

Maxisorp RAST

A sensitive method for detection of antigen-specific human IgE in culture fluids

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For determination of allergen-specific IgE in cell culture supernatants and other highly diluted IgE preparations a radioallergosorbent test (RAST) based on high adsorption polystyrene test tubes has been developed ("Maxisorp RAST"). *Cladosporium herbarum* extract was used as a model allergen but timothy grass pollen, house dust mite and dog dander showed similar results. The test showed specificities of both allergen and immunoglobulin isotype and significant correlations ($r = 0.67-0.88$) with established RAST procedures were found. Based on immunosorbent-purified allergen-specific IgE the estimated sensitivity was within the order of 150-300 pg allergen-specific IgE per ml. The within-assay variation was 4-9% and the inter-assay-variation 17-29%. The Maxisorp RAST is useful as an inhibition assay for quantitating allergenic activity down to 0.1 biological units/ml of allergen extracts.

Key words: allergen; allergen standardization; *Cladosporium herbarum*; IgE; RAST.

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CLINICAL ASPECTS

This paper describes a sensitive RAST-method for detecting allergen-specific IgE. Knowledge of the occurrence of specific IgE in cord blood and in preparations from leucocytes cultured *in vitro* might increase our understanding of IgE-producing cell clones and the regulation of specific IgE-synthesis.

Since the introduction of the Radio Allergo Sorbent Test (RAST) for determination of allergen-specific IgE (16), numerous variants have been described, including a widely used commercial design, in which allergen covalently coupled to paper discs is used as solid-phase (1). RAST is well established in clinical allergology for testing patient sera. Few attempts have been made to increase the sensitivity in order to quantitate specific IgE in low IgE experimental systems, such as lymphocyte culture supernatants or stripings from basophil granulocytes (13).

Based on previous development of an assay for total IgE (11) we designed a highly sensitive RAST method using as a model allergen *Cladosporium herbarum*. As it would be of interest to compare allergen-specific and total IgE in different systems, an attempt has been

made to determine allergen-specific IgE in nanograms.

MATERIAL AND METHODS

Allergens

A lyophilised allergen extract of the mould *Cladosporium herbarum* (Pharmalgen m2, reconstituted to 100,000 Biological Units/ml (BU/ml)) was obtained from Pharmacia, Uppsala, Sweden. The extract showed more than 60 precipitates in crossed immunoelectrophoresis (CIE). Other allergen extracts used were: timothy grass pollen (*Phleum pratense*) (Aquagen SQ 225, 100,000 SQ/ml) from ALK, Copenhagen, Denmark; house dust mite (*Derma-*

tophagoides pteronyssinus), and dog dander (Pharmalgen d1 and e5/e2, 10,000 BU/ml) from Pharmacia.

Sera

Serum from one patient (J.C.) allergic to *Cladosporium herbarum* was used throughout the study. The patient had not received immunotherapy but expressed moderate titers of allergen-specific IgG_{1,2,4} as determined by the IgG-subclass-RAST (7). Shown by crossed radioimmuno-electrophoresis (CRIE) the IgE in the serum had specificity to at least 7 components of the allergen extracts.

Seventy-nine sera from the Allergy Serum Bank of the department were chosen for comparison between different RAST methods. The sera were selected to represent a broad spectrum of titers [RAST class-0-4, as determined by the Phadebas RAST or by the standard method (AL-RAST, 10)] against the four allergens: timothy, mite, dog and *Cladosporium herbarum*. A pool of RAST-negative sera from blood donors was used as normal human serum (NHS) (10).

Maxisorp-RAST

To test tubes ("Maxisorp", NUNC, DK-4000 Roskilde, Denmark) were added 300 µl of the allergen extract appropriately diluted (see results) with PBS-7.4 (sodium phosphate 0.1 M, sodium chloride 0.15 M, sodium azide 10 mg/l, pH 7.4) and the tubes were incubated at room temperature overnight. After coating, the tubes were washed 4 times in PBS (sodium phosphate 1/15 M, sodium azide 10 mg/ml, pH 7.0) including 1% tween (v/v).

The samples to be tested were diluted (PBS + tween) and 300 µl were added to the tubes and incubated overnight at room temperature. On the next day the tubes were washed and 300 µl of ¹²⁵I anti-IgE (Pharmacia, Uppsala, Sweden) (diluted 1:12, about 10,000 c.p.m.) were added, incubated overnight and on the following day washed and counted in a gamma-scintillation counter. All incubations were performed on a vibrator in order to improve diffusion in the tubes.

Results were compared to a reference serum with IgE specific for the same allergen system (10) and expressed in sorbent units (SE) per ml. The reference sera used in the four allergen systems were all of RAST-Class 4 in AL-RAST and paper-RAST (see below), and were assigned the number of SE/ml found in the AL-RAST.

The test could be used as an inhibition assay for quantifying allergens. The standard serum in a suitable dilution was incubated with allergen extract 1:1 overnight at 4°C. On the next day the mixture was centrifuged, and the supernatant tested in the Maxisorp RAST as described. The result was expressed as

$$\% \text{ inhibition} = 100 - 100 * \frac{\text{sample-blank}}{\text{uninhibited-blank}}$$

Other RAST systems

Screening of sera was performed by means of the aluminium hydroxide RAST (AL-RAST) (10), in which the allergens are adsorbed on a gel of Al(OH)₃. ¹²⁵I rabbit anti-IgE from Pharmacia was used as detector antibody, and a positive timothy serum as reference.

Two forms of paper discs were used as allergosorbents in the "paperdisc RAST" (1). First, discs coupled with the same batches of allergen extracts as employed in the Maxisorp RAST, and second, discs supplied by Pharmacia (g6, d1, e2/e5 and m2, respectively). In both circumstances ¹²⁵I rabbit anti IgE (Pharmacia) was used as detector antibody. In the former RAST we used reference sera as described for the Maxisorp RAST, and in the latter, the reference serum supplied with the paper discs.

Assay for total IgE

This assay has previously been described (11). In short, anti-IgE (Behringwerke, Marburg, FRD) was coated on "Maxisorp" polystyrene tubes which were subsequently blocked by incubation with fetal calf serum in RPMI medium. The tubes were then washed thrice and incubated with serum and after washing the IgE was detected using ¹²⁵I rabbit anti human IgE (Pharmacia).

IgG-subclass RAST

This analysis was done according to Malling and Djurup (3, 7): Briefly, Maxisorp test tubes were coated with 500 μ l of the allergen extract. The sera to be tested were diluted at least 20 times and added to the tubes for 2 h, followed by incubation with monoclonal antibody against IgG-total or one of the IgG-subclasses. Finally, the tubes were incubated overnight with 500 μ l of ^{125} I-labelled rabbit anti-mouse IgG and counted in a gamma-scintillation counter.

Preparation of allergen-specific IgE

Cladosporium herbarum allergen extract (120,000 BU) was coupled to 3g of cyanogenbromide-activated sepharose (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. After extensive washing at alternating acid and neutral pH, the gel was incubated with a mixture of 1 ml serum J.C. which had previously been freed of IgG by incubation for 24 h with 6 ml of rabbit anti-human IgG (Code No. A 090, DAKOPATTS, Copenhagen, Denmark) and then centrifuged (1,500 g for 15 min). The mixture of serum and rabbit antibody incubated slowly rotating with the gel for 24 h, and after washing the gel, the bound immunoglobulin was eluted with potassium thiocyanate, 3 M, and dialysed and concentrated to 10 ml by ultrafiltration (Millipore "Immersible", Bedford, Mass., USA, Mw cut-off 30 kD). Several other elution buffers including glycine (pH 2.4) and carbonate (pH 11.0) were tried, but secondary incubation of the gel with ^{125} -I anti-IgE, showed that a significant part of the bound IgE remained adsorbed to the gel after elution with these buffers. With thiocyanate, however, the bound radioactivity was minimal.

Statistics

Correlations between different RAST-techniques were determined by Spearman's Rank Correlation using the MINITAB statistical software package on an IBM XT personal computer.

RESULTS

Coating of the tubes and test for specificity

In dose-response experiments (not shown) it was found that 5000 BU/ml was optimal for *Cladosporium herbarum* allergen in coating the surface of the tubes. Similar optimizations were performed in the three other allergen systems. Coated tubes could be stored at 4°C for at least 1 week. When patient serum (1:40) was incubated in the test tubes themselves (without allergen, but blocked with PBS + tween) no increase in radioactivity occurred as compared with buffer. Neither NHS (1:10) nor an IgE-rich serum of 2500 U/ml (1:10) without *Cladosporium herbarum* specificity, led to any significant binding compared with patient serum (1:640).

As shown in Fig. 1a the detection of specific IgE could be inhibited in a dose-related manner by preincubating serum with allergen extract. An allergenic activity as low as 0.1 BU/ml could be detected as it produced an inhibition of 38%. Control for interference from IgG "blocking" antibodies was performed by precipitation of IgG with rabbit anti-human IgG. The right side of Fig. 2 illustrates that the specific IgG has disappeared from the precipitated serum, as opposed to IgE which remains at the same level. In the IgE-maxisorp RAST the precipitated and the native serum produced identical dilution curves (Fig. 2, left side).

Precision and comparison with other RAST systems

When 31 preparations of IgE stripped from basophil granulocytes were run in duplicate, the mean of the coefficient of variation was 3.5% (SD = 2.2%). In the *Cladosporium herbarum* system the interassay variation, including the variation of the solid-phase preparation, was 16.9%; determined by testing 3 sera on 3 different days.

The precision of the allergen systems timothy, mite and dog is shown in Table 1. It appears from the table that the variations found in these three systems are somewhat poorer than the *Cladosporium herbarum* system. This is probably caused by the broader spectrum of titers of the sera included in Table 1.

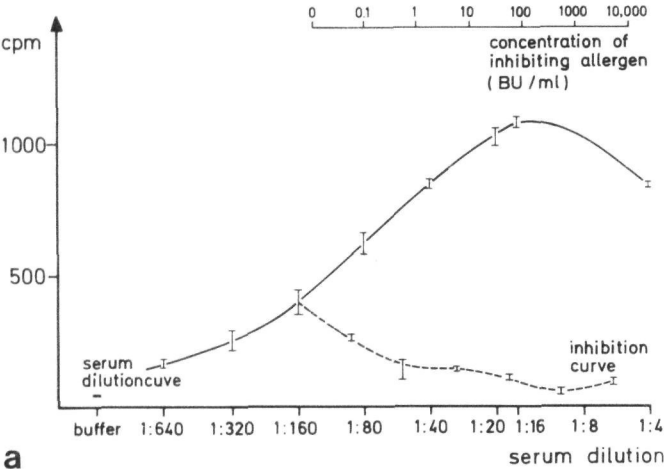
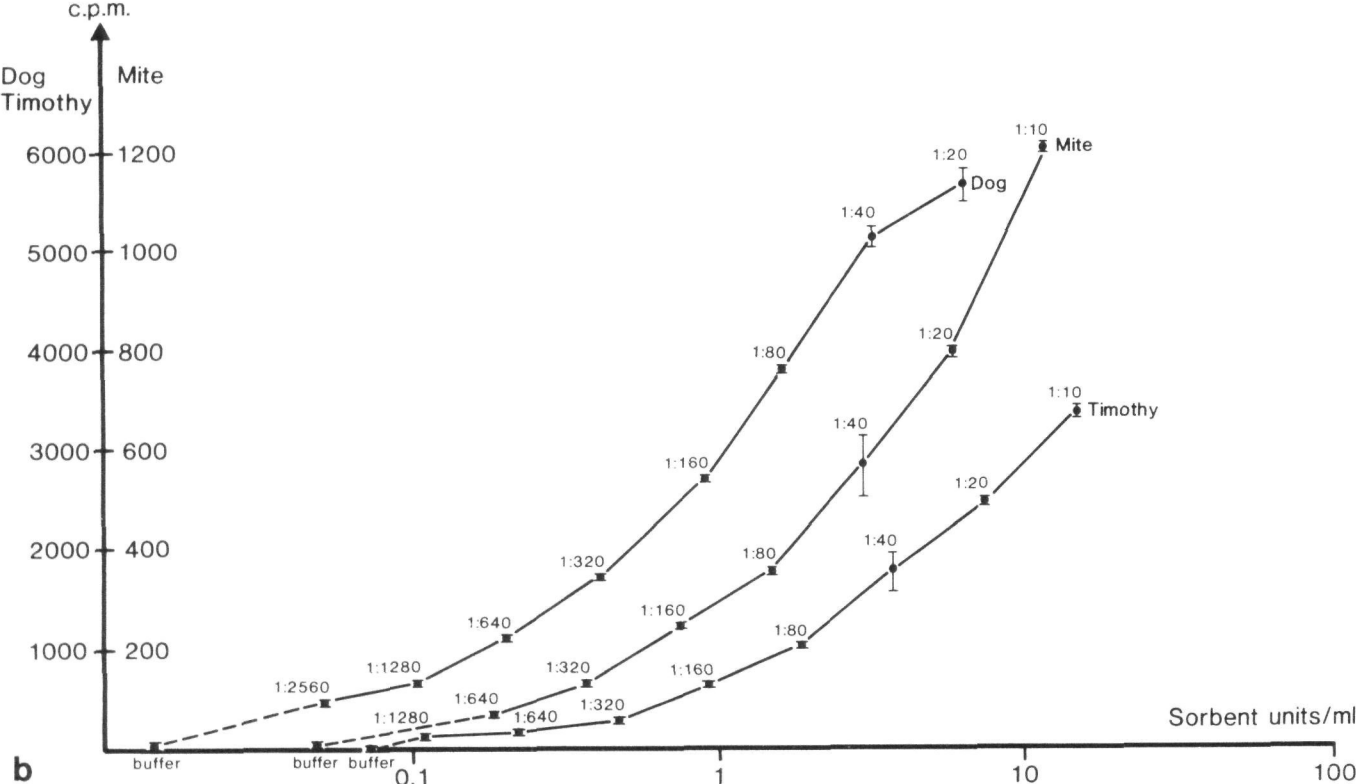


Fig. 1. Serum dilution curves and inhibition curve. a) Serum J.C. was tested in various dilutions (—) and the serum dilution 1:160 was inhibited with various concentrations of *Cladosporium herbarum* allergen extract (-----) as described in Material and Methods. b) Standard curves for grass pollen *Phleum pratense*, house dust mite (*Dermatophagoides pteronyssinus*) and dog dander. Sorbent units/ml refer to the response of the sera when tested in the AL-RAST.



Seventy-nine sera were tested in the allergen systems of timothy, mite, dog and *Cladosporium herbarum*. Sera > 100 SE/ml (as determined in the AL-RAST) were diluted 1:100, other sera were diluted 1:10. When the same sera were tested undiluted in the AL-RAST and the paper-RAST, significant correlations ($R = 0.67-0.88$) were obtained (Table 2). The paper-RAST was performed with the same batches of allergen as well as with the commercially available paper discs, but no consistent differences in correlation were found.

Range of measurements

Fig. 1a shows a serum dilution curve from a patient (J.C.) strongly allergic to *Cladosporium herbarum*. Dilutions more concentrated than 1:20 produce saturation, whereas dilutions of 1:5120 could still be distinguished from the blanc (Fig. 3, signal-to-noise-ratio = 3.5). This gives a dynamic range of the assay of 256 times or 2.4 decade. From Fig. 1b it appears that the three other systems have similar ranges of measurements.

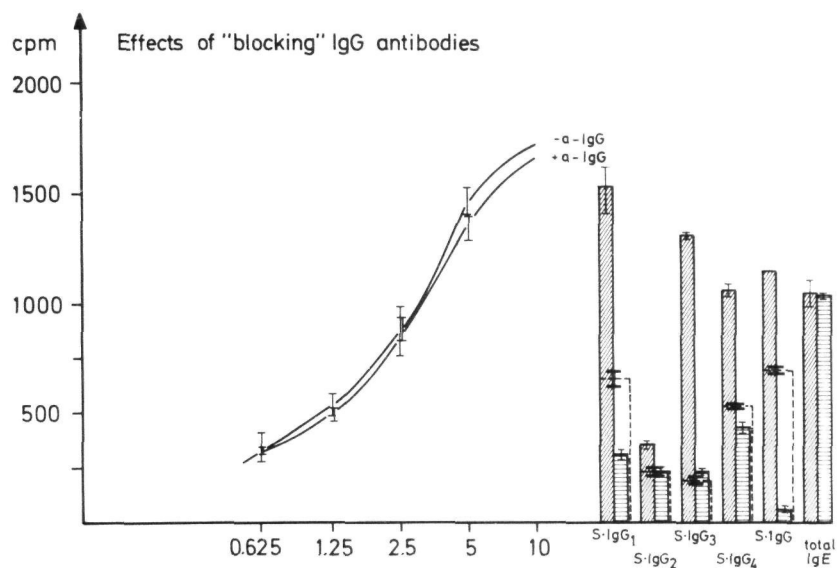


Fig. 2. Effect of "blocking" IgG antibodies. Serum J.C. was precipitated with rabbit anti-human IgG-antibody and then tested together with untreated serum. The bars show the response (in cpm) of the precipitated (horizontally hatched) and unprecipitated (skewly hatched) serum together with NMS (bars in broken lines) when tested in the assay for total IgE, and for allergen-specific IgG (total) and the four IgG subclasses (described in Material and Methods).

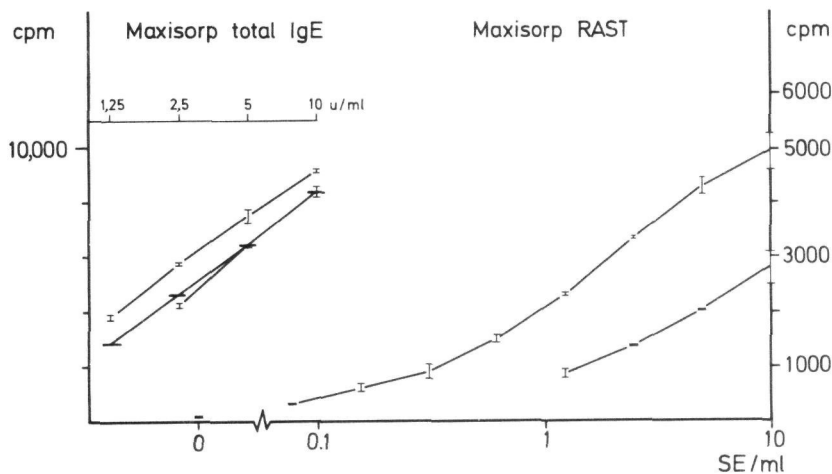


Fig. 3. Two-fold dilutions of serum J.C. (upper curves) and affinity-purified, specific IgE (lower curves) in the total IgE assay and RAST. As standards, IgE from Pharmacia and serum J.C., respectively, were used. The serum was initially diluted 1:40, the specific IgE was undiluted.

Absolute quantitation of specific IgE

To estimate the absolute amount of specific IgE, isolation of allergen-specific IgE was attempted. A dilution curve of this preparation in RAST and in the total IgE assay is depicted on the right and left sides of Fig. 3. Isolated IgE and the native serum show parallellity when tested in the total IgE assay. In RAST the native serum produced a slightly steeper curve

than the isolated fraction. However, in the more diluted samples the curves are parallel, thus making it possible to calculate the amount of specific IgE in SE/ml in the isolated specific IgE. Table 3 shows results of 4 purifications of specific IgE. Based on the results in Table 3, it was estimated that one sorbent unit in the RAST corresponds to 3.8 ng IgE. Allergen-specific IgE prepared from two other sera with about 10 times lower RAST-scores (40-50 SE/

ml) gave conversion units of 6.84 ± 3.20 (3 determinations) and 3.10 ± 0.70 ng/sorbent unit (2 determinations).

An inhibition experiment was performed to test the quality of the purified specific IgE. Preincubation of allergen with the purified IgE inhibited the RAST response up to 90 % in a dose-related manner. However, in the total IgE system inhibition was only 50 %. When the same experiment was performed with crude serum, the RAST activity was completely inhibited, whereas the total IgE content was only reduced about 20 %.

DISCUSSION

Using the principles of an assay of total IgE (11) we have developed a RAST-method using the same solid-phase (polystyrene tubes “Maxisorb”), especially prepared for binding proteins. The assay can be made from readily available commercial reagents. Neither IgE nor anti-IgE adhered unspecifically to the sorbent. The anti-IgE antibody of Pharmacia has previously been demonstrated to be specific for IgE (11), and this is further documented in Fig. 2, which shows that large amounts of specific IgG antibodies did not influence the serum dilution curve. We did not investigate the interference of specific immunoglobulins of other isotypes, but as the anti-IgE is known to be specific for

IgE, and there was no steric hindrance due to specific IgG, we did not consider this possibility very likely.

Recently, reports concerning *in vitro* synthesis of specific IgE in leucocyte cell cultures have appeared (14). From the data reported, it can be inferred that specific IgE detection limits in the picogram range are necessary in order to determine the net synthesis in cell cultures. Low detection limits (10–50 pg/ml) have been reported for methods other than RAST (6, 9). However, some of these methods lack the versatility of a RAST- and ELISA based system.

We found the detection limit of the assay to be 1:1250–1:5000 of the standard sera in the different allergen systems. Based on the estimate of 2–4 ng of IgE/SE in the *Cladosporium herbarum* system, the detection limit lies within 150–300 pg/ml. Using a fluorometric enzyme immunoassay, Underdown et al. measured standard sera specific against ragweed antigen E and rye grass in dilutions of more than 1:5000 and 1:2000 (15). Recently, Tamir et al. have described an assay in which dilutions of 1:10,000 of a standard serum (estimated to 70 pg/ml) were detected (14). This assay was based on nitrocellulose allergosorbent discs and prolonged incubation times (44 h in both incubation steps). It is difficult to compare assays not standardized in absolute quantities, but, basically, these detection limits seem to be of the same order. It is possible that prolonged incubation periods could increase sensitivity of the present assay with a factor 2–4, but other methods should be applied in order to increase sensitivity dramatically. The assay for total IgE based on two commercial preparations of anti-IgE (11) has a sensitivity of about 50 pg IgE/ml. Several reasons can be found for this higher sensitivity, but the most likely is a higher density of the binding protein on the solid-phase of the total IgE assay.

The precision of the assay is somewhat lower than claimed for the commercially available Phadebas RAST. However, included in the variability determined by us are, also the variations caused by different preparation of the allergosorbents. At this point, an additional step was introduced since it was necessary to

Table 1

Intra- and interassay coefficients of variation (in %) for maxisorp RAST involving the allergens timothy, mite and dog. The selected sera (see Material and Methods) were tested in duplicate for 3 separate weeks. Sera with titers > 100 SE/ml in the AL-RAST were diluted 100 times, other sera diluted 10 times. Intra-assay variation was determined as the mean of the individual CVs on duplicate determinations. Inter-assay variation was determined as the mean of the individual CVs obtained, when the same sera were tested for 3 separate weeks. Sera with titers below the detection limit were excluded from this analysis.

Allergen	Intra-assay		Inter-assay	
	CV	n	CV	n
Timothy	7.7	52	27.1	14
Mite	8.6	42	28.5	17
Dog	3.9	42	19.6	9

dilute serum 10–100 times before testing it in Maxisorp RAST. The correlation with the RAST tests established in clinical practice is shown in Table 2, and apparently there is no difference in the correlation with the AL-RAST (in which the allergens are adsorbed to a gel (10)) and the paper RAST (the allergens covalently bound to paper discs (1)). This suggests that all major allergens are in fact bound to the polystyrene tubes.

Absolute amounts of specific IgE can be determined by radioimmunoprecipitation of a radiolabelled antigen (8, 17). This technique, however, is dependent on one purified allergen

of known molecular weight. To circumvent this, several authors have tried to purify the specific IgE followed by a determination of the total IgE content (2, 12, 14). Problems inherent to this method are desorption of the IgE from the allergosorbent. Schellenberg et al. (12) demonstrated that only a fraction of IgE can be desorbed using non-denaturing pH conditions. This is in accordance with our findings (results not shown). Instead of pH extremes we used a chaotrope, but as not all IgE can be inhibited, it is possible that the allergen-specific part of IgE has been damaged. Thus, the conversion factor between sor-

Table 2
Comparison between Maxisorp RAST, AL-RAST, paper disc RAST. Coefficient of correlation (r) is determined using Spearman's Rank Correlation. The number of negative samples was less than 5 in all groups except that of dog sera tested in Maxisorp RAST, which produced 10 negative results

	Timothy	Mite	Dog	<i>Cladosporium herbarum</i>
n	19	20	20	20
r, AL-RAST	0.877***	0.838***	0.795***	ND
r, paper disc RAST, same allergen batch	0.875***	0.790***	0.674***	0.787***
r, paper disc RAST, Pharmacia discs	0.862***	0.859***	0.720 ¹ ** 0.822 ² ***	0.704**

P<0.01; *P<0.001; ¹Allergen disc e2; ²Allergen disc e5; ND = Not done

Table 3
Purification of specific IgE from serum J. C. IgG was precipitated with rabbit anti-human IgG (24 h), and after centrifugation (1500 g, 15 min) the serum was incubated on an allergosorbent (*Cladosporium herbarum* allergen extract coupled to cyanogenbromide-activated Sepharose). After wash the specific IgE was eluted with potassium thiocyanate, dialysed and concentrated to 10 ml (exp. I: 18 ml).

Experiment No.	I	II	III	IV	Mean	SD
Serum total IgE (ng/ml)	5443	6912	6528	ND	6294	762
Eluate total IgE (ng/ml)	6.24	10.56	10.27	24.12		
Yield (%)	2.1	1.5	1.6	3.8	2.2	1.1
Serum sp. IgE (SE/ml)			400 (by definition)			
Eluate sp. IgE (SE/ml)	2.80	2.20	1.64	13.20		
Yield (%)	12.6	5.5	4.1	33	13.8	13.3
1 SE = × ng	2.23	4.80	6.26	1.83	3.78	2.11
% specific IgE of total IgE in serum	16.4	27.8	38.4	11.6	23.6	12.0

ND = Not done. Calculations for experiment IV based on the mean of the serum total IgE determinations (6294 ng/ml).

bent units and ng IgE can only be estimated with some uncertainty.

Based on a conversion factor of 3.8 ng/SE the observed fraction of specific to total IgE was $24 \pm 12\%$ in the serum of a patient primarily allergic to *Cladosporium herbarum*. This was confirmed, as total inhibition of the RAST score in the serum caused a decrease of 20–27% in the total IgE. These figures are of the same order as found by several other authors (4, 8, 12, 13, 17).

The assay can be used as a highly sensitive method for detecting specific IgE or allergen analysis. The Maxisorp inhibition RAST has a potential for detecting airborne allergens, and with house dust mite it has been possible to detect RAST-activity in samples of airborne dust from indoor locations (4). Investigations of the *in vitro* synthesis of specific IgE are in progress.

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