

The potency and Group I antigen content of six commercially prepared grass pollen extracts

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Commercially prepared extracts of 6 different grass pollens, rye, fescue, sweet vernal, june, velvet, and redtop were compared for their skin reactivity, rye Group I allergen content, and protein nitrogen units (PNU) content claimed by the manufacturer and redetermined at the time the extracts were used. There was a high correlation between skin reactivity and Group I allergen content. There was no correlation between the PNU claimed and either skin reactivity or Group I allergen content. The PNU determined at the time the extracts were used showed an improved correlation with biologic activity.

In an initial study¹ of commercial short ragweed pollen extracts, biologic potency as measured in humans by skin test and histamine release, was found to correlate with the content of one well-characterized allergen, antigen E. The present study was initiated to determine whether this type of correlation could be extended to other extracts. For this purpose, grass pollen extracts were chosen because they are important allergens, and a purified allergen, Group I, isolated from the pollen of rye grass (*Lolium perenne*) appears to relate to many grass pollen extracts as antigen E does to short ragweed pollen extract.

The allergens of rye grass pollen have been studied in detail.²⁻⁷ The principal allergenic fraction (Group I) was shown to consist of a group of closely related proteins (isoallergens) of molecular weight about 27,000. These allergens are highly reactive as measured by skin test⁶ and by the histamine release test.⁷ The pollens of several common species of temperate grasses known to be important agents causing hay fever contain components closely related to rye Group I and show extensive immunologic cross-reaction with antisera raised against rye Group I.⁸ This antigen, therefore, has the potential for being a standard by which to measure the potency of more than one pollen extract. Furthermore, in recent comparative studies⁹ of the biologic potency of rye Group I relative to that of a crude mixed extract of 5 grass pollens in 87 patients, it was found that almost all grass-sensitive individuals were highly reactive to Group I, and that their sensitivity to Group I correlated highly significantly with their sensitivity to crude grass, as measured by quantitative histamine release or skin test assays.

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In the current study, 6 species of grass were selected for study on the basis that their principal allergen shows a strong cross-reaction with Group I of rye. Extracts of these grasses were purchased from 5 commercial suppliers. Biologic activity in sensitive humans was then compared with Group I determinations. As prior studies^{1, 10} have demonstrated that skin test and histamine release furnish virtually identical data, only skin tests were performed to assess biologic potency.

MATERIALS AND METHODS

Patient selection

Six patients used for the grass pollen extract study were grass-sensitive paid volunteers. They had been followed through several grass seasons and observed to have hay fever symptoms during the season of grass pollination. They had been treated symptomatically with drugs, but were not undergoing desensitization to grass pollens.

Extracts

Allergenic extracts were purchased from 5 commercial manufacturers by the Bureau of Biologics as concentrates of approximately 40,000 PNU per milliliter (or as near as possible to this figure). One laboratory did not perform PNU standardization; their extracts were obtained as 1:20 w/v concentrates.

The extracts purchased were: (1) June grass (*Poa pratensis*), (2) velvet (*Holcus lanatus*), (3) redtop (*Agrostis alba*), (4) perennial rye (*Lolium perenne*), (5) fescue (*Festuca elatior*), and (6) sweet vernal (*Anthoxanthum odoratum*).

Skin testing

Solutions for skin testing were made as serial tenfold dilutions in Tris-buffered physiologic saline containing 0.03 per cent (w/v) human serum albumin as a stabilizer. The skin of the back was employed, and 0.05 ml. of extract was injected intradermally from a disposable graduated 1.0 ml. syringe with a No. 25 needle. Results were read when responses were maximal (usually 15 to 30 minutes), and 2 diameters of wheal and erythema were measured with a millimeter rule. Responses were graded as follows:

	Wheal	Erythema
0	5 mm.	5 mm.
±	5-10	5-10
1+	5-10	10-20
2+	5-10	20-30
3+	10-15 or with pseudopods	30-40
4+	> 15 or with many pseudopods	> 40

The least dilution for a 1+ reaction was determined in the case of each extract. When this end point seemed to fall between two dilutions, i.e., the stronger gave a 2+, 3+, or 4+ reaction and the weaker a trace or negative reaction, the end point was considered intermediate between the two dilutions, and an interpolated value halfway (on a logarithmic scale) between the two dilutions was entered as an end point.

Determination of Group I antigen concentrations

Group I antigen from the pollen of perennial rye grass was purified according to the procedure of Johnson and Marsh.² Starch gel electrophoresis revealed that the purified preparation consisted mainly of the isoallergens I-B and I-C, with traces of I-A and I-D.³ A single precipitin line was obtained when the Group preparation was examined by immunodiffusion analysis against hyperimmune rabbit antiserum either to whole rye pollen extract or to a previously prepared Group I sample.

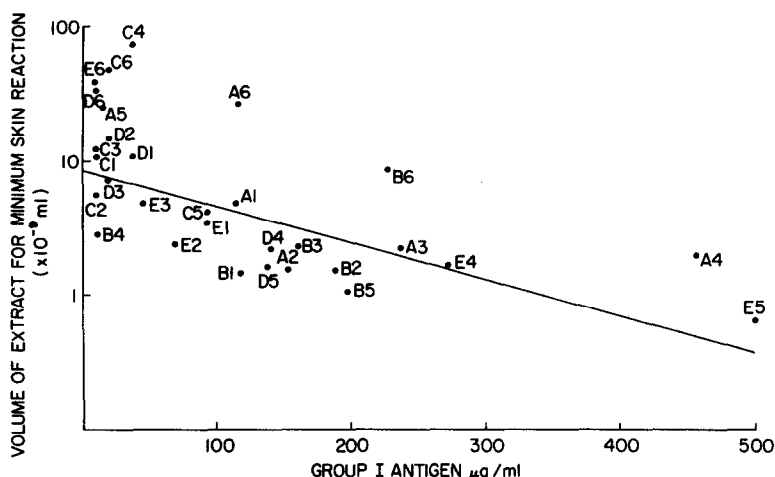


FIG. 1. Correlation of skin reactivity and Group I antigen content. 1-6 represent extracts: 1, June; 2, velvet; 3, redtop; 4, rye; 5, fescue; 6, vernal. A-E represent manufacturers.

Antiserum used in the present study was raised by multiple footpad immunizations of 2 rabbits with rye Group I adsorbed on alumina gel adjuvant. Bleedings taken 10 days after the second, third, and fourth courses were pooled and used at a final dilution of 1:5 incorporated into an agarose radial immunodiffusion gel plate using a 10 μ l Hamilton microsyringe. Ten microliter portions of rye Group I antigen solutions and the various commercial grass pollen extracts were dispensed in duplicate into wells cut in the gel. Group I antigen concentrations of 40 to 160 μ g per milliliter in borate-saline buffer at pH 8.4, containing 0.03 per cent human serum albumin as a stabilizer, were used. Two concentration levels were selected for each commercial pollen extract, such that they produced radial immunodiffusion circles with diameters falling within the range covered by the Group I antigen controls. Immunodiffusions were performed for 24 hours at 37° C. and terminated by immersion of the plates in saline at 4° C. The diameters of the precipitin circles were measured under a microscope equipped with dark-field illumination. Group I antigen concentrations of the pollen extracts were determined from standard plots of $\log_{10} (\text{Ag})$ versus mean precipitin circle diameter for the pure Group I antigen. (Corrections were made for small variations from plate to plate by including Group I controls on each plate.) Assays of each pollen extract were repeated as necessary until calculated values of Group I/10,000 PNU (labeled) determined at the 2 concentration levels were within 15 per cent of each other.

Protein nitrogen units (PNU)

The concentration of each extract, in terms of PNU per milliliter, was from washed precipitates obtained with phosphotungstic acid, modified from the method of Stull, Cooke, and Tennant,¹¹ and the nitrogen determined by a Nessler procedure.¹² Analyses were carried out in duplicate or triplicate, and the mean was recorded.

As described previously,¹ the results of biologic testing were freed from dependence on PNU or other laboratory-determined parameters by stating activity in terms of the minimum volume of each extract (as received from the manufacturer) required to produce a skin reaction. The minimum volume was computed from the dilution required to yield an end point.

RESULTS

Table I lists the extracts employed, their Group I antigen content, PNU as labeled by the manufacturer (claimed) and as redetermined (found), as well as the ratio of Group I antigen content to claimed and found PNU.

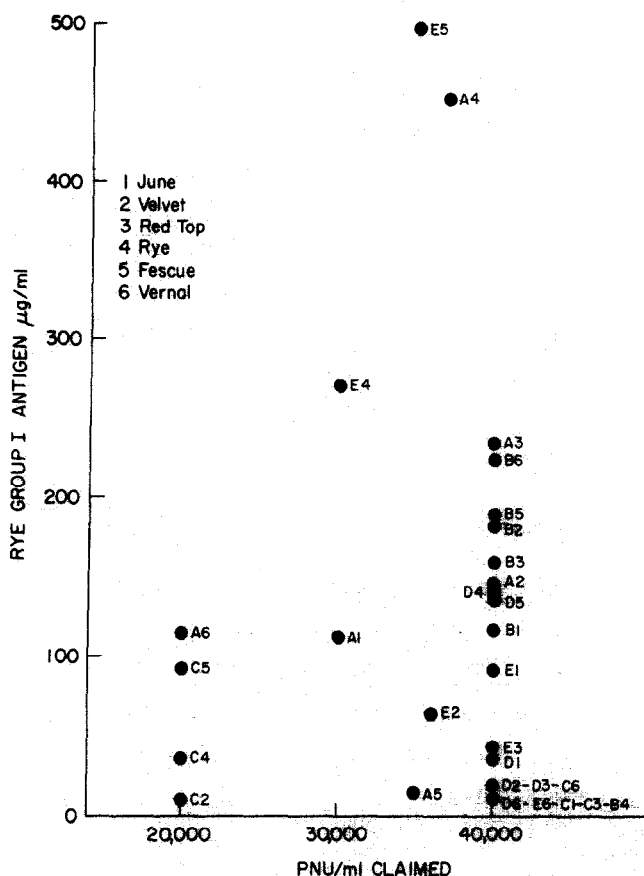


FIG. 2. Correlation of Group I antigen content and claimed PNU.

Although the extracts were purchased with very similar labeled PNU values, the redetermined values varied by as much as tenfold from the labeled value. The Group I content was also highly variable, ranging from no detectable value ($< 10 \mu\text{g}$ per milliliter) to $456 \mu\text{g}$ per milliliter.

Fig. 1 shows the relationship between the Group I antigen content and the skin reactivity of the extracts following dilution. The values for the two parameters appear to correlate well, the correlation coefficient between the Group I allergen content and the skin reactivity being -0.625 ($p < 0.05$) for all six grasses combined.

On the other hand, the correlation coefficient between Group I antigen and PNU claimed (Fig. 2) was $+0.0197$ ($p > 0.05$), and PNU found (Fig. 3) was $+0.447$ ($p < 0.05$); the correlation coefficient between the claimed PNU and skin reactivity (Fig. 4) was -0.220 ($p > 0.05$), and between found PNU and skin reactivity (Fig. 5) was -0.407 ($p < 0.05$).

DISCUSSION

It was shown earlier¹ that it was possible to correlate the biologic activity in humans of short ragweed pollen extract with the concentration of one important

TABLE I. Analysis of grass pollen extracts

Pollen	Source	Gp I content ($\mu\text{g}/\text{ml.}$)	PNU/ml.		Gp I/10,000 PNU (μg)	
			Claimed	Found	Claimed	Found
Rye	A	456	37,000	20,250	123	226
	B	< 10	(1:20)	23,200	< 2	< 4
	C	37	20,000	2,000	19	160
	D	272	30,000*	33,000	91	82
	E	140	40,000	20,500	35	68
Fescue	A	15	35,000	42,400	4	4
	B	195	(1:20)	23,300	49	84
	C	93	20,000	5,200	48	179
	D	500	35,000*	36,500	140	137
	E	136	40,000	23,300	34	58
Sweet vernal	A	115	20,000	17,800	58	65
	B	226	(1:20)	26,900	57	84
	C	20	40,000	12,900	5	16
	D	< 10	40,000	16,400	< 2	< 6
	E	< 10	40,000	24,300	< 2	< 4
June	A	113	30,000	16,800	38	67
	B	118	(1:20)†	19,800	30	60
	C	< 10	40,000	11,950	< 2	< 8
	D	93	40,000	19,900	23	47
	E	37	40,000	18,100	9	20
Velvet	A	146	40,000	26,700	37	55
	B	184	(1:20)	20,000	46	92
	C	< 10	20,000	7,700	< 5	< 13
	D	64	36,000*	28,900	18	22
	E	20	40,000	8,300	5	24
Redtop	A	236	40,000	23,000	59	103
	B	160	(1:20)	20,600	40	78
	C	< 10	40,000	9,700	< 2	< 10
	D	44	40,000	19,200	11	23
	E	20	40,000	16,700	5	12
Standard deviation $\pm$ 10-15%						

*These extracts contain 50 per cent glycerol.

†Values for extract B are based on the assumption that a 1:20 extract = 40,000 PNU per milliliter.

allergenic component, antigen E. The purpose of this study was to try to determine whether it was possible to use this type of correlation more widely. With the availability of highly purified rye Group I antigen, this study was carried out not only with rye grass pollen, but also with five cross-reacting species of grass. Fig. 1 shows that it is possible to obtain a high correlation between the concentration of this allergen and skin reactivity of 6 different species of grass pollen extracts commercially prepared. The results indicate that it is possible to predict the reactivity of an extract based on analyzing Group I content using a rye Group I-anti-rye Group I system. While we do not wish to imply that the Group I components of rye, June, redtop, fescue, velvet, and sweet vernal are identical they are sufficiently similar to enable the present immunoassay method to be used for standardization.

Although the extracts were ordered and arrived with similar PNU content on the labels, the Group I antigen content was very variable, ranging from 456 μg per milliliter to no detectable Group I allergen (< 10 μg per milliliter). The correlation coefficient between the labeled PNU and Group I allergen content was +0.0197, and skin test reactivity was -0.220. Thus, there was no statistically

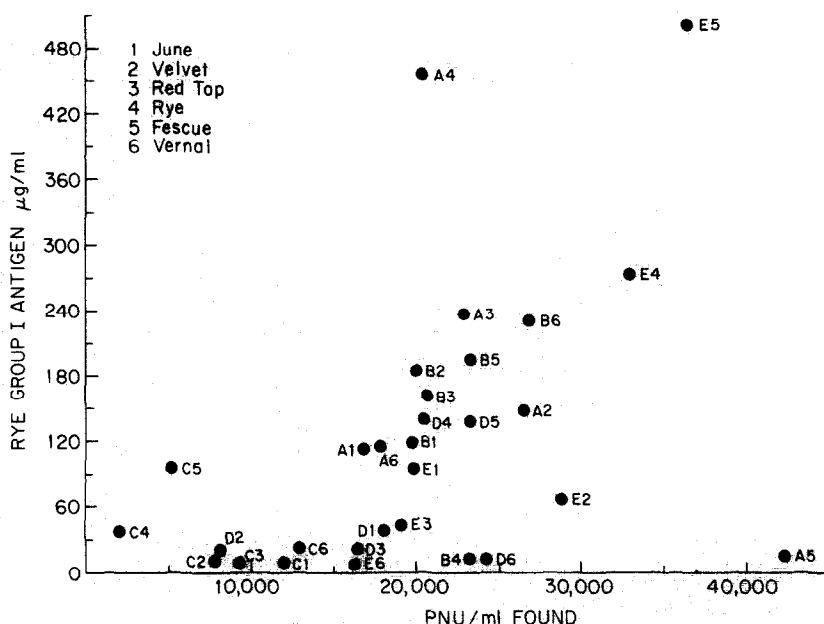


FIG. 3. Correlation of Group I antigen content and found PNU.

significant correlation between labeled PNU and Group I content and skin reactivity.

A redetermination of PNU showed some striking variations from the claimed values. The new values show an improved correlation with both Group I antigen content and skin reactivity. The correlation between PNU as determined at the time the skin testing was carried out and Group I antigen determined is, in fact, very high for most of the extracts. This is consonant with a recent finding for a group of short ragweed extracts.¹³ Why this was not true in an earlier study¹ we do not know.

It is interesting to note that the three extracts prepared in 50 per cent glycerol showed similar claimed and found PNU values (Table I), suggesting the possibility that some of the variability of PNU values upon redetermination could have resulted from loss of activity and PNU in less than optimum conditions of storage.

It should be pointed out that although the correlation between Group I and skin reactivity is high (-0.625) (Fig. 1), there are some peculiar outlying points, i.e., C4. This could be the result of a gross error of analysis, but suggests that an occasional patient or extract has an unusual reactivity or specificity. It may also be noted that velvet grass (*Holcus lanatus*) does not occur in the Baltimore area, yet all of our patients reacted to velvet extract, indicating cross-allergenicity between this grass and other hay fever grasses, as well as the cross-antigenicity displayed by double diffusion. This result confirms a previous report.⁸

Our results suggest that a rye Group I anti-rye Group I immunoassay can be used to standardize pollen extracts that possess a Group I component which

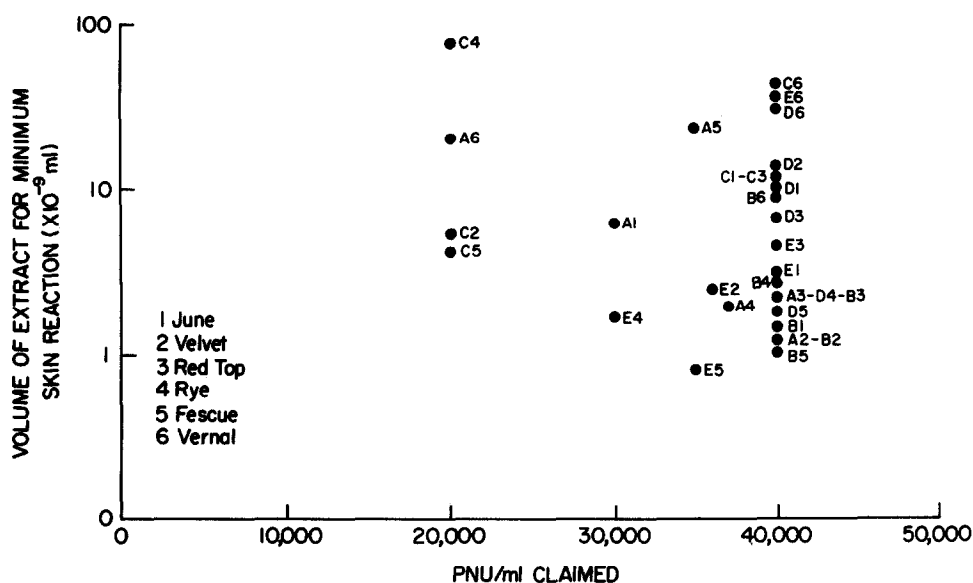


FIG. 4. Correlation of skin reactivity to claimed PNU.

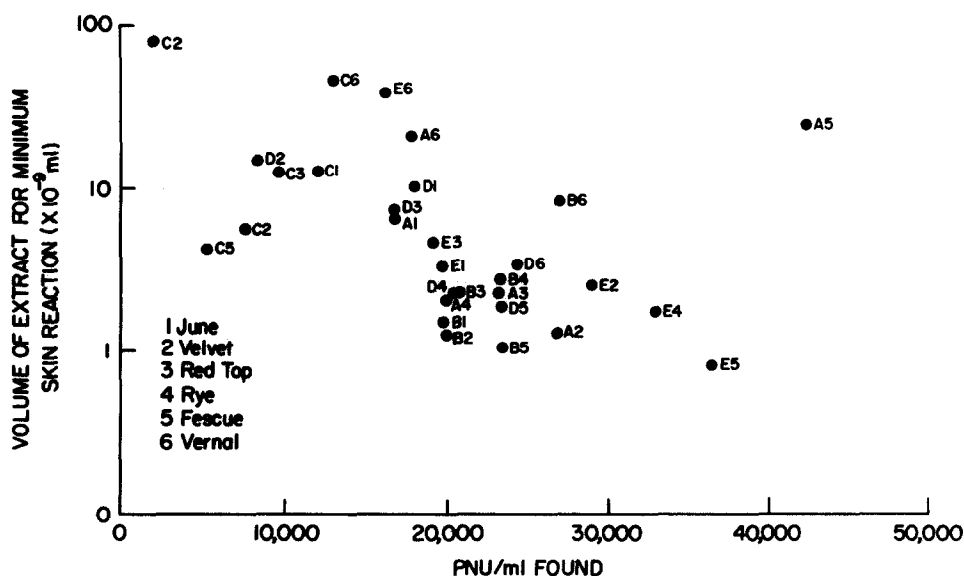


FIG. 5. Correlation of skin reactivity and found PNU.

cross-reacts sufficiently with rye Group I, hence obviating the need for isolating the major allergen from each species. Preliminary data indicate that timothy Group I does not cross-react sufficiently with rye Group I for it to be standardized by this method; on the other hand, extracts of orchard (*Dactylis glomerata*) can be readily analyzed due to the strong cross-reactivity between rye and orchard Group I components.⁸ In any event, purified allergens provide a satis-

factory standard, while PNU, as performed by commercial companies, showed virtually no correlations with biologic potency. Our results also show that when PNU is determined in a close temporal relationship to the use of the extract, it may be of some use in predicting potency.

REFERENCES

- 1 Baer, H., Godfrey, H., Maloney, C. J., Norman, P. S., and Lichtenstein, L. M.: The potency and antigen E content of commercially prepared ragweed extracts, *J. ALLERGY* **45**: 347, 1970.
- 2 Johnson, P., and Marsh, D. G.: The isolation and characterization of allergens from the pollen of rye grass (*Lolium perenne*), *Eur. Polymer J.* **1**: 63, 1965.
- 3 Johnson, P., and Marsh, D. G.: "Isoallergens" from rye grass pollen, *Nature* **206**: 935, 1965.
- 4 Johnson, P., and Marsh, D. G.: Allergens from common rye grass pollen (*Lolium perenne*). I. Chemical composition and structure, *Immunochemistry* **3**: 91, 1966.
- 5 Johnson, P., and Marsh, D. G.: Allergens from common rye grass pollen (*Lolium perenne*). II. The allergenic determinants and carbohydrate moiety, *Immunochemistry* **3**: 101, 1966.
- 6 Marsh, D. G., Milner, F. H.: and Johnson, P.: The allergenic activity and stability of purified allergens from the pollen of common rye grass (*Lolium perenne*), *Int. Arch. Allergy Appl. Immunol.* **29**: 521, 1966.
- 7 Lichtenstein, L. M., Marsh, D. G., and Campbell, D. H. In vitro studies of rye grass pollen antigens, *J. ALLERGY* **44**: 307, 1969.
- 8 Marsh, D. G., Haddad, Z. H., and Campbell, D. H.: A new method for determining the distribution of allergenic fractions in biological materials: Its application to grass pollen extracts, *J. ALLERGY* **46**: 107, 1970.
- 9 Norman, P. S., and Lichtenstein, L. M.: Capacity of purified antigens and whole pollen extracts to release histamine from leukocytes of hay fever patients, *J. ALLERGY CLIN. IMMUNOL.* **52**: 94, 1973.
- 10 Norman, P. S., Lichtenstein, L. M., and Ishizaka, K.: Diagnostic tests in ragweed hay fever. A comparison of direct skin tests, IgE antibody measurements, and basophil histamine release, *J. ALLERGY CLIN. IMMUNOL.* **52**: 210, 1973.
- 11 Stull, A., Cooke, R. A., and Tennant, J.: The allergen content of pollen extracts. Its determination and its deterioration, *J. ALLERGY* **4**: 455, 1933.
- 12 Natelson, S.: *Microtechniques of clinical chemistry*, ed. 2, Springfield, Ill., 1961, Charles C Thomas, Publisher, p. 304.
- 13 Gleich, G. J., Larson, J. B., Jones, R. T., and Baer, H.: Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test, *J. ALLERGY CLIN. IMMUNOL.* **53**: 158, 1974.

Select the ONE best answer for the following question from the Allergy Foundation of America Self-Assessment Program:

Question 2. If several batches of pollen extract are standardized by protein nitrogen units and weight/volume dilutions, the ratio of the former to the latter will be

- (A) 1:2
- (B) 2:1
- (C) 4:1
- (D) 6:1
- (E) no fixed relationship

The correct answer and bibliographic reference will be found on page 179 of this Journal.