

Comparison of in-house allergen extracts of *Phleum pratense* (timothy grass) pollen with the international standard and investigation of IgE specificities of a grass-pollen serum pool from West Germany and of the one recommended by the World Health Organization

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We compared our in-house reference extract (RE) and a production extract (PE) with the international reference preparation (IRP) of International Union of Immunological Societies of timothy-grass pollen, using various biochemical and immunochemical methods. Furthermore, we compared the IgE composition of our in-house grass-pollen serum pool (West Germany) with the serum pool recommended by the World Health Organization, using the RE for crossed radioimmuno-electrophoresis (CIE). Our extracts (RE and PE) were nearly comparable with the IRP. Only by CIE one more allergen could be detected in RE and PE than in IRP and in another extract purchased from an American company. This finding may depend on the region where the source materials were harvested and which source materials were used for the preparation of the extracts. Furthermore, by high-performance liquid chromatography in the low-molecular-weight range, two distinct peaks could be detected in the nondiafiltered IRP, which were detected in the diafiltered RE and PE as traces only. The IgE composition of our in-house grass-pollen serum pool was comparable with the serum pool recommended by the World Health Organization, as detected by CIE using RE. (J ALLERGY CLIN IMMUNOL 1989;84:448-56.)

In 1981, the Allergen Standardization Subcommittee of the International Union of Immunological Societies of the WHO initiated a program for the preparation of allergen IRPs. IRPs are now available for *Ambrosia artemisiifolia*,¹ *Dermatophagoides pteronyssinus*,² and *Phleum pratense*.³ The IRPs for *Betula verrucosa*, *Canis familiaris*, *Alternaria tenuis*, and

Abbreviations used

RE:	Reference extract
PE:	Production extract
IRP:	International reference preparation
CIE:	Crossed radioimmuno-electrophoresis
HPLC:	High-performance liquid chromatography
HPTLC:	High-performance thin layer chromatography
IEF:	Isoelectric focusing
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
RI:	RAST inhibition
WB:	Western blot
IP:	Immunoprint
P rels:	Relative potency
MW:	Molecular weight
<i>Phl p</i> :	<i>Phleum pratense</i>
WHO:	World Health Organization
pI:	Isoelectric point
SE:	Size exclusion

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Lolium perenne will be released in the near future or are in preparation.

The IRPs are not intended to be used as templates⁴; that is, commercially available extracts need not demonstrate the identical allergenic composition and activity as the IRP. The IRPs should rather be used as a yard stick for preparing in-house RE or national reference preparations. For example, an in-house RE or national reference could be compared with the IRP with RI. The relative total allergenic activity of the in-house RE could then be expressed in IUs. The IRP is arbitrarily assigned 100,000 IU.

As the amount of each IRP is limited and the IRPs are not intended for skin testing, allergen-extract manufacturers must compare their production lots with an in-house RE or national reference. The in-house RE can then be calibrated to the IRP.

In our investigation, we compared our in-house RE of timothy-grass pollens and a PE of the same species to the IRP of timothy-grass pollen, using various biochemical and immunochemical methods.

Our purpose was to examine whether our extracts were comparable to the IRP according to their protein, antigen, and allergen composition, and thus allergenic activity.

Furthermore, we investigated whether a serum pool from grass pollen-allergic patients recommended by WHO and a grass-pollen serum pool prepared from allergic patients' sera from West Germany would have comparable IgE compositions when both were used for CRIE.

MATERIAL AND METHODS

Preparation of the in-house RE and of a PE of the same species

For the preparation of the extracts, timothy-grass pollens were purchased from Allergon, Engelholm, Sweden, and were harvested in Engelholm (southern Sweden) by cutting the flowery heads. Heads were dried, sifted, and stored at 4° to 8° C. PE was prepared from one lot harvested in 1985. By microscopic analysis, the amount of timothy-pollen grains was 96%; of foreign pollens and molds, 0%; and of particles from plants, 4%. RE was also prepared from one lot harvested in 1979. By microscopic analysis, the amount of timothy-pollen grains was 95%; of plant fragments, 5%; and of molds and foreign pollens, 0%. The extracts were prepared by defatting the pollens with petroleum ether (boiling point, 40° to 60° C). A 1:10 wt/vol extract was prepared by stirring the defatted pollens with a bicarbonate-buffered saline (Coca's fluid)⁵ for 16 hours at 4° C. As Coca's fluid is a common extracting fluid for PEs, we used this because we wanted our reference to be as representative of our PE as possible. The extracts were centrifuged, diafiltered with a Romicon (Woburn, Mass.) hollow fiber cartridge with a nominal MW cutoff of 5 kd, lyophilized, and stored in 3 ml vials, each containing 1 ml under nitrogen at

–25° C. The moisture content was <1%. Before each diafiltration, the cartridges were cleaned with 4% sodium hypochlorite and were checked with a dextran blue solution. The factor of dilution was 100-fold. Completeness of diafiltration was examined by HPTLC.⁶ Diafiltration is a normal production step to remove low MW substances, like histamine or related compounds. Recently, the subcommittee of immunotherapy⁷ recommended the removal of low MW nonallergenic substances from allergen extracts.

Preparation of the IRP

Preparation and characterization of the IRP was described by Gjesing et al.³ The IRP was purchased from National Institute for Biological Standards and Control (London, U.K.). For the investigations, the lyophilized extract was dissolved in 1 ml of doubly distilled water according to the labeled recommendations.

Preparation of extract C purchased from Center Laboratories (Port Washington, N.Y.)

The extract was prepared from timothy-grass pollen collected in Minnesota in 1985. By microscopic examination, counting at least 10 fields and 1000 pollen grains, the grain was found to be 99.2% pure. No mold spores and foreign pollens were detected. It was stored at –15° C, with desiccant in a sealed container before extraction. It was extracted overnight at 4° C at 1:10 wt/vol with distilled water; 5% mannitol was added, and the extract was lyophilized. The final product contained <1% moisture and was stored at 2° to 5° C before the investigation.

Protein determination

Protein content was measured by a modified Lowry assay, the technique of which is reported elsewhere.⁸

HPTLC

The three extracts were tested for the presence of low MW substances containing free amino groups, that is, histamine or histamine-related compounds, with HPTLC.⁶

IEF

IEF was performed on the three extracts to separate the constituent proteins by their pIs (instruction No. 1804, LKB Pharmacia, Freiburg, West Germany). For comparison purposes, equal amounts of protein (125 µg) were applied onto the polyacrylamide gel for each extract. The pIs of the proteins were determined by pI-gradient marker proteins (Pharmacia). After focusing, the proteins were stained with Coomassie brilliant blue R 250 (E. Merck, Darmstadt, West Germany).

SDS-PAGE

SDS-PAGE (gradient 3% to 22.5%) was performed according to the manufacturer's prescription (LKB Pharmacia); 100 µg of protein of each extract was applied onto the gel. Determination of the relative MWs was performed with a protein marker (14.4 to 94 kd [Pharmacia]). The proteins were then stained with Coomassie brilliant blue R 250 (E. Merck).



FIG. 1. HPTLC of RE, PE, and IRP. H, histamine control solution. HPTLC plate, silicagel 60 (E. Merck); eluent, CHCl_3 :MeOH:25% ammonia: H_2O (58:32:8:2); detection, ninhydrin spray reagent (E. Merck).

HPLC

The distribution of the MWs of the three extracts was determined by SE HPLC.^{9, 10} 20 μg of protein in 20 μl of elution buffer was injected. The calibration of the system was performed with a commercial calibration kit (Bio-Rad Laboratories, Richmond, Calif.).

RI

The allergenic activity of the IRP, RE, and PE was determined by RI,^{11, 12} with allergen disks prepared from RE^{13, 14} and a serum pool of grass pollen-allergic patients ($n = 112$, RAST class 4, and dilution 1:10). Radiolabeled ¹²⁵I antihuman IgE (Pharmacia) was used as tracer. Estimations of P rels were done according to the method and statistical design of the Allergenic Products Branch, Bureau of Biologics, Food and Drug Administration.¹⁵

To investigate whether the serum pool recommended by WHO and the in-house serum pool from grass pollen-allergic patients elicited comparable results by RI, a six-grass pollen extract, mixture of equal amounts of dry-weight *Phleum pratense* L., *Lolium perenne* L., *Holcus lanatus* L., *Dactylis glomerata* L., *Festuca pratensis* HUDS., and *Poa pratensis* L. was measured versus the RE with allergen disks prepared with the mixture extract and with the in-house serum pool from grass pollen-allergic patients (1:10 diluted) and the serum pool recommended by WHO (1:10 diluted).

CRIE

CRIE was done according to the method of Weeke and Løwenstein.¹⁶ Along with the three extracts previously mentioned, a fourth U.S. lyophilized extract C was evaluated.

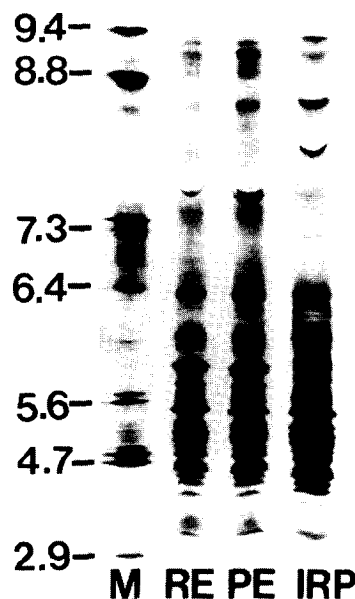


FIG. 2. IEF of PE, RE, and IRP; pl of the marker proteins (M).

Rabbit antithymothy-grass pollen serum was obtained from ALK (Copenhagen, Denmark); 10 μg of protein of each extract was applied onto the gel.

For CRIE, a serum pool from grass pollen-allergic patients (West Germany) ($n = 112$; dilution 1:24) and the serum pool recommended by WHO (dilution 1:24) were used. Additional 28 single sera from 28 grass pollen-allergic patients from West Germany (RAST class 2 to 4; dilution 1:24) with a positive clinical history and positive skin prick test were investigated by CRIE with the in-house RE.

For autoradiography, 3 kBq/ml of ¹²⁵I-labeled antihuman IgE (Pharmacia) and Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.) were used. Time of autoradiography was 3, 7, and 14 days at -20°C .

Specific IgE of all sera was determined by RAST with allergen disks prepared with RE.

WB

WB was done, as described by Burnette,¹⁷ slightly modified. Transfer of the proteins from SDS-PAGE onto nitrocellulose membrane (Schleicher & Schuell, Kassel, West Germany) was performed by electrotransfer. Free-binding sites were blocked with bovine serum albumin and Tween 20. For detection of specific IgE, the nitrocellulose membrane was incubated overnight with the serum pool from grass pollen-allergic patients (dilution 1:5) used for RI, washed, incubated with ¹²⁵I-labeled antihuman IgE (Pharmacia), and washed again. Autoradiography was performed as described in the CRIE section.

IP

IP was performed as described by Peltre et al.¹⁸ and was done with RE with the serum pool recommended by the

WHO and with some single patient's sera that were used for the preparation of the WHO serum pool (dilution 1:5). As negative control, a serum pool from nonallergic subjects ($n = 120$; dilution 1:5) was used. Transfer of the proteins from the agarose gel onto the nitrocellulose membrane (Schleicher & Schuell) was performed by capillary blotting. For detection of IgE binding sites, the nitrocellulose membrane was incubated with sera. Incubation, washings, and autoradiography were done as described by WB.

RESULTS

Protein determination

RE (1:10 wt/vol) contained 1.67 mg/ml; PE (1:10 wt/vol), 2.88 mg/ml; IRP, 3.3 mg/ml, and extract C, 1.71 mg/ml of protein.

HPTLC

Five weakly expressed bands could be detected in diafiltered extracts of RE and PE with a ninhydrin spray reagent for detection of substances with free amino groups. Ten strongly pronounced bands could be detected in the nondiafiltered IRP (Fig. 1).

IEF

The three extracts (RE, PE, and IRP) appeared nearly identical in their protein patterns (Fig. 2). Most proteins detected were grouped in a pI range of 4.5 to 6.4. Basic proteins with different staining intensity were also observed.

SDS-PAGE

As in the IEF, the three extracts demonstrated comparable patterns according to their relative MWs (Fig. 3, a). Most proteins were detected in an MW range of 14 to 94 kd. The proteins in the 60 to 84 kd were more pronounced in staining intensity in the IRP compared with RE and PE.

SE HPLC

RE and PE demonstrated nearly identical SE HPLC traces. In comparison, the HPLC trace of the IRP had two distinct peaks at ≤ 1.35 kd. These peaks could be detected only as small shoulders in RE and PE (Fig. 4).

RI

The three extracts by RI demonstrated nearly the same total allergenic activity on microliter and protein basis (Fig. 5). The RI regression lines of the assays of the three samples were parallel.

P rels of the RE and PE to the IRP were calculated on both an equal volume and on equal protein basis. With volume, allergenic activity of the RE and PE was 72,000 IU and 124,000 IU, respectively. On an

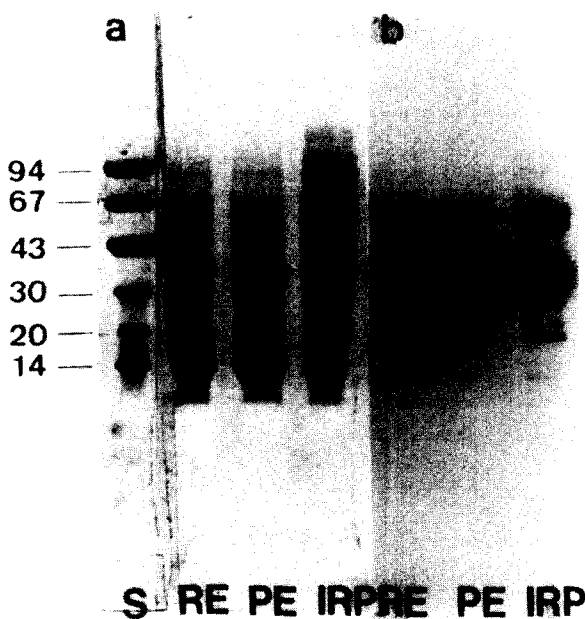


FIG. 3. SDS-PAGE. a, RE, PE, IRP and S, marker proteins and WB. b, Time of autoradiography. 14 days.

equal protein basis, RE was calculated at 142,000 IU and PE at 147,000 IU.

RI of a six-grass pollen-allergen extract versus RE with the in-house serum pool from grass pollen-allergic patients demonstrated P rel of 1.80, and with the pool serum recommended by WHO, P rel of 1.51.

CRIE allergogram

All four extracts by CRIE demonstrated almost the same allergen patterns. In each extract, the major allergens, *Phl p VI* (Ag 19), *Phl p V* (Ag 25), and the allergens, Ag 4 and Ag 27, could be detected.^{3, 19-23} In RE and PE only, an additional allergen could be detected. For purposes of discussion, this allergen will be called "allergen X" (Fig. 6).

CRIE investigation of RE with 28 sera of grass pollen-sensitive patients demonstrated that this allergen ("allergen X") is a major allergen.^{19, 24} The allergogram performed by summarizing the allergens that were detected in the 28 sera of grass pollen-sensitive patients by CRIE, with the in-house RE, is illustrated in Fig. 7. Allergen No. 3 is the so-called "allergen X."

This allergen could also be detected by CRIE of RE performed with the serum pool from grass pollen-allergic patients (Fig. 6). Furthermore, the allergen patterns were comparable. Slight differences could only be detected in the radiostaining intensity of some allergens.

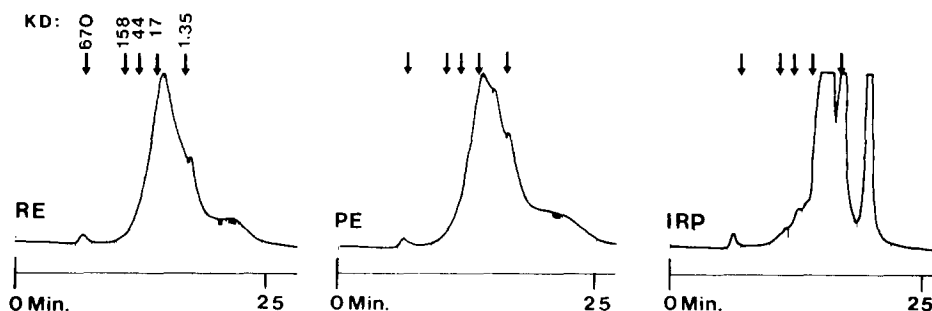


FIG. 4. SE-HPLC traces of RE, PE, and IRP (left to right). Arrows indicate the retention times of the calibration substances. Left to right: thyroglobulin (bovine), 670 kd; gamma globulin (bovine), 158 kd; ovalbumin (chicken), 44 kd; myoglobin (horse), 17 kd; vitamin B₁₂, 1.35 kd (Bio-Rad Laboratories). Column, TSK 250, 300 by 7.5 mm with guard column (Bio-Rad Laboratories); eluent, 0.5 mol/L of ammonium acetate buffer, pH 6.5; detection, 254 nm; running time, 27 minutes.

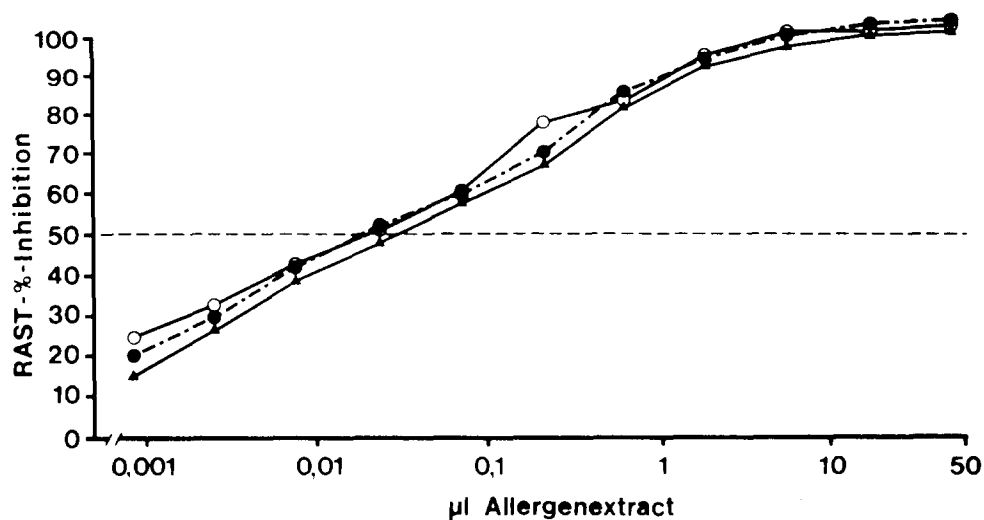


FIG. 5. RI of IRP (●—●), RE (—○—), and PE (○—○) on microliter basis.

WB

The three extracts by WB (RE, PE, and IRP) demonstrated comparable allergen patterns (Fig. 3, *b*). Most allergens were detected in the MW range of 16 to 60 kd. Allergens >94 kd were of weak intensity. Major allergens, as suggested by their radiostaining intensity, were found at relative MWs of 28, 30, 37, 50, and 58 kd. This finding, however, should be confirmed by WB with at least 20 sera from timothy pollen-allergic patients.

IP

IP was performed only with the RE with the serum pool recommended by WHO and some single patient's sera that were used for this preparation. As in the serum pool and in the individual's sera, most IgE specificities could be detected in a pI range of 4.4 to

6.6. Also, few allergens could be detected in the basic region (Fig. 8). With the serum pool from nonallergic patients as control, no IgE specificities could be detected.

DISCUSSION

Our purpose was to investigate whether an internal RE and PE of the same species were comparable to the IRP. Furthermore, we investigated whether our internal serum pool from grass pollen-allergic patients was comparable to the serum pool recommended by WHO. In most methods used, the results obtained with the PE and RE were comparable to results of the IRP. Some differences, however, could be detected between our extracts and the IRP by HPLC, HPTLC, and CRIE. By HPLC, the IRP demonstrated two prominent low MW peaks. They were only weakly

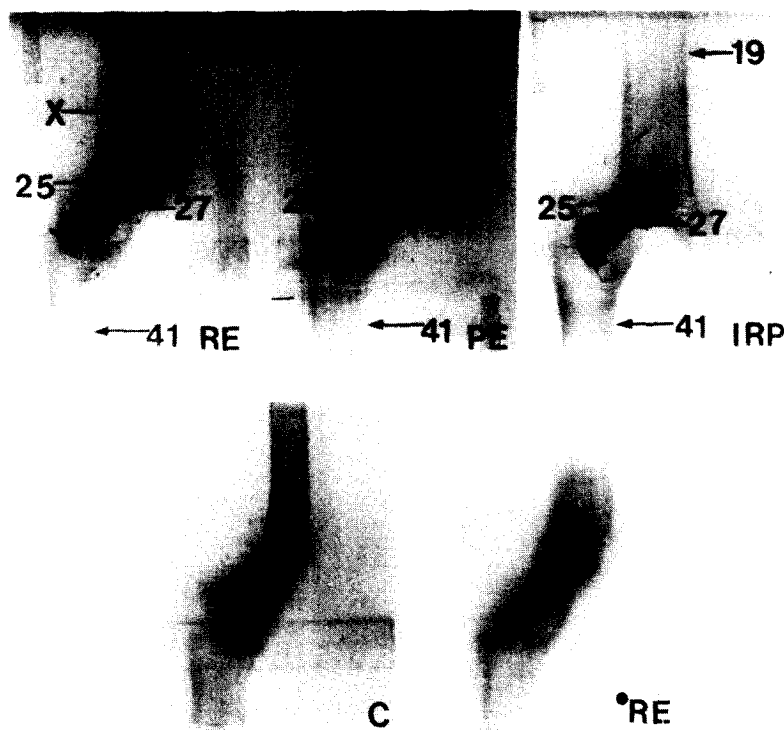


FIG. 6. CRIE of RE, PE, and IRP, and extract C with the in-house serum pool from grass pollen-allergic patients (West Germany) and CRIE of ●RE with the serum pool recommended by WHO. Arrows indicate the allergens, *Phl p VI* (Ag 19), *Phl p V* (Ag 25), Ag 27, Ag 41, and so-called "Allergen X." Time of autoradiography, 14 days.

detected in the RE and PE (diafiltered extracts). This result and the result of HPTLC demonstrated that IRP was not a diafiltered extract,³ since it contained a higher content of low MW extractables. In comparing CRIE patterns, the RE and PE contained one allergen, so-called "allergen X," which could not be detected in IRP and extract C by the methods used. The CRIE investigation was done with RE and 28 sera of grass pollen-allergic patients and demonstrated that this allergen ("allergen X") may be considered as a major allergen, according to the definition used by Løwenstein¹⁹ and Wahl and Maasch.²⁴ Despite the presence of IgE antibodies to this allergen in most patients' sera tested, immunochemical analysis is not sufficient to demonstrate that the allergen is responsible for symptoms.²⁵

Although it has not been fully investigated, it is quite possible that the differences in allergen composition observed by us may be due to differences in source pollen, that is, intraspecies variation, or it may also be due to extraction technique, that is, Coca's solution (RE and PE) versus distilled water (IRP and extract C). It is possible that "allergen X" is more extractable in physiologically salt dilution (Coca's solution) than in distilled water; pH may also play a

role. Those observations should be investigated further.

Since the IRP is not intended for in vivo use and there is only a limited quantity available, in vivo assessment of these compositional differences could not be investigated.

All extracts, including extract C, did contain the major allergens *Phl p VI* (Ag 19), *Phl p V* (Ag 25), and Ag 27 and Ag 41.^{3, 19, 23} By IEF, SDS-PAGE, WB, RI, and IP, comparable protein allergen patterns and allergen activity could be detected for extract RE, PE, and IRP.

Investigation of serum pools demonstrated that the serum pool recommended for use by the WHO and an in-house serum pool contained comparable IgE specificities, as detected by CRIE. Furthermore, nearly the same P rel could be measured by RI for a six-grass pollen-allergen extract with the in-house serum pool from grass pollen-allergic patients or the serum pool recommended by WHO.

In conclusion, our results demonstrated that an in-house RE was comparable to the IRP in total allergenic activity by in vitro methods, and the allergenic activity of the RE could be expressed in IUs. The standardization concept recommended for the International

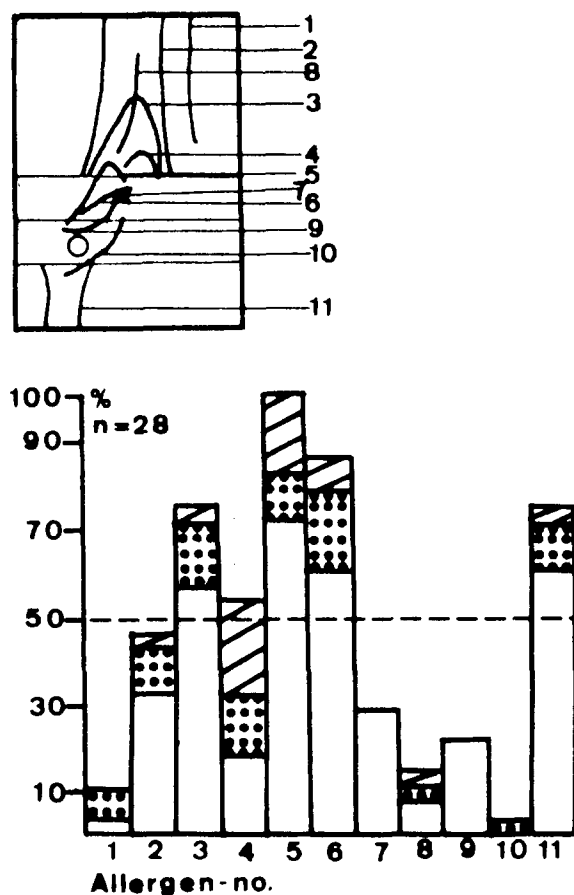


FIG. 7. Top, Summarizing of all allergens that were detected in the CRIEs of 28 sera of grass pollen-allergic patients with RE. Bottom, Allergogram: allergens that could be observed after 3 days of autoradiography (□), after 1 week (▨), and after 2 weeks of autoradiography (▤).

Union of Immunological Societies is feasible for timothy grass-pollen extracts.

Recently Tovey and Baldo,²⁶ Maasch et al.,²⁷ and Wahl et al.²⁸ demonstrated differences between the IRP of *Dermatophagoides pteronyssinus* extract and an extract prepared from another source material. Therefore, the source material used for the IRP must be considered by a company or regulatory authority when the IRP is compared to an internal or national reference preparation. By use of appropriate source material of high-quality, standardized procedures and calibration to the IRP, an in-house RE can then be a useful tool in the quality control of commercial allergenic extracts.

Because of differences in source materials, production procedures, and quality-control techniques used for the preparation of allergenic extracts throughout the world, physicians should be advised in the product labeling how the unitage was derived, for example, allergy units, biologic units, or IUs.²⁹ A

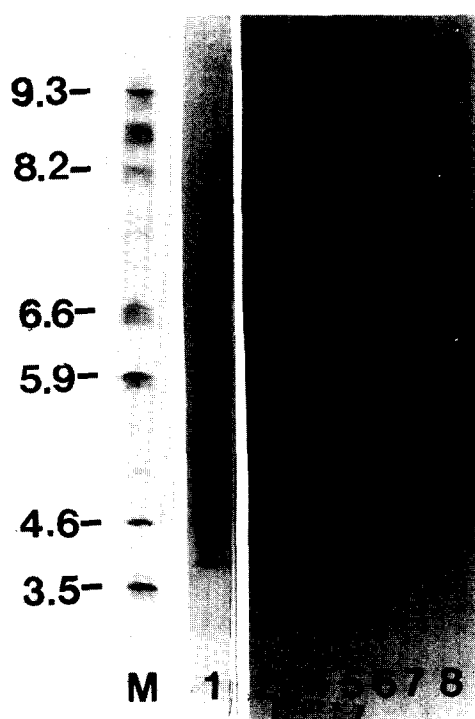


FIG. 8. IP of RE with the serum pool recommended by WHO⁸ and individual patient's sera that were used for the preparation of the WHO serum pool³⁷; negative control, nonallergic serum pool²; IEF¹; M, marker protein.

description of the assay systems used to assign allergenic activity should be included. Furthermore, when it is possible, a comparison of the product to the IRP should be included in labeling.⁷

Despite striking similarities, for example in IEF and RI among the extracts studied, differences in allergen composition were noted. Because biologic systems are dynamic, it is reasonable to assume that allergen source materials will vary³⁰ and that the extraction technique may influence the allergen composition of an extract.

Our demonstration of an allergen ("allergen X") not previously described and not present in the IRP, but present in IRPs and PEs, suggest that compositional differences exist. Compositional differences may not directly effect allergenic activity, as expressed on the label. For example, P rel of grass-pollen extracts in the United States are derived by RI. However, by our RI methods, these extracts behaved similarly. Furthermore, in the United States, cat extracts and short ragweed extracts have potency derived from single allergen content (*Fel d I* and *Amb a I*). Currently, there is no quantitation of minor allergens, and therefore, no specific compositional information is available on the label in these currently marketed (United States) extracts.

Manufacturers should be encouraged, worldwide,

to place as much information as possible regarding the potency expression on the label. The methods used to derive the potency expression, the limitations of these methods, and the relationship of the product to an IRP or national reference preparation could be included. Furthermore, where technology allows, individual manufacturers must be responsible for monitoring their own marketed extracts to minimize compositional differences. In this context, it is the manufacturer that should apply the template and define the extract with a national or IRP as the yardstick.

In spite of the limitations of the data derived from the methodologies currently used for standardization, worldwide, standardized extracts are less variable than previously observed with unstandardized allergenic extracts.³¹⁻³⁴

As we have not yet compared our in vitro findings to patterns of in vivo reactivity, we cannot predict how compositional differences among similarly labeled products may affect a timothy grass pollen-sensitive patient population.

However, it would appear prudent that allergists should consider these potential variances. When converting from one lot of allergenic extract to another lot of allergenic extract, intradermal skin testing at a safe equipotent dosage can provide guidance to compositional differences and their clinical relevance to individual patients undergoing immunotherapy with standardized allergenic extracts.³⁵

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