

Original article

Early effect of ultrarush venom immunotherapy on the IgG antibody response

Background: We have previously shown in several allergy models that allergic and tolerance status with respect to allergens is associated with a somewhat different dominant specificity of IgG antibodies. The objective was to test this hypothesis in the compelling model of ultrarush venom immunotherapy (VIT), which induces clinical tolerance after only a few hours of treatment.

Methods: Antibody titers and specificity were evaluated through solid-phase ELISA using streptavidin-biotin technology in 12 patients allergic to wasp venom before and during the ultrarush procedure (at 12 h, 24 h, and 15 days). The results were compared with those from another group of 20 patients treated with venom injections for at least 2 years.

Results: No significant change was observed in IgG titers during the early phase of VIT. The capacity of individual sera to prevent the antigen binding of pooled IgG from allergic patients changed rapidly, with mean percentage inhibitions falling from $80 \pm 15\%$, before starting VIT, to $26 \pm 14\%$, $35 \pm 15\%$, and $34 \pm 5\%$ after 12 h, 24 h, and 15 days of treatment, respectively ($P < 0.001$ by one-way ANOVA). The capacity of individual sera to prevent the antigen binding of pooled IgG from patients receiving prolonged VIT changed, with mean percent inhibitions increasing from $47 \pm 8\%$, before starting VIT, to $76 \pm 7\%$, $83 \pm 6\%$, and $87 \pm 6\%$ after 12 h, 24 h, and 15 days of treatment, respectively ($P < 0.001$ by one-way ANOVA).

Conclusions: During the initial phase of ultrarush VIT, a change in IgG specificity, i.e., a change in the set of epitopes dominantly recognized by IgG on wasp-venom antigens, occurred concomitantly with early clinical tolerance and was already detectable a few hours after the onset of treatment. Although it may be an epiphenomenon, this change represents the earliest humoral modification described so far during this procedure. The mechanism is unknown, but it appears to be a selective depletion of the highest avidity antibody fraction by the venom injected in large doses at this stage of therapy. Finally, our data now show the previously documented association between a particular IgG specificity and the clinical status (allergy vs tolerance) to be true also with ultrarush VIT, a model in which the clinical ability to display allergic symptoms is rapidly reversed.

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We have previously reported that patients allergic to house-dust mites (1) and grass pollen (2) who suffer from rhinitis and asthma display an IgG antibody with a dominant specificity somewhat different from that observed in healthy individuals. Similar findings were documented in children allergic to cow's milk when the specificity of their anti- β -lactoglobulin (BLG) IgG antibodies was compared with that from control children (3). Furthermore, when allergic children became tolerant to milk, their anti-BLG IgG antibodies acquired the dominant specificity exhibited by those from controls (3). Such a reversibility was also documented in the case of venom-allergic patients treated by immunotherapy

(IT) (4, 5). Taken together, these data suggest that allergic and tolerance statuses are associated with the preferential expression of distinct IgG specificities, which appear to be equally distributed over the IgG1 and IgG4 antibody subclasses (2, 5). Ultrarush venom immunotherapy (VIT) offers the opportunity to test this hypothesis in a model in which clinical tolerance, at least to subcutaneous injections of large amounts of venom, is induced in allergic patients after only a few hours of treatment (6–8). Therefore, the present study investigated the evolution of the IgG antibody specificity during the initial phase of ultrarush VIT in 12 allergic patients.

Table 1. Individual data on allergic patients

Patients	Age (years)	Sex (F/M)	Severity of sting reaction*	Serum specific IgE (iU/ml)	Intradermal response** (wheal size in mm)
1	43	F	IV	3.70	6
2	54	F	III	100.00	ND
3	36	M	III	15.80	8
4	34	M	III	1.72	6
5	20	F	III	4.82	10
6	37	F	III	3.85	6
7	52	F	III	4.75	7
8	58	M	IV	0.38	7
9	23	F	IV	1.28	7
10	39	M	IV	2.33	5
11	35	M	IV	ND	9
12	36	M	III	1.75	7

*According to Müller classification.

**Wasp venom (Pharmalgen, Pharmacia) 10^{-1} µg/ml.

Material and methods

Patients

Serum samples were collected from the following two groups of subjects.

Group 1: wasp-allergic patients. This group comprised 12 patients selected for VIT on the basis of exhibiting a systemic reaction to wasp stings. Reactions were graded according to the Müller classification (9) (Table 1). Immediate sensitivity was confirmed by intradermal skin tests with bee venom (Pharmalgen®: Pharmacia, Uppsala, Sweden) and by measurement of serum specific IgE antibodies by the radioallergosorbent test (RAST) (CAP System: Pharmacia, Uppsala, Sweden). Serum samples were collected before VIT (pre-IT), and after 12 h, 24 h (early VIT), and 15 days of treatment.

Group 2: long-term desensitized patients (VIT patients). This group comprised 20 wasp-allergic patients (mean age $\pm$ SD: 36 ± 9 years; nine women and 11 men) treated with wasp-venom injections (100 µg venom/month) for at least 2 years. Sera from these patients are referred to as VIT sera.

IT protocol

An ultrarush IT protocol (6) was used in this study. Ten incremental doses of venom were administered subcutaneously every 15 min. The initial dose was 0.01 µg of venom, and subsequent doses were increased until a cumulative dose of 68.5 µg had been administered on day 1. The maximum dose (100 µg) was reached on day 2, and the cumulative induction dose was approximately 180 µg venom. The maintenance dose (100 µg) was injected on day 15 and, thereafter, once a month.

Methods

Antigen extract. The antigen extract (from *Vespula germanica*) was purchased from Allergon®, Angelholm, Sweden, as a lyophilized powder containing 0.069 mg of phospholipase A (PLA) and 0.020 mg of Antigen 5 per mg total protein, as determined by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (the separated venom components were stained for the specific proteins). The extract was passed several times through a filter with a 10-kDa cutoff (Millipore®, Brussels, Belgium) to remove most traces of contaminating peptides possibly contained in the antigen extract.

IgG binding of venom extract. Specific IgG retention of native antigen was evaluated in a solid-phase enzyme-linked immunosorbent assay (ELISA). The wells of 96-well polystyrene microplates (Nunc®/TM-Immuno-plates; Gibco/BRL, Merelbeke, Belgium) were passively coated with the antigen extract (10 µg/ml in 10 mM carbonate buffer, pH 9.2), and additional nonspecific binding was prevented by saturation with phosphate-buffered saline (PBS) containing 1% w/v gelatin (Hemacel®: Hoechst, Belgium). Sera, diluted 1/20 in PBS containing 0.05% v/v Tween® 20 (PBS-Tween) and 0.1% w/v gelatin, were incubated for 3 h at 4°C with polyethylene glycol (PEG) (3.5% w/v) and then centrifuged (4000 rpm, 20 min); then, 100-µl aliquots of each supernatant were added to separate wells and incubated overnight at room temperature. After several washes with PBS-Tween buffer, 100 µl of a solution of peroxidase-conjugated mouse antihuman IgG (Sigma® Chemical Co.: 0.1% v/v in PBS-Tween) was added and incubated for 2 h at 40°C. After a further wash, the substrate solution (0.15% v/v H₂O₂, 0.3% w/v o-phenylenediamine in 0.2 M citric acid, and 0.4 M sodium phosphate) was added (100 µl/well). The enzyme reaction was stopped by the addition to each well of 100 µl of 2 M hydrochloric acid.

The binding activity of the antibodies of each serum was determined by reading the absorbance at 492 nm on a microtiter-plate reader (Dynatech MR 5000: Dynatech Laboratories, Chantilly, VA, USA). Antibody titers were expressed in arbitrary units (a.u.) according to a calibration curve made with serial dilutions of hyperimmune pooled sera. The procedure, performed in duplicate, was repeated twice for each serum. Nonspecific binding was evaluated in parallel by performing the same assay with microplates previously coated with blocking buffer only and no antigen. The zero control was evaluated with antigen-coated wells and diluent buffer only in place of serum.

Competitive binding tests: IgG antibody purification and biotin labeling

Purification. IgG fractions from pooled sera (12 wasp-allergic sera collected before [pre-IT IgG] and after 24 h

of IT [early VIT IgG], and 12 sera from patients treated for at least 2 years with venom injections [VIT IgG] were separated by affinity chromatography on recombinant protein-G Sepharose (10), after IgG rheumatoid factor had been removed by adsorption on sheep red cells sensitized with rabbit hemolysins. The purified IgG fraction was then dialyzed against carbonate buffer (50 mM, pH 9.5) to eliminate the glycine buffer (10 mM, pH 2.0) used for elution during the purification process. The concentration of the IgG-antibody solutions was evaluated by spectrophotometry.

Biotinylation of IgG fractions. The pooled IgG fractions were incubated for 4 h at room temperature with 250 µg biotin (NHS-LC-Biotin®, Pierce, Polylab, Antwerp, Belgium) per mg of IgG antibody in sodium bicarbonate buffer (50 mM, pH 8.5). Unreacted biotin was then removed by further dialysis against PBS (10 mM, pH 7.2). Biotinylated pooled IgG fractions were stored at 4°C until use.

Competition between biotinylated pooled IgG fractions and individual sera for binding to solid-phase-bound antigen. Ninety-six-well polystyrene microplates were passively coated with wasp-venom extract (1 µg/ml in carbonate buffer), and additional nonspecific binding was prevented by saturation with PBS containing 1% w/v gelatin. Samples containing individual sera (diluted 1/100) and biotinylated pooled-IgG fraction, diluted to 10 µg/ml in PBS-Tween containing EDTA (10 mM) and gelatin (1% w/v), were incubated for 3 h at 4°C with PEG (3.5% w/v) and then centrifuged (4000 rpm, 20 min); 100 µl of each supernatant was transferred, after vigorous mixing, to coated plates, and incubated overnight at room temperature. After the samples had been washed three times with PBS-Tween buffer, 100 µl of a solution of peroxidase-conjugated polystreptavidin (0.1% v/v in PBS-Tween) was added, and the plates were incubated for 3 h at 40°C. After an additional washing step, the binding activity was determined, as described above. The binding activity (OD) displayed by each of the biotinylated pooled-IgG fractions alone, served as the reference values. The results are expressed as the percent reduction of this (OD) value when individual sera were added to the biotinylated pooled-IgG fractions. The percent binding inhibitions were calculated as follows:

$$\% \text{ inhibition} = 100 \times \left(1 - \frac{OD_s - OD_{NS}}{OD_r - OD_{NS}} \right)$$

where OD_s, OD_r, and OD_{NS} represent, respectively, optical densities in the presence of serum, in the absence of serum (reference value), and corresponding to nonspecific binding. Nonspecific binding was evaluated in parallel by performing the inhibition assay exactly as described above (IgG fraction plus serum) with

microplates previously coated with blocking buffer but with no antigen. The zero control was evaluated with coated plates and peroxidase-conjugated polystreptavidin. A similar trend of results was obtained when different assay conditions were tested (e.g., different antigen-coating procedures, biotinylated IgG-antibody concentrations, and serum dilutions), but the most appropriate conditions were selected for the final experiments.

Specificity controls. To rule out any general phenomenon affecting IgG antibodies during ultrarush VIT, we performed similar experiments with a non-cross-reacting allergen (PLA₂ from bee venom) (11).

Functional affinity of IgG antibody

The functional affinity of anti-wasp-venom IgG antibodies from the different populations was compared in a competition ELISA by a method previously described (12, 13). After removal of antigen-antibody complexes by addition of PEG (3.5% w/v) followed by centrifugation, pooled IgG fractions were diluted to obtain a final OD of approximately 1.2. The diluted fractions were added to antigen-coated wells containing a range of concentrations from 0 to 100 µg/ml of free wasp-venom antigen in dilution buffer, and incubated overnight. The ELISA was then performed exactly as described above (Methods section). Percent binding was plotted for each concentration of free antigen. The concentration of free antigen eliciting 50% inhibition was taken as an estimation of the mean functional affinity. Increased antibody affinity for the solid-phase-bound antigen (i.e., functional affinity) was reflected by an increase in the amount of free antigen required to obtain 50% inhibition.

Statistics

The data were entered onto a PC and analyzed with the aid of the statistical package SPSS (Leuven, Belgium). Group comparisons were made by parametric tests where appropriate (one-way analysis of variance [ANOVA], with Scheffe's correction for multiple comparisons). Intragroup repeated data were compared by one-way ANOVA for repeated measures, and intragroup paired data were compared by the paired *t*-test. Spearman's correlation coefficients were calculated and tested for statistical significance to evaluate the relationship between clinical variables and both serum IgG titers and inhibition capacities.

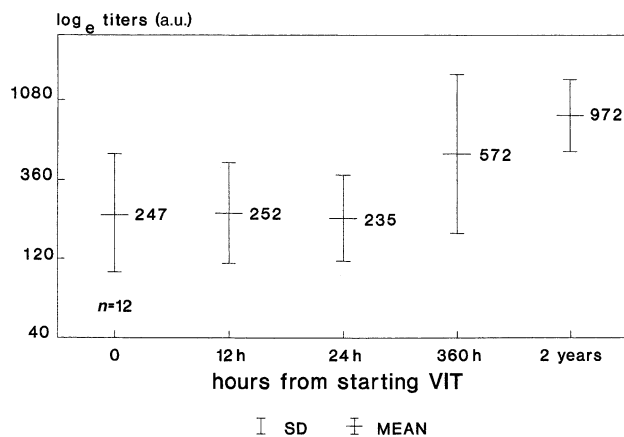


Figure 1. Stability of serum IgG titers during initial phase of ultrarush VIT.

Results

IgG binding to antigen extract

Fig. 1 shows the early evolution of IgG antibody titers during the ultrarush VIT procedure. No change was detected during the first hours of treatment (mean titers in a.u. $\pm$ SD: 247 ± 146 , 252 ± 131 , and 235 ± 126 before VIT, and after 12, and 24 h of treatment, respectively ($P > 0.1$ by one-way ANOVA for repeated measures)). A significant increase in titers was observed by day 15 with titers averaging $572 \text{ a.u.} \pm 396$ ($P < 0.01$ by Student's *t*-test for paired data). At this time, titers were not significantly different from those in long-term VIT patients ($P > 0.1$ by one-way ANOVA). Spearman's correlation analysis showed that age, sex, and grade of reaction had no influence on antibody titers ($P > 0.1$).

Binding inhibition of biotinylated pooled IgG fractions

Figs. 2–4 show the development of inhibitory capacities during the first hours of ultrarush VIT. The capacity of sera to prevent pre-IT allergic pooled-IgG binding to wasp antigen (Fig. 2a) changed dramatically, with the mean percent inhibition ($\pm$ SD) decreasing from $80 \pm 15\%$, before VIT, to $26 \pm 14\%$ and $35 \pm 15\%$ after 12 and 24 h of treatment, respectively ($P < 0.001$ by one-way ANOVA for repeated measures). No further significant change was observed after 15 days of VIT, with percent inhibitions at this time averaging $34 \pm 5\%$ ($P > 0.1$ by Student's *t*-test for paired data), similar to those demonstrated by sera from patients receiving VIT for over 2 years (mean inhibition capacity $\pm$ SD: $37 \pm 7\%$, $P > 0.1$ by one-way ANOVA). Fig. 2b also shows this to be true for each of the 12 sera individually.

In contrast, the capacity of sera to prevent VIT pooled-IgG binding to wasp antigen (Fig. 3a) increased from a mean percent inhibition ($\pm$ SD) of $47 \pm 8\%$, before VIT, to $76 \pm 7\%$ and $83 \pm 6\%$ after 12 and 24 h of treatment, respectively ($P < 0.001$ by one-way ANOVA

for repeated measures), and did not change further after 15 days (mean percent inhibition $87 \pm 6\%$, $P > 0.1$ by Student's *t*-test for paired data). This was not significantly different from the mean percent inhibition exhibited by sera from VIT patients ($75 \pm 7\%$, $P > 0.1$ by one-way ANOVA). Fig. 3b shows this to be true once again for each of the 12 sera individually. Finally, the mean inhibition capacity of sera to prevent early VIT IgG binding to wasp antigen (Fig. 4) decreased from $78 \pm 4\%$ before VIT to $36 \pm 12\%$ and $41 \pm 12\%$ after 12 and 24 h of treatment, respectively ($P < 0.001$ by one-way ANOVA for repeated measures), and did not change further after 15 days (mean percent inhibition $\pm$ SD, 30 ± 14 , $P > 0.05$ by Student's *t*-test for paired data). This is similar to the mean inhibition capacity demonstrated by sera from long-term VIT patients ($32 \pm 14\%$, $P > 0.1$ by one-way ANOVA).

Clinical variables such as age, sex, and severity of reaction were shown to have no influence on the inhibition capacities of sera ($P > 0.1$ by Spearman's correlation analysis).

Specificity control

When we used the PLA₂-binding capacity of the biotinylated pooled IgG fraction from patients allergic to bee venom as reference, there was no evolution in the inhibitory capacity of wasp-allergic sera during the first hours of the procedure, with percent inhibition averaging $43 \pm 7\%$ before VIT, and $46 \pm 5\%$ and $42 \pm 8\%$ after 12 and 24 h, respectively ($P > 0.1$ by Student's *t*-test for paired data), as compared to $82 \pm 7\%$ for bee-allergic sera ($P < 0.001$ by one-way ANOVA – data not shown).

Functional affinity

The concentration of free antigen necessary to achieve 50% binding inhibition was at least 10 times greater for IgG from pre-IT allergic patients and patients subjected to prolonged VIT than for IgG from allergic patients after only 12 and 24 h of treatment (Fig. 5). This indicates a lower affinity for the solid-phase-bound antigen in the two latter IgG populations.

Discussion

We have previously shown in several allergy models that allergic and tolerance status for allergens is associated with a particular dominant specificity of IgG antibodies (1–5). This specificity seems to be clinically linked, allowing almost complete discrimination between patient groups.

The present data confirm the association between the expression of a particular dominant IgG specificity and the clinical status even during a procedure which rapidly reverses the ability to exhibit allergic symptoms. Indeed, ultrarush VIT is known to result in nearly immediate

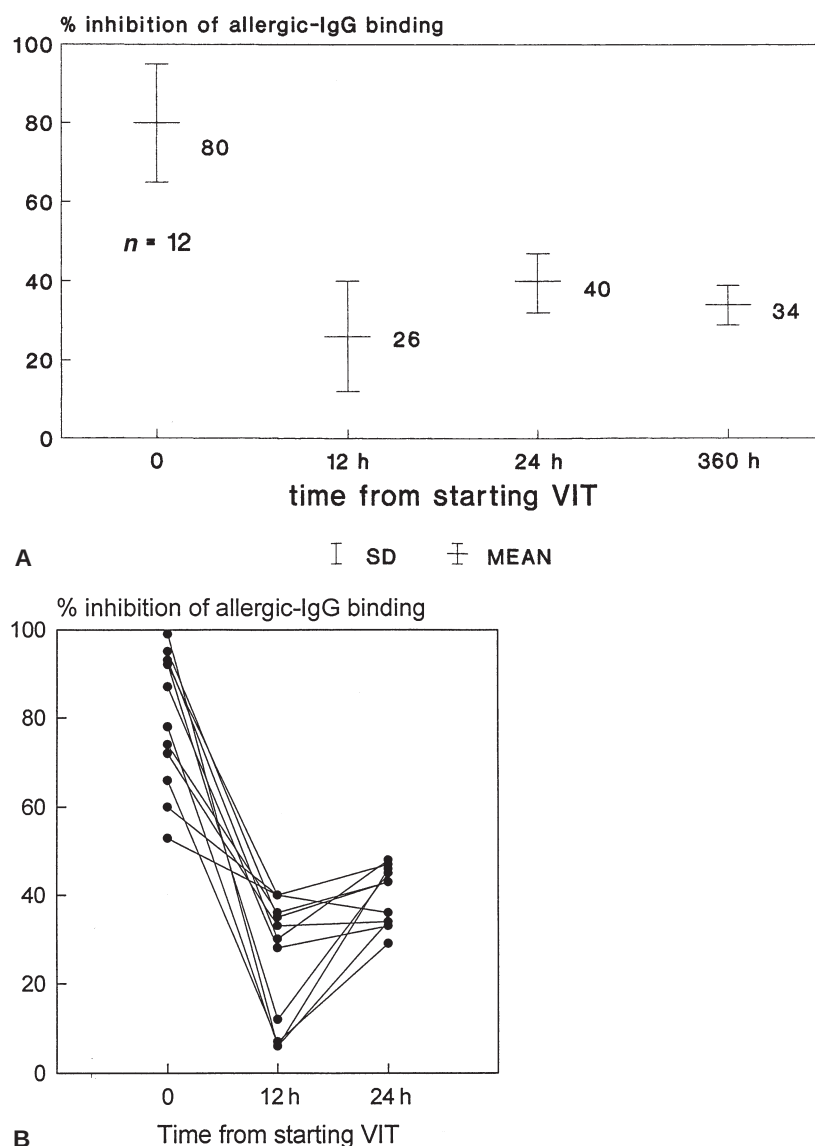


Figure 2a. Decrease of percent inhibitions of allergic pooled IgG binding to wasp antigens by sera after 12 h, 24 h, and 15 days of treatment. Figure 2b. Similar data for 12 sera individually.

tolerance, at least to injections of large amounts of venom, even though earlier studies showed that a subcutaneously tolerated dose of venom from the syringe does not always correlate with the tolerance to a live sting (9, 14). In any event, a change in the IgG antibody response was unlikely to occur in such a short period of time. However, while we were unable to detect any modification in antibody titers, our results did show a striking early change in antibody specificity. It must be emphasized that by “specificity” we mean the ability of sera to recognize a group of antigens present in wasp venom without any epitopic restriction. Differences in specificity were demonstrated through parallel experiments in which competition for antigen binding occurred between pooled IgG from different reference groups and individual sera. Pooled IgG were used to

provide the range of specificities of human IgG epitopes recognized on wasp antigens.

Remarkably, the initially strong capacity of allergic sera to prevent antigen binding by allergic pooled IgG was invariably reduced after only 12 h of treatment. Afterward, this inhibitory capacity remained low and was similar to that shown by sera from long-term desensitized patients. It must be pointed out that the initial capacity of allergic sera to prevent allergic IgG binding covers a wide range of values, suggesting, besides a common dominant pattern, individual variations in the affinity and specificity of IgG antibody in pretreatment allergic patients. Conversely, the initially low capacity of allergic sera to prevent antigen binding by VIT pooled IgG increased equally rapidly, reaching that shown by sera from patients treated by prolonged

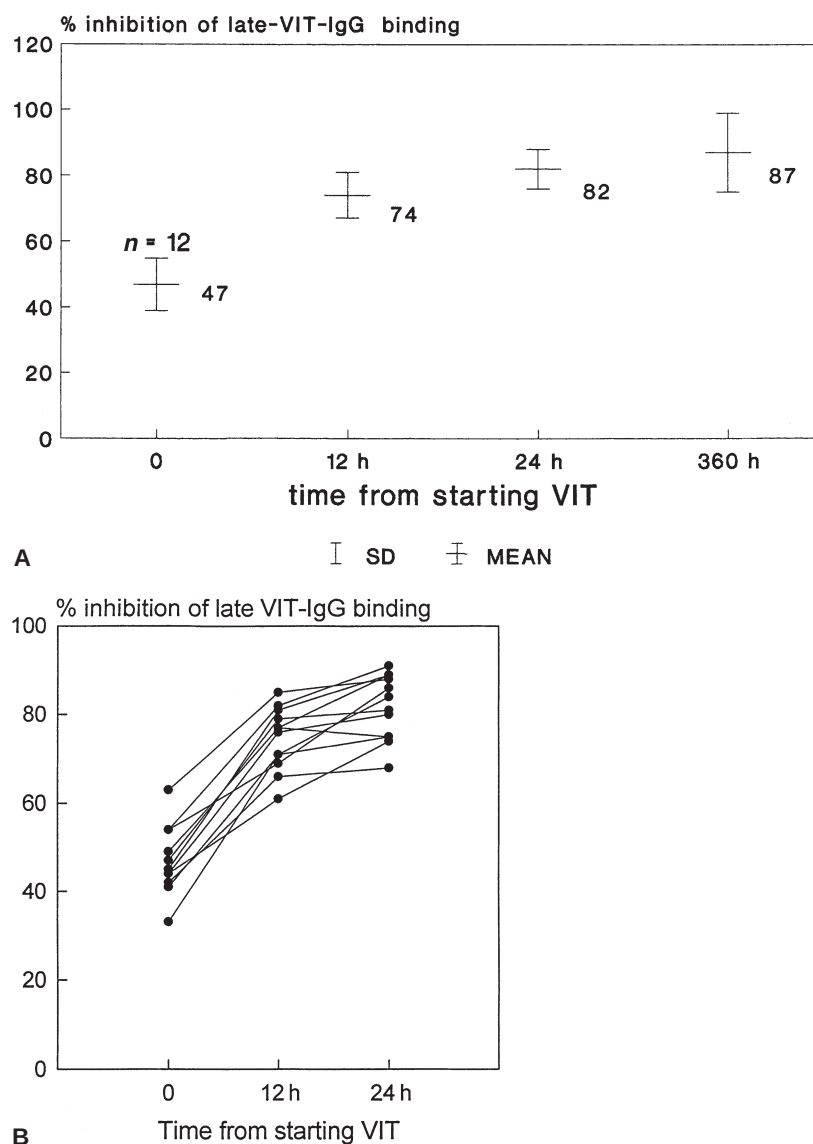


Figure 3a. Increase of percent inhibitions of VIT pooled IgG binding to wasp antigens by sera after 12 h, 24 h, and 15 days of treatment. Figure 3b. Similar data for 12 sera individually.

VIT in only 12 h. As antibody titers remain similar throughout the initial phase of treatment, these results cannot be explained by direct competition for binding to the same antigens. Therefore, the differences in inhibitory capacities of sera must reflect antibody populations with different specificities, including at least those of IgG antibodies, since competition experiments were performed with labeled pure IgG fractions.

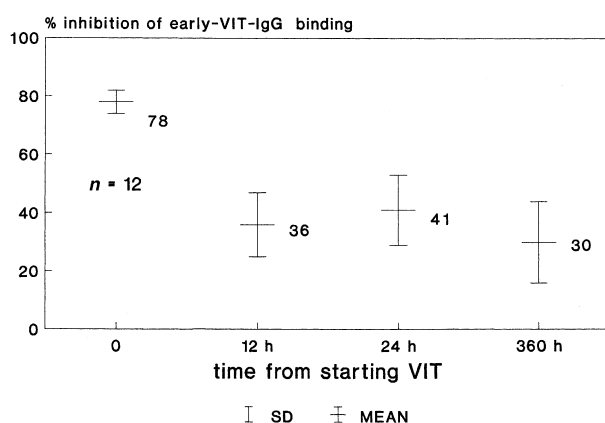
Interference in our competitive ELISA by autologous anti-idiotypic antibodies, restored in the serum of patients treated by VIT (15, 16), is unlikely after only 12 h of treatment and has been excluded previously (4). In addition, antigen-antibody complexes presumed to be present in the serum at this time were removed to prevent any interference in the competition test. Theoretically, the presence of venom-specific IgE, traces of venom, or even PEG in dilution buffer

could affect the results. However, such interferences should have been similar in all situations, but cross-experiments performed with the same sera in the presence of allergic or VIT IgG had completely different results. Finally, even the use of a crude antigen extract to include the broad range of human IgG epitopes recognized on wasp venom did not mask the observed change in specificity. This emphasizes the striking nature of the change, since the large number of antigens/allergens present in the extract could have prevented discrimination by enlarging the sets of potential epitopes.

The antigenic reactivity of pre-IT serum antibodies thus changes, very early during the initial phase of the ultrarush procedure, to cover, almost immediately, the specificity dominantly exhibited by antibodies from patients protected by prolonged VIT. A similar shift in

the pattern of immunodominant IgG epitopes recognized on PLA₂ has been reported for mice immunized with high, as opposed to low, immunogen doses (17). In our previous papers describing a progressive change in the antigenic reactivity of IgG antibodies during VIT (4, 5), we suggested that the high amounts of antigen repeatedly injected may change the established dominance of B clones, favoring the emergence of new antibody specificities. However, this mechanism is too slow to explain the almost immediate changes observed in this study. A more likely explanation would be a selective depletion of the highest avidity antibody fraction by the venom injected in large amounts at this stage of therapy.

However, these "high-affinity" antibodies must display a specificity somewhat different from that of the remaining "low-affinity" antibodies. Without such a difference, this mechanism cannot account for both the decrease in the capacity of sera to prevent allergic IgG binding after 12 h of treatment and the simultaneous increase in their capacity to prevent VIT IgG binding. This depletion of the high-affinity "allergy-associated" antibodies would leave a predominance of the lower affinity antibodies, including both low-affinity "allergy-associated" and low-affinity "tolerance-associated" antibodies already present in allergic sera. It must be pointed out that the terms "allergy-associated" and "tolerance-associated" antibody are used here to qualify specificities associated with different clinical states, and do not imply any operative mechanism. The change in dominance from high-affinity to low-affinity antibody populations at this time would explain both the strong capacity of pre-IT allergic sera and the relatively low capacities of early VIT and long-term VIT sera to prevent antigen binding by early VIT IgG. Indeed, allergic sera containing high- and low-affinity "allergy-associated" antibodies, as well as low-affinity "tolerance-associated" antibodies, show maximum inhibition of binding by early



24 h after starting VIT

Figure 4. Decrease of percent inhibitions of early VIT IgG binding to wasp antigens by sera after 12 h, 24 h, and 15 days of treatment.

VIT IgG containing low-affinity "allergy-associated" and "tolerance-associated" IgG antibodies.

With regard to antibody affinity, our results confirm that IgG from pre-IT allergic and long-term VIT-treated patients displays a higher affinity for the solid-phase-bound antigen than IgG collected from allergic patients after 12 and 24 h of ultrarush VIT (Fig. 4). Finally, the epitopes preferentially recognized by pre-IT allergic patients and long-term desensitized patients must be closely positioned on the allergens. Indeed, only a combination of steric hindrance and low affinity can explain the initial failure of "tolerance-associated" antibodies, already present in pre-IT allergic sera, to compete efficiently with VIT pooled IgG for antigen binding, as well as the rapid emergence (12 h) of this capacity after the high-affinity "allergy-associated" antibodies have been removed. Therefore, if we assume that binding to serum proteins or to a solid phase (18) changes the conformation of the antigen in a similar way, the following humoral hypothesis can be proposed. The initial phase of ultrarush VIT preferentially removes high-affinity "allergy-associated" antibodies from serum, leaving low-affinity "allergy-associated" and "tolerance-associated" antibodies. Further repeated injections boost the latter response, progressively increasing the affinity of "tolerance-associated" antibodies and changing the established dominance of B-cell clones. This is a reasonable hypothesis; however, it remains to be demonstrated.

In conclusion, during the initial phase of ultrarush VIT, a change in IgG antibody specificity that occurs

Inhibition of pooled IgG binding to solid-phase-bound antigen by increasing concentration of free antigen

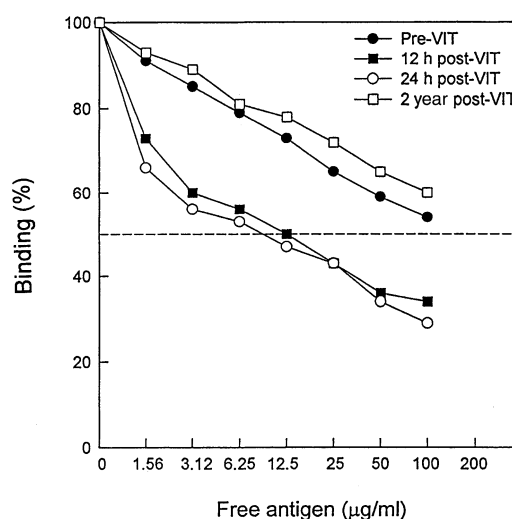


Figure 5. Pooled IgG from allergic patients at 12 h and after VIT shows at least 10-fold lower affinity for bound antigen, as indicated by concentration of free antigen needed to produce 50% binding inhibition.

concomitantly with early clinical tolerance is already detectable a few hours after the onset of treatment. Although it could be purely an epiphenomenon, this change represents the earliest humoral modification described so far during this procedure. The correlation documented previously between a particular dominant IgG specificity and the clinical status (allergy vs tolerance) has therefore been shown to be true also during a procedure which rapidly reverses the ability to exhibit allergic symptoms. Taken together, all these data suggest that investigating IgG specificity is a

promising diagnostic feature in allergology, where conventional tests usually document only the status of sensitization.

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