

## **Analysis of tobacco leaf allergens by crossed radioimmuno-electrophoresis**

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### **Summary**

The reactivity of eleven 'tobacco smoke sensitive' and eight 'non-sensitive' individuals to tobacco leaf allergens was tested by Crossed Radioimmuno-electrophoresis (CIE). All nineteen study subjects had IgE antibodies to tobacco leaf antigens as measured by Radioallergosorbent Test (RAST) and seventeen of the nineteen individuals were atopic. Of the thirty-seven tobacco leaf precipitins detected by Cross Immuno-electrophoresis (CIE), three were identified as allergens by CIE. All nineteen subjects reacted to at least one of the three allergens detected. However, neither the intensity nor the incidence of reactivity with any of the three allergens correlated with smoking or 'smoke sensitivity'.

### **Introduction**

Recently there has been increased interest in the immune response of man to tobacco antigens. This has been due to the supposition that tobacco smoke can stimulate a specific IgE response in man to tobacco allergens which causes 'tobacco smoke hypersensitivity' reported by some individuals [1–6]. However, this subject remains controversial and a relationship of 'tobacco smoke sensitivity' to either positive skin tests or IgE antibodies to tobacco allergens has not been demonstrated [7–9]. In contrast to these studies, an occupational IgE-mediated allergy has recently been described in one atopic tobacco worker to tobacco leaf allergens [10].

A problem with past studies is that no qualitative assessment was done of IgE reactivity to tobacco allergens. Thus if a subject reacts with multiple tobacco leaf allergens, it is possible that only one may be pathogenic for smoke sensitivity. In order to further evaluate the IgE antibody response of man to tobacco leaf allergens, and determine if there is a relationship of this response to 'smoke sensitivity', the following

investigation utilized the crossed radioimmuno-electrophoresis (CRIE) technique. In this study, the reactivity of 'smoke sensitive' and 'non-sensitive' study subjects to tobacco allergens was determined by CRIE and the incidence of these reactions assessed for any relationship to 'tobacco smoke sensitivity' or smoking history.

## Materials and methods

### *Test sera*

Sera were obtained from study subjects selected on the basis of clinical sensitivity to tobacco leaf as described in a previous report [9]. Briefly, study subjects were interviewed with a standard questionnaire for information on general health history, allergy history, smoking history, and types of symptoms, if any, encountered when exposed to tobacco smoke. Smoke 'sensitive' individuals were defined as those who claimed conjunctival, nasal, and/or bronchial tobacco smoke induced symptoms (i.e. intense lacrimation, rhinitis, sneezing, wheezing, or cough) on passive exposure to tobacco smoke. Individuals claiming no difficulty when exposed to tobacco smoke were classified as non-sensitive. Study subjects smoking at least one cigarette per day for the last 6 months were defined as current smokers. Ex-smokers were past smokers who had stopped smoking for at least the last 6 months. Non-smokers were individuals who had never smoked one cigarette per day or more for a six month period. Atopic individuals were study subjects with a personal and/or family history of allergy and two or more positive skin-prick tests to sixteen common inhalant allergens.

### *Tobacco leaf antigens and antisera*

A leaf extract was prepared from air-cured burley tobacco leaf (donated by D. L. Davis, Department of Agronomy, University of Kentucky, Lexington, KY) by extraction with phosphate (0.01 M) buffered (pH 7.2) saline (0.15 M NaCl), as reported in detail elsewhere [11]. Briefly, 100 g of the leaf was homogenized in 1.5 litres of phosphate buffered saline containing 0.1% sodium azide. Following overnight mixing at 4°C, residual leaf was removed by filtration through gauze and the filtrate centrifuged (40 000 × g). The lipid-like material at the surface of each tube was discarded and the remaining aqueous portion of the supernate precipitated with ammonium sulfate (50% saturation). Pellets were redissolved in and dialysed exhaustively against PBS. Rabbit antisera to leaf extract were prepared in 2.5 kg New Zealand white rabbits hyperimmunized with tobacco leaf extract as described previously [11,12]. All rabbit sera were concentrated ten-fold by ammonium sulfate precipitation (40% saturation) and ultrafiltration (Amicon UM 50 filter).

### *IgE assays*

The Radioallergosorbent test (RAST) utilizing cyanogen bromide activated discs coated with tobacco leaf extract [13] was performed as described previously [8,9]. Ratios  $\geq 2$  were arbitrarily considered to be positive. IgE determinations were done in duplicate using the PHADEBAS paper-radioimmunoabsorbent test (PRIST) kit, according to the directions of Pharmacia. IgE values (units/ml) were computed from a standard curve.

*Crossed immunoelectrophoresis (CIE) and crossed radioimmuno-electrophoresis (CRIE)* CIE was carried out in the first dimension on 10 × 10 cm glass plates, using a Tris-

Barbital buffer (pH 8.6) in 1% agarose (Eastman Kodak) essentially in accordance with the previously described procedure [14,15]. Five  $\mu$ l tobacco leaf extract (33.7 mg dry weight/ml) was run in the first dimension electrophoresis for 25 min. The second dimension electrophoresis was performed on  $5 \times 7$  cm glass slides at  $24^\circ\text{C}$  (cooled with tap water) for 20 hr with 2 V/cm using 50  $\mu$ l antiserum/slide (2.5  $\mu$ l per  $\text{cm}^2$  gel). The washed CIE plates were stained with Coomassie blue.

CRIE was performed in order to identify allergens in the CIE plates [15]. Unstained CRIE plates were incubated with 0.4 ml of the patient's serum diluted to 7 ml with the CRIE incubation buffer (70 mM phosphate buffer pH 7.0 + 15 mM  $\text{NaN}_3$ ) for 24 hr at  $24^\circ\text{C}$  in  $5.5 \times 7.5$  cm plastic boxes. The plates were washed as previously described, and incubated with approximately 250 000 cpm  $^{125}\text{I}$  anti-IgE (Phadebas RAST Isotope Reagent) in 7 ml total volume (0.05 M phosphate buffer, pH 7.5, 0.3% BSA, 0.15 M NaCl, 15 mM  $\text{NaN}_3$  + 2.7 mM EDTA  $2\text{H}_2\text{O}$ ) for another 24 hr. The plates were washed, dried, and placed on X-ray photographic film (Kodak XRP-1) for autoradiographic exposure.

All plates were exposed for varying time intervals (a few hours to several weeks) so that each exposure corresponded to one particular CRIE class, using a CRIE reference system [16]. The exposure periods were increased in a two-fold manner: the shortest period denoted CRIE class A and the longest exposure period denoted CRIE class G. Each precipitate giving rise to radiostaining, was said to be in a CRIE class corresponding to the shortest exposure period necessary to give visible radiostaining.

## Results

The CIE analysis of tobacco leaf allergen (Fig. 1) detected thirty-seven different precipitating antigens. Most of the antigens had anodic mobility, five appeared to have cathodic mobility. In order to determine which of these reacted with patients' IgE

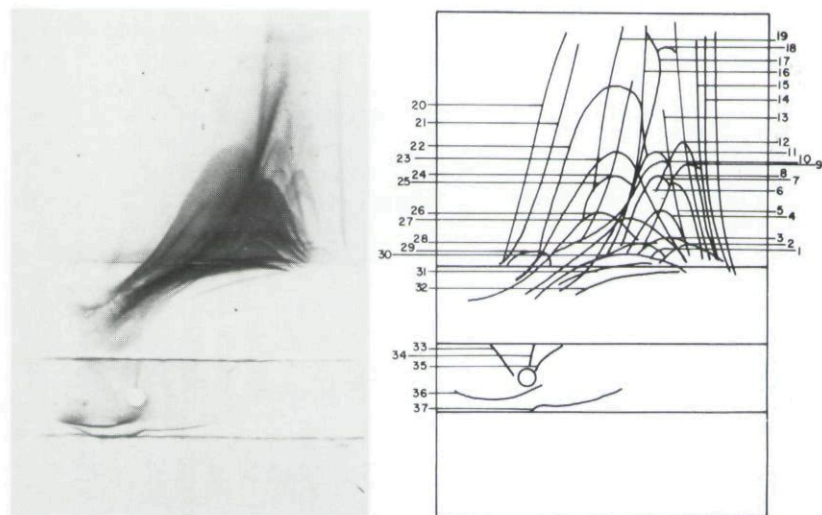


**Fig. 1.** CIE of tobacco leaf antigens. 169  $\mu$ g of tobacco leaf was electrophoresed for 25 min, 10v/cm followed by electrophoresis for 18 hr, 2v/cm into second dimension gel containing 50  $\mu$ l rabbit anti-tobacco leaf. CIE reference pattern of tobacco leaf with precipitins arbitrarily numbered.

antibodies, CRIE was performed. The results of representative CRIEs are summarized in Fig. 2. Here, using representative sera, it was demonstrated that precipitins 19, 23 and 27 reacted with patients' IgE antibodies, as demonstrated by the positive CRIE reaction.

The precipitin to which each sera reacted and the CRIE class of that reaction were determined and the results summarized in Tables 1 and 2. Individuals from whom sera

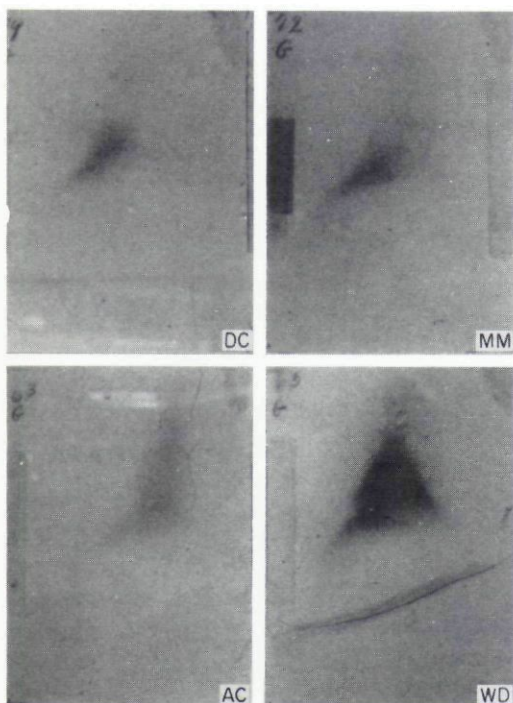


Fig. 2. CRIE of tobacco leaf allergens. The precipitins detected in these radiographs correspond to reference class G after 21 days exposure with 25 000 cpm<sup>125</sup>I. Three allergens were detected.

Table 1. Atopic status, IgE levels, smoking history and CRIE classes of smoke sensitive study subjects

| Study subjects | Total serum IgE | Smoking history | RAST  |              | CRIE classes precipitins |    |    |
|----------------|-----------------|-----------------|-------|--------------|--------------------------|----|----|
|                |                 |                 | Atopy | Tobacco leaf | 19                       | 23 | 27 |
| AC             | 4600            | Sm              | +     | 4.0          | F                        | F  | —  |
| TB             | 600             | Sm              | +     | 8.0          | —                        | G  | —  |
| SM             | 750             | Sm              | +     | 2.7          | —                        | —  | G  |
| WD             | 4100            | Ex-sm           | +     | 12.4         | C                        | B  | G  |
| PV             | 200             | Ex-sm           | +     | 8.0          | —                        | —  | G  |
| DC             | 490             | Ex-sm           | +     | 4.9          | —                        | —  | F  |
| BA             | 320             | Non-sm          | +     | 9.6          | F                        | F  | —  |
| MC             | 230             | Non-sm          | +     | 13.1         | —                        | F  | F  |
| RF             | 215             | Non-sm          | +     | 14.8         | F                        | —  | —  |
| MM             | 90              | Non-sm          | —     | 16.3         | —                        | —  | G  |
| MM             | 500             | Non-sm          | +     | 8.8          | —                        | F  | E  |

**Table 2.** Atopic status, IgE levels, smoking history and CRIE classes of non-sensitive study subjects to cigarette smoke

| Study subjects | Total serum IgE | Smoking history | RAST  |              | CRIE classes precipitins |    |    |
|----------------|-----------------|-----------------|-------|--------------|--------------------------|----|----|
|                |                 |                 | Atopy | Tobacco leaf | 19                       | 23 | 27 |
| PM             | 4               | Sm              | —     | 5.2          | —                        | —  | —  |
| GC             | 13              | Sm              | +     | 5.5          | —                        | F  | —  |
| BD             | 96              | Ex-sm           | +     | 7.9          | —                        | F  | —  |
| NN             | 540             | Ex-sm           | +     | 13.9         | —                        | F  | E  |
| CD             | 210             | Ex-sm           | +     | 12.1         | F                        | —  | G  |
| JW             | 66              | Non-sm          | +     | 7.3          | —                        | G  | —  |
| VT             | 9000            | Non-sm          | +     | 4.5          | —                        | G  | —  |
| SW             | 350             | Non-sm          | +     | 15.9         | —                        | —  | F  |

were obtained were almost all atopic, although their IgE levels and smoking history varied considerably. Two groups of sera were studied, those from individuals clinically 'sensitive' by history and those 'not sensitive' to cigarette smoke. All of the individuals had positive RAST values to tobacco leaf extract (although these values varied considerably) and were skin test positive to tobacco leaf extract. A similar RAST ratio range was observed for both the sensitive and non-sensitive groups of study subjects.

Sera from these study subjects reacted with three allergens, corresponding to precipitins 19, 23, and 27, as detected in CIE (Fig. 1). The majority of individuals reacted to precipitin 23, while many sera reacted to 19 and 23 or 23 and 27. One individual (WD) reacted to all 3 precipitins. The intensity of the reaction was generally not remarkable in that most individuals had CRIE classes F and G, with the exception of WD, whose serum had a reaction corresponding to CRIE classes B and C. Control sera from atopic individuals non-sensitive to smoke and RAST negative to tobacco leaf, did not react with any allergens by CRIE. Neither the overall prevalence nor the intensity of allergen reactivity related significantly with smoke sensitivity or smoking history, as determined by Fisher's exact test.

## Discussion

Rabbit antisera in the CIE reaction detected at least thirty-seven different precipitating antigens present in tobacco leaf extracts. Three of the precipitins were shown to be allergens in that they reacted with patient IgE antibodies as detected by the CRIE. In order to determine if these IgE antibodies are induced by cigarette smoke and/or are related to smoke sensitivity, the relationship of these reactions to tobacco smoke sensitivity and/or tobacco smoking was assessed. The results indicate that neither the intensity nor incidence of reactivity correlated with either smoking or 'smoke sensitivity'.

We have previously demonstrated in rabbits and mice that tobacco smoke extracts can stimulate an immune response to tobacco leaf antigens [8,11]. However, our current studies in man do not demonstrate a relationship of an immune response to tobacco leaf antigens and smoking or 'smoke sensitivity'. If these reactions to the tobacco leaf allergens identified by CRIE are not related to smoking, can we exclude tobacco smoke as a stimulating agent in this immune response? In this regard, it is

important to note that generally two types of tobacco smoke from cigarettes have been identified, main stream tobacco smoke, which is inhaled by the smoker, and side stream tobacco smoke, which is the smoke emanating from the lit cigarette tip. Side stream tobacco smoke is of great interest at this time since this is the smoke to which the non-smoking public is primarily exposed [2,4,17,18]. Since the public at large is exposed to both main and side stream tobacco smoke, we cannot exclude smoke exposure *per se* as the cause of this immune response to tobacco leaf allergens. However, the effect of this IgE response to tobacco leaf allergens as a factor in 'tobacco smoke hypersensitivity' is not substantial since there was apparently no relationship between this response and clinical smoke 'sensitivity'.

If an immune response to these tobacco allergens is not due to direct tobacco smoke exposure, or sensitivity to tobacco smoke, what is the origin of the antigens that stimulate production of these tobacco specific IgE antibodies? It is possible that the responses to these allergens are not related to tobacco leaf or smoke exposure, but rather exposure to other members of the plant family Solanaceae to which tobacco belongs. The recent report of Becker *et al* [19] that tobacco glycoprotein is present in many different vegetable products supports this possibility. Perhaps ingestion of any of these various plant antigens could stimulate the production of IgE antibodies. Thus, the response to tobacco allergens may reflect exposure to cross-reacting allergens of non-tobacco origin.

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