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In vitro Biphasic Effect of Honey Bee Venom on Basophils from Screened Healthy Blood Donors

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Apis mellifera L. bee venom is the most studied hymenoptera allergen, but many aspects of its action on human basophils remain unclear. Allergologists seek evidence of the effectiveness of bee venom immunotherapy as this approach is the chosen treatment for systemic allergic reactions. The effect of bee venom on human basophils *in vitro* has not been studied in detail for many reasons, including the paucity of basophils in peripheral blood, inter-individual basophil response variability, and the reliability and predictability of basophil activation tests. We conducted a brief preliminary survey of the effect of *Apis* bee venom on healthy asymptomatic (non-allergic) subjects. A dose of an aqueous commercial extract of *Apis* bee venom as high as 10 μg/mL activated resting basophils (CD63=+80-90%, CD203c=+30%), while it inhibited the expression of CD63 (-50%) following basophil stimulation by the soluble agonists formyl-Met-Leu-Phe or anti-IgE. The activation of resting basophils appeared to be dose-related. Only when basophils were activated with an IgE-mediated agonist, did bee venom extract exhibit a possible priming mechanism at the lowest doses used only via CD63, while it was ineffective via CD203c. Autocrine interleukin-3 may play a role in the observed biphasic behavior.

Key Words: Honey bee venom allergy; immunotherapy; basophil activation; CD63; CD203c; flow cytometry

Allergen immunotherapy is commonly used in the management of allergic diseases and it remains the only specific treatment for hymenoptera venom anaphylaxis. Venom immunotherapy (VIT) is recommended for patients with a history of IgE-mediated severe systemic allergic reactions, including anaphylactic reactions involving respiratory or cardiovascular symptoms.[1] The effectiveness of bee venom immunotherapy has been well established over the past 30 years and it is considered the treatment of choice for systemic allergic reactions induced by hymenoptera stings. Several studies have confirmed the effectiveness of VIT, demonstrating favorable patient responses to deliberate or field stings by the culprit insect after achieving the maintenance dose.[2] Desensitization by immunotherapy has been performed for several allergic situations throughout the last century, but its precise mechanism remains obscure. Many studies have focused on the roles of lymphocyte subsets in immunotherapy, while very few have examined the action of hymenoptera venom on basophilic cells directly. The basophil activation test (BAT) has not been used to evaluate the basophil response to hymenoptera bee venom. Consequently, many immunotherapy guidelines are based primarily on accumulated clinical experience, rather than on a genuine understanding of the cellular mechanism of the desensitization process. Additionally, some concerns remain about the relative safety of this form of therapy. There is some evidence of local allergic reactions associated with subcutaneous immunotherapy: side effects are fairly common and they are more frequent with honey bee VIT than with wasp treatment, especially during the maintenance phase compared with the increasing-dose phase.[1] Moreover, the advantage of using aqueous extracts is unclear, and aqueous preparations of honey bee venom have early local side effects, compared with depot extracts for VIT.[2] The occurrence of adverse systemic and local reactions in allergen immunotherapy remains a major problem for both patients and clinicians.[3] Is it possible to investigate the basal mechanism of immunotherapy and hymenoptera venom action by studying human basophils from control non-atopic subjects? VIT is used mostly for allergic symptomatic individuals, although preventive VIT is considered for populations at risk, such as beekeepers.[4] Sting-sensitized

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subjects can be enrolled from a healthy asymptomatic screened population, such as blood donors. For this population, the honey bee venom used for VIT has effects comparable to those described in individuals allergic to insect stings, constituting a good model for investigating the biology of VIT.

Subjects were recruited from regular plasma donors, who signed informed consent approved by the Ethics Committee. All individuals were screened for serum total IgE and subjects with abnormal plasma levels were excluded as donors and from this study. To assess the effects of hymenoptera immunotherapy on standard cell function, human basophils from 48 healthy screened blood donors (mean age 39.21±6.76 years, 48% female), with normal serum total IgE, who were non-atopic, asymptomatic, and had not undergone hymenoptera VIT, or any drug therapy in the previous 48 hours, were treated for 10 minutes at 37°C with an aqueous extract of honey bee (*Apis mellifera* L.) venom (HoBV; Pharmalgen-Alk Abelló, Horsholm, Denmark) and then activated with different agonists (N-formyl-L-Met-L-Leu-L-Phe, Sigma Aldrich, MO, USA or polyclonal goat anti-human IgE, Invitrogen Life Technologies, Carlsbad, CA, USA) for 30 minutes, according to published methods.[5] After activation in HEPES-buffered medium (pH=7.36) containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$, cell stimulation was stopped with HEPES-EDTA (2.8 mM) and the basophils were stained with fluorochrome-labeled monoclonal antibodies CD123-PECy5, HLADR-PECy7, CD45-APC-Cy7 (for cell electronic capture and phenotyping), and CD63-FITC and CD203c-PE, as activation markers, and then kept for 20 minutes at 4°C. The erythrocytes were then lysed with ammonium buffer (pH=7.2), and the basophils were resuspended in phosphate-buffered saline solution (pH=7.4) and evaluated by flow cytometry, according to published methods.[5] The flow analysis was performed using a two-laser BD FACScanto flow cytometer; this instrument has a 10,000 events/s capability, six-color detection, and 0.1% sample carryover. Analyses were performed with a mean flow rate of 300–500 events/s, setting an excess limit of 50,000 events to record in the basophil gate to evaluate the entire buffered suspension volume and properly estimate cell recovery and reproducibility. Compensation followed the manufacturer's instruction, according to an off-line procedure by applying automated electronics algorithms and preset templates, using bi-parametric logarithmic dot plots, gate-specific tubes and single-tube data analysis, and optimizing the forward scatter threshold and fluorochrome voltage as set-up parameters. To evaluate non-specific fluorochrome staining, isotype controls for anti-IgG_1 and anti-$IgG_{2\alpha}$ were introduced in the preliminary procedure to set up the photomultiplier and instrument technical parameters; this control used a staining procedure carried out without introducing the fluorochrome of interest to the assay system. The mean fluorescence intensity (MFI) for each fluorochrome-labeled monoclonal antibody was calculated automatically with the cytometer software by averaging the total fluorescence of the marker in the basophil gate. In addition, the software calculated the percentage of activated cells ($CD63^{expr}$%) by considering the CD63-FITC bright cells counted to the right of a threshold that was established including the main peak of fluorescence of a sample of resting cells. To reduce the standard deviation due to brightly fluorescent cells versus dimly fluorescent ones, a logarithmic scale and the coefficient of variation to measure variability dispersion were used. The percent effect of the dose-response curves was calculated by referring to 0 μg/mL HoBV as:

$[-100+(\text{mean } N_x/\text{mean } N_0)\times 100]$,

where N_x is the indicated values and N_0 the reference values. Statistics were determined using the Sigma Plot 10.0 software and SPSS 18.0 for data analysis, applying the Shapiro-Wilk test for normality and a non-parametric Wilcoxon's signed-rank test for data evaluation.

Figure summarizes the results of 12 separate triplicate experiments, expressed as the mean ± SEM, with the *P* values of significant effects ($^*P<0.05$; $^\dagger P<0.01$). In resting non-activated basophils (Figure, plots A-C), HoBV increased the expression of both the markers in a dose-dependent fashion. The greatest membrane marker up-regulation was at 10 μg/mL HoBV (+99.4 %, $P=0.003$, +81.8%, $P=0.046$, and +33.1%, $P=0.006$ for CD63-MFI, $CD63^{expr}$%, and CD203c-MFI, respectively) compared with HoBV non-treated cells. HoBV induced CD63 and CD203c up-regulation in resting, non-activated basophils and inhibited the expression of these molecules on the cell membrane in stimulated basophils at the highest honey bee venom concentration used. Agonists were able to down-regulate membrane markers expression at 10 μg/mL HoBV. The dose-response trend disappeared when basophils were treated with 100 nM N-formyl-L-Met-L-Leu-L-Phe (fMLP; Figure, plots D-F), but the highest HoBV concentration inhibited membrane marker expression elicited by bacterial formylated peptides (-67.2%, $P=0.0027$; -33.8%, $P=0.033$; -38.6%, $P=0.035$ for CD63-MFI, $CD63^{expr}$% and CD203c-MFI, respectively). This inhibitory effect was also observed when basophils were triggered with 2.0 μg/mL anti-IgE (-53.1%, $P=0.020$ for CD63-MFI, Figure, plot G; -49.6%, $P=0.048$ for $CD63^{expr}$%, Figure, plot H; -17.1 $P=0.480$ for CD203c, Figure, plot I). However, lower HoBV concentrations induced increased CD63 expression compared to HoBV non-treated cells and the increase was inversely proportional to the HoBV dose, reaching up-regulation exceeding 60% (+61.6%, $P=0.0224$ for CD63; +61.5, $P=0.0136$ for $CD63^{expr}$% at 0.01 μg/mL, Figure, plots G, H). No such effect was observed for CD203c (Figure, plot I). In an IgE-mediated activation pathway, HoBV stimulation was superimposed on that of anti-IgE. While the two markers showed a similar pattern for non-IgE-mediated activation, they behaved quite differently in basophils triggered with anti-IgE.

Activation of the ectoenzyme CD203c by HoBV was previously described in stung individuals with a diagnosed allergy to wasp or bee venom.[6] More recently, this marker was found to

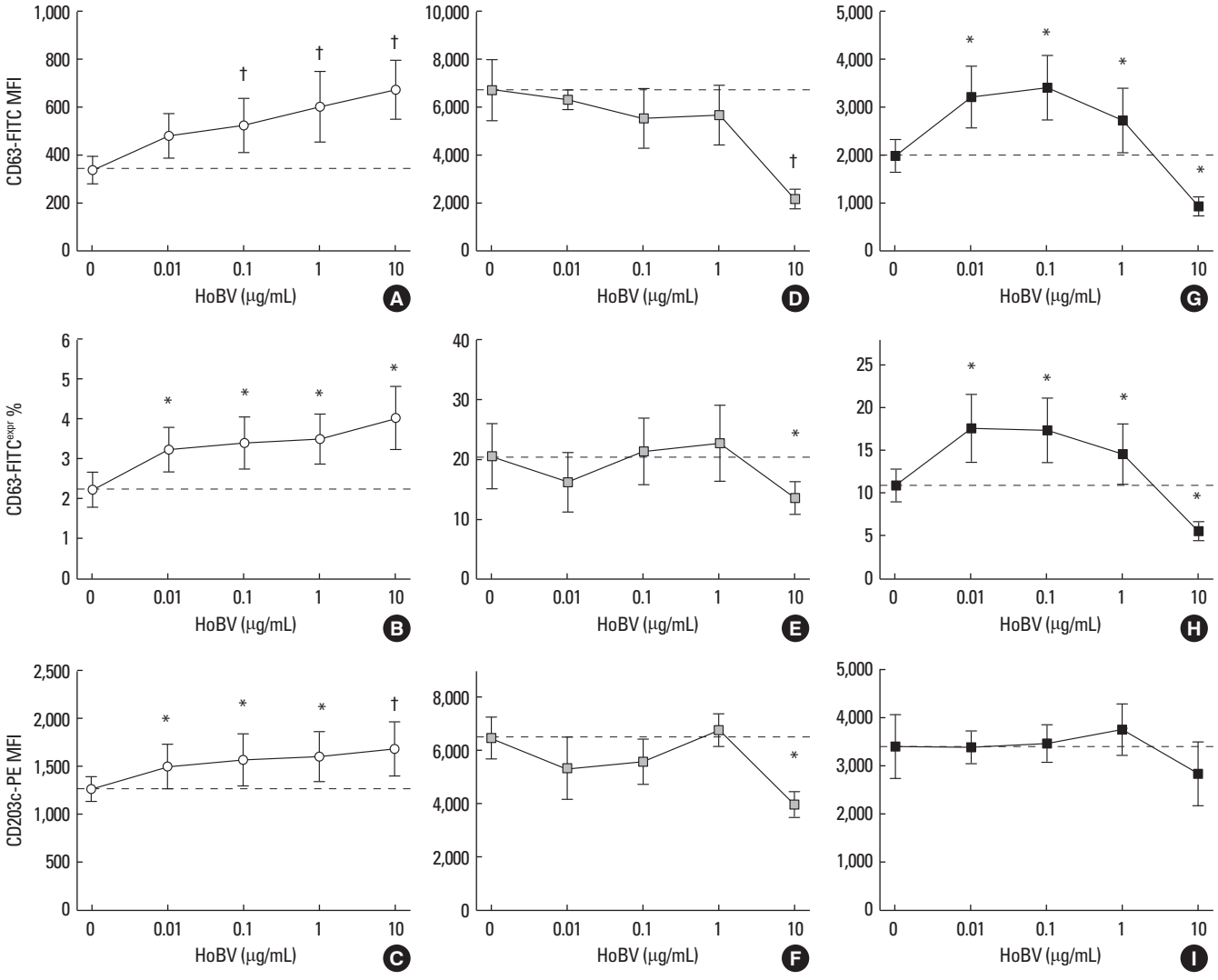

Figure. Dose response curves of honey bee venom (HoBV) on resting non-activated basophils (A-C) or cells stimulated with 100 nM fMLP (D-F) or 2.0 μg/mL anti-IgE (G-I). Values are expressed as mean±SEM of 12 separate triplicate experiments. See text for statistics.
*P<0.05; †P<0.01.

be related to the role of interleukin-3 in human basophils, because the cytokine modulates CD203c membrane expression.[7] A low level of IgE/FcεRI cross-linking in response to low doses of honey bee venom allergens can induce autocrine IL3 activity, leading to basophil priming.[8] In this study, we showed that HoBV induces membrane marker up-regulation from the basal level, confirming previous reports.[6,9] When IgE-FcεRI is triggered by an allergen, even at low concentration, basophils produce and bind the IL-3 they release, thereby inducing a primed state and augmenting the IgE-mediated response.[8] In fact, the cytokine can induce priming, but also sustains IgE-mediated activation.[10] Regular low doses of HoBV induce CD203c up-regulation, a mechanism in which IL-3 may play a role,[8] which in turn modulates basophil activation by two mechanisms, one of which is IgE- and calcium-dependent.[7,10] CD203c induced by autocrine IL-3 is not further up-regulated by the concentration of anti-IgE.[7] This concurs with evidence that HoBV increases basal CD203c, but it is not able to increase CD203c up-displacement on the basophil membrane following activation with anti-IgE.

Regarding the tetraspanin CD63, very low doses of HoBV induced synergistic marker up-regulation following stimulation with the anti-IgE agonist, while venom concentrations as high as 10 μg/mL triggered down-regulation in both activation pathways. Previous reports have shown that when IL-3 and anti-IgE are combined, the result is additive or supra-additive, depending on the basophil donor, leading to a clear priming phenomenon.[8] This seems to be the case, suggesting that HoBV induces autocrine IL-3 in basophils. fMLP cannot induce an increase via a synergistic effect on FcεRI, because it does not elicit IL-3

production,[8] but a high level of HoBV at IgE-FcεRI, which exerts an activation response at the basal level, might trigger a down-regulation pathway, probably involving a signaling pathway.[8] This evidence supports the hypothesis that high HoBV concentrations do not inhibit basophil function via a cytotoxic effect.

This hypothesis needs to be investigated further and our data are preliminary evidence. Others have extensively analyzed autocrine IL-3 mRNA levels and cytokine synthesis in basophils activated with anti-IgE of fMLP.[8] We were unable to find a better interpretation of the reported evidence.

What is the clinical relevance of these findings? Recent reports indicate that IL-3 is not essential for local or systemic anaphylaxis[11]; so our evidence is not directly related to systemic reactions following an insect sting involving an anaphylactic mechanism. Our evidence shows only that desensitization is a consequence of HoBV at an activated cellular state. However, the mechanism of action we suggest is a hypothesis, and we do not know whether this mechanism involves down-regulation mediated by IL-3, IgE-receptor internalization, protein tyrosine phosphatases, or histamine feedback on H_2 receptors; there may also be a role for intracellular modulation of the activating signal.[12-14] The aqueous HoBV used in immunotherapy appears to have biphasic (hormetic) behavior. Low doses of HoBV can diffuse into the systemic circulation and induce an activated state, while high doses elicit a primed state, but they induce IgE-FcεRIβ desensitization, as allergen/IgE cross-linking increases, a mechanism that allows us to suggest that the higher the priming is, the greater the desensitization. This occurs in all stung subjects, both sensitized asymptomatic and hymenoptera allergic subjects, raising issues about VIT maintenance time and doses.[4] VIT effectiveness is strictly dependent on allergy desensitization mechanisms, and an understanding of this is a major goals of immunotherapy. In this regard, many attempts to prevent side effects in immunotherapy, from induced cell-anergy with intracellular inhibitors[15,16] to IgG/FcγRIIb regulation of IgE/FcεRI cross-linking,[16] have been suggested, but their clinical feasibility is limited.[17] In this context, the observed biphasic behavior should be assessed further to ameliorate VIT in hymenoptera allergy and to gain new insights into the role of basophils in allergy inflammation.

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