LIST OF PUBLICATIONS & PRESENTATIONS


3. Lalit Kumar, S. Sridhara, B.P. Singh and S.V. Gangal. Immunobiochemical analysis of Cenchrus ciliaris pollen allergens and cross-reactivity with other tropical grasses. (Manuscript in preparation).


5. Lalit Kumar, S. Sridhara, B.P. Singh and S.V. Gangal. Allergy to Cogon grass (Imperata cylindrica) pollen: Cross-reactivity with four other tropical grass pollens. (Manuscript in preparation).

1. Lalit Kumar, S. Sridhara, B. P. Singh and S. V. Gangal. Purification and partial characterization of a major allergen from Bunch grass (*Cenchrus ciliaris*) pollen. 30th Annual Convention of Indian College of Allergy and Applied Immunology (ICAAI); December 13-15, 1996; Ahmedabad, India.

2. Lalit Kumar and S. V. Gangal. Pen t1: A new allergen from the pollen extract of *Pennisetum typhoides*. 65th Annual Meeting of Society of Biological Chemists, India (SBC), November 20-23, 1996; Bangalore, India.

3. S. Sridhara, Lalit Kumar, B. P. Singh and S.V. Gangal. Analysis of *Cenchrus ciliaris* pollen: Identification of IgE and IgG binding components by immunoblotting. 16th International Congress of Biochemistry and Molecular biology (IUBMB); September 19-22, 1994, New Delhi, India.

4. Lalit Kumar, S. Sridhara, B.P. Singh and S.V. Gangal. Immunobiochemical characterization of *Cenchrus ciliaris* pollen allergen extract. 16th International Congress of Biochemistry and Molecular Biology (IUBMB); September 19-22, 1994; New Delhi, India.

5. Lalit Kumar, S. Sridhara, B.P. Singh and S.V. Gangal. Immunobiochemical characterization of *Pennisetum typhoides* pollen allergens. 15th International Congress of International Association of Allergology and Clinical Immunology (IAACI) and Annual Conference of European Academy of Allergology and Clinical Immunology (EAACI)94; June 26-July 1, 1994; Stockholm, Sweden.

6. Lalit Kumar, S. Sridhara, B. P. Singh and S. V. Gangal. Identification and immunobiochemical characterization of allergens of whole pollen extract of *Sorghum vulgare*. 62nd Annual Meeting of Society of Biological Chemists, India (SBC); December 19-22, 1993; Madurai Kamraj University, Madurai, India.

7. S. Sridhara, B. P. Singh, Lalit Kumar and S. V. Gangal. Identification and immunological characterization of allergens of *Imperata cylindrica*. 26th Annual Convention of Indian College of Allergy and Applied Immunology (ICAAI); November 6-8, 1992; Banaras Hindu University, Banaras, India.

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Genic and allergenic relationships among borne grass pollens in India

Sridhara, PhD; B P Singh, PhD; Lalit Kumar; Jyotsna Verma; S N Gaur, MD*; and Sangal, PhD

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

Objective: The present study was undertaken to explore common antigenic Allergy, Asthma, & Immunology Vol. 74, Number 7, July 1995

tic components, if any, of five important grass pollens of India.

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

Results: Among 133 patients, Cenchrus extract elicited markedly positive skin test in most patients followed by Pennisetum, Imperata, Cenchrus, and Sorghum. A large number of patients showed markedly positive skin reactions and specific IgE levels to more than one grass pollen extract, ELISA inhibition tests showed different degrees of cross reactivity among the grass pollens. Rocket immunoelectrophoresis and ELISA inhibition using rabbit antisera against 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 difference suggest the presence of both common and specific allergens in the grass pollens studied. Based on the extensive immunologic reactivity, among the tropical grass species, it may be possible to use mixed preparations for allergy diagnosis and immunotherapy.

INTRODUCTION

are the most prolific of plant pollen in respect to numbers and obliges from grasses (Poaceae) are major aeroallergens causing sensitiv- pollen allergic individuals in Mediterranean area.1 In India also, there is no number of patients have hyperactivity to grass pollen.2-3 Being good

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 antigens 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 cross contamination. The pollen samples containing >95% pure pollens and ≤5% other plant parts from the flowers of the same species were defatted with diethyl ether. The extract was prepared in 0.05M ammonium bicarbonate buffer, pH 8.0 by continuous stirring for 18 hours on magnetic stirrer at 4°C. The extract was 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 dialyzate extracts were passed through millipore filter membrane (0.45 μm), lyophilized in different aliquots at stored at −20°C. The protein content of each extract was determined by the modified Lowry’s method.1

for Biochemical Technology, Mauli University Campus, Delhi, India. Received for publication February 25, 1994, and for publication January 11, 1995.
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 Shipwini and Agarwal. 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. 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 dilution 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 unbond 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), i.e., specific IgE level, of one grass extract was compared with another using Karl Pearson’s coefficient of correlation test taking paired data (N = 66 × 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 Immunoelectrophoresis

Rocket immunoelectrophoresis was performed on 1.8-mm thick glass plate with 1% agarose gel containing rabbit antisera in Svensden’s 1/10 buffer pH 8.6. Fifty micrograms of each grass pollen extract (protein) was applied wells punched into the gel phoresed for 10 to 20 hours at 4 °C. The gels were washed dry, and stained using Coomassie blue, R-250 (Sigma–USA).

IgE and IgG Specific ELISA Inhibition

Inhibition assays were performed by pre-incubating 50 μL of sera to rabbit antiserum for 16 hours at 4 °C, which was then added to assay wells. Normal human sera as well as pollen extracts or normal sera prior to the immunization regimen were used as negative controls. After overnight incubation, titrations of the molecules bound to the plate were tested with enzyme labeled IgE anti-rabbit IgG (Sigma Co., USA). Absorbance measurements were made after adjusting OD with negative control wells, and 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 1 Cynodon dactylon exhibited positive skin reactivity (2+ large number of the patient showed a positive reaction) with Petroselinum crispum var. Imperata cylindrica (26% ciliaris (22%), and Sorghum (16%). About 50% to 60% positive to Chenopodium shows positive skin reactions to Cynodon extracts. In positive patients, frequency of positive skin response was Chenopodium, Imperata than Cynodon extracts. A number of positive patients, elicited markedly positive reactions to the other four grass extracts (Fig 1).

Of 133 patients skin five grass pollen extracts,
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CENCHRUS

SORGHUM

CYNODON

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2

C

PENNISETUM

1. Comparative analysis of ID test results with five grass pollen extracts in 133 allergy patients. 

positivity (y-axis) to other four grass pollen extracts in CENCHRUS positive patients (group A), 

CYNODON positive patients (group B, n = 69), IMPERATA positive patients (group C, n = 35); 

SORGHUM positive patients (group D, n = 43) and SORGHUM positive patients (group E, n = 21).

2. Comparative account of ELISA results obtained with 66 patients sera positive to grass pollen 

Sera showing ≥5 times the OD of NHS were considered positive and compared in all possible 

ions. Percent positivity (y-axis) to other four grass extracts in CENCHRUS positive patients (group 

3). CYNODON positive patients (group B, n = 27). IMPERATA positive patients (group C, n = 32); 

SORGHUM positive patients (group D, n = 26), and SORGHUM positive patients (group E, n = 33).

to donate blood for further 

ents. Serum of each patient 

searcher by ELISA for the pres- 

pecific IgE to all five ex- 

tracts, individually. ELISA values 

(OD) ≥5 times of the NHS were 

considered positive and their fre- 

quency (%) in one extract was com- 

pared with another in different combi- 

inations (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 aller- 

genic 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 × 2 in all the cases. At 5% 

probability, the critical value of 0.242 (+) was recorded significant for 

2-tailed analysis. Critical values 

obtained in case of each grass in compar- 

ison with the other four grass pollen 

extracts showed a strong allergic 

correlation (0.656 to 0.906).

IgE: Specific ELISA Inhibition 

Competitive ELISA inhibition was 

performed using a serum pool of each 

extract to evaluate allergic cross re-

activity among five grass pollen aller-

gens. 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 pre-

sented in Table 2. The binding of spe-

cific IgE to CENCHRUS was substentially 

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. CENCHUS, IMPERATA, 

PENNISETUM, and SORGHUM were similar 

in their inhibitory capacity of spe-

ific 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 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 competitive (2+ to 4+) to one or more pollen extracts tested. Of 133 patients were recruited for ID tests with five grass pollen extracts tested. No such information is available about the grass species prevalent in Australia and other western countries.

In a random survey of 500 patients undergoing ID tests for allergy diagnosis and immunotherapy, 254 (44%) patients were found to be allergic to grass pollen among all the grass species prevalent in Australia and other western counties.

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

<table>
<thead>
<tr>
<th>Allergens</th>
<th>Correlation Matrix of All Variables at 5% Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cenchrus</td>
</tr>
<tr>
<td>Cenchrus</td>
<td>1.000</td>
</tr>
<tr>
<td>Cynodon</td>
<td>1.000</td>
</tr>
<tr>
<td>Imperata</td>
<td>1.000</td>
</tr>
<tr>
<td>Pennisetum</td>
<td>1.000</td>
</tr>
<tr>
<td>Sorghum</td>
<td></td>
</tr>
</tbody>
</table>

* Specific IgE was measured in 66 sera taking five extracts individually as solid phase antigens, 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 Inhibition with Sera of Grass Pollen Sensitive Patients**

<table>
<thead>
<tr>
<th>Allergens Used for Inhibition</th>
<th>Solid Phase Allergen*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cenchrus</td>
</tr>
<tr>
<td>Cenchrus</td>
<td>380</td>
</tr>
<tr>
<td>Cynodon</td>
<td>1000</td>
</tr>
<tr>
<td>Imperata</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Pennisetum</td>
<td>1600</td>
</tr>
<tr>
<td>Sorghum</td>
<td>&gt;5000</td>
</tr>
</tbody>
</table>

* Amount (ng) required for 50% inhibition.

**DISCUSSION**

Grass pollen allergens have been found to be important triggers of allergic disorders. Aerobiologic and clinical studies conducted in India have revealed the allergenic importance of certain grass species. As aqueous extracts prepared from the grasses Cenchrus, Cynodon, Imperata, and Sorghum have been routinely used for allergy diagnosis and immunotherapy for many years. The common allergenic proteins have been shown to be among phylogenetically related species, prevalent in Europe, Australia and other western countries. No such information is available about the grass species prevalent in tropical countries.

In a random survey of 500 patients undergoing ID tests for allergy diagnosis at V.P. Chest Institute, 254 (44%) patients were found to be allergic to grass pollen among all the grass species prevalent in Australia and other western countries.

Of 133 patients were recruited for ID tests with five grass pollen extracts tested. No such information is available about the grass species prevalent in Australia and other western countries.
The present study, Cynodon was the most reactive (ID positive) and Sorghum the least. It was reported that pollen of wild taxa show higher allergenicity than the cultivated species. Majority of the grass pollen allergens reacted to more than one pollen extract (Fig 1). This may be due to the presence of common proteins in the grasses. Presence of specific IgE to all grass pollen extracts was evaluated by ELISA in 66 subjects. Analysis of positive ELISA values for grass pollen extract in comparison to the other four showed elevated levels to more than one allergen in patients. The positive ELISA (OD ≥ 5 times of the NHS) of extract was taken as 100% and compared with the values of the other grass extracts (Fig 2). Statistical analysis of specific IgE values obtained for one grass extract when compared with another (OD, N = 66 × 2) showed significant correlations. The critical values obtained as a result of analysis for 2-tailed (paired) test indicate allergenic relationships among grass species (Table 1).

Figure 4. RIL of three grass pollen extracts using rabbit sera. The gels contained C: Ab = Anti Imperata antibodies, 1-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.

In order to quantitate the extent of reactivity, inhibition studies were carried out using pooled sera and serial dilutions of different grass extracts (Table 2 and Fig 3). The inhibitory activity of the extracts studied was distinct, depending on the pollen and the solid phase antigen used. This may be due to the presence of both common and unique proteins in these grass extracts. Cynodon and Pennisetum showed maximum inhibition in ELISA to Cenchrus whereas Imperata inhibited the binding to the pollen extract. Likewise, all the other four species inhibited the binding to Imperata, and Sorghum to some extent. Previous studies have shown that Cynodon dactylon reacts poorly with other grass species, and the present study, Cenchrus serata produced significant inhibition of Cynodon; however, these two grasses were not included in the previous studies. It has been reported that rabbit antibodies to Lol p 1 show affinity to 29 to 31 kD components of Cynodon pollen extract.

Monoclonal antibodies to Lol p 1 also exhibited binding to Cynodon antigens. In a recent study, anti-Cenchrus antibodies were found cross reacting with other Group I allergens, especially that of Dac g 1 and Poa p 1.

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 allergens. 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 antisera 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-
taking two groups of mixed allergen preparations comprising Cynodon with Cenchrus, Imperata and or Pennistum. 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 immuno-therapy.

ACKNOWLEDGMENTS

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

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cenchrus, μg</th>
<th>Imperata, μg</th>
<th>Pennisetum, μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cenchrus</td>
<td>0.250</td>
<td>0.250</td>
<td></td>
</tr>
<tr>
<td>Imperata</td>
<td>0.700</td>
<td>&lt;0.250</td>
<td>0.250</td>
</tr>
<tr>
<td>Pennisetum</td>
<td>1.100</td>
<td>3.400</td>
<td>1.400</td>
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</tbody>
</table>

<table>
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<tr>
<th>Table 3. Antigenic Cross Reactivity Among Grass Pollen Extracts as Measured by Inhibition Using Sera of Hyperimmunized Rabbits</th>
<th>Amount Required for 50% Inhibition</th>
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<tbody>
<tr>
<td>Antigen</td>
<td>Cenchrus, μg</td>
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<tr>
<td>--------------------------------------------------------------</td>
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<tr>
<td>Cenchrus</td>
<td>0.250</td>
</tr>
<tr>
<td>Imperata</td>
<td>0.700</td>
</tr>
<tr>
<td>Pennisetum</td>
<td>1.100</td>
</tr>
</tbody>
</table>

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