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Crossed Radio-Immunoisoelectric Focusing as a Method of Identifying Isoallergens: Identification of Isoallergens in Rat Urine Extracts

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A method of 2-dimensional radio-immunoelectrophoresis to detect directly the presence of 'isoallergens' in complex allergenic (skin test) extracts is described. This procedure, in which the components are separated by isoelectric focusing in agarose gel in the first dimension is therefore basically similar to that of crossed radio-immunoelectrophoresis, and hence has been termed crossed radio-immunoisoelectric focusing. The method has been applied to the allergens present in rat urine and has verified the presence of the cross-reacting α_2 -euglobulin and prealbumin components in (at least) 3 and 2 isoallergenic forms respectively.

Key words: *crossed radio-immunoisoelectric focusing – isoallergens – rat urine allergens*

Introduction

Crossed radio-immunoelectrophoresis (XRIE) was first introduced by Weeke and Lowenstein (1973) as a means of identifying the allergenic components present in the complex mixtures so characteristic of most allergenic (skin test) extracts. It has now been applied to a wide variety of such extracts, e.g., pollens, animal danders, fungi, house dust, etc. (Lowenstein, 1978, 1980) and has recently been recommended as an assay procedure for the standardization of such extracts (Lowenstein, 1980).

There has been increasing use of both analytical and preparative isoelectric focusing (IEF) methods for the characterization and purification of allergenic components. However, antigens which are immunologically identical may show different migration velocities, i.e., components with differing pI values, indicative of electrophoretic heterogeneity due to minor structural differences such as the nature of their carbohydrate moieties or degree of amidation (Marsh, 1975). Purified allergens that have been reported to exist as isoallergens include rye grass pollen, Rye I (Johnson and Marsh, 1965), ragweed pollen AgE (King et al., 1964), cod fish allergen M (Elsayed and Aas, 1971), *Cladosporium herbarum* Ag32 (Aukrust and

Borch, 1979) and house dust mite P1 (Chapman and Platts-Mills, 1980). In all but the latter case, the pI values of the isoallergenic components differed by less than 1 pI unit and in most instances the existence of the isoallergens was demonstrated after lengthy purification procedures.

A more direct qualitative IEF technique to identify allergenically active bands (Schroder et al., 1980; Yman et al., 1980) employed an enzyme-immunoassay system. This method, based on RAST (radioallergosorbent test), involved binding the focused proteins to cyanogen bromide activated paper, followed by patient's serum, anti-IgE enzyme conjugate, the enzyme reactions being developed with a zymogram staining procedure (Yman et al., 1980).

Although helpful in identifying allergenic bands this method does not give an indication of any antigenic relationship. The combination of crossed immunoisoelectric focusing with autoradiography (cf. XRIE), as applied in this paper to allergenic rat urinary components, allows for simultaneous comparison of the separated proteins for both their antigenicity (in rabbits) and their allergenicity (in man).

Materials and Methods

Antigen

Rat urine antigen was prepared as previously described (Longbottom, 1980) except that dialysis was against 0.05 M NH_4HCO_3 to prevent the precipitation of the α_2 -euglobulin component.

Human sera

These were obtained from rat allergic subjects who associated symptoms of asthma and rhinitis with exposure to rats and who were positive in skin prick tests and by RAST to the rat urine antigen (Longbottom, 1983).

Rabbit antisera

Rat urine antiserum was produced in rabbits (NZW) by repeated subcutaneous and intramuscular injections of the antigen emulsified with Freund's incomplete adjuvant. Initially 25 mg antigen was administered, repeated with 15 mg after 28 days and thereafter the rabbits were boosted with a saline solution (2 mg/ml) until a satisfactory response was obtained as determined in crossed immunoelectrophoresis (XIE) tests. A polyspecific antiserum pool was then prepared using such high titre sera from 3 rabbits. Anti-rat serum was obtained from Dakopatts A/S (Dako-immunoglobulins, Copenhagen).

Crossed immunoisoelectric focusing (XIIEF) methods

Isoelectric focusing (IEF) gels for the first dimension were prepared by dissolving 165 mg agarose IEF (Pharmacia Fine Chemicals, Uppsala) with 1.98 g D-sorbitol in 15.5 ml distilled water in a boiling water bath; the solution was allowed to cool to approximately 75°C and 1.05 ml ampholyte (pH 4–6.5 Pharmalyte; Pharmacia,

Uppsala) added. After mixing the gel was poured carefully onto the hydrophilic side of a pre-warmed GelBondTM plastic sheet, 11 cm × 12.5 cm, supported on a polystyrene pad on a level surface, allowed to set at room temperature and then harden at 4°C for 1 h before use. Five microlitre samples of antigen (10 mg/ml in 0.9% saline) were added to moist filter paper squares (5 mm × 5 mm) applied close to the cathodic side of the gel and focusing was carried out at a constant 8 W, i.e., 1500 V and unlimited current (Multiphor-R 2117 with DC power supply 2103, LKB, Sweden) for 90 min at 6°C with 0.05 M H₂SO₄ on the anode and 1 M NaOH on the cathode wicks. The final pH gradient across the gel was measured by a surface electrode.

Immediately focusing was completed, the gels were cut into strips (parallel to the IEF separation) for use in XIEF and 1 replicate fixed and stained as recommended by the manufacturer. Briefly, the gel is immersed in fixing solution (5% sulphosalicylic acid, 10% trichloroacetic acid in distilled water) for 30 min, then washed twice, i.e., 15 min in destain solution (35% ethanol/10% acetic acid in distilled water) and finally dried with 3–4 layers of filter paper (Whatman 3 MM) pressed onto the gel surface (2–5 g/cm²) for 10–15 min, and then in a current of warm air. The film is then stained (0.2% Coomassie blue R250 dissolved in destain solution) 5–10 min, and washed until the background is clear.

Whilst the samples were being focused, gels for the second dimension immunoelectrophoresis were prepared. Twelve millilitres 1% agarose (BDH England) in veronal buffer, pH 8.65, (stock solution: 20.6 g sodium barbitone, 3.68 g barbitone in 1 litre distilled water, $\mu = 0.1$, diluted 1 in 4 for the gel and 1 in 2 for the electrode compartments) were poured onto sheets of GelBondTM, 11 cm × 9 cm, prewarmed, etc., as described previously.

Areas of gel containing antiserum were incorporated by removing windows of gel and refilling with buffered agarose (56°C) containing appropriate antiserum, i.e., 8 cm × 3.5 cm for the main gel (MG) incorporating anti-rat urine, and 8 cm × 0.8 cm for the intermediate gel (IG) containing anti-rat serum separated by a 2 mm strip of the plain buffered agarose (Fig. 1). Depending on the relative titres of the rabbit antibodies, these antisera were incorporated at between 10–25 μ l antiserum/cm² gel. As with crossed immunoelectrophoresis (Longbottom, 1980; Walls and Longbottom, 1983a) an intermediate absorbing gel was employed in these experiments to distinguish the specific urinary from the serum-derived protein components in the urine antigens.

After cutting off the ends close to the electrode wicks one of the replicate strips of gel from the first dimension IEF was then laid gel surface down, parallel to and at a 2 mm distance from the 8 cm lower edge of the IG as indicated in Fig. 1. Continuous electrophoresis in the second dimension was carried out at 1.5–2 V/cm for 20 h. This method of laying on the IEF gel strip was found to give a more satisfactory result than attempting to lay the IEF strip gel side uppermost in an area where gel had been removed and using buffered agarose to mould the gels together according to the manufacturer's instructions for XIEF.

After completion of the second dimension electrophoresis, the gels were immersed in saline wash solution (0.9% NaCl with 0.05% sodium azide added) with 3 changes

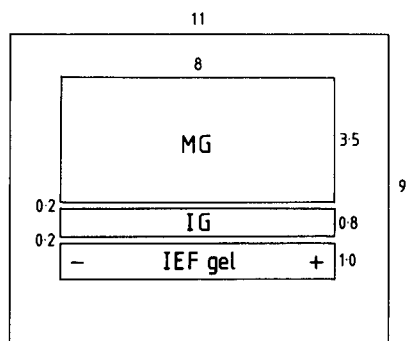


Fig. 1. Diagram giving dimensions (cm) and layout for the second dimension immunoelectrophoresis. MG = main gel, IG = intermediate gel, anode at the top.

of 30 min each, then dried as for the IEF gels except that the final drying was carried out in a current of cool air.

For the overlay of patient's serum, an approximately 1 cm outer border of wax crayon was applied to the dried agar side of the GelBond sheet, to render it hydrophobic, leaving clear the central area. Also, to ensure that the sheets remained flat during the subsequent overnight incubations, a few drops of distilled water were used to adhere them to glass plates by their hydrophobic underside. Seven millilitres of a 1 in 10 solution (in PBS, pH 7.3) of patient's serum was then carefully pipetted onto the central area and left to react overnight (18–20 h) at room temperature. It is necessary to ensure that the plates are on a level surface and covered to avoid drying.

The non-bound serum proteins were aspirated and the area washed with 7–8 ml saline wash solution at least 5 times for 12–15 min and blotted dry. Seven millilitres of ^{125}I -anti-IgE (Phadebas-RAST isotope; Pharmacia Diagnostics, Uppsala) reconstituted as directed and further diluted in PBS containing 0.3% w/v HSA to approximately 10,000 cpm/100 μl , were pipetted onto the same central area and left overnight (as previously). After carefully removing the non-bound anti-IgE, the sheets were washed, as before, and then dried in a current of warm air.

For autoradiography the dried agar surface was placed on X-ray film (Kodak no-screen film NS-2T) and left under applied pressure for 5–7 days. The exposed X-ray film was developed according to the manufacturer's instructions and the XIIEF sheet stained in Coomassie blue stain (0.5% in 45% ethanol; 10% glacial acetic acid in distilled water) (Pepys and Longbottom, 1977). As well as tests with allergic patients' sera a control test with a pool of sera from normal individuals was included to reveal possible non-specific absorption of either IgE and/or anti-IgE to the immune precipitates.

Results

The IEF profile of whole rat urine, pH 4–6.5 (Fig. 2a), shows the number of protein components separated. There are the 2 major bands in the prealbumin

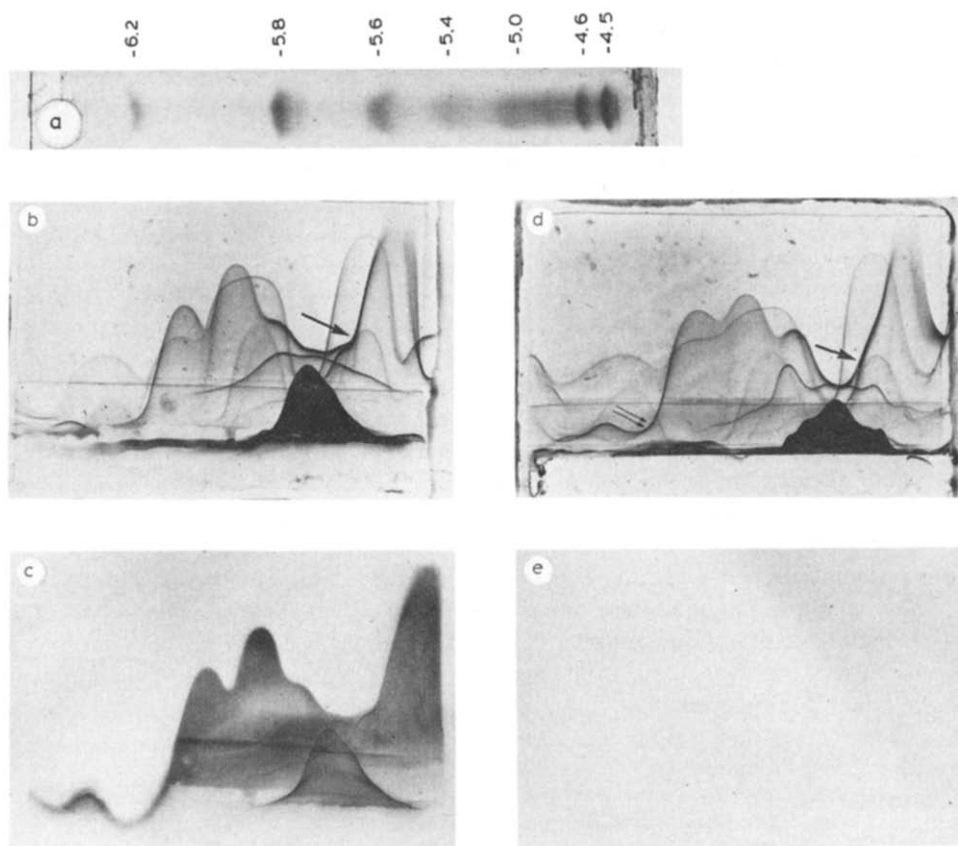


Fig. 2. (a) Reference IEF gel strip of rat urine (50 μ g) stained with Coomassie blue. (b–e) Crossed radio-immunoelectrophoresis patterns (XRIEF). (b) and (c) allergic serum; (d) and (e) control serum; (b) and (d) protein stained XRIEF and (c) and (e) the corresponding autoradiographs. Reaction of partial identity between prealbumin and α_2 -euglobulin indicated by arrow. In (d) a further reaction of partial identity is visible between the components with pIs 5.8 and 6.2, as indicated by the 2 arrows.

region, with pIs 4.5 and 4.6, and other components extending throughout the pH range, with major bands pI 5.6 and 5.8. The serum albumin, present in moderate amount in this particular rat urine extract (the amount of serum albumin in rat urine is largely dependent on sex and age) does not focus well and is seen as diffuse staining at approximately pI 5.

The XRIEF pattern (Figs. 2b and d) developed by using replicate lanes from the IEF as shown in Fig. 2a shows the reaction of partial identity between the prealbumin peak and other major protein peaks. These latter show a reaction of antigenic identity, i.e., the continuous line forming 2 distinct peaks corresponding with the position of the major protein bands with pIs 5.6 and 5.8 with a shoulder peak pI 5.4 and also possibly a fourth partially identical minor peak at pI $\approx$ 6.2 (Fig. 2d). The serum albumin present is seen as the densely stained peak absorbed in the

anti-rat serum intermediate gel with a peak maximum at approximately pI 5.0. Further minor peaks, some also showing reactions of antigenic identity between their components with different pIs, are also visible with these polyspecific rabbit antisera.

Figs. 2c and e are the autoradiographs obtained from Figs. 2b and d respectively, b/c with the serum of a rat allergic patient and d/e with the control serum pool. From Fig. 2c it is evident that specific IgE antibody has been absorbed to both the prealbumin peak and the 3 (possibly 4) α_2 -euglobulin peaks. Although with this patient's serum more radioactivity appeared to be associated with peak pI 5.6, this was not the case with the other rat-allergic human sera tested, where the radioactive uptake appeared approximately the same on all the peaks. However, as this was essentially a qualitative analysis, no attempt was made to semiquantify the activity by developing the autoradiographs for different periods of time.

Radioactivity present on the albumin peak may be non-specific due to coprecipitation of allergen within the dense immunoprecipitate (Lowenstein, 1978), as found in XRIE with rat allergen extracts (Walls and Longbottom, 1983b). None of the other minor peaks show any significant radioactive uptake, indicating the absence in this particular patient's serum of specific IgE to these components.

The pI values obtained here by agarose IEF were all slightly higher than those recorded previously (Longbottom, 1980) for polyacrylamide IEF in both systems a surface electrode was used to measure pH. No explanation for this apparent discrepancy can be given.

Discussion

Previous studies (Longbottom, 1980, 1983) showed that the 2 major (non-serum) allergenic components of rat urine, an α_2 -euglobulin and a prealbumin are antigenically related, the α_2 -euglobulin forming a reaction of partial identity with the prealbumin. A similar allergenic relationship, indicating possible shared allergenic determinants between these components, has also been demonstrated by crossed radio-immunoelectrophoresis with sera of rat allergic individuals (Longbottom, 1980; Walls and Longbottom, 1983b).

Separation on columns of Sephacryl S200 resulted in the preparation of 2 highly skin reactive fractions (skin prick tests positive at 10^{-2} mg/ml) the prealbumin MW 20–21,000 and α_2 -euglobulin 16–18,000 (Longbottom, 1980, 1983). These purified fractions gave virtually single antigen peaks on XIE with the polyspecific antisera, i.e., showed only trace contamination with each other (unpublished data), complete separation being difficult due to the similarity of their molecular weights. On isoelectric focusing, however, several protein bands with distinct pI values were associated with each purified fraction (prealbumin, pIs 4.5 and 4.6, and α_2 -euglobulin pIs 5.4, 5.6 and 5.8 (Longbottom, 1983) so that, as with previously isolated allergens (King et al., 1964; Johnson and Marsh, 1965; Elsayed and Aas, 1971; Aukrust and Borch, 1979; Chapman and Platts-Mills, 1980) these were considered to be 'isoallergenic' forms. The method described here has now confirmed that these

major components do exist in several isoantigenic/isoallergenic forms, and shown that such components may also be identified in crude extracts using polyspecific rabbit antisera.

As with RAST and XRIE, the method relies on a number of factors, the most important being immobilization of the allergen. In RAST this is achieved by chemically coupling the 'allergens' to an inert support and here, as in XRIE, by their precipitation as immuno-precipitates (antigen/antibody complexes), thus relying in the first instance on their being immunogenic in rabbits. Furthermore, it must be assumed that the precipitated allergenic molecules maintain their ability to combine with human IgE antibody although as yet it is not known whether this reaction is with the same or different antigenic determinants as the rabbit antibody.

Choice of the appropriate pH for the first dimension IEF separation must be determined initially by analytical IEF analysis, e.g., pH 3.5–9, either in agarose or polyacrylamide gel to identify the various protein components present in the extract. Better resolution of the 'isocomponents' may then be achieved by incorporation in the gel of a narrower range ampholyte appropriate for the components under examination, in the present case pH 4–6.5. The intermediate gel used here to distinguish the serum components from the specific urinary components, would not normally be required in other allergen systems. The application of a wax crayon border around the gel sheet provides a hydrophobic barrier so that an area of constant size may be used for each batch of tests, thereby ensuring a more quantitative assay and enabling smaller volumes of patient's serum and ^{125}I -anti-IgE to be used.

This method of crossed radio-immunoelectric focusing may be used not only to identify or confirm the existence of isoallergens, as here, but also as a guide for future isolation procedures by qualitatively assessing both antigenic and allergenic activities of the crude complex allergenic extract before attempting separation, particularly by preparative IEF or chromatofocusing. It may also enable IEF to become an even more useful procedure in allergen standardization.

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