

Measurement of IgG Antibodies against Timothy Pollen Allergens

A High Capacity Microtiter Assay Procedure

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This study describes a microtiter double antibody assay for the measurement of specific IgG, which is believed to be identical with blocking antibodies. The precipitates were harvested on glass fiber filters, and the activity from iodinated antigens fixed in the precipitates was used as a measure of the level of the serum blocking antibodies.

Key words. blocking antibodies; double antibody assay; microtiter assay; specific IgG-antibody; timothy pollen.

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CLINICAL ASPECTS

One of the effects of immunotherapy is the production of blocking antibodies, which to some extent are correlated to the relief of allergic symptoms following immunotherapy (2, 5, 7, 12). To be able to control the effect of immunization with allergen extracts it is important to be able to show changes of the titer of blocking antibodies in serum.

Blocking antibodies have been shown to have the characteristics of IgG (6, 15). Until recently these antibodies have been quantitated by their ability to inhibit histamine release from sensitized basophil leucocytes. As this is a costly and laborious method other procedures to quantitate allergen-specific IgG are desirable.

In this paper we report the assay conditions employed for measuring IgG antibodies against timothy pollen allergens in human sera by a high capacity microtiter assay procedure based on double antibody antigen-binding radioimmunoassay.

MATERIAL AND METHODS

Serum samples to be investigated originated from patients who had undergone immunotherapy with Allpyral® five-grass mix. Sera from subjects who were non-allergic, cutan and anamnestic negative as regards grass pollen allergy

served as normal control. The sera were stored at $\div 20^{\circ}\text{C}$ until analyzed.

The ^{125}I allergen tracer

A dialyzed aqueous extract from timothy pollen produced according to Løwenstein (8) and denoted WPP was used in the assay. WPP was iodinated by the chloramine-T technique according to Hunter as described by Skov et al. (3, 13). In brief, 10 μg of WPP were reacted with 1 mCi sodium ^{125}I and 20 μl chloramine-T (1 mg/ml). After 30 sec of agitation 20 μl ng freeze-dried WPP equal to 10^5 ct/min, on a Nonprotein bound iodine was removed by a Sephadex G-15 column (Pharmacia, Sweden). The pooled ^{125}I labeled allergens were 70% precipitable by 10% trichlore acetic acid. The specific activity achieved was 4×10^4 ct/min/ng.

The antibodies for precipitation were rabbit anti-human IgG (Fc-specific) antibodies obtained from DACO (Copenhagen, Denmark).

Double antibody assay

If not otherwise indicated the assay was carried out as follows: 50 µl serum diluted 1:40 in a test buffer (sodium borate 55 mmol/l, sodium chloride 100 mmol/l, BSA 1%, pH 8.0) was mixed with 50 µl ¹²⁵I-WPP corresponding to 2.5 ng freeze-dried WPP equal to 10⁻⁵ ct/min, on a Greiner microtiter plate and stored for 3 h at room temperature. 50 µl rabbit anti-human IgG diluted 1:2 was added and the samples were stored overnight at 4°C.

The amount of rabbit anti-human IgG necessary for optimal precipitation at different serum dilutions was estimated according to Sewell (11). The precipitates were collected and washed on glass fiber filters (Whatman GF/B) by means of a Scantron semi-automatic harvesting machine originally designed for cells.

The precipitates on the filter were then punched out into a tube. The fixed activity was counted in a gammacounter and expressed relatively to a standard reference positive serum sample.

Serum dilutions over 1:40 were performed in a test buffer containing a normal control serum diluted 1:40.

For comparison the assay was performed in tubes. The precipitates were washed four times by centrifugation before the activity precipitated was measured in a gammacounter. All samples were examined in duplicate, and the mean ct/min was used for calculations.

RESULTS

When the two methods for harvesting the precipitates were compared the results were almost identical (Table 1).

Sensitivity

Diluting the reference serum four-fold it was still possible to discriminate a 1/640 dilution of the reference serum from the non-specific

Table 1

Comparison of the results from measurement of specific IgG against timothy pollen allergens in serum by microtiter assay and tube assay

Patients	Tube assay (ct/min)	Microtiter assay (ct/min)	Microtiter tube (%)
HD	3432	3297	1.05
2/35	6720	7087	0.95
18/53	2577	2765	0.93
32	1496	1585	0.94
30	2505	2310	1.08

For comparison both methods were performed with 100 µl.

binding of normal serum (Fig. 1A), corresponding to approximately 0.02 ng/50 µl specifically bound antigen.

A decreasing ratio between ct/min precipitated in the reference serum and in the normal serum (non-specific binding) was found with decreasing concentrations of ¹²⁵I-WPP (Fig. 1B).

It was possible to dilute the antigen to 1/3200 (0.08 ng/50 µl) and still show a significant difference between reference serum and normal serum, corresponding to approximately 0.02 ng/50 µl ¹²⁵I-WPP specifically bound.

The analysis could be carried out with as little as 10 µl of each component. We found that 50 µl of each component was the smallest volume to give reproducible results with an acceptable error of the double determinations.

Reproducibility of the assay

During the course of the experiments four selected sera (three sera positive at different levels and a normal control serum) were studied eight times. Two different preparations of ¹²⁵I-WPP were used. The average coefficient of variation was 7.8%. Based on double determinations the error of the actual analysis was relatively 6%.

Quality of glass fiber filters

Five different glass fiber filters were tried. Specifications are given in Table 2. Showing the greatest recovery of the reference serum GF/B was chosen as the standard filter for the analysis.

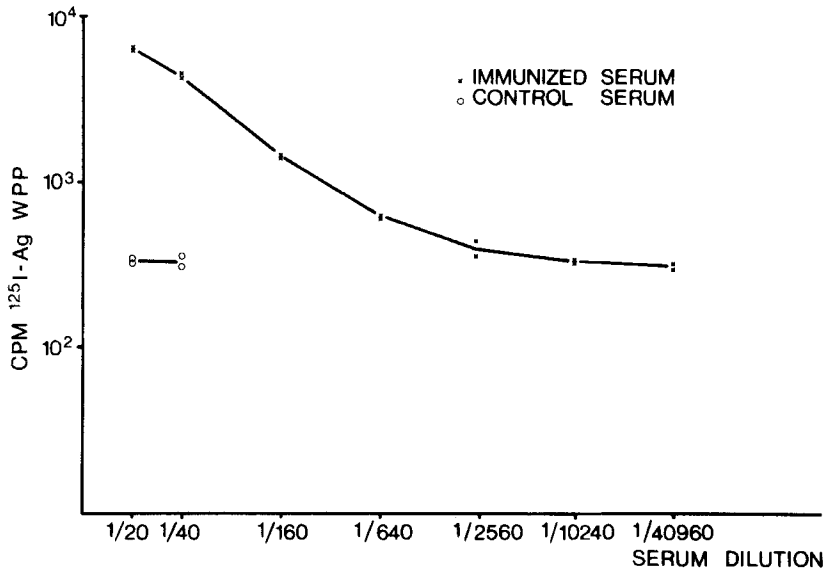


Fig. 1 A. Binding curve obtained in the radioimmunoassay using four-fold dilution of the reference serum with a constant quantity of ^{125}I whole timothy pollen allergen (WPP).

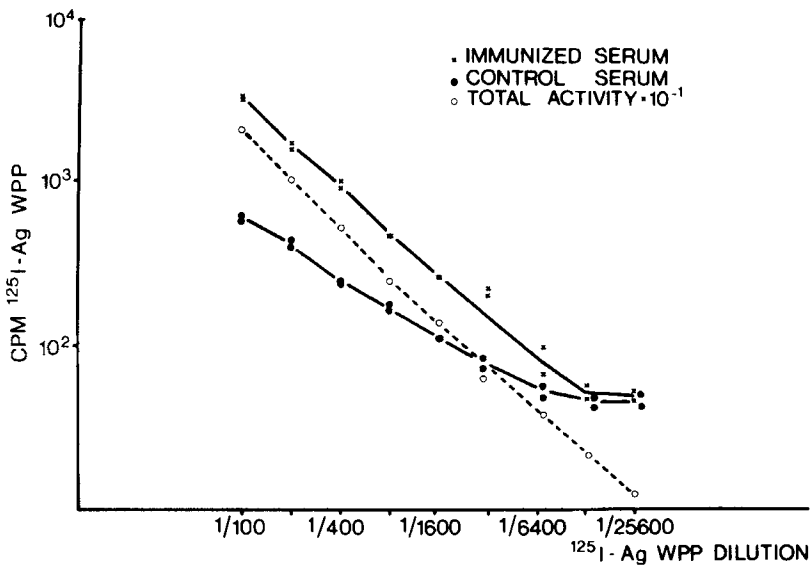


Fig. 1 B. Binding curve obtained in radioimmunoassay using four-fold dilution of the quantity of ^{125}I whole timothy pollen allergen (WPP) with a constant serum dilution.

Table 2

Specification of glass fiber papers used for the harvesting of precipitates

Glass fiber paper	Retention (μm)	Thickness (mm)	Results* (ct/min)
Watman GF/B	1.0	0.71	53909
Watman GF/F	0.7	0.44	49854
Watman GF/C	1.2	0.27	49100
Dynatec GA/100	1.0	0.30	31672
Titertec 934 AH	1.6	0.27	23342

* Mean of double determinations with serum HD.

DISCUSSION

The microtiter assay system described in this communication offers a reproducible, high-capacity procedure for assessing the binding potential of human IgG directed against a common inhalant allergen. Harvesting precipitates by a cell harvester has successfully been used earlier by Sevier & Reisfeld (10) in a double antibody radioimmunoassay of beta₂-microglobulin, by Bosma et al. (1) for a solid phase method for the assay of mouse Ig allotype, and by Jensenius in a modified Farr-type radioimmunoassay for rabbit Ig allotypes (4).

Several investigators have shown a statistically significant correlation between a high antigen neutralizing capacity of serum and a good clinical protection following immunotherapy (2, 5, 6, 14). It has been proven that blocking antibodies measured by inhibition of histamine release (antigen neutralizing capacity) are identical to those precipitated in an IgG-binding radioimmunoassay (8, 12).

This makes the IgG-binding radioimmunoassay the method of choice for the measurement of blocking antibodies. Compared with the repeated centrifugation and washings otherwise used in double antibody assays, the microtiter assay combined with a cell harvester seems to be an easier way to separate the precipitate from the supernatant. It is possible to wash and harvest 48 serum samples on one microtiter plate within approximately 10 min.

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