

Absolute Determination of IgE Antibodies to Grass Pollen Allergens

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Knowledge of specific IgE bound on immunosorbent as assayed by RAST is useful but cannot give a complete description of the biological states of patients concerning IgE antibodies. Using a computer program based on a least squares error method and knowing the amount of bound specific IgE antibodies to grass pollen antigens in a serum at each of the four tested dilutions it is possible to calculate the absolute value of specific IgE antibody level, the relative value of affinity constant and the relative proportion of IgE antibodies to the total amount of specific (IgE plus non-IgE) antibodies. It is found that specific IgE antibodies account for high percentages of the total IgE protein (mean of 20%). High differences in affinity constant and in the proportion of IgE antibodies to the total amount of antibodies could be found between sera. Severity of grass pollen allergy could be related to the absolute level of IgE antibody, but no definite conclusion could be made on the importance of affinity constant and the proportion of IgE antibodies to the total amount of antibodies.

Key words: grass pollen; IgE; radioallergosorbent test.

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The activity of IgE antibodies responsible for the typical weal and flare reactions induced in the skin of allergic individuals by injection of specific allergens can be measured by passive transfer test (17) or more simply by the radioallergosorbent test (RAST) first described by Wide et al. (22). In this procedure a given amount of allergosorbent (insolubilized allergen) is incubated with serum from allergic subjects. IgE antibodies bind to the solid phase allergen and are detected by addition of radioactive anti-IgE. It is currently assumed that the amount of radioactive anti-IgE retained by the allergosorbent gives a fair account of the specific IgE level in the assayed sera (22, 25). However, there must be competition among antibodies for allergenic determinants (1, 20) and the final measurement is a function of

the affinity of specific antibodies, the total concentration of (IgE and non-IgE) antibodies, the ratio of specific IgE antibodies, and the total number of antigen binding sites.

The basic principles of RAST have been studied in previous papers (5, 21) in order to provide a method which takes into account most of the equilibrium factors and uses more refined assumptions for the study and quantification of specific IgE antibodies.

In this work we intended to analyze sera from patients with allergic rhinitis or asthma who were clinically sensitive to grass pollen. We obtained a quantitative description of specific IgE by means of a set of parameters, and we tried to correlate these parameters with the clinical state of each patient.

MATERIAL AND METHODS

Patients

Sera from 13 patients with positive intra-dermal tests to grass pollen were collected during the pollen season and evaluation of clinical symptoms was made by dividing the patients into three groups as follows:

- Group 1: minimal clinical troubles requiring no treatment.
- Group 2: definite clinical symptoms requiring only antihistaminic drugs.
- Group 3: severe coryza or asthma requiring treatment with corticoids, beta-agonists, or theophyllin.

Allergen extracts

Aqueous grass pollen extract (a kind gift from Institut Pasteur, Paris) was prepared as follows: 10 g pollen grains were incubated with 50 ml carbonate buffer (CO_3 H Na 3 g and Cl Na 9 g diluted to 1 l with H_2O) at 4°C for 72 h. After centrifugation, the supernatant was used as aqueous pollen extract and total protein was assayed by the Folin technique. Specificity of the extract was tested *in vivo* and *in vitro*. Injection of 2/100 ml of a diluted extract (100 μg protein/ml) in the skin of 20 nonallergic persons produced no nonspecific reaction, but gave a weal and flare skin reaction in 20 patients allergic to grass pollen. *In vitro*, grass pollen extract, at a concentration of 1 mg protein/ml, gave no nonspecific histamine release from human leucocytes in five different experiments.

Total IgE determination

IgE determination was made using the PRIST technique (Pharmacia, Sweden). The serum IgE levels in I U/ml were converted to ng/ml by multiplying by 2.4 as determined by Bazaraal et al. (4).

Absolute determination of specific IgE bound on the allergosorbent

Three hundred paper discs, (Whatman, 1) 5 mm in diameter, were activated by cyanogen bromide and coupled with 200 μg aqueous grass pollen extract in 30 ml carbonate buffer according to the method of Ceska et al. (6). Fifty microliters of each serum were incubated with one paper disc in duplicate in serial two-fold dilutions (1/1, 1/2, 1/4, 1/8) using foetal calf serum as diluent. After washing with physiological saline, 50 μl of ^{125}I -labelled anti-IgE (Pharmacia, Sweden) were added and after 12 h incubation and a second washing step, paper discs were counted in a gamma counter for 4 min. As previously described (21), by comparing between known amounts of free IgE without antibody specificity for grass pollen and unknown amounts of IgE antibodies bound on the coupled discs for reaction with labelled anti-IgE it was possible to draw a reference curve between counts and quantitative amounts of IgE bound on the allergosorbent. It was then possible to obtain the absolute determination of specific IgE bound on the paper disc for each serum at each dilution. Results give the amount of (ng) specific IgE bound in 1 ml of serum.

Quantitative specific IgE present in sera using a set of parameters

Specific IgE antibodies present in a serum can be estimated using a semi-empirical formula (5):

$$\frac{\tau T}{(\tau G) d} = \frac{Eb}{\tau G} + \frac{1}{KG} \left(\frac{Eb/\tau G}{1 - Eb/\tau G} \right)^{1/a}$$

where

T = total antibody amount
(IgE + non-IgE).

$\tau = \frac{\text{IgE antibodies}}{\text{IgE + non-IgE antibodies}}$
is the proportion of specific
IgE antibodies.

d = serum dilution.

E_b = specific-bound IgE assayed as described above.

K = mean affinity constant of antibodies.

G = antigenic sites concentration on the allergosorbent.

a = heterogeneity index of antigen-antibody system.

Parameters were calculated in order to fit the experimental data with theoretical equation using a computer program based on a least squares error method (Appendix). Knowing the amount of specific-bound IgE antibodies in each of the four dilutions used for the assayed serum it is possible to find :

- the amount rT of specific IgE antibodies in the serum;
- KG , the relative value of affinity constant of antibodies (product of affinity constant by concentration of antigenic sites);
- rG , the relative proportion of IgE antibodies to the total amount of specific (IgE plus non-IgE) antibodies (product of proportion of IgE antibodies by concentration of antigenic sites).

RESULTS

Specific-bound IgE values for serial dilutions of 13 different sera from grass pollen allergic patients could be determined. Serial dilutions of 13 sera from different grass allergic patients were prepared. Each serum was compared with the others for specific IgE bound on the immunosorbent at each dilution. As shown in Table 1 the order (number in parenthesis) of the samples relative to the bound IgE may change if the dilution was changed. One example is serum 1. If the bound-IgE measurement was done with undiluted serum, this serum was third in rank order. If the bound-IgE measurement was done with the same patient's serum diluted to 1/8 the resulting quantitation ranked fifth. In some sera (Nos. 4, 11, 12) specific-bound IGE decreased much less if serum was diluted, than it did for others (sera 2, 3). Therefore specific IgE bound on the immunosorbent (as assayed with RAST technique) does not give a complete figure of the specific IgE content of each serum.

Levels of IgE antibodies in patients were assayed, and Table 2 shows the different

Table 1.
Specific anti-grass pollen IgE bound on immunosorbent

Patients	Serum dilutions			
	Pure	1/2	1/4	1/8
1	129.6 ^a (3) ^b	96 (3)	81.6 (3)	36 (5)
2	44.4 (12)	33.6 (12)	21.6 (12)	15.6 (12)
3	48 (10)	38.4 (11)	25.2 (11)	18 (11)
4	45.6 (11)	43.2 (10)	36 (10)	28.8 (8)
5	58.8 (9)	50.4 (9)	37.2 (9)	24 (10)
6	64.8 (8)	52.8 (8)	48.7 (8)	28.8 (8)
7	67.2 (7)	62.4 (6)	52.5 (6)	33.6 (6)
8	76.8 (5)	73.2 (5)	57.6 (5)	45.6 (4)
9	78 (4)	72 (4)	64.8 (4)	50.4 (3)
10	7.2 (13)	5.4 (13)	2.6 (13)	1.4 (13)
11	134.4 (2)	124.8 (2)	105.6 (2)	76.8 (2)
12	201.6 (1)	201.6 (1)	182.4 (1)	153.6 (1)
13	74.4 (6)	60 (6)	48 (7)	33.6 (7)

^a Value ng for 1 ml of serum.

^b Rank order.

Table 2.

Antibodies against grass pollen

Patients	Clinical status	Specific IgE level (ng/ml)	Total IgE level (ng/ml)	Specific IgE (%)	Affinity (relative value <i>KG</i>)	Proportion of IgE antibodies (relative value <i>rG</i>)
1	1	319	720	44	3.2	85.5
2	1	158	1,488	11	0.47	45
3	1	142	1,344	11	3.9	29.5
4	1	236	1,440	16	24.7	21.5
5	2	215	1,224	17	4.2	34
6	2	273	2,184	13	2.14	40.5
7	2	220	2,160	10	97.6	30.5
8	2	406	3,000	14	5.7	41
9	2	499	1,344	37	5.7	40
10	2	12	1,728	0.7	266	4.5
11	3	449	2,016	20	118	60.5
12	3	1,059	3,792	28	267	87.5
13	3	336	1,200	28	1.28	50.5

clinical and biological parameters which could be calculated by the method described above. The quantity of IgE antibody found in sera obtained during the pollinic season varied from 12 to 1,059 ng/ml. Except for serum 10, which has a very low level of specific IgE, reagents accounted for 7 to 44% of the total IgE with a mean of 20%. These results provide unambiguous determinations of the quantities of IgE antibodies to grass allergens in the sera of sensitive subjects, and indicate that in these sera IgE antibodies account for a high percentage of the total IgE protein.

The relative value of the mean affinity constant of antibodies could be measured for all sera as shown in Table 2. It is seen that serum 2 has a *KG* value of 0.47 and that serum 10 has a *KG* value of 266.7 which means a difference in affinity constant *K* of around 560 for these two sera.

Patients 2 and 4 have nearly equal specific-bound IgE values for undiluted sera as shown in Table 1, whereas the proportion of specific IgE antibodies to the total amount of (IgE and non-IgE) antibodies as expressed by the parameter *rG* is clearly different: serum 2 has a proportion of specific IgE which is double as high as that of serum 4.

Table 3 shows the different correlation coefficients between all studied parameters. Specific IgE bound (*Eb*) on the immunosorbent for undiluted sera was highly correlated to the absolute level of IgE antibodies present in sera ($r=0.90$, $P<0.001$) and was not significantly correlated to the relative value of affinity constant (*KG*) ($r=0.30$, $P<0.1$). However, if specific-bound IgE are assayed with diluted sera, the correlation coefficient between bound IgE and affinity constant improves ($r=0.48$). Total IgE content was significantly correlated ($r=0.67$, $P<0.01$) to specific IgE content but not to parameter *rG*, which represents the proportion of specific IgE antibodies to the total level of antibodies for the same antigen. The specific IgE level is significantly correlated to the clinical state ($r=0.57$, $P\leq 0.05$) and specific-bound IgE assayed for diluted sera (dilution 1/8) correlated better with the clinical state than specific-bound IgE assayed with undiluted sera.

DISCUSSION

If specific IgE bound on the immunosorbent are assayed in different dilutions of different sera, the rank order of each serum may

Table 3.

Correlations between several studied parameters

	1	2	3	4	5	6	7	8	9
1	1 ^a	0.99	0.98	0.92	0.49	0.90	0.50	0.30	0.91
2	0.99	1	0.99	0.97	0.55	0.94	0.61	0.39	0.84
3			1	0.97	0.55	0.95	0.62	0.41	0.82
4				1	0.60	0.97	0.71	0.48	0.71
5					1	0.57	0.51	0.46	0.29
6						1	0.67	0.36	0.24
7							1	0.56	0.24
8								1	0.05
9									1

^a r =correlation coefficient between variables x and y was obtained with a Texas SR 51 II Calculator; r is defined as:

$$r = \frac{\frac{(\sum xi)(\sum yi)}{N} - \sum xi yi}{\left(\left(\frac{(\sum xi)^2}{N} - \sum xi^2 \right) \left(\frac{(\sum yi)^2}{N} - \sum yi^2 \right) \right)^{1/2}}$$

Significance of obtained values was calculated using the theoretical distribution of r , (as tabulated in *Tables Geigy*, Geigy-Paris (1963) p. 61).

1. Specific-bound IgE for pure sera.
2. Specific-bound IgE for 1/2 diluted sera.
3. Specific-bound IgE for 1/4 diluted sera.
4. Specific-bound IgE for 1/8 diluted sera.
5. Clinical state.
6. Specific IgE level.
7. Total IgE level.
8. Affinity value (KG).
9. Proportion of IgE antibodies (rG).

change depending on which dilution is chosen. This observation, already reported (20) for sera of patients suffering from allergic bronchopulmonary aspergillosis, proves that the RAST technique (22) is not an exact semi-quantitative assay of specific IgE antibodies present in the sera.

The percentage of specific IgE antibodies to grass pollen found in this study (around 20%) is similar to that reported by Schellenberg & Atkinson (18) and Gleich & Jacob (10), but slightly higher than the percentage found by Zeiss et al. (24) who, in 15 patients, showed that a mean of 18% IgE protein was antibody-specific to ragweed antigen E. Nevertheless, it seems, as suggested earlier by Ishizaka et al. (12), that a high percentage of IgE protein possessed antibody activity in the sera of patients

allergic to pollen. This does not seem to apply to IgE antibodies in parasitic infections. Dessaint et al. (7) showed a mean percentage below 10% for IgE antibodies to hydatid fluid, and to schistosome antigens (8) related to serum IgE. One of the clearest explanations is the potentiation of IgE response to several unrelated antigens by parasitic infection as was demonstrated in rats (13). Few studies of this type have been conducted in experiments on animals or man. In humans, immunization with dextran results in low antibody levels, probably accounting for no more than 2% of the total immunoglobulin (14). After immunization of humans with hemocyanin, less than 2% IgG and IgA and 4% IgM classes could be considered as antibody during a secondary response (3). The finding that IgE antibody

accounts for high percentages of total IgE protein is probably reflected in the local deposition of pollen or pollen antigens in the respiratory tract, and the presence in these tissues of relatively large numbers of IgE-producing plasma cells (19).

Striking differences between constant affinity value could be found in the sera of patients allergic to grass pollen (Table 2) and must be taken into consideration. In immediate allergic symptoms, which are caused by the release of vasoactive amines from mast cells after an immunological reaction between the allergen and specific IgE antibodies attached to the surface of these cells, affinity constant would be a relevant factor. Until now, stress has been laid on the level of IgE antibodies, but as suggested by Ahlstedt et al. (2) avidity of antibodies is important and may change during immunotherapy. Clinical studies have shown striking differences between a patient's sensitivity to minimal doses of allergens. Such differences must be partly due to differences in specific IgE affinity constant, but as far as we know no report has been made on a possible correlation between individual patient sensitivity and specific IgE antibody affinity constant.

Experimental immunization of animals with pollen extract leads to the formation, not only of IgE antibodies, but also of IgG antibodies, and atopic patients have circulating non-IgE associated with IgE antibodies (11). During specific immunotherapy, several papers reported the decrease of IgE antibodies after a long-term treatment and an increase of IgG antibodies (9, 15, 16, 23). The proportion of specific IgE antibodies to total IgE and non-IgE antibodies as represented by the rG value may be of significance. Indeed, competition *in vitro* between IgE and non-IgE antibodies plays an important role in some allergic states such as bee venom allergy for example, and the efficiency of specific immunotherapy has been attributed partly to this phenomenon. (16). During immunotherapy, Lichtenstein et al. (16) and Evans et al. (9)

found, respectively, no relationship and a significant inverse relationship between the amount of IgE and IgG antibodies. Nevertheless, this problem is not completely resolved and Gleich et al. (11) found recently, during specific treatment, a positive relationship between concentrations of IgE and IgG antibodies. We have studied the evolution of the rG value in sera of three different patients submitted to immunotherapy: sera were periodically assayed during 2 years and the proportion of specific IgE antibodies was found to increase when immunotherapy began, and then it decreased until it reached a plateau below the initial level (unpublished data).

Correlation coefficients were calculated between the distinct parameters studied above. Activity of IgE antibodies is usually measured *in vitro* by the radioallergosorbent test which gives a semi-quantitative assay of specific IgE bound on the immunosorbent. This test provides a measure which is a function of both the quantity and the affinity of IgE antibodies and is not directly proportional to serum dilutions. For these reasons, study of each separate parameter might be useful for determinations of correlations, particularly with the clinical state. Severity of grass pollen allergy could then be related to the absolute level of IgE antibody. There was a good correlation between IgE antibody level and total IgE level. No definite conclusion could be made on the importance of affinity constant of antibodies. The proportion of IgE antibodies to the total amount (IgE plus non-IgE) of antibodies was poorly correlated to the clinical state of studied patients; it would be of interest to determine whether this conclusion remains valid during the course of immunotherapy. However, this finding contradicts the protective role of non-IgE antibody in grass pollen allergy. More extensive studies are planned to determine, if possible, which biological parameter would be the most useful for determination of clinical allergic sensitivity. Nevertheless, it is possible to conclude that specific IgE bound on

immunosorbent (as assayed by RAST) is useful but cannot give a complete description of the biological states of patients concerning IgE antibodies. Our method should substantially improve this situation.

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APPENDIX

Determination of equilibrium parameters of individual sera.

In a previous report basic equations were derived in order to calculate the following parameters:

rT : IgE antibody level;

rG : concentration of allergenic sites available for IgE antibodies;

KG : affinity constant multiplied by concentration of antigenic sites;

a : semi-empirical heterogeneity index.

Using a semi-empirical equation:

$$\frac{T}{G} = \frac{Eb}{rG} + \frac{1}{KG} \left(\frac{Eb/rG}{1 - Eb/rG} \right)^{1/a}$$

where Eb is the concentration of bound IgE antibodies on allergosorbent. Calculations were performed with a Wang 2200 computer using the following iterative method: Let x_1, x_2, x_3, x_4 be the Eb values obtained with a patient's serum diluted 1/1, 1/2, 1/4, 1/8 respectively. In order to fulfil the above equation we must have:

$$\frac{T}{G} = \frac{x_1}{rG} + \frac{1}{KG} \left(\frac{x_1/rG}{1 - x_1/rG} \right)^{1/a}$$

$$\frac{T}{G} = 2 \left\{ \frac{x_2}{rG} + \frac{1}{KG} \left(\frac{x_2/rG}{1 - x_2/rG} \right)^{1/a} \right\}$$

$$\frac{T}{G} = 4 \left\{ \frac{x_3}{rG} + \frac{1}{KG} \left(\frac{x_3/rG}{1 - x_3/rG} \right)^{1/a} \right\}$$

$$\frac{T}{G} = 8 \left\{ \frac{x_4}{rG} + \frac{1}{KG} \left(\frac{x_4/rG}{1 - x_4/rG} \right)^{1/a} \right\}$$

Therefore, the following sum S must be zero:

$$S = \left[\frac{x_1}{rG} + \frac{1}{KG} \left(\frac{x_1/rG}{1 - x_1/rG} \right)^{1/a} - 2 \left\{ \frac{x_2}{rG} + \frac{1}{KG} \left(\frac{x_2/rG}{1 - x_2/rG} \right)^{1/a} \right\}^2 + (2)^2 \left\{ \frac{x_2}{rG} + \frac{1}{KG} \left(\frac{x_2/rG}{1 - x_2/rG} \right)^{1/a} - 2 \left\{ \frac{x_3}{rG} + \frac{1}{KG} \left(\frac{x_3/rG}{1 - x_3/rG} \right)^{1/a} \right\}^2 + (4)^2 \left\{ \frac{x_3}{rG} + \frac{1}{KG} \left(\frac{x_3/rG}{1 - x_3/rG} \right)^{1/a} - 2 \left\{ \frac{x_4}{rG} + \frac{1}{KG} \left(\frac{x_4/rG}{1 - x_4/rG} \right)^{1/a} \right\}^2 \right]$$

Thus parameters rG , KG and a were chosen in order to make S minimum as follows: we made use of a tentative value for rG (not very different from x_1), then we calculated the minimum S value for various values of parameter a , ($1/a$ varying from 0.5 to 2 with 0.05 steps). This minimum was written as $M(rG)$, then we varied rG values in order to minimize $M(rG)$. The corresponding rG value enabled us to calculate a and KG as described above.

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