

19 | Preparing and Mixing Allergen Vaccines for Subcutaneous Immunotherapy

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COMMERCIALLY AVAILABLE ALLERGEN VACCINES

Allergen immunotherapy is appropriately performed with vaccines of inhalant allergens or the venom from stinging insects. The term vaccine refers to the solution administered to the patient. The materials used to prepare the vaccine are referred to as extracts. In the United States, allergen extracts are either standardized or nonstandardized extracts in a variety of formulations: lyophilized, adsorbed to aluminum, aqueous or 50% glycerin solutions. Potency is expressed as allergen units (AUs), bioequivalent allergen units (BAUs), content of the major allergen, weight by volume (wt/vol), or protein nitrogen units (PNU).

Standardized Extracts

The manufacturing and sale of allergen extracts in the United States is regulated by the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA) (1). CBER has established reference extracts and reference serum pools to be used by extract manufacturers to standardize certain allergen extracts. The potency of some of the CBER standard extracts was established by titrated intradermal skin testing (2). In this method, serial threefold dilutions of the extract are tested on the back of a group of subjects highly sensitive to that inhalant. On the basis of the dilution which yields an area of erythema with the sum of orthogonal diameters of 50 mm (the D50), the extract is assigned a BAU numerical value. Allergen extract companies then compare their extract with the CBER reference using radioallergosorbent test (RAST) or enzyme-linked immunosorbent assay (ELISA) inhibition and a potency is assigned. In contrast, the extracts of cat (3) and short ragweed (4) are standardized by their content of the major allergen, expressed in arbitrary FDA units, instead of standardization with quantitative skin testing.

The inhalant allergen extracts that are currently standardized (Table 1) are house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*), cat hair (which is low in cat serum albumin) and cat pelt (which contains substantial amounts of cat albumin), short ragweed (*Ambrosia elatior*), and eight grasses (Bermuda, June, meadow fescue, orchard, red top, rye, sweet vernal, and timothy).

While standardization of short ragweed, house dust mite and cat resulted in more consistently potent extracts than were previously available; the standardization in 1997 of the eight grasses resulted in a decrease, in some instances quite substantial, of the strength of the most potent extract available. This resulted from a CBER decision to reduce these grass extracts to a maximum testing potency of 10,000 BAU/mL and for treatment to 100,000 BAU/mL, which is considerably less than the potency of many of the previously available grass pollen extracts (5).

A second group of standardized extracts are the venoms of the stinging Hymenoptera (Table 1). These are standardized on the basis of a venom protein content of 100 µg/mL for all the individual species, but 300 µg/mL for the mixed vespids.

Physical Form and Diluent of Available Allergen Extracts

Standardized extracts are available in a lyophilized state (cat and Hymenoptera venoms), in 50% glycerin-saline (grasses, short ragweed, cat, house dust mites), and in aqueous solution (short ragweed). Nonstandardized extracts are available in either a 50% glycerin or an aqueous solution. The 50% glycerin contains equal parts of glycerin and normal saline. The aqueous extract consists of normal saline and 0.4% phenol. Glycerin at 50% concentration inhibits microbial growth and maintains the potency of allergenic extracts. Phenol, which is added to

Table 1 Allergen Extracts: CBER Basis for Standardization

Cat hair: 10,000 BAU/mL contains 10.0–19.9 units Fel d 1/mL and "little" cat serum albumin by isoelectric focusing
Cat pelt: 10,000 BAU/mL contains 10.0–19.9 units Fel d1/mL and substantial amounts of cat serum albumin by isoelectric focusing
Short ragweed: Designated weight by volume or protein nitrogen units, but with the Amb a 1 content in units/mL on the vial
House dust mite (<i>D. pteronyssinus</i> and <i>D. farinae</i>): 5000, 10,000, and 30,000 AU/mL determined by quantitative skin testing
Grasses: 10,000 and 100,000 BAU/mL determined by quantitative skin testing
Hymenoptera: Expressed as the venom protein content (100 µg/mL) for each individual insect species (honey bee, yellow jacket, wasp, yellow- and white-faced hornet)

Abbreviation: CBER, Center for Biologics Evaluation and Research.

aqueous extracts to inhibit bacterial and fungal growth, has an adverse effect on the potency of stored extracts. The choice between the two extracting and diluting fluids would favor glycerin were it not for the discomfort associated with injection of 50% glycerin (6).

A limited number of pollen extracts are available adsorbed to aluminum to delay their absorption from the injection site. When the initial extraction is performed with aqueous extracting fluids and the aluminum is subsequently added, the resulting vaccine is equally efficacious compared with aqueous vaccines (7) but is associated with a decreased incidence of systemic reactions (8). However, aluminum-precipitated vaccines, which have been initially extracted in pyridine, are markedly less potent than comparable aqueous vaccines (9,10).

Expressed Extract Potency

The traditional expressions of extract potency are weight by volume and protein nitrogen units. Neither provides precise information regarding the allergenic potency of the extract. However, it is likely that within broad limits the initial potency of many extracts obtained from the same commercial supplier have reproducible batch-to-batch potency (11). Thus it has been possible, as a general practice, to refill allergy treatment vaccines with new lots of the same stated potency from the same manufacturer without untoward reactions by reducing the first injection from the new vial by one-third to one-half of the previous dose.

Weight by volume is the simplest way to express the potency of allergen extracts. It is only necessary to weigh the material to be extracted and measure the volume of extracting fluid. Thus, 10 g of pollen extracted in 100 mL of extracting solution yields a final concentration of 1:10 wt/vol. One advantage of this method of expressing potency is that the extract need not be further diluted to achieve the desired level of potency.

PNU's were introduced in an attempt to more accurately express the allergen content of extracts (12). First, the protein nitrogen content is determined and then the content is converted to units (one unit equal to 0.00001 mg of protein nitrogen). The major allergens usually represent only a few percent of the total protein content of allergen extracts. Therefore, PNU offers little advantage as an expression of allergenic potency over weight by volume. The distinct disadvantage of PNU is that extracts are commercially available in specific concentrations (e.g., 20,000–40,000 PNU/mL). This requires that the extract be diluted from the strength obtained during the extraction process and therefore the most potent PNU extract available will be weaker than the most concentrated weight by volume for any given allergen.

The CBER BAU is based on quantitative intradermal skin testing with serial threefold dilutions of the extract in at least 15 highly allergic individuals (2). The endpoint is the dilution of the extract that results in an erythema with the sum of the largest and the midpoint orthogonal diameters of 50 mm. If the endpoint dilution is 9.0 to 10.9, the extract is assigned 1000 BAU/mL; if the endpoint dilution is 11.0 to 12.9, the concentration is 10,000 BAU/mL; and if it is 13.0 to 14.9, the concentration is 100,000 BAU/mL. Alternatively, the BAU potency can be determined by RAST- or ELISA-inhibition methods in comparison with the CBER reference product, whose BAU potency has been determined by quantitative intradermal skin testing. For a potency designation of 10,000 BAU/mL by the RAST-inhibition method, the relative potency in relation to the reference product must be 0.47 to 2.12 and for the ELISA-inhibition method 0.699 to 1.431. The CBER standardization method is likely more reproducible when content of major allergen is used as a basis. For Amb a 1, the major allergen of ragweed,

Table 2 Immunotherapy Dose and Outcome of Asthma

Treatment group	Number evaluable	Wheezing with exertion (%)	Wheezing with upper respiratory tract infections (%)
Highest tolerated dose (maximum 1:250 wt/vol)	43	9	9
1:5000 wt/vol	39	31	10
1:10,000,000 wt/vol	49	45	55
Saline	42	64	74

Source: From Ref. 13.

determinations by gel diffusion with a range of plus or minus 25% is allowed, as opposed to plus or minus 100% for quantitative skin testing.

ADEQUATE DOSING FOR DEMONSTRATED EFFICACY

Studies with Vaccines Prepared from Nonstandardized Extracts

Johnstone and Crump (13) conducted a study of the efficacy of allergen immunotherapy employing a broad range of doses. New patients with perennial bronchial asthma referred to the pediatric allergy clinic of Strong Memorial Hospital for immunotherapy were randomly assigned to receive treatment with buffered saline, or all inhalable allergens to which the child reacted on skin testing but administered to maximum concentrations of 1:10,000,000 wt/vol, 1:5000 wt/vol, or the highest tolerated dose up to a maximum of 1:250 wt/vol. The child, parent, and evaluator were unaware of the patient's assigned group. Two hundred children were randomized and 173 were available for evaluation during the winter of the fourth year of treatment. The results suggested that clinical improvement increased with increasing dosage of antigen (Table 2).

Lowell and Franklin (14) demonstrated, in a study meeting all the requirements for adequate blinding, that immunotherapy with ragweed pollen vaccine was clinically effective. They then applied the same study design to examine the effect of two doses of ragweed pollen vaccine on seasonal rhinitis symptoms (15). Twenty-five ragweed sensitive subjects were recruited who were still symptomatic during the ragweed pollen season despite receiving allergen immunotherapy that contained ragweed pollen extract. They were paired by severity of symptoms during the ragweed pollen season. One of each pair continued to receive ragweed vaccine at the customary level (median dose 0.3 mL of a 1:50 wt/vol concentration) while the other member of the pair received a dose reduced by 95% (median dose 0.3 mL of 1:1000 wt/vol). During the ensuing ragweed season, those receiving the reduced dose experienced significantly more symptoms of allergic rhinitis.

Studies with Vaccines Prepared from Standardized Extracts

One of the major advantages of using standardized vaccines is that proven treatment regimens from controlled studies can be more effectively applied by physicians to their clinical practices. There have been a number of double-blind, controlled studies employing the standardized vaccines that are now available in the United States (Table 3). In some instances only one concentration was employed, but the clinical benefit was demonstrable within a few months to a year and clinically relevant. In other studies, more than one dose was employed, and a dose response was demonstrated. In all of these studies, the dose of allergen employed was expressed in the concentration of one of the major allergens, since this is the only method of standardization recognized internationally. To allow general application of this information to standardized extracts available in the United States, representative values for the major allergen content of specific lots of extracts standardized in BAUs are given in Table 4. It must be appreciated, however, that standardized extracts, labeled with the same BAU potency may contain different amounts of the major allergens.

House Dust Mites

The study by Ewan et al. (16) demonstrated that a maintenance dose containing 11.9- μ g Der p 1 reduced symptoms and objective responses significantly after only three months, but with a

Table 3 Documented Optimal Effective Doses of Major Allergens

Allergen	First author	Optimal dose
<i>Dermatophagoides</i>	Ewan (16)	11.9- μ g Der p 1
	Haugaard (17)	7.0- μ g Der p 1
	Olsen (18)	7.0- μ g Der p 1
		10.0- μ g Der f 1
Cat dander	Van Metre (19)	13.8- μ g Fel d 1
	Alvarez-Cuesta (20)	11.3- μ g Fel d 1
	Hedlin (21)	17.3- μ g Fel d 1
	Varney (22)	15- μ g Fel d 1
	Ewbank (23)	15- μ g Fel d 1
	Nanda (24)	15- μ g Fel d 1
	Lent (25)	15- μ g Can f 1
Dog dander	Varney (26)	18.6- μ g Phl p V
	Dolz (27)	15- μ g Dac q V, Lol p V, Phl p V
Grass	Walker (28)	20- μ g Phl p V
	Van Metre (29)	11- μ g Amb a 1
Short ragweed	Creticos (30)	12.4- μ g Amb a 1
	Creticos (31)	6- μ g Amb a 1
	Furin (32)	24- μ g Amb a 1

Table 4 Representative Values for Major Allergen Content of U.S. Standardized Extracts^a

Allergen extract (<i>n</i> = number of extracts tested)	Expressed concentration	Major allergen	Mean content major allergen (μ g/mL)	Maximum content major allergen (μ g)	Minimum content major allergen (μ g)
Orchard <i>n</i> = 14	100,000 BAU/mL	Dac g 5	918	2,414	294
Fescue <i>n</i> = 12	100,000 BAU/mL	Fes p 5	152	204	75
Rye <i>n</i> = 14	100,000 BAU/mL	Lol p 5	337	526	157
Kentucky <i>n</i> = 23	100,000 BAU/mL	Poa p 5	300	482	118
Timothy <i>n</i> = 28	100,000 BAU/mL	Phl p 5	680	1,336	354
Short ragweed <i>n</i> = 21	1:10 wt/vol	Amb a 1	424	763	144
Mixed ragweed <i>n</i> = 10	1:10 wt/vol	Amb a 1	174	402	56
<i>D. pteronyssinus</i> <i>n</i> = 38	10,000 AU/mL	Der p 1	76	241	19
<i>D. farinae</i> <i>n</i> = 59	10,000 AU/mL	Der f 1	56	144	14
Cat hair <i>n</i> = 45	10,000 BAU/mL	Fel d 1	43	79	24
A-P dog	1:100 wt/vol	Can f 1	140		

^aValues provided by ALK-Abello (updated July, 2006).

Abbreviation: A-P, acetone precipitated.

Source: U.S. FDA reference extracts and extracts from ALK-Abello and other U.S. pharmaceutical firms.

high incidence of systemic reactions (approximately 15% of injections). The dose-response study by Haugaard et al. (17) demonstrated that there was marginal reduction in bronchial reactivity to mite allergen after two years of treatment with a maximum dose of 0.7- μ g Der p 1, but the reduction with a dose of 7 μ g was significantly greater. A higher dose (21 μ g) did not result in any additional benefit but caused more than twice as many systemic reactions per injection as the 7 μ g (7.1% vs. 3.3%). Therefore, the investigators concluded that a maintenance dose of 7- μ g Der p 1/injection appeared to be near optimal based on benefit/risk considerations. Olsen treated 23 adult patients with asthma for one year with a maintenance dose of 7.0- μ g Der p 1 or 10.0- μ g Der f 1 (18). Compared with patients who received placebo, those treated with mite vaccines had significantly decreased symptoms of asthma, and required less β -adrenergic agonists and inhaled corticosteroids.

Cat Dander

Four studies have demonstrated significant improvement employing a narrow range of doses. Treatment by Van Metre et al. (19) with a maximum Fel d 1 dose of 13.8 μ g/injection reduced both bronchial and skin reactions to cat dander extract. Alvarez-Cuesta et al. (20), treating with a maximum dose of 11.3- μ g/injection Fel d 1 for one year, also noted decreased skin,

conjunctival, and bronchial sensitivity, as well as a 90% reduction in symptom medication scores. Hedlin et al. (21), treating with a maximum dose of 17.3- μ g/injection Fel d 1 for three years, observed not only a reduced bronchial sensitivity to cat dander but also a significantly reduced response to bronchial challenge with histamine. Patients of Varney et al. (22), treated to a maintenance dose of 15- μ g/injection Fel d 1, had significantly reduced symptoms on exposure in a house contaminated with cat dander.

Ewbank et al. (23) compared the immunological response shortly after achieving maintenance doses containing 0.6- μ g Fel d 1, 3.0- μ g Fel d 1, or 15- μ g Fel d 1 by a cluster buildup. Both higher doses of vaccine produced significant decreases in prick skin test sensitivity and increases in cat-specific IgG₄, but only the vaccine containing a dose of 15- μ g/injection Fel d 1 produced a significant reduction in the percent of CD4⁺/IL4⁺ peripheral blood mononuclear cells. This study was duplicated in 28 additional cat-allergic patients, and outcomes were assessed both after reaching maintenance and again after one year of maintenance injections (24). Again there was a dose-related suppression of prick skin tests. Cat-specific IgG₄ was significantly increased with the two higher doses, but only the dose containing 15 μ g/injection of Fel d 1 produced sustained reduction in symptoms on nasal challenge. The conclusion of these two studies was that a maintenance dose of cat vaccine containing 15 μ g of Fel d 1 was optimal and superior to one containing 3 μ g of Fel d 1, while the results with a maintenance dose containing only 0.6 μ g/injection was similar to placebo.

Dog Dander Vaccine

Although dog dander extracts are not standardized in the United States, the major allergen of dog, Can f 1, can be measured in dog allergen extracts. Most of the commercial dog dander extracts contain very low levels of Can f 1; however, the acetone-precipitated (A-P) dog allergen extract 1:100 wt/vol manufactured by Hollister-Steir (Spokane, Washington, U.S.) contains an average of 140- μ g/mL Can f 1. A study, similar in design to those conducted with cat extract by Ewbank and Nanda, compared the response to Hollister-Steir dog vaccine containing 0.6 μ g/injection, 3.0 μ g/injection, or 15 μ g/injection of Can f 1 with placebo (25). There was a dose-related rise in dog-specific IgG₄, suppression of the titrated prick skin test, and reduction of in vitro secretion of TNF- α from allergen-stimulated lymphocytes. The conclusion was that the greatest and most consistent response was seen with a dose of dog extract containing 15 μ g of Can f 1.

Grass Pollen Vaccine

Varney et al. (26) conducted a preseasonal, double-blind trial of immunotherapy with timothy pollen vaccine in subjects with seasonal, grass pollen-allergic rhinitis. A maximum dose of 18.6- μ g/injection Phl p 5 reduced symptoms and medication use by more than 50% compared with placebo. This treatment also reduced conjunctival sensitivity and the delayed skin test response to timothy grass extract. Dolz et al. (27) treated allergic subjects for three years with a vaccine containing 15 μ g/injection of the major allergen of each component of a grass mixture. He observed a progressive decrease in ocular, nasal, and pulmonary symptoms over the three years of the study. Walker et al. (28) treated subjects with both seasonal allergic rhinitis and asthma with a timothy vaccine containing, at maintenance, 20 μ g of Phl p 5. Immunotherapy not only diminished rhinitis but also markedly reduced chest symptoms and blocked the seasonal increase in methacholine sensitivity.

In summary, grass pollen vaccines are effective for allergic rhinitis and conjunctivitis and may improve asthma. The lowest effective dose has not been determined but a maintenance dose of 15 to 18.6 μ g is effective.

Ragweed Pollen Vaccine

The most extensive experience with vaccines containing known amounts of the major allergens is with ragweed. Studies at Johns Hopkins University have included both single and multiple maintenance doses. However, the comparative dose studies have been either progressively increasing doses in the same individuals or the different doses have been administered for different number of years. There have been no studies in which groups of subjects receive different maximum doses for the same duration of treatment. Nevertheless, the data show that

clinical and objective benefits occur within months and are maintained with maximum maintenance doses of 11 μg (29) to 24.8 μg (30) of the major ragweed allergen, Amb a 1. Similar benefits were observed in a group who received a maintenance dose of 6- μg /injection Amb a 1 for three to five years (31). However, the response to 0.6 μg /injection (30) or to 2 μg /injection (32) was inconsistent and less than the response to higher doses.

Hymenoptera Venom Vaccines

Immunotherapy of insect-sensitive patients with Hymenoptera venom is effective in reducing reactions to intentional sting challenge (33). The original studies employed maximum doses of 100 μg /injection of the venom proteins, an amount exceeding the 50 μg that is usually injected by the sting of the insect. Treatment with the 100- μg dose has proven effective in the majority of sensitized subjects, therefore there have been few studies of alternative dosing.

CONSIDERATIONS IN FORMULATING AN ALLERGEN VACCINE FOR TREATMENT

The considerations in formulating an allergen vaccine for immunotherapy are (i) inclusion of an adequate dose of each extract in a vaccine to achieve an optimal response, (ii) utilization of allergenic relationships and cross-allergenicity to achieve similar effective doses for non-cross-reacting and cross-reacting allergens, (iii) combination of the individual extracts in vaccines to assure compatibility and stability, and (iv) selection of type of diluent.

Adequate Doses of Each Allergen

The optimal maintenance dose of the major allergens that are proven effective in placebo-controlled studies are listed in Table 3, and the approximate content of these major allergens in the U.S. standardized extracts are given in Table 4. From this information, it is possible to estimate the amount of standardized extract in a vaccine to be given per injection to achieve optimal dose. The amount will differ not only with different sources of the same extract but also with different extracts, as is suggested by different BAU and AU/mL values (e.g., grasses 100,000 BAU/mL, house dust mites 10,000 AU/mL).

To formulate a 10-mL maintenance treatment vaccine containing optimal concentrations of each standardized extract administered in a 0.5-mL maintenance injection, the mean effective dose, in major allergen content, for that allergen is multiplied by 20. This calculation provides the total amount of major allergen required in the 10-mL vial. There is a range of values for each vaccine depending on the major allergen content of that particular lot. An example of a vaccine mix containing optimal amount of the standardized extracts is given in Table 5.

What about the majority of allergens for which there is no information on optimal doses and no standardized extracts? Here it is necessary to work with the best clinical information available. The study by Johnstone and Crump (13) showed an inhalant allergen with a mix containing 1:250 wt/vol of each allergen was better than one with 1:5000 wt/vol of each allergen. In a similar fashion, the study by Franklin and Lowell (15) indicated that treatment with 1:50 wt/vol of ragweed was superior to treatment with 1:1000 wt/vol of ragweed.

Table 5 Representative Prescription for an Optimal Maintenance Dose Vaccine using U.S. Standardized Extracts

Extract	Concentration	Optimal dose, etc	Amount etc. (mL)	Amount expressed in U.S. CEFR units
Timothy	100,000 BAU/mL	18.6- μg Phl p 5	0.5	5,000 BAU
Short ragweed	1:10 wt/vol	12- μg Amb a 1	0.6	N.A.
<i>D. pteronyssinus</i>	10,000 AU/mL	3.5- μg Der p 1	0.9	900 AU
<i>D. farinae</i>	10,000 AU/mL	5- μg Der f 1	1.8	1,800 AU
Cat dander	10,000 BAU/mL	15- μg Fel d 1	6.2	6,200 BAU

This prescription is based on the mean documented optimal effective doses (Table 3) and the examples of the amount of major allergens contained in U.S. standardized extracts (Table 4). Major allergen content will vary among manufacturers for extracts of the same labeled potency.

Table 6 Representative Values for Major Allergen Content of Nonstandardized Extracts

Allergen	Expressed concentration	Major allergen	Major allergen concentration ($\mu\text{g/mL}$)	Reference
White birch	1:10 wt/vol	Bet v 1	390	ALK-Abello
English plantain	1:10 wt/vol	Pla l 1	30	ALK-Abello
European olive	1:20 wt/vol 50% Glycerin	Ole e 1	90	ALK-Abello
European olive	1:10 wt/vol Aqueous	Ole e 1	430	ALK-Abello
Sagebrush	1:10 wt/vol	Art v 1	1300	ALK-Abello
Dog	1:10 wt/vol 50% Glycerin	Can f 1	5-10	ALK-Abello
<i>Alternaria</i>	1:20 wt/vol 50% Glycerin	Alt a 1	1-5	ALK-Abello
<i>Alternaria alternaria</i>	1:10 and 1:20 wt/vol 50% Glycerin $n = 15$	Alt a 1	<0.01 to 6.1	34
<i>Alternaria alternata</i>	1:5 to 1:20 wt/vol $n = 7$	Alt a 1	0.04 to 2.04	35
<i>Aspergillus fumigatus</i>	1:10 and 1:20 wt/vol 50% Glycerin $n = 15$	Asp f 1	<0.01 to 64.0	34

Table 7 Representative Prescription for a Maintenance Vaccine Using Standardized and Nonstandardized Extracts

Extract	Concentration	Amount (mL)
Oak, White	1/10 wt/vol	1
Elm, American	1/10 wt/vol	1
Kochia	1/10 wt/vol	0.5 ^a
Russian thistle	1/10 wt/vol	0.5 ^a
Ragweed, short	1/10 wt/vol	0.5 ^a
Ragweed, giant	1/10 wt/vol	0.5 ^a
Timothy	100,000 BAU/mL	0.25 ^a
June grass	100,000 BAU/mL	0.25 ^a
Orchard grass	100,000 BAU/mL	0.25 ^a
Meadow fescue	100,000 BAU/mL	0.25 ^a
Diluent (to make 10 mL)	Saline with 0.03% HSA	5.0

The final concentration for each nonstandardized allergen group is approximately 1:100 wt/vol.

^aExtracts included in reduced amounts to compensate for significant cross-allergenicity (37). The rationale for a target of 1:100 wt/vol or a 10-fold dilution from the strongest available stock extract is by analogy with clinical studies on standardized ragweed (29-32) vaccines and published studies with nonstandardized pollen vaccines (13,15). Abbreviation: HSA, human serum albumin.

Limited data on major allergen content of nonstandardized pollen extracts suggests a range of major allergen content similar to that of standardized pollens (Table 6). This information would suggest that, at maintenance, a 1 to 10 dilution of the maximum concentration commercially available should be effective. Extracts that are less potent cannot be diluted to the same degree. Examples include cat dander and house dust mite extracts, for which substantially larger amounts of their concentrate must be added to the maintenance vaccine to provide adequate potency compared with ragweed or timothy. In the case of the very weak, nonstandardized extracts, such as dog dander (other than A-P dog) and various fungi (Table 6) and cockroach, it would be very difficult to deliver a major allergen dose in the range that has been shown to be clinically effective for other allergen extracts. The potencies of the 50% glycerin concentrate of a number of commercial extracts of German cockroach were found to be 10 to 782 BAU/mL and of American cockroach to be 10 to 250 BAU/mL (36). It is unlikely that effective doses can be attained in vaccines with many of these weak extracts. An example of a representative prescription for a maintenance vaccine containing nonstandardized vaccines is given in Table 7.

Botanical Relationships and Cross-Allergenicity

In formulating the example of a maintenance vaccine (Table 7), the calculated amount of each dust mite is reduced by half. This reflects the high degree of cross-allergenicity between these

Table 8 Patterns of Botanical Cross-Allergenicity

There is rarely significant cross-allergenicity between families.
 There is generally a degree of cross-allergenicity between tribes or genera of a family, but this is variable.
 There is generally a high degree of cross-allergenicity between species of the same genus.

Table 9 Patterns of Significant Cross-allergenicity Among the Tree Pollen

Birch family (39)
Birch
Alder
Hazelnut
Hornbeam
Olive family (40)
European olive
Ash
Privet
Russian olive (unrelated)
Cupressaceae (cypress) family (38)
Cedar
Cypress
Juniper
Arbor vitae
Fagaceae family (38)
Beech
Oak
Genus <i>Carya</i> (38)
Pecan
Hickory
Genus <i>Populus</i> (38)
Poplar
Aspen
Cottonwood

two species of *Dermatophagoides*. Cross-allergenicity among closely related plant pollen is also the rule (Table 8). If these relationships are not recognized, allergen vaccine mixtures may contain excessive amounts of some groups of allergens. This is likely to occur with the grasses, since most of the prevalent species in the United States fall into two non-cross-reacting botanical subfamilies (37), the first is northern pasture grasses typified by timothy and the second is Bermuda and related grasses. Other important cross-reacting groups are the individual members of the Ambrosia subtribe, the *Artemisia* genus, the Chenopod-Amaranth families of weeds, and members of certain tree groups, such as the genus *Populus* containing aspen, poplar, and cottonwood species and the junipers and cedars of the family Cupressaceae (38).

Patterns of Cross-Allergenicity

Trees. Among the trees there are few cases of cross-allergenicity sufficiently strong to restrict inclusion to only one representative in an allergen tree vaccine mix. These are listed in Table 9.

Grasses. Two non-cross-reacting subfamilies of grasses have been recognized. They are represented by the northern pasture grasses and Bermuda with its cross-reacting native prairie grasses (37). Each should be treated separately, using either timothy or a mixture for the northern pasture grasses and Bermuda for the other. There are also some regional grasses such as Bahia and Johnson that are in distinct subfamilies. Although they share allergens with the northern pasture grasses (Table 10), if locally important, they should be added as an additional component of the grass vaccine.

Immunotherapy with timothy and Bermuda grasses resulted in equivalent suppression of prick skin test reactivity to 10 different grasses from all three of the subfamilies listed in Table 10 (41). If more than one member of each of these subfamilies of grasses is to be included in a vaccine, the amount of each grass should be reduced to compensate for the marked cross-allergenicity.

Table 10 Botanical and Allergenic Relationships Among the Grasses

Festucoideae: Northern pasture grasses—orchard, timothy, June, red top, etc.
Eragrostoideae: Bermuda grass, grama, several western prairie grasses
Panicoideae: Bahia, Johnson

Source: From Ref. 39.

Table 11 Botanical and Allergenic Relationships Among the Weeds

Ambrosia
 Ragweeds
 Cocklebur
 Burweed
Artemesia
 Sages
 Wormwood
 Mugworts
Chenopods
 Russian thistle
 Kochia (burning bush)
 Lambs quarters
 Atriplex
Amaranth
 Pigweed
 Palmer's amaranth
 Western water hemp

Source: From Ref. 38.

Weeds. There are three major groups of weeds (Table 11). Two are in the *Compositae* family, the Ambrosia, which includes the ragweeds and related species, and the Artemesia, which includes the sages, wormwoods, and mugworts. The Chenopod-Amaranth families include many of the prominent weeds of the Western United States. The major ragweeds (short, giant, western, and false) are strongly cross-reactive, whereas southern and slender ragweeds are allergenically distinct (42). The locally most important of the four major ragweeds or a mixture should be used for treatment (43). There is no clinically important cross-allergenicity of the ragweeds with other members of the Ambrosia tribe, such as cocklebur and burweed, nor is there significant cross-reactivity between ragweeds and the other clinically significant group in the *Compositae* family, the *Artemesia* (43). Within the *Artemesia*, however, there is strong cross-reactivity and one representative species should suffice for treatment (43). The Chenopod-Amaranth families, which share some allergenicity, are best viewed as three groups: the *Atriplex* and the Amaranths, both of which are strongly cross-reactive, and the Chenopods, which share some allergens. If several species of Chenopods are locally important (e.g., Russian thistle and *Kochia*), it is better to include them as a mix rather than using one representative species to cover the whole family. Locally important weeds such as sorrel, dock, and plantain should be treated as distinct allergens (38).

House dust mites. The house dust mites, *D. pteronyssinus* and *D. farina*, are strongly cross-reactive (44). A mix of both major species is probably best employed if both are locally important.

Components of Allergen Vaccines That May Have a Deleterious Effect on Other Extracts with Which They Are Mixed in a Vaccine

Some extracts of pollen (45,46) contain enzymes that may cause autodigestion and contribute to loss of potency of a vaccine. Extracts of a number of fungi (molds) and insects contain proteases that are capable of degrading allergenic proteins in other extracts with which they may be mixed in a vaccine (47–50) (Table 12). Major allergens in American cockroach and house dust mites are gut derived and very likely are digestive enzymes (47,51). However, protease activity is lower in American house dust mite extracts than in European, since only mite bodies are extracted and not spent culture medium containing fecal particles (Robert Esch, Greer laboratories, personal communication). Detectable trypsin-like proteolytic activity is absent from extracts derived from animal dander and pollen (47) (Table 12).

Table 12 Protease Content of Allergen Extracts

Extract	Protease content ^a	Potency of ryegrass extract
Pollen		
Sagebrush	<1	1.18
Ragweed	<1	0.70
Oak	<1	0.89
Epithelia		
Cat epithelia	<1	0.95
Dog epithelia	<1	0.90
Insects/mites		
<i>D. pteronyssinus</i>	14 ^b	0.86
<i>D. farinae</i>	24 ^b	0.44
<i>P. americana</i>	168	0.17
Fungi		
<i>Alternaria alternata</i>	29	0.22
<i>Penicillium notatum</i>	242	0.19

The extract listed in the first column was mixed with perennial ryegrass extract and stored at 4°C for one month. Potency of the ryegrass extract was compared with a reference preparation of 1.0. Potency of ryegrass was determined by IgE ELISA inhibition.

^aµg Trypsin equivalent units/mL.

^bU.S. house dust mite extracts are derived from washed mite bodies without spent culture medium and contain less protease activity than is typical for European extracts.

Source: From Ref. 47.

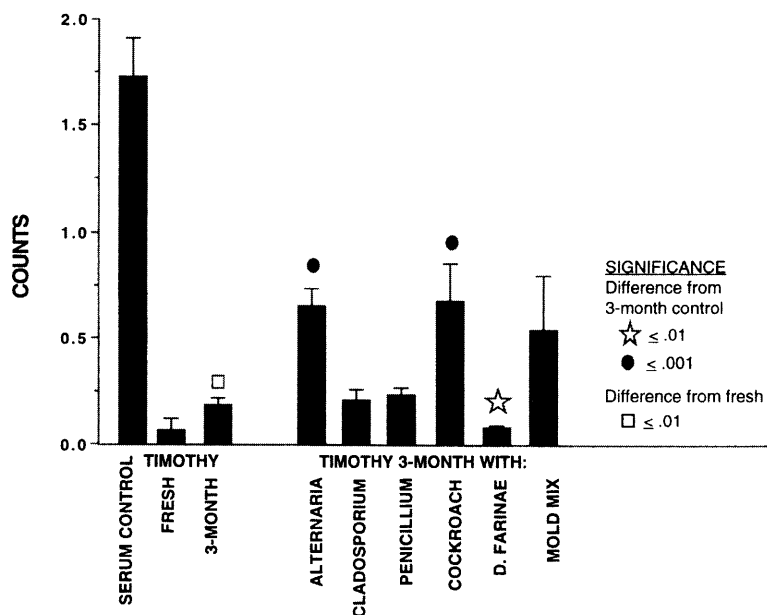


Figure 1 Stability of timothy grass alone and in mixtures. The potency of a 10-fold dilution of timothy grass stored under different conditions was compared by ELISA inhibition to that of a freshly diluted aliquot. After three months the diluted timothy extract had a significant decrease in potency compared with the fresh. In addition, those aliquots of timothy stored in combination with *Alternaria*, cockroach, and the mixture of *Alternaria*, *Cladosporium*, *Penicillium*, and cockroach showed significantly greater loss of potency than the timothy extract stored alone.

Grass pollen extracts are uniquely susceptible to these proteolytic enzymes (Fig. 1) (47,49,50,52). Birch pollen lost 70% of its allergenic potency over a period of 60 days when mixed with *Fusarium* (50). In a systematic assessment (Table 13), a number of pollen and animal dander extracts lost potency when mixed with one or more protease-containing extracts, while others were quite resistant (Fig. 2) (52). As illustrated in Table 13, the effect of mixing allergen extracts with potential protease-containing extracts is variable. *Alternaria*

Table 13 Effects of Mixing Extracts on Allergen Vaccines

Extract	Alt	Clad	PCN	CR	Mix	Mite	Overall <i>p</i>
Timothy	+	-	-	+	+	-	<0.0001
Bermuda	+	-	-	-	-	-	<0.0001
Short ragweed	-	-	-	-	-	-	0.64
Russian thistle							
1st	-	-	-	+	+	-	<0.0001
2nd	-	-	-	-	+	-	<0.01
White oak							
1st	+	-	-	-	-	-	<0.01
2nd	-	-	-	-	+	-	<0.02
Box elder							
1st	+	-	-	+	+	-	<0.0001
2nd	-	-	-	-	-	-	0.02
<i>D. farinae</i>							
1st	-	-	-	-	-	-	<0.01
2nd	-	ND	-	ND	ND	ND	0.49
Cat							
1st	-	+	-	-	-	-	<0.002
2nd	+	-	ND	ND	ND	ND	<0.001

The reference extracts listed on the ordinate were stored at a 10-fold dilution of the most concentrated available for three months either diluted in HSA-saline or combined with the extracts listed across the top of the Table. After three months, the residual allergenic activity of the reference allergen extract in the mixes was compared with that of the same extract stored alone. The *p* value is the overall difference among the seven conditions of storage (alone and six different combinations with other allergenic extracts). A (+) indicates significant degradation of the reference allergen extract due to mixing.

Abbreviations: Alt, *Alternaria*, Clad, *Cladosporium*, PCN, *Penicillium*, CR, cockroach, Mix, mixture of Alt, Clad, PCN, and CR. Mite, house dust mite; 1st, first of two studies with the same combinations; 2nd, second study; +, $p < 0.05$; -, $p > 0.05$; ND, not done.

Source: From Ref. 52.

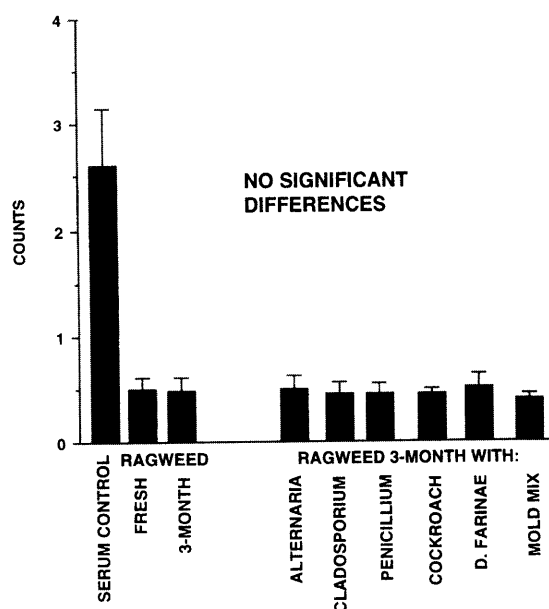


Figure 2 Stability of short ragweed alone and in mixtures. The potency of a 10-fold dilution of short ragweed stored under different conditions was compared by ELISA inhibition to that of a freshly diluted aliquot. After three months the diluted short ragweed extract and those aliquots of ragweed stored in combination with *Alternaria*, *Penicillium*, *Cladosporium*, and cockroach alone and in combination were all equal in potency to the freshly diluted aliquot of short ragweed.

significantly reduced the potency of five of eight extracts, cockroach reduced the potency of three of eight, while *Cladosporium* reduced the potency of only one extract. *Cladosporium* and cockroach reduced the potency of some extracts that were not affected by *Alternaria*. Furthermore, the effect of *Alternaria* extracts was inconsistent from lot to lot, suggesting varying quantities of protease activity were present in different lots of *Alternaria* extract.

The extracts which have deleterious effects on the potency of other extracts include *Alternaria* (52,53), *Cladosporium* (52,53), cockroach (49,52,53), *Helminthosporium* (49), *Penicillium* (47,53), *Aspergillus* (47,53), *Fusarium* (50), *Bipolaris* (53), and *Epicoccum* (53). House dust mite extracts have had no effect on other extracts (52,53) despite their content of proteases (47). This possibly results from their having low protease activity and having been tested in a diluent containing glycerin (52,53). While no single inhibitor will protect against all proteases (48), glycerin has been shown to have protective effects against some (47,53).

The degree of loss of potency due to mixing extracts in a vaccine may be marked. Allergenic activity of perennial ryegrass was reduced to 4% by mixing with *Helminthosporium* and to 11% by mixing with cockroach (49). Over 50% of timothy grass extract potency was lost within three days of being mixed with *Fusarium* (50). Some allergenic activity always remains suggesting that not all allergens in these extracts are susceptible to proteolytic digestion. On mixing with *Fusarium*, Bet v 6 and Phl p 5 were almost entirely degraded, while Bet v 1 and Phl p 1 remained relatively stable (50). However, even though there may be a significant amount of overall allergenic activity remaining, the selective reduction in certain allergens will make the vaccine less suitable for treatment and may place the patient at risk when they are treated with a freshly prepared vaccine that contains allergens no longer present in the mix that they had been receiving for immunotherapy. The results of testing of the effects of mixing extracts are not always consistent. Ragweed has been reported to be resistant (52) and susceptible (53), cat dander extract has been reported to be susceptible (52) and resistant (53), while mixing with *Fusarium* has been reported to cause loss of potency (50) and to have no effect (53). These differing results may reflect the marked variability in the composition of fungal and cockroach extracts and differing methods of assessing loss of potency.

In summary, many pollen and animal dander extracts are susceptible to accelerated loss of potency when mixed in a vaccine with protease-containing extracts. Despite the variable reports, the safest practice is to not include pollen and dander extracts in a vaccine with cockroach or fungal extracts. House dust mite extracts in even 10% glycerin appear to be neither susceptible to exogenous proteases nor to cause loss of potency due to their protease content (52,53). Degradation of fungal extracts by proteases in other fungal extracts when contained in a vaccine has not been demonstrated but has not been extensively investigated. Mixing cockroach, *Alternaria*, *Cladosporium*, and *Penicillium* extracts did not further reduce their potency in a vaccine (52). It is probable that the proteins susceptible to protease digestion had already been degraded by proteases in their own extract.

Diluents Employed in Mixing Allergen Vaccines

Because allergen extracts tend to lose potency with time, an effect that is enhanced by storing at higher temperatures and greater dilutions, a number of substances have been added to extracts both to preserve potency and prevent growth of microorganisms. The most effective preservation of extract integrity and potency is not by adding a preservative but by lyophilization (54,55). This is not routinely employed because the lyophilization process adds to the cost of the extracts.

Glycerin

Glycerin is the most effective preservative for allergen extracts (56). It is very effective at a 50% concentration. At this concentration it inhibits some, but not all, proteolytic enzymes (47,50,55). This may contribute to but does not completely explain its effectiveness as a preservative. Decreasing effectiveness as a preservative has been demonstrated with 25% and 10% concentrations of glycerin (53,56,57); however, even 10% glycerin is as effective as 0.03% human serum albumin. It is possible that the presence of glycerin accounts for the lack of proteolytic degradation of pollen extracts by the house dust mite extracts in several mixing studies (49,52,53).

Human Serum Albumin

The preservative effect of human serum albumin is likely due to reduction of adsorption of allergenic proteins to the vial surface (58) and protection of allergenic proteins from phenol denaturation (59). Human serum albumin has not been shown to have protective effects

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against proteolytic enzymes (47). Similar degrees of preservative effect were found with concentrations of 0.03%, 0.1%, and 1.0% human serum albumin (56).

Concern has been expressed that patients may become sensitized by repeated injections of human serum albumin possibly altered or aggregated in commercial processing (60). However, no cases of sensitization to human serum albumin in allergen vaccines has been reported, and one study which looked for evidence of positive skin tests or IgG antibodies directed toward human serum albumin was negative (61).

Phenol

Phenol is added to multidose vials of allergen extracts to prevent growth of microorganisms. Phenol denatures proteins, including those in allergen extracts (54) and the deleterious effect of phenol increases with increasing allergen dilutions (56). Phenol degrades extracts in vaccines that are in 50% glycerin solutions (59,62). Human serum albumin is relatively more protective than glycerin against the effect of phenol on extract potency (59,62).

Others

A number of other approaches to preserve extract potency have been suggested but have not found wide acceptance. Siliconization of vials has been suggested to decrease adsorption of proteins to their surface. Testing this method revealed it to be without effect (56). Polysorbate 80 in concentrations of 0.002% to 0.2% had a slight effect in preserving potency, but it was less effective than human serum albumin (56).

Epsilon-aminocaproic acid (EACA) has been suggested as a preservative (60) since pollens are known to contain enzymatic activity that may contribute to their loss of potency and EACA is a potent enzyme inhibitor. However, EACA was found to be ineffective against a variety of fungal proteases (47). In studies on preservation of extract potency, EACA was found to be less effective than glycerin (60). EACA was found to have less protective effect than human serum albumin on potency over the short term. This observation may be due to human serum albumin reducing adsorption of allergenic proteins to the walls of the vial whereas EACA provides protection from prolonged thermal denaturation (60).

In the absence of preservatives, extracts stored in saline buffered with bicarbonate lost potency to a greater extent than those stored in phosphate buffered saline or normal saline (55,57).

Mixing Extracts to Constitute a Vaccine

Extracts which are stored combined with several other extracts retain their potency to a greater extent than the same extract, at the same dilution, stored alone (57). This preservative effect is probably related to total protein content. In this instance, the proteins in the other extracts are functioning in a manner analogous to human serum albumin when combined in a vaccine.

CONDITIONS OF STORAGE

Maintenance of potency of a therapeutic allergen vaccine is a function of the dilution, the diluent, the effectiveness of preservatives, the temperature of storage, and the presence of proteolytic enzymes. The processes that lead to loss of vaccine potency and the measures that can be used to reduce the effect are given in Table 14.

Temperature

Allergen extracts and vaccines are susceptible to loss of potency if maintained at room rather than refrigerator temperature (57,62). Loss of activity with storage at room temperatures is likely

Table 14 Mechanisms of Loss of Potency of Allergenic Extracts

Mechanism	Favored by	Avoidance by
Adsorption	High dilution, high surface to volume ratio	Human serum albumin, glycerin
Thermal denaturation	High temperature	Storage at low temperature
Enzymatic autodigestion	Enzymes in extract	Glycerin, storage at low temperature

adsorption of from phenol ctive effects

caused by the proteases (54) while loss of potency with brief exposure to higher temperatures is probably related to heat lability of some of the allergenic proteins (63). Some extracts, such as cat (62), have been reported to be relatively resistant to this thermal lability effect. Other extracts, including white ash, elm, orchard grass, Bermuda grass (64), ragweed (63), and house dust mites (62), lose potency at high temperature (25°C–100°C). Since the loss of potency is a result of either protease susceptible or heat labile proteins, the stored extract will have an altered pattern of specificity due to the preferential persistence of the resistant proteins, resulting in an altered pattern of skin test reactivity and potential therapeutic efficacy (63).

Less extreme temperature exposure of allergen extracts and vaccines, such as exposure to room temperature for 13 hr/wk, resulted in significant loss of potency (57). The effect on allergen potency of repeated freezing and thawing has not been extensively studied but reports of reduced potency of ragweed (45) and dilute *Lolium perenne* (60) extracts are available.

Dilution

Extracts and vaccines are more susceptible to loss of potency when stored diluted rather than concentrated (52,56). The increased susceptibility of diluted extract is likely the result of lesser protein content and hence relatively greater adsorption of allergens to the container wall (54,57,58). However, addition of human serum albumin does not completely prevent this loss and all allergen extracts are not equally susceptible to this effect (52), suggesting other factors may be involved.

Occasional studies of diluted extracts report unexpectedly preserved potency for prolonged periods. Thus, intradermal skin test concentrations of timothy, birch, cat, and house dust mite preserved with human serum albumin showed preserved potency after 24 months' storage at 6°C (62). The explanation for these apparently aberrant results is unclear but may relate to the pattern of sensitivity of the population studied.

Loss of potency is related to the total protein content of the extract or vaccine. Not entirely filling a vial of extract has been reported to enhance loss of potency due to the greater surface area relative to the volume of solution from which protein is available for adsorption. This effect is diminished by including other extracts in a vaccine, thus increasing the total protein content (57). The same protective effect can be achieved by added extraneous protein, such as human serum albumin (57,58).

PATTERNS OF LOSS OF POTENCY

Assessment

A variety of methods have been employed to assess the residual potency of allergenic extracts (56). The two approaches most commonly employed, the RAST or ELISA inhibition (57) and skin testing (65), have been reported to yield similar results. In occasional studies, residual activity has been greater by skin testing than by RAST inhibition (54,55). However, a careful comparison of titrated intradermal skin testing and ELISA-inhibition yielded very similar results with multiple allergen extracts (49), suggesting that, properly done, the results with the two methods are comparable.

Individual Allergens

The stability of allergen extracts and vaccines to degradation can vary due to differing heat susceptibility of their components, different total protein content affecting the percent adsorbed to the container wall, and the content of proteolytic enzymes that may cause autodigestion (Figs. 3, 4). Stability varies with the addition of phenol as well as the presence of proteases in extracts used for the vaccine. As would be expected, studies have shown differing loss of potency for different extracts stored under similar conditions. Therefore, it is best to follow general principles that will protect the potency of the most susceptible extracts. These include (i) avoiding mixing fungal and insect extracts with pollen and dander extracts in formulating a vaccine (an exception appears to be house dust mite extracts), (ii) keeping the total protein content high by using concentrated extracts and adding human serum albumin to dilutions, and (iii) keeping the extracts and vaccines at refrigerator temperatures except when actually being used (for dilute vaccines left at room temperature consider using a refrigerated tray when exposed to room temperatures).

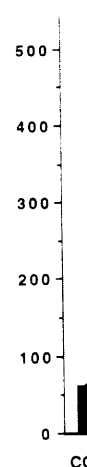


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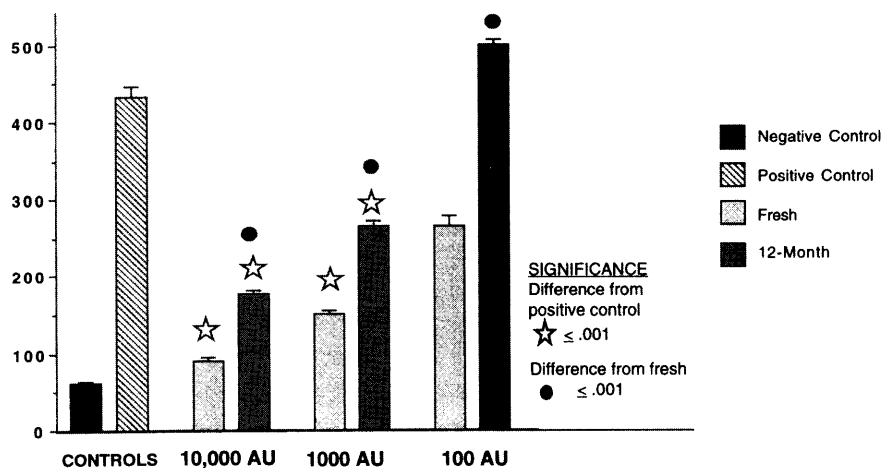


Figure 3 The potency of Bermuda grass extract stored at 4°C in concentrations of 100 AU/mL, 1000 AU/mL, and 10,000 AU/mL was compared after 12 months by ELISA inhibition to freshly diluted aliquots of the same Bermuda extract. There was, as indicated, significant loss of potency after 12 months in all dilutions. Negative control contained the Bermuda disc but no serum. Positive control contained Bermuda disc and mixed grass-allergic patients serum, but no Bermuda extract, while the tested aliquots contained Bermuda disc, Bermuda-allergic serum and dilutions of Bermuda extract.

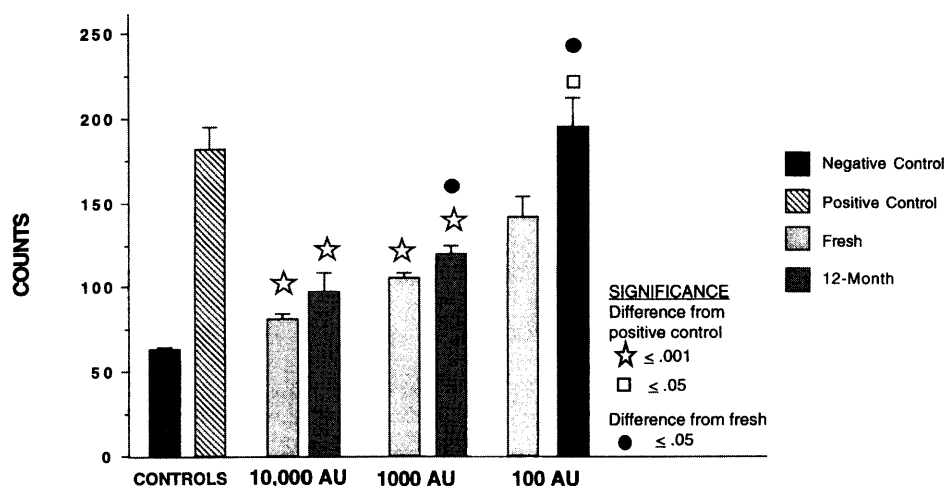


Figure 4 The potency of short ragweed extract stored at 4°C in concentrations of 100 AU/mL, 1000 AU/mL, and 10,000 AU/mL was compared after 12 months by ELISA inhibition to freshly diluted aliquots of the same short ragweed extract. There was no loss of potency after 12 months in the 10,000 AU/mL aliquot, but there was a significant loss of potency in the other two dilutions. Negative control contained the short ragweed disc but no serum. Positive control contained short ragweed disc and mixed ragweed-allergic patients serum, but no short ragweed extract, while the tested aliquots contained short ragweed disc, ragweed-allergic serum, and dilutions of short ragweed extract.

With attention to these details, some diluted vaccines will lose potency even after three months at concentrations used for maintenance immunotherapy (Figs. 1, 3), whereas others will not lose any potency at the same dilution over a year (Figs. 2, 4). Full strength extracts and vaccines are probably stable at refrigerator temperatures for their stated shelf-life. Full-strength extracts in 50% glycerin, as used for prick skin testing, are consistently stable until their expiration date (56). Diluted extracts, used for intradermal skin testing, have been found to be stable for prolonged periods by some investigators (62), but not by others (56). Some allergen extracts are susceptible to rapid loss of potency at high temperatures (25°C–100°C) (62–64), but the loss with exposure to room temperature is not rapid (57). It is unlikely that shipping

allergen extracts and vaccines through the mail would result in exposure to temperatures that would be deleterious.

SALIENT POINTS

- Standardized extracts of cat pelt and hair, house dust mites, short ragweed, eight grasses, and the venoms of four Hymenoptera are commercially available in the United States.
- Neither of the two expressions for potency used for nonstandardized extracts, weight by volume or protein nitrogen units, adequately reflects allergenic potency of a vaccine.
- There are two immunotherapy studies that relate dose to outcome employing nonstandardized vaccines. In one study employing multiple allergens, the outcome with 1:250 wt/vol vaccines was better than that with 1:5000 wt/vol. In another study, results with 1:50 wt/vol ragweed vaccine were significantly better than with 1:1000 wt/vol.
- Examples of representative values for the major allergen content of U.S. standardized vaccines are listed in Table 4. However, the major allergen contents in Table 4 are only representative and may vary significantly from one manufacturer to another.
- Placebo-controlled studies that demonstrated clinical effectiveness have been performed with the extracts that are standardized in the United States (house dust mites, cat dander, grass, and ragweed). In each instance, the effective dose of major allergen has been in the range of 7 to 20 μ g (Table 3).
- The information in Tables 3 and 4 allows formulation of an allergen vaccine mixture containing concentrations of the major allergens that approximate those that have been proven to be clinically effective (Table 5).
- Vaccines should contain effective quantities of each aeroallergen. If two or more components of the allergen mixture cross-react, the amount of each should be decreased so that the sum of the cross-reacting aeroallergens is equal to the optimal effective dose for a single allergen of the mixture.
- Most fungal and cockroach extracts contain proteases that are capable of degrading allergenic proteins contained in other extracts when constituted together in a vaccine. Therefore, mixing fungal and cockroach extracts with pollen or dander extracts in a vaccine is to be avoided (Table 12).
- Degradation of allergen extracts and vaccines is increased by dilution and by the time they are maintained at room temperature.
- Glycerin is the most effective preservative but is poorly tolerated by injection at 50% solution. Ten percent and 20% glycerin solutions cause some brief discomfort, but are usually tolerated. Human serum albumin is less effective but well tolerated. Glycerin or human serum albumin should be included in all dilute vaccines.

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