

Development of allergen-coated paper discs for allergy diagnosis by ELISA

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***In vitro* testing is useful for detecting pollen and fungal-allergen sensitivities in nasobronchial-allergy patients. However, allergen-coated discs or solid-phases supplied in kits do not contain all the relevant extracts. The present study was aimed to develop a safe and credible procedure of allergen coating on paper discs for allergy diagnosis by ELISA. Paper discs were coated with indigenous allergens using poly(vinyl alcohol) and glutaraldehyde. ELISAs were carried out to standardize the allergen-coated discs using skin-test-positive-patients' sera. The cut-off value (A) for ELISA was worked out by analysing normal human sera (NHS) and is based on the mean $\pm$ 2 S.D. for the NHS value. The allergen-coated discs, i.e. Centre for Biochemical Technology (CBT) discs, demonstrated excellent comparison with plate-binding method and Pharmacia discs [*Prosopis juliflora* (mesquite), critical value 0.879 at 5% probability $\pm$ 0.599, $n = 11$, is significant]. Inter- and Intra-assay coefficients of variations ranged from 2.5–14.05% to 4.8–11.5% with different allergens. Results of skin tests and ELISA with CBT discs showed 60–70% correlation.**

Introduction

Acute allergic symptoms such as asthma and rhinitis are due to raised specific IgE antibodies to atmospheric allergens [1,2]. These specific IgE antibodies can be measured by *in vitro* methods such as radioallergosorbent test (RAST) or immunosorbent assay (ELISA). However, *in vivo* tests [intra-dermal (ID)/prick test (PT)] are frequently used the world over for detecting offending allergens responsible for type I hypersensitivity disorders. Skin tests (ID/PT) possess several disadvantages, e.g. produce false-positive/negative reactions, chances of adverse systemic reactions (anaphylaxis) and are difficult to perform in children/persons with skin eruptions etc. Moreover, the skin tests cannot be practised during the patients' symptomatic days. *In vitro* tests such as RAST/ELISA are confirmatory tests and the titre of antibodies measured correlate well with weal/flare reactions

produced by skin tests with pollen and fungal-spore allergens [3–5].

Studies conducted previously have shown significant correlations in values of specific IgE estimations with RAST and ELISA [6,7]. ELISA is preferred over RAST, since it does not require the use of radiolabelled anti-IgE antibody [8]. Both the *in vitro* assays need an efficient solid-phase system containing sufficient allergen to bind all the specific IgE antibodies present in test sample. The IgG antibodies directed to the allergens in the natural course of events also compete and hinder the binding of specific IgE if the solid-phase allergen is in limited amount. The most commonly used methods for preparing solid-phase allergens are direct coating on poly(vinyl chloride) (PVC) membranes, polystyrene plates, agarose beads and CNBr-activated paper discs. Allergens prevalent in tropical countries are mostly different from those prevalent in Western world [9]. In the present study, allergen-coated paper discs were prepared by a new method and standardized with locally prevalent allergens for effective diagnosis of allergies by ELISA.

Material and methods

Collection of raw materials

Pollen grains were collected from known allergenic plants growing locally around the Delhi metropolis. The pollen samples were subjected to purification and microscopic analysis to check their quality. The samples with > 95% pollen (< 5% other floral parts of the same species) were selected for antigen extraction. Pure cultures of fungi were obtained from Indian Agricultural Research Institute, New Delhi, India. They were mass-cultured on Sabouraud's broth in the laboratory using standard protocols. The surface growth was harvested from the 12-day-old cultures, freeze-

Key words: cross-linking methods, *in vitro* test, respiratory allergy, solid support, specific IgE.

Abbreviations used: NHS, normal human sera; CBT, Centre for Biochemical Technology; CV, coefficient of variation; ID, intradermal; PT, prick test; RAST, radioallergosorbent test; PVC, poly(vinyl chloride); PTA, phosphotungstic acid.

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dried and powdered. All pollen and fungal raw materials were treated with diethyl ether, four or five times, to remove fat and other irritants prior to antigen extraction.

Extraction of antigens (allergens)

Extracts were prepared [10] by continuous stirring of pollen and or fungal raw materials for 16 h in ammonium bicarbonate buffer (0.05 M; 1:20, w/v) at 4 °C. The supernatant (extract) was separated by centrifugation (12000 g) for 30 min, freeze-dried and stored at -20 °C. The protein content of each extract was quantified by a modified Lowry method [11]. Phosphotungstic acid (PTA) reagent [15% (w/v) PTA in 10% (v/v) HCl] was used to precipitate the protein from the extracts. The biological potency of the extracts was defined on the basis of w/v (g/ml) ratio, protein estimation and biopotency tests on patients of known sensitivity.

ID tests and collection of human sera

Patients with nasobronchial allergy were selected on the basis of their clinical symptoms/history of illness, at the V. P. Chest Institute, Delhi, India, for ID tests with pollen and fungal extracts. For skin tests, about 0.02 ml of each extract was injected intradermally into the forearm of the patient, raising a bleb of 2–3 mm at the site of injection. As negative and positive controls respectively, PBS and histamine diphosphate (100 µg/ml) were also injected at separate sites. ID test reactions were graded after 20 min in comparison with the weal size of a negative control as described previously [10]. Skin tests with pollen/fungal extracts were also carried out on 25 healthy non-allergic volunteers (normal human sera, NHS). Blood was collected from patients showing positive ID tests (2+ to 4+) to the extract(s) tested and the persons (NHS) giving negative ID tests for use as negative control in ELISA. The consent of each patient and NHS was taken prior to skin tests and blood collection.

Allergen coating on solid supports

The coating of allergen (extracts) was carried out using a new method on Whatman-50 (W-50) filter paper. In this method, a mixture of each extract (proteins) with PVA was coated on to W-50 paper. Later glutaraldehyde was used in a suitable concentration to cross-link the amino group of protein (extract) entrapped on the W-50 paper [11a]. The discs were punched out after drying the allergen-coated paper using an office punch and called 'CBT discs'. Allergen-coated discs were also prepared by the CNBr-activation method described previously [12], taking the same allergen

extracts. For comparison, coating of extracts (proteins) was done by incubating wells of an ELISA plate with antigens in carbonate/bicarbonate buffer (0.05 M, pH 9.6) at 30 °C for 4 h.

Immunoassay for specific IgE

ELISAs were carried out [13] with allergen coated on paper discs and/or polystyrene high-binding microtitre plates (Corning). Unbound sites were blocked with 2% (w/v) BSA in PBS for 2 h at room temperature. Washing of discs/wells was performed three times with PBS/Tween 20 (0.05%). The discs/wells were incubated overnight at 4 °C with the patients' sera (1:10, w/v, in PBS; 200 µl/well). After washing with PBS/Tween 20, anti-IgE conjugated to alkaline phosphatase (1:2000, v/v, in PBS; 200 µl/well) was added and incubated overnight at 4 °C (Sigma). As a negative control, NHS was also tested at the same dilution. Washing was performed again and the colour was developed using *p*-nitrophenyl phosphate (1 mg/ml in glycine buffer). The substrate tetramethylbenzidine (TMB) was used for colour development when anti-IgE-horseradish peroxidase (1:2000, v/v, in PBS; 200 µl/well) was used as second antibody. The absorbance (A) was read at 405 nm (alkaline phosphatase)/490 nm (horseradish peroxidase) using a Dynatech ELISA reader.

Statistical analysis

Statistical analysis was performed using the ELISA results (A values) obtained with allergen-coated CBT discs, plate-binding method and allergen discs procured from Pharmacia, Uppsala, Sweden. Karl Pearson's coefficient-of-correlation test was employed, taking two paired samples for comparison.

Results and discussion

ELISA/RAST are useful techniques in the diagnosis of nasobronchial allergy disorders due to pollen and fungal-spore allergens [4,8]. The allergen is coated either on a solid support or on CNBr-activated paper discs and used in an ELISA or a RAST [14–16]. Initially we prepared allergen coated discs [*Amaranthus spinosus* (prickly amaranth) pollen extract] using CNBr method and tested by ELISA with positive ('ID+ve')-patients' sera. The discs worked well in ELISA, but there were problems on storage of the discs and in the handling of CNBr, a volatile carcinogenic chemical. Moreover, the activation of paper discs by CNBr method was found to be a cumbersome procedure. In the present study a process for immobilization of enzymes has been used

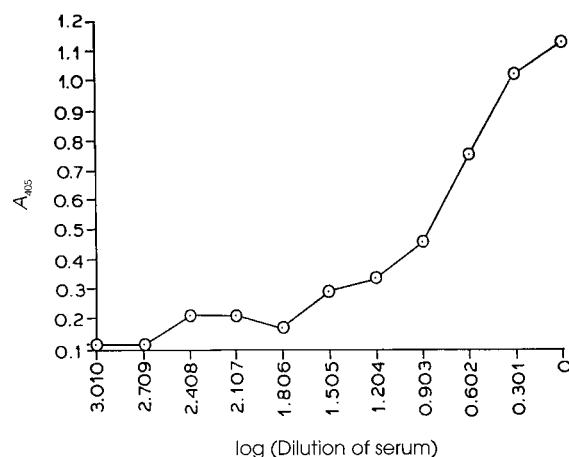


Figure 1 ELISA with *A. spinosus*-coated CBT discs

Serial dilutions of a strongly positive (ID) patient serum was used in the assay.

for the coating of pollen and fungal extracts with suitable modifications [17]. A number of gradient experiments were performed to find out the optimum amount of poly(vinyl alcohol), protein (extracts) for coating of paper discs, and glutaraldehyde for partial cross-linking of amino groups. Finally about $55 \mu\text{g}/\text{cm}^2$ of protein was coated on Whatman-50 filter paper, dried, and discs (2.5 mm diameter) were punched out using an office punch.

Dilution-dependent assays

To find out the working range of the assay, the dilution-dependent relationship was worked out by ELISA, taking CBT discs coated with *A. spinosus* allergens and positive-patient's serum in various dilutions (1:1–1:1024, v/v, in PBS). The assay (ELISA) worked well up to a serum dilution of 1:32 (w/v) in PBS (Figure 1). In the present study, serum dilution of 1:10 (v/v) in PBS was selected to carry out all the ELISAs with CBT discs and the results were compared with other methods. ELISAs with allergens coated on paper discs were carried out in tissue-culture plates. Similar results were obtained when the dilution-dependent assay was repeated with *Prosopis juliflora*-allergen-coated CBT discs and the positive serum of this extract (results not shown).

Defining ELISA results

ELISA results are expressed by comparing the absorbance of test samples with negative and positive controls. In the present study we have screened negative controls ('ID – ve', 20 NHS) by ELISA and positive-patients' sera with *A. spinosus*-allergen-coated CBT discs. Scattergrams were plotted, taking the A values obtained in ELISA. Means $\pm$ 2 S.D. of NHS results were calculated and marked on the graph as

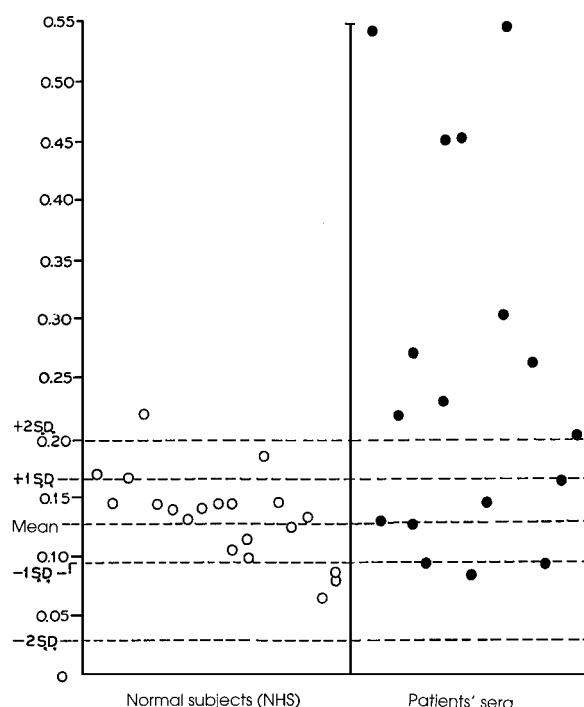


Figure 2 Scattergram plotted with A values obtained in ELISA to *A. spinosus*-coated CBT discs

Normal human sera (20) and test sera of ID+Ve patients are marked, along with the mean $\pm$ S.D.

per the criteria of Calenoff et al. [18]. The test sera showing an absorbance greater than 0.225 could be considered as ELISA-positive for *Amaranthus spinosus* (Figure 2). The same set of experiments were repeated with CBT discs prepared from other important pollen allergens such as those from *Holoptelea integrifolia* (Indian elm tree), *Imperata cylindrica* (cogon grass), *Prosopis juliflora* (mesquite) and *Putranjiva roxburghii* (Indian amulet tree). The cut-off values of these allergens ranged from A. 0.13 to A 0.16 in order to grade them as ELISA-positive. The positive controls, i.e. reference sera/discs [birch (*Betula verrucosa*) pollen], procured from Pharmacia, were run in parallel with the patient's sample in ELISA. The calibration curve was plotted with A values obtained for each reference serum. As per the grading criteria [19], sera showing 3.5 Phadebas RAST units/ml, A values corresponding to approx. 0.28 or more can be considered as ELISA-positive. McSharry et al. [20] recommended that sera with 4 Phadebas RAST units/ml (i.e. 10 ng/ml specific IgE) should be regarded as ELISA-positive.

Evaluation of allergen coated CBT discs by ELISA

(a) *Inter- and Intra-assay variations* To observe the intra- and inter-assay variations in ELISA, CBT discs coated with pollen extracts were screened individually (Tables 1a and

Table 1 Intra-assay (a) and inter-assay (b) variations in specific IgE estimations by ELISA using CBT discs coated with different allergens

(a)		
Allergen extract	A ₄₀₅ (mean±S.D.)	CV (%)
<i>A. spinosus</i>	0.51±0.052 (n = 38)	10.17
<i>I. cylindrica</i>	1.57±0.076 (n = 47)	4.87
<i>H. integrifolia</i>	0.27±0.031 (n = 44)	11.50
(b)		
Allergen extract	A ₄₀₅ (mean±S.D.)	CV (%)
<i>A. spinosus</i>	0.44±0.061 (n = 6)	14.05
<i>I. cylindrica</i>	1.54±0.038 (n = 9)	2.46
<i>H. integrifolia</i>	0.29±0.017 (n = 5)	5.76

1b). For inter-assay variations, six sets of assays (ELISA) were performed on 6 consecutive days using positive patients' sera to the respective allergen. Six assays (1:10, v/v, in PBS) were carried out in duplicate (40 wells). For intra-assay variations, ELISAs were done simultaneously in 40 wells with positive sera (1:10, v/v, in PBS) using the respective allergen-coated discs. Well-to-well variations in the microtitre plates were also determined by adding alkaline phosphatase-conjugated anti-IgE (100 µl/well) and the requisite substrate to develop the colour. S.D. and CV values were calculated by using standard formulae [19]. As evident from the results, CV values for inter-assay variation ranged from 2.5 to 14.05%, and intra-assay results were 4.8 to 11.5%, with CBT discs. A study [21] conducted elsewhere with allergen-coated discs of mite (*Dermatophagoides pteronyssinus*), timothy-grass (*Phleum pratense*) pollen and dog dander (skin scales) showed CV values of 4–9% for intra-assay variation, whereas it was 17–29% for inter-assay variation. Plebani et al. [22] reported a CV of 1.58–6.2% in within-assay experiments and it was 6.3–11.5% for inter-assay variation [22]. For independent validation of the assay with CBT discs, specific IgE was measured by ELISA in the same six sera samples independently by three researchers not involved in the present study. The absorbance values recorded were found to be similar, and the same four sera samples were assigned as ELISA-positive (A, mean±2 S.D. of NHS) by all three workers.

(b) *Skin-test grading versus ELISA* Specific IgE was measured using five important pollen (see above) and five fungal-allergen (*Alternaria alternata*, *Aspergillus fumigatus*, *Curvularia lunata*, *Helminthosporium anomalum* and *Epicoccum nigrum*) coated CBT discs in ID+ve patients' sera (30) to each respective extract. Analysis of skin tests and ELISA results showed a 60–70% correlation, i.e. sera collected from ID+ve cases proved ELISA-positive as well. Seltzer et al. [6] reported an 81% correlation between PT and modified RAST results in eight patients. Studies carried out in other

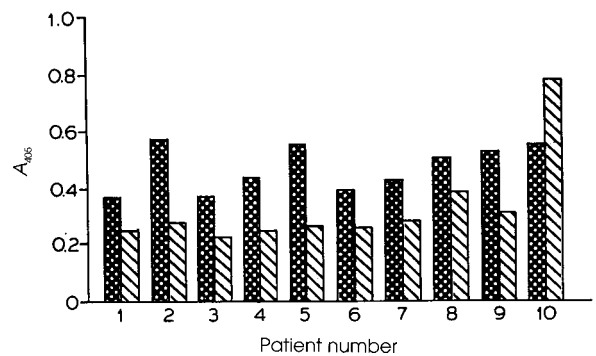


Figure 3 Comparison of specific IgE estimations by *A. spinosus*-coated CBT discs (▨) and the plate-binding method (■) with same extracts

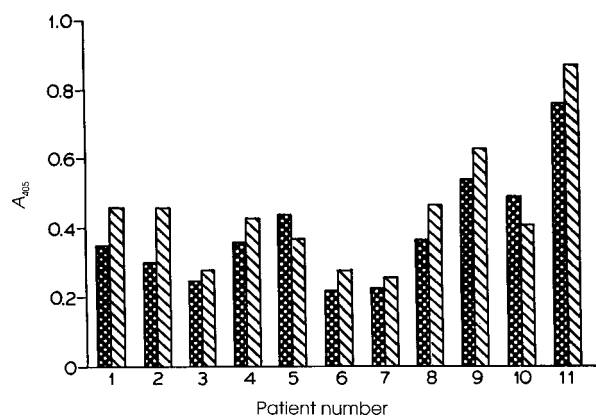


Figure 4 Comparison of specific IgE estimations by ELISA with *P. juliflora*-coated CBT discs (▨) and Pharmacia discs (■) with the same extract

countries [2–5] showed a 60–80% correlation between skin tests and RAST/ELISA results. ELISA has been recognized as an alternative to RAST for measurement of allergen-specific IgE [8].

(c) *ELISA with CBT discs and the plate-binding method* For comparison, specific IgE was estimated in sera samples by ELISA using *A. spinosus*- and *I. cylindrica*-coated CBT discs, individually, and the same extracts were coated on high-binding microtitre plates. The majority of the sera samples tested showed higher absorbances in ELISA with CBT discs than with antigen coated on to the high-binding plates (Figure 3). Similar results were obtained earlier by other workers, hence allergen-coated discs are favoured for ELISA [5].

(d) *ELISA with CBT discs versus Pharmacia discs* To compare, ELISAs were carried out with CBT discs and discs of the same allergen (*P. juliflora* and *A. fumigatus*) procured from Pharmacia (Figure 4). Statistical analysis of A values obtained with CBT discs and Pharmacia discs revealed an excellent

correlation (critical values 0.879 and 0.821 respectively). At 5% probability, the critical values ± 0.599 ($n = 11$) and ± 0.530 ($n = 14$) respectively were recorded as significant for a two-tailed analysis.

To determine non-specific binding, if any, ELISAs were carried out with NHS and *P. juliflora*-coated CBT discs and Pharmacia discs. The A values obtained with Pharmacia discs were higher (mean NHS 0.143) than CBT discs (mean NHS 0.065). Toorenenbergen et al. [23] have also reported non-specific IgE binding to cellulose discs (Pharmacia) in RAST.

(e) *Stability of allergen-coated discs* The shelf life of allergen-coated CBT discs was studied by ELISA using discs stored in dried form at three different temperatures (40, 30 and 4 °C). The allergen-coated discs stored at high temperature (i.e. 40 °C) for 1 month showed a sharp rise in A values (more than original) and this may be due to the exposure of more allergenic epitopes. However, A values slumped considerably from the second month onward for discs stored at 40 °C. The allergen-coated CBT discs were found to be quite stable for 3 months when stored at 30 and 4 °C respectively. However, a loss of 10–20% of the allergenic activity of the discs was recorded from the fourth month onwards. If we subtract the 10% average inter-assay variation, the disc-coated allergen prepared by the new method retained 90% of its allergenic potency for 6 months. The commercially available allergen discs claim a shelf life of about 1 year when stored at 2–8 °C.

In conclusion, allergen-coated discs were prepared by a simple, reliable method and were standardized for effective diagnosis of allergies by ELISA.

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