

Antigenic and allergenic relationships among airborne grass pollens in India

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Background: Pollen from grasses (Poaceae) are predominant aeroallergens throughout the world including tropical countries. Studies from USA, Europe, and Australia have shown extensive allergenic/antigenic cross reactivity among the grass pollen allergens prevalent there. No such information is available about airborne grass pollens of tropical countries.

Objective: The present study was undertaken to explore common antigenic/allergenic components, if any, of five important grass pollens of India.

Methods: Intradermal tests (ID) were performed with pollen extracts of *Cenchrus*, *Cynodon*, *Imperata*, *Pennisetum*, and *Sorghum* in patients with nasobronchial allergy. ELISAs were performed for estimating the allergen-specific IgE in sera of patients eliciting markedly positive ID response (2+ to 4+). To detect cross reactivity, ELISA inhibition experiments were carried out using pooled patient sera and five grasses, individually, as inhibitors with different solid phase antigens. To evaluate common antigenic components in *Cenchrus*, *Imperata*, and *Pennisetum* pollen extracts, rocket immunoelectrophoresis (RIE) and ELISA inhibition were conducted using rabbit antisera.

Results: Among 133 patients, *Cynodon* extract elicited markedly positive skin reactivity in most patients followed by *Pennisetum*, *Imperata*, *Cenchrus*, and *Sorghum*. A large number of patients showed markedly positive skin reactions and enhanced specific IgE levels to more than one grass pollen extract. ELISA inhibition experiments showed different degrees of cross reactivity among the grass pollens studied. Rocket immunoelectrophoresis and ELISA inhibition using rabbit antisera with homologous and heterologous pollen revealed the presence of shared antigenic components in *Cenchrus*, *Imperata*, and *Pennisetum* extracts.

Conclusion: The varied dose-response curves obtained with ELISA inhibition using different inhibitors suggest the presence of both common and specific antigens/allergens in the grass pollens studied. Based on the extensive immunologic cross reactivity, among the tropical grass species, it may be possible to use mixed allergen preparations for allergy diagnosis and immunotherapy.

INTRODUCTION

Grasses are the most prolific of plant families in respect to numbers and species. Pollens from grasses (Poaceae) are the major aeroallergens causing sensitivity in 85% of pollen allergic individuals in the Mediterranean area.¹ In India also, a large number of patients have hypersensitivity to grass pollen.^{2,3} Being good

pollen producers and wind pollinated, they are found among the first five dominant types of aeroallergens at several places in the country.^{4,5} About 1180 grass species belonging to 239 genera inhabit India.⁶

It is cumbersome to study the allergenic nature of each and every grass and include each in the testing kit for diagnosis of allergy. Studies with grasses in Europe, Australia, and USA have shown extensive cross-reactivity among grass pollen allergens.⁷⁻¹² There has been no such study of the antigenic and allergenic relationships of grasses prevalent in tropical countries.

The present study was undertaken to determine the allergenic relationships among five dominant grasses: *Cenchrus ciliaris* (bunch grass), *Cynodon dactylon* (Bermuda grass), *Imperata cylindrica* (Cogon grass), *Pennisetum typhoides* (pearl millet), and *Sorghum vulgare* (Sorghum, fodder grass) using various in vivo and in vitro methods. Attempts were also made to investigate antigenic relationships of three different grass pollen extracts using antisera raised against them in rabbits.

MATERIALS AND METHODS

Pollen Extracts

Pollen samples were collected from wild grasses (*Cenchrus ciliaris*, *Cynodon dactylon*, and *Imperata cylindrica*) and cultivated types (*Pennisetum typhoides*, and *Sorghum vulgare*). The collected samples were processed carefully to avoid any cross contamination. The pollen samples containing >95% pure pollen and <5% other plant parts from the flowers of the same species were defatted with diethyl ether. The extracts were prepared in 0.05 M ammonium bicarbonate buffer, pH 8.00 by continuous stirring for 18 hours on a magnetic stirrer at 4 °C. The extracts were centrifuged (10,000 rpm, 4 °C) and supernatant brought to 90% saturation with ammonium sulphate. The precipitate was dissolved in distilled water and dialyzed using 'Spectrapor' membrane, molecular weight cut off 2,000. The dialyzed extracts were passed through millipore filter membrane (0.45 µm), lyophilized in different aliquots and stored at -20 °C. The protein content of each extract was determined by the modified Lowry's method.¹³

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Intradermal Tests (ID) and Collection of Sera

Intradermal tests were performed with pollen extracts of five grasses in 133 patients (aged 17 to 42 years) attending the Clinical Research Centre, V.P. Chest Institute, Delhi (India), according to the method of Shivpuri and Agarwal.¹⁴ For skin tests, lyophilized extracts were reconstituted (1:500 wt/vol) in phosphate buffered saline (PBS). About 0.01 mL of each extract was injected intradermally in the forearm, raising a bleb of 2 to 3 mm at the injection site.

Simultaneously, PBS was injected as a negative control. The skin tests were graded after 20 minutes, based on the wheal size produced in comparison to the negative control. The ID reactions more than 3 times of the negative control were graded as 2+, 3 to 4 times the control with 1 to 2 pseudopodia as 3+, and multiple pseudopodia as 4+. Histamine diphosphate, 100 µg/mL, was also injected as a positive control, which produced skin reactions in the range of 11 to 15 mm (graded as 2+).

Sera were collected from the patients showing markedly positive (2+ to 4+) skin reactions to any of the five extracts tested. None of the patients was receiving immunotherapy when ID tests were performed or sera collected. Normal human sera were obtained from nonallergic subjects with negative ID tests. The consent of every patient was obtained prior to skin testing and sera collection.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were performed to measure the specific IgE levels for each grass in individual patient sera by the method of Voller et al.¹⁵ Antigenic extracts of all the five grass pollen types were diluted in carbonate coating buffer, pH 9.6. To each well of the microtiter plate, 100 µL of the diluted extract (1 µg protein/100 µL) was added followed by overnight incubation at 4 °C. After washing thrice with phosphate buffered saline (PBS) Tween, the plates were incubated with 100 µL (1/10

diluted in 0.1 M PBS, pH 7.4) of individual patient's serum. As a control, normal human serum (NHS) from subjects with negative ID tests to grass pollen extracts was also tested. The washing was carried out again with PBS-Tween.

Alkaline phosphatase labeled anti-human IgE (ε-specific, Sigma Chemical Co, USA) was added to each well of the plate and incubated overnight at 4 °C. The unbound anti-IgE was washed off and 100 µL of enzyme substrate (0.1% *p*-nitrophenyl phosphate in glycine buffer, pH 9.6) was added. The color development process was stopped after 40 minutes by adding 50 µL of 3 N NaOH to each well of the microtiter plate. Absorbance was measured at 410 nm with a NUNC ELISA reader. Sera showing ≥5 times the OD obtained with normal human serum were considered as ELISA positive. Whereas, sera showing ≥10 times ELISA values (OD) of the NHS were pooled separately and used for inhibition assays.

Statistical Analysis

ELISA values (OD), ie, specific IgE level, of one grass extract was compared with another using Karl Pearson's coefficient of correlation test taking paired data ($N = 66 \times 2$) in all possible combinations for five extracts.

Immunization for Rabbit Sera

Rabbits were immunized, each with 1 mg of protein (extract) emulsified with Freund's complete adjuvant (Difco). Booster injections were given at intervals of 4 weeks with the same protein emulsified with Freund's incomplete adjuvant. The rabbits were bled seven days after the third booster to check the presence of antibodies by immunodiffusion. The sera obtained from three rabbits immunized with each antigen were mixed in equal volumes to obtain polyvalent sera for each grass extract.

Rocket Immuno-electrophoresis

Rocket immuno-electrophoresis was performed on 1.8-mm thick glass plate with 1% agarose gel containing rabbit antisera in Svendsen's¹⁶ buffer pH 8.6. Fifty micrograms of each grass pollen

extract (protein) was applied into the wells punched into the gel and electro-phoresed for 10 to 20 hours (1.5 v/cm, 4 °C). The gels were washed, pressed dry, and stained using Coomassie brilliant blue, R-250 (Sigma Chemical Co, USA).

IgE and IgG Specific ELISA Inhibition

Inhibition assays were performed by pre-incubating 50 µL of either pooled patients sera or rabbit antisera with 50 µL of inhibitor in various concentrations for 16 hours at 4 °C. The mixture was then added to antigen-coated wells. Normal human serum from subjects with negative skin tests to grass pollen extracts or normal rabbit serum prior to the immunization with antigens were used as negative controls. After overnight incubation at 4 °C, antibodies bound to the plates were detected with enzyme labeled anti-human IgE/anti-rabbit IgG (Sigma Chemical Co, USA). Absorbance measurements were made after adjusting the background with negative control. Percent inhibition was calculated as:

$$\% \text{ inhibition} = \frac{\text{OD of the test sample} - \text{OD of the inhibited sample}}{\text{OD of the test sample}} \times 100$$

RESULTS

In the test population of 133 patients, *Cynodon dactylon* exhibited markedly positive skin reactivity (2+ to 4+) in a large number of the patients (52%) followed by *Pennisetum typhoides* (32%), *Imperata cylindrica* (26%), *Cenchrus ciliaris* (22%), and *Sorghum vulgare* (16%). About 50% to 60% of patients positive to *Cenchrus* showed markedly positive skin reactions to *Pennisetum* and *Cynodon* extracts. In *Pennisetum* positive patients, frequency of marked positive skin response was more to *Cenchrus*, *Imperata* than to *Cynodon* and *Sorghum* extracts. Among *Sorghum* positive patients, 24% to 42% elicited markedly positive skin reactions to the other four grass pollen extracts (Fig 1).

Of 133 patients skin tested with five grass pollen extracts, 66 subjects

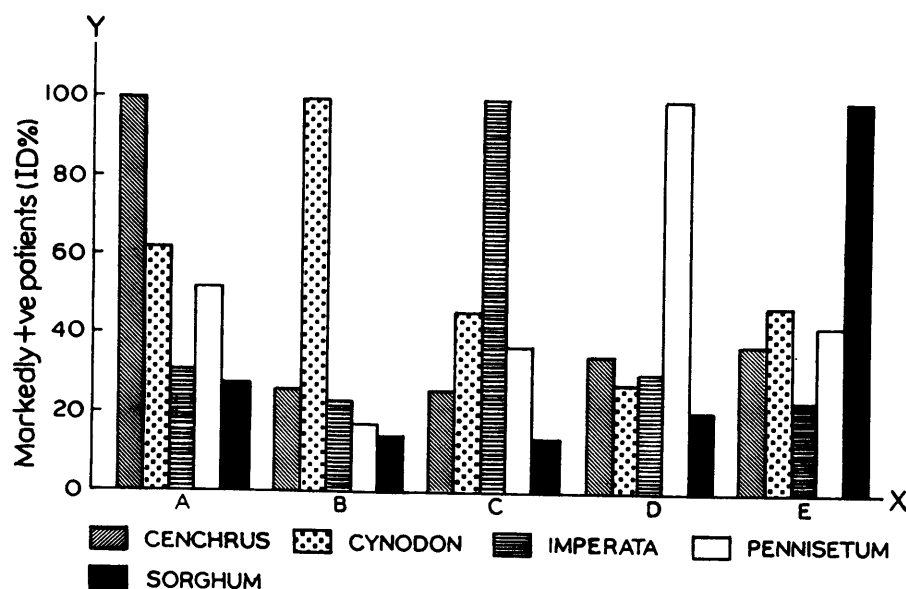


Figure 1. Comparative analysis of ID test results with five grass pollen extracts in 133 allergy patients. Percent positivity (y-axis) to other four grass pollen extracts in Cenchrus positive patients (group A, n = 29); Cynodon positive patients (group B, n = 69); Imperata positive patients (group C, n = 35); Pennisetum positive patients (group D, n = 43) and Sorghum positive patients (group E, n = 21).

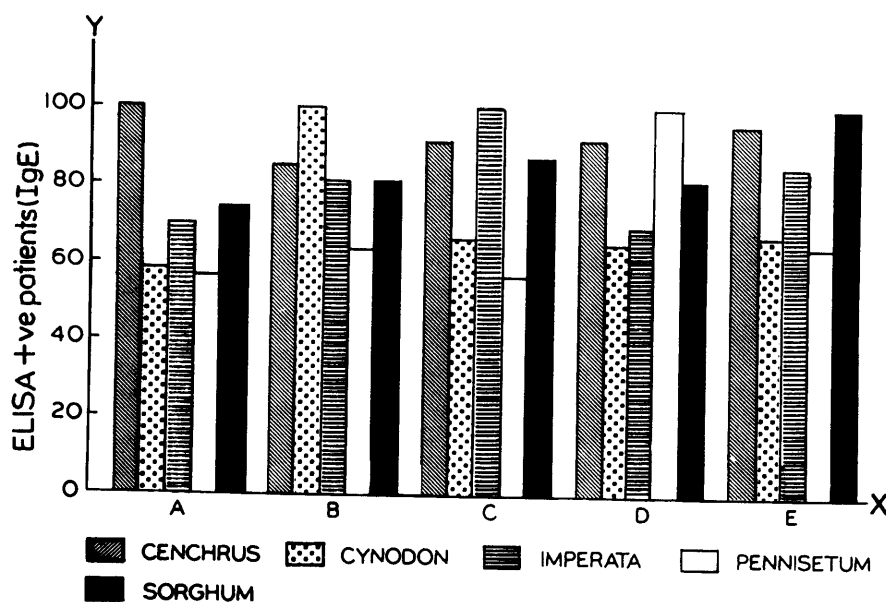


Figure 2. Comparative account of ELISA results obtained with 66 patients sera positive to grass pollen extracts. Sera showing ≥ 5 times the OD of NHS were considered positive and compared in all possible combinations. Percent positivity (y-axis) to other four grass extracts in Cenchrus positive patients (group A, n = 43), Cynodon positive patients (group B, n = 27), Imperata positive patients (group C, n = 32), Pennisetum positive patients (group D, n = 26), and Sorghum positive patients (group E, n = 33).

agreed to donate blood for further experiments. Serum of each patient was screened by ELISA for the presence of specific IgE to all five ex-

tracts, individually. ELISA values (OD) ≥ 5 times of the NHS were considered positive and their frequency (%) in one extract was com-

pared with another in different combinations (Fig 2). It is evident from the findings that sera from $>60\%$ of the patients possess significant levels of specific IgE to the five grass pollen extracts tested.

Statistical analysis of ELISA values was also carried out using 'correlation matrix' to find out the extent of allergenic relationship in different grasses (Table 1). The specific IgE data of one grass pollen extract was compared with another taking paired samples, where $N = 66 \times 2$ in all the cases. At 5% probability, the critical value of 0.242 ($\pm$) was recorded significant for 2-tailed analysis. Critical values obtained in case of each grass in comparison with the other four grass pollen extracts showed a strong allergenic correlation (0.656 to 0.906).

IgE Specific ELISA Inhibition

Competitive ELISA inhibition was performed using a serum pool of each extract to evaluate allergenic cross reactivity among five grass pollen allergens. A dose-dependent inhibition of binding was observed, when serum pools were pre-incubated with serial dilutions of homologous grass extracts.

Interestingly some heterologous grass extracts also produced significant dose-related inhibition (Fig 3). The amount of each allergen required for 50% inhibition was calculated and presented in Table 2. The binding of specific IgE to Cenchrus was substantially inhibited by Cynodon and Pennisetum, while Imperata and Sorghum showed the least inhibition. The binding to Cynodon was significantly inhibited by Imperata, Cenchrus, and Sorghum, while even 5 μg of Pennisetum failed to produce 50% inhibition. Cenchrus was almost as potent as Imperata to inhibit the binding of specific IgE to solid phase Imperata. The other three grass pollen extracts were quite similar in their inhibitory capacity to Imperata yielding 50% inhibition within a factor of 2 of each other. Cenchrus, Imperata, Pennisetum, and Sorghum were similar in their inhibitory capacity of specific IgE binding to Pennisetum, while Cynodon was approximately 1/10th as

active. All the grass extracts were active inhibitors of specific IgE binding to solid phase Sorghum, requiring less than 10 ng for 50% inhibition.

RIE Using Rabbit Antibodies

All the experimental rabbits gave good antibody response to three grass pollen extracts namely Cenchrus, Imperata, and Pennisetum. In RIE all the three

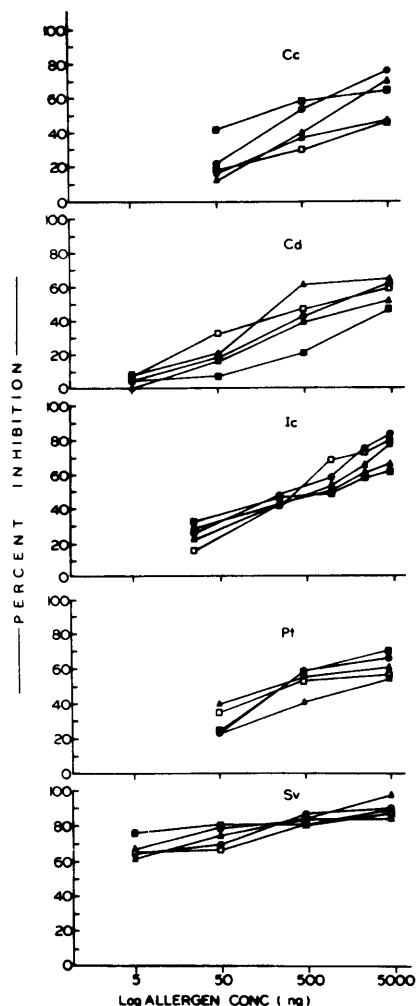


Figure 3. ELISA inhibitions demonstrating the extent of allergenic cross-reactivity among five grass pollen extracts. Solid phase used: Cc = *Cenchrus ciliaris*; Cd = *Cynodon dactylon*; Ic = *Imperata cylindrica*; Pt = *Pennisetum typhoides*, and Sv = *Sorghum vulgare*. The binding of IgE antibodies in sera from allergy patients to these allergosorbents was inhibited with Cenchrus ●—●, Cynodon △—△, Imperata □—□, Pennisetum ■—■, or Sorghum ○—○.

Table 1. Allergenic Relationships Among Five Types of Grass Pollen Allergens Based on Specific IgE Levels*

Allergens	Correlation Matrix of All Variables at 5% Probability				
	Cenchrus	Cynodon	Imperata	Pennisetum	Sorghum
Cenchrus	1.000	0.769	0.792	0.842	0.869
Cynodon		1.000	0.719	0.656	0.801
Imperata			1.000	0.670	0.906
Pennisetum				1.000	0.799
Sorghum					1.000

* Specific IgE was measured in 66 sera taking five extracts individually as solid phase antigen.

Critical value 1—Tail, 0.05: ± 0.204 .

Critical value 2—Tail, 0.05: ± 0.242 .

N = 66.

Table 2. Allergenic Cross Reactivity Among Grass Pollen Extracts as Measured by ELISA Inhibition with Sera of Grass Pollen Sensitive Patients

Allergens Used for Inhibition	Solid Phase Allergen*				
	Cenchrus	Cynodon	Imperata	Pennisetum	Sorghum
Cenchrus	380	1010	410	300	<10
Cynodon	1000	265	1100	2200	<10
Imperata	>5000	700	390	320	<10
Pennisetum	1600	>5000	1250	280	<10
Sorghum	>5000	2800	750	195	<10

* Amount (ng) required for 50% inhibition.

grass extracts produced maximum numbers of precipitin bands with homologous antibodies. In addition, at least two to four precipitin bands were obtained with the other two grass extracts. An antigenic component in Pennisetum reacted to the maximum extent, as measured by rocket height with all the three antibodies (Fig 4).

IgG-Specific ELISA Inhibition

Antigenic relationship among the three grasses namely Cenchrus, Imperata, and Pennisetum was also evaluated by competitive ELISA inhibition using rabbit antibodies. Each of the three grass extracts was used individually to compete for binding to rabbit antibodies raised against homologous pollen antigen coated on ELISA plate (Table 3 and Fig 5). Cenchrus and Imperata gave an identical inhibition pattern, when Cenchrus was used as a solid phase antigen and antibodies to Cenchrus were employed. When Imperata or Pennisetum antibodies were used Pennisetum and Cenchrus showed identical inhibition curves depicting their close relationship.

DISCUSSION

Grass pollen allergens have been found to be important triggers of allergic disorders. Aerobiologic and clinical studies conducted in India have recognized the allergenic importance of the several grass species.^{2,6,17,18} As a result, aqueous extracts prepared from pollen of Cenchrus, Cynodon, Imperata, Pennisetum, and Sorghum have been used routinely by various allergologists for allergy diagnosis and immunotherapy for many years. The common allergenic proteins have been reported among phylogenetically related grass species, prevalent in Europe, USA, Australia and other western countries.^{19,20} No such information is available about the grass species prevalent in tropical countries.

In a random survey of 565 patients undergoing ID tests for allergy diagnosis at V.P. Chest Institute, Delhi, 249 (44%) patients were found to be positive (2+ to 4+) to one or more grass pollen extracts tested. Of 249 cases, 133 patients were recruited for repeat ID tests with five grass pollen extracts.

In the present study, *Cynodon* was found to be the most reactive (ID positive) grass and *Sorghum* the least. It has been reported that pollen of wild growing taxa show higher allergenic potential than the cultivated species. The majority of the grass pollen allergic individuals reacted to more than one grass pollen extract (Fig 1). This may be due to the presence of common allergenic proteins in the grasses.

The presence of specific IgE to all the five grass pollen extracts was evaluated by ELISA in 66 subjects. Analysis of the positive ELISA values for each grass pollen extract in comparison to the other four showed elevated IgE levels to more than one allergen in many patients. The positive ELISA values ($OD \geq 5$ times of the NHS) of one extract was taken as 100% and compared with the values of the other four grass extracts (Fig 2). Statistical analysis of specific IgE values obtained for one grass extract when compared with another ($OD, N = 66 \times 2$) using 'correlation matrix,' showed significant correlations. The critical values obtained as a result of analysis for every 2-tailed (paired) test indicate close allergenic relationships among all five grass species (Table 1).

In order to quantitate the extent of cross reactivity, inhibition studies were carried out using pooled sera and serial dilutions of different grass pollen extracts (Table 2 and Fig 3). The inhibitory capacity of the extracts studied was found distinct, depending on the serum pool and the solid phase antigen employed. This may be due to the presence of both common and unique allergenic proteins in these grass extracts. *Cynodon* and *Pennisetum* showed maximum inhibition in ELISA binding to *Cenchrus* whereas *Imperata* and *Cenchrus* inhibited the binding to *Cynodon*. Likewise, all the other four grasses inhibited the binding to *Imperata*, *Pennisetum*, and *Sorghum* to some extent. Previous studies^{7,8,11,21} have shown that *Cynodon dactylon* extract cross reacts poorly with other grasses. In the present study, *Cenchrus* and *Imperata* produced significant inhibition of *Cynodon*; however, these

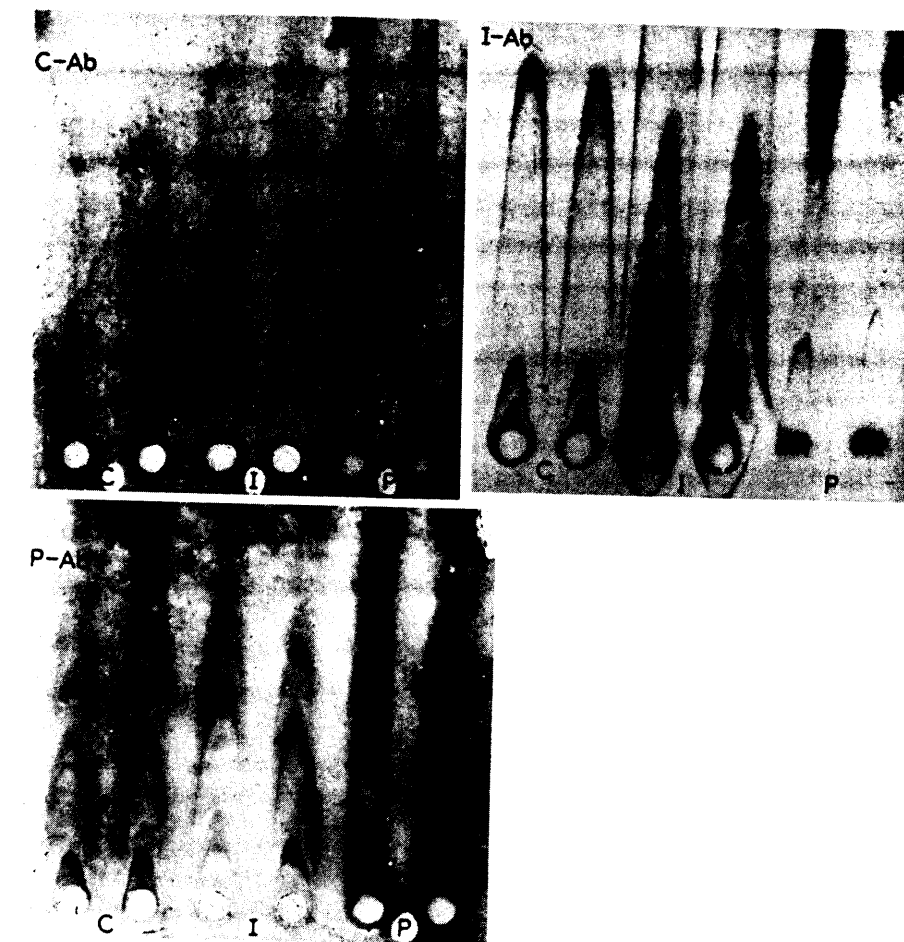


Figure 4. RIE of three grass pollen extracts using rabbit sera. The gels contained C-Ab = Anti *Cenchrus* antibodies, I-Ab = Anti *Imperata* antibodies, and P-Ab = Anti *Pennisetum* antibodies. The extracts electrophoresed are in lane 1, 2 (C) *Cenchrus*; lane 3, 4 (I) *Imperata* and lane 5, 6 (P) *Pennisetum*.

two grasses were not included in the previous studies. It has been reported that rabbit antibodies to *Lol p I* show affinity to 29 to 31 kD components of *Cynodon* pollen extract.

Monoclonal antibodies to *Lol p I* also exhibited binding to *Cynodon* antigens.^{22,23} In a recent study,²⁴ anti-*Cyn d I* antibodies were found cross reacting with other Group I allergens, especially that of *Dac g I* and *Poa p I*.

The observations using pooled patients' sera do not rule out the possibility that the reactivity observed may be due to varying degree of exposure to the different grass pollen aeroallergens. Hence, the antibodies were raised in rabbits against *Cenchrus*, *Imperata*, and *Pennisetum*. The antisera obtained were used to study the

shared antigenicity among the three grasses. Maximum numbers of precipitin rockets were observed with homologous antisera. In addition, each antiserum showed a few precipitin rockets with other two extracts, suggesting the presence of cross reacting antigens in all the three grass pollen extracts (Fig 4). ELISA inhibition (specific IgE) experiments using anti-*Cenchrus* antibodies showed that serial dilutions of *Imperata* and *Pennisetum* were able to inhibit its binding to solid phase *Cenchrus*. The quantity of *Imperata* required for 50% inhibition was very little compared with that required by *Pennisetum* for the same degree of inhibition. Similar results were obtained with anti-*Imperata* antibodies and anti-*Pennisetum* antibodies indi-

cating high degree of cross reactivity among the three grass pollen extracts.

The high degree of immunologic cross reactivity observed among the grass pollen allergens studied suggests the presence of common antigenic/allergenic components. Based on these findings, clinical trials are suggested

Table 3. Antigenic Cross Reactivity Among Grass Pollen Extracts as Measured by ELISA Inhibition Using Sera of Hyperimmunized Rabbits

Antigens	Amount Required for 50% Inhibition		
	Cenchrus, μg	Imperata, μg	Pennisetum, μg
Cenchrus	<0.250	0.250	2.000
Imperata	0.700	<0.250	0.700
Pennisetum	1.100	3.400	1.000

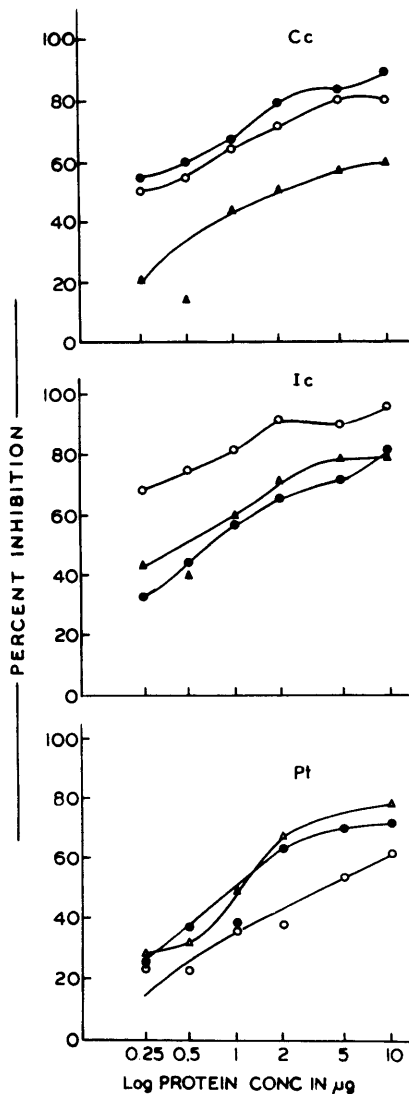


Figure 5. ELISA inhibitions showing the antigenic relationship among the three grass pollen allergens. Inhibition experiments were carried out using antisera raised in rabbit. Solid phase used: Cc = *Cenchrus ciliaris*, Ic = *Imperata cylindrica*, and Pt = *Pennisetum typhoides*. The binding of respective antibodies to these allergens was inhibited with Cenchrus ●—●, Imperata ○—○ or Pennisetum △—△.

taking two groups of mixed allergen preparations comprising *Cynodon* with *Cenchrus*, *Imperata* and or *Pennisetum*, *Sorghum* to evaluate their allergenic potential in a large number of cases sensitive to grass allergens. Further studies with dominant cross reacting proteins/isolated fractions of grass allergens are also required before the number of allergens are actually reduced for allergy diagnosis and immunotherapy.

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WOMEN USE METERED DOSE INHALERS LESS ADEQUATELY THAN MEN

In order to determine how adequately patients with asthma or chronic obstructive pulmonary disease were using metered dose inhalers the authors studied 26 women and 33 men 20 to 81 years of age. Adequacy of inhalation technique was evaluated with a metered dose inhaler adapter that contained a miniature sensing system. The authors determined inspiratory flow at the moment of actuation of the inhaler, the volume at actuation as a fraction of total inspiratory volume, breath-holding time, and inspiratory volume as a fraction of FVC. The patients were told to use the inhaler with their normal technique. Acceptable technique was defined as inspiratory flow between 25 and 90 L/min at actuation, actuation during early inspiration (at not more than 20% of inspiratory volume), breath-holding for more than 4 seconds, and a deep inhalation (inspiratory volume more than 50% of FVC).

Overall only 25% of inhalations met all four criteria for adequacy. Significantly more men than women demonstrated acceptable technique (43% versus 4%, $P < .001$). There were no significant differences by age or diagnosis.

Inadequate technique in use of metered dose inhalers may contribute to the increased morbidity from asthma among women. Use of spacers or automatic delivery devices may facilitate delivery of medications from metered dose inhalers to both women and men.

—RMS

Goodman DE, Israel E, Rosenberg M, et al. The influence of age, diagnosis, and gender on proper use of metered-dose inhalers. *Am J Respir Crit Care Med* 1994;150:1256-61.