

Immunotherapy with short ragweed fraction A:D-glutamic acid:D-lysine polymer in ragweed hay fever

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We report the first human trial of immunotherapy employing the nonimmunogenic carrier, D-glutamic acid:D-lysine linked to short ragweed (SRW) fraction A (fraction A:D-GL). Twelve SRW-sensitive patients with no immunotherapy during the previous 19 yr received a 2-mo (7/79 to 9/79) course of fraction A:D-GL (average dose 49.5 mg, range 21 to 78 mg). We compared their symptom scores and serologic changes with two control groups of SRW-sensitive patients. Patients receiving fraction A:D-GL demonstrated at least a tenfold decrease in skin-test sensitivity to SRW and had statistically lower mean seasonal symptom scores ($p < 0.02$) than untreated controls. Mean seasonal symptom scores did not differ statistically from those of control patients on year 4 of immunotherapy. In contrast to the expected suppression of IgE, we found that fraction A:D-GL stimulated both IgE and IgG responses to SRW and SRW-antigen E. These increases in IgE and IgG antibodies were significantly greater than in the control groups and appeared to be due largely to injection of fraction A:D-GL. Though fraction A:D-GL was generally well tolerated, we noted mild generalized urticaria in three patients, and large local reactions in five others. The differences between our results and the earlier results in mice may reside in the particular characteristics of this preparation of fraction A:D-GL.

The interaction of short ragweed (SRW) pollen and mast cell-fixed antibody leads to the immunologic release of the mediators of anaphylaxis and results in the clinical symptoms of hay fever. The efficacy of conventional immunotherapy has been ascribed to a combination of immunologic effects on the host's response to antigen, including a blunting of the seasonal rise in IgE antibody and an increase in blocking IgG antibody.¹⁻⁵

Poly:D-glutamic acid:D-lysine (D-GL), a copolymer of molecular weight approximately 50,000, is nonimmunogenic in various animals and man.⁶⁻⁸ Animal experiments indicate that injection of a hapten or a purified protein coupled to D-GL will induce

suppression of the IgE response to the hapten or antigen.⁹⁻¹⁸ The suppression is antigen specific, relatively long-lasting, and develops whether or not sensitization has already occurred.

We evaluated the safety and efficacy of immunotherapy with SRW fraction A¹⁹ linked to D-GL (fraction A:D-GL) to induce reaginic suppression in a group of SRW-sensitive patients. We measured serum levels of IgE and IgG antibodies to SRW and SRW-antigen E (AgE) in these patients, as well as in a group of patients receiving their fourth year of parenteral immunotherapy with aqueous SRW, and in nonimmunized SRW-sensitive patients. We found statistically significant increases in both IgE and IgG antibodies to SRW and AgE in fraction A:D-GL-treated patients compared with the two control groups.

MATERIALS AND METHODS

Immunotherapeutic drugs

Fraction A:D-GL. Fraction A:D-GL (Lot 6-79) was provided as a sterile off-white lyophilized powder by Miles Laboratories (Elkhart, Ind.). Fraction A was prepared from SRW pollen, *Ambrosia elatior* (Greer Laboratories, Lenoir, N. C.), by precipitation with ammonium sulfate and gel

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filtration over a Sephadex G-25 column. Fraction A was conjugated to D-GL by reacting m-maleimidobenzoyl-fraction A with S-acetyl biotin:D-GL in the presence of hydroxylamine. The fraction A:D-GL conjugate was purified by affinity chromatography over an avidin-Sepharose 4B column, a method that has been shown to yield conjugates free of detectable nonconjugated protein.¹⁵ AgE content was determined by the Mancini technique. One microgram AgE equivalence was equal to 630 μ g of fraction A:D-GL as assayed by Miles Laboratories. The copolymer of D-GL has a molecular weight on average of at least 50,000 and a molar ratio of glutamic acid:lysine of 6:4. Tolerogenic activity of the compound used (Lot 6-79) was determined in an animal test system. Briefly, CAF₁ mice were either given fraction A:D-GL conjugate, 500 μ g, administered by intraperitoneal injection four times at 3-day intervals (days -12, -9, -6, -3), or phosphate-buffered saline (PBS) of equal volume to that used with the tolerogen. Animals were immunized with 10 μ g fraction A with 1 mg alum on days 0 and 21 and were bled by retroorbital puncture on days 10, 14, 21, 28, and 31. IgE was determined by passive cutaneous anaphylaxis (PCA) in rats by challenge with 10 mg of fraction A. Miles Laboratories determined the stability of a separate lot (Lot 4-24-79) of fraction A:D-GL polymer by radial immunodiffusion for AgE content and by thin-layer chromatographic (TLC) analysis. Lot 4-24-79 was prepared in the same fashion as the one used in this study. AgE content remained stable at 40°C for 14 days, and no breakdown of the conjugate was found by TLC. Fraction A:D-GL was diluted in Sorensen's phosphate buffer containing 0.5% albumin for use in the present study.

SRW. One group of control patients received monthly injections of aqueous SRW extract (Greer Labs., Lenoir, N. C.) Lot No. L56-38, diluted to 10,000 PNU/ml with Sorensen's phosphate buffer containing 0.5% albumin and 0.4% phenol.

Experimental design

Patients were drawn from a pool of SRW-sensitive individuals who had responded to local notices for volunteers. All had participated in previous studies of SRW hay fever at the Mayo Clinic.

The group receiving fraction A:D-GL included 12 patients, 18 yr of age and older, who had positive histories of ragweed hay fever and positive skin tests to SRW at a concentration of 1 PNU/ml (1×10^{-5} mg/ml AgE equivalence). All patients were skin-test negative to the coseasonal allergen, *Alternaria*, at a concentration of 100 PNU/ml. Women of childbearing potential, patients with a history of connective tissue disease, and patients who had received immunosuppressive therapy or systemic corticosteroids within the past year were excluded. Only one patient had ever received immunotherapy previously (1960). Informed consent was obtained from each patient prior to study. The medical histories of all patients receiving fraction A:D-GL were reviewed, and each patient was given a complete physical examination prior to treatment (7/79) and at cessation of treatment (9/79). Blood and urine tests were performed

TABLE I. Clinical characteristics of the three study groups

	Fraction A:D-GL	Aqueous SRW	No treatment
Number	12	13	14
Age (yr)			
Mean	37	47	46
Range	19-51	28-73	24-66
Sex			
Male	9	9	6
Female	3	4	8

prior to the study (7/79), at the termination of injections (9/79), and 3 wk following cessation of fraction A:D-GL treatment (10/79). These included Na, K, Ca, PO₄, total protein, glucose, alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT), total/direct bilirubin, uric acid, creatinine, creatine kinase, white blood cell count and differential, platelet count, hemoglobin/hematocrit, and urinalysis. Immunologic studies included measurement of total IgG, IgA, IgM, IgE, and of IgE and IgG antibodies to SRW, AgE, and D-GL.

Patients receiving fraction A:D-GL were skin tested intradermally with serial tenfold concentrations of SRW and fraction A:D-GL solutions beginning at 1×10^{-8} mg/ml AgE equivalence. Skin tests using diluent, histamine (1 mg/ml), and *Alternaria* (100 PNU/ml) were also performed. Skin tests were repeated at cessation of immunotherapy (9/79) and 3 wk following treatment (10/79).

Patients maintained daily symptom diaries, semiquantitatively grading sneezing, stuffy nose, runny nose, red itchy eyes, itchy nose/itchy throat, and cough/wheeze. In addition, chlorpheniramine maleate, 4 mg, was supplied, and the number of tablets taken recorded.

Control groups consisted of 14 patients with seasonal SRW hay fever not receiving immunotherapy, and 13 SRW-sensitive patients on their fourth year of standard immunotherapy with aqueous SRW at maximal tolerated doses (average monthly dose 8,538 PNU). The clinical characteristics of the three study groups are listed in Table I. Both control groups were tested in July and October for total IgE, IgE to SRW and AgE, and IgG to SRW and AgE. They kept symptom diaries and medication counts. Neither control group was skin tested during the study.

Immunotherapy with SRW-D-GL

The initial dose of fraction A:D-GL was 0.1 ml of the dilution giving a 5 $\times$ 5-mm wheal by intradermal testing. Starting doses were either 0.1 ml of a 1×10^{-8} mg/ml or a 1×10^{-7} mg/ml AgE equivalence; these doses contained 6.3×10^{-7} and 6.3×10^{-6} mg of fraction A:D-GL, respectively. Each patient was seen twice weekly and at each visit received two injections, each followed by a 30-min observation period. Patients tolerating the initial 0.1-ml injection were given 0.2 ml of the same dilution in the opposite arm and observed for 30 min. On the second weekly

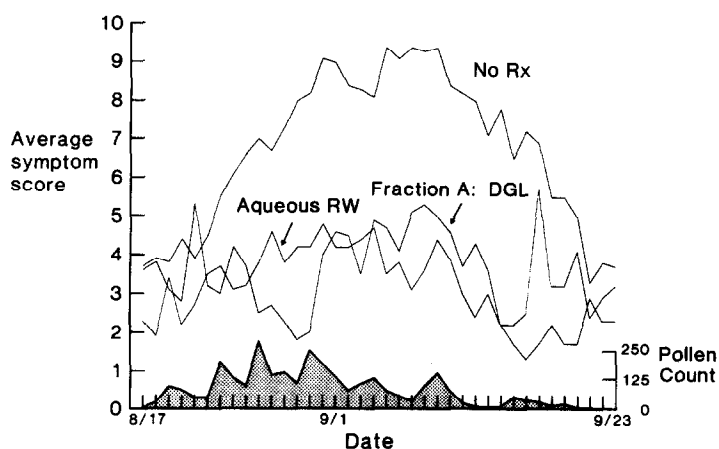


FIG. 1. Average daily symptom scores for fraction A:D-GL, aqueous SRW, and untreated groups. The daily pollen counts (8/17/79 to 9/23/79) are indicated by the shaded area.

TABLE II. Total dose of fraction A:D-GL and side effects (7/79 to 10/79)

Patient No.*	Fraction A:D-GL total dose (mg)	Side effects
11	21	Local swelling
9	21	Urticaria, local swelling
5	26	Urticaria, local swelling
1	27	Local swelling
8	34	Urticaria, local swelling
7	52	Local swelling
2	52	Local swelling
13	65	None
3	71	Local swelling
6	71	Local swelling
10	78	None
12	78	None

*Patient 4 dropped from study.

visit, side effects were reviewed and unless contraindicated, a dose of 0.4 ml of the initial dilution was given, and if no adverse reaction occurred, 0.1 ml of a tenfold higher concentration was injected after 30 min. Each patient, therefore, received doubling doses of fraction A:D-GL approximately twice weekly. Dosage modification was made as indicated by local or systemic side effects. When 2×10^{-2} mg AgE equivalence (12.6 mg fraction A:D-GL) was reached, dosage was held constant. The total time of therapy was 2 mo, from July 19 through September 21, 1979.

Pollen counts

Pollen counts were monitored daily through the study period using a rotoslide sampler.²⁰

Immunologic tests

Total IgG, IgA, and IgM levels in all patients were measured by laser nephelometry²¹; total IgE levels were mea-

sured by radioimmunoassay.²² IgE antibody levels to SRW, AgE, and D-GL were measured by the radioallergosorbent test (RAST) as described previously.²³ IgG antibodies to SRW and AgE were measured by a RAST interference procedure.²⁴

IgG antibodies to D-GL were assayed by two methods. IgG antibodies were first measured by radioimmunoprecipitation. Briefly, D-GL was radiolabeled with ¹²⁵I by the Bolton-Hunter method²⁵ and free ¹²⁵I was removed by dialysis. Over 80% of the radioactivity was precipitated with 10% phosphotungstic acid. The radiolabeled D-GL was then used in a radioimmunoprecipitation assay similar to that described previously.²⁶ In the second method, 0.05 ml of a solid-phase D-GL suspension was incubated overnight with 0.05 ml of test serum. After two washes we added 5 ng of radiolabeled staphylococcal protein A (Pharmacia Fine Chemicals, Piscataway, N. J.) followed by a second incubation. After two washes, radioactivity bound was determined and compared with sera from nonatopic patients, with sera from untreated SRW-allergic patients, and with sera from SRW-sensitive patients on their fourth year of immunotherapy.

Statistical analysis

Mean and total symptom scores were determined for each group and analyzed for significance by the t test. Mann-Whitney rank sum correlation was done to compare initial levels and changes in IgG and IgE antibody levels between groups.²⁷

RESULTS

The injections of fraction A:D-GL were generally well tolerated. Mean total dose injected was 49.5 mg (0.79 mg AgE equivalence), range 21 to 78 mg (0.335 to 1.25 mg AgE). Major side effects consisted of mild episodes of generalized urticaria in three patients (Table II). Minor side effects in five patients consisted of edema, redness, and pain at the site of injection,

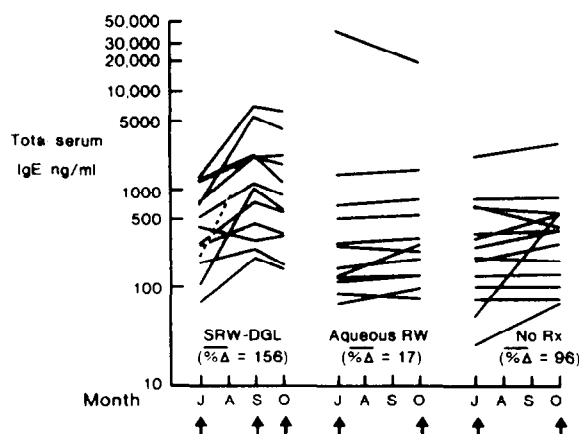


FIG. 2. Changes in levels of total serum IgE and mean percent change (% Δ) in total serum IgE for each of the three groups, July through October, 1979. In this and the following figures the fraction A: D-GL patient leaving after 1 mo of therapy but prior to SRW pollination is indicated by the dotted line. Arrows indicate the sampling dates for each group; the fraction A: D-GL patients were bled three times (July, September, October); the controls were bled twice (July, October).

generally subsiding within 24 hr. No patient discontinued therapy because of side effects. There was no correlation between dose received and occurrence of urticaria. Patient No. 4 of the fraction A: D-GL group left the study in mid-August just prior to ragweed pollination. His symptom scores and skin-test data are not reported. However, his serologic parameters, represented by a dotted line in Figs. 2 to 6, are of central interest. The symptom score data of patient 12 of the fraction A: D-GL group were omitted from 8/17 to 9/6, as he was exposed to a known allergen, cat dander, for this period. Two patients had borderline high calcium values that did not change during the study. In addition, one of these two patients also had asymptomatic hyperbilirubinemia, 2.1 mg% (7/79) and 1.7 mg% (9/79). The remaining routine chemistry, hematologic, and urinalysis results were normal at each sampling.

Clinical effects of SRW-D-GL

Fig. 1 shows the pollen counts and mean daily symptom scores for all three groups. Although this was an open trial, we found that the average seasonal symptom score for the fraction A: D-GL group did not differ significantly from the control group being treated for the fourth year with aqueous SRW. Both the fraction A: D-GL patients and aqueous SRW-treated control patients had statistically lower ($p < 0.02$) mean seasonal symptom scores than the untreated controls.

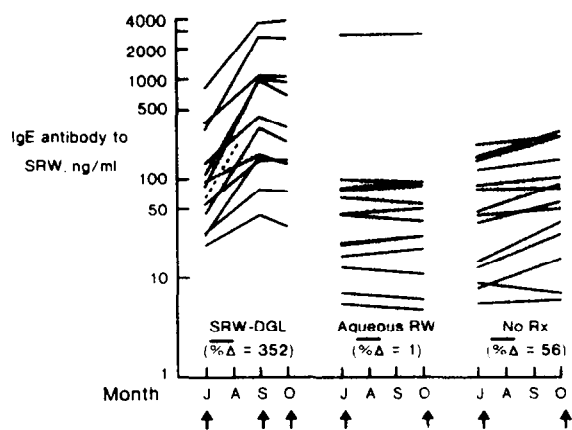


FIG. 3. Changes in IgE antibody levels to SRW and mean percent change (% Δ) in IgE antibody to SRW for each group, July through October, 1979. See legend for Fig. 2.

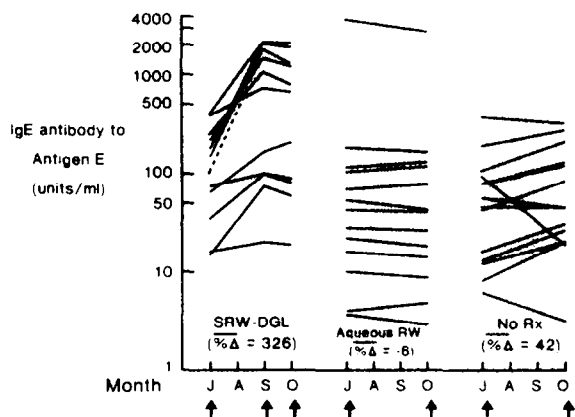


FIG. 4. Changes in IgE antibody levels to AgE and mean percent change (% Δ) in IgE to AgE for each group, July through October, 1979. See legend for Fig. 2.

Skin tests

We found the concentration of SRW required to produce a 5 × 5-mm wheal increased (7/79 to 10/79) by at least tenfold in all patients. The concentration of fraction A: D-GL required to produce a 5 × 5-mm wheal increased tenfold in seven of 12, 100-fold in three of 12, and did not change in two of 12. All patients were therefore less sensitive to SRW (and 10 of 12 less sensitive to fraction A: D-GL) by skin test after treatment with fraction A: D-GL.

IgE and IgG antibodies to SRW

Figs. 2, 3, and 4 show the respective changes in levels of total IgE, IgE to SRW, and to AgE. There was no statistical difference between the group receiving fraction A: D-GL and either control group in the initial (7/79) mean levels of total serum IgE, or IgE antibody to AgE. The fraction A: D-GL group did

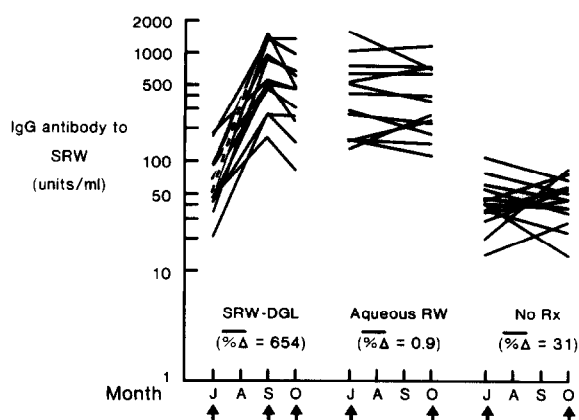


FIG. 5. Changes in IgG antibody levels to SRW and mean percent change ($\% \Delta$) in IgG antibody to SRW for each group, July through October, 1979. See legend for Fig. 2.

have a higher initial level of IgE antibody to SRW than did the group on aqueous immunotherapy ($p < 0.025$) but not when compared with the untreated control group. The group receiving fraction A:D-GL had significantly higher ($p < 0.02$) mean percent increases in total IgE when compared with the control group already receiving immunotherapy but not when compared with the untreated controls. The fraction A:D-GL-treated group demonstrated significantly higher mean percent increases in IgE antibody both to SRW and to AgE than either control group ($p < 0.001$).

Figs. 5 and 6 show the respective changes in levels of IgG antibody to SRW and AgE. The fraction A:D-GL group had a statistically higher initial mean level of IgG antibody to SRW than the SRW-sensitive untreated controls ($p < 0.025$), and a significantly lower level of IgG antibody to AgE than the untreated controls ($p < 0.03$). The fraction A:D-GL group had statistically lower mean levels of IgG antibody to SRW and AgE (7/79) than the control group already receiving immunotherapy ($p < 0.001$). The fraction A:D-GL group had significantly greater increases in IgG to both SRW and AgE than either control group ($p < 0.001$). The October levels of these IgG antibodies to SRW and AgE did not differ statistically from those of the control group already receiving immunotherapy.

We found no correlation between the quantity of fraction A:D-GL injected and subsequent changes in either IgE or IgG antibody levels to SRW or AgE. Table II summarizes individual patient dose and side effects data for the fraction A:D-GL group.

The patient illustrated by the dotted line in Figs. 2-6 provides strong evidence for the immunogenic activity of fraction A:D-GL. This patient demonstrated considerable rises in both IgE and IgG titers

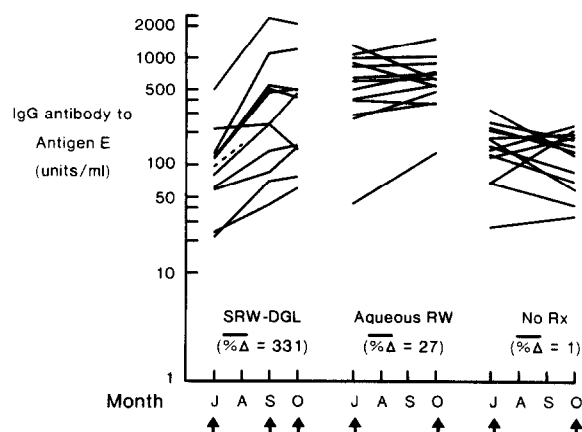


FIG. 6. Changes in IgG antibody levels to AgE and mean percent change ($\% \Delta$) in IgG antibody to AgE for each group, July through October, 1979. See legend for Fig. 2.

following 1 mo (July to August) of fraction A:D-GL administration, even though he was not exposed to SRW pollen by inhalation. His rises in these antibody levels were comparable to the July to October rises in the fraction A:D-GL group as a whole.

The possibility that administration of fraction A:D-GL stimulated an immune response to D-GL was tested by measurement of IgE and IgG antibody levels to D-GL in fraction A:D-GL-treated patients, in nonatopic patients, and in patients in the two control groups. None showed evidence of IgE or IgG antibody to D-GL.

DISCUSSION

The SRW fraction A:D-GL polymer used in this study stimulated significant increases in both IgG and IgE antibodies to SRW and AgE. That these increases were not due solely to seasonal SRW pollination itself is supported by our finding of comparable elevations of these antibodies in one patient who received fraction A:D-GL for 1 mo but left prior to SRW pollination. We found no correlation between total dose of fraction A:D-GL administered and subsequent antibody rises or changes in skin-test reactivity to SRW. Several potential explanations may be offered for the discrepant findings of our study and those of prior animal models.

First, we used a mixture of SRW proteins (fraction A) purified from SRW pollen by Sephadex G-25 gel filtration. The use of protein antigens as tolerogens coupled to D-GL presents technical difficulties because of the need to separate the unconjugated molecules, which are antigenic in their own right, from the D-GL proteins. Liu et al. have utilized affinity chromatography on avidin-Sepharose to separate D-GL proteins from unconjugated proteins.¹⁶

They first coupled biotin to D-GL, then reacted the protein with the biotin-D-GL, separated the biotin-D-GL-protein conjugate from unconjugated protein by affinity chromatography on Sepharose-avidin, and finally recovered the biotin-D-GL-protein conjugate by desorption with free biotin. This method provided them a means to obtain conjugates that are devoid of detectable unconjugated protein. In the case of SRW fraction A:D-GL, one must assume that all free fraction A was removed as in the model systems using ovalbumin and insulin. Katz has shown that conjugates of fraction A to D-GL are tolerogenic in mice,* and so it does not seem likely that the use of a mixture of ragweed proteins per se is an explanation for our results.

Second, even though AgE as a marker of SRW allergenic activity was bound to the D-GL, one must question whether the ratio of AgE to D-GL was appropriate. Hapten-D-GL conjugates containing a low epitope density have been shown to stimulate an immune response, while conjugates containing a high epitope density induce tolerance.²⁸ In their experiments Liu and Katz utilized AgE-D-GL containing a ratio of AgE to D-GL between 0.05:1 and 0.1:1 based on the antigenic activity of AgE in the conjugate.¹⁷ The fraction A:D-GL we employed contained a ratio of AgE to D-GL of 0.003:1 based on the finding of 116 μ g AgE antigenic activity per 50 mg total protein. Thus it would appear that as far as AgE is concerned the epitope density of the material used by Liu and Katz was considerably higher than that of the material we employed. Analysis in mice of the tolerogenic activity of fraction A:D-GL conjugate, Lot 6-79, tested at Miles Laboratories showed an IgE antibody titer of 1:80 at day 28 while untreated controls had a titer of 1:640. Analysis of the tolerogenic activity of fraction A:D-GL prepared at Scripps and tested at Scripps showed an IgE antibody titer of 1:10 at day 28 while untreated controls had a titer of 1:1,280. Thus, Lot 6-79 appears less tolerogenic than does the Scripps' preparation. Furthermore, after the completion of our clinical trial, Lot 6-79 was tested at Scripps and found at day 10 after primary immunization to stimulate an immune response in mice; the Scripps' fraction A:D-GL was still tolerogenic. Whether this difference is due to degradation of Lot 6-79 as a consequence of storage or due to the difference in epitope density is not clear.

Third, it is possible that proteolytic enzymes in vivo may degrade fraction A:D-GL to yield free SRW antigens. We cannot eliminate such a possibility. Studies of fraction A:D-GL in vitro indicated that the

AgE content was stable at 40° C for 14 days and TLC did not reveal a breakdown of D-GL as shown by the absence of free D-glutamic acid and D-lysine.*

Fourth, we began with minute starting doses of fraction A:D-GL, based on skin-test reactivity. We gave approximately doubling doses twice weekly through the SRW pollination season, as tolerated by the patients, and we gave total doses of 21 to 78 mg of fraction A:D-GL. The doses of AgE:D-GL needed to tolerize mice have ranged from 0.2 to 1.0 mg. Assuming the mass of a mouse as 30 gm, the comparable doses in humans on a per mass basis for a 50-kg person are 333 to 1667 mg.^{12, 17, 18} In animal studies, injections of tolerogen were given before a primary immunization or else repeated once or twice at intervals during the experiment.^{9, 12, 15, 17} Also, the animals were subjected to discontinuous exposure to their allergens, whereas our patients had daily exposure to SRW pollen for over 30 days. Furthermore, SRW pollen stimulates immune responses after inhalation, presumably by triggering lymphoid cells in the upper respiratory tract. Whether such cells are accessible to fraction A:D-GL or whether these cells can be tolerized by fraction A:D-GL is not known.

Finally, we cannot rule out a fundamental difference in the responses of normal mice and atopic humans to the injection of protein copolymer conjugates.

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