

Enzyme-linked immunosorbent assay (ELISA) of changes in specific IgG antibodies to five venoms during venom immunotherapy

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Summary

An enzyme-linked immunosorbent assay (ELISA) was developed that could measure titres of human IgG antibodies to five different venoms (honeybee, yellow jacket, yellow hornet, white-faced hornet, and wasp), and to honeybee phospholipase A. Changes in specific IgG anti-venom titres were measured in twenty patients that had systemic anaphylactic reactions to insect stings, and ten non-allergic controls. After being stung and prior to treatment all patients had anti-venom IgG titres greater than controls. Treatment with small doses of venom over 1-2 months resulted in prompt rises in anti-venom IgG titres that may represent secondary anamnestic responses primed by prior stings. All patients undergoing venom immunotherapy showed at least 2-fold increases in IgG antibody to the venoms they were treated with by the time maintenance doses of 100 mcg were achieved, with one exception. Significant cross-reactive increases in anti-vespid IgG antibodies to venoms not used for treatment occurred in nine of eighteen treated patients. Overall, ELISA of IgG antibodies to five venoms allowed clear evaluation of the considerable variation of IgG responses among different patients. We conclude that serial determination of venom-specific IgG titres by ELISA offers an important adjunct to evaluating the results of venom immunotherapy.

Introduction

Anaphylactic reactions to insect stings are considered due to venom-specific IgE antibodies that sensitize mast cells and basophils. Although immunotherapy with *Hymenoptera* venoms has been shown to be highly efficient in preventing such reactions, the mechanisms by which success has been achieved are still understood incompletely (Hunt *et al.*, 1978; Lichtenstein, Valentine & Sobotka, 1979). Venom immunotherapy is associated with increases in venom-specific IgG antibodies and this has correlated with the efficacy of treatment (Golden *et al.*, 1980; Hoffman *et al.*, 1981).

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Indeed, an increase in venom-specific IgG antibody of at least 2-fold above a patient's pre-treatment level, or the attainment of certain minimum levels set in terms of the IgG assay employed, seem to be needed in order to attain clinical protection from a subsequent sting reaction (Lessof, Sobtka & Lichtenstein, 1978; Golden *et al.*, 1982).

Several methods have been employed for the determination of venom-specific IgG antibodies and recent reports indicate that an enzyme-linked immunosorbent assay (ELISA) may be the most advantageous (Grant *et al.*, 1981; Reid, Kwasnicki & Cheung, 1982). In the present report we used an ELISA for the simultaneous determination of IgG antibodies to honeybee, yellow jacket, yellow hornet, white-faced hornet, and wasp venoms, and to purified honeybee phospholipase A. This method allows for the first time evaluation of the cross-reactivity that occurs in the venom-specific IgG antibody responses of patients undergoing immunotherapy with different venoms. In addition, it effectively demonstrates the considerable patient-to-patient variation in response to venom immunotherapy and points the way toward the acquisition of important new clinical information.

Materials and methods

Patients and immunotherapy regimen

Twenty patients who had experienced systemic anaphylactic reactions from insect stings and ten skin-test-negative controls who had never experienced such reactions were evaluated. Sensitivity to insect venom was demonstrated by immediate weal and flare intradermal skin tests with venom (Pharmacia Diagnostics, Piscataway, NJ) and graded according to the package insert. Patients undergoing immunotherapy started with subcutaneous venom doses of 10–40 mcg and proceeded to maintenance levels of 100–400 mcg/month by doubling of doses at 2–4-week intervals in a manner similar to the 'rush' regimen of Golden *et al.* (1981, 1980).

Elisa

A modification of the ELISA described by Grant *et al.* (1981) was performed in polystyrene microtitre plates (Immulon II, Dynatech Laboratories, Alexandria, VA). Honeybee (HB), yellow jacket (YJ), yellow hornet (YH), white-faced hornet (WH), and polistes wasp (W) venom (Vespa Laboratories, Spring Mills, PA) and honeybee phospholipase A (PLA) (Sigma Chemical Co., St Louis, MO) were radiolabelled with ^{125}I using the Choramine T method (Hunter & Greenwood, 1962). Various concentrations of each radiolabelled venom were placed in the wells of the microtitre plates and counts bound to the wells after 24 hr incubation at 4°C were divided by the total counts added to determine an optimal coating concentration of venom (see Results, Fig. 1).

For routine ELISA, wells were coated for 24 hr at 4°C with 0.15 ml of carbonate buffer (0.06 M NaHCO_3 , 0.06 M Na_2CO_3 , pH 9.6) containing 10 mcg/ml of one of the five venoms or PLA.

At the time of assay, wells were washed twice with phosphate-buffered saline (PBS)-Triton 0.05% (0.05 M Na_2HPO_4 , 0.05 M NaH_2PO_4 , 0.15 M NaCl, 0.05% Triton X-100). Duplicate 0.15-ml aliquots of sera diluted 1:20, 1:200 and 1:2000 in PBS-Triton were placed into wells that were precoated with each of the five venoms and PLA. Covered plates were then incubated over night at room temperature.

The next day, plates were emptied and washed twice with PBS-Triton. Wells were filled with 0.15 ml peroxidase-conjugated goat antihuman IgG (Tago Laboratories,

Burlingame, CA) diluted 1:3000 in PBS-Triton and incubated overnight at room temperature. Plates were again emptied and washed twice with PBS-Triton. To each well 0.15 ml of substrate, ortho-phenylene diamine (0.1 mg/ml), was then added and allowed to react for 30 min at room temperature. The reaction was stopped by the addition of 0.05 ml 4 N H_2SO_4 and the optical density of each well was measured at 492 nm on an eight-channel photometer (Titertek Multiskan; Flow Laboratories, Mclean, VA). On each plate, the first row of venom-coated wells that had not been exposed to serum was used as the photometer blank.

For each serum a titre of specific IgG was determined by linear regression analysis of the dilution curve for each venom. Absorbance values for the 1:20, 1:200 and 1:2000 serum dilutions were plotted on a scale using the negative logarithm to the base ($-\log_2$) to express serial 2-fold dilutions. A titre was defined as the dilution of serum having an absorbance value equal to the mean of absorbance values from the ten control sera at a dilution of 1:20 (Fig. 2). The resulting titre values expressed as $-\log_2$ had a standard deviation (s.d.) of ± 0.4 and titre changes of > 2 s.d. were taken as significant.

Results

Determination of conditions for coating wells with venoms

Triplicate wells of microtitre plates were coated with 0.15 ml of different concentrations of radiolabelled HB, YJ, YH, WH and W venoms, and with honeybee phospholipase A and the quantity of each venom bound to the wells was calculated. Figure 1 shows that binding of venom proteins to the wells reached a plateau amount

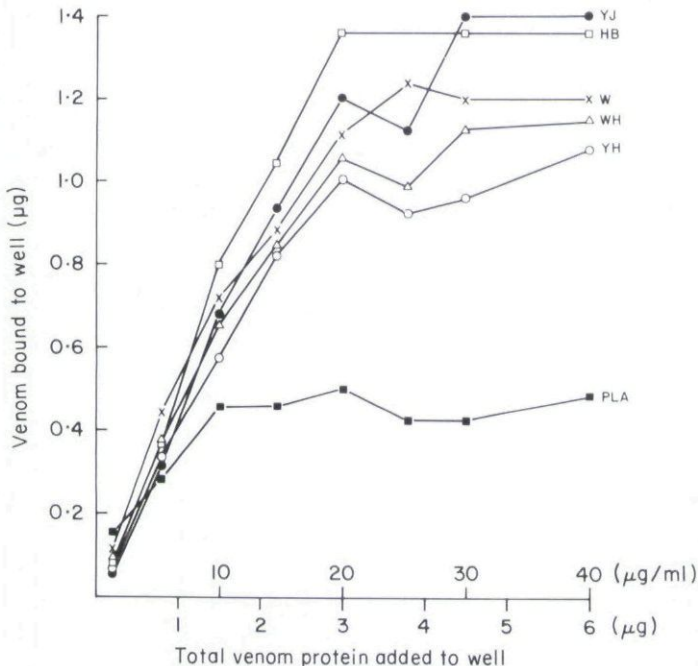


Fig. 1. Binding of increasing amounts of radiolabelled venoms to polystyrene wells.

for all venoms at concentrations of 20 mcg/ml or more, while the plateau for PLA was achieved at 10 mcg/ml. At the 20 mcg/ml concentration 40% of the total of each venom added to the wells was bound to the well after 24 hr incubation and the percent of the total bound decreased with increasing concentration up to 40 mcg/ml (Fig. 1). A 2-hr incubation yielded 25% binding for the 20 mcg/ml concentration while a 6-week incubation resulted in a 7% decrease in binding. Chequerboard titration using 5, 10 and 20 mcg/ml coating concentration of venom with dilutions of reference sera from patients undergoing venom immunotherapy showed that the 10 and 20 mcg/ml venom concentrations yielded essentially the same IgG titres, so 10 mcg/ml was selected as the coating concentration for all subsequent studies to economize on the use of the expensive venoms.

Determination of IgG anti-venom titres

The results of a typical ELISA titration for 2-fold dilutions of serum containing IgG antibodies against yellow jacket venom and a negative control serum are shown in Fig. 2a. Sigmoid curves were obtained with a semilog plot using the negative logarithm to the base 2 ($-\log_2$) to express serial 2-fold dilutions of serum. The slopes of curves for patient sera were generally parallel and their mid-portions were linear over a wide range of antibody concentrations. Because of this, linear regression analysis was used to mathematically describe the mid-portions of the dilution curve. This information was converted into a titre value by calculating the dilution at which the curve of 2-fold dilutions (Fig. 2a) or of 10-fold dilutions (Fig. 2b) intersected with the mean absorbance value of 1:20 dilutions of the ten control sera. The correlation coefficient (r) for the linear regression analysis of the dilution curves averaged 0.97 with a range of 0.94–0.99, indicating that the slopes were indeed linear. The resulting titre values expressed as $-\log_2$ had a standard deviation of ± 0.4 . When a single serum was assayed on different occasions, the resulting titre values showed little variation.

Changes in venom-specific IgG titres over time with various venom immunotherapy regimens

Figure 3 shows the IgG levels to all the venoms and PLA at four different times over 3 years in one of two untreated patients with similar findings. There is little change in the levels of venom-specific IgG over this period of time. However, it is noteworthy that all levels are greater than controls and that the skin tests became negative. Figure 4 depicts the effect of YJ-venom immunotherapy in a patient that had a systemic anaphylactic reaction to a sting. A prompt rise in YJ-venom-specific IgG (8-fold) occurred in the first month of immunotherapy and then declined gradually to pre-treatment levels by 1 year. There was no significant change in IgG antibody levels to any other venom, except for a gradual decline over 2 years.

Figure 5 shows the results of mixed vespid (YJ, YH, and WH) venom immunotherapy in a patient who experienced large local reactions from early injections. Maintenance doses were eventually achieved and the IgG antibody titres to the treatment venoms (YJ, YH, WH) increased significantly while titres of IgG antibody to venoms not being administered declined.

Figure 6 shows a dramatic rise in IgG antibodies to YJ in a patient receiving equal doses of four venoms (YJ, YH, WH, W). While the increase of IgG to the other treatment venoms were significant, they were of much smaller magnitude than the response to YJ. When treatment was interrupted for 2 years, there was a drop off in the

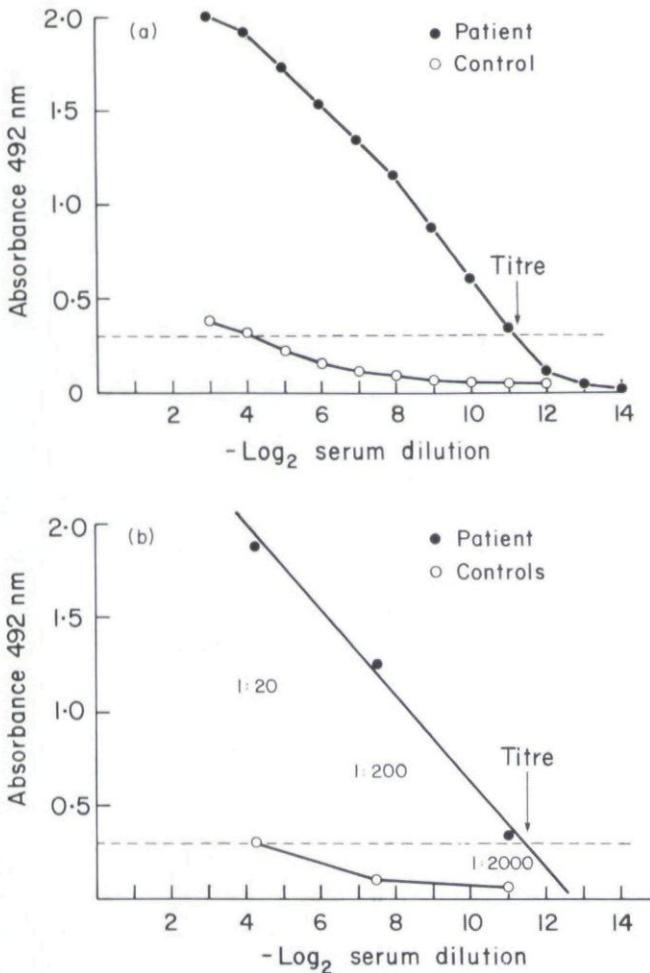


Fig. 2. (a) ELISA titration of IgG antibodies against YJ venom in a patient (●) treated with YJ venom, and a non-allergic control (○). Sigmoid-shaped curves with linear mid-portions result. (b) ELISA of IgG antibodies against YJ venom in a patient (●) treated with YJ venom and measured at dilutions of 1:20, 1:200 and 1:2000, and the mean of ten control sera (○) at these dilutions. The antibody titre is the serum dilution determined by linear regression analysis (straight line) which has the same absorbance value as the mean of ten control sera at dilutions of 1:20.

titres of antibody that had been achieved under treatment (Fig. 6). Another patient (Fig. 7) that received equal doses of four venoms (YJ, YH, WH, W) demonstrated considerable IgG antibody increases to three venoms—YJ, YH, WH—but no increase at all to wasp (W) despite 100-mcg doses every 6 weeks for 1 year.

Of the twenty patients who were evaluated, all, including those that were not treated (see Fig. 3), had pre-treatment amounts of venom-specific IgG antibody to most venoms that were greater than controls. There was considerable individual variation in response to venom immunotherapy as Figs 4–7 depict, and this has been noted before (Golden *et al.*, 1981b). Taken together, all patients undergoing venom immunotherapy showed at least a 2-fold increase in IgG antibody levels to the venoms

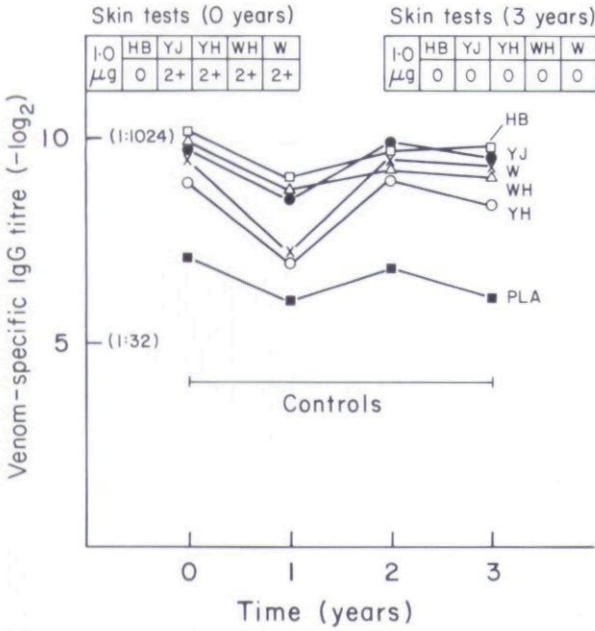


Fig. 3. Changes of venom-specific IgG antibody titres in an untreated patient followed for 3 years. Note change in skin-test reactivity to negative while IgG titres showed little change.

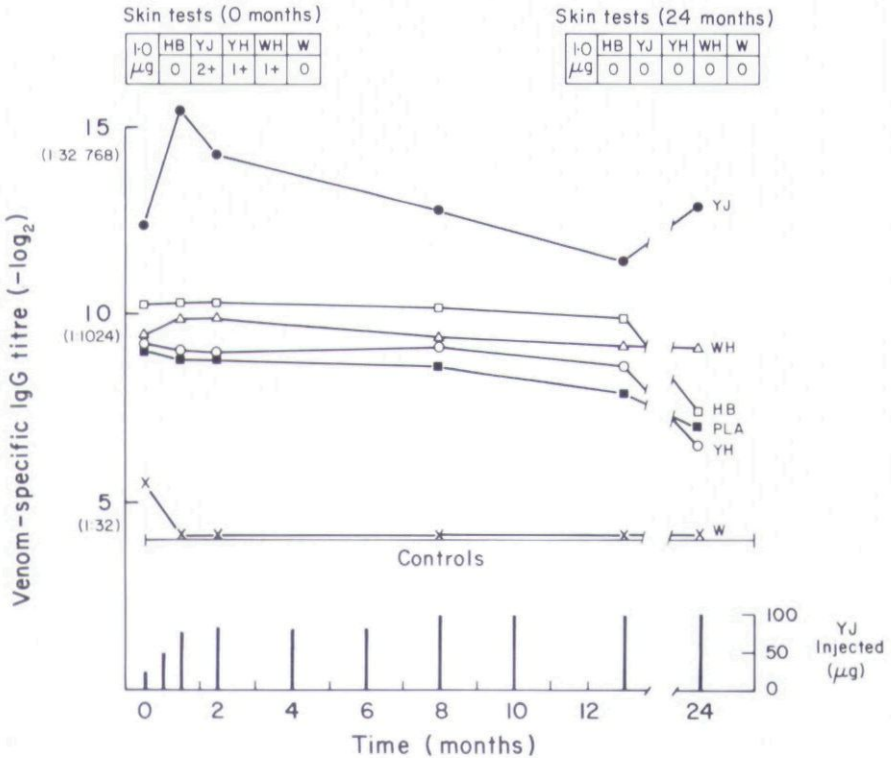


Fig. 4. Changes of venom-specific IgG antibody titres in a patient treated with YJ venom for 2 years. There is an 8-fold rise in IgG to YJ in the first month but no cross-reactive changes in IgG to any other venom.

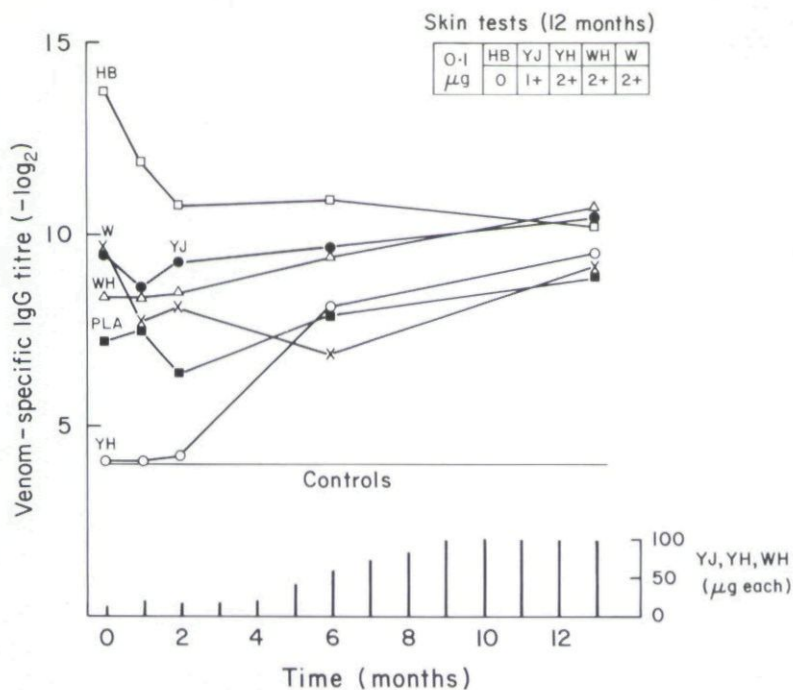


Fig. 5. Changes of venom-specific IgG antibody titres in a patient treated with mixed vespid (YJ, YH, WH) venom for 13 months. IgG for untreated venoms (HB, W, PLA) shows some decline while IgG for treated venoms show varying increases.

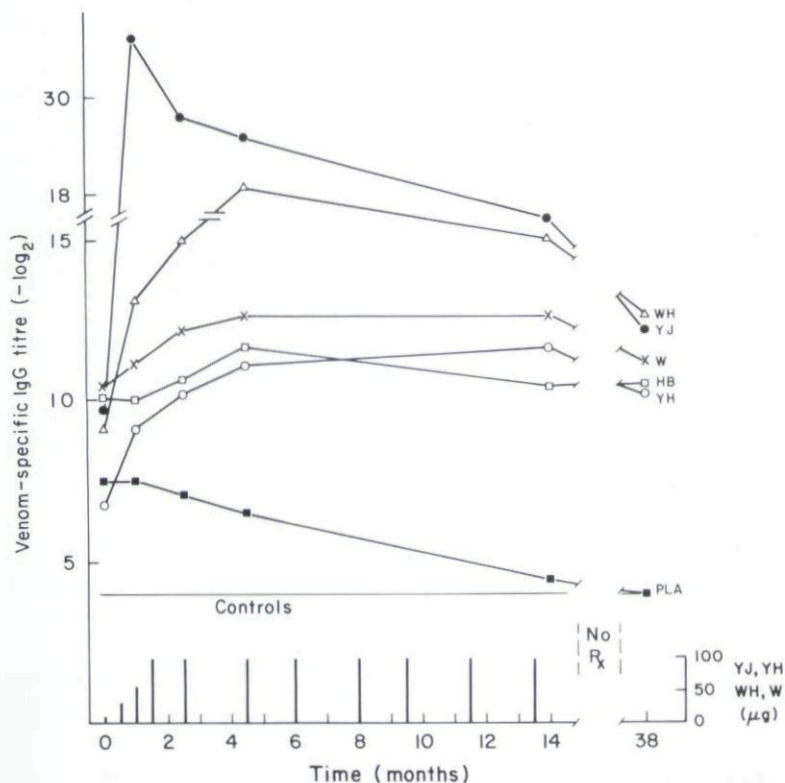


Fig. 6. Changes of venom-specific IgG antibody titres in a patient treated with mixed vespid (YJ, YH, WH) and wasp (W) venoms. Note dramatic IgG responses to YJ and decline in titres of all treatment venoms when immunotherapy was stopped.

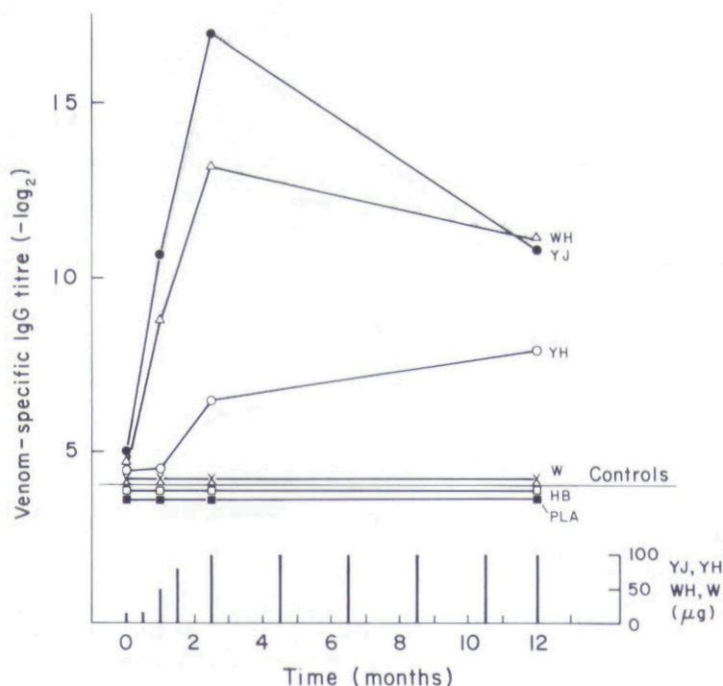


Fig. 7. Changes of venom-specific IgG antibody titres in a patient treated with mixed vespid (YJ, YH, WH) and wasp (W) venoms. There are substantial increases in IgG antibody to YJ, YH, WH venoms but none to W venom, even though equal amounts of each venom were given for 1 year.

they were treated with by the time maintenance doses of 100 mcg were achieved, except for one venom in one patient (Fig. 7). Often peak antibody titres were achieved at this point and were then followed by slow declines. Nonetheless, titres of IgG antibody to treatment venoms at the end of 1 year of immunotherapy were still well above pre-treatment levels in most instances.

Cross-reactivity to anti-venom IgG responses

In this study, neither HB venom nor PLA was used for immunotherapy, and IgG antibody titres to HB and PLA generally declined over time or remained stable. In a few instances (Figs 5 and 6) modest IgG increases to either HB or PLA were noted in patients being treated with vespid venoms that were not specifically treated were often unchanged or slightly increased, indicating some cross-reactivity, but this was an inconstant finding. Figure 4 does not show any increase in IgG titre for any vespid venom other than the YJ which was used for treatment. On the other hand, Fig. 8 shows an increased titre of IgG antibody to WH and W along with YJ in a similar patient that was treated just with YJ venom and this may have been enough to provided protection from a wasp sting that occurred during immunotherapy (Fig. 8). Significant cross-reactive elevations in anti-vespid IgG antibodies occurred in nine of the eighteen patients (50%) undergoing venom immunotherapy that we studied, but no pattern of association was apparent.

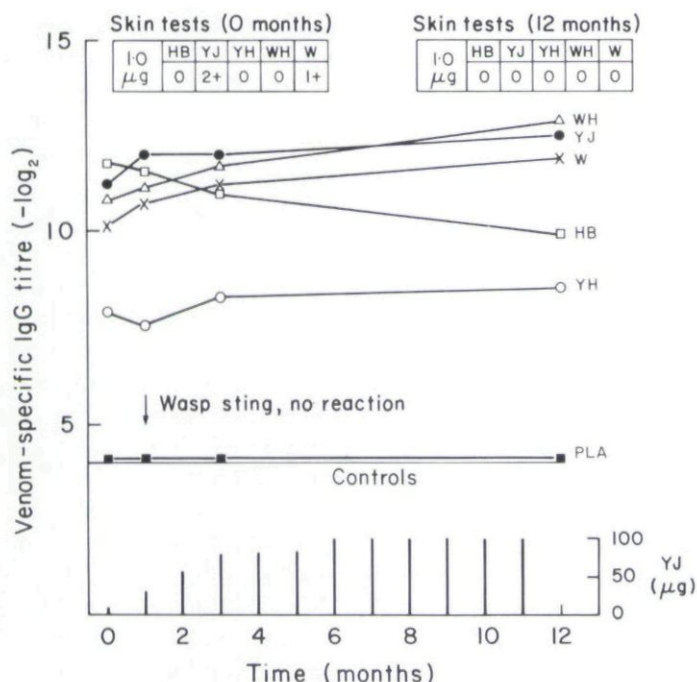


Fig. 8. Changes of venom-specific IgG antibody titres in a patient treated with YJ venom. Increasing titres of IgG antibody to WH and W in addition to YJ indicate some cross-reactivity.

Discussion

This study demonstrates that the ELISA employed is reproducible and sensitive enough to quantitate venom-specific IgG antibodies to all treatment venoms used in immunotherapy. This is an extension of previous work (Grant *et al.*, 1981; Reid *et al.*, 1982) and confirms the value of ELISA as a tool for measuring venom-specific IgG antibodies.

Several findings emerge from this study that have clinical relevance. Following anaphylactic reactions, and prior to immunotherapy, patients who all had positive immediate skin-test responses, all had titres of venom-specific IgG to most venoms well in excess of the skin-test negative controls. While this is not a new finding, its significance may not have been fully appreciated. In a study of venom immunotherapy utilizing a placebo group (Hunt *et al.*, 1978), measurable amounts of IgG antibody to HB PLA and YJ were found in the pre-treatment sera of all groups. While venom immunotherapy produced significant increases in the amounts of venom-specific IgG, the amounts initially present may have been sufficient to account for the lack of reaction to a challenge sting in 42% of the placebo group. The two patients in this study who were not treated, but were followed over 3 years were children. Both patients maintained relatively stable titres of venom-specific IgG to most venoms well in excess of controls (Fig. 3), but less than the levels achieved in treated patients. This finding may provide some explanation as to why untreated insect allergic children have such a low reaction rate to subsequent stings (Schuberth *et al.*, 1982).

What is readily apparent from our data is the patient-to-patient variability. This is not due to technical factors involved in the ELISA since all determinations were done

at the same time under the same conditions, and similar findings were made when assay of sera of some patients was repeated. The standard deviation of $\log_2$ titres (± 0.4) was reasonable, and allowed clear discrimination of changing titres. The variability, then, is really due to the differing immune responses of individual patients to different venoms. This patient-to-patient variability has long been recognized and has been one of the factors complicating our understanding of how to judge the efficacy of venom immunotherapy. While previous studies have shown increases in venom-specific IgG with venom immunotherapy, only IgG antibodies to HB, YJ and HB PLA were measured. While it may be reasonably assumed that IgG antibodies to other treatment venoms would behave similarly, this study provides direct evidence of this for the first time.

We also found that the same dose of individual venoms can produce vastly different titres of specific IgG at a given time in individual patients. This could mean that clinical protection would be achieved for some venoms but not others even though the same dose of each venom was administered. While recent reports would indicate the need to obtain certain amounts of venom-specific IgG to be assured of protection (Golden *et al.*, 1982), the exact amount needed for each venom would still seem to be in question in light of this variability. Although 2-fold rises in IgG levels are usually achieved by the time a 100-mcg dose of venom can be given (generally within 3 months) some patients seem to take longer to reach this level (Fig. 5), and most patients show a decline in antibody titre from peak values at the end of 1 year, despite persistent injections of 100-mg doses of venoms. Although this decline may not be associated with any decline in clinical protection from a sting (Golden *et al.*, 1981), it does make the assignment of clinical protection to a particular laboratory value at a particular time a more uncertain task. It also raises the question that the mechanism of the protection afforded by venom injections may differ at different times during the course of immunotherapy.

Cross-reactivity among the vespid venoms has been demonstrated by different investigators (Kern *et al.*, 1976; Reisman *et al.*, 1982) but the degree of this cross-reactivity has been variable. The results of the present study demonstrate this aspect of variability on a larger scale. While significant cross-reactive elevations of anti-vespid IgG antibodies occurred in half of the patients undergoing immunotherapy with at least one vespid venom, the combination and associations between the venoms varied with each individual patient. The unpredictable nature of the cross-reactivity seen in the present study leads us to conclude that the most reliable way of achieving protection with immunotherapy should consist of using the specific venoms that produce positive skin tests rather than relying on cross-reactive protection from a single venom.

Another interesting finding was the change in skin-test results over time. Five patients undergoing immunotherapy showed a 10-fold decline in the amount of venom required to evoke a positive skin test and five other patients had negative skin tests after 1–2 years. One patient that did not require immunotherapy, and had no changes in IgG levels, reverted to negative skin tests (Fig. 3). The purpose of this study centered on venom-specific IgG antibodies during venom immunotherapy. However, these changes in weal and flare skin-test responses emphasize that IgG is only one of the changing variables in these patients and that venom-specific IgE antibodies and mast-cell responsiveness should also be considered when assessing clinical protection from insect sting reactions.

We also demonstrated prompt and at times dramatic rises in venom-specific IgG after administration of relatively small amounts of venom (10–40 mcg) in the first 1–2 months of treatment. This is characteristic of a 'secondary' (anamnestic) immune response where lower threshold doses of antigen, shorter lag phases, and greater antibody synthesis and persistence are seen. This may have implications for consideration of other regimens of venom immunotherapy than are used currently. In the evaluation of other regimens of venom immunotherapy, an ELISA for venom-specific IgG would seem to be a simple, convenient and valuable tool. The present study demonstrated the reliability and sensitivity of this assay and its capability for providing important clinical information.

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