

Polymerized insoluble bee venom

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Using a polymerization process previously used for ragweed allergens, honeybee venom was polymerized. Instead of soluble polymers, an insoluble precipitate, polymerized insoluble bee venom (PIBV), is the result. A major allergen of honeybee venom, ^{125}I phospholipase A (PL-A) incorporated into PIBV, was shown to have decreased dissemination from subcutaneous injection sites. After thorough mixing, samples of PIBV can be withdrawn from a vial with a syringe with no more than 10% error. Approximately 90% of PL-A was incorporated with PIBV. The soluble PL-A was removed during subsequent washing of the PIBV and this soluble PL-A was shown to be polymerized mainly to high-molecular weight PL-A. PIBV (20 mg) injected in each of six rabbits resulted in formation of precipitating antibody in all rabbits. The rabbit antisera bound ^{125}I PL-A and reacted with PL-A in whole bee venom. These physical and immunologic characteristics of PIBV suggest further study of its potential for human use.

Previous studies from this laboratory have shown that allergens responsible for IgE-mediated human disease can be polymerized to produce a high-molecular weight preparation that has retained immunogenicity but reduced reactivity in the allergic human.^{1, 2} In addition to ragweed antigen E, the polymerization process has been applicable to grass³ and tree pollen allergens.⁴ Clinical efficacy and safety of polymerized ragweed antigen E and partially purified, more allergenically complete preparations of ragweed have been shown first in limited studies^{5, 6} and finally in a multicenter study.⁷

Studies of venom immunotherapy have shown that preparations of appropriate venoms are indicated for patients with a history of systemic reactions to Hymenoptera stings and positive skin tests to one or more venoms.⁸ Although this therapy is effective in reducing risks of reactions after stings, the problems of therapy in terms of immediate reactions and prolonged dosage regimens to achieve and sustain maintenance therapy are not dissimilar to aqueous pollen extract therapy. Thus it seemed appropriate to determine if a polymerized form of venom could be prepared that was highly immunogenic since it could be

expected to be less reactive with antibody than monomer.

METHODS AND MATERIALS

Preparation of polymerized insoluble bee venom (PIBV)

A solution of honeybee venom (Sigma Chemical Co., St. Louis, Mo.) was prepared from powdered venom dissolved in 0.15 M phosphate-buffered NaCl, pH 7.35 (PBS). This solution was sterilized by filtration through a Nalgene, 20- μ filter unit (Nalge, Sybron Corporation, Rochester, N. Y.) and standardized by optical density (O.D.) at 280 nm. The stock solution of the venom was diluted to an O.D. of 10 to 30.

Polymerization was done under sterile conditions, using sterile vials, and all reagents were filtered through Nalgene filter units. A solution of glutaraldehyde 25% solution (Sigma Chemical Co., St. Louis, Mo.) was added to venom, using 0.31 mg glutaraldehyde/mg venom protein, estimating the protein concentration of the venom as 1 O.D. unit approximating 1 mg protein/ml.

Ten to 100 mg bee venom were used for different polymerization experiments. In each case, the mixture of glutaraldehyde and bee venom protein was agitated at 25° C for 2 hr. A visible precipitate formed, which is the PIBV. A sterile solution of excess glycine (50 mg/mg glutaraldehyde) was added to combine with any free glutaraldehyde binding sites. The precipitate was sedimented by centrifugation at 1,000 $\times$ g for 30 min. The supernatant was removed and the precipitate washed three times using 5 ml of sterile PBS containing 0.4% phenol and 0.005% Tween 80 (Sigma Chemical Co., St. Louis, Mo.). The precipitate was sedimented by centrifugation after each wash.

The PIBV was exposed to ultrasound for 30 min using an

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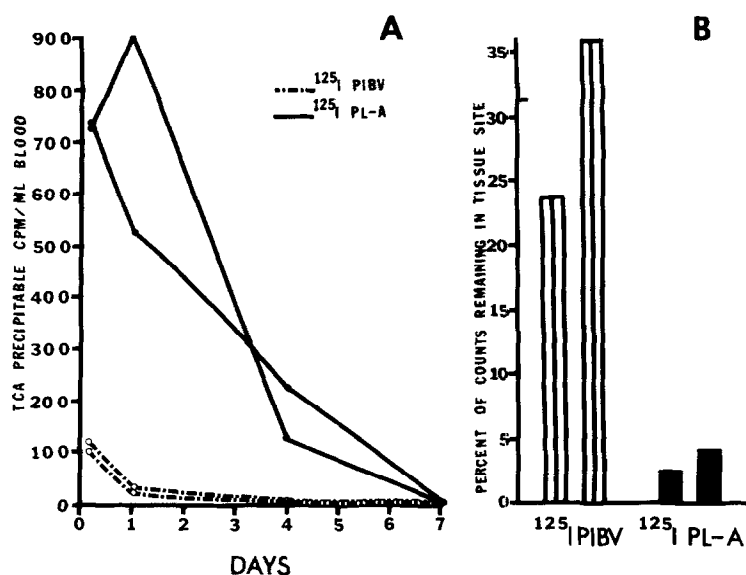


FIG. 1. Comparison of distribution of ¹²⁵I polymerized insoluble bee venom (¹²⁵I PIBV) or unpolymerized bee venom mixed with trace ¹²⁵I bee phospholipase A (¹²⁵I PL-A) after multiple injections of each into the forepaws of guinea pigs. TCA, Trichloroacetic acid; cpm, counts per minute. *A*, Counts per minute in blood after injections; *B*, Counts per minute in tissue 7 days after injection.

ultrasonic cleaner (Mettler Electronics Corporation, Anaheim, Calif.), washed three times, and finally suspended in 5 ml PBS with 0.4% phenol and 0.005% Tween 80.

The nitrogen concentration of the preparation of PIBV was determined by micro-Kjeldahl determination and protein content was calculated. The final solution of PIBV was prepared by diluting to the desired protein concentration. A sample of the final preparation was cultured for sterility.

Immunization of rabbits with PIBV

Six young adult rabbits, weighing 3 to 4 kg each, were used for the immunization with PIBV. Two rabbits each received a total of 20 mg PIBV divided in three subcutaneous doses given at intervals of 1 wk. One week after the last immunization, serum samples were collected and stored at -20° C until used. Four rabbits were immunized with a single dose of 20 mg PIBV per rabbit in multiple sites. Rabbits were bled before immunization and 2 and 4 wk after immunization.

Radioiodination of bee phospholipase A (PL-A)

Bee PL-A was obtained from Sigma Chemical Company (St. Louis, Mo.) and was labeled with ¹²⁵I utilizing the chloramine-T method of Gleich et al.⁹

Incorporation of PL-A into PIBV

Three PIBV preparations were done on different occasions to evaluate the incorporation of PL-A into PIBV. Polymerization was performed as described above using 10 mg bee venom mixed with less than 1 ng PL-A. The percent

¹²⁵I PL-A incorporated into PIBV was calculated by the following formula:

$$\frac{\text{cpm of } ^{125}\text{I PL-A added to bee venom}}{\text{cpm in final PIBV}} \times 100 = \text{\%PL-A incorporated into PIBV}$$

(where cpm is counts per minute), ¹²⁵I PIBV was used for further studies.

Determination of the extent of incorporation of ¹²⁵I PL-A into ¹²⁵I PIBV

In four experiments, bee venom was polymerized as described above but in the presence of trace-labeled ¹²⁵I PL-A. Radioactivity was determined in all supernatants, washes, and in the solid ¹²⁵I PIBV. The extent of incorporation was then calculated. Further characterization of the soluble components after polymerization was done in one experiment. Polymerization was performed in the presence of ¹²⁵I PL-A. After centrifugation to sediment ¹²⁵I PIBV, the supernatant was saved. To this was added the first supernatant obtained after sonication of the solid ¹²⁵I PIBV. No appreciable radioactivity was found in the other washes. This pool was then applied to a Sephadex G-200 column and the elution profile of the radioactive components was determined.

Comparison of retention of unpolymerized venom and PIBV at site of injection

Four guinea pigs, each weighing about 1 kg, were used. Unpolymerized bee venom mixed with ¹²⁵I PL-A and ¹²⁵I

PIBV, prepared as described above, were used. These contained 6 mg protein/ml and about 6×10^7 cpm/ml. Two guinea pigs were injected subcutaneously in the front paw with ^{125}I PIBV and two with ^{125}I PL-A mixed with unpolymerized bee venom. Blood samples were obtained at 3 hr, and 1, 4, and 7 days to determine trichloroacetic (TCA) precipitable counts in serum samples. After 7 days, animals were killed, forelegs previously injected with bee venom mixed with ^{125}I PL-A or ^{125}I PIBV removed, fragmented, and radioactivity remaining at the injection site was determined.

Immune response to PIBV: Gel double diffusion

The sera of rabbits immunized with PIBV were evaluated for precipitating antibody against bee venom using gel double diffusion. Glass slides were layered with 0.8% Ionagar in PBS. Undiluted rabbit serum was diffused against the optimum concentration of bee venom (1 mg/ml).

Quantitative measurement of antibody against PL-A in rabbit sera

For this assay, a modification of the ammonium sulfate technique of Lidd and Farr was used.^{10, 11} Aliquots of 0.02 ml of sera from four rabbits taken at the time of immunization with PIBV and 4 wk following immunization were studied. Serum samples were incubated with 10, 25, 50, 100, and 500 ng of ^{125}I -labeled PL-A in a total reaction volume of 0.6 ml. The mixture was incubated for 2 hr at 37°C and 16 hr at 4°C. Four-tenths milliliter of saturated ammonium sulfate (pH adjusted to 7.0) was added in the cold, drop-wise, with constant stirring. The precipitates formed for 2 hr at 4°C and were centrifuged at $1000 \times g$ for 15 min. The supernatant solution was separated and radioactivity of supernatant and precipitate was determined. The percent ^{125}I PL-A bound to globulin was plotted on the ordinate of semilog paper and the nanograms of PL-A added were plotted on the log $\times$ axis. The nanograms of PL-A bound at the 20% binding point were multiplied by the dilution factor. The result is the nanograms of PL-A bound by 1 ml of serum in great antigen excess.

Several correction factors were used to determine the true percent PL-A bound. It was found by experimentation that over the range of labeled antigen added the precipitate would entrain 10% of the supernatant counts. Additionally, ammonium sulfate at 40% saturation will precipitate 10% of the total counts added. Before each assay, the percent immune reactivity of the ^{125}I -labeled PL-A was assessed by the ability of rabbit anti PL-A to precipitate the radiolabeled antigen in antibody excess. The final percent PL-A bound is calculated after making these corrections.

Measurement of PL-A in bee venom

This was done using an extension of the radioimmunoassay for ^{125}I PL-A (described above) as follows. ^{125}I PL-A with minimal (<1 ng) of protein was added to bee venom and increasing concentrations of the mixture of ^{125}I PL-A and bee venom were added to rabbit anti-PIBV and binding

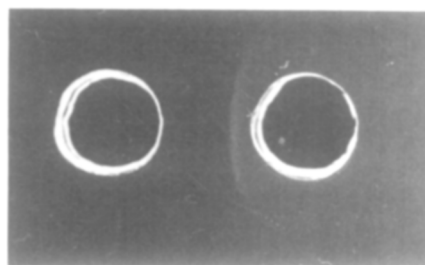


FIG. 2. Photomicrograph of an Ouchterlony plate showing a precipitin band formed between undiluted rabbit anti-PIBV (*left well*) and 1 mg/ml bee venom (*right well*).

of ^{125}I PL-A determined exactly as described above. This assay demonstrated that the PL-A in bee venom reacted with antibody against PIBV resulting in dose-related inhibition of binding of ^{125}I PL-A analogous to the inhibition produced by unlabeled PL-A in Fig. 3.

Inhibition of binding of PL-A by PIBV

To demonstrate that antigenic determinants of PL-A were present on or in PIBV particles, PIBV was preincubated with antiserum against bee venom and then binding of ^{125}I PL-A by the antiserum determined as described above.

RESULTS

General characteristics of PIBV

PIBV is a light brown suspension of particulate material that can be drawn into a syringe with a 27-gauge needle. Repeated withdrawal of 0.1 ml of ^{125}I PIBV from a vial using a 1-ml syringe and measurement of the radioactivity withdrawn in each sample demonstrated that there was no more than 10% variation in counts per minute of the samples. Thus after complete mixing, aliquots of PIBV can be withdrawn in a manner analogous to that in clinical use with minimal risk of significant variation in quantities of PIBV delivered.

Incorporation of ^{125}I PL-A into PIBV

In three separate experiments, the incorporation of ^{125}I PL-A into PIBV was measured. The results demonstrated that 85%, 83%, and 95% of ^{125}I PL-A was incorporated into PIBV with a mean of 87.6%. These results were obtained by measuring the radioactivity of all supernatants and washes and of the solid final product, PIBV.

In another experiment, the supernatants were characterized further by passage through a Sephadex G-200 column. Of the total supernatant radioactivity, 66% of the TCA precipitable counts were excluded by Sephadex G-200. The ^{125}I PL-A used as a marker contained at most traces of high-molecular weight components since over 99% of its precipitable counts were included in the calibrated Sephadex G-200 col-

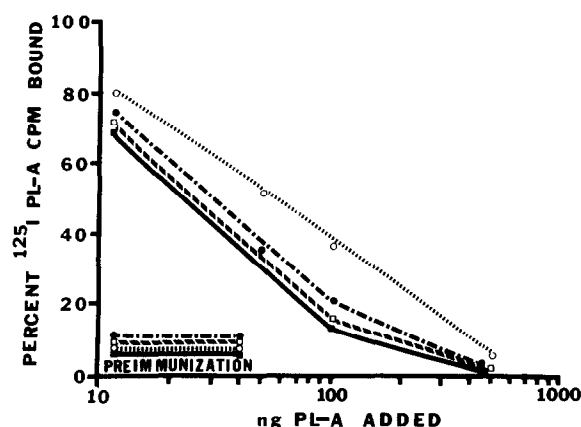


FIG. 3. Binding of ^{125}I bee phospholipase A (^{125}I PL-A) by sera of rabbits immunized with polymerized insoluble bee venom (PIBV).

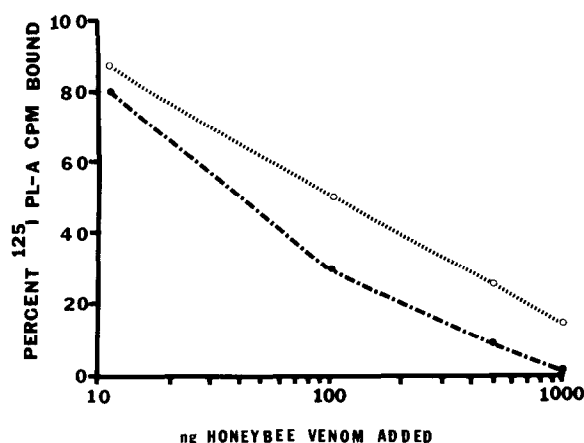


FIG. 4. Binding of ^{125}I phospholipase A (^{125}I PLA) mixed with bee venom by two rabbit antisera against polymerized insoluble bee venom. The symbols for the two rabbit sera are the same as in Fig. 3.

umn. This indicates that two thirds of the soluble PL-A after glutaraldehyde treatment has actually been polymerized and has a molecular weight in excess of 200,000 daltons. In this experiment, overall incorporation of ^{125}I PL-A into PIBV was 97%. It must be emphasized that essentially all of the soluble ^{125}I PL-A was removed by the three washes before and the three washes after sonication in the preparation of PIBV analogous to that which might be considered for potential human use.

Distribution of radioactivity after subcutaneous injection of ^{125}I PIBV and bee venom mixed with ^{125}I PL-A

Two pairs of guinea pigs were injected with radiolabeled PIBV or unpolymerized bee venom mixed with PL-A in multiple sites in forelegs and blood radioactivity determined for 7 days. Results

shown in Fig. 1, A, demonstrate the lower levels of radioactivity in animals injected with ^{125}I PIBV than those injected with ^{125}I PL-A mixed with unpolymerized bee venom. After 7 days, the injected tissue was removed and remaining radioactivity determined. The results (Fig. 1, B) show considerably more ^{125}I PIBV retained at the injection sites than ^{125}I PL-A mixed with unpolymerized bee venom, a finding inversely related to initial blood levels.

Immune response to PIBV: Precipitating antibody

Six rabbits each received 20 mg of PIBV protein either as three equal divided doses given at weekly intervals or a single 20-mg dose. All rabbits were bled at 4 wk after initial injection. All six rabbits made precipitating antibody by 4 wk against whole bee venom. An example of the precipitin bands against bee venom using serum from one rabbit is shown in Fig. 2.

Binding of ^{125}I PL-A by rabbit anti-PIBV

Fig. 3 shows the binding of ^{125}I PL-A by four different rabbit antisera against PIBV. These results are compared with pretreatment sera of the same rabbits. The two sera with the highest binding activity (Fig. 3) bound 2,600 and 1,000 ng/ml at the 20% binding point.

Inhibition of anti-PIBV binding of ^{125}I PL-A by bee venom

Trace amounts of ^{125}I PL-A were mixed with bee venom and increasing concentrations of the mixture added to two rabbit antisera against PIBV. The binding of ^{125}I PL-A under these conditions are shown in Fig. 4. There is decreased binding of ^{125}I PL-A with increasing concentrations of bee venom added to the antisera. The differences in binding curves in Figs. 3 and 4 reflect the fact that PL-A is only one of the constituents of bee venom and, therefore, more venom is needed to produce an inhibition of binding of ^{125}I PL-A compared with that of PL-A.

Absorption of anti-PL-A by PIBV

Two rabbit antisera that bound 2,600 and 1,000 ng, respectively (Fig. 3), were absorbed with 1 mg PIBV to confirm that antigenic determinants of PL-A were in or on the particular PIBV. After the absorption of antisera with PIBV no binding of ^{125}I PL-A occurred using the technique shown in Fig. 3.

DISCUSSION

Our experience with soluble polymerized ragweed allergens extended from basic laboratory studies^{1, 2} to

a multicenter clinical study demonstrating safety and efficacy.⁷ Preparations of grass³ and tree polymers⁴ have solubility characteristics and elution profiles similar to ragweed.

It was our expectation that a soluble polymer of bee venom would be obtained with use of our standard polymerization process.^{1, 3, 4} Repeated experiments showed that we were able to prepare only an insoluble polymer by glutaraldehyde treatment of bee venom. Multiple experiments ($n = 33$) using variations in concentration of glutaraldehyde, reaction time, and temperature produced two results, either no polymerization (as evaluated by the elution pattern of bee venom through Sepharose 4B) or formation of a solid product. The inability to prepare a soluble polymer of bee venom led to the current evaluation of the insoluble precipitate, PIBV, to determine whether or not it had suitable characteristics for potential use in patients with anaphylactic sensitivity to bee venom.

The current studies utilize preparations of PIBV, some containing ¹²⁵I PL-A. PL-A was used as marker because studies have reported PL-A as an antigen in bee venom that releases histamine^{12, 13} and against which IgE antibody is directed. Although PL-A has been suggested as the major allergen in honeybee venom,¹³ it is not the only one.¹⁴

The current studies demonstrate that the PIBV, prepared as described here, can be dispensed by syringe as in common clinical practice and is retained at tissue injection sites longer than soluble, unpolymerized venom. In these studies, the use of ¹²⁵I PL-A as a marker provides important information because PL-A has been suggested as the major allergen of honeybee venom; also, because of its relatively low molecular weight of 14,000 to 16,000, it might be expected to diffuse more rapidly from an injection site than higher-molecular weight allergens.

The PIBV was immunogenic as shown by production of precipitating antibody in rabbits. Further, the rabbits immunized with PIBV produce antibody against PL-A as shown by direct antibody binding of ¹²⁵I PL-A and confirmed by inhibition of PL-A binding by antisera using bee venom in which PL-A is a major allergen.

PIBV will neutralize antisera against PL-A as demonstrated in these studies. Thus the PIBV has available antigenic determinants. PIBV is a particulate suspension with grossly visible particles. Such particles likely contain exposed antigenic determinants

within a lattice-like structure through which antibodies may diffuse. Whether or not all antigenic determinants are exposed externally or internally or both, the PIBV is antigenic but, because of its physical, particulate nature, should have a marked reduction in ability to diffuse through tissue, react with tissue mast cells, and result in significant mediator release. These properties appear to be appropriate for an agent with immunotherapeutic potential in patients who are anaphylactically sensitive to bee venom.

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