Chapter V

CROSS-REACTIVITY OF ALLERGENS/ANTIGENS OF *Pennisetum typhoides* WITH THOSE OF OTHER GRASS POLLENS
INTRODUCTION

Pollen of the grass family Poaceae represent an important source of aero-allergens. Grasses are the most prolific of the plant families in respect to numbers and species. Being excellent pollen producers and wind pollinated, they are among the first five dominant types of aero-allergens in India \([42,43]\). Allergenic pollen producing species include both wild as well as cultivated types. Most of the grass pollen types share morphological features. It is sometimes difficult to identify the primary allergen source due to the presence of various grass pollen species in the atmosphere. Each type of grass pollen may carry a number of allergenic components. It is, therefore, cumbersome to study the allergenic nature of each and every grass pollen and include each one in the testing kit for the diagnosis of allergy. This makes it necessary to explore the presence of shared or cross-reactive allergens in various grass pollen extracts and to study the possibility of using single allergenic protein for diagnosis and therapy of all the grass pollen sensitive patients.

Studies in USA, Europe and Australia have indicated extensive cross-reactivity amongst different grass pollens. Bernstein et al. (1976) studied the cross-reactivity among major allergenic pollens prevalent in United States. Pollen allergens from different grass species were shown to be immunochemically similar when assayed by RAST-inhibition \([6]\). Similarly, Martin et al. (1985) demonstrated botanical classification based allergenic relationships in 14 different grass pollens prevalent in various regions of the USA \([28]\). Besides RAST and RAST-inhibition, various other in-vivo and in-vitro methods like skin tests, cross immunoelectrophoresis, ELISA and ELISA-inhibition, immunoblot and immunoblot-inhibition by using patient IgE, specific rabbit and monoclonal antibodies were employed in the study of cross-reactivity among various grass pollens. Chakraborti et al. (1981) also demonstrated a great deal of cross-reactive allergens in various grass pollens \([8]\). Smith et al. (1994) identified isoforms of the two major allergens of rye grass pollen, Lol p 1 and Lol p 5 and cross-reactive allergens in other grasses by means of affinity purified specific IgE antibodies. Group 1 proteins were identified in grasses from two sub-families of the Poaceae, while the group 5 allergens were identified only in pollens of grasses from one sub-family, the Pooidae \([46]\). IgE from the sera of allergic individuals is a useful probe for allergens. The use of IgE-13 R has provided information about the
conservation of cross-reactive allergenic epitopes of group 1 allergens in different grasses. Cross-reactive epitopes have been identified in 14 of the 16 grass pollens studied. IgE-12R/I9B probe recognized cross-reactive epitopes in 13 out of 16 grass pollens from the sub-family Pooidae [46]. By using specific monoclonal antibodies, Fahlbusch et al. (1993) showed the existence of shared group IV and V allergenic epitopes in 14 different temperate grass pollens by using techniques such as ELISA-inhibition and dot-blot [17]. Considerable evidence in the literature indicates extensive cross-reactivity amongst grass pollen allergens.

Monoclonal antibodies against 14 kDa fragment (P3) of group 1 allergen (Fes e 1) recognized cross-reactive determinants on group 1 antigens isolated from five different grass pollens (viz. Poa, Lolium, Anthoxanthum, Agrostis, and Festuca), suggesting that P3 is probably a conserved portion of the grass group 1 allergens [16]. Cross-reactive group 5 allergens were also found in 10 different grass pollens [30]. By the use of monoclonal antibody PpV4 raised against Phleum pratense group 5 allergen, cross-reactive 25-28 kDa members of the same group were isolated from related grass species i.e. Lolium, Poa and Dactylis. Information regarding both amino-acid composition and NH₂-terminal sequence were obtained from each group 5 allergen. Common traits such as high alanine content and presence of modified amino acid (hydroxyproline) were observed by Klysner et al. (1992) [24].

Extensive cross-reactivity amongst allergens derived from non-grass sources has also been investigated by various groups. Fernandez et al. (1993) analyzed the possible cross-reactivity between pollens of Sunflower and other species of the family Compositeae, by means of RAST and immunoblot-inhibition. Parallel regression line of RAST inhibition were observed which together with inhibition of IgE binding by Sunflower pollen in immunoblot inhibition experiment suggested a very close relationship between allergens of Sunflower and Mugwort pollen [18]. Barletta et al. (1996) explored the relationship between Cupressus arizonica and Cupressus sempervirens pollen extract by polyclonal rabbit anti-sera and human IgE. Two cross-reactive allergens with molecular weight 43 kDa and 36 kDa were observed in both extracts with patients sera. A number of common epitopes were identified by means of rabbit polyclonal anti-sera [5]. Using purified monoclonal antibody produced against Ole e 1, a major tree pollen allergen, Martin-Orozco et al. (1994) analyzed the
common epitopic determinants in olive and different Oleaceae pollens (like *Fraxinus excelsior*, *Ligustra vulgaris*, *Syringa vulgaris*, and *Forsynthia suspensa*). 18-20 kDa molecular weight protein was observed in all pollens except *Forsynthia suspensa* [29].

Strong cross-reactivity of Timothy and Birch pollen extracts with Kiwi fruit allergens by means of immunoblot-inhibitions were demonstrated by Pasterallo et al. (1996) [34]. Cross-reactive IgE were found to be responsible for allergic reactions in patients allergic to pollen on ingestion of food. Bet v1 and birch profilin have been identified as relevant cross-reactive allergens by Heiss et al. (1996) who used monoclonal antibodies specific to Art v1, a 60 kDa glycoprotein, to detect cross-reactive allergens in other pollens and other plant derived food. It recognized similar bands in fruits and vegetables. They defined a novel and cross-reactive 60 kDa pan allergen, identical to Art v1, present in various pollen and plant food, distinct from Bet v1 and profilin [20]. Allergenic cross-reactivity among three *Fusarium* species were studied by Verma and Gangal (1994) by CIE, ELISA-inhibition and immuno-print inhibition assays. Dose response inhibition of 14 kDa allergen in immuno-print inhibition of culture filtrate by extracts of 3 different *Fusarium* species demonstrated significant cross-reactivity. Both shared as well as unique allergens were observed [52]. A number of shared allergens (molecular weight range of 14-70 kDa) were identified when the same group studied 11 common allergenic fungal extracts. A 45 kDa protein was found to be common among these fungi both by IgE and rabbit IgG immunoblot analysis [53]. Doeke et al. (1993) showed a highly significant correlation between levels of ant *P. ovale* IgE reacting with extract of *Candida albicans* due to a marked cross-reactivity as shown by ELISA inhibition. Fluid phase pre-incubation of double positive sera either of the two yeast extracts resulted in a dose dependent inhibition of the IgE reaction with both coated *P. ovale* and *Candida albicans* allergens [10-11].

Knowledge of patterns of cross-reactivity has application for *in-vivo* standardization of allergenic extracts, diagnostic testing and treatment of sensitive patients. Elucidation of the allergenic epitopes of the allergenic proteins is critical for the development of immunotherapeutic strategies. This goal with grass pollens may be simplified by the likely presence of structurally similar, cross-reactive allergenic
determinants. India, being a country with varied climatic conditions, hosts a number of allergenic grasses which are different from the one reported from other parts of the world. There is, however, very little information available on the antigenic and allergenic nature of grass pollens prevalent in India [47]. The present investigation is aimed at gaining an insight into the antigenic and allergenic relationships among various grasses inhabiting India and western countries of the world.

**MATERIAL AND METHODS**

**Preparation of allergenic pollen extracts from eight different grasses**

Highly pure pollen samples were collected from wild grasses (*Cenchrus ciliaris, Cynodon dactylon* and *Imperata cylindrica*) and cultivated types (*Pennisetum typhoides* and *Sorghum vulgare*) growing in and around Delhi. Pollens of the grasses *Lolium perenne, Phleum pratense* and *Poa pratensis* were purchased from MIS Allergon (Pharmacia). Allergenic extracts from respective pollen were prepared as described previously (Chapter II). Protein content in the pollen extracts was determined by modified method of Lowry et al. (1951) [27] using phosphotungstic (PTA) reagent (15% PTA in 10% HCl) to precipitate the proteins [44]. Bovine serum albumin (BSA) was used as a standard.

**Selection of Pennisetum typhoides specific patient and pooled rabbit sera**

Serum samples from a group of 26 *Pennisetum typhoides* pollen sensitive patients [chapter II] were selected for this study. A pool of patients' sera was prepared by mixing equal volume of serum from each of these patients. This pooled sera was considered as *Pennisetum* specific pooled patients sera. Preparation of pooled *Pennisetum typhoides* pollen specific rabbit sera has been described elsewhere [chapter II].

**Specific IgE and IgG antibody ELISA inhibitions**

Inhibition assays were performed by pre-incubating 50 μl of either pooled patient sera (1 : 5 v/v diluted) or pooled rabbit anti-sera (1 : 5000 v/v diluted) with 50 μl of inhibitor (pollen extract of *Pennisetum typhoides* and other grasses) in various concentrations ranging from 0 to 10 μg. The mixture was then added to crude *Pennisetum typhoides* pollen extract coated wells of a polystyrene microtitre plate.
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(Corning™). NHS from subjects with negative ELISA and ID response to grass pollen extracts and pre-immune rabbit sera were used as negative control in IgE and IgG ELISA inhibition, respectively. A standard ELISA inhibition procedure was followed by using rabbit anti-human IgE (ε-chain specific) peroxidase conjugate (Sigma) and goat anti-rabbit IgG (γ-chain specific) peroxidase conjugate (Sigma) in the case of IgE ELISA inhibition and IgG ELISA inhibition, respectively, and the percent inhibition was calculated as described in chapter II.

**Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE of different grass pollen extracts (at equal protein concentrations) was carried out on 12% acrylamide gel using the discontinuous buffer system as described earlier (chapter II) on midget apparatus (Pharmacia). After the completion of run, gel was fixed for 1 hour in fixing solution [methanol: acetic acid: water, 45: 45: 10; (v/v), respectively] and stained for one hour in 0.1% Coomassie Brilliant Blue R-250 (CBB) prepared in destaining solution containing ethanol: acetic acid: water, 45: 10: 45; (v/v), respectively. Excess of stain was removed by washing the gel in destaining solution and the gel was preserved in 0.7% acetic acid solution. Simultaneous staining of glycosylated and non-glycosylated proteins of allergen extract of all grass pollens was carried out essentially as described in chapter II.

**IgE and IgG immunoblot analysis**

Pollen extracts were separated on 12% SDS-PAGE and electrophoretically transferred on to nitrocellulose membrane as described earlier (chapter II). The membrane was stained with Ponceau 'S' in order to estimate the extent of protein transfer from the gel to nitrocellulose membrane. After complete destaining with distilled water, the free sites on nitrocellulose membrane were blocked with 2% gelatin (prepared in PBS) solution. Following washing three times for five minutes each with PBST, the membrane was incubated with appropriate primary antibodies (either with 1 : 10 (v/v) diluted pooled patient's sera for the detection of IgE binding allergenic proteins; or with 1 : 10000 (v/v) diluted *Pennisetum typhoides* specific pooled rabbit anti-sera for the detection of cross-reactive antigenic proteins. The membrane was also incubated with 1:500 (v/v) Phl p 1 specific monoclonal antibody (IG 12) overnight for the detection of grass group I allergens; or with 1 : 40 (v/v) Phl p 5 specific monoclonal antibody (Bo1) for the detection of group V allergens). The
monoclonal antibodies IG 12 and Bo1 were received as a kind gift from Dr. G. Schramm, Forschungsinstitute, Borstel, FRG [39]. The nitrocellulose membrane was then washed with PBST and again incubated with appropriate secondary antibody conjugates (either 1:500 (v/v) diluted rabbit anti-human IgE antibodies conjugated to peroxidase (Sigma), or with goat anti-rabbit IgG antibodies conjugated to peroxidase (Sigma); or 1: 1000 (v/v) rabbit antimouse IgG1-Alkaline conjugate (for both group 1 and 5 allergen detection), respectively. After washing the membrane with PBST, the cross-reactive allergenic and antigenic proteins were detected by keeping the membrane in appropriate substrate solutions for few minutes. The colour reaction was stopped by rinsing the membrane several times in distilled water.

RESULTS

All grass pollen extracts resolved into a number of CBB stained bands when subjected to 12 % SDS-PAGE (figure 1). Most of the bands appeared in the molecular weight range of 10-100 kDa. A total of 21, 40, 24, 28, 24-26, 30, 32 and 21 CBB stained bands were observed in Cenchrus ciliaris, Cynodon dactylon, Imperata cylindrica, Lolium perenne, Pennisetum typhoides, Phleum pratense, Poa pratensis and Sorghum vulgare pollen extract, respectively. Many of these bands were found to possess similar electrophoretic mobility.

SDS-PAGE separated profiles of these grass pollen extracts were electrophoretically transferred on to nitrocellulose membrane and subjected to protein / glycoprotein staining. Carbohydrate components with very high (above 100 kDa) molecular weight were observed in all grass pollen extracts (figure 2). Most of these glycan components did not appear as well defined bands. Only Cynodon, Imperata and Lolium extracts showed the presence of 3, 1 and 2 sharp glycan (blue stained) bands, respectively in the region above 100 kDa. Pollen extracts of Cenchrus, Imperata and Sorghum showed strong blue staining indicating high levels of glycosylation of their SDS-PAGE separated proteins. Most of the bands in the lanes corresponding to Cynodon, Lolium, Pennisetum, Phleum and Poa extracts, appeared relatively less glycosylated.

By using Pennisetum specific pooled patients sera in immunoblot experiment, a number of IgE binding bands were observed in each of the grass pollen extracts studied (figure 3). Most of these allergenic bands appeared in the molecular weight
FIGURE 1:
SDS-PAGE profile of eight different grass pollen extracts.
FIGURE 2:
DIG based simultaneous detection of glycosylated and non-glycosylated SDS-PAGE separated proteins of 8 different grass pollen extracts after their transfer on to nitrocellulose membrane.
FIGURE 3:
IgE immunoblot analysis of different grass pollen extracts by *Pennisetum typhoides* specific pooled patients sera demonstrating allergenic cross-reactivity among various grass pollens.

range of 10-90 kDa. Maximum number (14) of IgE binding bands were observed in the Poa pratensis pollen extract, followed by Lolium which showed the presence of 13 IgE binding bands. Pennisetum, Phleum, Sorghum, Cenchrus, Cynodon and Imperata extract revealed 12, 11, 9, 8, 7 and 5 IgE binding components, respectively. An allergenic band with molecular weight ~12 kDa was observed in Cenchrus, Lolium, Poa and Sorghum extracts. However, this band was most prominent in case of Sorghum and Cenchrus pollen extracts. IgE binding bands with molecular weights 57, 43, and 30 kDa were observed in all grasses (table 2). It possibly indicates the presence of shared allergenic epitopes on these proteins.

Shared antigenicity among different grass pollens was visible when immunoblot was carried out using Pennisetum typhoides specific pooled rabbit antisera (figure 4). Fourteen IgG binding bands were observed in the Poa pratensis and Phleum pratense pollen extracts. Cynodon dactylon pollen extract showed the presence of only two weak antigenic bands. Pennisetum typhoides, Lolium perenne, Sorghum vulgare, Cenchrus ciliaris and Imperata cylindrica pollen extracts showed 12, 12, 12, 6 and 8 IgG binding components, respectively, in the molecular weight range of 14-85 kDa. Antigenic bands with molecular weights 70, 50, 43, 34 and 14 kDa were detected in 6 out of 8 grass pollen extracts (table 3).

Monoclonal antibody IG 12 identified group 1 allergens in pollen extracts of Phleum pratense, Lolium perenne, Poa pratensis and Pennisetum typhoides when eight different pollen extracts were screened by immunoblot (fig. 7). No other grass pollen extract showed the presence of group 1 allergens. However, when these pollen extracts were screened by monoclonal antibody Bo1, group 5 allergen was not detected in any of the Indian grass pollen extracts (fig. 8). On the other hand, Bo1 identified Lol p 5, Poa p 5 and Phl p 5 allergens from the pollen extracts of Lolium perenne, Poa pratensis and Phleum pratense, respectively.

Competitive ELISA inhibition was performed using Pennisetum specific pooled patients sera to evaluate allergenic cross-reactivity amongst 8 grass pollens. Two common allergenic weed and one allergenic tree pollen were also included in the study. A dose dependent inhibition of IgE binding was observed, when pooled sera was pre-incubated with serial dilution of homologous and heterologous grass pollen extracts (figure 5). As it can be seen from this figure, except Cynodon, all
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heterologous grass pollen extracts produced significant inhibition of specific IgE binding to solid phase *Pennisetum typhoides* pollen extract. On the other hand, tree and weed pollen extracts produced no significant IgE inhibition. The amount of each extract required to produce 50% inhibition (ID$_{50}$) was calculated and presented in table 4. The binding of IgE to solid phase *Pennisetum typhoides* pollen extract was substantially inhibited by *Poa* and *Sorghum* extracts for which ID$_{50}$ was estimated as 280 and 285 ng, respectively, less than the ID$_{50}$ value obtained with homologous inhibitory pollen extract (i.e. *Pennisetum typhoides*). This suggests that *Poa* and *Sorghum* extract carry most of the allergenic epitopes present in *Pennisetum* extract. However, it may also be due the the presence of some unique allergenic epitopes present in *Poa* and *Sorghum* since patients are generally exposed to many types of airborne pollen allergens which manifest in the development of specific IgE antibodies. Similar results obtained from RAST inhibition assays have been discussed by Bernstein et al. (1976) [6].

Antigenic relationship among these grasses was also evaluated by competitive ELISA inhibition by using *Pennisetum typhoides* specific rabbit anti-sera. Here also only grass pollen extracts could produce 50% or more inhibition of binding of specific IgG antibodies to solid phase *Pennisetum* pollen antigen. Tree and weed pollen extract could produce only upto 36% inhibition. Except *Cynodon* all other grass pollen extracts showed close antigenic relationship as they produced similar lines of specific IgG inhibition (figure 6). Their respective ID$_{50}$ values are given in table 3.

DISCUSSION

Substantial evidence provided by various in-vivo and in-vitro studies, indicates that there is extensive cross reactivity among various temperate grass pollen allergens [8,26,28,30,55]. However, very little information is available regarding the cross reactive nature of grasses prevalent in tropical and sub-tropical regions of the world [47]. The airborne pollen of family Gramineae has been recognized as a cause of respiratory allergy for many decades in India and other parts of the world with similar climatic conditions. The present study also reveals substantial cross reactivity of pollen allergens of *Pennisetum typhoides*, a major contributor of allergenic pollen in India, with that of other tropical and temperate grass pollens.
FIGURE 4:
IgG immunoblot analysis of different grass pollen extracts by using *Pennisetum typhoides* specific pooled rabbit anti-sera demonstrating antigenic cross-reactivity among various grass pollens.

FIGURE 5:
IgE ELISA-inhibition dose response curves of eleven (8 grasses, 2 weeds and 1 tree) different allergenic pollen extracts by using *Pennisetum typhoides* specific pooled patients sera with *Pennisetum typhoides* pollen extract as solid phase allergen.
FIGURE 6:
IgG ELISA-inhibition dose response curves of eleven (8 grasses, 2 weeds and 1 tree) different allergenic pollen extracts by using *Pennisetum typhoides* specific pooled rabbit anti-sera with *Pennisetum typhoides* pollen extract as solid phase antigen.
FIGURE 7:
Identification of group 1 allergen homologues in different grass pollen extract on immunoblot by using Phl p 1 specific monoclonal antibody (IG 12).

FIGURE 8:
Identification of group 5 allergen homologues in different grass pollen extract on immunoblot by using Phl p 5 specific monoclonal antibody (Bo 1).
Table 1:
Taxonomic classification* of clinically important grass species used in the present study.

<table>
<thead>
<tr>
<th>FAMILY</th>
<th>SUB-FAMILY</th>
<th>TRIBE</th>
<th>SCIENTIFIC NAME</th>
<th>COMMON NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gramineae</td>
<td>Chloridoideae</td>
<td>Chlorideae</td>
<td>Cynodon dactylon</td>
<td>Bermuda grass</td>
</tr>
<tr>
<td>or Poaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panicoideae</td>
<td></td>
<td>Andropogoneae</td>
<td>Imperata cylindrica</td>
<td>Cogon grass</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sorghum vulgare</td>
<td>Sorghum</td>
</tr>
<tr>
<td>Paniceae</td>
<td></td>
<td>Cenchrus ciliaris</td>
<td>Bunch / buffel grass</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pennisetum typhoides</td>
<td>Pearl millet</td>
</tr>
<tr>
<td>Pooidae</td>
<td>Agrostideae /</td>
<td>Phleum pratense</td>
<td>Timothy grass</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Avenae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Festucae /</td>
<td>Loliurn perennne</td>
<td>Perennial rye grass</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poeae</td>
<td>Poa pratensis</td>
<td>June grass or</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kentucky blue grass</td>
<td></td>
</tr>
</tbody>
</table>

*References
Pohl R W: How to Know the Grasses, ed 3. Dubuque, WC Brow Co. 1978
Kaul M K: Weed Flora of Kashmir Valley, Scientific Publisher, Jodhpur, 1986
Table 2:
IgE binding components present in different grass pollen extracts identified by using serum pool of patients sensitive to *Pennisetum typhoides*.

<table>
<thead>
<tr>
<th>Pollen extract</th>
<th>Number of bands</th>
<th>Molecular weight (kda)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cenchrus ciliaris</em></td>
<td>8</td>
<td>85, 80, 70, 57, 43, 34, 25</td>
</tr>
<tr>
<td><em>Cynodon dactylon</em></td>
<td>7</td>
<td>85, 80, 70, 57, 43, 34, 25</td>
</tr>
<tr>
<td><em>Imperata cylindrica</em></td>
<td>5</td>
<td>85, 75, 70, 57, 43, 34, 25</td>
</tr>
<tr>
<td><em>Lolium perenne</em></td>
<td>13</td>
<td>85, 75, 70, 57, 43, 34, 25, 18, 14, 12</td>
</tr>
<tr>
<td><em>Pennisetum typhoides</em></td>
<td>12</td>
<td>85, 80, 70, 57, 43, 34, 25, 18, 14</td>
</tr>
<tr>
<td><em>Phleum pratense</em></td>
<td>11</td>
<td>75, 70, 57, 43, 34, 25, 18</td>
</tr>
<tr>
<td><em>Poa pratensis</em></td>
<td>14</td>
<td>85, 75, 70, 57, 43, 34, 25, 18, 14, 12</td>
</tr>
<tr>
<td><em>Sorghum vulgare</em></td>
<td>9</td>
<td>85, 75, 70, 57, 43, 34, 25, 18, 14, 12</td>
</tr>
</tbody>
</table>

Table 3:
IgG binding components present in different grass pollen extracts identified by using *Pennisetum typhoides* specific pooled rabbit anti-sera.

<table>
<thead>
<tr>
<th>Pollen extract</th>
<th>Number of bands</th>
<th>Molecular weight (kda)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cenchrus ciliaris</em></td>
<td>6</td>
<td>80, 67, 50, 43, 34, 26</td>
</tr>
<tr>
<td><em>Cynodon dactylon</em></td>
<td>2</td>
<td>80, 67</td>
</tr>
<tr>
<td><em>Imperata cylindrica</em></td>
<td>8</td>
<td>70, 50, 48, 34, 26, 16, 14</td>
</tr>
<tr>
<td><em>Lolium perenne</em></td>
<td>12</td>
<td>85, 80, 70, 54, 43, 36, 34, 26, 16, 14</td>
</tr>
<tr>
<td><em>Pennisetum typhoides</em></td>
<td>12</td>
<td>85, 80, 70, 67, 54, 43, 34, 26, 18, 16, 14</td>
</tr>
<tr>
<td><em>Phleum pratense</em></td>
<td>14</td>
<td>85, 80, 70, 67, 54, 43, 34, 26, 18, 16, 14</td>
</tr>
<tr>
<td><em>Poa pratensis</em></td>
<td>14</td>
<td>85, 80, 70, 67, 54, 43, 34, 26, 18, 16, 14</td>
</tr>
<tr>
<td><em>Sorghum vulgare</em></td>
<td>12</td>
<td>85, 80, 70, 67, 54, 43, 38, 35, 30, 26, 22, 14</td>
</tr>
</tbody>
</table>
TABLE 4:
Amount of protein required to produce 50 % inhibition (ID_{50}) of binding of *Pennisetum typhoides* specific antibodies to solid phase homologous pollen extract in ELISA-inhibition assays.

<table>
<thead>
<tr>
<th>Inhibitory protein</th>
<th>ID_{50} value (μg)</th>
<th>IgE ELISA inhibition</th>
<th>IgG ELISA inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cenchrus ciliaris</em></td>
<td>430</td>
<td>620</td>
<td></td>
</tr>
<tr>
<td><em>Cynodon dactylon</em></td>
<td>2400</td>
<td>1380</td>
<td></td>
</tr>
<tr>
<td><em>Imperata cylindrica</em></td>
<td>620</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td><em>Lolium perenne</em></td>
<td>370</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td><em>Pennisetum typhoides</em></td>
<td>290</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td><em>Phleum pratense</em></td>
<td>500</td>
<td>630</td>
<td></td>
</tr>
<tr>
<td><em>Poa pratensis</em></td>
<td>280</td>
<td>610</td>
<td></td>
</tr>
<tr>
<td><em>Sorghum vulgare</em></td>
<td>285</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Artemisia scoparia</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Prosopis juliflora</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Ricinus communis</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*detected by using *Pennisetum typhoides* specific pooled patients sera.*

*detected by using *Pennisetum typhoides* specific pooled rabbit anti-sera.*

*Allergenic weed pollen as a negative control.*

*Allergenic tree pollen as a negative control.*

*Allergenic weed pollen as a negative control.*

*ND* not determined.
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Other pollen extracts except Cynodon produced 50% inhibition in a range of 520-800 ng (table 3). In agreement to the previous reports, the present study confirms the different antigenicity of the Cynodon pollen proteins [41,45,48]. Here also Sorghum, Lolium and Poa antigens competed closely for Pennisetum specific antibodies and produced ID_{50} at 520, 600 and 610 ng, respectively. The observed antigenic closeness between Pennisetum and Sorghum clearly indicate the more obvious relationship between the tribes Paniceae and Andropogoneae of the same sub-family i.e. Panicoideae (table 1), due to the presence of similar antigenic/allergenic components in these pollens. These observations suggest that at least in some individuals multiple sensitization to different grass pollen allergens follows exposure and sensitization by a single grass pollen. If this is correct then it might be possible to reduce the number of grass pollen used as therapeutic extracts. This possibility emphasize the need to identify and characterize cross reactive allergenic proteins from various grass pollen extracts.

SDS-PAGE analysis of 8 different grass pollen extracts revealed the presence of proteins with almost equal electrophoretic mobility. Immunoblotting analysis of crude extract is a powerful tool for detecting such cross reactive components. Immunoblotting with pooled patients sera (collected against Pennisetum typhoides pollen) demonstrated the presence of extensive cross reactivity among various grass pollen extracts (figure 3). Poa, Lolium and Pennisetum extracts showed the presence of 14, 13 and 12 IgE binding components, respectively, with pooled patients sera. Strong IgE (from pooled patients' sera) binding to proteins with molecular weight 75, 70, 57, 33, 25, 18, 14 and 12 kDa in the pollen extract of Poa pratense was observed. On the other hand, proteins with molecular weights 80, 57, 54, 48, 43, 34 and 23 kDa in Lolium extract exhibited strong IgE binding. Phleum extract also showed 11 allergenic components with 57, 54 and 33 kDa components demonstrating strong IgE binding. Among tropical grasses, Sorghum showed 9 allergens with 43, 40 and 30 kDa as strong allergenic components, however, IgE binding components with high molecular weights did not appear as clear bands. At the same time, a 12 kDa band, which showed strongest IgE binding, was observed in Sorghum and Cenchrus extracts. A weak allergen with similar molecular weight has been identified in some grass pollens and described as cytochrome 'C' [14]. Interestingly, this band did not appear as strong in other grasses including
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*Pennisetum*. These results demonstrate the extensive cross-reactivity of *Pennisetum* pollen with other grass pollens most notably with *Sorghum, Poa, Lolium* and *Phleum*, irrespective of their temperate or tropical origin. This is in contrast to earlier reports with *Cynodon* pollen, the only tropical grass pollen compared so-far with temperate grasses [26,28,40]. Minimum number of allergenic bands were detected in *Cynodon* pollen extract with pooled patients sera. All 7 IgE binding components in *Cynodon* were observed as faint bands. Potter et al. (1993) studied the cross-reactivity of *Pennisetum clandestinum*, a close relative species of *Pennisetum* prevalent in South Africa, with that of *Cynodon* and found weak cross-reactivity of *Pennisetum clandestinum* pollen allergens with those of *Cynodon*. Only 32 kDa allergen from *P. clandestinum* was found to cross-react with *Cynodon* pollen. However, they observed no cross-reactivity between *Pennisetum clandestinum* and *Phleum pratense* grass pollen allergens [38]. This is in contrast to the results obtained in the present study. The difference in the results may be attributed to factors like different *Pennisetum* species having different allergenic/antigenic make up, difference in the assay conditions, and inadequate exposure of patients to these pollens.

In line with other reports, the present study suggested that *Cynodon* grass pollen contains few allergens cross reactive with those of *Pennisetum*’s than other grasses. Alternatively, it is also possible that allergic patients selected for this study had not had significant sensitization by *Cynodon* grass pollen and reactivity seen to this grass was only due to the presence of allergens cross reactive with *Pennisetum typhoides*.

It is interesting to note that none of these temperate grasses are indigenous to Indian sub-continent and the serum pool used in these experiments could have been deficient in specific reactivity to these temperate allergens. This extensive cross-reactivity may be explained by various factors such as the presence of conserved carbohydrate moieties shared by various proteins in different allergen sources as observed mainly in fungal and vegetable allergenic sources [1,10-11,21,32,36-37,54]. Most of the grass pollen extracts, in the present study, showed the presence of a number of glycosylated proteins (figure 2). Results with other pollen (*C. arizona*) suggested that most of the pollen components contain glycosidic residues, mainly mannose, glucose, fucose, fructose, N-acetyl glucosamine, and N-acetyl galactosamine [9]. The presence of high proportion of carbohydrate in some of the
pollen could be of great relevance in regard to the high degree of cross-reactivity at the IgE level reported for some of the closely related as well as unrelated pollens. The presence of conserved epitopes on different proteins of these grass pollens is one of the reasons for the observed cross-reactivity among various grass pollen allergens. It may also be due to the less affinity maturation of IgE response due to a lack of evolutionary pressure [1]. Cross-reactive priming of the patients from other sources may also be responsible for the observed cross-reactivity among different allergenic proteins [1].

The pattern of cross-reactivities based upon related allergenic determinants in allergenic proteins from different sources has implication in clinical management of allergic patients. Where IgE antibodies are directed against the same or similar determinants in the different protein sources, they must be regarded as potentially involved in the sensitization of the subject and avoidance of these sources might be considered as a good therapeutic measure, especially when seen in conjunction with the clinical histories of the allergic subjects [33]. It may also be anticipated that desensitization with one of the allergenic proteins will result in desensitization to other. Selection of suitable highly cross-reactive allergenic proteins may reduce the number of allergens used in diagnosis and therapy of such diseases.

The occurrence of shared antigenic/allergenic components among grass pollen extract for a particular protein/glycoprotein band could either be due to the presence of co-migratory proteins or to the antigenic similarities of epitopes between the two extracts. 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 rabbit antibodies were raised against *Pennisetum* pollen extract to study the shared antigenicity among different grasses. *Poa* and *Phleum* showed 14 antigenic proteins with *Pennisetum* specific rabbit anti-sera, whereas *Pennisetum*, *Sorghum* and *Lolium* showed 12 antigens each. Antigenicity of the *Phleum* pollen components appeared weak as these bands were faintly stained on immunoblot. Uniqueness of *Cynodon* antigens was once again confirmed as only 2 components could be identified with *Pennisetum* specific rabbit anti-sera (Table 2). Shared antigenic bands with molecular weights 80, 26 and 70, 67, 50, 43, 34, 14 kDa were observed in 7 and 6 different grass pollens.
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Cross-reactivity of Pennisetum pollen with other grass pollen respectively by Pennisetum specific rabbit anti-sera. A combination of SDS-PAGE, western blotting and immunodetection with rabbit antibodies against rye grass group 1 (R7) allergen has been used by Standring et al. (1987) to demonstrate the distribution of this cross reactive allergen in 11 different grass pollens [48].

Monoclonal antibody IG 12 [39] identified highly cross reactive group 1 allergen homologues in pollen extracts of Pennisetum, Phleum, Poa and Lolium. No other Indian grass pollen was found to have representative of group 1 allergens (fig. 7). Similarly, group 5 allergen homologues were identified by monoclonal antibody Bo1 [39] only in the grasses prevalent in temperate regions of the world (fig. 8). Recently, Ledece-Brodard et al. (1996) have demonstrated the absence of group 4 allergen (another important group of grass pollen allergens) homologues in pollen extract of Pennisetum typhoides, by using monoclonal antibodies raised against Dac g 4 (from pollen extract of Dactylis glomerata) [25]. These results demonstrate the distinct nature of epitopes present on allergenic proteins of tropical grass pollens.

Patients with food allergies can be sensitive to pollen as well [15,34]. Food allergens from mustard, wheat, barley and rice seed have also been characterized [12,19]. Birch pollen profilin has also been identified in both, the pollen and the vegetative tissues of many crop species [51]. Thus, it is obvious to anticipate that the pollen of agricultural crops represent an additional source of allergenic proteins. John et al. (1995) from their cross reactivity studies concluded that no clinically significant cross reactivity exists between grasses and cereal grains [22]. On the contrary, Singh et al. (1985) demonstrated that a number of agricultural species including maize oats and cultivated rye contain protein antigens which are weakly cross reactive with rye grass allergens [45]. Poa, Lolium and Phleum pollen allergens are shown to cross react with many of the agricultural crops. Astwood et al. (1995) demonstrated that barley pollen expresses protein capable of cross reacting with antibodies. The cross-reactive proteins were of exactly the same molecular weight as those found in Poa pratense pollen. They suggested that pollens from agricultural crop may be an additional occupational hazard for subjects with grass pollen allergies and dealing with agricultural crops [33]. Kalveram and Forek (1978), observed a close immunological relationship between corn and grass pollen antigens. They suggested that grass pollen extract contained all antigenic determinants of the corn pollen and
recommended the exclusive use of grass pollen extract in diagnosis and possible therapy of combined corn and grass pollen allergy [23].

In conclusion, a high degree of cross reactivity among various grass pollens at the allergenic as well as antigenic level has been observed. Taxonomic closeness correlates well with the degree of cross reactivity as demonstrated by the related allergenicity among the members of sub-family Panicoideae and Pooideae. The study demonstrate the presence of at least three shared allergens with molecular weights 57, 43 and 30 kDa in all the grass pollens studied (table 1). A highly allergenic protein with molecular weight 43 kDa from the pollen extract of *Pennisetum typhoides* has been purified and characterized (chapter IV). Elucidation of the allergenic epitopes present this protein may be helpful in designing peptide based vaccine for the treatment of grass pollen induced type I allergic diseases.
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