ALLERGENICITY AND CROSS-REACTIVITY OF BUFFALO GRASS (*STENOTAPHRUM SECUNDATUM*)

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Background. 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 immunoglobulin E (IgE) epitopes of Bermuda grass extracts are known to be distinct from those of the Pooid pollen extracts. Following our demonstration of sensitivity in 43% of patients grass-allergic to the Panicoid, Kikuyu grass, we have studied the closely related buffalo grass, *Stenotaphrum secundatum*, indigenous to the Western Cape region, the east coast of Africa and the oceanic islands such as Mauritius; and *Eragrostis*, another common indigenous grass with a wide distribution.

Objective. To partially characterise the allergens of buffalo pollen, and examine its immunological relationships with local common grasses such as *Eragrostis* and Kikuyu.

Methods. Grass-allergic patients were evaluated clinically, and skin prick tests (SPTs) and radio-allergosorbent tests (RASTs) to Bermuda and grass mix were performed. Sera of timothy grass-sensitive patients from Belgium were also included in this study. Pollen extract from buffalo grass was characterised by specific IgE binding by means of immunoblotting and enzyme-linked immunosorbent assay (ELISA). Cross-reactivity between the grasses was studied by means of inhibition of IgE binding.

Results. More than 90% of grass-sensitive patients were found to have IgE antibodies to Buffalo and *Eragrostis* pollen. Inhibition of ELISA and immunoblots revealed that extracts of these grass pollens could significant inhibit IgE binding to the local grass pollens, Kikuyu, buffalo, *Eragrostis* and Bermuda on solid phase, but 100% inhibition was never achieved, indicating that cross-reactive but also unique epitopes are present. We also identified a subset of patients with negative RASTs to Bermuda, and minimal inhibition by Bermuda pollen extract.

Conclusion. Buffalo and *Eragrostis* are important aero-allergens in the Cape, dispersed during the long dry, windy summer. Our data suggest that the local grasses are major sensitisers, and that South African diagnostic panels should include extracts of buffalo and *Eragrostis* grasses.

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The prevalence of allergic rhinitis and asthma in urban areas is increasing worldwide. Susceptibility in children is associated with atopy, and is characterised by increased immunoglobulin E (IgE) production in response to common allergens. Aero-allergens are prime initiators of an immunological response that culminates in airway inflammation. Grassland and savannah constitute important biomes in southern Africa, and a long summer period, high temperatures and wind are major factors contributing to the production of large amounts of anemophilous grass pollen for most of the year. Grass pollen was reported by David Ordman[1] to be the major aero-allergen responsible for upper airway allergic disease in South Africa.

In southern Africa there are 967 species of the grass family *Pooaceae*. Of these, 847 species are indigenous, and 115 are naturalised. The related subfamilies of the Panicoideae and Chloridoideae have a wide distribution in South Africa, comprising 566 species, compared with the Pooideae with 133.[2] Panicoids comprise many of the grasses cultivated for lawns, such as *Pennisetum clandestinum* (Kikuyu), *Cynodon dactylon* (Bermuda), and *Stenotaphrum secundatum* (buffalo); for pastures (*Digitaria erianthe*); garden ornamentals (e.g. *Pennisetum villosum*); and for erosion control, *Pennisetum* and *Eragrostis*. *Lolium perenne* (rye grass), of the subfamily Pooideae, has been introduced and is widespread in the Cape Peninsula, where it has adapted to the Mediterranean climate.

Both urban and rural populations are exposed to these pollens for the greater part of the year, although interestingly very little pollen allergy is seen in rural areas. The importance of Bermuda grass in this region was first reported by Orren and Dowdle.[3] A study by Potter *et al.*[4] showed that 43% of patients had a clinical sensitivity to Kikuyu grass (*Pennisetum clandestinum*), a Panicoid naturalised from Kenya. In the present study we have examined the allergen profile of the local indigenous grass, buffalo grass (*Stenotaphrum secundatum*), of the same tribe, namely Paniceae. This is a hardy, drought-resistant grass found down the east coast of Africa, as well as on Mauritius and other islands. It grows extensively over the Cape Peninsula and is commonly used for lawns in this region. The flowering period begins in late October and continues through February. It is believed to play a major role in seasonal rhinitis in late spring and summer. Also included in the study is a related and common grass, *Eragrostis curvula*, known as the weeping love grass, which is of the Chloridoideae subfamily, tribe *Eragrosteae*, and is related to Bermuda grass (*Cynodon dactylon*) (Fig. 1). It has a wide distribution throughout South Africa, readily colonising wasteland and verges. It has been introduced as a pasture grass throughout the tropics and East Africa. It forms large perennial tufts, and has an exceptionally long flowering period, starting to produce pollen in August, peaking after the rainy season, and continuing through the summer into the winter months of June and July.

Bermuda grass (*Cynodon dactylon*, subfamily Chloridoideae), an important source of allergens in subtropical climates, has been shown to have limited immunological cross-reactivity with the clinically significant grasses of the Pooideae,[5-7] and therefore separate diagnostic tests and immunotherapy extracts are required for allergic patients. It is important to establish which diagnostic testing panels are appropriate for the taxonomically related and clinically unrelated indigenous grasses of this region in order to prepare the most appropriate immunotherapy extract. In this communication we report the results of our studies on the prevalence of specific IgE responses to buffalo grass pollen in allergic individuals, and our evaluation of the presence of epitopes that are cross-reactive between this grass and other common indigenous grasses of southern Africa.

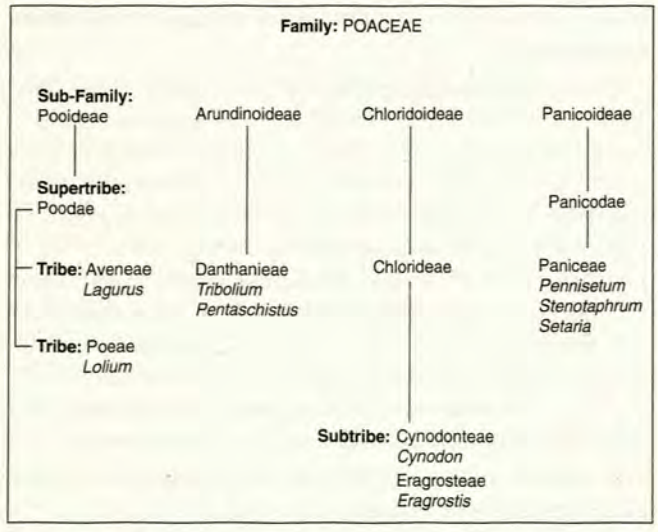

Fig. 1. Classification of grass species investigated.

METHODS

Sera

There were four patient groups: (*i*) group 1 — sera from 100 allergic patients, children and adults confirmed in the laboratory to have positive CAP radio-allergosorbent tests (RASTs) to the Pharmacia grass mix gx2 and/or specifically to Pharmacia g2, *Cynodon dactylon* (Bermuda grass); (*ii*) group 2 — a cohort of 32 adult volunteers from the Cape Town area who had presented with seasonal allergic rhinitis and/or conjunctivitis with confirmed sensitivity to Bermuda grass using CAP RAST, and who had not undergone previous immunotherapy; (*iii*) group 3 — a cohort of 14 laboratory staff with no clinical history of allergic disease, with negative CAP RAST and skin prick tests to Bermuda grass pollen; and (*iv*) group 4 — 5 cord blood sera and sera from 8 non-atopic individuals, confirmed by skin prick tests (SPTs) and CAP RASTs, were used as controls for immunoblotting.

RASTs and SPTs

SPTs were performed in groups 2 and 4 using the Bayer Bermuda grass and Bayer grass mix (M5), consisting of extracts of rye, timothy, orchard, red top, Johnson and Bahia pollens. RASTs for Bermuda, rye and timothy grasses, and gx2 grass mix were performed on the UniCAP system for groups 2 and 4. Grass mix gx2 consists of Bermuda (*Cynodon dactylon*), rye (*Lolium perrenne*), timothy (*Phleum pratense*), Kentucky blue (*Poa pratensis*), Johnson (*Sorghum halepense*) and Bahia (*Paspalum notatum*) grasses.

Preparation of grass pollen proteins

Pollens were stored as dry material at –80°C and extracted in a 1:10 (weight/volume) dilution in 100 mM ammonium bicarbonate buffer, pH 8.3, plus 1 mM phenyl methyl sulphonyl fluoride (PMSF), 0.015M sodium azide (NaN_3), by rotation overnight at 4°C, centrifuged at 12 000 *g* for 30 minutes, and the supernatant recovered. Protein concentrations were determined using a Pierce BCA-protein assay kit with bovine serum albumin (BSA) as the protein standard.

Preparation of in-house antibodies

Polyclonal rabbit anti-buffalo and anti-*Eragrostis* antibodies were raised by injecting crude pollen extracts in complete Freunds adjuvant, boosting after 4 and 8 weeks, and screening for antibody by enzyme-linked immunosorbent assay (ELISA).

Monoclonal antibodies to buffalo pollen were generated by the method of Kohler and Milstein,[8] using myeloma cells. Fifty µg of pollen extract in complete Freunds adjuvant were injected intraperitoneally into male Balb/c mice, with boosting at 2-week intervals in incomplete Freunds adjuvant, and a final booster after 1 week. The spleen was removed and the fusion performed with SP2 myeloma cells.

Characterisation of natural grass pollen extracts by immunoblotting

Ten µg/lane of each pollen extract were loaded onto 12% weight/volume polyacrylamide gels, in sodium dodecyl sulphate (SDS) sample buffer, under reducing conditions and after boiling for 90 seconds, and run for 3 hours. The separated proteins were electroblotted onto polyvinyl difluoride (PVDF) membrane (Amersham International, Buckinghamshire, England) in a semi-dry system at 200 mA for 1 hour. The membranes were probed with patient serum, and the bound IgE antibodies detected using the enhanced chemiluminesence (ECL) technique.

Two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed by iso-electric focusing in the first dimension, using a pH gradient of 3 - 10. This tube was then run horizontally on a 12% SDS gel, and electroblotted onto Hybond-PVDF membrane (Amersham). Immunoblotting with a pool of positive serum was used to indicate the allergenic isoforms.

ELISA and ELISA inhibition

Pollen extracts were coated onto microtitre plates (Nunc Polysorp, Denmark) overnight. Plates were blocked with 2% weight/volume bovine serum albumin (BSA) in phosphate-buffered saline, 0.1% Tween 20 buffer (PBS-T); 100 µl/well of patient serum, diluted 1:10 in 0.5% BSA blocking buffer, was added for 2 hours at room temperature. Bound IgE antibodies were detected by adding a monoclonal anti-human IgE, followed by alkaline phosphatase-labelled rabbit anti-mouse antibody. The colour reaction was developed by the enzyme substrate p-nitrophenyl phosphate.

Shared epitopes were identified by inhibition of IgE binding. Pre-absorption of serum from a grass-sensitive individual with an appropriately diluted pollen extract, overnight at 4°C before incubation in the natural extract-coated plates or immunoblots, removed cross-reacting IgE antibodies to shared allergens.

Identification of common epitopes

Monoclonal antibodies to Bermuda grass (*Cyn d 1*) and rye grass (*Lol p 1*, clones 2 and 14) were kindly supplied by ALK-Abello (Denmark), and were used to investigate the presence of these epitopes in indigenous grasses by ELISA, using plates coated with extracts of buffalo, Kikuyu, *Eragrostis* and Bermuda pollens. Inhibition of binding by pre-absorption of the monoclonal antibodies with local pollen extracts was used to demonstrate the presence of cross-reactive and unique epitopes.

Basophil histamine release

To confirm the allergenicity of buffalo and *Eragrostis* pollens in the *in vivo* situation, fresh blood basophils from confirmed grass-sensitive patients with IgE antibodies and non-allergic control subjects were subjected to incubation with different concentrations of pollen extracts in pipes/calcium buffer for 60 minutes at 37°C. The histamine released was measured by radio-immunoassay (Pharmacia).

Lymphocyte proliferation of peripheral blood mononuclear cells of grass-sensitive patients and non-atopic controls was performed, using pollen extracts of buffalo and *Eragrostis*.

RESULTS

Characterisation of pollen allergens

SDS-PAGE and immunoblotting of buffalo pollen extract revealed a spectrum of IgE-binding bands, ranging from 10 to 90 kilodaltons (kD), of which the dominant one was a 34 kD MW band. Other IgE-reactive bands on SDS-PAGE were found at MWs 11, 14, 39, 46, 52, 60 and 68 kD (Fig. 2). Two-

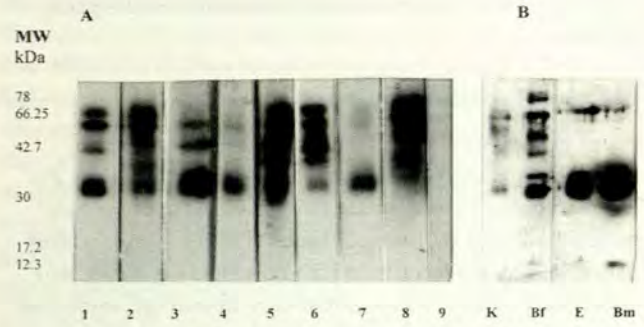

Fig. 2. A: Profiles of IgE binding to blotted buffalo extracts. Lanes 1 - 8 represent different patients and lane 9 is a non-allergic control, detected by autoradiography. B: Analysis of IgE binding to transferred Kikuyu (K) (lane 1), buffalo (Bf) (lane 2), Eragrostis *(E) (lane 3) and Bermuda (Bm) (lane 4) pollen extracts, detected by enhanced chemiluminesence.*

dimensional PAGE was used to resolve the major 34 kD band of buffalo grass into its component isoforms, each with a different iso-electric point. About 9 - 10 isoforms in the pH range 4 - 7, of the major 34 kD group 1 allergen were revealed by immunoblotting using a pool of positive sera. The basic group V allergen, found in Pooid but not Bermuda pollens, was not present. Isoforms were also found in the 14 kD, 44 kD and 57 kD bands.

Distribution of specific IgE antibodies to buffalo grass pollen

In group 1, immunoscreening on Western blots revealed IgE antibodies to buffalo pollen extracts in > 95% of the patients in this group. Further screening showed that more than 90% had concordant sensitivity to *Eragrostis* pollen extract.

In group 2, IgE sensitivity to buffalo pollen extract was found in all but 1 of the cohort of 32 volunteers with seasonal symptoms, and in 30:32 with sensitivity to *Eragrostis*.

In group 3, 12:14 of this clinically asymptomatic group with negative Bermuda grass SPTs surprisingly showed IgE binding on immunoblots of buffalo and *Eragrostis* pollen extracts. Subsequently 6:14 reacted to the Bayer 5 grass mix SPT extract, with a wheal diameter of 3 - 4 mm, and had a positive gx2 CAP RAST.

Negative controls showed negligible IgE binding.

Cross-reactivity

A high degree of concordance of patient IgE binding between the two Paniceae grasses, Kikuyu and buffalo, and the two Chloridoidae grasses, *Eragrostis* and Bermuda, was evident in ELISA inhibition experiments. All four extracts were able to produce inhibition, in all the combinations, as solid phase or as inhibitor, although greater than 75% inhibition was not achieved. Equivalent IgE inhibition by *Eragrostis* and Bermuda at the lower concentrations confirms the presence of cross-

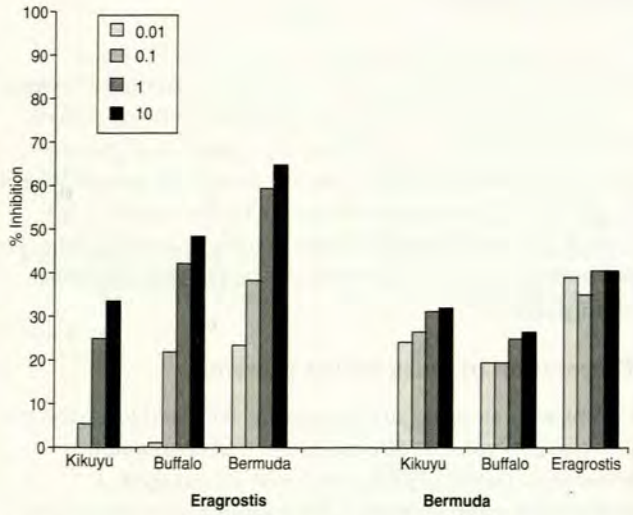

Fig. 3. Comparison of IgE inhibition of Eragrostis *pollen extracts, using a pool of Bermuda-positive serum. Concentrations of inhibitor ranged from 0.001 to 10 µg/ml.*

reactive epitopes (Fig. 3), but Bermuda did not inhibit all the epitopes on *Eragrostis*.

We also identified a subset of patients who were Bermuda-negative, but whose IgE bound strongly to *Eragrostis*, buffalo and Kikuyu. Binding to these indigenous grasses was not inhibited by pre-absorption with Bermuda pollen extract, confirming that these members of the Panicoideae have unique IgE-binding epitopes not found on Bermuda.

Inhibition of the highly specific Bermuda CAP RAST by buffalo and *Eragrostis* extracts, using a pool of Bermuda-reactive sera, is shown in Fig. 4.

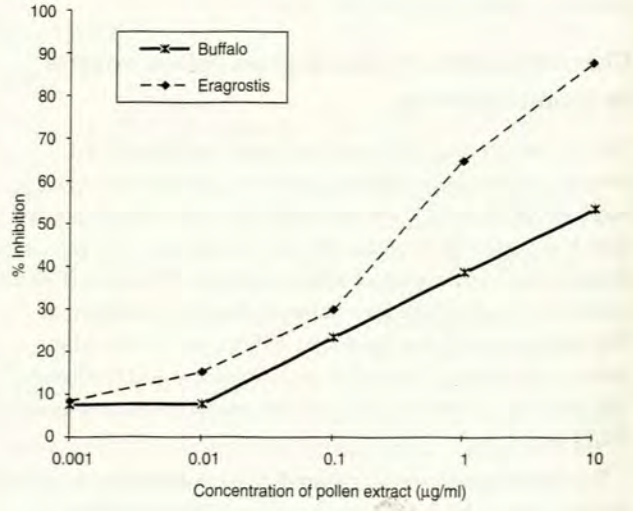

Fig. 4. Inhibition curves of Bermuda CAP RAST by buffalo and Eragrostis *pollen extracts, using a pool of Bermuda-positive serum. Concentrations of inhibitor ranged from 0.001 to 10 µg/ml.*

Attenuation of IgE binding by all four pollen extracts was obtained in immunoblot inhibition experiments, in particular to the major 34 kD allergen. Buffalo was able to demonstrate effective inhibition of binding to all the grasses on immunoblots in most sera. When *Eragrostis* was used as the inhibitor, abrogation of IgE binding was shown to all the blotted extracts in many grass-sensitive sera. In a subset of sera, however, Bermuda was not able to inhibit the major buffalo allergen, even at a concentration of 100 µg/ml (Fig. 5).

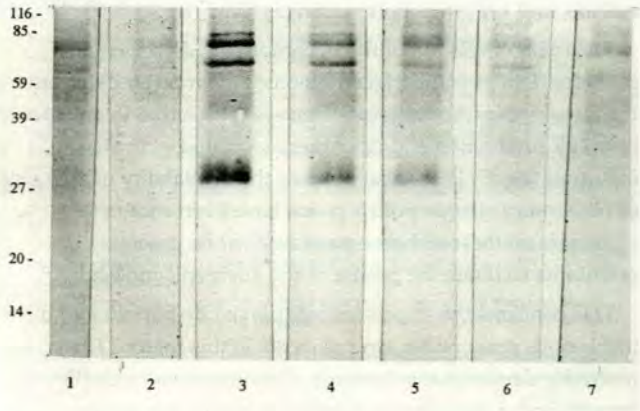

Fig. 5. Inhibition of immunoblot of buffalo allergen with 5 and 50 µg/ml of Bermuda and Kikuyu pollen extracts. Lanes 1 and 2 are cord and non-allergic control sera; lane 3 shows serum binding in the absence of inhibitor; lanes 4 and 5, in the presence of 5/100 µg/ml Bermuda as inhibitor; lanes 6 and 7 with 5/100 µg/ml Kikuyu as inhibitor.

Identification of epitopes common to buffalo and Bermuda

Cross-reactivity was further detected by testing the ability of the whole buffalo extract to inhibit the monoclonal *Cyn d 1* antibody binding to the local grass extracts. The anti-*Cyn d 1* Mab showed some binding to extracts of buffalo and Kikuyu grass (Fig. 6), which was not inhibited by pre-absorption with buffalo extract up to concentrations of 10 µg/ml. Also shown is the binding of the anti-*Lol p 1* (clone 2) monoclonal antibody to the four extracts, with reduced values for buffalo and Kikuyu, while the anti-*Lol p 1* (clone 14) reacted to Kikuyu and buffalo antigens with greater affinity than to *Eragrostis* or Bermuda-coated allergen.

Basophil histamine release

Basophils of grass-sensitive individuals, when incubated with different concentrations of pollen extract, demonstrated a specific and dose-dependent histamine release to pollen extracts of Buffalo, Kikuyu and *Eragrostis* grasses (Fig. 7).

Sensitisation to the local grasses was confirmed by the dose-dependent proliferation of the T-cells of grass-allergic individuals when stimulated by pollen extracts of buffalo and

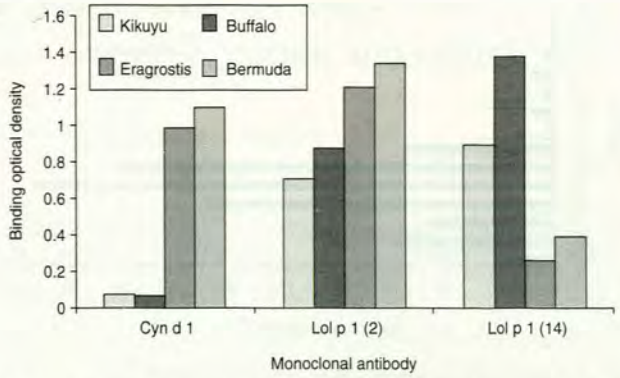

Fig. 6. Cross-reactivity of monoclonal antibodies, α-Cyn d 1 and α-Lol p 1, clones 2, 14 to four local pollen extracts as solid phase, shown by ELISA.

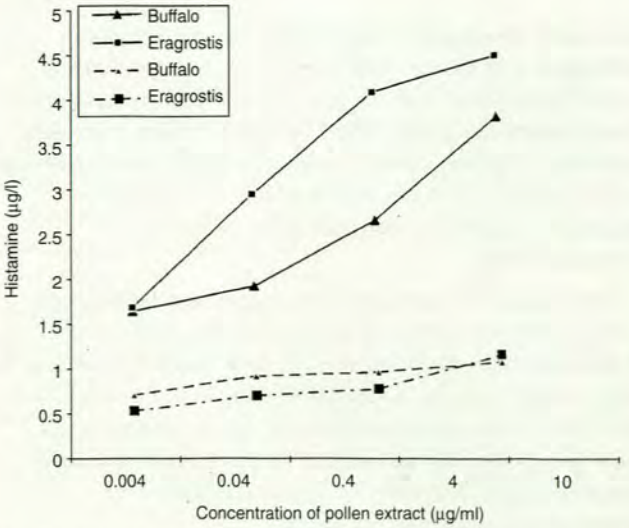

Fig. 7. Histamine release by basophils of one patient when incubated with pollen extracts of buffalo and Eragrostis *grasses; dose response curve, compared with release by a non-allergic control serum.*

Eragrostis, in short-term culture, compared with that of non-allergic controls (Fig. 8).

Discussion

Increasing urbanisation has resulted in a concomitant increase in allergic diseases in southern Africa. Quality of life may be severely impaired in allergic rhinitics and asthmatics. The effective diagnosis of the causes of upper airway disease is cost-effective in the context of health care delivery. With this in mind, we have identified major sensitising allergens important for the development of appropriate allergen diagnostic panels and effective immunotherapy in the Cape.

Our study has shown that 95% of grass-sensitive individuals have IgE antibodies to buffalo pollen extracts on immunoblots. These patients have a concordant IgE response to *Eragrostis*,

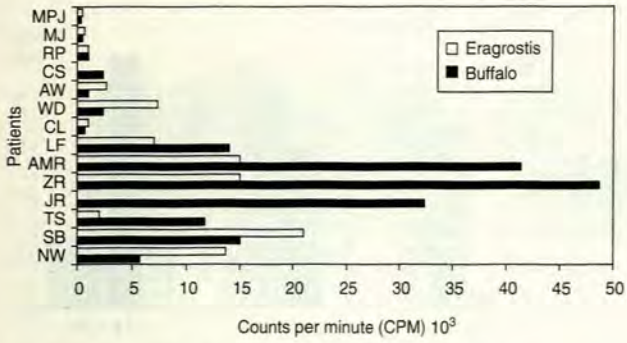

Fig. 8. T-cell proliferation to pollen extracts of buffalo and Eragrostis *grasses. Values shown are mean cpm of triplicate wells as determined by ^{3}H-Th incorporation. The top 7 individuals are asymptomatic, SPT +ve, while the rest are grass-sensitive individuals.*

and many also react to Kikuyu grass. The major buffalo allergen is a 34 kD periodate-sensitive glycoprotein, pI 5-7, which is consistent with the principal allergenic component of most grasses, the group 1 (Gp 1) allergen.[9-12] None of the sera exhibited a binding pattern unique to a single species, although 4:32 volunteers, with negative Bermuda SPTs, exhibited negligible binding to a Bermuda grass extract on immunoblotting.

Cross-reactivity among the four species studied has been clearly demonstrated by inhibition of ELISA, RAST, and immunoblotting. *Eragrostis* was able to abrogate IgE binding by most allergic sera, and to abolish binding in some patient sera. It is therefore an important allergenic grass, considering its year-long pollination period and widespread distribution in southern Africa. *Eragrostis* is a logical candidate for a desensitising vaccine for the region.

It was interesting to find that Belgian grass-sensitised individuals had cross-reactive IgE that binds to components on immunoblots of the African indigenous buffalo, Kikuyu and *Eragrostis* grasses. These individuals had not been exposed to the subtropical grasses. It has been found that 80% of the South African patients sensitive to buffalo have concordant sensitivity to rye. Local subjects also have specific IgE to timothy grass pollen in CAP-RASTs, confirming the presence of cross-reactive epitopes since timothy does not occur locally.

Recent reports have implicated cross-reactive sugar molecules in IgE binding,[13,14] as first reported for the major allergen of Bermuda grass, *Cynd1*,[10] and the 60 kD allergen, BG 60,[15] of Bermuda grass. We have demonstrated carbohydrate moieties in Kikuyu, buffalo and *Eragrostis* extracts, and periodate treatment of these local pollen extracts resulted in reduced patient IgE binding, as well as the monoclonal antibody, anti-*Lol p 1* (2) in ELISAs. The carbohydrate moiety of B-cell epitopes may conceivably contribute to the cross-reactivity between these Panicoid and the unrelated Pooideae grasses.

Interspecies allergens have been proposed to account for the cross-reactivity between unrelated fruits and vegetables,[16-18] such as the ubiquitous panallergen profilin, an actin-binding protein, which plays an important role in pollen germination and tube growth, which is recognised by 20% of pollen-sensitive patients. Reactivity to the 14 kD protein in buffalo and *Eragrostis* extracts has been shown by many of our allergic sera. The presence of interspecific calcium-binding allergens was also demonstrated by inhibition of patient IgE binding in the presence of ethylenediamine-tetraacetic acid (EDTA) to Kikuyu, buffalo, and *Eragrostis* pollen extracts (data not shown).

The rising levels of pollution, comprising diesel exhaust particles (DEPs), industrial emissions and wood smoke from the burgeoning informal settlements, are reported to enhance both IgE production and cytokines associated with airway inflammation.[19,20] DEPs also increase the availability of allergens of respiratory size, as pollen grains have been shown to aggregate on these airborne particles,[21] while gaseous pollutants facilitate the release of the allergenic molecules.[22]

This confirms the important role played by buffalo and other indigenous grass pollens in pollinosis in this region. The cross-reactivity demonstrated between all four grasses underlies the importance of the dominant Panicoid family as a pollen sensitiser in southern Africa. Current testing panels and immunotherapy vaccines for this region are deficient in representatives from these important grass pollen families. Diagnostic and therapeutic strategies should take this into consideration. We propose that buffalo, Kikuyu and *Eragrostis* be included in the SPT panels, and further studies are underway to develop appropriate desensitising vaccines for the region.

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