

The preparation and testing of the proposed International Reference (IRP) Bermuda grass (*Cynodon dactylon*)–pollen extract

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*A lyophilized candidate extract of Bermuda-grass (*Cynodon dactylon*) pollen, intended for use as an International Reference Standard, was prepared by pooling four individual candidate extracts. In preliminary investigations, the four candidate extracts encompassed a variety of extraction methods. The collaborative testing program simultaneously analyzed the proposed reference and the individual extracts and included 11 laboratories performing RAST inhibition, histamine release, crossed immunoelectrophoresis, crossed radioimmunoelectrophoresis, isoelectric focusing, sodium dodecyl sulfate gel electrophoresis, and protein determinations with a variety of reagents and methods. The four candidate extracts and the pooled reference were found to be equivalent. The stability of this extract has also been studied. This International Reference Preparation of Bermuda grass–pollen extract should be useful for research and industry. (J ALLERGY CLIN IMMUNOL 78:624-31, 1986.)*

A proposed IRP of Bermuda grass (*Cynodon dactylon*)–pollen extract was prepared from a pool of four candidate extracts. This pooled extract and the four candidate extracts were tested by the authors in an international collaborative study with the sponsorship of the Allergen Standardization Subcommittee of the International Union of Immunological Societies.

Eleven laboratories participated in the international effort to evaluate these extracts. Tests included protein

Abbreviations used

CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
IEF:	Isoelectric focusing
IRP:	International Reference Preparation
OBRR:	Office of Biologics Research and Review of the U.S. Food and Drug Administration
PNU:	Protein nitrogen unit
RP:	Relative potency
SDS:	Sodium dodecyl sulfate

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determination, RAST inhibition, IEF, CIE, CRIE, SDS electrophoresis, and histamine release; no single laboratory performed all tests. Common reagents were used by some laboratories, whereas others chose to use in-house test systems and reagents. Six coded extracts were distributed to each participating laboratory. One of the six was a duplicate sample and was included to examine intralaboratory test variability. The data produced in this study evaluated the proposed IRP Bermuda grass–pollen pooled extract and the individual component extracts. Comparison of the in-

terlaboratory variability caused by differences in technique and methodology was also made possible by the structure of this study.

MATERIAL AND METHODS

Preparation of the test materials

Four Bermuda grass-pollen extracts were produced by manufacturers in the United States, Spain, and Sweden. Two of the four extracts were received as freeze-dried bulks, and two extracts were aqueous. The individual candidate extracts were accompanied by a history of extraction, methodology, and source material origin. Each extract was subjected to a qualitative analysis by IEF and a quantitative examination by RAST inhibition. No differences were detected among the four candidate extracts by these methods in the preliminary review.

Equal volumes of glycerol were added to 50 ml portions of the two aqueous extracts. These became extracts D and E in the collaborative study. The remainder of the two aqueous extracts was pooled, freeze-dried in bulk, and stored at -20°C .

Samples of the two freeze-dried candidate extracts were rehydrated with water for the preliminary analysis. On completion of the IEF and RAST inhibitions, the extracts were rehydrated to their original volumes. Fifty milliliter portions of these two candidates, B and C, were then freeze-dried at OBRR in glass-sealed ampules for use in the collaborative study.

At this point, the freeze-dried pool of aqueous extracts D and E was rehydrated to original volume and combined with the rehydrated freeze-dried extracts B and C, filtered with an $0.22\ \mu\text{m}$ filter, and freeze-dried in 0.5 ml aliquots in glass ampules at the American Type Culture Collection facility in Rockville, Md. After a 72-hour drying cycle, 1943 ampules were sealed under dry nitrogen and designated extract A. The rehydration, pooling, filtering, and initiation of the freeze-drying procedure of this pooled extract were completed in less than 24 hours, during which time the extract was maintained at 4°C .

Sterility and general safety tests of extract A were performed by members of the Division of Product Quality Control of the OBRR in accordance with the procedures outlined in 21 Code of Federal Regulations 610.12 and 610.11, respectively.¹ Dr. Joan May of the Analytical Chemistry Branch, OBRR, analyzed extract A for moisture content with the thermogravimetric method² and determined the accuracy of fill by weighing the contents of 12 randomly selected vials. These procedures were not performed on the retained portions of the individual extracts.

A serum pool was prepared from 28 Bermuda grass-allergic individuals by Dr. Michael Schumacher of the University of Arizona. Individual serum samples from untreated patients with histories of Bermuda-grass allergy were tested for allergenic activity by direct RAST. Tests for the presence of hepatitis B antigens were negative in all samples. The serum pool was freeze-dried at OBRR in 2 ml aliquots and tested by direct RAST to confirm the required minimum 5

to 1 count ratio of positive serum pool to background. Microparticulate support was prepared by coupling extract A (aqueous extract, retained from bulk to be freeze-dried) to Matrex (Amicon Corporation, Lexington, Mass.) on the day that the pooled extract was prepared for freeze-drying at American Type Culture Collection facility.³

Methods used by participating laboratories

Protein. The protein content of the test extracts was determined with PNU,⁴ Lowry or Folin assays, Bio-Rad dye binding (Bio-Rad Laboratories, Richmond Calif.), and Ninhydrin⁵ and Kjeldahl analyses for total nitrogen. Bovine serum albumin, human serum albumin, or in one case, an in-house Bermuda-grass extract, was reported to have been used for the protein standard. Total protein of sample A was also determined from an amino acid analysis.

IEF. Flat-bed IEF with commercially prepared polyacrylamide gels was performed by five laboratories, agarose IEF by four, tube polyacrylamide gels by one laboratory, and an SDS polyacrylamide gel electrophoresis by one laboratory.

Histamine release. One laboratory performed this assay with the six extracts of the study with peripheral leukocytes from allergic patients.⁶ A modification of the immunochemical study of histamine from leukocytes of peripheral blood⁷ was used to assess the ability of the six coded Bermuda-grass samples to release leukocyte histamine with blood from four individuals, one of whom was nonallergic and three who were known to be allergic to grasses. Heparinized blood samples were collected, and the leukocyte plasma layer was allowed to separate from erythrocytes. The leukocytes were then pelleted, washed, and aliquoted at 10 per assay tube. The six coded Bermuda-grass extracts were prepared in serial tenfold dilutions from frozen stock sources (reconstituted as directed and stored at -20°C), and varying concentrations were used to challenge the leukocytes. The histamine released was measured spectrophotometrically, and the percent of histamine calculated was based on the total histamine content, determined by lysis with perchloric acid. A positive control with burro anti-IgE antibody to challenge leukocytes was included to document the "releasability" of leukocyte histamine. The relative potency of the candidate extracts was expressed as the ratio of the 50% leukocyte histamine release of the candidate extract and the 50% leukocyte histamine release of extract A.

CIE/CRIE. Since a precipitating animal serum was not available for distribution, only one laboratory performed this procedure.⁸

RAST inhibition. All 11 laboratories performed RAST-inhibition studies on the proposed reference and the four candidate extracts. An acceptable test consisted of at least four inhibition points between 10% and 90%, with a computed regression line having a correlation coefficient of ≥ 0.9 . The relative potency of extracts B, C, D, and E to A (the reference) was calculated by a parallel line bioassay procedure.³ Data submitted by two laboratories failed to include inhibition values surrounding the 50% midpoint

TABLE I. Extraction conditions of candidate Bermuda grass-pollen extracts

Extract	W:V	Time (hr)	Temperature (C)	Extraction fluid
B	1:10	1	18	50 mmol/L NH ₄ HCO ₃
C	1:10	16 to 18	4	H ₂ O
D	1:15	24/1*	4	25 mmol/L NH ₄ HCO ₃
E	1:10	20	4	H ₂ O

All pollen was reportedly collected in Arizona during the 1981 season.

*Pollen was initially extracted for 24 hours. After removal of this extract, pollen cake was reextracted for 1 hour, and the extracts were pooled.

TABLE II. Protein content (milligrams per milliliter) of the proposed IRP (extract A) and the four candidate extracts

Lab	Method	Extracts				
		A	B	C	D	E
1	I	2.8*	2.9	3.1	3.6	5.4
1	II	4.0	2.5	2.8	—	—
2	IV	2.4	1.8	1.5	3.0	4.4
3	III	1.6	1.0	1.0	0.9	1.6
5	V	3.2	2.0	2.0	2.4	3.0
6	V	1.3	1.0	1.9	0.8	1.8
7	IV	3.3	2.8	2.7	2.5	4.0
8	IV	1.4	1.6	1.2	1.3	2.4
9	III	2.1	1.6	1.5	—	—
9	IV	4.1	3.2	3.2	—	—
10	IV	6.0†	5.3†	5.5†	5.0	7.0
11	IV	2.8	2.4	2.6	—	—
Mean protein		2.92	2.34	2.42	2.32	3.82
SD		1.29	1.12	1.17	1.60	1.74
2 SD range		0.10	-0.21	-0.13	-0.88	0.34
Low						
High		5.74	4.91	4.95	5.52	7.30

I = Ninhydrin procedure; II = (Total nitrogen by Kjeldahl) $\times$ 6.25; III = PNU value $\times$ 16000; IV = Lowry method; V = Bio-Rad dye-binding assay.

*Laboratory 1 also determined the total protein of sample A with data obtained from an amino acid analyzer; this value was 2.8 mg/ml.

†Value outside the 2 SD range of reported values.

value as required by the statistical criteria for the parallel line bioassay method. Data from these laboratories were not included in the evaluation of the products. Of the nine laboratories that submitted statistically acceptable raw data, one laboratory used a serum pool other than the one offered by OBRR; no differences were observed in the activities of the two serum pools. Three laboratories performed RAST inhibition by use of paper discs, and six used the micro-particulate support prepared at OBRR and the methodology outlined in the OBRR Manual of Methods³ available on request from OBRR. Information concerning the nature or quality of the extracts coupled to the discs was not provided by the laboratories concerned. Two laboratories used commercially prepared discs, and one laboratory prepared its own disc supports. Radiolabeled antihuman IgE was pur-

chased by most of the laboratories, but some facilities labeled their own antibody preparations.

RESULTS

Table I summarizes pollen source and extraction methods of extracts B, C, D, and E. All the candidate extracts were prepared from pollen collected in the same geographical region of the United States during 1981. Extract A, the proposed IRP, was prepared by pooling extracts B, C, D, and E and passed sterility and general safety tests as outlined in 21 Code of Federal Regulations 610.11 and 610.12. Extract A had a mean weight of total solids per vial of 16.54 ± 1.17

TABLE III. Mean and range of reported protein values by different methods*

Method	Extracts				
	A	B	C	D	E
Lowry/Folin (six labs)	3.3 1.4 to 6.0	2.7 1.6 to 5.3	3.0 1.2 to 5.5	2.8 1.3 to 5.0‡	4.3 2.4 to 7.0‡
Kjeldahl/PNU (three labs)	2.6 1.6 to 4.0	1.7 1.0 to 2.5	1.8 1.0 to 2.8	0.9 ‡	2.6 ‡
Bio-Rad (two labs)	2.3 1.3 to 3.2	1.5 1.0 to 2.0	2.0 1.9 to 2.0	1.2 0.8 to 2.4	2.4 1.8 to 3.0
Ninhydrin (one lab)	2.8	2.9	3.1	3.6	5.4
Amino acid analysis (one lab)	2.75	ND	ND	ND	ND

ND = Not done.
*All values expressed as milligram per milliliter.
‡Five reported values.
‡One reported value.

TABLE IV. RAST RP of the four candidate extracts when extract A = 1.0

Lab	Method	Extracts			
		B	C	D	E
1	I	1.15	1.79	0.98	1.44
2	II	0.82	1.05	4.01*	6.11*
3	I	1.20	0.86	0.57	1.11
4	I	1.20	0.98	0.82	1.17
5	I	1.36	0.82	0.47	0.97
6	I	1.61	1.42	0.07*	0.83
7	I	1.00	0.92	0.37	1.07
8	II	1.30	1.30	0.85	1.10
10	II	1.04	0.90	1.31	1.33
	Mean RP	1.19	1.12	1.05	1.68
	SD	0.23	0.32	1.17	1.67
	2 SD range				
	Low	0.73	0.48	-1.29	-1.66
	High	1.65	1.76	3.39	5.02

I = RAST inhibition, microparticulate support; II = RAST inhibition, disc support.
*Value outside the 2 SD range of reported values.

mg, and the moisture content of this extract was 1.74%.

The protein content reported by each participating laboratory for the coded extracts is presented in Table II. The overall mean values of the tested products are similar, and a 2 SD range was calculated to assess the variability in the protein analyses. Only two reported values were found to be outside the range for the individual extracts. The effect of the method used to assay protein is presented in Table III. Six laboratories reported Folin or Lowry assay results, and although

the mean value for extract A in these assays was 3.3 mg/ml, individual laboratories reported values ranging from 1.4 to 6.0 mg/ml for this sample. PNU (one value) and total nitrogen (two values) results were averaged. The resulting value was similar to the Lowry/Folin mean, but the individual reported values for extract A ranged from 1.6 to 4.0 mg/ml in these tests.

Laboratories that used the dye-binding assay for protein determination used different standards (bovine serum albumin or a Bermuda extract), and the reported

TABLE V. Mean and range of RAST-inhibition methods

	Extracts				
	A	B	C	D	E
Microparticulate support (six labs)	1.0	1.2 1.0 to 1.6	1.13 0.82 to 1.8	0.54 0.07 to 0.98	1.09 0.83 to 1.4
Discs (three labs)	1.0	1.05 0.82 to 1.3	1.08 0.9 to 1.2	0.85 0.85 to 4.0	2.84 1.1 to 6.1

TABLE VI. Duplicate extract* protein and Rast inhibition RP values

Lab	Duplicates	Protein		RAST RP	
1	B = F	2.9 2.5	3.1 2.7	1.2	1.85
2	C = F	1.5	1.5	1.05	2.44
3	C = F	1.0	1.4	0.86	0.96
4	B = F	ND	ND	1.20	1.23
5	C = F	2.0	2.2	0.82	0.71
6	C = F	1.9	2.2	1.42	1.18
7	B = F	2.8	2.7	1.0	0.94
8	C = F	1.2	1.3	1.3	0.76
9	B = F	1.6 3.2	1.8 3.2	Invalid tests	1.45
10	B = F	5.3	4.9		
11	C = F	2.6	2.7	Invalid tests	

ND = not done.

*Duplicate samples of either extract B or C were sent labeled as extract F.

TABLE VII. Mean protein and RP values of Bermuda grass-pollen extracts

Extract	Protein (mg/ml)	RP		RP	
		RAST	Histamine	RAST (mg prot)	Histamine (mg prot)
A	2.94	1.00	1.00	0.34	0.34
B	2.32	1.24	0.80	0.53	0.34
C	2.59	1.15	0.90	0.44	0.35
D	2.42	1.12	1.90	0.46	0.79
E	3.81	1.68	1.40	0.44	0.37

values ranged from 1.3 to 3.2. With one exception, all reported protein values fell within a 2 SD range of the mean value of extract A of 3.3 mg/ml. Single values for extracts B and C were also outside the computed 2 SD range for each extract (Table II). The mean total protein of extract A, the proposed IRP as determined from two amino acid analyses, was 2.8 mg/ml.

RAST-inhibition data that provided at least four points between 10% and 90% inhibition with values surrounding 50% inhibition were entered in a parallel

line bioassay program. Extract A, the proposed IRP, was assigned a RAST RP of 1.00 and used as the reference extract for individualized calculations of each laboratory. Table IV contains the calculated RAST RP value for each extract that was determined from the data submitted from each laboratory. Although RP values for extracts B and C varied from 0.82 to 1.60, RP values for extract E ranged from 0.83 to 6.11, and values for extract D varied from 0.07 to 4.01. Three RP values were found to be outside the 2 SD limit for extracts D and E.

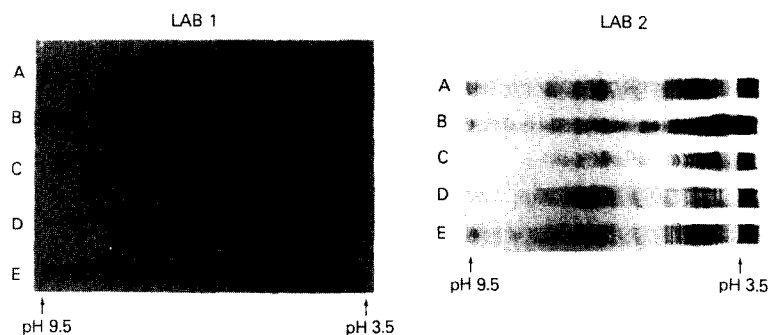


FIG. 1. Interlaboratory comparison of IEF patterns.

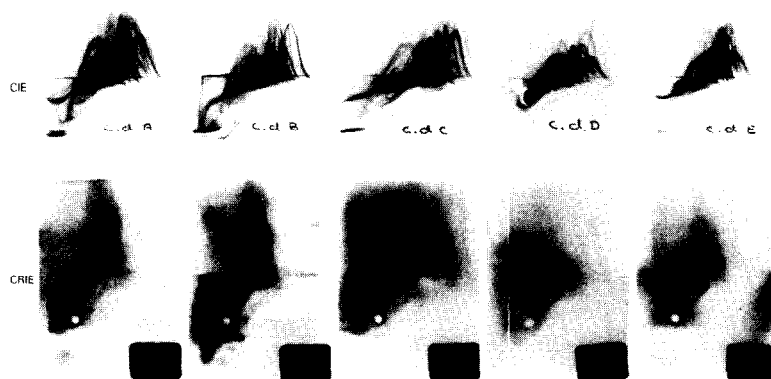


FIG. 2. CIE and CRIE of Bermuda grass extracts.

When the RAST-inhibition results were separated on the basis of test methods, as displayed in Table V, variability that could be related to methodology was less than that found in the measurement of protein content. Extract D, which had the greatest range of RAST RP values, varied from 0.07 to 0.98, or fourteenfold, with the microparticulate support, and from 0.85 to 4.0 with disc support. Extract E, which had values from 0.83 to 6.11 in pooled RAST RP, varied from 0.83 to 1.4 in microparticulate assay, and from 1.1 to 6.1 in disc RAST RP.

A comparison of the analyses of the duplicate samples within the participating laboratories revealed a high degree of internal consistency in both protein and RAST testing. Table VI presents the reported protein and RAST RP values for duplicate samples in the individual laboratories.

One laboratory determined the RP of the reference and the candidate extracts by the release of histamine from the peripheral leukocytes of two allergic individuals. The results are presented in Table VII, and the proposed reference (extract A) was again assigned an RP of 1.00. The RP of the four candidate extracts was not found to be significantly different from extract

A. When the RPs of the four extracts as measured by RAST inhibition or histamine release are compared, the differences in the two values are <0.5 in all samples. The mean protein values for each extract are also reported in Table VII to illustrate the similarities in RAST and histamine RP per milligram of protein for the proposed IRP and the four candidate extracts.

IEF was performed by nine of the 11 participating laboratories; the results of two laboratories are presented in Fig. 1. All the extracts demonstrate the presence of at least 25 bands in the acidic and basic isoelectric point regions, and the bands are distinct and sharply focused. The laboratory that performed SDS gel electrophoresis also demonstrated no visibly detectable differences in either the test or candidate reference extracts (not presented).

CIE/CRIE was performed by one laboratory, and the results are presented in Fig. 2. CIE demonstrates at least 25 antigens in each of the test extracts, with a few of the antigens migrating to the cathode. The CRIE patterns indicate approximately 14 of these antigens are allergens; these include some of the antigens that migrate to the cathode.

Storage Conditions of freeze-dried amps		Rast R.P.	IEF	
Time (months)	Temperature (°C)		pH 9.5 	pH 3.5
18	-20	1.0		
8	25	0.8		
8	35	*		
1	4	1.2		
1	25	1.1		

FIG. 3. Stability of proposed Bermuda grass-pollen extract. *Denotes invalid t test; therefore no RAST RP was computed.

Stability studies

The stability of the proposed reference has been evaluated by the coordinating laboratory. Freeze-dried ampules were stored at -20° , 4° , 24° , and 35° C for given periods, and the IEF and RAST RP of the reconstituted extracts were examined. Fig. 3 presents the IEF patterns and the mean RAST RP values of three assays for extracts stored at intervals as long as 14 months at -20° C and as short as 2 weeks at 35° C. No significant changes as measured by IEF and RAST inhibition occurred in extracts stored at temperatures of 24° C or less. At 35° C, the extract could no longer be compared to the reference by RAST inhibition because of a significant difference in slope of the best fit regression line. The IEF pattern of this extract (Fig. 3) revealed a loss of distinct bands in the 7 to 8 isoelectric point regions.

DISCUSSION

The Allergen Standardization Committee of the International Union of Immunological Societies recommended that each laboratory evaluate the proposed IRP and the candidate extracts with the methods that they usually perform. This resulted in the detection of multiple antigens in the proposed IRP by IEF and CIE, and multiple allergens, or IgE binding proteins, by CRIE. The allergenic activity of the proposed IRP and the candidate Bermuda extracts was quantified by RAST inhibition and histamine release. Only for RAST inhibition testing was a specific procedure and reagents made available to the collaborators.

Since an IRP was to be used as a calibrator for other extracts, the apparent fourfold variability of reported values for protein was larger than anticipated. To resolve the question of the most accurate value for total

protein, the protein content of the proposed IRP, sample A, was calculated from an amino acid analysis. The results of this determination illustrates that some of the most well-known and widely used methods for protein analysis are either not applicable to this product or laboratories have not carefully standardized their method of protein analysis.

The analysis of RP by RAST inhibition demonstrated little variability among the reference and the test extracts when the means of all tests were calculated in a similar manner. Although microparticulate support, disc supports, and two different serum pools were used, there is not enough data to determine whether these differences had an effect on the determination of RP by this method. Of the three RP values that appear to be outside the 2 SD limit of the mean (Table IV), two of these values were generated by a single laboratory by use of paper discs. It would appear that evaluation of the RP of an extract by RAST inhibition requires the adherence to well defined procedures if data among laboratories are to be compared.

The summary of all analytical values (Table VII) indicates that the five extracts are comparable in terms of their protein content and RP whether they are measured by RAST or histamine release. The ratios of RP to protein by RAST has small variability (0.34 to 0.53) because the values are means of data obtained from multiple laboratories. The wider variability in the ratio of protein to histamine RP is probably related to the fact that only one laboratory assayed the extracts for RP by histamine release.

CONCLUSIONS

These collaborative studies reveal that the proposed IRP, extract A, should be a suitable reference for

Bermuda grass-pollen extracts. The stability of this preparation has been studied, and the extract has demonstrated no loss of potency during 1 year of continuous storage at -20°C . The allergenic activity of this extract has been demonstrated, making this IRP a useful reference for research and industry.

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Myocardial specific creatine phosphokinase isoenzyme elevation in children with asthma treated with intravenous isoproterenol

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We retrospectively reviewed admissions that required intravenous (IV) isoproterenol in an intensive care unit setting for management of severe childhood status asthmaticus. Elevation of the cardiac-specific serum creatine phosphokinase MB (CPK-MB) isoenzyme was noted in 15 of 19 admissions. The mean peak elevation of serum CPK in those 15 admissions was 204 IU/L with a mean peak MB band of 6.05%. Nine of the 15 admissions with elevated CPK-MB level had follow-up levels measured after IV isoproterenol had been stopped. In all nine cases, the follow-up serum CPK-MB level was 0%. In six of those nine cases, follow-up serum with undetectable CPK-MB level was obtained while the patients continued to receive IV aminophylline and corticosteroids, and inhaled but not intravenous β -adrenergic agonist medications. These observations suggest that elevated serum CPK-MB levels that may be indicative of myocardial injury are associated with IV isoproterenol therapy of severe childhood asthma. Therefore, we recommend caution along with serial monitoring of serum CPK-MB levels when IV isoproterenol is administered in the therapy of childhood asthma. (J ALLERGY CLIN IMMUNOL 78:631-6, 1986.)

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