

A Double-Antibody Assay for Grass Pollen-Specific IgG in Human Serum

Comparison between Double-Antibody and Histamine Liberation Inhibition Technique

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A double-antibody radioimmunoassay for measurement of grass pollen antigen-specific IgG in serum is described. Grass pollen antigens were used to show a correlation between the results obtained by this method and those obtained by measuring blocking antibodies by inhibition of antigen-induced leukocyte histamine release. The new technique described is convenient, sensitive, specific and reproducible and can be recommended for clinical use.

Key words: antigen-neutralizing capacity; blocking antibody; double-antibody technique; histamine release; timothy pollen.

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CLINICAL ASPECTS

Immunotherapy with allergen extracts is a well-documented treatment of patients with allergic asthma and rhinitis. One of the immunological changes following this therapy is the increase in allergen-specific IgG antibodies, which is the only immunological change to date to show some, albeit rough, correlation with symptom scores. It is therefore important to be able to measure these antibodies. Until recently allergen-specific IgG has been quantitated by its ability to inhibit allergen-induced histamine release from sensitized basophil leucocytes. As this is a laborious and costly method, the simple assay presented in this study is advantageous.

Patients with hay fever are improved but seldom cured by immunotherapy, a procedure introduced by Noon 70 years ago (13). One of the effects of this therapy is to induce the production of blocking antibodies of the IgG type (7, 16). Although these antibodies can inhibit type I reactions in biological test systems such as the Prausnitz-Küstner neutralization test (2) and the self-neutralization test developed by Loveless (9, 10) it remains to be clarified whether there is any connection between the occurrence of blocking antibodies and the clinical improvement of the patients following immunotherapy.

A prerequisite for such a study is a reliable assay for quantitation of the antigen-specific IgG. So far pollen-specific antibodies have for the most part been quantitated indirectly by measuring the amount of antigen that can be neutralized by the serum to be tested (6, 8).

The aim of the present study was to develop a simple and reliable double-antibody assay for quantitation of antigen-specific IgG (blocking antibodies), in which IgG is bound to labelled antigen and then precipitated with anti-human IgG (the second antibody). In the present study this assay was found to compete favourably with the more laborious histamine liberation

inhibition technique. The double-antibody assay has been used in patients immunized with timothy pollen antigen and some preliminary results are presented.

MATERIAL AND METHODS

Antigens

Two aqueous allergen preparations were used: timothy pollen extract, SQ-quality (PP) and a mixture of two isolated major timothy pollen allergens – antigens 19 and 25 (Ag 19/25) obtained from ALK, Copenhagen. In crossed immunoelectrophoresis PP shows 20 antigens with relative molecular masses in the range of 5×10^3 to 10^6 . As judged by crossed radio-immunoelectrophoresis 14 of these antigens are able to bind IgE in humans (11). Among the antigens, Nos. 19 and 25 account for about 90% of the total allergenic activity of the whole extract, judged by the RAST inhibition test (12). The mixture of the antigens 19 and 25 was composed of 90% antigen No. 25 and 10% antigen No. 19 (11). As judged by quantitative immunoelectrophoresis the relative content of Ag Nos. 19 and 25 was identical in the PP and Ag 19/25 preparations.

Antibodies

Rabbit anti-human IgG (Fc-specific) was obtained from DAKO (Copenhagen, Denmark).

Iodination of antigens

The chloramine-T technique according to Hunter (4) was used for the incorporation of ^{125}I into the antigens. The specific activity was approx. 50,000 cpm/ng PP and 100,000 cpm/ng Ag 19/25. The ^{125}I -labelled antigens were 70–80% precipitable by 10% trichloroacetic acid. They were found to be stable for at least 4 months when kept at -20°C .

Double-antibody assay

Unless otherwise stated, the procedure for quantitation of antigen-specific IgG in serum

was as follows: 0.1 ml of patient serum diluted 1:40 in test buffer (sodium borate 55 mmol/l, NaCl 100 mmol/l, BSA 1%, NaN_3 0.1%, pH 8.0) and 0.1 ml of ^{125}I -labelled PP (approx. 5 ng) or Ag 19/25 (approx. 2.5 ng) were mixed and incubated at room temperature for 3 h. Serum dilutions over 1:40 were performed in the test buffer containing non-allergic serum diluted 1:40. Thereafter 0.1 ml rabbit anti-human IgG diluted 1:2 with test buffer was added, and the samples were left overnight at 4°C . This resulted in optimal precipitation, as tested according to Sewell (17). To remove non-bound ^{125}I -Ag the precipitate was washed four times with 2.5 ml test buffer and centrifuged each time for 15 min at 3,000 g, and the radioactivity was counted in a gamma counter. All samples were examined twice, each time in duplicate. The mean value was used for calculations.

The IgG bound ^{125}I -Ag was expressed as the percentage of a positive reference serum, which precipitated between 10 and 15% of the cpm added. The reference serum was obtained from a 26-year-old female with allergic rhinitis to grass pollen. She was immunized with Allpyral® "five grass mix" for 3 years and showed serum IgE 970 U/ml and RAST class 4 to timothy. This comparison was made in order to correct for minor fluctuations in the specific activity from iodination to iodination. The precipitated radioactivity was corrected for background, i.e. the amount of cpm precipitated by a non-allergic serum pool, which precipitated 0.8 and 1.7% of the counts from PP and Ag 19/25, respectively.

Specificity of the method

To examine whether the method was specific for the determination of IgG, sera from four patients were assayed before and after removal of IgE, IgA, and IgM. The removal of these non-IgG antibodies was performed by precipitation, i.e. serum was incubated for 24 h at 4°C with rabbit anti-human IgA or IgM or with sepharose-coupled immuno-adsorbed rabbit anti-human IgE.

Histamine liberation inhibition technique

The level of blocking antibody was estimated as the antigen neutralizing capacity of serum according to Skov & Norn (18). In an excess of timothy grass pollen extract, part of the antigen was neutralized by preincubation with patient serum and the remainder of free antigen was measured by histamine liberation in leucocytes from the same patient. The level of blocking antibody is expressed by the ratio between the allergen concentration causing a 30% release in the presence or absence, respectively, of patient serum.

Patients and treatment

Patients with grass pollen hay fever were diagnosed by anamnesis, cutaneous prick testing, RAST, and histamine liberation or bronchial provocation test. Two groups of patients were studied. A) Six patients immunized with Allpyral "five grass mix", including timothy pollen allergens (Dome Laboratories Ltd., England). Following the schedule recommended by the manufacturers, injections were given weekly from the middle of March to the end of May. B) Two groups of 20 patients each were given weekly injections of A1 (OH)₃-adsorbed Ag 19/25 or PP (Alutard® SQ, ALK, Copenhagen, Denmark) from January to June with increasing doses, standardized in biological units according to Aas et al. (1). This was followed by injections every fourth week all the year round. During the grass pollen season the doses were reduced to approximately one third. The maintenance dose was the highest tolerated, i.e. the dose just below the one causing one relapse of major general side effects or four relapses of local reactions of more than 10 cm in diameter with the same dose.

The effect of the immunotherapy was tested by clinical scores based on patient diaries and from changes of the patients' sensitivity to repeated skin prick and nasal provocation tests (20).

Controls

As controls, 13 normal individuals were used without any history of allergy, with negative cutaneous test and RAST to timothy pollen and with normal serum IgE.

Blood samples

Venous blood from six patients in group A was drawn before and during the immunization and during and after the pollen season. Blood samples from the two patient groups of 20 each in group B treated with PP or Ag 19/25 were obtained in January before the treatment had started, after 8 weeks of hyposensitization, and just before and after the grass pollen season. Blood samples were obtained from the controls in February/March and October/November. The sera were kept frozen (−20°C) until analysed.

Statistical method

The Spearman rank correlation coefficient was employed in the statistical analysis of the data.

RESULTS

Methodological evaluation

The time course for the equilibrium of the antigen-antibody reaction is given in Fig. 1a. Patient serum diluted 1:40 and Ag 19/25 were mixed and left at room temperature for different periods of time before precipitation with rabbit anti-human IgG. It appears that equilibrium was obtained within 2–3 h. This was also true for the PP pollen extract. Following precipitation of the antibody-bound ¹²⁵I-antigen, the non-specifically attached radioactivity could be completely removed by four washing cycles (Fig. 1b). The immunized patient sera tested (diluted 1:40) bound between 7 and 15% of the added radioactivity, whereas the control sera bound only between 0.6 and 1.4% of PP and Ag 19/25, respectively.

The method was found to be specific for the determination of IgG, since identical results

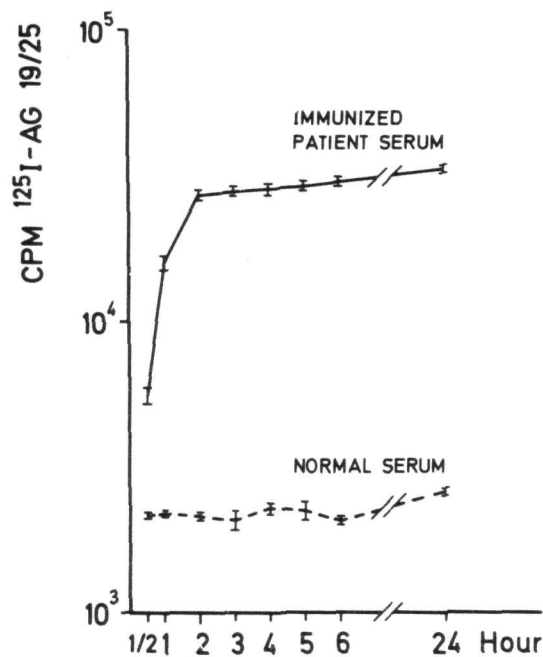


Fig 1a. Time course for the equilibrium of the antigen-antibody reaction. The mixture of serum and Ag 19/25 was incubated for different periods of time before precipitation with rabbit anti-human IgG.

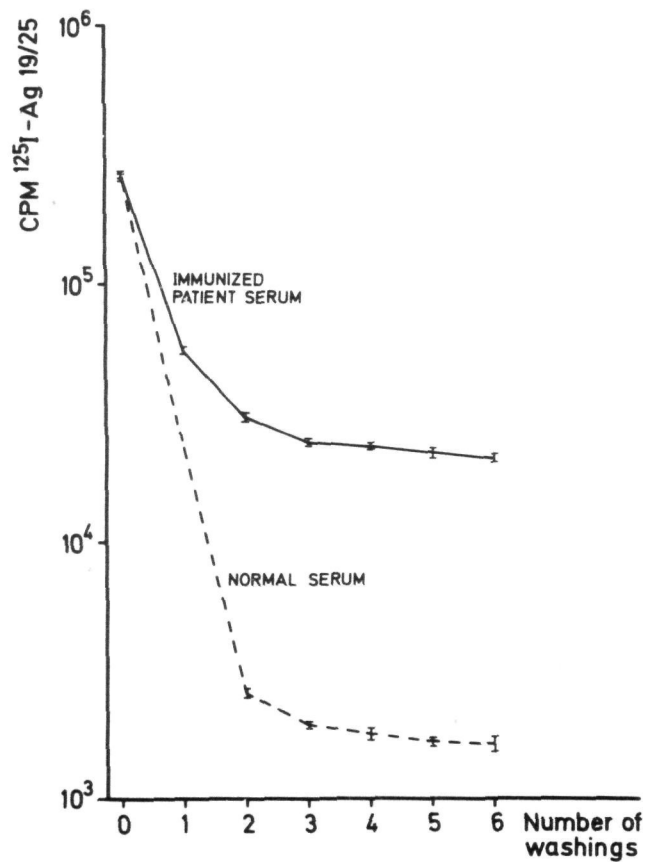


Fig. 1b. Removal of non-specific radioactivity by washing procedures.

Table 1			
Precision of double-antibody assay for timothy pollen allergen-specific IgG			
Patient sera	PP* %	Ag 19/25** %	Ratio Ag 19/25:PP
2/35 n = 20	122.8 ± 14.4	198 ± 13.6	1.6
J.T. n = 8	97.2 ± 6.1	176 ± 19.0	1.8
18/53 n = 20	58.5 ± 5.3	94.5 ± 11.6	1.6

The IgG-bound ¹²⁵I-Ag is expressed as a percentage of a positive reference serum. (Mean ± SD).

* PP: whole extract of timothy pollen.

** 19/25: purified timothy pollen extract consisting of the major allergens Nos. 19 and 25.

were obtained by assay of patient sera before and after the removal of IgA, IgM, and IgE.

The mean coefficient of variation describing day-to-day precision was about 10 %. This was estimated from three patient sera, which were examined 8 to 20 times during a 4-month period using three different preparations of iodinated antigens (Table 1). Higher values (cpm) were obtained with Ag 19/25 than with PP, but the ratio between these values was constant, i.e. 1.6 to 1.8.

The effect of serum dilution

The influence of the dilution of patient sera is given in Fig. 2. For all six immunized patients a serum dilution from 1:20 to 1:80 showed a level of radioactivity which was significantly higher than that of the control group consisting of six normal individuals. Fig. 2 shows serum dilution curves for five sera of group B patients (immunized with Ag 19/25) and for the positive reference serum.

The five sera were obtained just before the start of the grass pollen season. Although the curves show the same pattern, they are not exactly parallel, thus indicating a qualitative difference of the antigen-specific IgG of the individual sera.

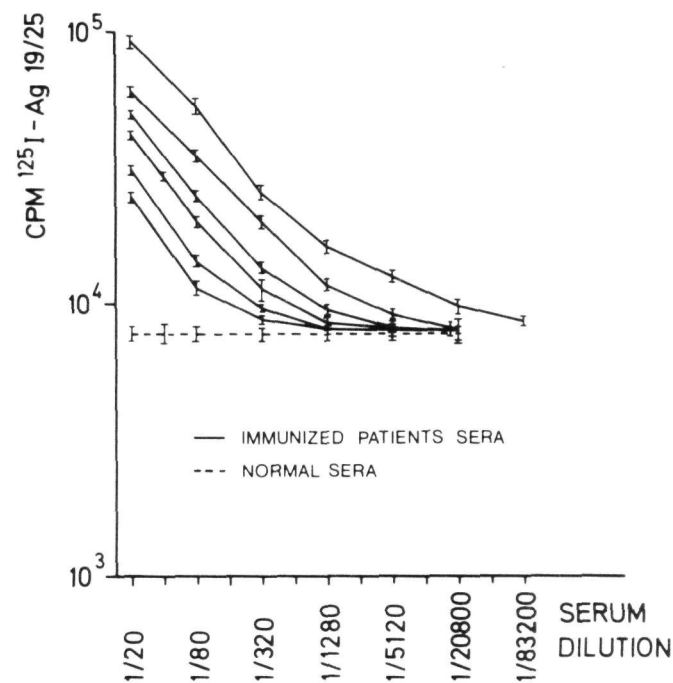


Fig. 2. Serum dilution curves for sera from five patients immunized with Ag 19/25 and for the positive reference serum. (No. 3 from the top).

Use of ¹²⁵I-PP versus ¹²⁵I-Ag 19/25

For practical purposes the use of ¹²⁵I-PP is preferable to ¹²⁵I-Ag 19/25 for the determination of pollen-specific IgG levels in serum. The correlation between the IgG levels obtained with PP and Ag 19/25 was highly significant ($r_s = 0.93$, $P < 0.001$) in sera from PP-immunized patients.

Table 2
Displacement of labelled PP in the IgG-bound ¹²⁵I-Ag by unlabelled PP or Ag 19/25

	Before displacement	Displaced by*	
		PP	Ag 19/25
Patient	3769 cpm	376 cpm	602 cpm
Normal	377 cpm	358 cpm	361 cpm

* Unlabelled PP or Ag 19/25 were added to serum together with ¹²⁵I-PP before the precipitation procedure.
cpm: mean of duplicate determinations.

Comparison between methods for determination of allergen-specific IgG

Six patients immunized with Allpyral “five grass mix” (group A) had earlier been examined for blocking antibodies by the histamine liberation inhibition technique. For comparison, these sera were assayed by the double-antibody assay. A highly significant correlation was found between the IgG levels obtained by these two methods ($r_s = 0.82$, $P < 0.001$), which is shown in Fig. 3.

Clinical application of the double-antibody assay

Two groups of patients were studied, each consisting of 20 patients. One group was immunized with PP and the other with Ag 19/25. In both groups the mean antigen-specific IgG level

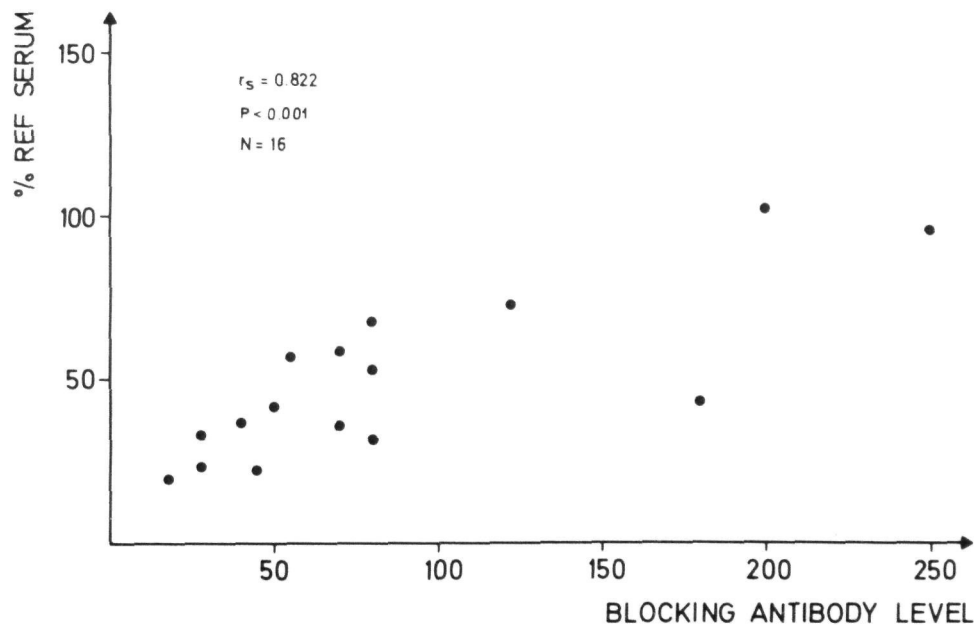


Fig. 3. Comparison of the serum IgG levels obtained with double-antibody assay and histamine liberation inhibition technique in patients immunized with Allpyral® “five grass mix”.

before immunization was slightly above that in normal sera. The titre reached a steady state level before the grass pollen season in both groups and stayed there throughout the season. The group of patients immunized with PP showed a statistically significantly greater increase of allergen-specific IgG and a higher degree of clinical improvement than those immunized with Ag 19/25. However, within the groups no significant correlation was found between the individual IgG levels and the symptom scores. Detailed information is given by Østerballe et al. (21).

DISCUSSION

We have presented an easy double-antibody radioimmunoassay for the measurement of specific serum IgG against timothy pollen antigens. Similar assays have earlier been described for other antigens (14, 15, 19). Two preparations of timothy pollen antigens have been used, one consisting of the whole extract (PP) and the other of a purified mixture of the two major allergens (Ag 19/25). For practical purposes the use of ^{125}I -PP instead of purified ^{125}I -Ag 19/25 is preferable for the determination of specific IgG.

A highly significant correlation was found between the IgG levels obtained by the double-antibody assay and the histamine liberation inhibition technique. In bee venom-allergic patients a similar correlation was found by Sobotka et al. (19). Therefore the double-antibody technique competes favourably with the more costly and laborious histamine liberation inhibition technique.

The method has been used in grass pollen hay fever patients. Preliminary results are presented. Hyposensitization with high doses of PP or Ag 19/25 increased the titre of specific IgG to reach a steady state level before the grass pollen season. PP caused a higher increase in both IgG titre and clinical improvement than Ag 19/25. However, within the groups no significant correlation was found between the individual titre or increase of allergen-specific IgG and the clinical improvement by immunothera-

py, which is also in accordance with the findings of other authors (3, 5).

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