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Underestimation of specific immunoglobulin E by microtiter plate enzyme-linked immunosorbent assays

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We developed an ELISA by use of monoclonal anti-IgE to measure allergen-specific IgE. We measured perennial ryegrass (PRG) specific immunoglobulin E (sIgE) with conventional single incubations and with the recently reported transfer method to decrease interference in the assay. Paired sera in 10 patients before and during immunotherapy with PRG extract were analyzed for PRG sIgE with single serum incubations and with the sum of five serial serum incubations. A standardized reference serum and patient sera were incubated 12 hours and then transferred to a second well, incubated 12 hours more, and transferred again. The process was repeated until five pairs of wells had been incubated with each serum dilution. Color was developed, and optical density was measured. PRG sIgE was calculated by interpolation from the reference curve. Evidence of interference was inferred when less PRG sIgE was detected by single incubation than by transfer. Inhibition of 71.3% occurred in the sera drawn during immunotherapy. Inhibition of 32.9% was found before immunotherapy. Also, a late peaking of the well-to-well curve of optical density was observed in nine of 10 sera drawn during immunotherapy (the exception had not reached full strength), whereas all of the sera drawn before immunotherapy had normal curves. This suggests that sIgE determinations performed in microtiter plate format with single incubations will usually underestimate the sIgE level. This drawback needs to be kept in mind by the clinician interpreting the results of these assays. (J ALLERGY CLIN IMMUNOL 76:172-6, 1985.)

Microtiter plate ELISAs have been used for measuring sIgE in research because of their sensitivity, specificity, convenience, and low cost and are now

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Abbreviations used

sIgE:	Specific immunoglobulin E
sIgG:	Specific immunoglobulin G
HSA:	Human serum albumin
PRG:	Perennial ryegrass
PBS:	Phosphate-buffered saline
NGS:	10% normal goat serum in PBS
OD:	Optical density
MSPRIA:	Microtiter solid-phase radioimmunoassay
M-anti-HIgE:	Mouse anti-human IgE antibody
G-anti-MIgG:	Goat anti-mouse IgG antibody
OPD:	o-phenylenediamine

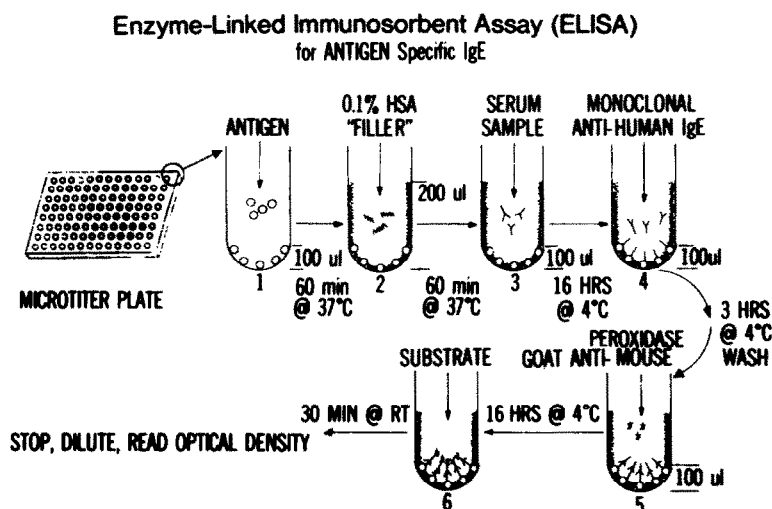


FIG. 1. Schematic representation of the ELISA.

being introduced commercially to diagnose patients with allergy.^{1,2} The ELISA has many advantages over other in vitro assays for sIgE. Among these advantages are nonradioactive tracers, physical rather than chemical binding of antigen to the solid phase immunosorbent, and the ease of assaying multiple allergenic specificities with microtiter plates. These features make this system very attractive.

The occurrence of interference in the detection of sIgE in several in vitro systems has been reported.³⁻¹⁰ This interference is probably caused in part by competitive specific IgG antibody.¹¹ The interference could be overcome in the MSPRIA by serially transferring sera from well to well. The present study was undertaken to confirm if this phenomenon exists in the ELISA, as expected, and if it did exist, to attempt to quantitate it. We not only found that patients undergoing immunotherapy with induction of high levels of sIgG had profound interference in the sIgE measured by the ELISA, but also found interference in sera from patients before undergoing immunotherapy that resulted in a reduction in the sIgE that the system could detect.

This phenomenon has important implications for the interpretation of results obtained by use of microtiter plate immunoassay systems for the measurement of sIgE in allergic individuals.

MATERIAL AND METHODS

Antigens

PRG pollen extract 1:20 w/v in 50% glycerine (lot no. L577738, Hollister-Stier, Spokane, Wash.) was used in appropriate dilutions throughout the study for patient immunotherapy. It was also used to provide the solid-phase antigen in the ELISA.

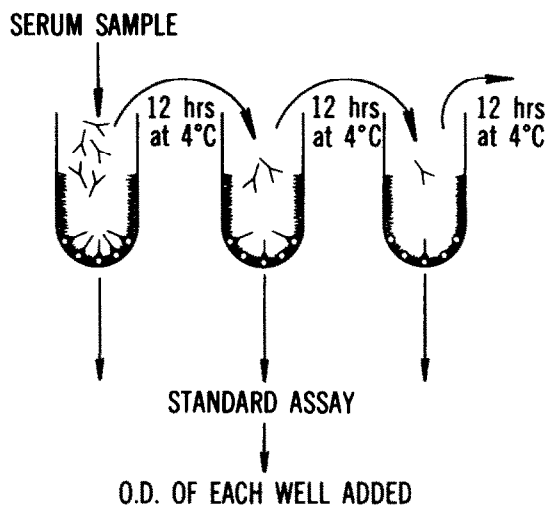


FIG. 2. Schematic representation of the serial serum transfer technique used to reduce interference in measuring sIgE.

Immunoglobulins

Monoclonal M-anti-HIgE was purchased from Hybridtech, La Jolla, Calif., lot no. D20505A. Affinity column purified G-anti-MIgG-peroxidase was purchased from Tago Inc., Burlingame, Calif., lot no. 501404.

Sera

Sera was obtained from 10 volunteers, five male and five female, aged 21 to 55 years, before and during immunotherapy for grass pollen-induced seasonal allergic rhinitis. When the second specimen was drawn, all but one of the subjects had reached a 1/100 dilution of aqueous PRG. PRG sIgE was measured both before and during immunotherapy. Measurement was done by use of a microtiter plate ELISA with and without transfers to reduce the inhibition caused

TABLE I. Calculated sIgE (international units per milliliters)

Patient	Before immunotherapy				During immunotherapy			
	Non-transfer	Transfer	% Inhibition	Curve	Non-transfer	Transfer	% Inhibition	Curve
1	163.5	288.9	43.4	A	81.0	212.0	61.8	A
2	95.9	170.6	43.8	A	21.1	80.5	73.8	B
3	35.5	53.4	33.5	A	20.6	55.2	62.7	B
4	27.1	43.8	38.1	A	7.4	21.3	65.3	B
5	25.3	51.8	51.2	A	0.9	5.1	82.4	B
6	11.5	24.3	52.7	A	3.1	11.7	73.5	B
7	8.9	12.4	28.2	A	2.5	8.6	70.9	B
8	6.6	7.6	13.2	A	1.1	7.3	84.9	B
9	6.0	8.0	25.0	A	4.5	13.4	66.4	B
10	1.6	1.6	0	A	1.7	5.9	71.2	B
Average	38.2	66.2	32.9		14.4	42.1	71.3	

by PRG sIgG. PRG sIgE was quantitated by use of a reference serum whose PRG sIgE was established in international units per milliliters by use of a previously reported standardization method that will be described below.¹¹ PRG sIgE was calculated on the same samples by use of both conventional single incubations and the transfer method. In the single incubation method, both the reference curve and the unknown sera were incubated only once. In the transfer method, final ODs were obtained by summing the ODs derived from five serial incubations for both the reference curve and the unknown sera. The percent inhibition was derived from the following formula:

$$(\text{transfer sIgE} - \text{single incubation sIgE}) / \text{transfer sIgE} \times 100.$$

Buffers

Coating and washing buffer, as previously described,¹² was PBS, pH 7.2. Tween 20, 0.05%, was added to the washing buffer. "Filler" protein was 0.1% globulin-free HSA (Sigma Chemical Co., St. Louis, Mo.) in PBS. Citrate buffer, as previously described,¹² was pH 6.0 with 38.9 ml of 0.1 mol/L citric acid, 63.1 ml of 0.1 mol/L NaHPO₄, and 20 ml of 30% H₂O₂. All buffers except citrate that was mixed fresh contained 0.01% thimersol. OPD was obtained from Sigma Chemical Co.

ELISA procedure for PRG sIgE

A schematic representation of the assay is illustrated in Fig. 1: 0.1 ml of 20 mcg/ml PRG in PBS was placed in pairs of wells in 96-well, flat bottom, Immulon II microtiter plates (Dynatech Industries, Alexandria, Va.) and was incubated for 1 hour at 37° C. The solutions were aspirated, and 0.2 ml of 0.1% HSA in PBS was added to the wells and incubated for 1 hour at 37° C. The HSA was aspirated, and the wells were washed three times with washing buffer and dried. Serum, 0.1 ml, diluted appropriately in NGS was added to each well and incubated overnight at 4° C. The serum was aspirated, and the wells were washed three times

in washing buffer and dried. When the transfer technique was used, the aspirated serum was transferred to a second well, incubated, aspirated, and transferred to a third well where the procedure was repeated until the desired number of transfers were done. After washing the wells, 0.1 ml of 2.5 mcg/ml of M-Anti-HIgE in NGS was added and incubated for 3 hours at room temperature. The wells were again aspirated, washed three times, dried, and 0.1 ml of a 1/5000 dilution of G-anti-MIgG-peroxidase was added to each well and incubated overnight at 4° C. The wells were again aspirated, washed three times, dried, and 0.15 ml of OPD 0.5 mg/ml in citrate buffer was added to each well. After 30 minutes at room temperature, the color reaction was stopped with 0.05 ml 5 N H₂SO₄ and read by a Dynatech MR580 Microelisa Autoreader at 492 nm. The sIgE concentration was calculated by interpolation from a reference curve derived from a standardized pooled high-titer serum. In the plate transfer method the reference serum was transferred and calculated as described above.

PRG sIgE standardization

This technique, which has been previously reported,¹¹ will be briefly described. A reference serum with high PRG sIgE and low PRG sIgG at various dilutions was assayed by the ELISA for PRG sIgE with four serial transfers (total of five successive assays). In parallel, an IgE standard from National Institute of Allergy and Infectious Disease was also serially diluted, and 0.1 ml volumes were assayed by ELISA for total IgE with four serial transfers until less than 10% OD was left. They were developed with M-anti-HIgE, G-anti-MIgG-peroxidase, and OPD in the usual manner. The total cumulative OD for the sIgE and total IgE dilution curves were then plotted on a semilog scale, OD versus dilution of the reference and international units per milliliter of the IgE from National Institute of Allergy and Infectious Diseases. The PRG sIgE in the reference could thus be standardized in terms of the international units per milliliter of World Health Organization. This reference was then used to standardize all unknown serum samples.

Interference in measurement of sIgE

Evidence that the detection of sIgE had interference was inferred from the following data: (1) if a lower concentration of sIgE was measured with the single incubation technique than with the transfer method, and (2) if a late peaking (type B) curve was produced by graphing the serial incubations of a particular serum.

RESULTS

Table I summarizes the results of the experiment. Patient 5 had a sharp drop in sIgE during immunotherapy as measured both by single incubation and by transfer. This patient was skin tested at the time both serum samples were obtained. The prick test initially was 3+ and fell to 0 during immunotherapy. This suggests that the drop in sIgE was real and not due to a technical error.

The measured mean PRG sIgE concentration before immunotherapy was 38.2 IU/ml by single incubation and 66.2 IU/ml by transfer. During immunotherapy, the measured PRG sIgE was 14.4 IU/ml by single incubation and 42.2 IU/ml by transfer. The mean percent inhibition rose significantly from 32.9% to 71.3%, $p < 0.001$, before and during immunotherapy. Note also that when the single incubation technique was used, only one of 10 patients had a rise in measured PRG sIgE during immunotherapy, but when the transfer method was used, the PRG IgE concentration rose in three of 10 patients.

When the ODs of the individual incubations are graphed serially (Fig. 3), most sera from unimmunized patients gradually drop from transfer to transfer (type A curve). When sera from individuals receiving immunotherapy are examined in a similar manner, the transfer curves often rise to levels higher than the initial incubation (type B or late-peaking curve).

"Normal" or type A curves were found in all 10 patients before immunotherapy (Fig. 4). During immunotherapy, late-peaking or type B curves were found in all but one patient. This was the only patient who had not reached 1/100 extract. The difference was highly significant by chi-square analysis, $p < 0.0001$.

DISCUSSION

Microtiter plate ELISAs are becoming a common method for determining sIgE. We have found evidence that lower sIgE is measured by conventional single incubation than by transfer both before and during immunotherapy. Although the interference in measuring sIgE is greater during immunotherapy, evidence is presented that there may be inhibition before immunotherapy is begun.

Further support for this hypothesis is derived from

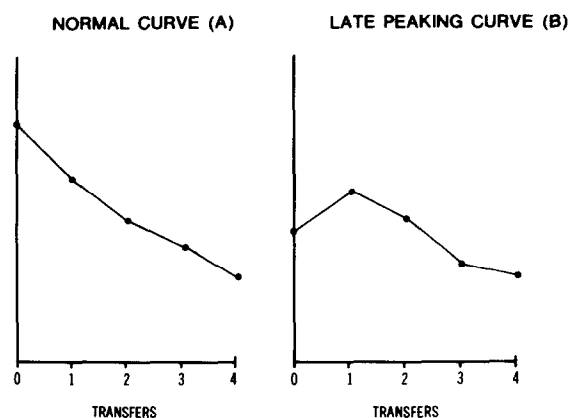


FIG. 3. Serial transfer curves in which the OD of each successive transfer is plotted. **A**, Type A or normal curve in which the OD drops from transfer to transfer. **B**, Type B curve or late-peaking curve in which the OD of later incubations rise to a level higher than the initial incubation.

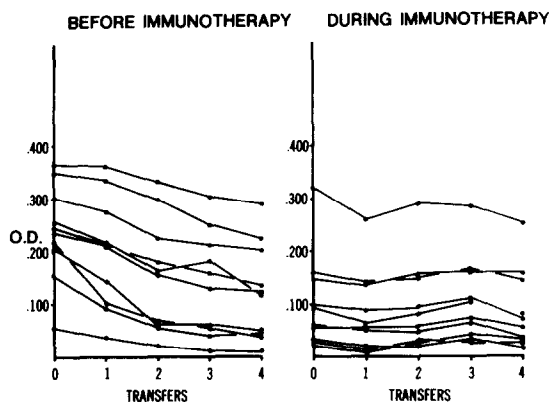


FIG. 4. Actual serial transfer curves done before and during immunotherapy on our 10 patients.

analyzing the shape of the curves produced when the OD resulting from each of the five incubations in the transfer method is measured. In a "normal" or type A curve, the amount of sIgE bound by each incubation gradually drops as would be expected since the absolute amount of sIgE available for binding drops. This type of curve is usually observed before immunotherapy. During immunotherapy, nine of 10 patients had late-peaking or type B curves in which the amount of sIgE bound rose to a higher level in the later incubations than was observed in the first incubation. This analysis demonstrates that interference increases during the course of immunotherapy. The interference has been demonstrated to correlate with allergen-specific IgG.

The wells of microtiter plates bind limited amounts of antigen. This may cause apparent "interference"

in the detection of sIgE in high-titer sera because of plateauing of the amount of antibody bound caused by the antibody excess in the system. Another possible cause of variance between the single step assay and the transfer assay is the nonlinear relationship between OD and sIgE concentration. We believe that the use of a standardized reference curve to calculate the sIgE concentration minimizes this problem.

An interesting question is whether clinical response to immunotherapy correlates with either interference that would reflect sIgG to presumably relevant determinants, or to the actual lowering of sIgE concentration, or to neither of these.

More importantly, this evidence demonstrates that sIgE determinations performed in microtiter plate format with single incubations can underestimate the sIgE level. This phenomenon may be present before immunotherapy when the interfering sIgG is presumably stimulated by natural exposure to allergen. This is more than enough interference to make the microtiter plate single incubation method nonquantitative and may be enough to completely miss patients with low levels of sIgE. Our data need to be taken into consideration when the clinician interprets sIgE levels measured by a single incubation microtiter plate system. For example, the transfer method used in a microtiter plate radioimmunoassay analogous to this ELISA has been demonstrated to correlate with skin test results in honeybee venom-sensitive patients better than the single incubation technique.¹¹

This limitation, which is probably caused by the finite quantity of allergen bound by plastic microtiter plate wells,¹³ may be overcome by the use of the transfer method to increase the amount of allergen in the system. Alternatively, one may use immunosorbents that bind greater quantities of allergen.

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