a systemic reaction after a sting, they were skin test positive to one or more of the venoms. Our results indicate that insect venom RAST are useful diagnostically, but that a significant percentage of both false-negative and positive results occur when compared to skin tests. The majority of the sera yielding "false" results contain

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³ Abbreviations used in this paper: Radioallergosorbent test, RAST(s); Phospholipase A₂, Phos A; Radioimmunosorbent test, RIST; Cyanogen bromide, CNBR.

small quantities of IgE antibodies approximating the values found in normal sera.

MATERIALS AND METHODS

Antigens. Honeybee venom was collected as previously described (15). Venom sacs of yellow jackets were teased from the abdomen and freed of surrounding anatomical structures. The yellow jacket venom was eluted into a mildly acidic buffer, after multiple needle punctures of the sacs. Sac fragments were removed by Millipore filtration and the nonvenom proteins, precipitated by the acidic buffer, were removed by centrifugation. The precipitation and removal of nonvenom proteins are reported to occur by this procedure as judged by electrophoretic analysis against pure, aspirated venom (A. Benton, personal communication). Based on Dr. Benton's protein determination by Lowry, the venoms were diluted (10 mg in 10 ml) in coupling buffer (0.1 M sodium carbonate, 1.0 M NaCl, pH 8.5) for use in the RAST or in mg/ml solutions in Tris-buffered saline containing 0.03% human serum albumin for use in skin tests and leukocyte histamine release (16). Phos A, Grade I from honeybee venom (Sigma Chemical Company, St. Louis, Mo.), was diluted similarly. All antigens were stored at -20°C until used.

Antisera. The goat anti-IgE used for histamine release was kindly supplied by Dr. K. Ishizaka as was the rabbit anti-IgE used to measure total IgE in the radioimmunosorbent test (RIST) and specific IgE antibodies by RAST (17, 18). The latter was purified by elution from an IgE Sepharose column with a pH 2.8 glycine buffer and made ϵ -Fc specific by passage, sequentially, over affinity columns of insolubilized IgG and Fab₂ fragments. Iodination of the rabbit anti-IgE was performed by the chloramine-T method (19).

The reaginic serum used as the standard in the direct RIST contained 14,500 ng/ml IgE and had been standardized by using as reference WHO standard 68/341. One international unit (I.U.) of IgE was equated to 2.4 ng.

Sera. Sera obtained from 167 patients before their entering a program of immunotherapy for insect allergy were stored at -20°C until analyzed. Sera from atopic patients without histories of insect allergy were selected from those used in previous studies and sera from nonatopic, never stung controls were obtained from healthy laboratory volunteers. Informed consent was obtained from both patients and controls before skin testing and/or venipuncture.

Skin tests. Endpoint titrations with honeybee venom, Phos A, and yellow jacket venom were performed as previously described (20). In most cases the skin tests and serum collection for RAST were performed on the same day. At the extreme, 4 weeks occurred between skin testing and serum collection for RAST.

Histamine release. The methods used have been published in detail (21, 22). The assay was usually performed on the same day as the skin testing and the sera for RAST were obtained. At most, 1 month elapsed between skin testing, RAST, and histamine release.

Sorbent. Sepharose 4B (Pharmacia, Piscataway, N. J.) was used for both the RAST and the RIST. Activation by cyanogen bromide (CNBR) and coupling of antigen were performed following the method of Cuatrecasas and Anfinsen (23). Briefly, 20 ml of packed Sepharose 4B beads were washed thoroughly with distilled water and suspended as a 50% aqueous solution. Four grams of CNBR were added and the pH was adjusted to 11.0 and maintained by using 2 N NaOH. The temperature was maintained at 20°C by addition of ice to the mixture and the

reaction was continued until all CNBR crystals were dissolved. The reaction was stopped by lowering the temperature and the activated beads were washed in a Buchner funnel with 500 ml coupling buffer. Antigen (10 mg) in 10 ml coupling buffer was added immediately to the activated beads and incubated for 18 hr at 4°C with continuous, gentle agitation. The antigen-coated beads were washed with water and incubated with 1.0 M ethanolamine, pH 8.0, for 3 hr. The beads were then washed four times alternatively with high pH (0.1 M sodium borate, 1.0 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 1.0 M NaCl, pH 4.0) buffers with 15-min incubations. The allergen-sorbent was brought to 50% (v/v) in RAST buffer and stored at 4°C until diluted for use.

Buffer. RAST buffer, used to dilute allergosorbents, sera, and for washes in the RAST and RIST, contains 0.05 M Na_2HPO_4 , 0.5 M NaCl, 0.2% (w/v) bovine serum albumin, 0.5% (w/v) NaN_3 , and 0.5% (v/v) Tween 20 adjusted to pH 7.5 with 6 N HCl.

RAST and RIST assays. Techniques for performing the RAST and RIST assays are very similar and have been reported in detail (24-26). Briefly, 0.1 ml of a serum dilution was added to 0.5 ml of allergosorbent in a 15×75 mm plastic tube (Falcon, Oxnard, Calif.). Duplicate or triplicate determinations were made at each serum concentration. The tubes were placed on an orbital rotator and incubated at 25°C for 4 hr followed by centrifuging at 2000 rpm and washing three times with RAST buffer to remove unbound immunoglobulins. Subsequent to removal of the final wash fluid by gentle suction, 0.5 ml ^{125}I rabbit anti-IgE (5 to 15 ng, specific activity $\approx 70,000$ cpm/ng on the day labeled) was added and the tubes were placed on an orbital rotator and incubated for 18 hr to 20 hr at 25°C . The allergosorbent was then washed four times with RAST buffer (3 ml) and, subsequent to the final centrifugation and removal of ≈ 3.2 ml fluid, the specifically-bound ^{125}I anti-IgE was determined by using a gamma scintillation counter (Serle Analytic Inc., Des Plaines, Ill). The controls included in each assay are tubes containing only ^{125}I anti-IgE (Totals), sorbent without serum, and sorbent incubated with normal human serum before the addition of anti-IgE (Blanks). Also, sorbent-coated maximally with E myeloma protein is reacted with ^{125}I anti-IgE to determine the quantity of immunoreactive antibody bound and thus the maximal binding possible under the assay conditions (B_{max}). In practice, our results are calculated both in terms of total cpm bound and of percent maximal bound by E myeloma (B_{max}).

RESULTS

Methodology. Variations in the RAST assay usually involve the use of different antigen-coated sorbents such as paper discs, walls of plastic test tubes, and carbohydrate polymers made from cellulose, agarose, or Sepharose. Although we did not analyze all sorbents by using venom proteins, for most clinical diagnostic purposes all of these polymers are suitable. However, measurement of low levels of IgE antibodies requires maximal assay sensitivity and the study of sera containing increased amounts of allergen-specific IgG blocking antibodies also necessitates the use of a sorbent with maximal binding capacity. Since, in our laboratory, the RAST was to be used not only as a routine diagnostic test but also to assess both the minimal quantity of IgE antibody diagnostically relevant in insect allergy and to monitor changes in antibody titer during specific immunotherapy, we selected a carbohydrate polymer, Sepharose 4B (27).

For routine use, two allergens were coupled to Sepharose

beads, unfractionated, dialyzed yellow jacket venom and Phos A, a major allergen of honeybee venom (14).

To use the RAST as a diagnostic technique several factors must be considered: the amount of allergosorbent to be used, the reproducibility and sensitivity of the assay, and criteria as to what value(s) constitute a positive result.

To assess what quantity of allergosorbent should be used in the RAST, several dilutions of the allergen-coated Sepharose were tested with sera from allergic patients. In practice, allergosorbent concentrations covering a range of 0.1 to 10% v/v were evaluated with 0.5 ml of sorbent and 0.1 ml of serum. As shown in Figure 1, using the Phos A RAST "plateaus" of antibody binding were reached, in this case, at sorbent concentrations of 0.5% v/v and greater. Increasing the sorbent concentration above 0.5% did not increase binding of specific IgE antibody by the standard serum (which contains little IgG antibody), whereas increasing the sorbent did slightly affect the cpm bound by a serum from a patient with low specific IgE antibody and a significant amount of IgG, blocking antibody (36 μ g/ml) (28). No significant increase in cpm bound, however, occurred for this patient's serum above a 1% sorbent concentration. Supernatants from the first stage of both the Phos A and yellow jacket RAST were retested for specific binding. No detectable binding to the same sorbents was noted for either assay in the "plateau" region. The assessment of the supernatants was performed within 24 hr of the original absorption by using the same ^{125} I a-IgE for both assays. Reciprocal plots, extrapolated to infinite dilution also suggest that more than 90% of the IgE antibody was removed during the initial Phos A RAST. In addition, absorption of IgE antibodies with the Phos A sorbent indicate the $\geq 90\%$ of IgE antibodies are removed as judged by passive leukocyte sensitization followed by histamine release with crude honeybee venom (data to be published).

Also shown are sorbent binding curves obtained by using a serum from an unstung, nonatopic person (serum blank) and one obtained with sorbent to which only RAST buffer was added (sorbent alone). Nonspecific binding of radiolabeled IgE antibody is quite low in both situations, 1 to 5% of the total counts bound by the standard serum. Due to decay of the isotope for 25 days, the total cpm ^{125} I a-IgE added per tube was 47,802. Of the total counts added the blank serum bound 288 cpm at 1% and 473 cpm at 3% sorbent. The sorbent alone

contributes to the nonspecific binding of the radiolabeled IgE antibody and different allergosorbents vary significantly in this regard: sorbent coupled with yellow jacket venom nonspecifically bound up to 10% of the cpm bound by the standard serum. With the information shown in Figure 1, a concentration in antigen-sorbent excess, 1% v/v, was chosen for use in the Phos A RAST to minimize the effect of competition by blocking antibodies, which also bind to the antigen-coated sorbent. Although this concentration is inadequate for some sera this can be detected by serum dilution (see below). At this sorbent concentration little nonspecific binding occurs and a large number of assays (≈ 5000) can be performed with a single sorbent preparation. Yellow jacket venom-sorbents were evaluated with similar results although an absolute plateau was not obtained; rather, slight increases in binding were observed above 5.0% sorbent. A concentration of 1% was chosen for use in that RAST, since any increased binding at 5 or 10% sorbent was minimal. For all sorbents used in this study nonspecific binding was less than 10% of the maximal bound by the standard sera, usually 1% of the total cpm added.

Figure 2 records dose-response curves obtained in several assays during 6 weeks using a single serum from a honeybee allergic patient in the Phos A RAST. The reciprocal of the serum dilution required for 40% binding was 12.8 ± 2.7 (mean $\pm$ S.D.). The yellow jacket RAST yielded a similar degree of precision (data not shown).

Using a RAST elution technique developed in this laboratory, we measured a value of 850 ± 73 ng/ml for the specific IgE content of the standard serum used in the Phos A RAST. The RAST elution technique has been used to evaluate similarly sera in the ragweed system; the validity of using a known standard serum and extrapolating the antibody titers (ng/ml) of unknown sera has been shown (29). The standard curves in Figure 2 indicate that 10% binding, obtained by using 0.1 ml of a 1/100 dilution of the standard serum was different from nonspecific binding (which has been subtracted from all values). The Phos A RAST, therefore, measures less than 1 ng of specific IgE. By using 850 ng/ml as the titer of the standard serum, IgE antibody for unknown sera in the Phos A RAST are reported in ng/ml. The elution technique has not been applied to evaluate similarly the yellow jacket RAST since we questioned the validity of antibody quantitation with a venom of unknown antigenic composition. With a mixture of proteins it is not possible to know whether the binding reflects the

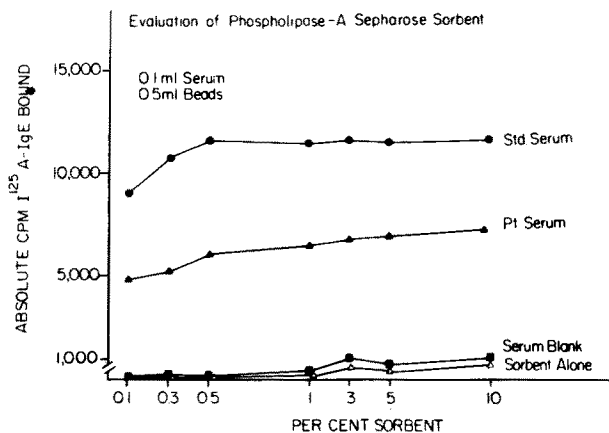


Figure 1. Evaluation of the binding of the IgE anti-Phos-A antibody to various concentrations (v/v) of Phos-A coupled sorbent. The standard serum contains no detectable, specific IgG antibody whereas the patient's serum has 39 μ g/ml IgG anti-Phos A. The serum for the blank was obtained from an unstung volunteer. The total cpm ^{125} I anti-IgE added were 47,802. The cpm bound by the blank were 221,288, and 473 at 0.5, 1, and 3% sorbent, respectively (mean of triplicates).

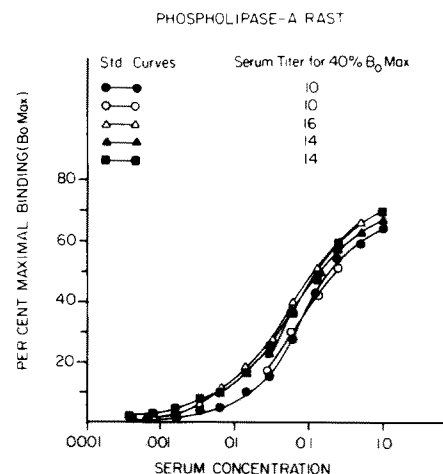


Figure 2. Dose response curves obtained with a single standard serum (with two allergosorbent preparations) over a 6-week period. The average serum titer for 40% $B_{0\max}$ was 12.8 ± 2.7 (mean $\pm$ S.D.).

relative concentration of the different proteins in the venom. Results in arbitrary units (cpm minus background), therefore, are given for the yellow jacket RAST.

Although the use of an antigen-excess sorbent usually results in reproducible values for IgE antibodies in undiluted sera, exceptions occur necessitating the routine analysis of several serum dilutions. As shown in Table I, similar values for IgE antibody were obtained in the Phos A RAST with four sera at several dilutions. The fifth serum presents two problems that can distort RAST results; it contains almost 6 times as much specific IgE antibody as the standard serum and large quantities of specific IgG antibodies (1.5 mg/ml) (28). To overcome these effects, the serum was assayed at dilutions until reproducible data were obtained. The sixth serum contains sufficient specific blocking antibodies to compete effectively for the antigen on the sorbent and cause an apparent titer, in undiluted serum, 2-fold less than that obtained after dilution. Data using the yellow jacket RAST (No. 7 through No. 10) are presented to show that these parameters are not unique to the Phos A RAST but must be considered in general when determining IgE antibody titers. Note that the titer of No. 8 increases more than 4-fold as the serum is diluted.

Another consideration when establishing a RAST is the decision as to what constitutes the background or nonspecific binding. The level chosen is of critical importance for diagnostic use of the RAST and should be evaluated for each antigen-sorbent system. We approached this problem in several ways: 1) A serum from a nonatopic subject, who had never been stung by a Hymenopteran, was included in all assays and the variation in binding was determined (Table II), 2) Sera that contained elevated total IgE levels, obtained from pollen allergic patients, were evaluated with the venom RAST, and 3) sera from patients with a history of a systemic reaction after the insect sting but who were skin test negative to the allergen used in the RAST were tested in a single assay. The results shown for the single nonstung control are for six separate assays including the assay testing other sera. Results with both the Phos A and yellow jacket RAST are shown in Table II. The serum from the nonstung control bound the lowest amount of ^{125}I anti-IgE in both assays; this binding was not significantly different from the average value obtained with sera from noninsect allergic, atopic patients. In the yellow jacket RAST, sera from 46 skin test-negative patients with histories of insect allergy bound, on average, more of the labeled anti-IgE suggesting low levels of specific antivenom IgE antibodies. The values obtained, how-

TABLE II

Insect venom RASTs: evaluation of nonspecific binding (cpm $\pm$ S.D.)

Patients	Yellow Jacket RAST	Phos-A RAST
Insect history positive skin test	634.9 $\pm$ 254.4 N = 46	246.3 $\pm$ 104.7 ^a N = 43
negative		
Inhalant allergy insect history negative	457.6 $\pm$ 204.1 ^a N = 12	274.0 $\pm$ 46.2 ^a N = 6
Nonatopic-never stung ^b	376.8 $\pm$ 158.7 ^a N = 6 ^c	200.5 $\pm$ 32.9 ^a N = 6 ^c

^a For each RAST assay, nonspecific binding did not differ significantly by *t*-test.

^b Normal serum blank for all assays, lowest nonspecific binding of all sera evaluated.

^c One serum used in six assays.

ever, were not significantly different from the 12 sera from the inhalant allergy group. In contrast, no marked increase in binding of Phos A sorbent was observed with similar sera. Based on these data a value greater than 300 cpm bound was considered positive in the Phos A RAST. At this level of binding, three standard deviations (S.D.) above the nonstung control, the majority of skin test-positive patients were also RAST positive. The same types of control sera were assayed in a single RAST with the yellow jacket sorbent except that sera from patients skin test negative to this venom comprised one control population. The choice of a value of 3 S.D. above the normal control (850 cpm) as positive seemed valid for the yellow jacket RAST since it also encompassed nonspecific binding 2 S.D. above the atopic control sera. However, the increased binding observed with sera from patients skin test negative to yellow jacket venom led us to compare this nonspecific binding with RAST values obtained with sera from skin test-positive patients. The data are shown graphically in Figure 3. The fewest false-positive and false-negative RAST values (based on skin tests) occurred as the cpm bound increased from 500 to 1000; therefore, the selection of values above 850 cpm as positive, determined with various control sera, seemed appropriate.

In addition to the marked differences in background cpm bound in the yellow jacket RAST (376-634) and that for Phos A sorbents (200 to 274), the maximal amount of ^{125}I anti-IgE bound by standard and unknown sera in the two assays differed significantly. In practice a value of 6000 to 8000 cpm was maximal with yellow jacket allergosorbents, whereas 2000 to 3000 cpm was the maximal binding obtained using Phos A; the quantity of anti-IgE used was the same for both assays. The ordinates used to present RAST values in Figures 4, 5, and 6 exemplify this difference between the two assays.

Diagnostic evaluation. Sera from patients who were skin test positive to ≤ 1 $\mu\text{g}/\text{ml}$ venom protein were evaluated by RAST for venom-specific IgE antibodies. In Figure 4, the results for 57 sera using the yellow jacket RAST are presented in absolute cpm (background not subtracted). All sera were tested in triplicate in a single assay. The mean total ^{125}I anti-IgE per tube was 41,505 cpm. The nonatopic control serum bound 460 cpm (mean). Forty-nine sera are positive and eight sera negative with, as significant, binding values above 850 cpm. The mean cpm $\pm$ S.D. for the three control populations, obtained in a separate assay (Table II), are represented by bargraphs.

The leukocytes of 51 of 57 skin test-positive patients were tested for histamine release after challenge with yellow jacket venom (Fig. 4b). The eight RAST-negative patients were tested;

TABLE I

Phospholipase A RAST (Ng IgE antibody/ml serum)

Serum No.	Serum Dilution									
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
1	23.0	—	28.0	—	—	—	—	—	—	—
2	4.0	—	4.0	—	—	—	—	—	—	—
3	32.0	—	32.0	—	—	—	—	—	—	—
4	63.0	—	—	67.0	66.0	—	—	—	—	—
5	>STD	—	>STD	3239.0	—	3525.0	—	4837.0	4844.0	4863.0
6	349.0	—	372.0	438.0	596.0	596.0	—	—	—	—

Yellow Jacket RAST (cpm $\times 10^3$)										
7	3.2	5.0	6.9	6.9	6.8	—	—	—	—	—
8	3.8	6.3	9.3	11.8	16.8	16.6	—	—	—	—
9	1.1	—	1.2	—	—	—	—	—	—	—
10	1.4	—	1.5	—	—	—	—	—	—	—

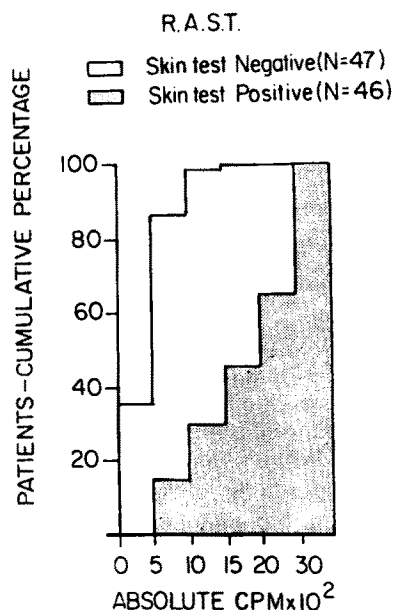


Figure 3. Analysis of the yellow jacket RAST by comparison of the cumulative percentages of skin test negative (□) and skin test-positive patients (■) with regard to the absolute cpm ^{125}I IgE bound by their sera. The least incorrect diagnoses with respect to skin tests occur between 500 and 1000 cpm.

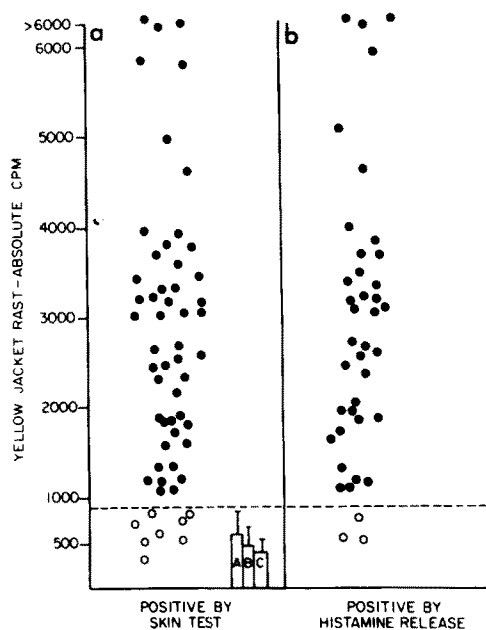


Figure 4. *a*, The cpm ^{125}I anti-IgE bound in the RAST by pretreatment sera from 57 patients skin test positive to yellow jacket venom are shown. The nonspecific binding $\pm$ S.D. by sera from three control populations is represented by bar graphs. ●, RAST-positive patients; ○, RAST-negative patients (see text and Table II for explanation). *b*, Fifty-one skin test-positive patients were tested for yellow jacket venom induced leukocyte histamine release; 37 of 43 RAST positive patients released $\geq 30\%$ histamine. Of the eight RAST-negative patients tested, the cells of only three released histamine.

only three released greater than 30% of their cellular histamine. Of the 43 RAST-positive patients tested, 37 were also positive by histamine release. Four of the RAST-positive patients, who did not release histamine upon venom challenge, also released less than 10% histamine when their leukocytes were exposed to anti-IgE.

Data on 31 patients skin test positive to Phos A, the major

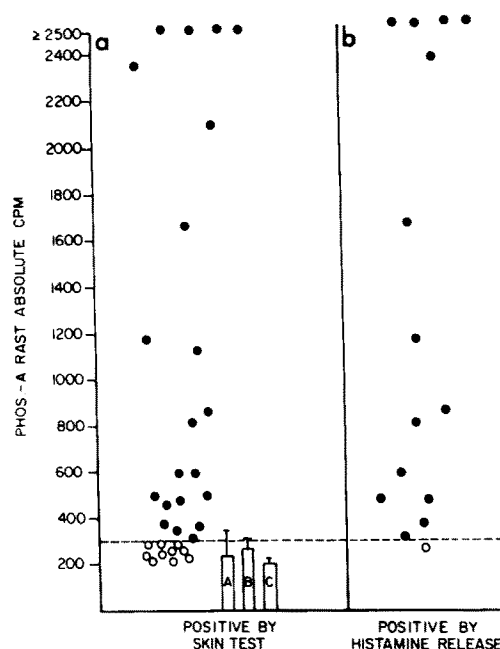


Figure 5. *a*, The RAST titers in pretreatment sera from 31 patients skin test positive to honeybee venom are shown. Bar graphs, the nonspecific binding $\pm$ S.D. of three control populations. ○, ● RAST negative and RAST positive patients, respectively. (See text and Table II for explanation). *b*, The leukocytes of 27 of 31 patients skin test positive to honeybee venom were challenged with the antigen; only one RAST negative patient released histamine. Three RAST positive patients released $\leq 10\%$ histamine to antigen or anti-IgE.

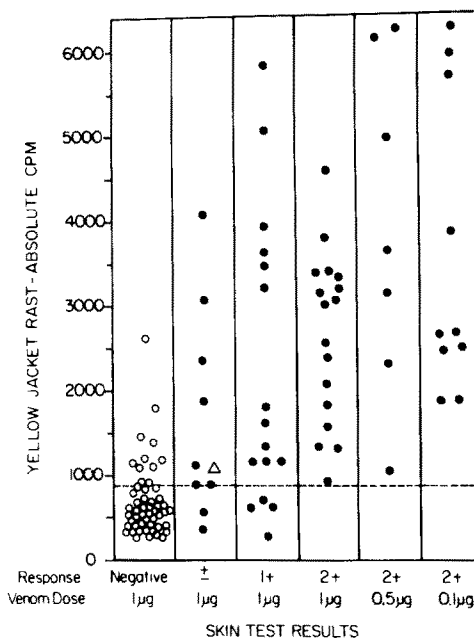


Figure 6. The scattergram compares the cpm bound in the yellow jacket RAST with skin test responses for 139 patients. Skin test negative patients (○); skin test positive patients (●). ----, 850 cpm cutoff for background binding. Δ, patient who responded systemically to an inhospital sting. A trend is seen toward increasing cpm bound in the RAST with increasing skin sensitivity, but no significant correlation was obtained between the two parameters.

allergen of honeybee venom, as well as to the whole venom at $\leq 1 \mu\text{g}/\text{ml}$ protein are shown in Figure 5. In a separate report, we will present data indicating that more than 90% of patients skin test positive to honeybee venom also respond to Phos A (A. K. Sobotka and L. M. Lichtenstein. Allergy to insect stings.

Comparison of skin test responses to honeybee venom and its major allergen, Phospholipase A, manuscript in preparation). All sera were run in a single RAST. The mean total cpm added per tube was 34,912. The nonatopic serum bound 224 cpm. Panel A, again, shows the absolute cpm obtained in the Phos A RAST and panel B the histamine release results when using honeybee venom as antigen in about half of the skin test-positive patients. As was found using the yellow jacket RAST, many (10) false-negative diagnoses by RAST occurred with relation to skin tests. Of these 10 RAST-negative patients tested, only one released histamine upon venom challenge. Three Phos A RAST-positive patients were also "nonreleasers"; i.e., neither antigen nor anti-IgE challenge led to basophil histamine release.

No correlation was found between values obtained with either the yellow jacket or Phos A RAST and the dose of antigen necessary to elicit a positive skin response. As shown in Figure 6, with the yellow jacket assay as an example, a trend exists toward higher RAST values in the sera of more sensitive patients but the wide range in the amount of IgE antibodies found in sera from patients of equal skin sensitivity (e.g., 2+ at 1 μ g; RAST range 950 to 4,600 cpm) obviates the probability of a direct relationship between the two diagnostic tests. It should be noted that the range of antigen concentrations eliciting positive skin tests is very narrow for yellow jacket venom (10^{-2} to 1 μ g/ml); this could contribute to the poor correlations observed. Although the data presented here show that most sera with borderline positive or negative RAST values were obtained from patients with negative or marginal skin responses, a negative diagnosis based on these criteria alone is not possible. The patient, indicated by the symbol (Δ), experienced a severe systemic reaction to a yellow jacket sting that occurred 2 months after his skin tests and RAST. The patient had been hospitalized on two occasions after a sting and was highly sensitive to yellow jacket venom by histamine release.

It is clear from these data, however, that the majority of incorrect diagnoses by yellow jacket RAST, both positive and negative, fall in a range of 500 cpm to 1500 cpm. At a slightly higher level, approximately 2000 cpm, only one patient positive by RAST was negative by skin test; conversely, 19 additional sera would now be considered equivocal. Evaluation of the false-negative sera for total IgE by RIST showed that about half had less than 100 ng/ml; the total IgE content of four such sera was less than 50 ng/ml.

Attempts to correlate cell sensitivity with the quantity of specific IgE antibodies as measured by the yellow jacket RAST also failed ($r = 0.3$). In contrast a marginal but significant correlation ($r = 0.65$ $p < 0.01$) was obtained when cell sensitivity to honeybee venom was compared to the ng/ml IgE anti-Phos A levels as determined by RAST. Data on 22 patients are presented in Figure 7. This difference in results may be influenced by the fact that the dose range for both skin tests and histamine release with honeybee venom and Phos A (10^{-5} to 1 μ g protein/ml) is broader than that for yellow jacket venom (10^{-2} to 1 μ g protein/ml).

A summary of the RAST data obtained on all insect allergic patients studied is presented in Table III. All patients were not skin tested with both venoms. Of 139 patients evaluated 39% were skin test positive to honeybee venom. Only one such patient failed to respond to Phos A. A larger percentage of patients skin tested (70 of 122) were sensitive to yellow jacket venom. By using a positive skin test as our criteria for the existence of insect allergy, in both the Phos A and yellow jacket

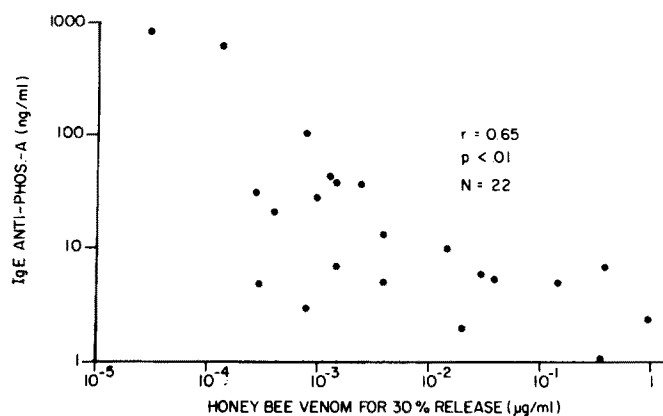


Figure 7. The relationship between cell sensitivity to honeybee venom and IgE antibody to Phospholipase A for 22 patients skin test positive to bee venom.

TABLE III

Diagnosis of insect hypersensitivity: comparison of skin test and RAST in patients with histories of systemic reactions

	Honeybee		Yellow Jacket	
	N	%	N	%
Patients Tested	139		122	
Skin Test Positive	54	39	70	57
RAST Positive ^a	44	81	57	81
RAST Negative ^a	10	19	13	19
Skin Test Negative	85	61	52	43
RAST Positive	13	15	9	17

^a Of the skin test-positive patients.

RASTs 81% of skin test-positive patients were also RAST positive; 19% were negative. In both RAST assays positive results were obtained in approximately 16% of skin test-negative patients.

DISCUSSION

The introduction of the RAST as a serologic test for diagnostic used in diseases mediated by IgE antibody has been rapidly accepted because of its potential convenience and accuracy. RAST have been utilized for the diagnosis of sensitivity to a wide variety of allergenic extracts often with little critical evaluation of the individual allergens, the precision and sensitivity of the assays, or the predictive value of a positive or negative result.

In these studies we have developed RAST for use in insect hypersensitivity. In one case we were able to utilize a highly purified honeybee venom antigen, Phos A; in the other instance we used unfractionated yellow jacket venom. Our RAST technology was carefully evaluated in order to minimize difficulties that might occur because of low total or specific IgE antibody levels, high IgE antibody levels, or high levels of IgG antivenom antibodies that markedly decrease the absorptive capacity of the RAST sorbents.

Considerable emphasis has been placed on defining positive and negative RAST values. For this purpose we evaluated three types of control sera from 1) nonatopic individuals, 2) patients with inhalant allergy but no history of insect hypersensitivity, and 3) patients with a positive history of insect allergy but negative venom skin tests. The latter two groups did not differ significantly with regard to nonspecific binding in the RAST;

the first gave the lowest binding in all instances. In spite of this effort, a distinct overlap was observed between RAST titers in skin test-positive patients and the various control groups. We found approximately 16% false-positives and 20% false-negatives in both RAST assays. Our basis for the diagnosis of insect allergy was a positive skin test with the appropriate venom. Skin tests as a criterion for clinical sensitivity were evaluated in other studies of venom immunotherapy: about 60% of skin test-positive patients respond systemically to a single sting. In data to be published elsewhere, we found that neither histamine release assays nor the RAST proved more accurate in predicting a reaction. It seems reasonable, therefore, to take a positive skin test as the best index of clinical sensitivity. In penicillin allergy, a similar clinical situation, skin tests are useful for defining a group of patients who have a greater probability of developing hypersensitivity reactions. Several studies have shown that positive skin tests predict a reaction in 60 to 70% of patients, whereas less than 1% of history positive patients with negative skin tests respond to the parenteral administration of penicillin (30, 31).

Aside from the ability to assess clinical sensitivity in this disease, our RAST data show about the same degree of positivity relative to skin tests as has been reported by others. We will, therefore, discuss primarily why limitations in RAST technology exist and attempt to point out errors that may accrue to incomplete studies.

There is a marked advantage to carrying out the RAST assay with purified antigens. Our use of a Phos A RAST rather than unfractionated honeybee venom was a deliberate choice. Although we and others have found that hyaluronidase and melittin and, perhaps a high m.w. allergen, exist in bee venom, our skin test studies have shown only one patient positive to honeybee venom who did not also react to Phos A (14, 32-35). Further, in the quantitative histamine release assay there is a very high correlation between cell sensitivity to honeybee venom and Phos A (14). The RAST with whole yellow jacket venom, in common with other crude allergens, was more marked by nonspecific binding by normal sera than was the Phos A assay. In our hands, the honeybee RAST was not dependable due to excessively high (25 to 50% of cpm added) nonspecific binding. Whatever difficulties exist with purified allergens in the RAST are magnified by the use of a mixture of allergens.

These studies demonstrate that each RAST requires its own blank and allergen-specific standard serum. An intelligent evaluation of RAST data and a definition of positive and negative results can only be judged against internal blanks and a standard serum specific for each sorbent used in a RAST. Both the background cpm and total counts bound by positive sera differ markedly among allergosorbents. The commercial practice of carrying out RAST with the binding in a single serum-allergen system as the basis for grading antibodies to other allergens seems seriously lacking. For example, a value of 1000 cpm in our Phos A RAST is highly positive whereas the same binding in the yellow jacket assay is only marginally above background.

Our methodology was chosen to maximize sensitivity and to minimize the effects of IgG-blocking antibodies and elevated specific IgE levels. The Phos A assay was capable of measuring less than 1 ng of specific IgE antibody. The yellow jacket RAST, although not quantitative, is probably of equal sensitivity. Despite this sensitivity, a significant percentage of false-negative RAST, as judged by positive histories and skin tests, occurred in both assays. In the majority of instances, the false-

negative RAST occurred in patients whose total IgE level was quite low and, thus, the RAST carried out in conjunction with total IgE levels may alert the investigator to this problem. Also, the possibility that IgG reagins could be involved cannot be excluded (36, 37). However, these data do not indicate a major role for these antibodies in allergic diseases.

As is true for most diagnostic tests, there is, in the RAST assay, an area of overlap within which it is not possible to distinguish allergic from nonallergic patients. That our results were truly falsely negative was established when two of our RAST-negative, skin test-positive patients experienced a systemic reaction to an inhospital sting. Low or negative RAST titers do not preclude a systemic reaction. This is not unexpected since it is the basophil/mast cell-bound IgE, rather than the serum IgE, which is of clinical importance. Recent studies suggest, moreover, that at a relatively low degree of basophil receptor saturation with specific IgE antibody, the cells become maximally sensitized (38). Thus, even with respect to target cells themselves, a very low level of IgE antibody is adequate to arm the cells for release. These observations effectively eliminate the use of the RAST as a quick diagnostic scan, except in highly positive sera, and this conclusion, we feel, necessitates the use of other tests before a decision to declare a patient negative can be entertained.

Difficulties with the RAST not only occur due to low levels of IgE but also due to high levels of this or other classes of specific antibody. The use of a Sepharose sorbent leads to a high allergen-binding capacity (compared to the paper disc method, for example) and enables one to choose, by dilution, allergosorbent concentrations in high allergen excess (27). Nonetheless, very high levels of IgE antibody or the evaluation of immune sera containing microgram amounts of specific IgG blocking antibody make assessment of sera at a single dilution impractical even if only relative titers are sought. For example, in the presence of elevated IgG antibody titers, undiluted sera that apparently contain approximately 300 ng of specific IgE antibody have been found after extensive dilution, actually to contain 3- to 5-fold more IgE antibody per milliliter (Table I). All methods used should, we feel, be carefully assessed and appropriate dilutions employed to obtain accurate titers; this is especially true when following IgE antibody during immunotherapy, since an apparent fall in IgE antibody may, in reality, be due to an increase in IgG antibody. It is our experience that a minimum of two dilutions should be evaluated on each serum to determine the IgE antibody titer.

The RAST is a good screening assay and it is possible to set a limit whereby, in insect allergy, a positive RAST is diagnostic, i.e., above a certain level with both the Phos A and yellow jacket venom RAST, (e.g., 600 cpm and 2000 cpm, respectively) there is a greater than a 95% concordance with skin tests. If, however, the RAST is negative, it is of much less diagnostic utility. The most definitive means for diagnosis at the present time, based on these data, remains the venom skin test. Even this is not ideal but represents the best that is presently available. Whether it will be possible to develop better diagnostic tools is not clear because the immunologic status of a patient at risk for systemic anaphylaxis is only one parameter of importance. Given a similar immunologic state, it has been possible to show that the ability of patients to release mediators differs widely (39-41). Even when this variable is considered, there remain the checks and balances to the physiologic response to released mediators, which clearly differ from patient to patient. We would, therefore, suggest that definite diagnosis

of anaphylactic sensitivity may well not be possible but rather, by using several diagnostic tests, it is possible to eliminate patients not at risk and to develop criteria that better define a group that has the greatest potential for anaphylactic reactions.

REFERENCES

1. Prausnitz, C., and H. Küstner. 1921. Studie über die ueberempfindlichkeit. *Zentralbl. Bakteri.* 86:160.
2. Ishizaka, K., and T. Ishizaka. 1967. Identification of separate γ E antibodies as a carrier of reaginic activity. *J. Immunol.* 99:1187.
3. Norman, P. S., L. M. Lichtenstein, and K. Ishizaka. 1973. Diagnostic tests in ragweed hay fever. *J. Allergy Clin. Immunol.* 52:210.
4. Lichtenstein, L. M., P. S. Norman, and J. T. Connell. 1967. Comparison between skin sensitizing antibody titers and leukocyte sensitivity measurements as an index of the severity of ragweed hay fever. *J. Allergy* 40:160.
5. Wide, L., H. Bennich, and S. G. O. Johansson. 1967. Diagnosis by an *in vitro* test for allergen antibodies. *Lancet* 2:1105.
6. Yunginger, J. W., and G. J. Gleich. 1973. Seasonal changes in IgE antibodies and their relationship to IgG antibodies during immunotherapy for ragweed hay fever. *J. Clin. Invest.* 52:1268.
7. Johansson, S. G. O., H. Bennich, and T. Berg. 1971. *In vitro* diagnosis of atopic allergy III. Quantitative estimation of circulating IgE antibodies by the radioallergosorbent test. *Int. Arch. Allergy Appl. Immunol.* 41:451.
8. Foucard, T., K. Aas, and S. G. O. Johansson. 1973. Concentration of IgE antibodies, PK titers, and chopped lung titers in sera from children with hypersensitivity to cod. *J. Allergy Clin. Immunol.* 51:39.
9. Lichtenstein, L. M., K. Ishizaka, P. S. Norman, A. K. Sobotka, and B. M. Hill. 1973. IgE antibody measurements in ragweed hayfever. Relationship to clinical severity and the results of immunotherapy. *J. Clin. Invest.* 52:472.
10. Aas, K., and S. G. O. Johansson. 1971. The radioallergosorbent test in the *in vitro* diagnosis of multiple reaginic allergy. A comparison of diagnostic approaches. *J. Allergy* 48:134.
11. Eriksson, N. E. 1977. Diagnosis of reaginic allergy with house dust, animal dander and pollen allergens in adult patients. III. Case histories, skin test and the radioallergosorbent test, RAST, compared with provocation tests. *Int. Arch. Allergy Appl. Immunol.* 53:441.
12. Sobotka, A. K., M. D. Valentine, A. Benton, and L. M. Lichtenstein. 1974. Allergy to insect stings. I. The diagnosis of IgE-mediated Hymenoptera sensitivity by venom-induced histamine release. *J. Allergy Clin. Immunol.* 53:170.
13. Hunt, K. J., M. D. Valentine, A. K. Sobotka, and L. M. Lichtenstein. 1976. Diagnosis of allergy to stinging insects by skin testing with Hymenoptera venoms. *Ann. Intern. Med.* 85:56.
14. Sobotka, A. K., R. Franklin, N. F. Adkinson, Jr., M. D. Valentine, and L. M. Lichtenstein. 1976. Allergy to insect stings. II. Phospholipase A: The major allergen in honeybee venom. *J. Allergy Clin. Immunol.* 57:29.
15. Benton, A. W., R. A. Morse, and J. D. Stewart. 1963. Venom collection from honeybees. *Science* 142:228.
16. Lowry, O. H., N. J. Rosebrough, A. L., Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.
17. Ishizaka, K., T. Ishizaka, and E. H. Lee. 1970. Biologic function of the Fc fragments of E myeloma protein. *Immunochemistry* 7:687.
18. Ishizaka, T., H. Tomioka, and K. Ishizaka. 1971. Degranulation of human basophil leukocytes by anti- γ E antibody. *J. Immunol.* 106:705.
19. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131} I labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89:114.
20. Norman, P. S., L. M. Lichtenstein, and K. Ishizaka. 1973. Diagnostic tests in ragweed hay fever. *J. Allergy Clin. Immunol.* 52:210.
21. Lichtenstein, L. M., and A. G. Osler. 1964. Studies on the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leukocytes by ragweed pollen antigen. *J. Exp. Med.* 120:507.
22. Siraganian, R. P. 1974. An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal. Biochem.* 57:383.
23. Cuatrecasas, P., and C. B. Anfinsen. 1971. Affinity chromatography. *Methods Enzymol.* 22:351.
24. Adkinson, N. F., Jr. 1976. Measurement of total serum immunoglobulin E and allergen-specific immunoglobulin E antibody. *In* Manual of Clinical Immunology. Edited by N. R. Rose and H. Friedman. American Society for Microbiology, Washington, D.C. P. 590.
25. Gleich, G. J., and R. T. Jones. 1975. Measurement of IgE antibodies by the radioallergosorbent test. I. Technical considerations in the performance of the test. *J. Allergy Clin. Immunol.* 55:334.
26. Gleich, G. J., and B. S. Jones. 1975. Measurement of IgE antibodies by the radioallergosorbent test. II. Analyses of quantitative relationships in the test. *J. Allergy Clin. Immunol.* 55:346.
27. Aalberse, R. C., E. M. Hoorweg, and E. E. Reerink-Brongers. 1974. The coupling of allergens to cellulose or Sepharose for use in the radioallergosorbent test (RAST). *In* Immunoglobulin E, Allergens and Their Interaction. Edited by R. C. Aalberse. N. V. Rodopi, Amsterdam. P. 73.
28. Sobotka, A. K., M. H. Lessof, and L. M. Lichtenstein. 1976. Absolute blocking antibody levels in passively and actively treated honeybee-sensitive patients. *J. Allergy Clin. Immunol.* 57:247.
29. Schellenberg, R. R., and N. F. Adkinson, Jr. 1975. Measurement of absolute amounts of antigen-specific human IgE by a radioallergosorbent test (RAST) elution techniques. *J. Immunol.* 115:1577.
30. Levine, B. B. 1966. Immunologic mechanisms of penicillin allergy. *Seminars in Medicine, Beth Israel Hospital, Boston* 275:1115.
31. Levine, B. B., and D. M. Zolov. 1969. Prediction of penicillin allergy by immunological tests. *J. Allergy* 43:231.
32. Shepherd, G., W. B. Elliott, and C. E. Arbesman. 1974. Fractionation of the bee venom. I. Preparations and characterization of four antigenic components. *Prep. Biochem.* 4:71.
33. Hoffman, D. R., and W. H. Shipman. 1976. Allergens in bee venom. I. Separation and identification of the major allergens. *J. Allergy Clin. Immunol.* 58:551.
34. Paull, B. R., J. W. Yunginger, and G. J. Gleich. 1977. Melittin: An allergen of honeybee venom. *J. Allergy Clin. Immunol.* 59:334.
35. King, T. P., A. K. Sobotka, L. Kochoumian, and L. M. Lichtenstein. 1976. Allergens of honeybee venom. *Arch. Biochem., Biophys.* 172:661.
36. Parish, W. E. 1970. Short-term anaphylactic IgG antibodies in human sera. *Lancet* 2:591.
37. Bryant, D. H., M. W. Burns, and L. Lazarus. 1975. Identification of IgG antibody as a carrier of reaginic activity in asthmatic patients. *J. Allergy Clin. Immunol.* 56:428.
38. Conroy, M. C., N. F. Adkinson, Jr., and L. M. Lichtenstein. 1977. Measurement of IgE on human basophils: Relation to serum IgE and anti-IgE-induced histamine release. *J. Immunol.* 118:1317.
39. Siraganian, R. P., and P. A. Siraganian. 1975. Mechanism of action of concanavalin A on human basophils. *J. Immunol.* 114:886.
40. Grant, J. A., E. Dupree, A. S. Goldman, D. R. Schultz, and A. L. Jackson. 1975. Complement-mediated release of histamine from human leukocytes. *J. Immunol.* 114:1101.
41. Kern, F., and L. M. Lichtenstein. 1976. Defective histamine release in chronic urticaria. *J. Clin. Invest.* 57:1369.