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Regulation of the Immune Response to Allergens by Immunosuppressive Allergenic Fragments

1. Peptic Fragments of Honey Bee Venom Phospholipase A₂

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Abstract. Nine patients with anaphylactic sensitivity to honey bee venom (HBV) were treated with P-1, a pepsin derived fragment of HBV phospholipase A₂ (PLA₂). P-1 caused only rare reactions with doses of 100 µg/injection. Treatment resulted in a substantial decrease in specific anti-PLA₂ IgE and IgG antibodies as well as a decline in skin test sensitivity to PLA₂. Another group of HBV-sensitive patients was treated with unaltered PLA₂. Doses greater than 20 µg/injection were not tolerated. PLA₂ injections caused an increase in anti-PLA₂ IgG and IgE antibodies as well as increase in skin test sensitivity. This study demonstrates that a non-immunogenic fragment derived from an allergen can downregulate immune responses and thus offer a new modality for therapy of allergic diseases.

Introduction

Allergen immunotherapy is an empirical medical treatment which results in alleviation of clinical IgE-mediated immediate hypersensitivity diseases. This treatment consists of a series of subcutaneous injections of the offending allergen with gradually increasing potency. Although this type of immunotherapy causes clinical improvement, the precise immunological mechanisms responsible for benefit are unclear. Among the several immunological events that occur during this treatment, and to which part of the therapeutic benefit is frequently attributed, is a gradual rise of allergen-specific IgG 'blocking' antibodies. The relationship between the effectiveness of immunotherapy and the level of specific IgE antibodies is even less clear. Initially, these antibodies rise and only with very prolonged high-dose treatment they fall but often not below the baseline level [1, 2]. A large decline in these antibodies would be expected to increase the efficacy of immunotherapy. The present agents available for immunotherapy are the unmodified etiologic

allergens. Various alterations of these allergens have been suggested and tested by a number of investigators to make immunotherapy more effective and safer [3-5]. One ideal modification of immunotherapeutic allergens would be to confer a molecular change which would cause decreased specific IgE antibody levels. Another favorable allergen molecular change for immunotherapy would be a structural alteration, which would result in restriction of allergen binding to IgE antibodies. This change would decrease the likelihood of systemic allergic reactions to injections, one of the limitations presently encountered using high doses of allergens for immunotherapy. Also such lessened antibody binding would permit more rapid attainment of therapeutically effective doses.

We have been successful in creating enzymatically derived fragments of protein allergens which seemingly have both these qualities. As shown in studies in mice, these allergenic fragments are poorly immunogenic themselves but have the capacity for down-regulating specific IgE immune responses [unpubl. data].

While they bind to specific IgE antibodies, they do not elicit immediate hypersensitivity reactions in the form of passive cutaneous anaphylaxis. To evaluate the potential usefulness of such allergenic fragments in treatment of human immediate hypersensitivity diseases, we elected to utilize P-1, an enzymatically derived fragment of phospholipase A₂ (PLA₂), the major allergen in honey bee venom, to treat humans with anaphylactic sensitivity to honey bee stings. Our prior studies with P-1 in mice had demonstrated that while P-1 bound to anti-PLA IgE antibodies, it would not elicit an immediate hypersensitivity response in the form of a passive cutaneous anaphylaxis reaction [unpubl. data]. Also, injections of P-1 into mice with high levels of IgE anti-PLA₂ antibodies produced a marked decline of these levels.

In this initial human study, injections of P-1 had striking immunoregulatory effects which were similar to those observed in the mouse. This pilot project demonstrates the potential usefulness for similar immunoregulatory fragments to treat allergic diseases.

Materials and Methods

(1) Purified PLA₂ was prepared by dissolving crude honey bee venom in water and removing undissolved particulates by centrifugation at 3,300 g for 30 min. This clarified solution was passed through a Sephadex G75 column. Fractions were eluted with distilled water. The second peak containing only PLA₂ was lyophilized. For treatment purposes, lyophilized PLA₂ was reconstituted in a sterile human serum albumin-saline diluting solution (Albay, Hollister-Stier, Spokane, Wash.) and suitable dilutions were made.

(2) The immunoregulatory fragment of PLA₂, P-1, was prepared by limited pepsin digestion. One hundred micrograms of PLA₂ were dissolved in 10 ml of water and the pH adjusted to 3.0 with HCl. Digestion was initiated by adding 5 mg of porcine pepsin and the reaction allowed to proceed for 6 h at 25 °C. Proteolysis was terminated by the addition of 1 N NaOH raising the pH to 8.0, which destroyed any residual pepsin activity. The digested fragments of PLA₂ were separated into individual components by Sephadex G50 chromatography. The first peak contained one polypeptide, P-1, molecular weight 13,000–15,000 daltons, whose biochemical properties will be described in a separate publication [unpubl. data]. Following chromatography, P-1 was lyophilized. For human use it was solubilized in human serum albumin-saline solution (Albay, Hollister-Stier, Spokane, Wash.).

(3) Lyophilized honey bee venom for skin testing was obtained from Hollister-Stier, Spokane, Wash.

(4) *Experimental subjects.* Patients were admitted into the study only if they had a history of an unequivocal anaphylactic reaction to a honey bee sting. Identification of the offending Hymenoptera species was done by the individual being able to either positively identify a honey bee or noting that a stinger had been left behind in the area of the sting. In addition, each individual was re-

quired to have an immediate positive skin test to whole honey bee venom at a concentration of 1 µg/ml or less as well as a positive skin test to PLA₂ at a concentration of 1 µg/ml or less. Volunteers were limited to ages 21 through 45 years, had to have a negative history of cardiovascular disease as well as no evidence on physical examination of any cardiovascular abnormality. Physical examinations, complete blood counts, sedimentation rates, urinalyses, biochemical profiles were done prior to initiation of the study and repeated every 6 weeks during the study. This study was approved by the Human Research Committee, College of Medicine at the University of Cincinnati. All patients signed an informed consent.

(5) *Skin test end point titrations.* End point titrations were performed by intradermal injections of 0.02 ml of 10-fold dilutions of PLA₂, P-1, and whole honey bee venom. The end point was defined as the logarithm of that dilution which caused a reaction of 6 mm or greater induration than that observed with a saline control. The skin test end point titrations to PLA₂ were redetermined after each patient had received an injection of 20 µg of their respective allergen and again after 100 µg.

(6) *Serological assays.* PLA₂ IgE antibodies were determined by a RAST procedure using paper discs to which PLA₂ had been coupled by cyanogen bromide [6]. The discs were graciously supplied by Dr. Donald Hoffman, East Carolina School of Medicine. RAST results are expressed as counts per minute. The specificity of the RAST results were confirmed by specific inhibition with PLA₂. All pretreatment sera showed significant RAST inhibition with PLA₂. PLA₂ IgG antibodies were determined by an ELISA assay [7]. ELISA results are expressed as arbitrary units (AU) based on a control serum which had been obtained by mixing sera from three different bee keepers who had tolerated numerous stings without incurring any allergic manifestations.

(7) *Experimental plan.* After initial evaluation, each patient was placed in either the P-1 or the PLA₂ treatment groups. As the patients were acquired sequentially rather than at one time, each was arbitrarily assigned to one of the two treatment groups upon entrance to the study. Thus, the assignment of patients in a treatment group was based on their clinical sensitivity rather than on skin tests or serological findings. Such a selection method is supported by the experience of many investigators who have found that patients with Hymenoptera hypersensitivity frequently have a poor correlation between clinical and serological sensitivity as measured either by skin test end point titrations or in vitro determination of IgE antibodies [8]. The study was single-blinded. After each patient was placed in either the P-1 or PLA₂ treatment group, they received weekly subcutaneous injections of their respective allergen beginning with 0.10 ml of 0.10 µg/ml P-1 or 0.1 ml of 0.001 µg/ml PLA₂. The dose of each allergen was doubled at each subsequent visit. The dosage regimen was modified to more conservative increments when a large local reaction or a systemic reaction occurred after the preceding injection. Our initial plan was to attain a final injection dose of 20 µg of each allergen. Because of the dramatic serological changes observed in P-1-treated patients, it was decided to try to raise the final injected dose to 100 µg of allergen in each treatment group. After completion of the study, each patient was offered conventional immunotherapy with whole honey bee venom.

Statistical analyses were performed using the Clinfo system. To normalize the data, log transformation was done. The paired t test was primarily employed for assessing most data using each patient as their own control. When the data were nonparametric, the sign test or the Wilcoxon signed rank test was used.

Table 1. Reactions to immunotherapy

Group	Allergen dose μg	Reaction type	
		local (> 20 mm induration)	systemic
P-1	0-20	0	0
	20-100	0	1
PLA ₂	0-10	8	0
	10-20	6	3

Each group consisted of 9 patients.

Table 2. End point skin test titration to PLA₂

	Pre-treatment	Treatment dose	
		20 μg	100 μg
P-1	-3.8	-2.0	-1.8
PLA ₂	-1.4	-1.9	-

End point titration is expressed as the logarithm of the dilution of a 1 $\mu\text{g}/\text{ml}$ solution which caused 6 mm or greater induration.

Results

Clinical Course of Patients

Nine patients completed the treatment regimen in each group. Because of our sequential allocation of patients into the two treatment groups, the patients treated with P-1 were by skin test end point titration more sensitive to PLA₂. The mean PLA₂ end point titration for P-1-treated patients was -3.8 logs whereas for PLA₂-treated patients, it was -1.4 logs. However, the initial PLA₂ IgE RAST titers did not differ significantly between the two groups. Although our initial intention has been to achieve a final injection dose of each allergen of 20 μg , the dramatic decline in PLA₂ IgE antibodies noted in P-1-treated patients caused us to attempt to increase the dose of allergen in each patient group to 100 μg . This was successful in P-1-treated patients but not in PLA₂-treated patients. The latter could not tolerate a dose higher than 20 μg because of an undue number of local and systemic reactions to the injections. The incidence of local and systemic reactions in the two treatment groups is depicted in table 1. There is a marked difference

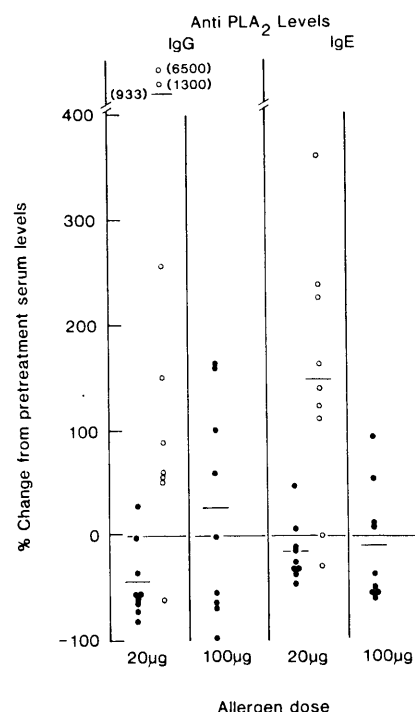


Fig. 1. Anti-PLA₂ IgG and IgE antibody responses. ● = P-1-treated patients; ○ = PLA₂-treated patients. The mean value in each column is indicated by a horizontal line.

between the two groups for both types of reactions. The only systemic reaction to P-1 occurred after injection of 50 μg of P-1 in 1 patient. This patient subsequently tolerated all further injections. In PLA₂-treated patients, 6 of 14 local reactions and the 3 systemic reactions occurred while the patients received doses between 10 and 20 μg of PLA₂.

The results of the sequential biochemical analyses, complete blood counts sedimentation rates, and urinalyses remained normal in all patients.

Skin Test End Point Titrations

Prestudy Results. As described above, the initial end point to PLA₂ in the two patient groups was -3.8 logs in P-1-treated patients and -1.4 logs in PLA₂-treated patients. Honey bee venom skin test titers were 1 log less reactive than that found with PLA₂ with one exception. This was expected as PLA₂, the major allergen in honey bee venom, constitutes approximately 12% of honey bee venom proteins [9]. In both groups, the initial skin test reactivity to P-1 was far less than that found with PLA₂. Indeed, in 13 pa-

tients, it was entirely absent up to a concentration of 1 $\mu\text{g}/\text{ml}$. In the 4 patients who did react, it was 2, 4, 5, and 6 logs less reactive than the PLA₂ skin test titer. One patient in the P-1 patient group was not tested as she was dermatographic. However, she was included in the study as she has a positive history and a significant PLA₂ RAST titer.

Sequential Study of PLA₂ Skin Test Titers. Table 2 depicts the skin test titer results obtained in the two treatment groups. PLA₂-treated patients, after receiving a dose of 20 μg of PLA₂, showed an increase in sensitivity to PLA₂ with end point titers rising from a mean of -1.4 to -1.9 logs. In contrast, in patients receiving P-1 treatment, sensitivity to PLA₂ declined with skin test mean end point titers of PLA₂ decreasing almost 2 logs after each patient received 20 μg of P-1 and 2 full logs after the 100- μg dose. These differences were highly significant statistically ($p < 0.01$).

IgE PLA₂ Antibodies

The pretreatment mean PLA₂ IgE antibodies in the two groups was 1,872 cpm in P-1-treated patients and 1,779 cpm in PLA₂-treated patients. This difference was not statistically significant. Figure 1 represents the percent changes produced by treatment on PLA₂ IgE antibody levels in both patient groups. After an injection of 20 μg , 7 of 9 P-1-treated patients exhibited a decrease in IgE antibodies to PLA₂ (mean -16.2%, range -44.6 to +45%). The opposite effect was found in PLA₂-treated patients (mean +146.5%, range -0.5 to +360%). In these patients, 7 of 9 developed an increase in IgE antibodies to PLA₂. The intragroup change in PLA₂ IgE titers within each patient group was statistically significant (pretreatment vs. 20 μg) ($p < 0.05$) although the change in each group was in opposite directions. Comparing the intergroup percent change in PLA₂ IgE antibody levels between P-1-treated patients and PLA₂-treated patients after the 20- μg dose, a statistical difference ($p < 0.05$) was also found. After raising the dose of P-1 to 100 μg , 5 P-1-treated patients had a further decline in PLA₂ IgE antibodies while in 4 they unexpectedly rose (mean -11.2%, range -60 to +93%). However, because of this increase in titer in some of the patients, the intragroup statistical difference in P-1 patients (pretreatment vs. 100 μg) was now lost, although comparison of the percent change in IgE titers between the P-1 patients who had received 100 μg and the PLA₂ patients who had received 20 μg was still statistically significant ($p < 0.05$).

IgG Antibodies to PLA₂

The PLA₂ IgG antibody response in both treatment groups is shown in figure 1. The mean pretreatment values were 21.8 AU in the P-1 group and 10.2 AU in the PLA₂ treatment group. In patients receiving P-1, these antibodies were partially suppressed in 7/9 patients after they had received 20 μg of P-1 (mean -43%, range -80 to +28%). This change was statistically significant ($p < 0.02$). Raising the dose of P-1 to 100 μg caused a further decline in PLA₂ IgG antibodies in 3 patients, an increase over the baseline levels in another 3 patients, and no change in 3 others (mean +26%, range -98 to +186%). Because of this loss of suppression in some patients, there was no longer a statistical difference between the pretreatment PLA₂ IgG levels and the levels found after these patients had received 100 μg of P-1. In contrast to P-1-treated patients, a definite increase in IgG anti-PLA₂ antibodies occurred in 8/9 of PLA₂-treated patients (mean +933%, range -67 to +6,500%). This difference was statistically significant (sign test; $p < 0.05$). Also, as the direction of change in IgG PLA₂ antibody level in the two groups after 20 μg of antigen was opposite, a comparison of the percent change between the two groups (-43% in P-1 group and +933% in PLA₂ group) was statistically significant ($p < 0.01$) (Wilcoxon rank sum).

Discussion

The peptic fragment of PLA₂, P-1, while close to PLA₂ in size, displayed dramatically altered immunological properties. It bound poorly to skin mast cells even when considerable quantity of specific anti-PLA₂ IgE antibodies were available for binding. Thus, intradermal injections of P-1 up to a concentration of 1 $\mu\text{g}/\text{ml}$ failed to elicit any immediate wheal and flare reactions in 13/17 patients, all of whom had a positive response to unaltered PLA₂. Subcutaneous injections of P-1 into honey bee venom-sensitive patients only rarely produced significant local or systemic reactions, even when the injected dose was 8-fold higher than that used therapeutically in individuals receiving conventional immunotherapy with whole honey bee venom (12 μg PLA₂ in 100 μg honey bee venom) and 5 times the dose of PLA₂ used in the PLA₂-treated patients in this study. It should be noted that the dose of unaltered PLA₂ used in this study was

limited to 20 µg by the occurrence of frequent systemic and large local reactions.

The second and the most important change that pepsin proteolysis produced in PLA₂ was the change from an immunogen that produced upon subcutaneous injection increases in specific IgE and IgG antibodies to that of downregulator of both of these classes of antibodies. This down regulating property seemingly has a plateau effect in that in some individual patients very large doses of P-1 caused an additional fall in antibody levels but in other produced significant increases. Thus, there would seem to be an optimum dose for each individual to produce a fall in antibody levels. An alternative interpretation would be that pepsin hydrolysis altered the immunodominant epitope(s) of PLA₂ but when very large doses of injected P-1 were used, immune responses were increased to minor unaltered epitopes. Thus, when the whole anti-PLA₂ antibody response was measured as was done in this study with whole PLA₂ attached to filter paper discs or on ELISA plates, an increase in the total antibody level of that isotype (either IgE or IgG) would be found. However, it should be noted that skin tests for wheal and flare reactions showed only continued downregulation with increasing doses of P-1.

The level of immunoregulation that is affected by the molecular change in PLA₂ cannot be determined from our study in which only the final B cell pathway, IgE and IgG antibodies, was determined. Prior studies from our laboratory indicated that pepsin-derived fragments of bovine serum albumin (BSA) upon injection into mice suppressed a subsequent BSA challenge by preferential induction of T suppressor cells [10]. The suppressed immune response included not only epitopes present in the fragment but all epitopes on the entire BSA molecule. What precise molecular change is induced by pepsin hydrolysis is unclear and remains under investigation. One appealing postulate is that helper and suppressor epitopes are located at different sites on the protein molecule. Pepsin hydrolysis would yield some fragments, which contains only a suppressor epitope or more suppressor than helper epitopes.

The question of the clinical benefit of P-1 is not addressed in this study. Its profound immunoregulatory properties included not only a decline in specific IgE antibodies, but also a decline in specific IgG antibodies. This latter change could be unfavorable as some investigators claim that a minimum level of specific

IgG antibodies is required for conventional venom immunotherapy to be protective [11]. Further study will be required to resolve this question.

Finally, it could be argued that the drop in IgE and IgG levels following immunotherapy with P-1 was due primarily to the lack of antigenic stimulation since Reisman et al. [12] observed that shortly following a sting there is a gradual decline in IgE levels in some of the sensitized individuals. However, the IgE and IgG titers in our patients who were treated with P-1 for relatively a short period of time (mean 10.7 weeks to reach 20 µg/injection) fell more dramatically than could be expected from a natural decline. Moreover, 6 of 9 of the P-1-treated patients were stung more than 1 year before initiation of therapy, and therefore the levels of their specific IgE and IgG were likely to be at a steady state. Therefore, we believe that the decline of IgE and IgG antibodies produced by P-1 injection was the result of immunoregulatory suppression and not a natural catabolism.

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