

Methods to assess RAST results in patients exposed to chemical allergens

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RAST is routinely used to assess the presence of IgE antibodies against environmental allergens in the sera of symptomatic patients. With exposure to low-mol.-mass (chemical) allergens, RAST is also done, but the interpretation of results is more difficult. A study was undertaken to assess methods which have been employed to determine the positivity of sera and to develop an objective, statistically based procedure for determination of RAST positivity to chemical allergens. Sera were obtained from 14 nonsensitized (control) subjects and examined for total IgE by radioimmunoassay, and for cyanuric chloride (CyCl)-specific IgE by RAST. Linear regression analysis revealed the correlation between the tests to be 0.94. Equations for the 95% and 99% predicted intervals were obtained. Sera from 19 cyanuric chloride workers were tested for total and specific IgE. Positivity of RAST results was determined by three methods: RAST inhibition, RAST ratio (binding to CyCl-human serum albumin (HSA) disks/HSA disks), and the regression equation where values outside the 99% predicted interval were considered to be positive and those $>95\% <99\%$ were considered to be borderline values. Determination of CyCl RAST positivity by the regression method gave results comparable to those obtained from RAST inhibition, whereas the RAST ratio resulted in many more false positive conclusions. This statistical approach to RAST analysis was also used to assess sera from patients exposed to the unrelated chemical allergens, toluene diisocyanate and formaldehyde. Conclusions based on the regression method were in good agreement with those from RAST inhibition assay. Moreover, use of one standard curve may be sufficient for interpretation of RAST results for diverse haptenic allergens. These results indicate that the regression method provides a statistical basis from which to determine positivity of RAST analyses while eliminating the need for RAST inhibition assays.

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Several low-mol.-mass chemicals are known to cause respiratory hypersensitivity (8). Although the mechanism(s) of sensitization to such chemicals remains uncertain, IgE antibodies against the chemicals have been detected in a small percentage of sensitized subjects (1, 2). One reason for the low detection rate may be inadequate method.

In vitro tests for allergen-specific IgE were developed more than 20 years ago (17). For chemicals, the RAST method is the most commonly used and typically employs a hapten conjugate in which human serum albumin (HSA) is covalently attached to the chemical hapten. The basic principle of RAST is that hapten-specific IgE in the serum binds to the hapten-HSA conjugate immobilized on a disk. The

bound IgE is detected with an enzyme or a radio-nuclide-labeled anti-IgE antibody reagent. Although the mechanics of RAST has been quite uniform, preparation of the hapten conjugates, and analysis of assay results, including the establishment of criteria for ascertaining positivity, have been highly variable. Some of the criteria currently employed to ascertain RAST positivity are summarized in Table 1. Several of these methods require a separate RAST to assess binding of IgE to HSA.

We sought to obviate one cause of the uncertainty regarding the role of IgE antibody in chemical hypersensitivity by developing an objective method for assessment of RAST data and determination of RAST positivity. The purpose of this study was to

Table 1. Criteria for RAST positivity

- RAST binding $> \bar{x} + 2$ SD of control values
- RAST binding $> \bar{x} + 3$ SD of control values
- RAST binding $> \bar{x} + 4$ SD of control values
- RAST binding $> \bar{x} + 2$ SD and RAST inhibition assay

- Ratio cpm $\frac{\text{conjugate}}{\text{HSA}} \geq 2$
- Ratio cpm $\frac{\text{conjugate}}{\text{HSA}} \geq 3$
- Ratio cpm $\frac{\text{conjugate} - \text{HSA}}{\text{HSA}} \geq 2$
- Regression method (values outside 95% predicted interval for total IgE based on IgE-RAST regression)

develop a method which was statistically based, easy to use, and applicable to all chemical allergens.

Material and methods

Serum samples

Sera were obtained from occupational physicians. Donors were employed in the production of either cyanuric chloride ($n = 14$) or toluene diisocyanate (TDI) ($n = 25$), or were thought to have been exposed to formaldehyde ($n = 6$). Control sera ($n = 48$) were from subjects employed at the TDI facility but who were not exposed to the chemical. All sera were stored at -20°C until assayed. A clinical history of each subject was obtained from the attending physician.

Antigens

Cyanuric chloride is recognized to be a dermal and respiratory tract irritant and sensitizer. It is a white powder, insoluble in water, which hydrolyzes to cyanuric and hydrochloric acids. A cyanuric chloride (CyCl)-HSA antigen was prepared using a 50:1 molar ratio of CyCl to HSA for reaction. Specifically, 0.188 g of CyCl in 1 ml acetone was added dropwise to a rapidly stirred solution of 1 g HSA dissolved in 200 ml of 0.05 M NaPO_4 , pH 7.4. After 30 min, the reaction was terminated by addition of a molar excess of monoethanolamine (Fisher Scientific, Pittsburgh, PA, USA). The product was purified by filtration with Whatman no. 4 paper, followed by isoelectric precipitation. The conjugate was dissolved in 0.1 M ammonium carbonate, dialyzed against distilled water, and then lyophilized. Calculation of the mol CyCl bound per mol HSA was based on analysis with 2,4,6-trinitrobenzene sulfonic acid (15). Analysis indicated 43 mol CyCl/mol HSA.

A TDI-HSA conjugate was prepared in an analogous manner, but without acetone. Specifically, 100 μl TDI (TD_{80} (Miles Chem. Corp., Pittsburgh,

PA, USA), 80:20 mixture of 2,4- and 2,6-TDI isomers) was added dropwise to a vigorously stirred solution of 1 g HSA dissolved in 200 ml 0.05 M NaPO_4 , pH 7.4. The solution was stirred for 3 h at 37°C . The reaction was terminated by the addition of monoethanolamine, and the product was isolated as described above. Spectral analysis of the conjugate (10) indicated 35 mol TDI/mol HSA.

A conjugate of formaldehyde with HSA was prepared as previously described (11). Briefly, 175 μl formaldehyde (40% solution) was added to a rapidly stirred solution of 0.5 g HSA and 1.175 g sodium cyanoborohydride in 500 ml 0.05 M NaPO_4 , pH 7.4. The mixture was stirred for 2 h at 37°C . The reaction was terminated by addition of 100 μl monoethanolamine, and the product was purified as described above. Analysis of the antigen (15) indicated 55 mol formaldehyde per mol HSA.

Serologic assays

Total serum IgE. Serum IgE level was determined by a radioimmunoassay kit (Hycor, Ventrex Division, Portland, ME, USA).

RAST. Antigen-coated disks were prepared by reacting the hapten-HSA conjugates with cyanogen bromide-activated paper disks, as described previously (7). For assay, a disk was placed in 50 μl of serum and incubated for 16 h at ambient temperature. The disks were then washed and 50 μl ^{125}I -labeled antihuman IgE (Hycor-Ventrex reagent) was added. After 16 h, the disks were washed and then counted in a gamma scintillation counter. Titers were calculated as the percentage of the added cpm which remained bound to the disk (% binding).

RAST inhibition assay. A volume of 25 μl serum was added to 25 μl solution containing 100–1000 μg of hapten-HSA conjugate, and the mixture was kept at 37°C for 2 h. A hapten-HSA-coated disk was added, and RAST was done as described above. The degree of inhibition of reaction was calculated as follows:

$$\% \text{ inhibition} = 100 \left(1 - \frac{\text{net cpm with inhibitor}}{\text{net cpm with buffer}} \right)$$

Inhibition $\geq 25\%$ was considered to be positive indication of the presence of specific IgE antibody.

Statistics

Linear regression analysis was done with MINITAB.

Results

Sera from the 48 control subjects were assessed for total IgE. From this group, 14 sera were selected.

The sera ranged in total IgE from 2 to 820 IU/ml. They were then assessed by RAST for IgE binding to CyCl-HSA-coated disks. The values for IgE and RAST are shown in Fig. 1. Linear regression analysis yielded the following values: $y = 1.60 + 0.0062x$; $r = 0.94$. The 95% confidence interval (C.I.) is shown in Fig. 1A and the predicted intervals (P.I.) in 1B. All points but one were within the 95% P.I.; all points were within the 99% P.I.

This association between IgE and RAST was used to assess RAST results of sera obtained from 19 workers employed in CyCl production. The total IgE level of each serum was determined, and then the sera were assessed for specific IgE by RAST with CyCl-HSA disks. The data were compared with the regression statistics obtained for the control population. As shown in Fig. 2, five of the points were outside the 99% P.I. These were concluded to be RAST positive. Four points were within the 95–99% interval. They were concluded to be borderline values.

RAST positivity was determined by RAST inhibition assay of these sera. Results of all analyses are given in Table 2. There was a clear association be-

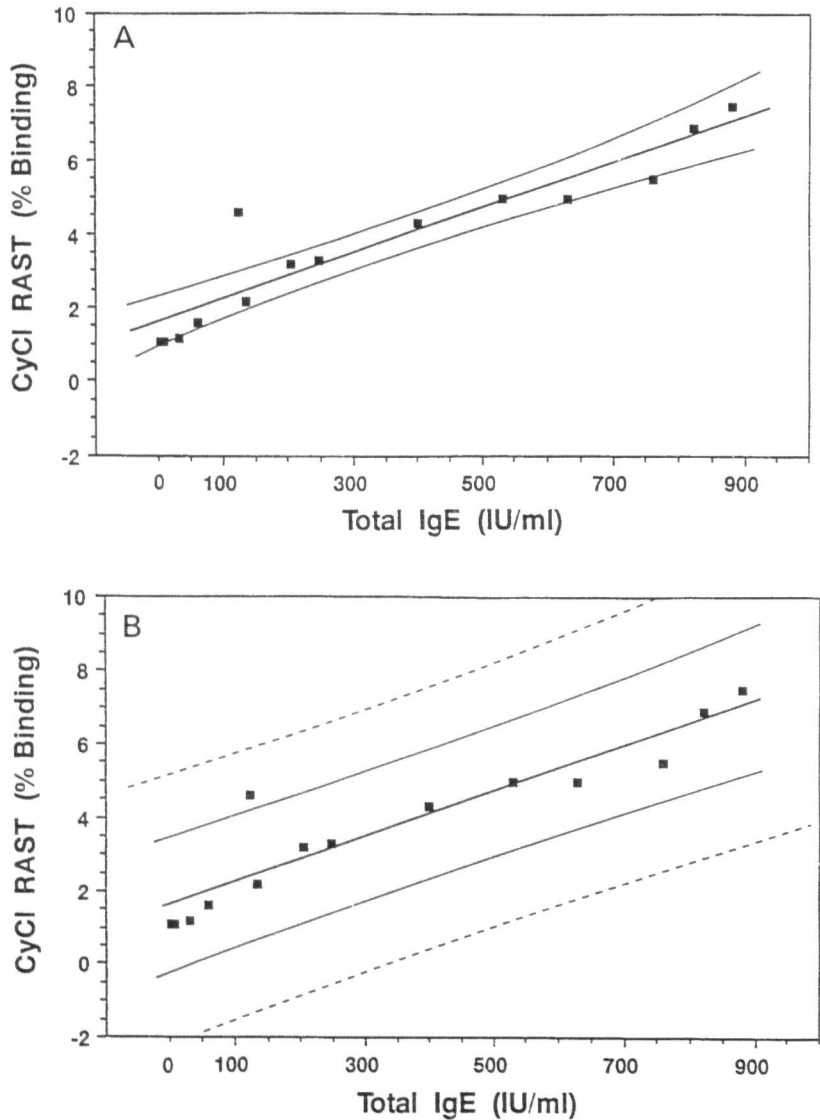


Fig. 1. Total IgE and CyCl-HSA RAST. Assays were done on 14 control sera. Linear regression analysis yielded $y = 1.60 + 0.0062x$; $r = 0.94$. A) 95% C.I.; B) 95% P.I. (solid lines), 99% predicted interval (broken lines).

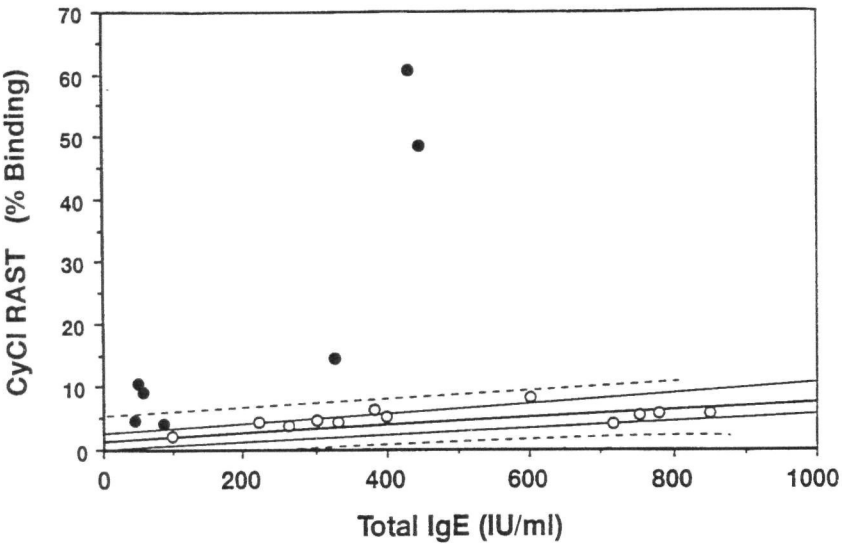


Fig. 2. Serologic assessment of total and CyCl-specific IgE of 19 subjects exposed to cyanuric chloride. Sera were also assessed by RAST inhibition assay. Closed circles indicate samples which were positive by RAST inhibition assay; open circles indicate samples which were negative by RAST inhibition. Lines represent linear regression analysis with 95% and 99% C.I., as given in Fig. 1B.

Table 2. Comparison of RAST inhibition and regression analysis to indicate positive cyanuric chloride RAST results

Serum	Total IgE (IU/ml)	CyCl RAST (% bound)	RAST inhibition (%)	Regression analysis
E1	44	4.8	51	BL
E2	49	10.8	68	POS
E3	57	9.3	59	POS
E4	84	4.3	29	BL
E5	98	2.2	8	NEG
E6	220	4.4	11	NEG
E7	262	4.0	7	NEG
E8	300	5.4	5	NEG
E9	325	14.7	49	POS
E10	330	4.5	0	NEG
E11	380	6.5	4	BL
E12	397	5.3	12	NEG
E13	430	60.7	33	POS
E14	445	48.6	59	POS
E15	600	8.5	15	NEG
E16	716	4.9	9	BL
E17	752	5.5	18	NEG
E18	780	5.8	0	NEG
E19	850	6.1	3	NEG

Numbers in bold indicate positive RAST inhibition ($\geq 25\%$); see Material and methods. Abbreviations: POS, positive; NEG, negative; BL, borderline result.

between results obtained by RAST inhibition and by regression analysis. In every case where RAST inhibition was positive, the regression method gave a positive result. In two instances with negative inhibition (E11, E15), the regression method indicated a borderline positive result.

RAST ratio

A third procedure which has been employed to determine RAST positivity for hapten-specific IgE is

the RAST ratio. Six of the sera listed in Table 2 were additionally tested for RAST positivity by the RAST ratio method. The six sera were selected to span a wide range in total IgE levels. The sera were tested for IgE reactive with HSA-coated disks. The ratio of binding to each type of disk was calculated. In addition, the ratio was calculated after subtraction of HSA binding from binding to CyCl-HSA disks. Results are presented in Table 3. The RAST ratios ranged from 1.8 to 19.4. If we assume a RAST ratio > 2.0 to be a positive result, several of the sera which were negative by both RAST inhibition assay and regression analysis were positive for CyCl-specific IgE by the RAST ratio method of analysis. The regression method and RAST ratio after subtraction of HSA binding gave results consistent with the RAST inhibition procedure and showed that only serum E14 was positive. This serum gave a RAST ratio of 19.4.

TDI RAST

The regression method was tested for applicability to other haptenic allergens. A standard curve was generated, depicting the relationship between total IgE and TDI RAST for 43 sera from control subjects. The data, together with regression analysis, are presented in Fig. 3. The regression equation for TDI was as follows: $y = 1.86 + 0.0041x$; $r = 0.89$. Comparison of the regression equations obtained for the two chemical allergens, CyCl and TDI, indicated a high degree of similarity.

With sera from 25 workers who were exposed to TDI, a study was conducted to assess the ability of the regression method to detect samples positive for TDI RAST. Results from the regression analysis were compared with those obtained from RAST inhibition analysis. Values for the sera are displayed in Fig. 4. Values for nine of the 25 sera exceeded the upper 95% P.I. of the standard curve; six of the values were at or above the 99% P.I. Comparison of the interpretation of RAST results for the nine sera by regression analysis with that obtained from RAST inhibition analysis is presented in Table 4.

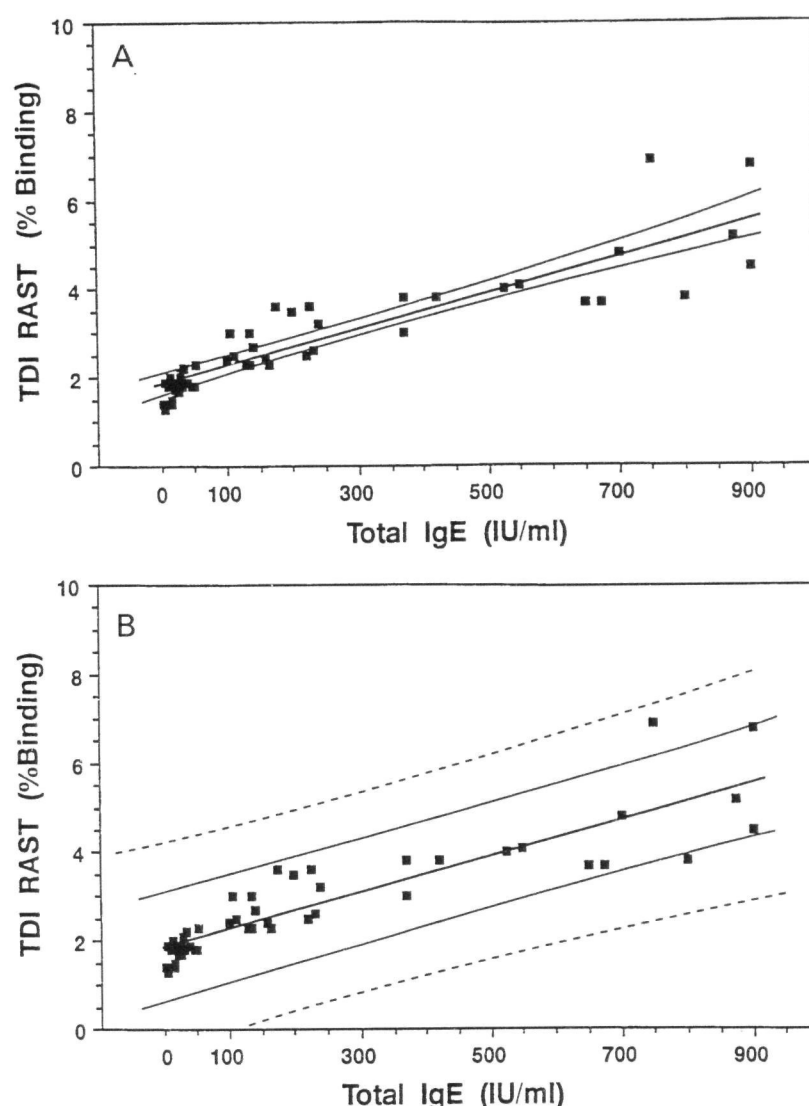


Fig. 3. Total IgE and TDI-HSA RAST. Assays were done with serum samples from 43 control subjects without TDI exposure. Linear regression analysis yielded $y = 1.86 + 0.0041x$; $r = 0.89$. A) 95% C.I.; B) 95% P.I. (solid lines), 99% P.I. (broken lines).

Five of the sera were positive by RAST inhibition analysis. Of these, four were scored positive by regression analysis. The fifth serum was scored as a borderline result. Of the four sera which were negative by RAST inhibition analysis, the regression method scored two as negative, and two as positive.

Formaldehyde-HSA RAST

The similarity of the regression equations for CyCl-HSA RAST and TDI-HSA RAST data prompted

Table 3. Comparison of three methods for determining RAST positivity

Serum	Total IgE (IU/ml)	% Binding CyCl-HSA	RAST		Inhibition assay	Regression analysis
			CyCl-HSA HSA	CyCl-HSA-HSA HSA		
E5	98	2.2	1.8	0.96	NEG	NEG
E7	262	4.0	2.5	1.6	NEG	NEG
E12	397	5.3	2.8	1.8	NEG	NEG
E14	445	46.6	19.4	18.3	POS	POS
E16	716	4.9	2.2	1.2	NEG	NEG
E17	752	5.5	2.2	1.2	NEG	NEG

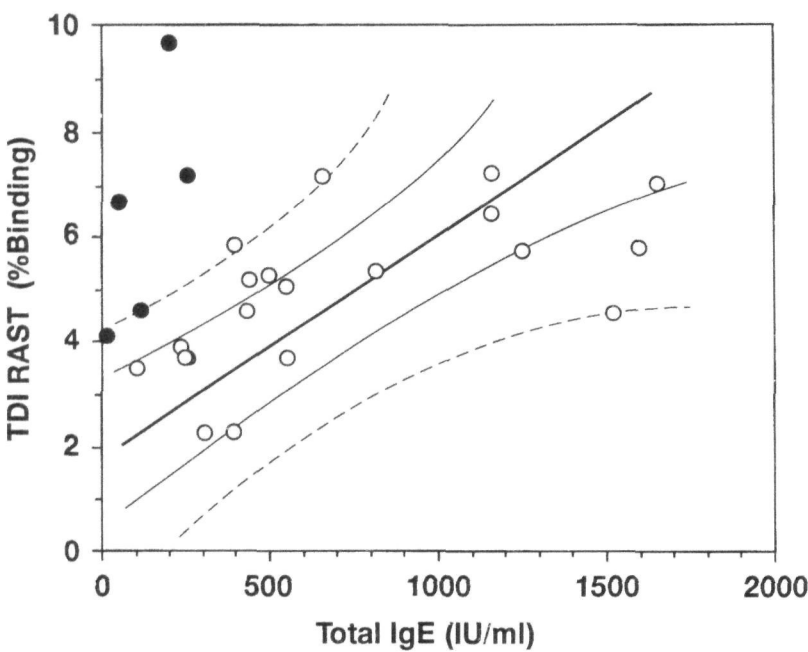


Fig. 4. Serologic assessment of total and TDI-specific IgE of 25 subjects exposed to TDI. Sera were also assessed by RAST inhibition assay. Closed circles indicate samples which were positive by RAST inhibition assay; open circles indicate samples which were negative by RAST inhibition analysis. Lines indicate linear regression analysis with 95% and 99% P.I., as given in Fig. 3B.

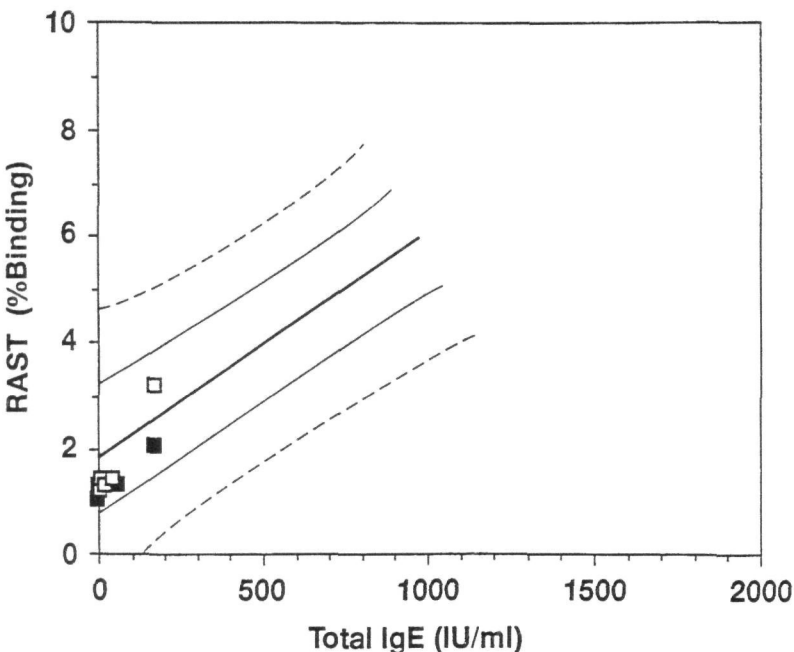


Fig. 5. Interpretation of formaldehyde-HSA RAST results by standard regression equation derived from TDI control samples. Sera from three control subjects (closed squares), and six subjects with possible exposure to formaldehyde (open squares) were assessed for total IgE; then they were tested for RAST binding to formaldehyde-HSA disks. Data were compared with regression statistics obtained for TDI-HSA (as in Fig. 3). All points were within 95% P.I. of TDI standard curve.

Table 4. Comparison of methods to interpret RAST for IgE antibodies against TDI

Serum	Total IgE (IU/ml)	TDI RAST (% bound)	RAST* inhibition (%)	Regression analysis
T ₁	16	4.1	39	BL
T ₂	48	6.7	54	POS
T ₃	115	4.6	50	POS
T ₄	200	9.7	39	POS
T ₅	250	7.2	47	POS
T ₆	396	5.9	0	POS
T ₇	430	4.6	16	NEG
T ₈	550	5.1	0	NEG
T ₉	650	7.2	0	POS

* Numbers in **bold** indicate positive inhibition results.
BL: borderline value.

a study to determine whether these regression equations were applicable to other, unrelated haptenic RAST evaluations. Sera from three control subjects and six subjects with possible formaldehyde exposure were assessed for total IgE and for IgE binding to formaldehyde-HSA disks. Results are shown in Fig. 5. Values for the control sera were within the 95% P.I. for both CyCl and TDI standard data. Each of the patient sera was also within these limits.

Discussion

RAST is routinely used to determine allergen-specific IgE where allergens are of high mol. mass. Its asso-

ciation with clinical disease has been extensively discussed (2). RAST for low-mol.-mass allergens has been less successful. Only a small percentage of subjects with clinical diagnosis of chemical sensitivity are found to have allergen-specific IgE. It is uncertain whether the low percentage of positive sera reflects the absence of such antibodies (9), or the varied methods used for assessment and evaluation of results (12).

The difficulties encountered in designing a RAST for low-mol.-mass chemical allergens have been discussed (9) and include the selection of a protein "carrier", as well as the extent of adduction of the low-mol.-mass allergen to the carrier, in addition to consideration of the contribution to RAST of carrier-specific antibody.

Czuppon & Baur (3) reported finding false positive RAST values with the standardized test kit of Pharmacia (Phadebas), which employs RAST classes. In the standardized method, 50 µl serum is pipetted onto an allergen-coated disk in a test tube. The sample is incubated at ambient temperature for 3 h, and then is washed three times at 10-min intervals. ¹²⁵I-labeled rabbit antihuman IgE is then added, and the tubes are again incubated at room temperature for 26–28 h. The disks are washed and then counted in a gamma counter. These authors found that it was important to transfer the disks to unused tubes after the final washing to obtain valid counts for the disks.

Total IgE has been found to influence not only RAST but also the skin prick test (6). In the latter,

a study of 593 hay fever patients revealed a positive correlation for eight tree pollen allergens. The correlation between total IgE and RAST results for each of the allergens ranged from 0.28 to 0.47 ($P < 0.05$).

It has been noted that false positive RAST may be obtained for patients with a total IgE in excess of 1500 kU/l (13) or greater than 3000 kU/l (4). The explanation has been assumed to be excess background binding. However, false positive RAST was found with IgE in the range of 50–300 kU/l (6). It is clear that total IgE must be taken into account when either *in vitro* or *in vivo* test results are analyzed.

Factors affecting RAST results for a haptenic conjugate antigen were analyzed (16). Sera were obtained from six patients with diagnosis of asthma and positivity to bronchoprovocation challenge with diphenylmethane-4,4'-diisocyanate (MDI). All subjects had specific IgE when the RAST used a conjugate with a low molar ratio of isocyanate:carrier. When the molar ratio of MDI:HSA in the conjugate exceeded 30, RAST values were lower. Control sera gave elevated RAST with conjugates of 30 or more MDI:HSA composition. Total IgE values in excess of 200 IU/ml affected RAST results.

In 1979, a modified RAST (MRT) was introduced as an alternative to the standard procedure (14). This MRT simplified the calculation and reporting of results. The assay uses 100 μ l of a 25 kU/l total IgE reference sample, which is incubated with a disk to which anti-IgE has been covalently coupled. After appropriate washing procedures, 50 μ l ^{125} I-anti-IgE is added. The disk is again washed and then placed in a gamma counter and counted until 25 000 counts are obtained. The counting time is determined. A calibration curve is set by five points after assay. The relationship between counts is defined as follows: 750 counts = 0.02 U/ml, 1600 counts = 0.01 U/ml, etc. The curve is linear when plotted in a log-log coordinate system, and has an invariable slope and intercept. The test is used for high-mol.-mass allergens and reports results in units of IgE. The method was found to be inaccurate when assaying samples containing both high and low IgE content (5). Furthermore, the assay depends on accurate and precise assessment of one sample. If these assumptions are not met, the calibration curve will be faulty, as will all results which are dependent on it. Another problem with the MRT method is that as isotopically labeled reagents decay they require more time to achieve preset counts. It was concluded (5) that the MRT could be used as a semiquantitative assay for allergen-specific IgE, employing appropriate negative controls to minimize the false positive rate of the assay. The test is not appropriate in a research or clinical situation.

The association between total IgE and RAST titers was used to develop a method for determination of RAST positivity. As seen in Figs. 1 and 3, this association was found to hold over at least a three-log range of total IgE values (1–1000 IU/ml) and yielded correlation coefficients of approximately 0.9. Subsequent tests were done with 10 normal sera having total IgE of 1000–3000. Each of the 10 sera gave a value within the 95% P.I. of the regression equation, indicating that the equation should also be predictive for allergic patients with total IgE in the 1000–3000 IU/ml range.

The similarity of regression equations obtained for the two chemically unrelated haptens, cyanuric chloride and toluene diisocyanate, indicates that the correlation was largely independent of the particular RAST antigen employed. Because of the strong association between the two serologic assays, the possibility that positive RAST samples could be identified as those which had values outside the range predicted from the correlation was explored.

RAST inhibition was selected as the standard method by which to judge the positivity of sera. This was done because this method provides information relative to the specificity of the antibodies assessed. The criteria for conclusion of a positive RAST inhibition result were evidence of dose-dependent RAST inhibition, and inhibition of at least 25% magnitude.

For CyCl RAST, the regression method correctly identified as positive all sera scored as positive by RAST inhibition (Table 2). It also indicated two negative sera as borderline readings. For TDI RAST (Table 4), the regression method correctly identified as positive each of the five samples positive by RAST inhibition assay. It also scored as positive two of the remaining 20 negative sera, thus yielding a low percentage of false positive results. Assessment of the performance of the regression method with the formaldehyde RAST data was limited because of the small number of samples and the finding that none of the sera revealed formaldehyde-specific IgE antibody. However, all of the data were found to lie within the C.I. predicted by both the TDI and CyCl regression equations (for example, Fig. 5). Therefore, it is possible that a single regression equation may be used to determine positivity of RAST results for any haptenic allergen. Such a possibility requires further testing with various haptenic structures.

In comparing methods currently used to determine RAST positivity for low-mol.-mass chemical allergens, there are clear advantages with the regression method. The RAST ratio procedure has the disadvantage of requiring two RAST assays; one for RAST binding to HSA disks, and the other for binding to hapten-HSA disks. Moreover, the decision as to which ratio of hapten-HSA:HSA indi-

cates a positive RAST result is arbitrary. As shown by Table 3, a RAST ratio of 2 can yield numerous false positive results. However, the RAST ratio method, in which HSA binding is subtracted from conjugate binding before calculating the ratio, gave results consistent with RAST inhibition and the regression method.

The RAST inhibition assay is currently the preferred method by which to determine RAST positivity. However, this method is lengthy. It requires that a RAST be done, and then that a series of confirmatory assays be undertaken if RAST results appear to be positive. The procedure is not only time-consuming, but also requires increased use of reagents, including radionuclides.

The regression method requires two serologic assays for each serum sample: total IgE and RAST. These assays may be done simultaneously. Importantly, both assays yield information relevant to diagnosis of symptomatic patients. Moreover, the possibility that a single regression curve can be used to assess data for all haptenic allergens suggests that RAST interpretations can be obtained rapidly for any allergen by this method. The method has a tendency to a low percentage of false positive readings. More importantly, no false negative results have been obtained.

In summary, this report presents a new approach to determination of RAST positivity to haptenic allergens. The method is based on the demonstrated association between RAST and total serum IgE. The method offers the promise of rapid, accurate interpretation of RAST data for any haptenic allergen.

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