

Analysis of the potency of extracts of June grass pollen by their inhibitory capacities in the radioallergosorbent test

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The potencies of 11 commercial extracts of June grass pollen were analyzed by skin test end point titrations and compared to potencies as determined in vitro (1) by the radioallergosorbent test (RAST), (2) by Group I antigen content, and (3) by protein nitrogen units (PNU). RAST potencies were determined by the capacity of the extract to inhibit the binding of IgE antibody to solid-phase allergen, and they were expressed as the quantity of extract required for 50% inhibition of binding. Potencies determined by skin testing in 8 patients were significantly related among the various patients in 19 of 27 comparisons and showed differences of up to 95,000-fold in the strengths of the extracts. Estimation of potencies by RAST inhibition showed approximately a 100-fold difference among the extracts and in 5 of 8 cases these were significantly related to potencies measured by skin tests. Similarly, PNU determinations and Group I determinations were also significantly related to potencies by skin test titration in 5 of 8 and in 4 of 8 comparisons, respectively. Comparison of the geometric mean skin test potencies with RAST, PNU, and Group I potencies revealed that all were significantly related to skin test potencies although the correlation of RAST and skin potency was the highest. The results indicate that measurement of potency by RAST inhibition compares favorably with other in vitro measurements of potency. These results are compared with those of a prior study with extracts of short ragweed, and the reasons for the differences between the results in the two studies are discussed.

At the present time there is no United States standard of potency for allergy extracts, and the potency of these materials is usually listed as the number of protein nitrogen units (PNU) per milliliter of extract or simply in terms of weight per volume used to prepare the extract. The potency of extracts can be measured by their content of individual allergenic moieties and in the case of ragweed and grass extracts, antigen E^{1, 2} and Group I antigen,^{3, 4} respectively, have been used for this purpose. Also, in the case of cod allergens⁵ and birch allergens,⁶ individual allergenic moieties have been identified. For the remaining numerous extracts no in vitro measure of potency is presently available.

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In a prior study we measured the potency of extracts of short ragweed by their inhibitory capacities in the radioallergosorbent test (RAST) and showed that measurements of potency by this procedure correlated remarkably well with estimates of potency by skin test end point titrations, by antigen E content, and finally by PNU.⁷ In the present study we have continued our tests of the usefulness of the RAST for the measurement of allergen potency and have analyzed the potency of 11 extracts of June grass pollen by RAST inhibition, by their content of the Group I antigen, by their content of PNU, and finally by skin test end point test titrations.

MATERIALS AND METHODS

Allergens and allergenic extracts

Extracts of June grass (*Poa pratensis*) were purchased from 11 different manufacturers in the United States. The manufacturer's claimed potency in terms of weight per volume or PNU measurement of potency for these extracts is shown in Table I. Extracts 1 and 3 were supplied in 50% glycerin. All extracts were tested prior to the expiration date listed on the bottle.

Solid-phase June grass allergen

The solid-phase June grass allergen used in these studies was prepared by reaction of a June grass extract with cyanogen bromide-activated cellulose. Undefined June grass pollen, 10 gm (Lot RM 710695 from Hollister-Stier Laboratories, Spokane, Wash.), was extracted in 90 ml distilled water with magnetic stirring at room temperature for 8 hr. The pH was maintained at approximately 6.5 during this time by addition of 1N NH_4OH . Samples of the extract were dried overnight in an oven at 55° C; the dry weight of the extract was 44.6 mg per milliliter.

The details of the coupling procedure have been described previously.⁸ Briefly, 1 gm of microcrystalline cellulose was activated at pH 11 by reaction with 2 gm cyanogen bromide dissolved in N, N-dimethylformamide. The activated cellulose was washed twice with 0.2 M H_3BO_3 -0.04 M NaOH-0.16 M NaCl, pH 8.0 (borate-saline), suspended in a volume of 11.5 ml of June grass extract containing a total of 500 mg of dissolved solids, and the pH was adjusted to 8.5. The suspension was tumbled in a 40-ml tube at 4° C for 72 hr. The cellulose was repeatedly washed with borate saline until the absorbance of the washings at 280 nm was 0.028. A total of 527 absorbance units (absorbance at 280 nm per milliliter multiplied by total volume) was exposed to the cellulose and 79.6% of the absorbance units was recovered in the initial supernatant and the borate saline washings.

Performance of RAST

Serum IgE antibody to June grass pollen allergens was measured by RAST essentially as described elsewhere.⁹ Solid-phase grass allergen, 0.5 mg, suspended in 0.5 ml 0.1 M pH 7.4 KH_2PO_4 - K_2HPO_4 containing 0.2% bovine serum albumin, 1% Tween 20, and 0.1% sodium azide (RAST diluent), was added to 0.05 ml patient's serum and brought to a final volume of 0.75 ml with RAST diluent in 10 × 62 mm tubes (Luckham, Ltd., Sussex, England). The tubes were capped and were slowly rotated overnight at room temperature, after which they were centrifuged and the supernatant aspirated. The solid-phase grass allergen was washed twice with 1 ml of 0.1 M pH 7.4 K_2HPO_4 - KH_2PO_4 containing 1% Tween 20. Radiolabeled affinity chromatography purified sheep anti-IgE, 20 ng, in a volume of 0.1 ml, was added to each tube. The volume was adjusted to 0.75 ml with diluent consisting of 0.1 M KH_2PO_4 - K_2HPO_4 , pH 7.5, 470 ml; fetal calf serum (Grand Island Biologicals, Grand Island, N. Y.), 20 ml; 10% NaN_3 , 5 ml; and Tween 20, 5 ml (FCS diluent). The tubes were rotated overnight and washed again. Radioactive counts associated with the final washed polymer were measured in a gamma spectrometer and the results were expressed as counts bound divided by counts added × 100. All serum samples were tested in duplicate and the results averaged. The results for the individual patients participating in the study are shown in Table I. Normal human serum controls yielded a value of 0.45% binding.

Measurement of the potency of June grass extracts by RAST inhibition

The RAST inhibition procedure was carried out essentially as described previously.⁷ A pool of sera from 5 individuals highly allergic to northern grass pollens was used as the source of IgE antibody in the first step of the procedure. Eighteen patients responded to an advertisement in an institutional newsletter seeking individuals with symptoms of allergic rhinitis in May-June, and 5 patients' serum having the greatest reactivity with solid-phase June grass allergens were selected for use in the study. All patients had positive skin tests to June grass extracts. None of these patients had been treated by hyposensitization with June grass allergens. When reacted with 0.5 mg solid-phase allergen, 0.05 ml of the serum pool yielded a maximum of 17.3% of total counts bound while normal human serum yielded a maximum of 0.7% binding.

In preliminary experiments, 0.1 ml samples of each extract were tested over a 1,000-fold range of dilutions to determine that amount which yielded approximately 50% inhibition of binding. In the actual performance of the RAST inhibition procedure, 0.05 ml of the allergic serum pool was added to the various inhibitors and the volume brought to 0.5 ml, 0.5 mg of the solid-phase June grass cellulose was added in a volume of 0.5 ml and the tubes were rotated at room temperature for 24 hr. After washing, 20 ng of radiolabeled affinity chromatography-purified sheep antibody to IgE was added and the volume was brought to 0.75 ml. After rotation for another 24 hr, the solid-phase allergen was rewashed and the radioactivity was determined in a gamma scintillation counter. The percent of total counts bound by the normal serum control was subtracted from all of the tests.

Skin testing

The *in vivo* potency of the June grass extracts was estimated by the skin test end point titration method as described previously for the extracts of short ragweed.⁷ Patients with varying degrees of sensitivity to grass pollen extract were selected for skin testing. All patients except J. S. had histories of spring hay fever, positive skin tests to grass pollen extracts, and a significantly positive RAST for June grass. Patient J. S. denied a history of spring hay fever even though the other criteria of sensitivity were present. Sera from Patient J. A. and R. H. were also used as constituents of the serum pool used for measurement of potency by RAST inhibition.

Radial immunodiffusion for measurement of Group I antigen and measurement of protein nitrogen

The concentration of Group I antigen in each extract was measured by radial immunodiffusion using rabbit antibody to Group I antigen as described elsewhere.⁴ The measurement of the Group I antigen of June grass extracts is possible because of the cross-reactivity of the Group I antigen from rye and June grass with antiserum to rye Group I antigen. In the performance of the test, a calibration curve was constructed by analyzing 8 standardized solutions of rye Group I varying from 5 to 80 μ g protein per milliliter. For rye Group I antigen $E_{1\text{cm}}^{1\%} = 15.1$. The coefficient of variation in the assay was 10.4%. The results listed in Table I are the mean of three or four determinations. The number of protein nitrogen units was determined by precipitation with phosphotungstic acid and analysis of the dissolved precipitate.⁴ The results listed in Table I are the mean of three determinations.

Statistical tests

Results were evaluated by tests of rank correlation and by analysis of covariance.¹⁰ A programmable Hewlett-Packard 9810A calculator was used for the statistical evaluations.

RESULTS

The results of the various *in vivo* and *in vitro* analyses of the June grass allergens are summarized in Table I. Only three allergen preparations had a PNU content designated by the manufacturer. Comparison of these values with those

TABLE I. Potency of extracts of June grass as measured in vitro by PNU, Group I, and RAST inhibition, and in vivo by skin test end point titration

June grass extract*	Designated potency	PNU/ml†	Group I (μg/ml)†	Potency in vitro 50% RAST inhibition‡
1 ♦§	1:20 (5%)	36,075	197	1,008
2 +	1:30	13,200	97	665
3 ●	1:20	15,875	100	614
4 ▲	1:10	10,797	87	362
5 ×	1:10	33,150	243	347
6 □	1:20	10,850	92	332
7 ■	40,000 PNU/cc	11,265	107	311
8 ○	1:20	23,125	160	194
9 ⊙	40,000 PNU/cc	2,335	44	112
10 △	20,000 PNU/cc	12,025	50	89
11 ◇	1:10	14,150	61	11

*All of the extract solutions contained 0.5% phenol except ♦ and ●, which contained 50% glycerin.

†Determined in Dr. H. Baer's laboratory.

‡Potency in vitro is defined as the number of 50% inhibition units per milliliter. For example, if 5 μl yielded 50% inhibition, then 1 ml of the extract contains $\frac{1,000}{5} = 200$ RAST inhibition units.

§Symbols used to represent the various extracts in the figure.

||Counts bound/counts added × 100.

TABLE II. Relationship between measurements of potency of June grass extracts by skin testing in 8 patients

Patient	Rank correlation coefficients*						
	B. P.	C. L.	S. B.	W. N.	J. C.	J. S.	J. A.
R. H.	0.636	0.782	0.546	-0.091	0.573	0.582	0.864
J. A.	0.655	0.873	0.791	-0.264	0.673	0.609	—
J. S.	0.436	0.600	0.718	-0.200	0.773	—	—
J. C.	0.755	0.500	0.836	0.118	—	—	—
W. N.	0.191	-0.291	-0.018	—	—	—	—
S. B.	0.645	0.682	—	—	—	—	—
C. L.	0.518	—	—	—	—	—	—

*Eleven grass extracts were tested in each patient, and the extract potencies between patients were compared by the Spearman rank correlation test.¹⁰ Numbers are correlation coefficients, r_s . For $n = 11$ and a 1-tailed test, r_s values greater than +0.521 are significant at $p < 0.05$; r_s values greater than +0.685 are significant at $p < 0.01$; and r_s values greater than +0.847 are significant at $p < 0.0005$.

measured by us revealed striking discrepancies. For example, extract 9 was listed as 40,000 PNU per milliliter, whereas we found only 2,335 PNU per milliliter. The actual PNU content of the 11 extracts varied from a low of 2,335 to a high of 36,075. All of the extracts contained measurable Group I antigen varying from a low of 44 μg per milliliter to a high of 243 μg per milliliter. The number of RAST inhibition units per milliliter in the extracts varied from a low of 11 to a high of 1,008. The potencies of the extracts as determined by skin test end point titrations differed by as much as 95,000-fold in Patient C. L. to as little as 26-fold in Patient W. N.

To determine whether the skin test end point titration procedure yielded reliable estimates of the potency of the extracts among the patients, we determined the correlations among the potencies in the 8 patients and the results are shown

Potency in vivo (skin test units $\times 10^6$)							
R. H.	J. A.	S. B.	B. P.	C. L.	J. C.	J. S.	W. N.
6,100	8,560	19,800	4.10	1,220	0.0531	0.00227	0.00834
20,400	6,210	9,750	6.54	316	0.0722	0.00289	0.00356
30,500	3,360	2,560	22.9	30.6	0.140	0.00498	0.0575
1,710	685	14.4	0.0498	0.172	0.0275	0.000345	0.0117
156	2,440	2,440	4.52	0.156	0.0808	0.00154	0.00625
6,100	244	1.95	0.391	0.0794	0.00234	0.000112	0.0279
144	93.3	3.98	0.0966	0.289	0.000851	0.000381	0.00246
3,310	6,100	5,370	15.3	1.72	0.0438	0.000287	0.0410
140	26.6	5.23	0.625	0.164	0.0250	0.000040	0.0323
0.733	1.93	5.31	0.131	0.0129	0.0178	0.000160	0.0242
97.7	3.91	233	0.104	0.0438	0.0308	0.000476	0.0634
Serum IgE antibody to June grass							
17.48	12.27	8.81	5.58	5.44	1.32	1.09	1.05

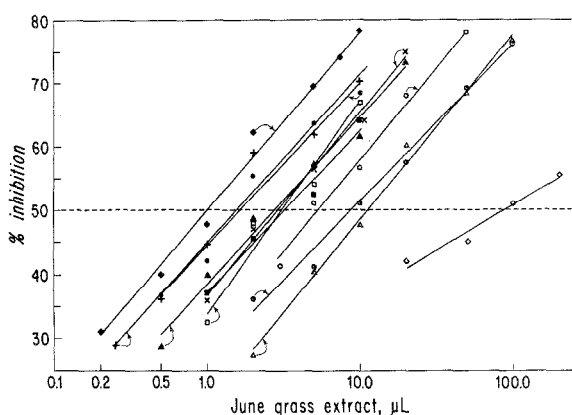


FIG. 1. Measurement of the potency of extracts of June grass by RAST inhibition. Potency is defined as the number of microliters of extract required to produce a 50% reduction in the RAST. The symbols for the various extracts are listed in Table I. The lines are the best fit to the experimental points by the least squares method.¹⁰

in Table II. In 18 of 28 comparisons the correlations of the potency estimates were significant at $p < 0.05$, while in 10 the rank correlation was not significant. Of these 10, 7 were accounted for by comparisons of Patient W. N. with the other patients. Because all of the skin test procedures were performed in a double-blind fashion and Patient W. N. did not appear to be unusual in his response to skin testing, we have no ready explanation for the anomalous results found in this particular patient.

The measurements of the potencies of the 11 grass extracts by RAST inhibition are shown in Table I and Fig. 1. Ten of the 11 extracts were within approximately a 10-fold range of each other in potency, whereas the remaining extract differed by another 10-fold factor. By analysis of covariance, the slopes of the 11 extracts differed [$F(10,35) = 2.97$; $p < 0.01$], whereas for the 10 extracts

TABLE III. Comparison of potencies of June grass extracts as determined by skin testing and by RAST, Group I, and PNU

Patient	Rank correlation coefficients*		
	RAST	Group 1	PNU
R. H.	0.855	0.509	0.346
J. A.	0.846	0.727	0.591
J. S.	0.655	0.555	0.655
J. C.	0.546	0.491	0.709
S. B.	0.444	0.527	0.782
C. L.	0.782	0.555	0.382
B. P.	0.391	0.464	0.591
W. N.	-0.464	-0.400	0.009

*For significance levels, see caption to Table II.

TABLE IV. Comparison of the geometric means of the skin test end point titrations with the in vitro estimates of potency

Rank correlation coefficients*		
RAST vs skin test	PNU vs skin test	Group 1 vs skin test
+0.809	+0.655	+0.627

*For significance levels, see caption to Table II.

with 50% inhibition points between 1 and 11 μ L the slopes did not differ [$F(9,34) = 1.22$; $p > 0.25$]. Results comparable to those shown in Fig. 1 were obtained in a separate experiment.

Comparisons of the in vitro and in vivo estimates of potency are presented in Tables III and IV. As shown in Table III, the potencies of the extracts by skin test end point titrations were significantly related ($p < 0.05$) to PNU in 5 of 8 tests, to Group I antigen content in 4 of 8 comparisons, and to RAST inhibition potency in 5 of 8 tests. Again, Patient W. N. is remarkable in that we found negative correlations in many instances between the in vitro and in vivo potency estimates. The above results pertain to comparisons of potency by skin test in the individual patients. Comparisons of the geometric mean skin test potencies of each extract with the in vitro measurements are shown in Table IV. Here RAST emerged as a better correlate of in vivo potency than either PNU or Group I antigen content, although all of the in vitro estimates of potency were related to geometric mean skin test end point titrations. We also analyzed the correlations among the in vitro comparisons of potency of the grass extracts. The rank correlations of measurements of Group I content and PNU, $r_s = +0.764$, and RAST inhibition and Group I content, $r_s = +0.564$, were significant at $p < 0.01$ and $p < 0.05$, respectively, while the relationship between RAST inhibition and PNU, $r_s = +0.336$, was not significant.

Finally, we compared the levels of IgE antibody to June grass as determined by RAST with the degree of skin sensitivity to the individual extracts. The rank correlations for these comparisons are shown in Table V. All of these were significant at $p < 0.05$, and 9 of 11 were significant at $p < 0.01$. The two extracts with the lowest correlation coefficients were those with the lowest potencies as determined by RAST inhibition.

TABLE V. Relationships between serum IgE antibody to June grass allergens and skin sensitivity

Extract	Rank correlation coefficient, r_s *
1	0.857
2	0.929
3	0.952
4	0.952
5	0.899
6	0.929
7	0.905
8	0.905
9	0.929
10	0.738
11	0.786

*All rank correlation coefficients are positive. For $n = 8$ and a 1-tailed test, r_s values greater than +0.621 are significant at $p < 0.05$; r_s values greater than +0.789 are significant at $p < 0.01$; and r_s values greater than +0.925 are significant at $p < 0.0005$.

DISCUSSION

In this study we have analyzed the potency of extracts of June grass pollen by skin test end point titrations and have compared these estimates of potency with three in vitro estimates of potency, namely RAST inhibition, Group I content, and PNU. In general, the results confirm those which we found previously for analysis of extracts of short ragweed pollen.⁷ In the cases of the grasses, however, the correlations of in vitro and in vivo results were not as intense as with ragweed and in many patients they did not reach statistical significance. This appeared to hold for all in vitro estimates of potency. Two explanations may be entertained to explain the differences between the results in this study with grass extracts and the prior study with extracts of short ragweed. First we noted in the skin test procedure that estimation of end points was considerably more difficult than in the case with the ragweed extracts. Becker and Rappaport¹¹ found that the skin reaction to graded doses of ragweed allergen had a broad plateau region and an abrupt decrease at lower concentrations of the injected ragweed extract. Our findings with the ragweed skin test end point titrations appeared similar. However, in the present study we found that the determination of the 6 x 6 mm end point wheal size was exceedingly difficult in that the skin tests in most patients with the 5-fold dilutions gave very similar responses over a broad range. Comparison of these skin test results with those from the prior study of ragweed extracts suggests that the slope of the dose-response curve in the case of the grass extracts is less than with ragweed extracts. Further, 1 of our patients, W. N., appeared anomalous in that his end point titrations results did not correlate with the results in any of the other patients. This patient was a local resident who had a history of allergic rhinitis in June and a RAST that was significantly positive (twice the percent of counts bound by normal serum). His skin tests to June grass extracts were positive and he had a positive provocation test, i.e., he experienced typical symptoms of allergic rhinitis when June grass extract was nebulized into the nasal cavity. Thus we cannot exclude the results of the end point titrations obtained in his case. A second possibility for the failure to confirm the striking correlations of the prior study with ragweed extracts is the

finding that in general the potencies of the grass extracts did not differ as greatly as those of ragweed extracts. For example, with the ragweed extracts there was a 1,000-fold range in the potencies both in the skin test end point titrations and by RAST inhibition. In the case of the grass extracts there was only a 100-fold range in this potency and 10 of the 11 extracts were within 10-fold of each other in potency. Clearly, in testing the usefulness of methods to determine potency it is desirable to have marked differences in activity among the extracts, especially because the skin test end point titration procedure is probably rather imprecise. Further, because of the overall similarity in the potencies of the 10 grass extracts and the variance in the assays, it is probable that on repeat analysis some of the extracts would yield slightly different values for the Group I and the RAST measurements of potency. Thus the correlations between measurements of potency by skin test and by Group I content and RAST would in turn vary slightly. In spite of these problems it is remarkable that the geometric means of the end point titrations in all patients for a given extract were significantly correlated with the in vitro measurement of potency and, as shown in Table IV, the RAST inhibition potencies correlated especially well with the in vivo potencies.

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