

## CRITICAL EVALUATION OF AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY FOR IgG ANTIBODIES TO *DACTYLIS GLOMERATA*•

SERGIO BONINI \*, PETER BOULTON \*\*, JONATHAN BROSTOFF \*\*

*I Clinica Medica \**, Università 'La Sapienza', Policlinico Umberto I, Roma

*Department of Immunology \*\**, The Middlesex Hospital Medical School, London

IgG antibodies to allergens are known to have an important role in allergic rhinitis and asthma. 'Blocking' antibodies belonging to the IgG class are in fact detectable after immunotherapy and may contribute to a reduction in the clinical sensitivity to specific allergens<sup>1,25</sup>. Short-term sensitizing IgG (S-TS IgG) have also been described<sup>27</sup> and may define particular forms of clinical allergy<sup>8,17</sup>.

Several immunoassays have been reported for the detection of serum IgG and IgG-subclass antibodies to allergens. These include red cell-linked antigen antiglobulin reaction (RCLAAR)<sup>11</sup>, defined antigen substrate spheres (DASS) immunofluorescence<sup>36</sup> and several radioimmunoassays, such as the solid-phase method with <sup>125</sup>I-*Staphylococcus* protein-A<sup>10, 18-20, 31, 32</sup>, a sandwich technique using Sepharose protein-A for adsorption of IgG<sup>23</sup> and, as a method of choice, various modifications of the double antibody or antigen-binding technique<sup>1, 9, 30, 35, 40</sup>. However, none of these methods for IgG has yet attained a prominent place in clinical practice in the way the radio-allergosorbent test (RAST) for specific IgE has.

Diversified applications of enzyme-linked immunosorbent assay (ELISA) have been employed both for research and clinical practice in various branches of medicine<sup>37</sup>, including allergy<sup>2,5, 12, 13, 15, 16, 21, 34, 39</sup>. Although ELISA has been used for measuring IgG antibody to several antigens (and allergens) and, more recently, for detecting IgG subclass antibody to different allergens (including pollen allergens)<sup>22, 28, 33</sup>, as far as we know, it has not yet been applied successfully to the detection of total IgG antibody to grass pollen, apart from a preliminary communication<sup>24</sup>.

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We describe the experimental attempt to set up an indirect ELISA for the detection of IgG antibodies to *Dactylis glomerata* (DG), one of the pollen antigens more frequently responsible for allergic hay fever and asthma.

## MATERIALS AND METHODS

The test is based upon the reaction of sera with an antigen immobilized by passive adsorption on a solid-phase. After the removal of unreacted serum, an antiglobulin-enzyme conjugate is added. The bound conjugate is then revealed by the addition of the specific substrate. The microplate ELISA method of VOLLER et al.<sup>37</sup> was used as follows.

### Materials

*Solid-phase* - Disposable flat-bottomed polystyrene plates (Micro-ELISA, Dynatech Labs) were used as a solid-phase.

*Antigen* - One gram of lyophilized crude extract of DG (Bencard) was dissolved in 1 l of 0.1 M NaHCO<sub>3</sub>. Further dilutions in carbonate-bicarbonate buffer (pH 9.6) were prepared from this stock solution.

*Sera* - Sera for testing were collected from 40 selected patients allergic to grass as proven by history, skin tests and RAST. Twelve of these had been desensitized with alum-precipitated pyridine grass extracts (Allpyral) for a period ranging from one year to four years (mean 2.2 years). Reference sera, highly positive, weak positive and negative for pollen-specific IgG antibodies, were kindly given by Dr. K. Huggins of Dome Labs and by Dr. T. A. E. Platts-Mills of the Clinical Research Centre, Harrow (Great Britain).

*Antisera and conjugates* - A rabbit was immunized successively at 2-week intervals with 1 mg/ml of DG in complete Freund's adjuvant, incomplete Freund's adjuvant and on an alum precipitate. A test bleed showed a precipitation in double-diffusion in agar. In all subsequent tests, the pre-bleed was used as a control. The gammaglobulin fraction of this antiserum was used for checking the adsorption of DG onto the plates.

A goat anti-rabbit immunoglobulin was prepared using the same immunization schedule and was purified by ammonium sulphate precipitation followed by ion-exchange chromatography on Whatman DE52 cellulose, to give the IgG fraction. These antibodies and a commercial anti-human IgG were then conjugated with horseradish peroxidase (type VI, Sigma Chem. Co.) by the sodium periodate two-step procedure<sup>36</sup> and by the glutaraldehyde two-step procedure<sup>6</sup>. A commercial anti-human IgG-peroxidase conjugate was also used.

Reference class-specific anti-human IgG-peroxidase conjugates were a gift of Dr. A. Voller of Nuffield Laboratory of Comparative Medicine, London and of Dr. G. Bahr (Middlesex Hospital, London).

*Substrate* - The substrate was prepared at the time of the test by adding 40 mg of o-phenylenediamine (Sigma Chem. Co.) and 40 µl of hydrogen peroxide (20 vol.) to 100 ml of 0.1 M citrate-phosphate buffer, pH 5.0.

*Method* - 200 µl of antigen at the optimal dilution (see below) in carbonate-bicarbonate buffer, pH 9.6, were incubated in each well overnight at 4 °C. After three washings of the plates by adding PBS-Tween and emptying, sera were

added. After a further washing, each well was filled with 200  $\mu$ l of conjugate for incubation at room temperature. Dilutions of sera and conjugate and incubation times were varied in the different experiments, as reported below.

The enzymatic reaction was elicited by adding 200  $\mu$ l of freshly prepared substrate and stopped by the addition of 50  $\mu$ l of 2.5 N  $H_2SO_4$ . Different times and temperature for the enzymatic reaction are reported below. Readings were obtained at 492 nm by a Multiskan through-the-plate reader (Flow Labs).

#### *Sepharose protein-A test for IgG antibodies to DG*

The test was performed by the method of JOHANSSON et al.<sup>23</sup>. IgG is captured by staphylococcal protein-A, coupled to a solid-phase (Sepharose 4B, Pharmacia Ltd.), and the specificity of the bound IgG is revealed by adding a radiolabelled antigen, in this instance  $^{125}I$ -pollen.

## RESULTS

### *Optimization of the test*

*Coating of the plates with DG pollen extract* - In order to check whether DG was suitable for coating the plates and to find the optimal concentration for coating, a direct double-layer ELISA was performed. An overnight incubation of different solutions of antigen was followed by a 3-h incubation with rabbit anti-DG. A 1:500 dilution of goat anti-rabbit IgG-peroxidase conjugate was used for revealing the reaction with the specific substrate. A non-pollen antigen (ovalbumin) at a comparable dilution, and normal rabbit serum (pre-bleed) acted as controls; 100  $\mu$ g/ml of the DG antigen solution were chosen as suitable for further experiments, as this concentration was the lowest to give maximal readings within the range of the photometer, as well as the highest ratio between positive and negative control samples.

*Conjugate dilutions* - Dilution curves of the various conjugates in a direct ELISA using 100 ng/ml human IgG on the plates were comparable. The optimal dilution for obtaining an absorbance of 1.0 ranged from 1:2,000 to 1:3,000, depending on the conjugate used; 1:2,500 dilution of the conjugate prepared by sodium periodate conjugation was used in succeeding experiments.

*Serum dilutions* - A checker-board titration of various dilutions of sera showed that a 2-h incubation with 1:50 dilution gave the best discrimination between high positive, weak positive and negative reference sera when 100  $\mu$ g/ml of coating antigen and a 1:2,500 dilution of conjugate were used. Further dilution beyond 1:50 reduced the discrimination without lowering the background.

*Specificity* - The specificity of the system was tested by an inhibition ELISA using soluble DG antigen. Dose-dependent inhibition (up to 50% at 1 ng/ml) was obtained, whereas no inhibition was seen using ovalbumin at the same concentrations.

*Reproducibility* - When results were expressed as absolute readings of absorbance, the intra- and inter-assay variations in ten determinations of the refer-

ence sera had coefficients of variation of 18.2 and 23.2 for high positive reference serum, 21.6 and 29.4 for weak positive serum, and 7.1 and 21.4 for negative serum.

Expressing the results as a percentage of the reference, weak positive serum reduced the inter-assay variation to a coefficient of variation of 16.4 for the high positive serum and 12.2 for the negative reference.

*Sensitivity* - While the background value (using buffer instead of serum for testing) was not relevant, a high non-specific binding was sometimes observed when uncoated plates, or plates coated with a non-pollen antigen, were used with sera from atopic or normal subjects in the test. However, the mean ratio between the value of the high positive reference serum to the non-specific binding value was  $4.7 \pm 2.2$ . Non-specific binding was sometimes as high as the negative reference.

Three modifications of the test were examined to determine whether non-specific binding could be reduced:

1. addition of 1% human serum albumin to the buffer;
2. 'blocking' the pollen-coated plates with 50 µg/ml human serum albumin;
3. reduction of the incubation time of the conjugate to 3h.

Of these three modifications, only the third gave a significant increase in the ratio between a positive reference serum and non-specific binding.

Useful information was obtained by studying the effect of different times and temperatures for the conjugate-substrate reaction (fig. 1). The ratio between positive values and non-specific binding values was higher in the early stage of the reaction. This was more easily seen if the reaction was slowed by a low temperature.

On the basis of these determinations, final conditions chosen for the assay were: coating overnight at 4 °C using 100 µg/ml DG, incubating 1:50 dilutions of sera for 2h at room temperature, adding a 1:2,500 dilution of conjugate for 3h (room temperature), developing the colour reaction at 4 °C for 30 min.

### *Applications*

*Detection of IgG antibodies to DG in allergic subjects* - Figure 2 shows the results of DG-specific IgG antibody determinations in 28 untreated allergic patients and in 12 allergic patients receiving immunotherapy. Hyposensitized patients had higher pollen-specific IgG than untreated patients, the mean levels being 43.7% of reference  $\pm 17.4$ , and 28.6%  $\pm 9.9$ , respectively ( $p < 0.005$ ). However, there was no obvious correlation between individual antibody levels and the degree of individual clinical improvement.

### *Correlation with Sepharose protein-A test*

Figure 3 shows the correlation between ELISA and Sepharose protein-A test for the detection of DG-specific IgG. No significant correlation was found ( $p > 0.05$ ). When sera were considered as positive or negative for each test (values above or below 2 SD from the mean of 23 normal sera, respectively), it was shown that sera positive in the protein-A test were usually positive in ELISA. However, some sera were positive in the ELISA and not in the protein-A test.

## DISCUSSION

ELISA has been said to be a technique offering the same accuracy, specificity and reproducibility as other immunoassays, but with greater facility<sup>37</sup>. These are obvious reasons for attempting to apply it to the detection of IgG antibodies to DG in allergic patients. Accordingly, a microtitre plate was chosen to allow an easily performed procedure, which would allow automation at a later date.

The concentration of antigen required for coating the plates was higher than that usually used for other antigens<sup>37,38</sup>. The biochemical characteristics and relative impurity of the antigen could possibly account for this.

Preliminary experiments (not reported here) led us to choose peroxidase as a label rather than alkaline phosphatase as, although results were broadly comparable, peroxidase gave a more intense colour easily read at 492 nm, and it is cheaper. Although there was no significant difference in the activity of conjugates prepared by different methods, the sodium periodate two-step con-

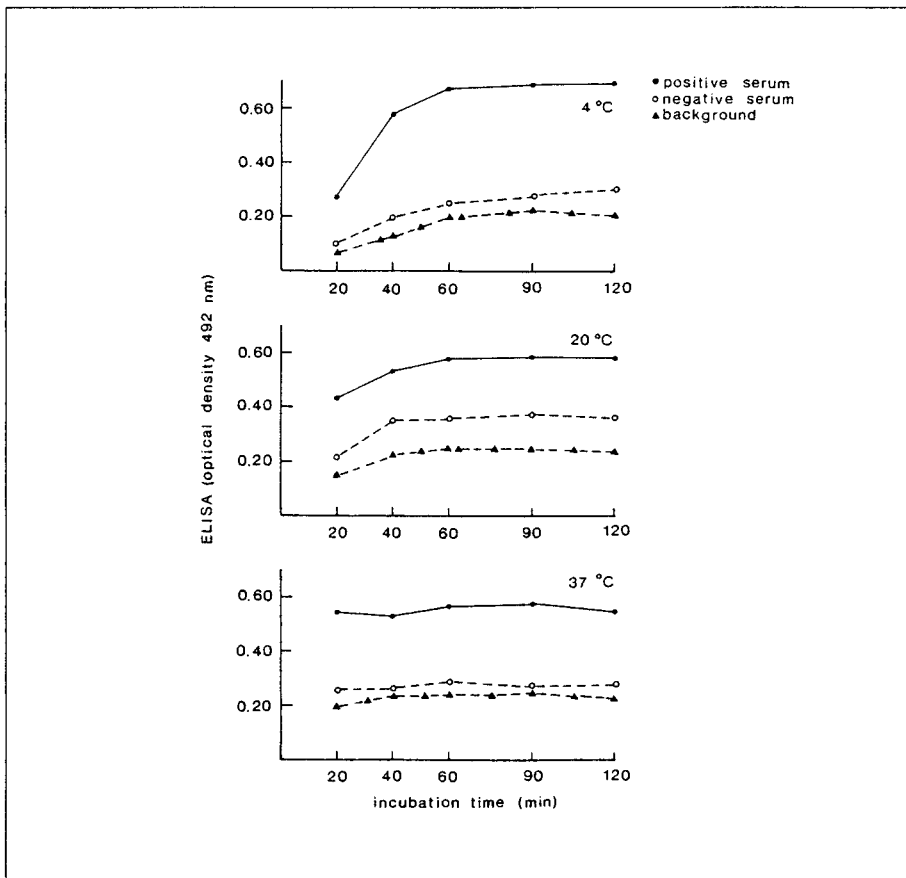


Fig. 1 - Changes of ELISA readings as a function of incubation time and temperature.

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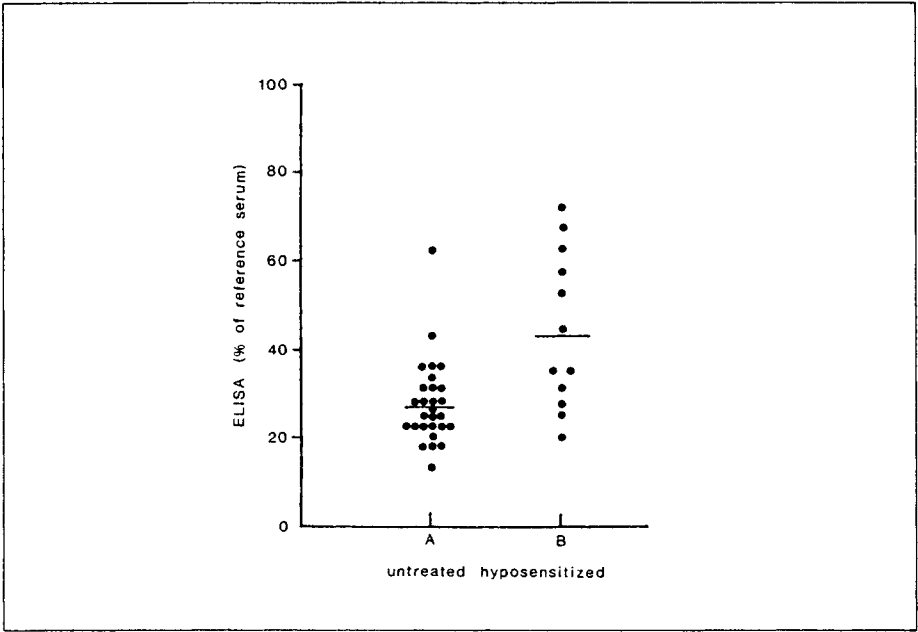


Fig. 2 - ELISA values (expressed as a percentage of reference serum) in 28 untreated allergic patients (A) and in 12 allergic patients receiving immunotherapy (B).

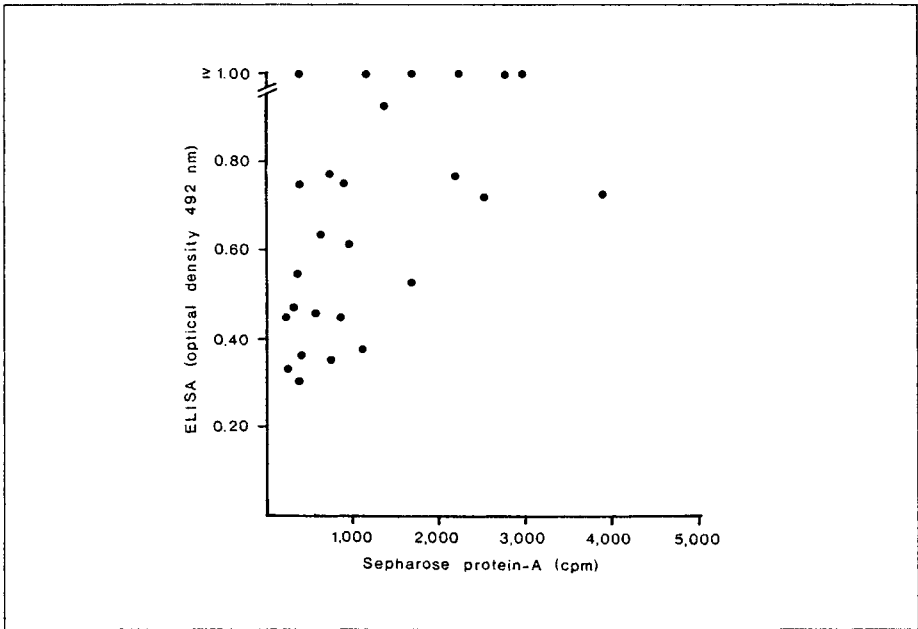


Fig. 3 - Comparison of IgG antibodies to *Dactylis glomerata* as determined by ELISA and by Sepharose protein-A test.

jugation was preferred, as it yielded a greater amount of conjugate per ml of antibody, further reducing the cost of the test.

When the test was optimized, as outlined in the *Results* section, a satisfactory degree of accuracy was found.

Confidence in the specificity was supported by the inhibition of positive reactions when soluble-phase pollen was added.

If the discordance between positive ELISA and negative protein-A test results is not attributable to a high percentage of IgG3, which is not bound by protein-A<sup>23</sup>, another explanation must be found for these high values and for the high non-specific binding observed for some sera.

When an indirect ELISA is used, the unsuitability of the solid-phase, poor quality of antigen and antiserum, interference by specific antibodies of other immunoglobulin classes and a low ratio of specific and total antibodies of the same class should be regarded as potential causes for unsatisfactory results.

The solid-phase itself and the antiserum were usually proved satisfactory.

Purified antigen is highly desirable in every immunoassay. However, the purification may require sophisticated procedures, unsuitable for application in large-scale tests. The DG used in this ELISA is regularly used in RAST, and seemed to be acceptable in experiments for checking the coating of the plates as well as the specificity of the assay.

The relative paucity of IgG antibodies to DG in relation to total serum IgG is a relevant consideration when exploring the less satisfactory aspects of the test and indeed is an important aspect to bear in mind when critically considering previous reports by other workers for different tests for IgG antibodies to allergens. The ratio of DG-specific IgG to total serum IgG has been reported as very low<sup>29</sup> as compared with IgE, where the proportion of specific to total IgE may be very high.

The influence on non-specific binding by non-specific IgG has received little attention in most previous reports of tests for IgG antibodies to allergens; for instance, it was not included among the controls in a successful report of an indirect ELISA for IgG antibodies to house dust<sup>13</sup>. The specific IgG content of sera from normal subjects is often not reported, or seems to have been overestimated and due to non-specific binding when compared with results provided by an accurate antigen-binding assay<sup>30</sup> or, as in our own tests, with results from a test where labelled antigen, rather than labelled anti-IgG, is used. Accordingly, IgG RAST is not satisfactory<sup>9,23,29</sup>. On the contrary, RAST's suitability for specific IgE determinations is enhanced by the high ratio between total and specific IgE<sup>14,29</sup>.

From our findings it appears that the indirect ELISA may offer an easily performed and inexpensive test for the detection of IgG antibodies to pollen antigens, which can be caused by most laboratories to monitor, at least semi-quantitatively, changes in specific IgG following immunotherapy. The inclusion of reference and sequential samples in each test avoids the problem of inter-assay variability discussed above. However, it must be mentioned that the clinical relevance of specific IgG measurements is not yet clear, since a definite correlation between the degree of clinical improvement and a rise in IgG antibodies following immunotherapy is usually lacking<sup>1</sup>.

IgG antibodies are a topic of expanding interest in several research areas in allergy. The low sensitivity of the ELISA, mainly due to non-specific IgG-

binding, makes the test perhaps less useful for accurate research application in pollen allergy. In fact, experiments reported suggest that non-specific binding should warrant a more careful consideration as a possible misleading factor in techniques employing solid-phase adsorption of antigen.

#### SUMMARY

Experiments are reported for the evaluation of an indirect ELISA using peroxidase conjugate for the detection of IgG antibodies to *Dactylis glomerata* (DG), a grass pollen antigen frequently responsible for allergic rhinitis and asthma. When optimized, the test proved to be useful in clinical practice for a semiquantitative, cheap and simply-performed determination of IgG antibodies to DG. The advantages and disadvantages of this method, compared with those of elaborate antigen-binding assays, are discussed.

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*Requests for reprints should be addressed to:*

SERGIO BONINI  
I Clinica Medica  
Università 'La Sapienza'  
Policlinico Umberto I  
00161 Roma - Italia