

IMMUNOCHEMICAL CHARACTERISATION OF GRASS POLLEN ALLERGENS IN SOUTH AFRICA

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ABSTRACT

In the subtropical climate of South Africa, grasses of the subfamily Panicoideae are predominant. Bermuda grass has previously been shown to be an important local allergen, and IgE epitopes of Bermuda grass extracts are known to be distinct from those of the Pooid pollen extracts. The immunological relationships of the closely related Panicoid grasses, kikuyu and buffalo grass, and *Eragrostis*, another common indigenous grass, related to Bermuda were examined. More than 95% of grass-sensitive patients were found to have IgE antibodies to buffalo and *Eragrostis* pollen extracts. Inhibition of enzyme-linked immunosorbent assays (ELISA) and immunoblots revealed that extracts of these grass pollens could significantly inhibit IgE binding to each of the pollens, kikuyu, buffalo, *Eragrostis*, and Bermuda on solid phase, but never achieved 100% inhibition, indicating that cross-reactive but also unique epitopes are present. We also identified a subset of patients who had negative radioallergosorbent tests (RASTs) to Bermuda, and minimal inhibition by Bermuda pollen extract. Buffalo and *Eragrostis* pollens are major sensitisers and should be included in South African diagnostic panels.

Because of their worldwide distribution and profuse pollen production, grasses represent a major aeroallergen source for 40% of allergic patients. In Southern Africa there are 94 genera (10% of the world total) and 967 species of grass. Of these, 847 species are indigenous, and 115 are naturalised.¹ Southern Africa comprises both subtropical and temperate regions, where grassland and savannah biomes predominate. The most significant pollens associated with pollinosis in South Africa are those produced by grass. The important tree pollens implicated in pollinosis are mostly from introduced species, and have an important role in early spring, but have a limited duration. Weed and dicotyledon pollens are produced through the summer, but make a lesser contribution.

Grass pollen aeroallergens are among the most potent elicitors of IgE-mediated type 1 hypersensitivity in South Africa.² The pollen grains are small and light, and, triggered by humidity, are shed from anthers in large quantities into the atmosphere where they are wind-dispersed (anemophilous). Furthermore the antigens are rapidly released upon contact with oral, nasal and eye mucosa.

Pollen production and dispersal are influenced by climatic factors. Temperature and rainfall patterns preceding the pollen season are related to the onset, intensity and duration of the pollen season. In the Cape region, relatively high temperatures throughout the long summer, together with the wind factor, ensure large amounts of anemophilous grass pollen in the atmosphere, with peaks after rain. The long summer with rain on the Highveld also ensures abundant pollen production in these more northern grasslands. Fresh pollen production is noted after showers during the summer in some species of grass, notably important sensitisers.

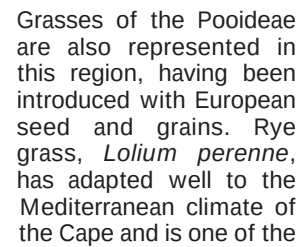
There is a peak in grass pollen concentration in October, following the tree pollen period in early spring. This coincides with the major peak of 'hay fever' symptoms of allergic rhinoconjunctivitis. However, not all the grasses pollinate at that time and symptoms often persist through the long summer. Certain grass species, such as *Eragrostis*, produce low concentrations of pollen from August through until July, particularly after rain. *Eragrostis* gives a very strong positive skin-prick test (SPT) reaction in sensitised subjects, who remain symptomatic throughout the season.

The prolonged pollen season of up to 10 months each year is relevant to sensitisation and the primary IgE-antibody response in susceptible individuals. Ongoing allergen exposure to low concentrations leads to a memory B-cell response, followed by cross-linking of mast cell- and basophil-bound IgE and consequent mediator release. Late inflammatory responses are triggered by the influx of activated basophils, eosinophils and T-cells. High concentrations of larger and heavier pollens, such as *Pinus* and *Acacia*, induce symptoms in only a few individuals living in close proximity. Thus the appearance and severity of symptoms depend on the quantity of pollen inhaled and the allergenic potency of antigenic proteins released.

The Panicoideae and Chloridoideae families comprise the dominant grass families in South Africa (Fig. 1). Orren *et al.*³ were the first to characterise allergens of Bermuda pollen in the Western Cape. It is a major tropical allergenic grass, as 80% of patients with allergic rhinitis are sensitive to Bermuda grass pollen on SPTs.² An assessment of the potential allergenicity of indigenous pollens was relevant in the context of diagnosis and immunotherapy in the region.

Potter *et al.*⁴ undertook a study of an African indigenous grass, and found 43% of rhinitis patients in the Cape to be sensitive to kikuyu grass pollen of the Paniceae tribe. Kikuyu grass, *Pennisetum clandestinum* (Fig. 2), was introduced from Kenya and has been widely planted as lawn, playing fields and pasture grass. We further investigated the potential cross-reactivity with the related Panicoid, broad-leaved *Stenotaphrum secundatum* or buffalo grass (Fig. 3), a coastal indigenous grass that migrated down the eastern coast of Africa, including Madagascar and Mauritius, and grows extensively over the Cape Peninsula. The study included a related and abundant indigenous grass, *Eragrostis curvula* (Fig. 4), a member

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The grass pollen peak occurs in October in this region. However buffalo grass produces pollen from November to the end of February, and Bermuda has been seen pollinating mainly from December to April. *Eragrostis* produces pollen throughout the year, and the sedge grasses pollinate in July.



Pollen from buffalo, kikuyu and *Eragrostis*, *Lolium* and Bermuda species was collected under controlled conditions, dried and stored at -80°C . Extraction of aller-



SDS-PAGE was performed on the pollen extracts, using 7-18% acrylamide gels; thereafter the separated proteins were transferred electrophoretically to polyvinylidene-difluoride (PVDF) membranes for immunodetection of IgE in serum of sensitised subjects bound to allergenic proteins.



Fig 4a & b. Boer love grass (*Eragrostis curvula*).

Major or minor allergens were classified on the frequency of recognition by IgE from selected sera from allergic subjects.

We have confirmed, on immunoblotting, that 95% of the grass-sensitive individuals in the Cape have IgE antibodies to buffalo pollen extracts, and 95% have a concordant IgE response to *Eragrostis*, and conclude that buffalo and *Eragrostis* pollen are common sensitizers of grass-allergic subjects in this region (Fig. 5). We have partially characterised these allergens and observed similarities in the physicochemical parameters and immunological reactivity within the Panicoid and the Chloridoid families. The major buffalo allergen, defined as binding IgE from most subjects, is a 34 kDa glycoprotein, pI 5-7, which is consistent with the principal allergenic component of most grasses, the group 1 (Gp 1) allergen.⁵ Other IgE-binding allergens were found at 12, 14, 39, 56 and 68 kDa. The 14 kDa allergen on immunoblots, which binds 30% of sera, may have homology to profilin, a panallergen present in many grass pollens and foods, and in humans.⁶ Kikuyu extract exhibited a very similar pattern to that of buffa-

lo. The main IgE-binding allergen of *Eragrostis* has a 30 kDa MW, similar to that of Bermuda pollen, Cyn d 1.⁵ IgE binding to Bermuda extract was found in 68% of sera, but it was about 95% to rye, buffalo and *Eragrostis* pollen extracts.

To confirm the specificity of the patient sensitivity to these pollen extracts, basophil histamine release was measured by RIA (Pharmacia), following incubation of blood samples from sensitised subjects with decreasing concentrations of buffalo and *Eragrostis* pollen extracts for 60 minutes at 37°C. This is a close approximation of the *in vivo* situation and demonstrated a specific and dose-dependent release, for different concentrations of pollen extracts of buffalo and *Eragrostis*.

Peripheral blood mononuclear cells (PBMCs) were also separated from grass-sensitive subjects and gave specific and dose-related responses in lymphocyte proliferation assays, when stimulated with buffalo and *Eragrostis* pollen extracts for 6 days before the addition of ³H-thymidine for 18 hours. T-cells of non-atopic controls reacted in a less specific manner. SPTs to fresh extracts of the indigenous grass pollens also show strong wheal and flare responses in patients with allergic rhinitis.

Isoallergenic variations of the B-cell epitopes, comprising a single amino acid substitution, have also been identified in our studies of the indigenous grasses, using 2-dimensional PAGE, and reactivity with IgE of a pool of sera from allergic subjects. A number of such isoforms, being highly conformation-dependent, were revealed in the group 1 allergens, with pIs ranging from 5 to 8, and in the group IV allergens, pI 7-9, of buffalo, *Eragrostis* and kikuyu pollen extracts, which may account for their heterogeneous immunological and biochemical properties.⁷

CROSS-REACTIVITY

Most grass pollens have a high degree of cross-reactivity, as many IgE-binding B cell epitopes are common to most species.⁸ However, as the dominant families in South Africa differ from those of the northern hemisphere, from where the allergen testing panels and immunotherapy vaccines are derived, it was important to determine the relevance of the latter in the context of the cross-reactive epitopes in the sera of our sensitised subjects.⁹ A comparative evaluation of various pollen extracts by inhibition of IgE binding *with crude extracts* in ELISA, RAST, and immunoblotting has demonstrated that not only does cross-reactivity exist between kikuyu, buffalo, *Eragrostis* and Bermuda, but

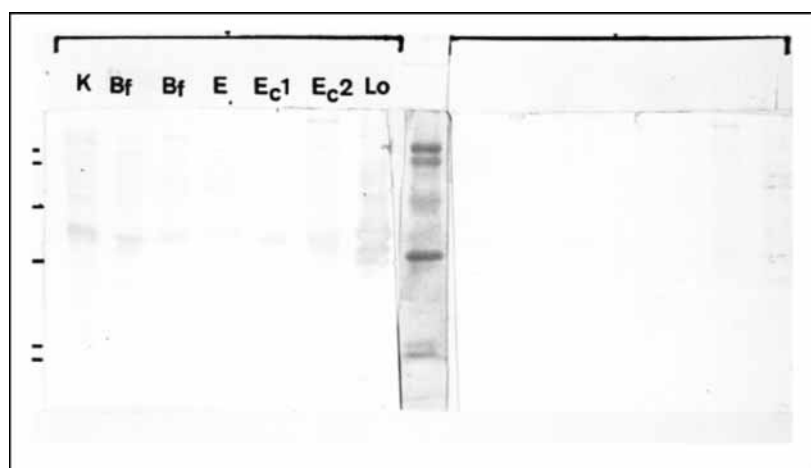


Fig. 5. IgE binding (on left) to kikuyu, buffalo, *Eragrostis* and Lolium, and inhibition by preabsorption with *Eragrostis* extract (on right).



Fig 6. *Ficinia radiata* (*Cyperus*) pollinating in July.

also with the Pooid grass species, *Cynodon*, *Lolium*, *Lagurus* and *Phleum*, and with indigenous fynbos grasses such as *Tribolium* (Arundineae) and *Cyperus* (Cyperaceae) (Fig. 6).

Each pollen extract is able to variably abrogate the IgE binding to one or more of the other three immobilised pollen extracts in ELISAs, when preabsorbed with a pool of patient serum. *Eragrostis* is a highly efficient inhibitor of most allergic sera, and is able to abolish all of the IgE binding to allergens of the other three pollens on immunoblots in some patient sera (Fig. 7).

Eragrostis consistently achieved greater inhibition than Bermuda at lower concentrations in ELISA competition assays, suggesting the presence of unique epitopes in this species, and thus cross-sensitisation rather than co-sensitisation with Bermuda.

Timothy grass (*Phleum pratense*) does not naturally occur in this region. However, sera from Belgian timothy pollen-sensitised individuals (kindly provided by Prof Stevens, Antwerp), who have not been exposed to the subtropical grasses, possess cross-reactive IgE that binds to components on immunoblots of buffalo, kikuyu and *Eragrostis* grasses, which was abolished by preabsorption with the local extracts. Timothy pollen contains a comprehensive range of allergens, which are cross-reactive with our subtropical grass pollens.

Inhibition of the specific Bermuda CAP-RAST assay by buffalo and *Eragrostis* extracts was dose-related, and achieved as much as 85% inhibition in some patient sera, at a concentration of 50 µg/ml of inhibitor.

The inability of Bermuda extract to inhibit IgE binding of some patient sera on immunoblots¹⁰ suggests the exis-

tence of unique B-cell epitopes in the indigenous Paniceae grasses and *Eragrostis*. Kikuyu pollen extract was found to contain species-specific IgE-binding components of 48 and 70 kDa using Bermuda pollen inhibition on immunoblots.⁴ A monoclonal antibody, Cyn d 1, raised to Bermuda pollen (very kindly supplied by ALK laboratories, Horsholm, Denmark) shows very little recognition of kikuyu and buffalo pollen extracts in ELISA, and there is limited recognition of *Eragrostis* extract (see Fig. 5), confirming the presence of unique epitopes in these indigenous grasses.

Analysis of nucleic and amino acid sequences has confirmed that various pollen extracts share common allergens, including pan-allergens with structural similarities, such as profilin and calcium-binding allergens,^{6,11} from both related and more distant species, and also cross-reacting carbohydrate moieties linked to proteins.¹¹ We have detected carbohydrate moieties in kikuyu, buffalo, *Eragrostis* and Bermuda extracts, using a carbohydrate-labelling kit (Amersham). These may well be similar to the glycoprotein allergens, Cyn d 1 and BG60.^{5,12} The involvement of such components in allergenicity is demonstrated by the decreased IgE affinity in ELISAs and immunoblotting after periodate treatment of the extracts and blots.

The presence of molecules containing a number of domains able to bind calcium was confirmed by the reversible inhibition of IgE binding in ELISAs by 25% in the presence of ethylenediaminetetraacetic acid (EDTA) to kikuyu, buffalo, *Eragrostis* and Bermuda pollen extracts.

Highly cross-reactive pollen-derived calcium-binding allergen has the potential for creating a very effective allergen vaccine, as shown by a study which engineered hypoallergenic variants by disrupting the three-dimensional structure and used them to generate blocking IgG antibodies.¹³

DISCUSSION

Allergenicity of pollens

Epidemiological data indicate that the frequency of sensitisation is higher than pollenosis. Experimental studies have suggested that both environmental and genetic factors influence the induction of an IgE response to an inhaled aeroallergen. Thus in susceptible individuals, if the integrity of the airway epithelial barrier is breached at the time of initial exposure, for example by infection or exposure to environmental pollutants, an IgE response may be stimulated.¹⁴ Enzymes

including proteases in pollens are released in high concentration upon deposition on the upper respiratory mucosal surfaces, which are able to disrupt epithelial integrity and probably thus facilitate access of allergenic protein components to the subepithelial antigen-presenting dendritic cells.¹⁵ Pollutants such as diesel particles and other airborne matter which adsorb pollen particles and starch granules from ruptured pollen act similarly, rendering city-dwellers more susceptible to pollenosis.^{16,17} Further the allergenicity of urban as opposed to rural pollen appears to be greater.¹⁸

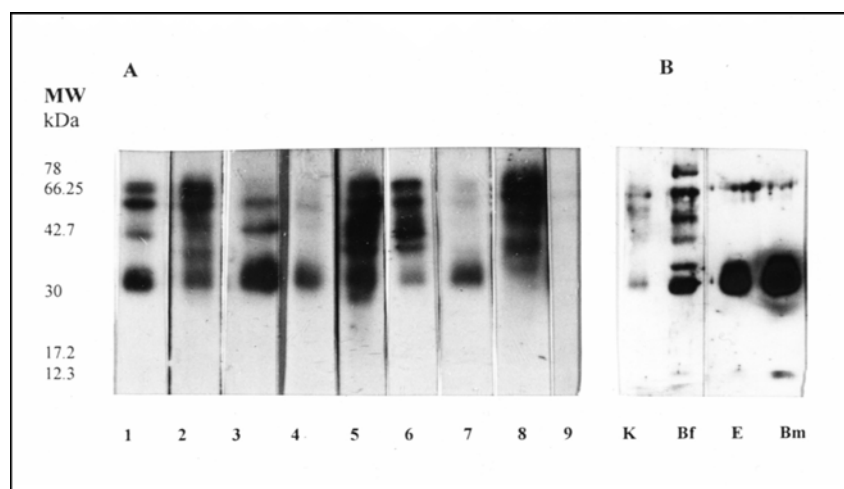


Fig 7.A. Individual sensitivity patterns to buffalo pollen extract on immunoblotting; B. IgE binding of one individual to kikuyu (K), buffalo (Bf), *Eragrostis* (E), Bermuda (Bm).

Interestingly pollen allergy is not seen to any great extent in the rural areas, despite exposure to large concentrations of grass pollen.

The principal allergenic component of most grasses, the Gp 1 allergens, which are glycoprotein isoallergens, are the major elicitor of symptoms in grass-sensitive individuals, and have been extensively characterised. The considerable sequence homology revealed by amino acid and nucleotide sequences has provided a molecular basis for the widely-documented antigenic and allergenic cross-reactivity of IgE responses.⁹ However, the immunological cross-reactivity between *Cynodon* (Bermuda) and Pooideae grass pollen is limited.¹⁰ Antibodies to allergens of *Cynodon* and *Lolium* grass pollens have assisted in identifying the cross-reactive IgE responses between members of the Panicoideae and Chloridoideae subfamilies studied, the common indigenous local buffalo, kikuyu and *Eragrostis* pollens.

It follows that such cross-reactive epitopes may lead to the hypersensitisation of individuals to many tree and grass pollens other than those to which they have been exposed. Cross-reactivity to whole grains and foods is also documented in such subjects.

The number of IgE epitopes on an allergen molecule and their spatial clustering¹⁹ determine its allergenic activity, and, together with the concentration of allergen-specific IgE antibodies, have been demonstrated to determine the extent of effector cell degranulation.¹⁴ *Eragrostis* pollen may contain a more potent allergen and thus be the major sensitiser, rendering it a candidate for a desensitising vaccine suitable for the Panicoid and Chloridoid species of local grasses.

These observations have diagnostic and therapeutic implications, which have been addressed in this study of the local related indigenous grass pollens. The very individual IgE-binding profiles and absence of total inhibition of IgE binding point to the presence of unique B-cell epitopes in both the Panicoid grasses and *Eragrostis*, a Chloridoid in this region. The inability of Bermuda extract to abrogate IgE binding in a subset of grass-sensitive subjects confirms this observation, and emphasises the need to include the local grass pollens in our SPT panels.

CONCLUSIONS

In addition to Bermuda grass, the indigenous *Eragrostis*, kikuyu and buffalo grass pollens are clearly major aeroallergens in this region, showing IgE reactivity in >90% of patients. The IgE-binding profiles of the four grass pollens demonstrate different profiles of responses of grass-sensitive patients. A major Group 1 allergen, of 32-35 kDa, binds a high proportion of IgE, but unique B-cell epitopes present in the indigenous grasses have been revealed by inhibition of ELISA and immunoblotting.

The local indigenous grass pollens contain allergens that have B- and T-cell epitopes that cross-react both with each other and with northern hemisphere grass pollens.

Considering its widespread distribution in Southern Africa and abundant year-long pollen production, *Eragrostis* may well be the most important sensitising

grass, responsible for the long period of seasonal allergy noted in many subjects. It is a logical candidate for a regional grass pollen vaccine. Furthermore, allergen diagnostic screening *in vitro* and *in vivo* panels should include the local indigenous grasses whenever possible, when investigating allergic patients in Southern Africa.

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