

Polymerized soluble venom—Human serum albumin

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Extensive previous studies have demonstrated that attempts to produce polymers of Hymenoptera venoms for human immunotherapy resulted in insoluble precipitates that could be injected with safety but with very limited immunogenicity in allergic patients. We now report soluble polymers prepared by conjugating bee venom with human serum albumin with glutaraldehyde. The bee venom-albumin polymer (BVAP) preparation was fractionated on Sephacryl S-300 to have a molecular weight range higher than catalase. ¹²⁵I-labeled bee venom phospholipase A was almost completely incorporated into BVAP. Rabbit antibody responses to bee venom and bee venom phospholipase A were induced by BVAP. Human antisera against bee venom were absorbed by BVAP. No new antigenic determinants on BVAP were present as evidenced by absorption of antisera against BVAP by bee venom and albumin. BVAP has potential immunotherapeutic value in patients with anaphylactic sensitivity to bee venom. (J ALLERGY CLIN IMMUNOL 75:382-7, 1985.)

Because previous controlled studies from this laboratory had demonstrated safety and efficacy of polymerized ragweed^{1,2} and, subsequently, polymerized grass immunotherapy,³ we conducted extensive experiments to produce polymerized venoms. Experimental techniques that resulted in soluble preparations of polymerized ragweed,⁴ polymerized grass,⁵ and polymerized trees⁶ were applied to venoms in attempts to produce polymerized soluble venoms, but the result was a precipitate of venoms. In spite of multiple experimental variations in the polymerization technique including changes in glutaraldehyde concentration, time of glutaraldehyde exposure, temperature, and pH, the results were always precipitation or absence of polymerization as demonstrated by molecular sieve chromatographic analysis. Because of the potential therapeutic value of a polymerized venom, we investigated the polymerized insoluble venom and demonstrated that it was antigenic in rabbits.⁷ We subsequently made similar preparations of all five clin-

Abbreviations used

BVAP: Bee venom-albumin polymer
PLA: Phospholipase A
OD: Optical density
HSA: Human serum albumin

ically relevant Hymenoptera venoms and initiated human clinical trials. A normal control subject (one of the investigators) and subjects with Hymenoptera anaphylaxis were treated. An IgG response to the respective venoms used occurred in the sensitive patients without local or systemic allergic reactions, but the IgG response was of such low magnitude and of such short duration that we concluded that polymerized insoluble venoms were safe but had not demonstrated efficacy, and we terminated this approach.

After various other unsuccessful attempts to prepare a soluble venom polymer, we now report the successful development of a soluble human serum BVAP with characteristics that should provide a safe, effective, and less tedious immunotherapeutic regimen.

MATERIAL AND METHODS

Preparation of honey bee venom-human albumin polymer

The methodology for venom-albumin polymer was derived from multiple preliminary experiments designed to

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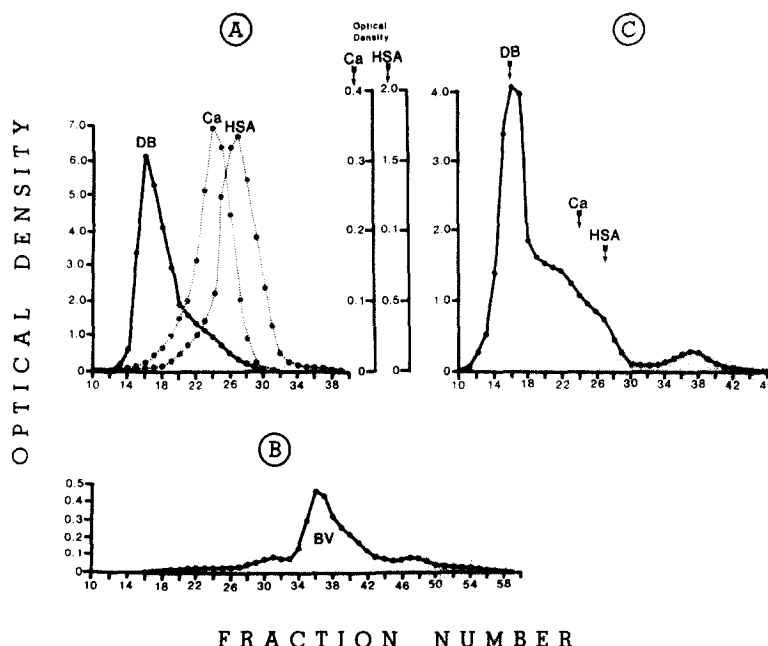


FIG. 1. Sephacryl S-300 column chromatography fractionations. **A**, Profiles of dextran blue (DB), catalase (Ca), and HSA. **B**, Profile of monomeric bee venom (BV). **C**, Profile of BV and HSA after polymerization with glutaraldehyde. Arrows mark the location of DB, Ca, and HSA peaks that were obtained in separate fractionations on the standardized columns, as demonstrated in **A**.

obtain a soluble polymer with the highest venom to albumin ratio. The final preparation is described.

Honey bee venom (Sigma Chemical Co., St. Louis, Mo.), 5 mg, as estimated by OD at 280 nm, and 27 mg (by OD at 280 nm) of chromatographically purified human albumin (Cappel Labs., Cochranville, Pa.) were dissolved in 0.15M of phosphate-buffered NaCl, pH 7.35 (phosphate-buffered saline). The venom and albumin were polymerized with a 25% solution of glutaraldehyde at a final concentration of 0.008M. Total volume was 4.3 ml. Polymerization was carried out at room temperature for 3 hr, and 95 mg of glycine was added to stop further polymerization. The solution was mixed for 20 min and centrifuged for 10 min at $1200 \times g$. The supernatant was chromatographed on a Sephacryl S-300 superfine (Pharmacia Fine Chemicals, Piscataway, N. J.) column previously calibrated with dextran blue 2000 (Pharmacia), chromatographically pure HSA, and catalase. Polymers used had a molecular weight higher than catalase (240,000).

Multiple preparations of BVAP demonstrated the same characteristics by chromatography.

Immunization of rabbits with BVAP

BVAP was used to immunize rabbits with seven weekly subcutaneous injections of 2.6 mg of protein (estimated by OD at 280 nm). Blood samples for studies of the immune response were taken at zero time and biweekly for 14 wk. Sera were obtained by centrifugation and were stored at -20°C .

ELISA for measurement of antibody against bee venom

A micro-ELISA method⁸ was used with slight modifications. Briefly, Immulon-microplates (Dynatech Labs., Alexandria, Va.) were used. Coating buffer was 0.1M Tris and 0.15M NaCl, pH 7.9. Wells were coated with 100 μg of venom or polymer per milliliter. Other buffers were the same as described previously.⁸ The only other modifications were: (1) plates were reacted with antibody dilutions for 1 hr at 37°C and (2) alkaline phosphatase-conjugated goat anti-rabbit IgG or rabbit anti-human IgG was incubated for 1½ hr at 37°C instead of at room temperature. Plates were read for an OD at 410 nm on a micro-ELISA minireader MR 590, Dynatech Instruments, Inc., Santa Monica, Calif.

Inhibition of human and rabbit anti-bee venom by bee venom

A human antiserum pool against bee venom from beekeepers and two additional sera from persons with antibody to bee venom were supplied by Dr. John Yunginger, Mayo Clinic. Rabbit antisera prepared as described above were used. These antisera were titrated by the ELISA technique. The highest dilutions of antisera were selected that produced the greatest OD separation of positive sera from negative control sera. To these antisera dilutions and normal human or normal rabbit serum dilutions, increasing amounts of either bee venom or BVAP were added. Volumes were kept constant by the addition of buffer. All antisera and control

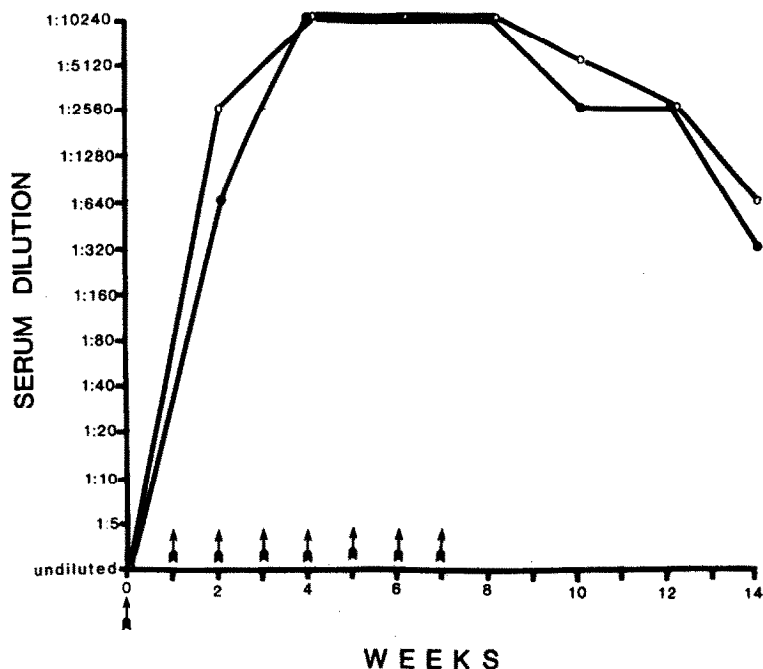


FIG. 2. Antibody response to bee venom induced in two rabbits by polymerized venom-albumin. Immunization schedule is demonstrated by arrows. Endpoint titers are two times control by use of the ELISA technique.

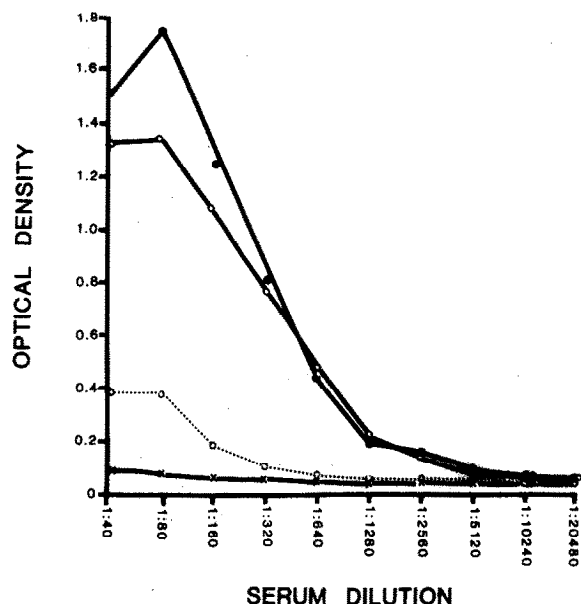


FIG. 3. Human antibody titers against BVAP. ELISA wells coated with BVAP. Beekeeper sera pool (●—●), human anti-venom serum A (○—○), human anti-venom serum B (○---○), and normal human serum (x—x). Endpoint titers are two times control by use of the ELISA technique.

sera were incubated at 37° C for 30 min and were incubated overnight at 4° C with the inhibiting antigens. Varying dilutions of these mixtures were added to the ELISA wells precoated with bee venom or BVAP, and the residual antibody activity was measured as described above.

TABLE I. Inhibition of human anti-bee venom antisera pool by BVAP with ELISA technique

	Antigen used for inhibition* (μg)		
	1	50	100
Bee venom	82†	100	100
BVAP	44.6	87.9	97

Wells coated with bee venom; antisera pool from beekeeper sera, 1:1000 dilution; antigens were bee venom or BVAP; calculations:

$$\text{percent inhibition} = \frac{100 \times \text{OD antibody alone} - \text{OD after antigen inhibition}}{\text{OD antibody alone}}$$

*Calculated from OD at 280 nm.

†Percent inhibition at each concentration of antigen.

Radioiodination of bee PLA

Bee PLA was obtained from Sigma Chemical Co. and labeled with ^{125}I with the use of the chloramine-T method of Gleich et al.⁹

Incorporation of ^{125}I -labeled bee PLA into BVAP

Approximately 0.2 μg of ^{125}I -labeled PLA was added to a mixture of 5 mg of bee venom and 27 mg of HSA, and polymerization of this mixture was carried out in the manner described above. Each Sephacryl S-300 chromatographic

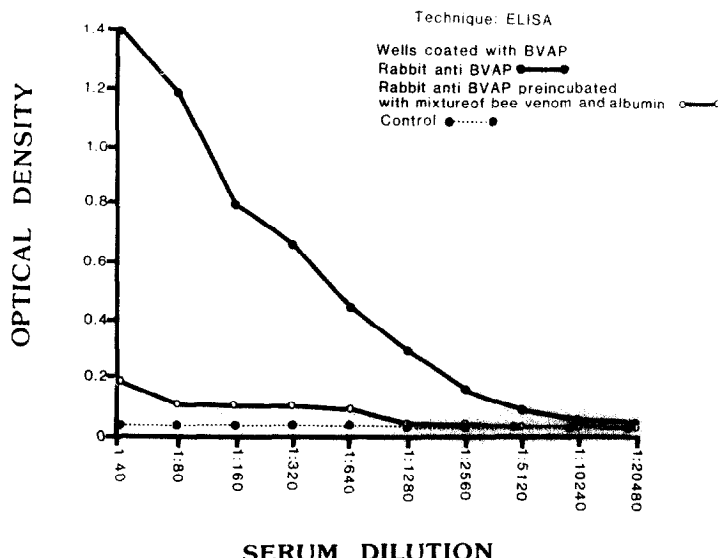


FIG. 4. An ELISA titer of rabbit anti-BVAP against BVAP and change in titer after the anti-BVAP was preincubated with a mixture of bee venom and HSA.

fraction was checked for OD at 280 nm and total radioactivity, and the appropriate fractions were combined as in the preparation of BVAP. Protein bound counts were determined after 10% trichloroacetic acid precipitation. From the data obtained the extent of incorporation of ^{125}I -labeled PLA into the BVAP could be determined.

Measurement of antibody against bee PLA in rabbit anti-BVAP

The modification of the ammonium sulfate technique of Lidd and Farr was used as previously described in detail for this assay.⁷

RESULTS

Chromatographic profiles of dextran blue, bee venom, HSA, catalase, and BVAP with Sephacryl S-300

Fig. 1, A illustrates dextran blue with the peak in excluded volume (tube 16). Catalase and HSA are in the S-300 included volume. Untreated bee venom has a wide profile (Fig. 1, B). After polymerization of bee venom and HSA, the polymers are in both the excluded and included fractions of S-300 (Fig. 1, C). Only the fractions with higher molecular weights than catalase (MW 240,000) are used for the BVAP preparation.

Immune response of rabbits immunized with BVAP

Rabbits were immunized with BVAP prepared from the S-300 chromatographic separation illustrated in Fig. 1, C. Both rabbits demonstrated an immune response to bee venom as demonstrated by the ELISA technique (Fig. 2). The antibody responses were of

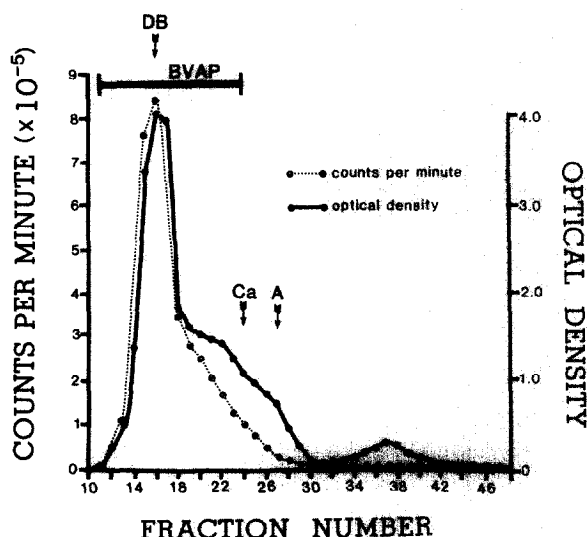


FIG. 5. Sephacryl S-300 profiles of BVAP by OD and radioactivity by use of ^{125}I -labeled PLA incorporated into BVAP during the polymerization process. Peaks of dextran blue (DB), catalase (Ca), and albumin (A) on the same column are demonstrated.

similar levels in both rabbits. The antibody levels declined after immunization was terminated.

Antibody activity of human anti-bee venom sera against BVAP

The ELISA technique was used with wells coated with BVAP. Three human sera were analyzed for antibody activity against BVAP. These sera were from a beekeeper sera pool (141 μg of IgG antibody against PLA) and two individual sera with antibody against

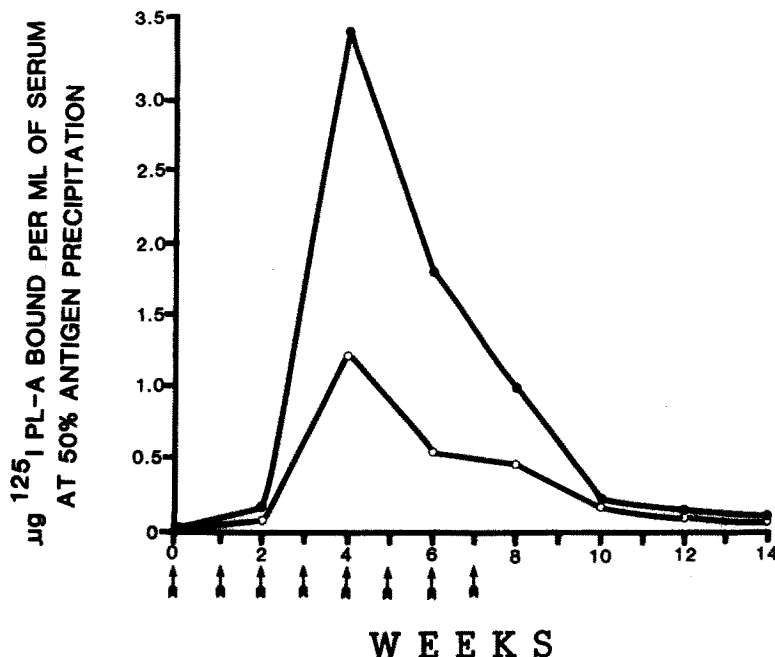


FIG. 6. Antibody binding of ^{125}I -labeled PLA after immunization of two rabbits with BVAP. Immunization schedule is demonstrated by arrows.

bee venom. The results (Fig. 3) demonstrate antibody activity in all three human sera against BVAP.

Inhibition analysis demonstrating antigenic completeness of BVAP

This study was done to determine if the antigens in BVAP would inhibit human antibody reactions against bee venom. The technique and results are summarized in Table I. These results demonstrate that preincubation with BVAP will almost completely inhibit the subsequent reaction of human antiserum with bee venom. Higher concentrations of BVAP as compared with bee venom are necessary to achieve similar degrees of inhibition.

Neutralization of antibody against BVAP by bee venom and HSA

This study was done to determine if there was evidence of new antigenic determinants on BVAP as evidenced by failure to inhibit anti-BVAP with bee venom and albumin. The titer of a rabbit anti-BVAP was determined by use of ELISA with wells coated with BVAP. Simultaneously, the titer was determined after the anti-BVAP had been preincubated with a mixture of 50 μg each of bee venom and HSA. The titer of the neutralized anti-BVAP was reduced to 1:640 (two times normal rabbit serum control) from the unneutralized anti-BVAP titer of 1:10,240 (Fig. 4). If new antigenic determinants were present on BVAP as a result of the polymerization process, absorption

of the anti-BVAP with bee venom plus albumin should not have reduced the titer to near control levels.

Incorporation of ^{125}I -labeled PLA into BVAP

^{125}I -labeled PLA was mixed with bee venom and HSA. This mixture was polymerized and fractionated on S-300. The results are illustrated in Fig. 5. Bee venom PLA has a molecular weight of about 20,000 daltons.¹⁰ More than 96% of the radioactivity was associated with molecules of higher molecular weight than albumin, demonstrating that polymerization of most of the PLA occurred. Since polymers larger than catalase are used for BVAP, 92% of the PLA of monomer was incorporated into polymers with molecular weights in excess of 240,000 (Fig. 1).

Antibody responses to PLA in rabbits immunized with BVAP

Results are illustrated in Fig. 6. The antibody levels declined from peak levels during continued immunization.

DISCUSSION

The safety and efficacy of ragweed polymers were demonstrated,^{1,2} and subsequently, polymerized grass allergens were demonstrated to be safe and effective.³ Only a small number of immunizing injections were required for clinical benefit lasting at least 3 yr (longest period that therapy was evaluated).¹¹ The potential benefit of venom polymers for patients with anaphy-

lactic venom reactivity was obvious. This encouraged continued research directed toward preparation of soluble antigenic venom polymers in spite of the tendency of venoms to form insoluble polymers. The soluble BVAP should provide a useful immunotherapy for venom anaphylaxis. The molecular weight of the BVAP is in excess of that of catalase (Fig. 1) and thus ranges from 240,000 to the polymers excluded by Sephacryl S-300 that would be in excess of 1,500,000 daltons. The BVAP produces an immune response against bee venom in rabbits. Human-IgG antibody against bee venom reacts with BVAP, and BVAP can absorb the human antibody against venom. HSA is already required in diluents for venoms for human immunotherapy. In the BVAP we believe the HSA constitutes an inert carrier that permits the formation of soluble BVAPs. We demonstrated no evidence of new antigenic determinants on BVAP as evidenced by neutralization of anti-BVAP by a mixture of bee venom and HSA. These results are consistent with BVAP being a potential immunotherapeutic agent for bee venom anaphylaxis. A major antigen of bee venom, PLA, is incorporated into BVAP as demonstrated by the studies with ^{125}I -labeled PLA incorporation. An unexpected result was the decline in antibody to PLA during continued immunization with BVAP in contrast to the total antibody response that fell only after immunization was completed. The possibility that this may limit the usefulness of this antigen in human venom immunotherapy must be considered if immune responses are not maintained. However, a similar decline in antibody responses occurred when rabbits were immunized with AgE.¹² These antibody declines occurred during immunization with polymeric and particularly with monomeric forms of ragweed by use of similar immunization regimens.⁴ Because both the monomeric and polymeric^{1, 2} forms of ragweed are proved to be effective immunotherapeutic agents in man, the rabbit studies with BVAP do not preclude longer term efficacy in man. Possibly the BVAP may only be useful as a safer priming an-

tigen for subsequent monomeric venom boosters. In contrast, it may be possible that a form of tolerance was induced by BVAP that in man might be present for both IgG and IgE immune responses.

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