

Studies on Bermuda Grass Pollen Allergens

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SUMMARY

Two allergens, BGP-1 and BGP-2, were identified in crude Bermuda grass pollen extract. BGP-1 was a major allergen and elicited a skin reaction in all subjects sensitive to the crude extract, whereas BGP-2 was a minor allergen and elicited a reaction in only 75% of such subjects.

Calibrated column chromatography was used to determine the molecular weights and Stokes radii of the allergens. For BGP-1 they were 30 000 daltons and 24.3×10^{-7} cm respectively and for BGP-2 they were 14 000 daltons and 18.1×10^{-7} cm respectively. The sedimentation coefficient ($S_{20,w}$) of BGP-1 on sucrose density gradient ultracentrifugation was 3.1 and on iso-electric focusing its iso-electric point was found to be pH 5.4.

The reason why only certain individuals sensitive to BGP-1 are sensitive to BGP-2 is discussed. It is possible that the former is a dimer of the latter.

S. Afr. med. J., 51, 586 (1977).

Bermuda grass (*Cynodon dactylon*; fine kweek) is commonly encountered in South Africa and other parts of the world and is extensively cultivated as domestic lawn and pasture. In a recent survey of 268 randomly selected blood donors, we found that 16% showed immediate cutaneous hypersensitivity to an aqueous extract of Bermuda grass pollen,¹ indicating that this grass is an important environmental source of allergenic material.

Little is known of the immunochemistry of the allergens found in this pollen. Marsh *et al.*² showed that there is little antigenic cross-reaction between Bermuda grass antigens and other grass antigens and Meyers *et al.*^{3,4} have reported a 'hapten-like' dialysable component in Bermuda grass pollen extract.

In this article we present the results of experiments designed to define some of the molecular characteristics of those compounds present in Bermuda grass pollen that elicit immediate cutaneous hypersensitivity reactions in sensitive human subjects. To avoid semantic confusion we refer to these substances as 'allergens' without presuming or implying that they are able to act as primary immunogens.

MATERIALS AND METHODS

Skin Testing for Allergenic Activity

Allergenic potencies of solutions of raw or fractionated Bermuda grass pollen extract were tested by the conven-

tional prick test method in 30 subjects sensitive to Bermuda grass. These subjects all had seasonal symptoms characteristic of inhalant pollen allergy and gave informed consent to the test procedure.

Discrete drops of test solutions were placed on the skin of the anterior surface of the forearm and a minute amount was inoculated into the superficial layers of the epidermis by pricking through the drop with a sharp needle. Results were read at the time of maximum response, that is approximately 20 minutes after challenge, and were graded on the basis of the diameter of the resultant wheal or flare (whichever was greatest) (Table I).

TABLE I. GRADING OF RESPONSE TO ALLERGEN

	Wheal	Flare
0	< 3 mm	< 5 mm
+	≥ 3 mm, < 5 mm	≥ 5 mm, < 10 mm
++	≥ 5 mm, < 8 mm	≥ 10 mm, < 20 mm
+++	≥ 8 mm, < 10 mm	≥ 20 mm, < 26 mm
++++	≥ 10 mm	≥ 26 mm

Bermuda Grass Pollen Extract

A '6%' crude extract of Bermuda grass pollen containing 0.45% NaCl, 50% glycerol and 0.5% phenol was a generous gift from the Bencard Allergy Unit, Worthing, England. The '6%' refers to the weight of pollen originally used to prepare the extract. Pollen extracts lose biological activity unless glycerol or some other preservative is present. We found that purified fractions could be stabilized for storage at -20°C by the addition of sucrose to a final concentration of 20%. Preservatives were removed from the pollen extracts by dialysis. Preliminary experiments showed that the allergenic material present in the extract was retained during dialysis.

One millilitre of 6% extract yielded approximately 50 μg protein containing the major allergenic factors after dialysis, Sephadex G100 and diethylamino-ethyl (DEAE) cellulose chromatography. As the extract was available in limited quantities this low yield limited the number of studies that could be performed. All pollen antigens studied have been shown to consist mainly of protein.⁵⁻⁷ We accordingly used the techniques of protein biochemistry for the isolation of Bermuda grass pollen allergens.

Column Chromatography

Sephadex G100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and DEAE cellulose (Whatman's, Kent, England) were prepared according to the manufacturers' recommendations. All columns and test tubes were siliconized to prevent loss of allergens through adsorption to glass. Protein concentrations of pooled fractions were measured by the method of Lowry *et al.*⁸ Details of individual experiments are given in the 'Results' section.

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Date received: 16 November 1976.

Sucrose Density Gradient Ultracentrifugation

Sucrose density gradients from 5% to 20% in 0.05M pH 7.5 sodium phosphate buffer were prepared in 4.4-ml cellulose nitrate tubes (Beckman Instrument Co., Palo Alto, California) and allowed to stand at 4°C for 2 hours before the samples dissolved in 150 μ l of gradient buffer were layered. The tubes were centrifuged (16 h, 4°C, 145 000 g) in a No. SW 39 rotor in a Beckman Model L centrifuge. The tubes were then punctured and the gradients collected in fractions of 8 drops (approx. 150 μ l) each for skin testing. Protein markers with known sedimentation coefficients ($S_{20,w}$) were centrifuged simultaneously.

The following markers were used: horse heart cytochrome c; horse skeletal muscle myoglobin; human serum albumin (HSA) (all from Miles Seravac, Cape Town); and alcohol dehydrogenase (Sigma Chemical Co., St Louis). The HSA was labelled⁹ with ¹³¹I and its presence was detected by gamma scintillation counting. Alcohol dehydrogenase activity was measured by the rate of increase in optical density at 340 nm of a 1.0-ml reaction mixture containing 170 μ mol ethanol, 50 μ mol Tris-HCl at pH 8.5, 15 μ mol NAD⁺, and 5-20 μ l enzyme solution. The $S_{20,w}$ of the allergenic material was determined by interpolation from the linear relationship between $S_{20,w}$ and distance travelled into the gradient for the marker substances according to the method of Martin and Ames.¹⁰

Iso-electric Focusing

Ampholyte solution (pH range 5-8; LKB Produkter AB, Sweden) was incorporated, at a final concentration of 1%, into a gradient of 0%-50% sucrose in a 110-ml capacity LKB iso-electric focusing column. The pollen extract was dialysed overnight against 1% glycine in water and introduced into the gradient by substitution for an equal volume of light solution. The anode solution was 10 ml of 1% v/v phosphoric acid and the cathode solution was prepared by adding 0.4 ml ethanolamine and 12 g sucrose to 14 ml distilled water. Three hundred volts were applied across the column until the current had stabilized after approximately 48 hours. The column was then drained and 4.5-ml fractions were collected. After pH measurement each fraction was dialysed for 36 hours against 4 changes of 0.005M pH 8 sodium phosphate buffer. Sucrose (final concentration 20%) was added to the fractions which were then used for skin testing.

RESULTS

For each chromatographic run 4 ml of 6% crude pollen extract was concentrated by ultrafiltration to half its original volume and applied to a 1 $\times$ 150-cm Sephadex G100 column, pre-equilibrated and run with 0.005M pH 8 sodium phosphate buffer. The column was eluted by downward flow at 4 ml/h and 2-ml fractions of the eluate were collected for skin testing. As can be seen from the elution profile depicted in Fig. 1, 3 peaks of allergenic activity, eluting at 40 ml, 65 ml and 80 ml, could be detected; these we have designated fraction I, fraction

II and fraction III, respectively. Sixteen subjects sensitive to Bermuda grass pollen were skin tested with the eluate fractions from 5 chromatographic runs. Not all subjects reacted to all 3 peaks. The data summarized in Table II show that all 16 subjects were sensitive to fraction II. The intensity of the responses were such that individual wheal and flare reactions tended to be weaker with fraction I than with fraction II. Fraction I eluted with the approximate void volume of the column and its activity may have been due to aggregate formation. King *et al.*⁶ have reported such aggregation with ragweed antigen E. In 12 out of 16 subjects fraction III gave responses that were as intense as those seen with fraction II, whereas in the other 4 subjects it elicited no reaction whatsoever. This 'all or none' pattern of response with fraction

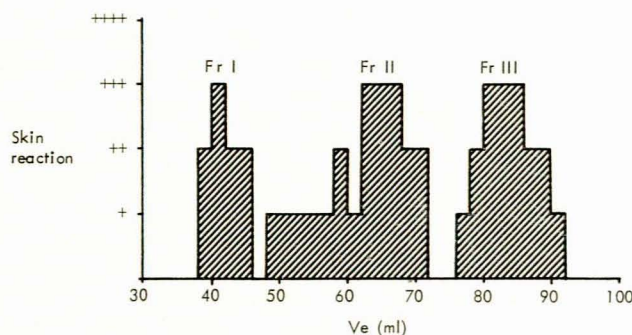


Fig. 1. Elution profile of skin reactivity of samples obtained by Sephadex G100 chromatography of dialysed crude Bermuda grass pollen extract.

TABLE II. SKIN TEST REACTIONS OBTAINED ON TESTING 16 BERMUDA GRASS-SENSITIVE INDIVIDUALS WITH IMMUNOREACTIVE MATERIAL OBTAINED BY SEPHADEX G100 CHROMATOGRAPHY OF DIALYSED BERMUDA GRASS POLLEN EXTRACT

Subject	Skin test reactions*		
	Fraction I	Fraction II	Fraction III
1	+	++++	—
2	—	++++	+
3	+++	++++	++++
4	++	++++	—
5	++	+++++	+++++
6	++++	+++++	+++++
7	++++	+++++	+++++
8	++	++++	+++++
9	+	+++++	+++++
10	++	++++	—
11	++	+++++	++++
12	++	+++++	+++++
13	++++	+++++	+++++
14	++++	+++++	+++++
15	++	++	—
16	+	+++++	+++++
Number positive	15	16	12

* The maximum reaction to any of the three fractions is recorded.

III indicated that it contained a determinant against which certain subjects had formed reaginic antibodies while others had not.

It is convenient to adopt the terminology of Johnson and Marsh,¹¹ who defined a major allergen as one that elicits a skin reaction in all subjects sensitive to the unpurified parent material and a minor allergen as one that gives reactions as intense as those observed with the major allergen, but only in a limited number of sensitive subjects. By these criteria, fraction II contained a major, and fraction III a minor Bermuda grass pollen allergen.

Pooled samples of fraction II from Sephadex G100 chromatography in 0,005M pH 8 sodium phosphate buffer were adsorbed to a 1,5 × 40-cm column of DEAE cellulose that had been equilibrated with the same buffer; a linear gradient 0,005M - 0,2M pH 8,0 sodium phosphate buffer (total volume 200 ml) was applied. Fractions of 5 ml each were collected in siliconized glass tubes. These were used for skin testing in 11 sensitive subjects and were analysed for buffer concentration by sodium measure-

ment on an atomic absorption spectrometer (Perkin Elmer Model 303).

Skin testing of sensitive subjects identified a major allergen that eluted with approximately 0,05M buffer. This we have called fraction IIB. Seven subjects reacted weakly to an allergen that eluted with 0,1M buffer (fraction IIC) and 1 subject reacted to an allergen that eluted with the starting buffer (fraction IIA). These data are summarized in Table III and a typical elution profile is shown in Fig. 2.

Fraction IIB (total protein approximately 50 µg) was concentrated by negative pressure ultrafiltration and re-chromatographed on a calibrated Sephadex G100 column. Fractions were collected as before and were used to skin-test 3 sensitive subjects. In all cases skin testing revealed a peak of allergenic activity that eluted with the elution volume of fraction II. The response of 1 subject, however, indicated a peak of allergenic material with the elution volume of fraction III. Since only fraction II was taken for the initial DEAE cellulose chromatography, the presence of fraction III on re-chromatography of the major allergen from DEAE cellulose indicated that fraction II had dissociated in solution to give rise to a minor allergen with the physical characteristics of fraction III.

TABLE III. SKIN TEST REACTIONS OBTAINED ON TESTING 11 BERMUDA GRASS-SENSITIVE INDIVIDUALS WITH THE ELUATES OF DEAE CELLULOSE CHROMATOGRAPHY OF BERMUDA GRASS FRACTION II

Subject	Skin test reactions*		
	Fraction IIA	Fraction IIB	Fraction IIC
1	—	+++	—
2	—	++++	+++
3	—	++++	++
4	—	+++	+
5	—	+++	++
6	—	+	—
7	—	++	—
8	—	++++	++
9	—	++++	+
10	++	+++	—
11	—	++	++
Number positive	1	11	7

* Reactions recorded are the maximum obtained to any particular fraction.

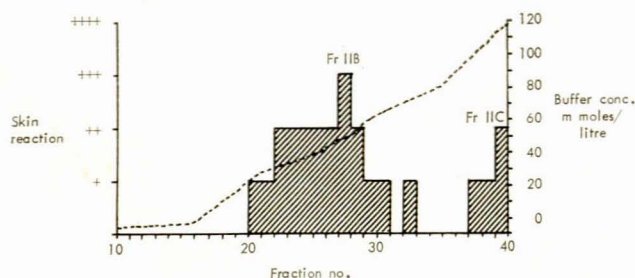


Fig. 2. Elution profiles of skin reactivity and buffer concentration of samples obtained by DEAE cellulose chromatography of fraction II (i.e. the major allergenic peak obtained by Sephadex G100 chromatography of the crude extract) (... = buffer concentration).

Characterization of the Allergens

The Sephadex G100 column was calibrated by determining the elution volumes (V_e) of 3 protein markers (horse heart cytochrome *c*, horse skeletal muscle myoglobin and HSA). Blue dextran (Pharmacia, Uppsala) and tyrosine were used to determine the void volume (V_o) and total column volume (V_t) respectively (Table IV).

The relationships described by Andrews¹² were then used to compute molecular characteristics for the allergens.

Molecular weight. It can be shown empirically that there is a linear relationship between the logarithm of the molecular weight ($\ln MW$) of a macromolecule and its elution volume (V_e), provided the markers and unknown samples elute in the central region of the working range of the column. Since our samples fulfilled these criteria, V_e was plotted for the markers as a function of $\ln MW$ and a least squares regression line was fitted to give the parameters m and c in the equation:

$$MW = e^{\frac{V_e - c}{m}}$$

The data summarized in Table IV and Fig. 3 gave molecular weights of 30 000 and 14 500 daltons for the allergens in fraction IIB and fraction III respectively.

Stokes radii. The distribution coefficient (K_d) for a macromolecular solute is that fraction of the internal volume ($V_i - V_o = V_i$) of the column that is accessible to the solute molecules. Hence one may write:

$$V_e = V_o + K_d V_i$$

$$\text{or } K_d = \frac{(V_e - V_o)}{V_i}$$

Using this equation, K_d could be calculated for the marker proteins and for the allergens (Table IV). Porath¹³ has

TABLE IV. CALIBRATED SEPHADEX G100 CHROMATOGRAPHY

Sample	Molecular weight (daltons)	Stokes radius (cm $\times 10^{-8}$)	Elution volume (V_e) (ml)	Distribution coefficient (K_d)
Blue dextran	$\pm 2 \times 10^6$		36*	—
Tyrosine	< 1 000		110†	—
Cytochrome c	12 400	16,4	90	0,729 7
Myoglobin	17 800	20,7	76	0,540 54
HSA	69 000	35,5	46	0,135 14
Immunoreactive peak				
Fraction II (BGP-1)			66	0,405 4
Fraction IIB re-chromatographed (BGP-1)			66	0,405 4
Fraction III (BGP-2)			84	0,648 7

The table gives the known molecular weights and Stokes radii of the markers and the elution volumes and distribution coefficients of the markers and allergens.

* V_0

† V_t

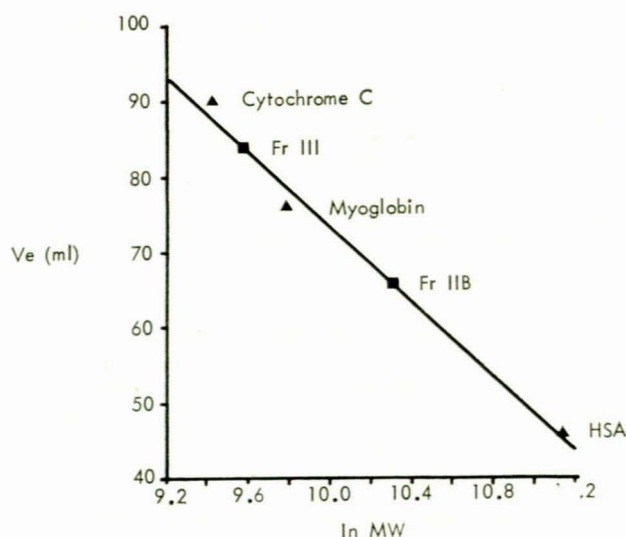


Fig. 3. Calibrated Sephadex G100 chromatography. Values of V_e for the markers are plotted against the natural logarithm of the known molecular weights (Δ = marker points; $\blacksquare$ = antigen points).

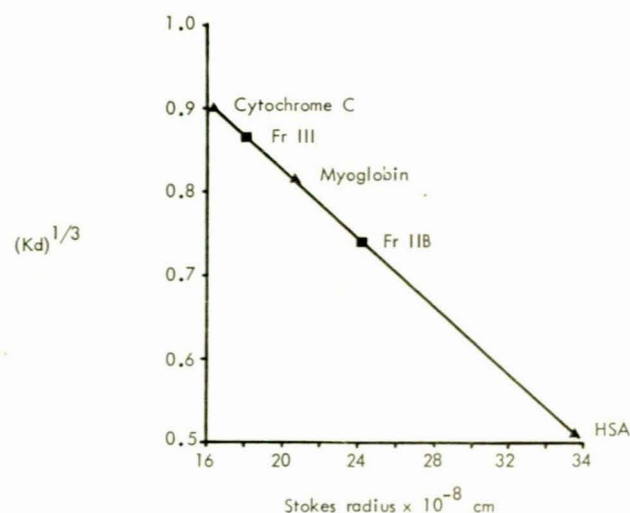


Fig. 4. Calibrated Sephadex G100 chromatography. Values $(K_d)^{1/3}$ for markers are plotted against the known values for their Stokes radii (Δ = marker points; $\blacksquare$ = antigen points).

described the relationship, $(K_d)^{1/3} = n - ma$, where n and m are constants and a is the Stokes radius.

Accordingly, $(K_d)^{1/3}$ was calculated for the protein markers and plotted as a function of their Stokes radii. The parameters of the least squares regression line obtained (Fig. 4) were used to calculate Stokes radii of $24,3 \times 10^{-8}$ cm for the allergens in fraction IIB and $18,1 \times 10^{-8}$ cm for the allergens in fraction III.

Sedimentation coefficients. Velocity sedimentation of fraction IIB and marker proteins on linear sucrose density gradients gave, on 4 separate occasions, $S_{20,w}$ values of 3,2, 3,3, 2,9 and 3,1 for the major allergen in fraction IIB (mean value 3,1). A plot of the results obtained on one experiment is shown in Fig. 5. On one occasion, when a subject sensitive to the minor allergen was used for skin testing, a second allergenic fraction could be detected

in the sucrose gradient that sedimented with a velocity corresponding to an $S_{20,w}$ of 2,2. This provides further evidence for the fact that the major allergen had dissociated in solution to give rise to a smaller minor allergen.

Iso-electric points. Iso-electric focusing of fraction IIB on a pH gradient of 5 - 8 revealed the presence of 3 allergenic components when gradient fractions were used to skin test 2 sensitive subjects. One of the profiles obtained is shown in Fig. 6. The 2 lesser components had iso-electric points of 5,0 and 6,6. Jacobs¹⁴ has recently shown that minor degrees of oxidation of macromolecules may take place during iso-electric focusing, giving rise to artefactual heterogeneity; hence we are unable to comment on the minor components seen. However, the allergens present in the fractions of pH 5,4 are clearly the most abundant or most reactive and therefore we consider pH 5,4 the iso-electric point of the major allergen.

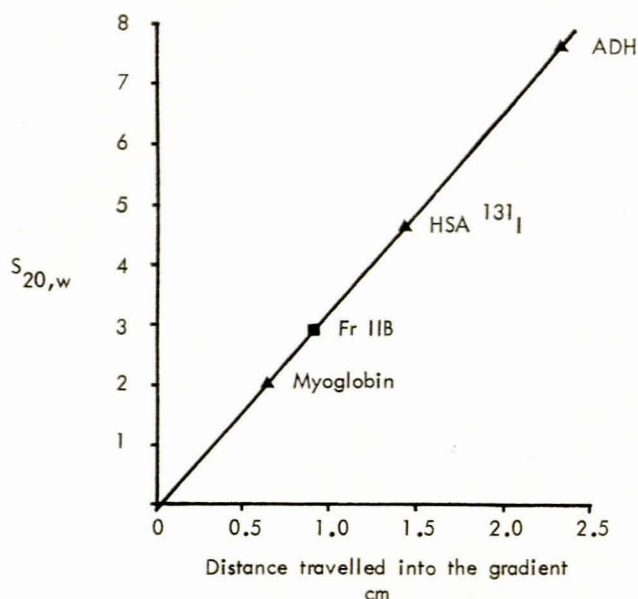


Fig. 5. Sucrose density gradient ultracentrifugation. The sedimentation coefficients ($S_{20,w}$) of the markers were plotted against the distance they travelled into the density gradient ($\blacktriangle$ = marker points; $\blacksquare$ = antigen points).

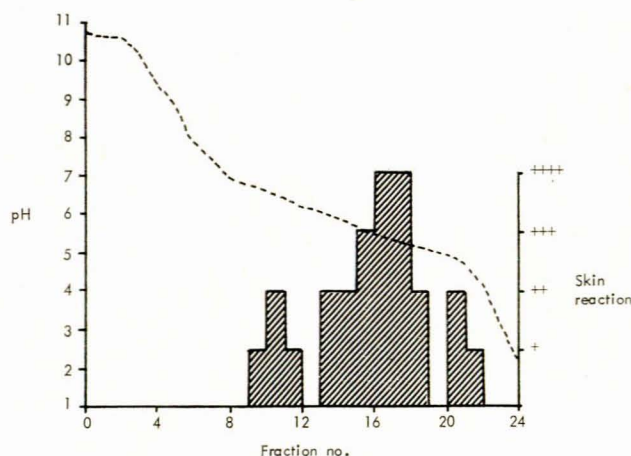


Fig. 6. Elution profiles of skin reactivity and pH of samples obtained after iso-electric focusing in the presence of ampholytes (pH range 5-8) (... = pH).

DISCUSSION

The studies reported here have demonstrated the presence of 2 allergens in the crude extracts of Bermuda grass pollen. In studying any biologically active material it is desirable to have satisfactory assays for quantitating the compound concerned, both in terms of its biological activity and in terms of its chemical mass. In the work reported in this article we were unfortunately unable to achieve this ideal. The prick test is extremely sensitive and in certain reactive individuals is capable of detecting as little as 10^{-8} - 10^{-7} μ g of allergenic material.¹⁵ On the

other hand, as a means of measuring the amount of allergenic material present, it is at best only semiquantitative. Squire¹⁶ demonstrated a linear relationship between the diameter of prick test reactions and the logarithm of the concentration of either (i) histamine or (ii) horse dander protein (in horse-sensitive individuals) used to elicit the reactions. However, he found that the quantitative error of the method was 22% and that the linear relationship did not hold true for wheals greater than 6 mm in diameter. Johnson and Marsh¹⁷ confirmed the linear relationship described by Squire but, owing to the inherent error in the method, used 'quantitative prick testing' only to estimate relative potencies of rye grass pollen solutions. We have followed a similar policy and have not attempted to use the prick test to determine the absolute concentrations of our solutions. Furthermore, the mass of the active components in the material available to us was too small for complete identification or chemical studies.

Fractionation of crude extracts has revealed more than one allergen in all allergenic pollens investigated thus far.^{2,11,15,17,18} The results of our studies show that Bermuda grass pollen is no exception to this apparently general rule. We have shown that Bermuda grass pollen contains a major allergen, to which all sensitive subjects gave a positive cutaneous reaction, and at least one minor allergen to which most sensitive subjects reacted. The former we have called BGP-1 and the latter BGP-2.

Re-chromatography of BGP-1 after purification and storage revealed the presence of allergenic material with the elution volume, not only of BGP-1 but also of BGP-2. This fact, coupled with the observation that the molecular weight of BGP-1 was, within the limits of experimental error, twice that of BGP-2, indicates that the major allergen may simply be a dimeric form of the minor allergen and that spontaneous dissociation of dimer to monomer takes place in solution. Unfortunately we lacked sufficient pure material to undertake the complete structural and chemical analyses that might have provided the conclusive evidence for such a dimer-monomer relationship.

While allergenic pollens from other grasses tend to have antigens which cross-react, the Bermuda grass pollen antigens show antigenic uniqueness.² It is therefore of interest to compare the physicochemical properties of Bermuda grass pollen allergens with those of other grass pollens and ragweed pollen. The data are summarized in Table V, from which it can be seen that pollens from Bermuda grass, rye grass, ragweed, timothy grass and cocksfoot grass all possess major allergens with molecular weights in the region of 30 000 - 40 000 daltons, and sedimentation coefficients in the vicinity of 3 Svedberg units, allowing one to draw the circumstantial inference that molecular size plays an important part in determining allergenicity. It is also worthy of note that apart from ragweed antigen K, the minor allergens are all smaller than the major allergens.

There is no completely satisfactory explanation for the observation that persons who have been sensitized by contact with Bermuda grass pollen include a proportion who are non-reactive to minor determinants present in

TABLE V. SUMMARY OF SOME DATA AVAILABLE ON THE PHYSICAL CHARACTERISTICS AND RELATIVE IMPORTANCE OF SOME POLLEN ALLERGENS

Pollen	Number of allergen groups	Name of allergen (if any)	Molecular weight	S _{20,w}	Relative importance
Bermuda grass	2	BGP-1	30 000	3,1	Major
		BGP-2	14 400	2,2	Minor
Rye ¹¹	2	Group I	32 000	2,89	Major
		Group II	9 300	1,36	Minor
Ragweed ^{6,18-20}	4	Antigen E	37 800	3,05	Major
		Antigen K	38 200	NA	Minor
		Ra3	15 000	1,8	Minor
		Ra5	5 200		Minor
Timothy grass ¹⁷	2	A	NA	3,0	NA
		B	10 500	2,0	NA
Cocksfoot grass ²¹	2		30 000	NA	NA
			17 000	NA	NA

NA = data not available.

the parent substance. If, as seems likely, BGP-2 is a dissociation product of BGP-1, it is possible that the dimer presents a strong, steric immunogenic determinant while immunogenicity of the weak, monomeric determinant is restricted to individuals constitutionally endowed with the ability to degrade the pollen and/or to respond to the minor antigen.

It has been suggested that the ability to respond to allergens is controlled by autosomal genes linked to the major histocompatibility locus in a manner analogous to that found for synthetic peptides in mice and guinea pigs²² or for reaginic antibody responses in mice.²³

The evidence for such a genetic control system in man, however, is unconvincing. Marsh *et al.*²⁴ concluded that sensitivity of ragweed-sensitive individuals to the minor allergens of ragweed pollen was significantly associated with the possession of a histocompatibility antigen belonging to the HL-A7 'Greg' cross-reacting group. The authors, in analysing the data, failed to recognize the statistical difficulties emphasized by McDevitt and Bodmer²⁵ that arise in studies of this kind where associations between disease and HL-A types are examined. If, for example, one studies 20 associations, one might expect on the basis of chance alone, to find that one of them is 'significant' at the 5% level. If one applies the compensating correction factor they suggest to the data of Marsh *et al.*, one finds that the alleged association is no longer significant.

Levine and co-workers²⁶ studied 46 individuals and concluded that inheritance of ragweed hay fever is controlled by autosomal genes closely linked to the HL-A haplotype. Complete details were given for only one large family of 16 individuals among whom there were 4 allergic individuals. The remaining 30 subjects came from 6 different families and included 16 who were sensitive to ragweed. Therefore, in some families, the hay fever associated haplotype must have been chosen on the basis of only 2 individuals with hay fever and it is difficult to accept that such data constitute sound evidence

for linkage. Neither in this article nor in the article by Blumenthal *et al.*,²⁷ which claims to have demonstrated similar linkage, do the authors report the statistical methods used to establish linkage.

Therefore we feel that the question of why certain individuals react to different antigens remains completely open. Progress in the study of immune responsiveness to different allergens would be likely to follow the complete characterization of the allergens themselves. Hopefully, sufficient raw material will become available to permit this.

This work was supported by grants from the Chris Barnard Fund and the South African Medical Research Council.

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