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 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 Panicoideae 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.
most abundant pollens with a high allergenic potential. It was found that 80% of the patients sensitive to Buffalo have concordant sensitivity to rye and also to timothy grass pollen CAP-RASTs.

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.

**CHARACTERISATION OF ALLERGENS**

Serum was collected from 35 volunteers with allergic rhinitis, confirmed by positive SPTs to Bayer grass mix (M5), consisting of extracts of rye, timothy, orchard, red top, J johnson and Bahia pollens, and to Bayer Bermuda pollen, although 4/32 subjects were not sensitive to Bermuda pollen on SPT and radioallergosorbent test (RAST). A pool of serum from 8 subjects was used for inhibition studies. One hundred sera sent for CAP-RAST over a period of 3 years were also screened for IgE to buffalo, kikuyu and Eragrostis pollens. Sera from asymptomatic individuals with negative SPTs and CAP-RASTs for grasses were used as controls for immunoblotting.

Pollen from buffalo, kikuyu and Eragrostis, Lolium and Bermuda species was collected under controlled conditions, dried and stored at -80°C. Extraction of allergens was in an aqueous 0.125M NH₄HCO₃, pH 8.3 buffer, with added protease inhibitors, overnight at 4°C, and the extract was centrifuged at 10 000 g for 30 minutes. The protein content was measured.

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.
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 sensitisers 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. 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. Kikuyu extract exhibited a very similar pattern to that of buffalo. The main IgE-binding allergen of *Eragrostis* has a 30 kDa MW, similar to that of Bermuda pollen, Cyn d 1. 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 pl 7-9, of buffalo, *Eragrostis* and kikuyu pollen extracts, which may account for their heterogeneous immunological and biochemical properties.

**CROSS-REACTIVITY**

Most grass pollens have a high degree of cross-reactivity, as many IgE-binding B cell epitopes are common to most species. 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. 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...
also with the Pooid grass species, Cynodon, Lolium, Lagurus and Phleum, and with indigenous fynbos grasses such as Tribolium (Arundinaceae) 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 pollen extracts 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 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.

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 immunoblots suggests the existence 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. 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, from both related and more distant species, and also cross-reacting carbohydrate moieties linked to proteins. 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. 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.

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. Enzymes including proteases in pollen 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. 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. Further the allergenicity of urban as opposed to rural pollen appears to be greater.

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. However, the immunological cross-reactivity between Cynodon (Bermuda) and Pooidae grass pollen is limited. 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 clustering determines its allergenic activity, and, together with the concentration of allergen-specific IgE antibodies, have been demonstrated to determine the extent of effector cell degranulation. 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 Panicoideae and Chloridoideae 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 Chloridoide 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.

REFERENCES


Current Allergy & Clinical Immunology, November 2007 Vol 20, No. 4 193