

An Automated Particle Counting Immunoassay (PACIA) for Determination of Blocking Antibodies against Timothy Grass Pollen in Sera from Desensitized Allergics

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An automated particle counting immunoassay (PACIA) for measurement of blocking antibodies (antigen neutralizing capacity) against timothy grass pollen extract in sera from desensitized allergics is described.

Latex particles coated with F(ab')₂-anti-timothy are agglutinated by timothy. Serum containing anti-timothy antibodies inhibits the agglutination. Non-agglutinated particles are counted in a modified AutoCounter. Nineteen of 20 sera from timothy allergics who had undergone immunotherapy with purified timothy extract for 30 weeks, showed significant agglutination-inhibition. None of 42 normal human sera gave significant inhibition. The inhibiting antibody could be removed by absorption with protein A and was thus of non-IgE nature, i.e. blocking antibody. The results obtained correlated statistically significantly with those found with a double-antibody method ($r_s = 0.62$, $n = 20$, $t = 3.35$, $P < 0.01$) and with the cumulated dosage of timothy allergen extract administered to the individual patient ($r_s = 0.56$, $n = 20$, $t = 2.87$, $P < 0.02$). Between-assay coefficient of variation was from 6.4% to 18.3%. The capacity is 40 samples per hour. The method has also been applied to measurement of blocking antibodies to honey bee and wasp venom.

Key words: blocking antibodies; immunotherapy; latex agglutination.

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Immunotherapy (hyposensitization) is an important part of the treatment of allergic patients. To further improve the efficacy of this therapy and to reduce its side effects and costs, various hyposensitization regimens are continually under evaluation (1, 18). As a part of the beneficial effect of immunotherapy seems to be due to development of "blocking antibodies" (i.e. allergen-specific non-IgE antibodies) (2, 18), measurement of the titer of these antibodies is generally considered an important supplementary parameter in the evaluation of

the efficacy of different regimens (18). Thus, an increase in the amount of blocking antibody is at least evidence for ongoing stimulation of the immune system.

For patients undergoing immunotherapy with insect venoms, the blocking antibody (BA) response (13), or the ratio between BA and IgE (10), may even permit an estimation of whether the individual patient has achieved sufficient protection. For other allergens, e.g. pollens, the correlation between clinical improvement and rise in BA titer has been found to be less

satisfactory (18), although only a few studies with a limited number of patients have been conducted.

To get a more definitive understanding of the protective role of BA against other allergens than insect venoms, a great number of patients undergoing various hyposensitization treatments should be examined with an optimal, reliable test, which ideally should permit interlaboratory studies. As a first attempt to reach this goal, we have developed an automated immunoassay for determination of BA against a complex allergen mixture, timothy grass pollen, using a very sensitive latex agglutination technique, named PACIA (particle counting immunoassay) (3, 17).

MATERIAL AND METHODS

Sera

Sera were obtained from 20 timothy allergic patients undergoing immunotherapy with alum-adsorbed timothy (Alutard SQ 225, Allergologisk Laboratorium, ALK, Copenhagen, Denmark). The patients and the therapy procedure have been described in detail elsewhere (25, 26). Samples were collected just before treatment and after 8, 12 (just before season) and 30 (just after season) weeks of treatment. Normal human sera were kindly supplied by the hospital's blood bank. No history concerning the donors could be obtained.

Treatment of sera. Sera were stored at -20°C until use. To remove particulate material and precipitated lipoproteins, which may interfere in particle counting (17), all sera were treated with Freon 113 (du Pont de Nemours, Delaware, USA): 2 vol. serum were mixed vigorously with 1 vol. Freon. After centrifugation for 10 min (3,200 g) the clear supernatant was kept.

To inactivate IgM antibodies which may cause undesired agglutination (14), the supernatants were treated with dithiothreitol (DTT) (Sigma, St. Louis, Missouri, USA). In the present work, the DTT treatment was done manually (semi-automated method) to make sparing use of the patient sera. In the routine version of the assay,

the DTT treatment may be done in the DIAS unit of the PACIA between station 1 and 2 (see below, equipment). The manual DTT treatment is done as follows: 50 μl Freon-treated serum is diluted with 170 μl GBS (0.17 M glycine and 0.1 M NaCl adjusted to pH 9.2 with NaOH, containing 40 mg/l sodium azide) and mixed with 15 μl DTT (5 mg/ml distilled water). After 1 h at room temperature the excess of DTT is oxidized with 15 μl of 0.2% (v/v) H_2O_2 .

Equipment

The assay was carried out in a PACIA instrument purchased from Technicon International Division, Geneva, Switzerland. This instrument consists of a DIAS (Diluter-Incubator-Agitator-Sampler), a Pump III, a modified AutoCounter and a Hewlett Packard HP 85. This instrument and the PACIA technique is described in detail elsewhere (3, 17). Our DIAS has an extra station for predilution and DTT treatment of the serum sample (see below, Methods). The DIAS unit dilutes the samples, adds all reagents, provides the rather strong agitation required to accelerate the agglutination process and predilutes the latex mixture before counting. The pump III aspirates and dilutes the latex particles and feeds them to the AutoCounter, which counts only the non-agglutinated (0.8 μm) particles. It should be stated that the assay may be done with much more simple and correspondingly less expensive equipment which should be generally available (3, 17).

Reagents

GBS: 0.17 M glycine, 0.1 M NaCl, pH 9.2, 0.04 g/l NaN_3

GBS-BSA: 10 g bovine serum albumin/l GBS

NRS-BSA: 100 ml normal rabbit serum (Gibco) plus 900 ml GBS-BSA.

Diluent. As some sera may contain antibodies (serum agglutinators (14)) against rabbit F(ab')_2 used for coating the latex (see below), F(ab')_2 fragments obtained by pepsin digestion of normal rabbit immunoglobulins (DAKO, Copenhagen, Denmark) is added (0.2 mg/ml) to

the NRS-BSA used for dilution of the timothy and as diluent in DIAS unit.

Additive. To increase the agglutination of the latex particles and to decrease non-specific interaction, an additive consisting of 20 g/l Dextran T500 (Pharmacia, Uppsala, Sweden) in GBS containing 50 mM EDTA and 3 M NaCl (16) is added in the DIAS.

Allergens: The timothy used as agglutinator in this assay was a partially purified aqueous extract (Aquagen SQ 225, Allergologisk Laboratorium, ALK).

Anti-timothy serum. Antiserum against timothy was raised in three rabbits using a standard procedure (9). Timothy alpha fraction kindly supplied by Dome-Hollister Stier (England) was used as immunogen. Each animal received 0.2 mg timothy per injection. Serum drawn after about 1 year's immunization was used. The IgG fraction of this antiserum was isolated by use of protein A Sepharose (Pharmacia) as described in (5). After concentration by ammonium sulphate precipitation (75% saturation), the IgG fraction was resuspended in 2.5 ml distilled water and transferred to acetate buffer, 0.1 M, pH 4.5, by use of a PD 10 column (Pharmacia), for pepsin digestion. In a separate experiment, the IgG fraction was isolated as described in (9). Crossed immunoelectrophoresis of partially purified timothy (alpha fraction) against this IgG fraction confirmed that the antiserum had reactivity against 20 of the 30 identified antigens in timothy (15, 19), among these all the major allergens as identified by crossed-radio-immunoelectrophoresis (CRIE) (15, 24).

F(ab')₂ fragments from IgG anti-timothy

The IgG fraction obtained by protein A Sepharose chromatography was digested by pepsin (Sigma P 7012) in acetate buffer, 0.1 M, pH 4.5, for 24 h at 37°C at an enzyme/IgG ratio of 1:50 (w/w). The reaction was stopped by addition of solid Tris until pH 8.0. The F(ab')₂ fragments were isolated by gel-filtration, concentrated, transferred to borate buffered saline

(0.02 M borate, 0.15 M NaCl, pH 8.1, 0.1 g/l NaN₃) on a PD 10 column and stored at -20°C.

Latex preparation

F(ab')₂ fragments of the anti-timothy serum were covalently coupled to carboxylated latex particles (0.8 µm, PSI 480, Rhone-Poulenc, Courbevoi, France) by *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide HCl (Merck, Darmstadt, Germany), using 100 mg carbodiimide and 4 mg F(ab')₂ anti-timothy per ml latex (10% w/v) essentially as described in (17). After the final wash, the latex particles were resuspended in 20 ml GBS-BSA per ml latex and stored in small aliquots at -20°C until use. On the day used, the particles were thawed, diluted 1:8 with GBS-BSA and sonicated 10 s to disrupt aggregates.

METHODS

PACIA (semi-automated version)

The PACIA technique has been described in detail elsewhere (17). The principle of our assay is illustrated in Fig. 1. In brief, 70 µl DTT-treated serum (diluted 1:10 in the standard assay) is incubated with 70 µl diluent containing 300 µg/l timothy extract for 39 min at approximately 30°C. Then 30 µl of this mixture is incubated with 30 µl additive and 30 µl latex coated with F(ab')₂ anti-timothy for 44 min at 37°C. The reaction is terminated by addition of 1.09 ml GBS and 0.10 ml of this mixture is aspirated into the Manifold (Pump III), in which the suspension is further diluted 20-fold with GBS containing 1 ml Tween 20/l. The number of non-agglutinated latex particles (0.8 µm) is counted by the AutoCounter and read out as a peak height (0 to 100). All determinations were done in duplicate.

The cycle-time in the semi-automated version is 90 s, corresponding to a capacity of 40 samples per hour.

All data presented in this work were obtained with the semi-automated version of assay, in which the DTT-treatment is done manually. However, it is possible to incorporate

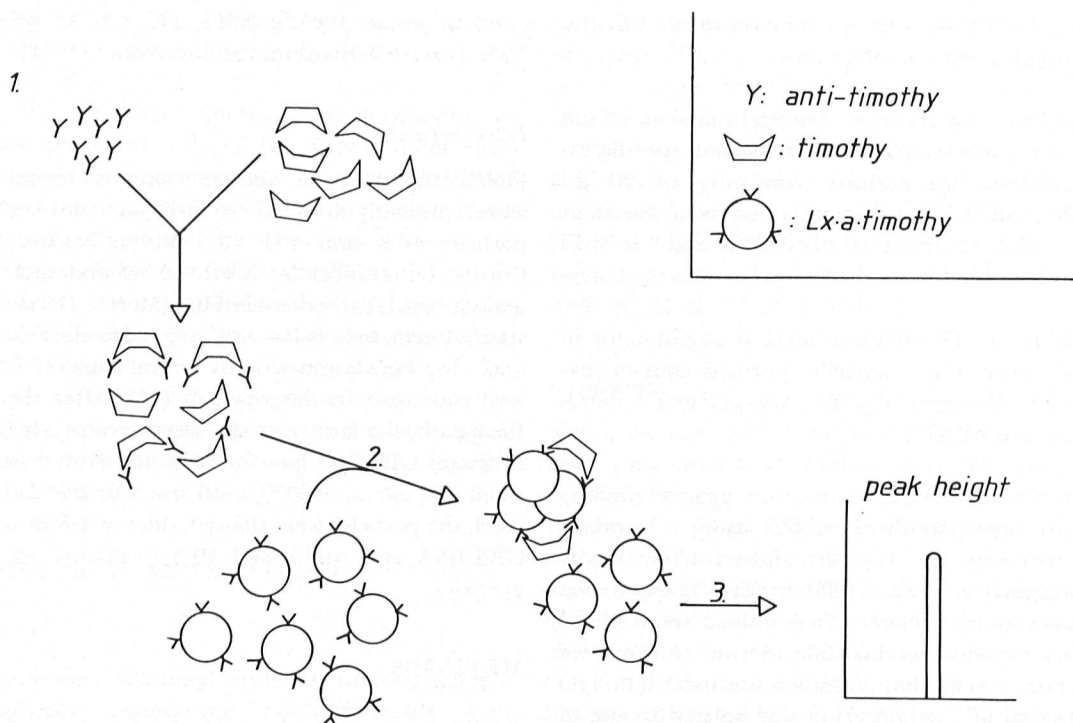


Fig. 1. Principle of the assay. 1. Patient serum is incubated with timothy pollen extract in excess. The anti-timothy antibodies neutralize a part of the timothy. 2. Latex particles, coated with F(ab')₂-anti-timothy, are added in excess. The remaining timothy agglutinates some of the particles. 3. The remaining free particles are counted in a modified AutoCounter. The value is read out as a peak height.

even the DTT-treatment in the automated handling of the samples. Our preliminary results with the fully automated version has shown that performance characteristics comparable to those found with the semi-automated version can be obtained.

Double-antibody method (DAM)

This was performed exactly as described previously (21).

Absorption with protein A-Sepharose CL-4B

Five sera obtained after 30 weeks of immunotherapy were absorbed with protein A-Sepharose CL-4B (Pharmacia). Sepharose 6B (Pharmacia) was used as a control for non-specific absorption. Absorption was done for 1 h at room temperature with vortex mixing and then

for 16 h at 4°C without mixing. For the absorption 0.15 ml sedimented gel was reacted with 0.4 ml 1:10 diluted serum, thus resulting in great excess of protein A in relation to the IgG amount normally found in human serum. After removal of the protein A-Sepharose by centrifugation (10 min, 3,200 g), the absorbed sera were tested in a final dilution of 1:60.

Expression of blocking antibody concentration

The peak height was electronically adjusted to 95 in absence of timothy. The amount of timothy added, corresponding to 50 µg/l final reaction volume (i.e. reaction volume after addition of latex particles), gave an agglutination corresponding to approximately peak height 30 (Fig. 2). In the standard assay all sera were tested in the dilution 1:60. The blocking antibody concentration (i.e. the antigen neutralizing

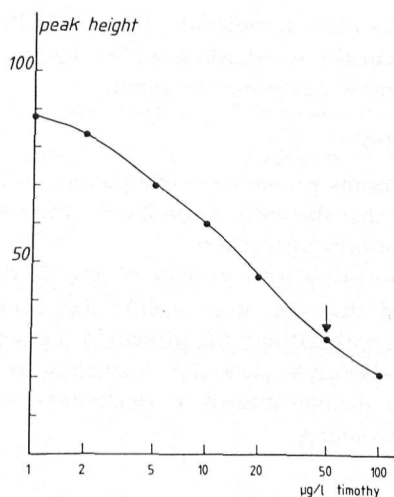


Fig. 2. Agglutination of $F(ab')_2$ -anti-timothy coated latex particles by timothy pollen extract. The arrow marks the timothy concentration selected for the agglutination inhibition assay for blocking antibody activity.

capacity) was then expressed as the agglutination inhibition as percent of the maximal obtainable inhibition with a potent human reference serum (run in 1:30) (11):

$$\% \text{ inhibition} = \frac{P_S - P_M}{P_R - P_M} \times 100$$

where p_S is the peak height for the sample, p_M the peak height in absence of serum (maximal agglutination) and p_R the peak height for the reference serum (maximal agglutination inhibition). The inhibition is expressed in units, 1 U = 1% inhibition.

Detection limit

The detection limit of the assay was arbitrarily defined as the mean of $p_M + 2$ SD. Agglutination inhibition below this limit was considered insignificant.

RESULTS

Agglutination of anti-timothy coated latex by timothy pollen extract

The latex particles coated with $F(ab')_2$ -anti-timothy were agglutinated by timothy pollen

extract as shown in Fig. 2. The agglutination showed a clear dose-dependence. Another botanical allergen, potato extract, did not cause any agglutination.

To obtain a steep inhibition curve, we selected a timothy concentration which caused about 70% agglutination (Fig. 2) (16).

Inhibition of agglutination by sera from desensitized timothy allergics

Nineteen of the 20 sera obtained after 30 weeks of immunotherapy with timothy pollen extract inhibited the agglutination of the anti-timothy latex caused by timothy. Increasing the serum dilution decreased the inhibition (Fig. 3).

Serial dilution curves for seven of these sera, selected to represent the whole range of inhibiting capacity found, are shown in Fig. 4. The curves are not parallel. However, allowing for experimental error, they do not cross in dilutions from 1:60 to 1:240.

Interferences

To test for non-specific interference, especially in the form of agglutination caused by serum agglutinators (i.e. antibodies with reactivity against $F(ab')_2$) (14, 17), the assay was run with 42 normal human sera (NHS) and the patient sera obtained after 30 weeks' immunotherapy in the dilution 1:30, omitting timothy extract as

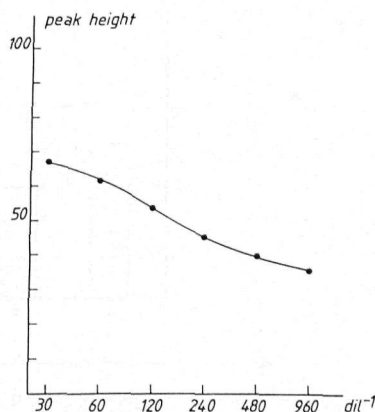


Fig. 3. Inhibition of agglutination by a serum from a desensitized timothy allergic. Abscissa: Reciprocal dilution.

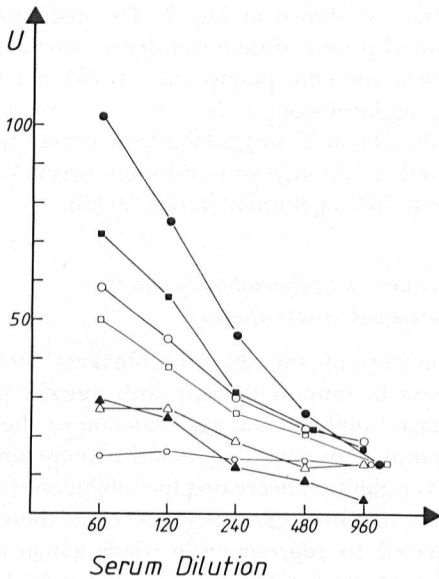


Fig. 4. Serial serum dilution curves for seven sera. The sera were obtained after 30 weeks of immunotherapy with timothy. Abscissa: Reciprocal dilution. Ordinate: Units inhibition.

agglutinator. Significant agglutination was not observed.

To test for non-specific and specific (i.e. due to BA against timothy) agglutination inhibition

in a normal population, the 42 NHS were tested in the standard assay. No significant agglutination inhibition was found.

Specificity

The results presented above provide clear evidence that the assay is specific for the timothy-anti-timothy interaction.

Absorption with protein A (Fig. 5) demonstrated that the assay mainly measures antibodies with affinity for protein A, i.e. non-IgE antibodies (BA) (7, 8, 22). A single serum contained mainly antibodies which did not react with protein A.

Precision

The within-assay (intra-assay) and between-assay (inter-assay) precision for sera with low, medium and high amount of BA is shown in Table 1.

Effect of immunotherapy on BA concentration

Before treatment low, but significant BA activity was found in only five patient sera. Significant BA activity was found in 10 sera after 8 weeks' immunotherapy, in 16 after 12 weeks' and in 19

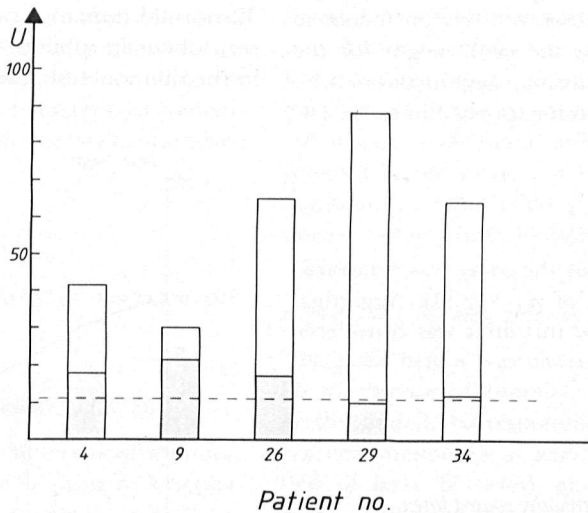


Fig. 5. Absorption with protein A-Sepharose CL-4B and uncoupled Sepharose 6B. The total area indicates the amount of BA against timothy after absorption with Sepharose 6B. This amount did not differ from the amount found in unabsorbed sera, tested in the dilution 1:60. The shaded area is the amount of BA remaining after absorption with excess protein A-Sepharose-CL-4B. The dotted line is the detection limit of the assay.

Table 1

Precision of PACIA for determination of BA against timothy

Within-assay precision ^a		Between-assay precision ^b	
Mean $\pm$ SD	CV(%)	Mean $\pm$ SD	CV(%)
14.6 $\pm$ 3.1	21.0	27.5 $\pm$ 5.2	18.3
37.8 $\pm$ 3.2	8.5	31.6 $\pm$ 4.7	14.9
56.6 $\pm$ 4.0	6.1	56.6 $\pm$ 3.6	6.4
68.8 $\pm$ 4.2	7.1	83.2 $\pm$ 8.2	9.9

^a Seven determinations in duplicate run on the same day.^b Five determinations in duplicate run on five different days with the same batch of latex and timothy.

after 30 weeks' immunotherapy. The rise in BA for six patients is shown in Fig. 6.

After 30 weeks' immunotherapy the patients had received between 11,300 and 76,900 biological units of the timothy extract (25). The BA concentration as determined by the PACIA method showed weak, but statistically significant correlation to the cumulated dosage administered to the individual patient ($n = 20$, $r_s = 0.56$, $t = 2.87$, $P < 0.02$).

The results obtained with DAM did not correlate with the cumulated dosage ($n = 20$, $r_s = 0.19$, $t = 0.82$).

Correlation of PACIA with DAM

The sera obtained after 30 weeks' immunotherapy were examined both with DAM (X) and PACIA (Y). The XY-plot is shown in Fig. 7. The correlation was statistically significant ($n = 20$, $r_s = 0.62$, $t = 3.35$, $P < 0.01$).

DISCUSSION

Timothy pollen extract is a very complex allergen mixture. Thus, the timothy preparation used for immunization of the patients in this study and for the agglutination of the latex particles in the PACIA method contained at least 20 individual allergens as determined by means of crossed immunoelectrophoresis against a rabbit anti-timothy antibody. Studies with CRIE (15, 24) have shown that timothy allergics have IgE antibodies to many of these allergens. Very likely, the non-IgE antibodies (mainly IgG antibodies) formed during immunotherapy may consist of many different subpopulations as regards allergen specificity. It follows that it might be difficult to obtain antigen excess for each of these subpopulations without sacrificing assay sensitivity (8). In this study we found that none of the sera could

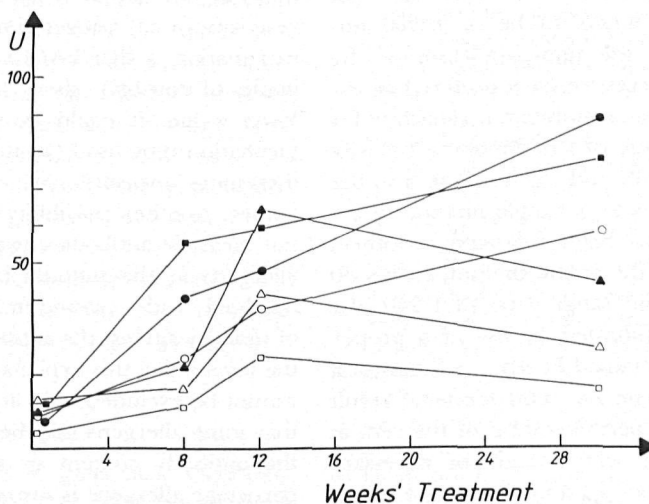


Fig. 6. Effect of immunotherapy on BA as determined by PACIA. Abscissa: Number of weeks' immunotherapy. Ordinate: Units inhibition.

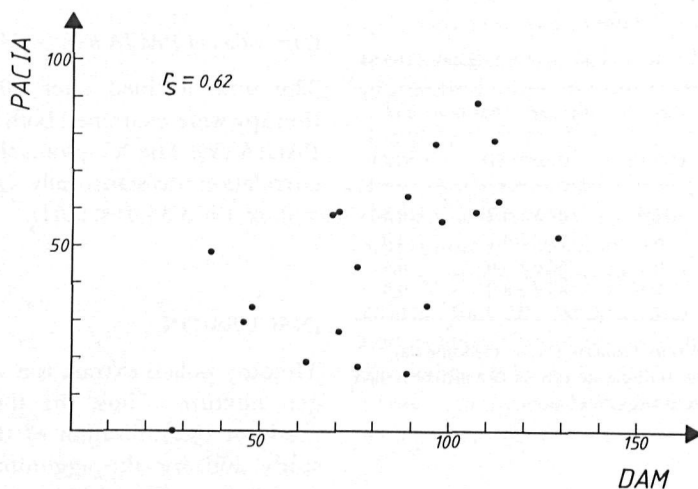


Fig. 7. Correlation between PACIA and DAM. Twenty sera obtained after 30 weeks' immunotherapy were tested with both methods for BA concentration.

inhibit more than 55% of the agglutination, caused by 50 µg/l timothy extract, even in the dilution 1:30. This might be taken as evidence for antigen excess. However, the non-parallelism of serum dilution curves (Fig. 4) indicates that the assay is not working in antigen excess for every antibody specificity present in the patient sera. Nonetheless, allowing for experimental error, the serum dilution curves did not intersect in the range 1:60 to 1:240. Thus, the rank order of the serum samples remains unchanged in this interval and the slight deviation from parallelism seems to be of trivial importance. Due to the non-parallelism of the serum dilution curves we have preferred to express the agglutination inhibition capacity of a serum in percentage of the inhibiting capacity of a standard serum and to run all sera in the same dilution. This very simple method of result expression has been validated by others (11). However, as the serum dilution curves do not intersect in the range 1:60 to 1:240, the assay might be calibrated by use of a proper standard curve, obtained by serial dilution of a human reference serum. This mode of result expression would permit testing of the sera at different dilutions, which might be necessary for reliable evaluation of sera with very high or very low anti-timothy antibody content.

The DAM, which is the method of choice for

determination of BA to purified, individual allergens (7), seems to be less suited for measurement of BA to complex allergen mixtures (7, 8). We found a statistically significant, but weak correlation between PACIA and DAM. Thus, most of the variation (62% = $1 - r^2$) found in the PACIA method cannot be ascribed to the variation found in DAM. When PACIA gives a higher value than DAM, the most likely explanation is that DAM underestimate antibodies to some allergens due to: i) poor iodination, ii) iodination-induced altered immunoreactivity or iii) antigen-limitation (8). Another explanation is that PACIA also measures antibodies of non-IgG class. When PACIA gives the lower value, it could be due to the shorter incubation time used (40 min versus 3 h) which disfavours measurement of low avidity antibodies. Another possibility is that PACIA does not measure antibodies against some individual allergens in the timothy mixture. The results obtained with crossed-immunoelectrophoresis of timothy against the antibody used for coating the latex make this explanation less likely, but it cannot be excluded. Still another explanation is that some allergens may be highly iodinated, so the antibody content in serum against these particular allergens is overestimated with DAM (7, 8).

As the results obtained with PACIA correlat-

ed statistically significantly with the cumulated dosage given to the individual patient, and as such correlation was not found with DAM, we think that PACIA gives a more reliable measure for the specific immunostimulation obtained by the immunotherapy than the DAM (21) used for comparison in this study.

In our opinion the main advantage of the PACIA method is that it measures the "true" capacity of the patient sera to "block" or neutralize antigen within a reasonably short time, and not only – as most other assays (6, 8, 20, 21, 23) – the primary interaction between antigen and antibody. Thus, PACIA measures in principle the same parameter as the histamine liberation inhibition technique (12). Another advantage is that PACIA uses the same, unmodified allergen mixture for measurement of BA activity as has been used for desensitizing the patients. Thus, the immunoreactivity is not altered due to iodination or coupling to a solid-phase. Furthermore, the precision is good and much better than that recently reported for an ELISA technique (6), the reagents are stable for at least 1 year at -20°C (16) and the assay may be fully automatized. The assay is easily adapted to measurement of BA to other allergens (16).

The only drawbacks with the PACIA system are that the advanced equipment (used in the present work) which allows full automatization, is very expensive and requires meticulous daily maintenance and regular service. Furthermore, it is at least theoretically a drawback that the assay measures both IgE and non-IgE (BA) antibodies. In this context it should be noted that IgE antibodies only contribute a small part of the antigen-binding capacity of a serum from a desensitized patient (20).

In conclusion, we have shown that PACIA is a valuable method for measurement of blocking antibodies to complex allergen mixtures.

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