Paradoxical inhibition of basophil degranulation by excess binding of a cedar pollen allergen, Cryj1

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Abstract

Background: Repeated exposure to allergens leads to tolerance in type I allergy. However, its mechanism is largely unknown at cellular level. Basophil stimulation test serves as a sensitive method to evaluate allergen-specific activation of basophils. It has been reported that basophil stimulation results in typical bell-shaped dose-response characteristics with inhibition of activation at high concentrations. However, there has been no study evaluating the dose-effect of allergen binding directly. Objectives: To disclose relationships between allergen dose and binding, parameters of basophil activation and severity of the clinical symptoms. Methods: Peripheral blood was obtained from volunteers with proved cedar pollen allergy. Basophil stimulation test was performed with different concentrations of biotin-conjugated cedar pollen antigen, Cryj1. Cryj1 binding, expression of surface antigens, and release of mediators were determined simultaneously. Severity of symptom was evaluated by scoring nasal, eye and throat symptoms and QOL of the subjects. Results: In all cases, bell-shaped dose-response was obtained for the expression of basophil granular proteins LAMP1, LAMP2 and CD63 on cell surface and release of mediators. In contrast, expression of the surface antigen CD203c and Cryj1 binding increased dose dependently without high-dose inhibition. Basophil activation was induced at very minute level of Cryj1 binding and the differences in dose-response profile reflected the level of inhibition at higher Cryj1 concentration. The levels of inhibition inversely correlated with the severity scores of the clinical symptoms. Conclusions: Basophil activation is induced at very low level of allergen binding and inhibited at high allergen level by individually different manner.

Key words Cryj1, basophil, activation, CD63, CD203c

Introduction

Clinical symptoms of type I allergy or immediate type hypersensitivity is generally evoked when allergen-sensitized individuals are exposed to specific allergens[1]. On the other hand, it has been known that certain fraction of pollen allergy patients can be successfully desensitized by immunotherapy[2], or infants with food allergy often cease to show symptoms when they grow older[3]. These phenomena are usually explained by "tolerance", through which the patients acquired clinical non-responsiveness toward the responsible allergens[4]. Tolerance is at least partly explained by alteration of host immune status or by maturation of mucosal barrier systems. For example, it is shown that the immunotherapy for cedar pollen allergy leads to reduction of allergen-specific IgE to IgG4 ratio after repeated intradermal injections of allergens[5][6]. In contrast to IgE-mediated activation of basophil/mast cell, IgG4-mediated signal is known to elicit activation of inhibitory pathway within these cells. Part of the mechanism of food allergy tolerance can be explained by the maturation of mucosal barrier and

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Abbreviations : ITAM: immunoreceptor tyrosine-based activation motif, ITIM: immunoreceptor tyrosine-based inhibitory motif, LAMP: lysosome associated membrane protein, QOL: quality of life, RPMI: Roswell Park Memorial Institute

development of local immune system including secretory IgA within the infant's intestine[7]. However, precise mechanism responsible for tolerance induction at cellular level is largely unknown.

Basophils are activated after cross-linking of high-affinity IgE receptor, FcεRI when the relevant antigens, or allergens bind the receptor bound IgE. The allergen-induced cross-linking of FcεRI leads to the series of intracellular changes, including the tyrosine phosphorylation of immunotyrosine activatory motifs (ITAM) in the cytoplasmic domain of the β and γ chains of FcεRI. Immediate reflection of this basophil activation is the upregulation of surface CD203c expression[8] and subsequent de novo expression of basophil granular protein CD63 on the cell surface[9]. The expression of CD63 reflects degranulation and closely associated with the release of the granular contents, such as leukotriene and histamines. In basophil activation test, the characteristic alteration of surface antigen expression is used as the sensitive indicator of in vitro basophil activation to certain antigens and evaluated by a flow cytometry[10].

In the present study, we stimulated the peripheral blood from allergic patients in vitro with the cedar pollen antigen Cryj1, and examined the levels of allergen binding, activation and degradation in basophils simultaneously by highly sensitive flowcytometric analysis[10]. By this method, dose-dependent allergen binding, induction of basophil activation, and degranulation were determined at the same time utilizing biotin-conjugated allergen Cryj1, CD203c and CD69 as the specific indicators, respectively[8][11][12]. The results were compared with the clinical symptoms of the patients.

Materials And Methods

Subjects

Six adult volunteers confirmed as cedar pollen allergy by basophil activation test were selected for this analysis. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

No specific season was selected for the study, because preliminary experiments revealed that there exist little seasonal variations in basophil response profiles.

Basophil activation test

Peripheral whole blood was obtained and washed twice in phosphate-buffered saline (PBS), once in RPMI-1640 medium containing 10% fecal bovine serum, and then suspended in medium. Subsequently, suspension was stimulated with different concentration of biotin-conjugated cedar pollen antigen Cryj1 (Hayashibara Biochemical Laboratories, Inc. Okayama, Japan) at 37℃ for 40 minutes. Cryj1 concentration was set at 8 levels from 10^{-6} to 10^{0} μg/ml in tenfold dilution. In some experiments, culture supernatant was obtained and stored at −20℃ before processing for flow cytometry.

Flow cytometry

After incubation, red blood cells were lyzed and washed in PBS, and the samples were incubated with monoclonal antibodies for 15 minutes on ice. Fluorescein isothiocyanate (FITC)-conjugated anti CD63 antibody was obtained from Immunotech (Marseille, France). Phycoerythrin (PE)-conjugated anti-CD203c antibody and R-phycoerythrin-Cy5-conjugated streptavidin were the products of Beckman Coulter (Tokyo, Japan). PE-conjugated anti CD107a (LAMP1) antibody and FITC-conjugated anti CD107b (LAMP2) antibody were purchased from BD Pharmingen (San Diego, CA, USA). After washing in PBS, cells were analysed with a flow cytometer [fluorescence activated cell sorter (FACSCalibur); Becton Dickinson, San Diego, CA, USA]. The resulting data were analysed using CELLQUEST software (Becton Dickinson).

After gating lymphocyte region on a cytogram, basophils were selected as CD203c-positive cells. Expression of basophil markers and Cryj1 binding were evaluated simultaneously and the levels of these parameters were expressed as mean fluorescence intensity (MFI).

Histamine and leukotriene release

Histamine and leukotriene concentrations in the culture supernatant samples were determined by commercial enzyme-linked immunosorbent assay kits (Oxford Biomedical Research Inc., Oxford, MI, USA).

Clinical data

Clinical severity of patients was assessed with questionnaires evaluating nasal symptoms (blowing nose, sneezing, blocked nose, itching), eye symptoms (itching, tearing, redness, mattering), throat symptoms (itching, tickle) and QOL (difficulty of daily life, use of rescue medication). Each symptom

was scored on a 0-4 scale, the maximum severity score was 48.

Patients' blood was analysed for allergen-specific IgE and IgG4 to cedar pollen[13].

Statistical analysis

Within-group comparisons were analyzed by One-way ANOVA, followed by Dunnett's multiple comparison. Correlations were expressed using the Spearman rank correlation coefficient. For the analyzed measures, p values less than 0.05 were considered significant.

Results

Expression profiles of basophil antigens after Cryj1-induced basophil activation

Cryj1 binding increased dose-dependently with increasing Cryj1 concentration as shown in the representative figure (Fig.1, upper panel). Similary, CD203c levels increased dose-dependently (Fig.1, lower panels). In marked contrast to the dose-dependent increase of Cryj1 binding and CD203c expression, fraction of $CD63^{high}$ cells reached the maximum level at $10^{-3}\mu$g/ml and the levels became lower at $10^{-1}\mu$g/ml. Essentially similar profiles of basophil activation were obtained with different individuals (Fig.2). Although the levels of the binding varied from individuals to individuals, Cryj1 binding increased dose-dependently and reached the maximum levels at $10^{0}\mu$g/ml (Fig.2A). CD203c expression reached the maximum at much lower concentration of $10^{-3}\mu$g/ml and remained at similar levels with higher Cryj1 concentration (Fig.2B). Although the maximum levels of $CD63^{high}$ cells differed significantly, the peak value was reached at $10^{-2}\mu$g/ml in most cases and the levels decreased significantly at higher antigen concentration (Fig.2C).

To determine if $CD63^{high}$ cells truly represent degranulated basophils, we examined expression profiles of other granule-associated proteins LAMP1 and LAMP2. Expression of both antigens were rapidly induced upon Cryj1 binding and the dose-dependent profiles were identical to that of CD63 (Fig.3).

Histamine and Leukotriene release

Next we examined release of basophil granular contents, histamine and leukotriene. We measured their concentration in the culture supernatant samples after basophil stimulation. Both histamine and leukotriene release perfectly matched with the dose-dependent profile of CD63 expression (Fig.4). Release of histamine and leukotriene reached maximum at Cryj1 level of $10^{-3}\mu$g/ml and the levels sharply declined at higher antigen

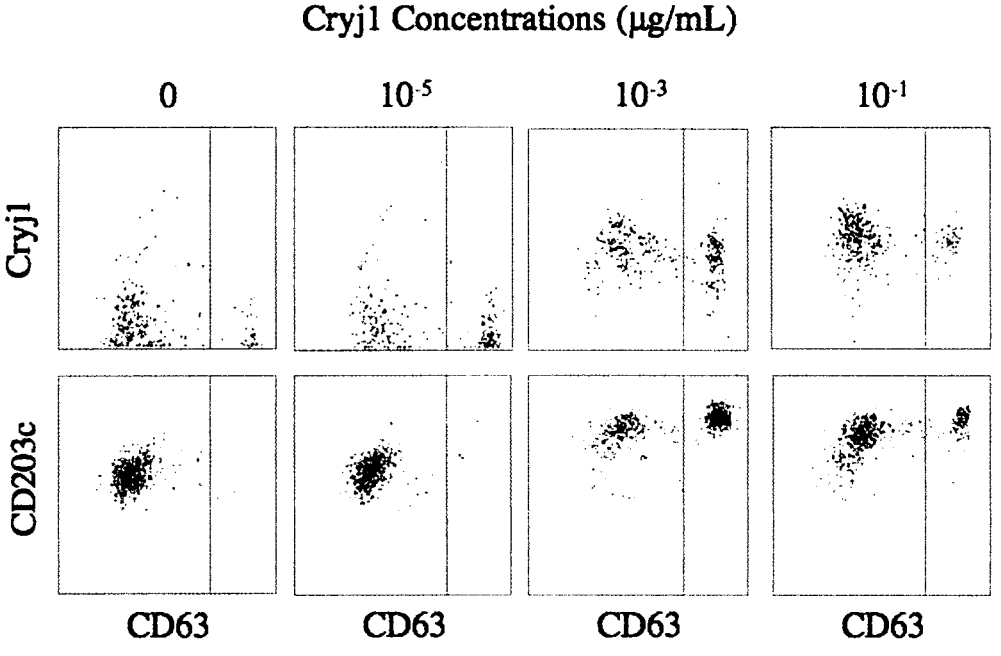

Fig. 1. Dose-dependent basophil activation in response to biotin-conjugated Cryj1.
Biotin-conjugated Cryj1 induced basophil activation in a dose-dependent manner. Culture was performed without or with 10^{-5} to 10^{-1} μg/ml of Cryj1. After 40 min of culture, basophil surface antigens were analyzed. CD63 expression was compared with Cryj1 binding (upper panel) or with CD203c expression (lower panel). Vertical lines delineate $CD63^{low}$ and $CD63^{high}$ basophils.

concentration.

Consistency of individual variations

Profiles of dose-dependent basophil activation were obtained repeatedly to see if different patters of Cryj1 responses reflect true individual differences or simple experiment-to-experiment variations. Fig.5 shows the result from three different individuals. It is clear from these results that the distinct profile of the dose-response curve is reproducible for each individual.

Relationship between Cryj1 binding and CD63 expression

To see if there are individual differences in the relationship between Cryj1 binding and CD63 expression, we plotted Cryj1 binding against ratio of $CD63^{high}$ cells at different Cryj1 concentrations. The plot data for each individual were obtained from multiple (3 to 5) experiments on different occasions. Approximation and curve fitting was performed with eye guide. The profiles differed markedly among 6 individuals (Fig.6). However, it was intriguing that in every subjects CD63 expression reached maximum levels with extremely low level of Cryj1 binding (MFI, mean fluorescence Intensity=2.0). Furthermore, excessive Cryj1 binding resulted in paradoxical inhibition of CD63 expression. The profiles of "high-dose" inhibition also differed

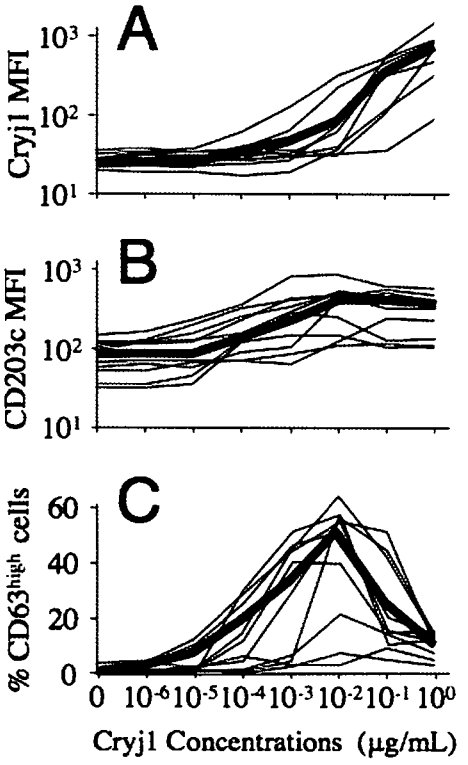

Fig. 2. Variations of individual profiles of basophil stimulation tests.
Cryj1 was added at ten fold dilutions (from 10^{-6} to 10^0 μg/ml) and cells were cultured for 40 min. Level of Cryj1 binding (A) and intensity of surface CD203c expression (B) were determined by mean fluorescence intensity (MFI). Fractions of $CD63^{high}$ cells (C) were evaluated at the same time. Bold lines indicate the mean values.

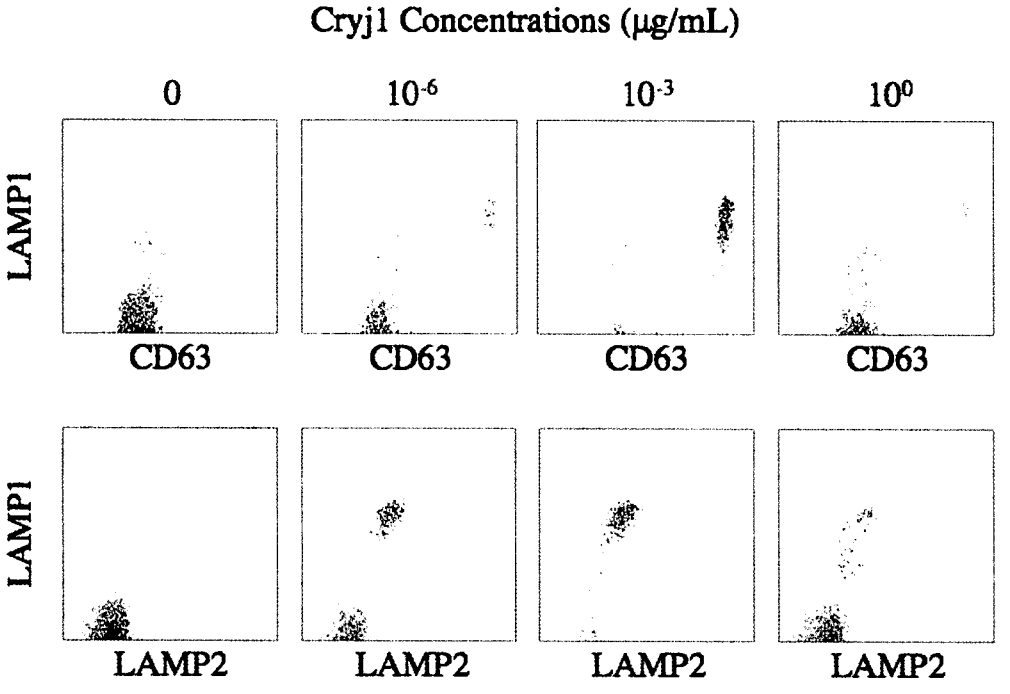

Fig. 3. Comparison of granule-associated molecules on activated basophils.
Surface expression of lamp1 was compared with CD63 (upper panel) or with lamp2 (lower panel) after stimulation with different doses of Cryj1.

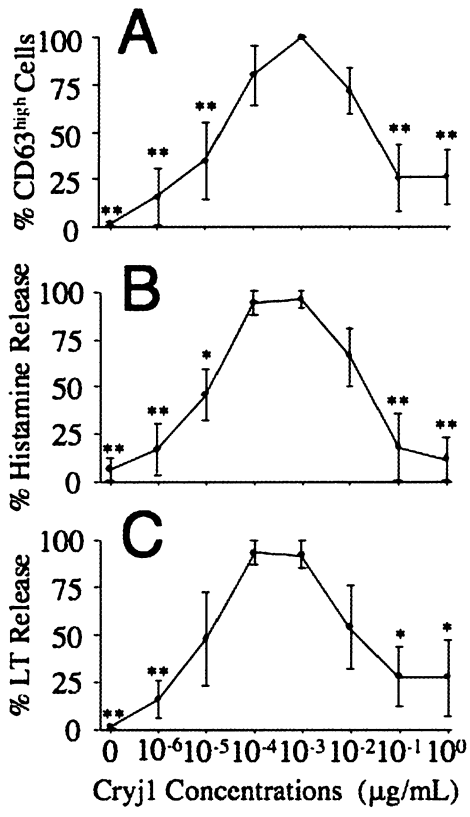

Fig. 4. Dose-dependent profiles of basophil degranulation.
Basophil degranulation profiles were evaluated simultaneously by $CD63^{high}$ cell fractions (A), histamine release (B) and leukotriene (LT) release (C). Data represent the means of 5 independent experiments. Vertical bars indicate SD of the mean values. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the peak values.

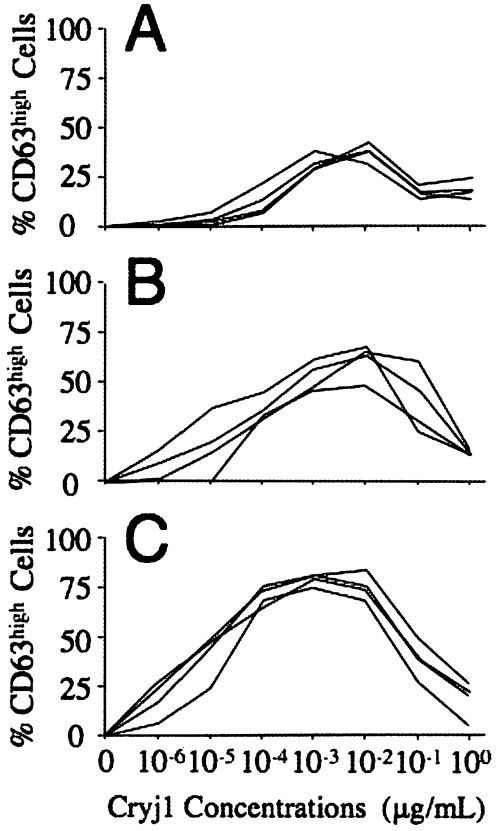

Fig. 5. Individual variations in dose-dependent basophil degranulation.
Basophil stimulation tests were repeated four times for three different individuals (A, B and C) who showed distinct profiles of CD63 expression after Cryj1 stimulation.

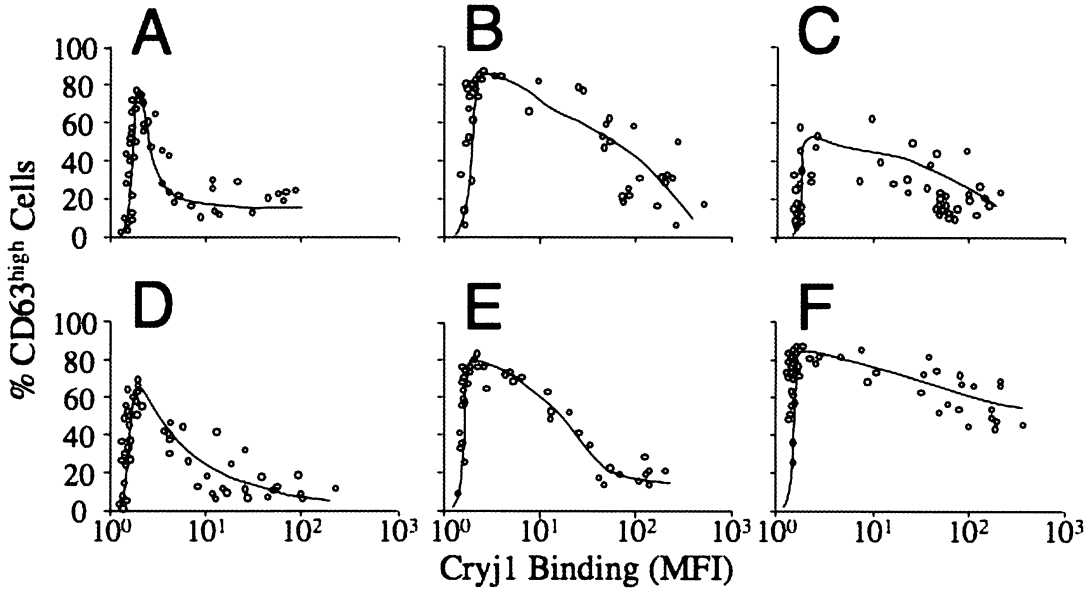

Fig. 6. Relationships between Cryj1 binding and basophil degranulation.
Levels of Cryj1 binding and $CD63^{high}$ ratio were determined at the same time. Experiments were repeated multiple times for each individuals and the data were plotted on each panel. Curve fitting was performed and expressed by solid lines. Each panel represents data from different individual.

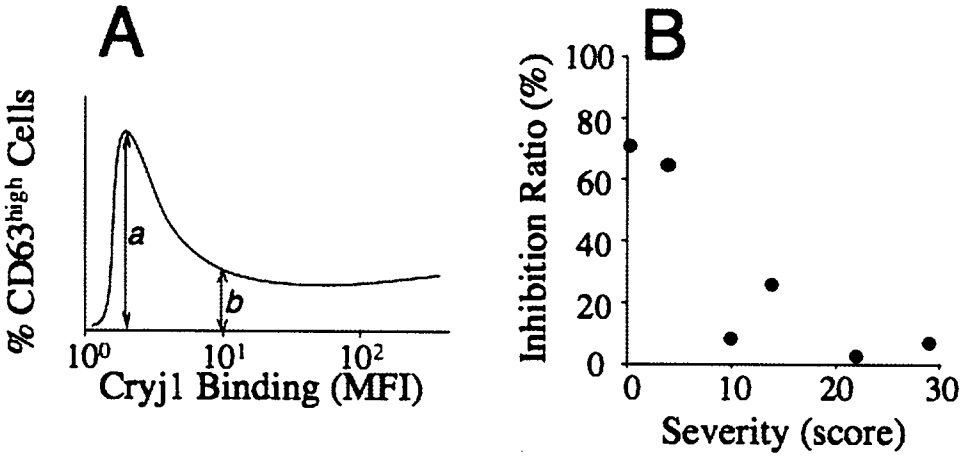

Fig. 7. Inhibition ratio and clinical severity scores.
From the curve fitting data, inhibition ratio of basophil degranulation was calculated as a-b/a x 100 (%) for each individual (A). Clinical severity score was calculated at the same time based on the clinical symptoms shown in Table. Inhibition ratio and severity score was compared and plotted on a panel (B). $r = -0.838$, $P > 0.05$

Table1. Inhibition ratio of basophil activation and clinical profiles in pollen allergy subjects

Case	1	2	3	4	5	6
Nasal symptom	0	3	9	6	7	10
Eye symptom	0	1	1	5	8	6
Throat symptom	0	0	0	0	6	8
QOL	0	0	0	3	1	5
Severity score	0	4	10	14	22	29
Inhibition ratio (%)	70.5	64.1	8	25.6	1.9	7.1
Specific IgE (UA/mL)	32.7	25.9	5.9	44.9	13.2	177.1
Specific IgG4 (mU/mL)	180	33	5	245	5	5

QOL; quality of life.

among individuals. Some individuals showed sharp decline of basophil activation above the threshold concentration (Fig.6A and D), whereas certain individuals exhibited rather slow decline at higher Cryj1 concentration (Fig.6C and F).

Inhibition ratio and clinical severity

Next we examined if the distinct profiles of Cryj1 bindings and CD63 expression have any clinical relevance. To do this, we compared "inhibition ratio" and clinical scores. Clinical scores were determined as the sum of nasal, eye and throat symptoms and QOL scores (Table1). After curve-fitting the plot data, inhibition ratio (%) was defined as percent inhibition of the maximum ratio of $CD63^{high}$ cells at the Cryj1 binding level of 10^1 MFI, i.e., (a-b)/a x 100 in Fig.7A.

Neither antigen-specific IgE value or antigen-specific IgG4 value as independent parameter showed positive correlation with the clinical scores. There seemed to be an inverse correlation between the inhibition ratio and the severity scores, although Spearman correlation was not statistically significant ($r = -0.838$, $P > 0.05$) (Fig.7B).

Discussion

It has been known that stimulation of basophil or mast cells through FcεRI by either allergens or anti-IgE antibodies result in bell-shaped dose-dependent activation curve. Presence of certain inhibitory signals at high allergen concentrations is the most likely explanation for this phenomenon. However, precise mechanism of high-dose inhibition and its clinical relevance is largely unknown because most of the studies are performed using mast cells and studies using basophils are mostly animal experiments.

In this study, we analyzed the parameters of basophil activation and the levels of allergen binding at the same time. Furthermore, we established a novel way of determining the level of high-dose inhibition and compared the inhibition ratio with actual clinical severity scores of pollen allergy. Cryj1 served as a useful tool for this experiment because biotin-conjugated Cryj1 was available commercially and clinical scores of cedar pollen allergy could be easily determined by simple clinical parameters[14)15)].

Expression of CD63 and release of granular proteins showed typical bell-shaped dose-response curves as expected. On the contrary, CD203c expression increase as the allergen concentration increased and remained at the maximum level when

the allergen concentration exceeded the optimum level of degranulation. These results confirmed the previous experiments using human mast cells and murine basophils. At the same time, the results clearly indicated that the high-dose inhibition is not the inhibition of basophil activation as represented by CD203c upregulation, but inhibition of the specific cellular event, degranulation. It is possible to assume that the inhibition of degranulation is induced by the reduced Cryj1 binding at higher antigen concentration. Altered affinity of membrane-bound specific IgE to Cryj1 or shedding of allergen-IgE complex from the cell surface could explain the paradoxical decrease of FcεRI-mediated basophil activation signal. However, the results shown here clearly indicate that the high-dose inhibition of granulation is not associated with the reduction of Cryj1 binding at higher allergen concentration, suggesting that excess of allergen binding leads to selective inhibition of degranulation through certain cellular mechanism.

It might be dangerous to assume that CD63 expression directly reflect basophil degranulation, although it has been frequently used as the sensitive indicator of basophil activation and degranulation[10]. Simultaneous and identical surface expression profiles of two other granule-associated proteins LAMP1 and LAMP2 support the view that CD63 expression truly reflect basophil degranulation[16]. It was further confirmed by the experiment that concentrations of representative granular contents, histamine and leukotriene, showed identical profiles of dose-dependent stimulation with CD63 expression[17].

It was also shown in this study that only small amount of Cryj1 is required to obtain maximum basophil activation/degranulation, regardless of the profiles of high-dose inhibition. Significant differences in allergen stimulated basophil activation is thus observed not in low-dose activation phase, but rather in high-dose inhibition phase, suggesting that the individual differences in molecular mechanism of basophil inhibitory signals exist. And this individual difference in the levels of inhibitory signals are important to determine the *in vivo* behavior of the basophils to certain level of allergen exposure, and subsequently the clinical symptoms evoked by the exposure.

The profiles of basophil stimulation test may vary from time to time with a single individual unless the characteristic profile reflect certain genetically determined factors. Repeated experiments with three individuals who show distinct profiles proved that the individual characteristics do not change significantly but rather maintained their typical profiles. Thus it is suggested that the profiles of basophil stimulation test is determined by factors unique to the individuals, but not by experimental error or by simple variation. Factors which may influence the profiles are, levels of Cryj1-specific IgE and Cryj1-specific IgG, levels of activating and inhibitory signals for basophil stimulation, and the regulatory network of basophil activation.

Although the number of the cases is small and the study design is limited, comparison of clinical significance and the level of high-dose inhibition suggest that the *in vivo* activation of basophil/mast cell is modified by the inhibitory mechanism and the symptom is altered by this cellular event. The presence of active inhibitory mechanism at cellular level suggest that it is possible to induce effector cell anergy by repeated exposure to certain amount of allergens and modulating the levels of the innate inhibitory signals. We need to monitor patients in a prospective fashion before and after immunotherapy to prove this possibility. If significant change in basophil stimulation profile is observed after immunotherapy, it may serve as a very sensitive and reliable marker to evaluate the effect of the treatment.

The molecular mechanism through which basophil degranulation is inhibited at high allergen-dose is not clear. Two possible mechanisms have been indicated by previous experiments. One mechanim is through inhibitory signal via inositol 5'phosphatase on basophil, SHIP. Basophil surface expresses not only FcεRI but also FcγRIIb. When the former receptor binds specific IgE and serves as the positive regulator, the latter recepor binds IgG4. At lower allergen concentrations, allergen binds IgE predominantly because of its higher affinity. On the contrary, allergen may bind IgG4 at higher concentrations when FcεRI is saturated and maximum stimulation is delivered through the receptor. Binding of allergen to IgG4 leads to stimulation through its receptor FcγRIIb, and subsequent activation of ITIM. The activation of ITIM results in the association of SHIP and its phosphorylation, leading to inhibition of basophil degranulation[18][19]. In particular, Gibbs et al

elegantly showed that human circulating basophils behaved differently with suboptimal and supraoptimal antingen concentrations. They further disclosed that SHIP plays major role in these distinct response patterns of basophil responses[20]. To support this view, cells from SHIP deficient mast cells do not show bell-shaped pattern of allergen stimulation[21]. Furthermore, chimeric IgE-IgG2 exhibit inhibition of basophil activation indicating that simultaneous activation of ITAM and ITIM results in the inhibition of basophil function[22)23)]. Increase of allergen-specific IgG4 after repeated exposures may explain its role as the regulator of basophil activation through FcγRIIb[24)25)].

Alternative explanation for high-dose inhibition is involvement of src-family tyrosine kinase, Lyn-mediated inhibitory signal. FcεRI is composed of α, β and γ subunits and β subunit is known to be associated with Lyn within lipid raft upon exposure to excess amount of allergens. Lyn phosphorylate β subunit of FcεRI and thereby inhibitory signals are delivered intracellularly[26)-28)]. In other word, Lyn serves as a regulatory molecule by associating with β subunit of FcεRI depending on the level of allergen exposure. Practically, both mechanisms are likely involved in the high-dose inhibition and the regulatory system is closely related to the induction of anergy at cellular level.

Neither of these possibilities is directly examined in this experiment because of the technical difficulty. We would like to prove the direct association of the basophil stimulation profile and the phosphorylation profiles of Lyn or SHIP in the future study, possibly using antibody against phosphorylated proteins and sensitive flow cytometry assay.

Conclusions

This study shows for the first time the direct relationship between allergen-binding and basophil activation. Basophil activation is induced at very low level of allergen binding and inhibited at high allergen level by individually different manner. The results indicate that judicious manipulation of allergen exposure leads to safe introduction of allergen-specific tolerance at cellular level. Furthermore, they may offer a rationale for allergen immunotherapy.

Acknowledgements

We thank Ms. Harumi Matsukawa and Ms. Shizu Kouraba for excellent technical assistance.

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