

# Pretreatment with Low Levels of FcεRI-Crosslinking Stimulation Enhances Basophil Mediator Release

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## Key Words

Allergen · Basophil · Degranulation · Immunoglobulin E · Mast cell · Subthreshold stimulation

## Abstract

**Background:** Basophils and mast cells are important initiator/effector cells capable of rapidly responding to IgE-mediated stimulation, but the precise mechanisms regulating their functions in vivo have not been fully identified. In this study, we assessed whether low levels of antigen can modulate activation of basophils and mast cells. **Methods:** Human basophils and cultured mast cells were pretreated with low concentrations of anti-FcεRI α-chain mAb (CRA-1 mAb), and their cell functions were assessed. **Results:** Basophils preincubated with CRA-1 mAb at as low as 1 ng/ml for 1 h showed significantly enhanced degranulation in response to various secretagogues such as MCP-1, FMLP, leukotriene B<sub>4</sub> and Ca ionophore A23187. FMLP-induced leukotriene C<sub>4</sub> production by basophils was also enhanced by CRA-1 mAb pretreatment. Degranulation was further enhanced when CRA-1 mAb-pretreated basophils were additionally treated with IL-3, IL-33 or leptin before stimulation with MCP-1. Priming by

subthreshold CRA-1 mAb was a slow process, since 1 h of pretreatment was needed for maximal enhancement. Basophil priming also resulted from preincubation with subthreshold doses of an allergen, Der f 2. In parallel mAb experiments, CRA-1 mAb showed weak priming effects on human umbilical cord blood-derived cultured mast cells; a higher dose, 100 ng/ml, was necessary for this priming. **Conclusion:** These results indicate that subthreshold doses of CRA-1 mAb or allergens can prime basophils and induce exaggerated responses to various IgE-independent stimuli. This may be a potentially important mechanism that explains environmental allergen-induced exacerbation of IgE-mediated allergic diseases such as asthma.

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## Introduction

Mast cells and basophils abundantly express a high-affinity receptor for IgE (FcεRI) on their surface [1]. Crosslinkage of cell surface-bound IgE by multivalent antigens induces immediate activation of these cells. Both types of cells are able to release the contents of their in-

tracellular granules including histamine, and are also able to newly synthesize mediators such as leukotrienes. Through the release of these mediators, mast cells and basophils are thought to be actively involved in the pathogenesis of immediate-type allergic diseases such as bronchial asthma and anaphylaxis.

Using experimental models in humans and rodents, mast cells and/or basophils have been shown to be responsible for the local appearance of histamine and leukotrienes during immediate and late-phase allergic reactions occurring in the lung, nose and skin [2–5]. Recently, basophils were clearly demonstrated to be especially important in the pathogenesis of certain types of allergic reactions in mice, such as IgE-dependent very-late-phase responses and IgG-mediated anaphylactic shock [6, 7]. Such findings imply that basophils may also play a central role in some situations in humans. Our understanding of the separate roles of basophils and mast cells is rapidly increasing.

In clinical situations, the symptoms of allergic diseases do not always manifest as antigen-induced monophasic or biphasic responses. Although it is clear that provocation with high-dose allergens can elicit immediate and late-phase reactions at local sites, including the bronchial and nasal mucosa, the minimal dose of allergens that elicits allergic symptoms has not been clearly established in clinical settings. Importantly, the *in vivo* effects of very low levels of allergens on the clinical manifestation of allergic diseases are largely unknown, although such situations with weak allergen exposure may be common. We often see worsening of disease control due to environmental allergen exposure, but the underlying mechanisms of functional modification of FcεRI-expressing mast cells and/or basophils have not been clearly identified. In our studies of various IgE-mediated biological actions, we recently found that subthreshold levels of antigen exposure can efficiently modulate cellular activation profiles, including enhancement of surface CD69 expression on human basophils [8]. In this study, we show that pretreatment of human peripheral basophils with subthreshold levels of allergen or anti-FcεRI mAb can enhance degranulation in response to subsequent stimulation with various IgE-independent secretagogues.

## Materials and Methods

### Reagents

The following reagents were purchased as indicated: Percoll (GE Healthcare, Uppsala, Sweden), PIPES, TPA and FMLP (Sigma, Mo., USA), MCP-1, IL-3, IL-4, IL-5 and GM-CSF (PeproTech,

London, UK), IL-33 (Adipogen, Incheon, South Korea), leptin (R&D, Minn., USA), recombinant Der f 2 (Asahi Brewery, Tokyo, Japan), calcium ionophore A23187 (Calbiochem-Behring, Calif., USA), actinomycin D and cycloheximide (Wako Pure Chemicals, Osaka, Japan), mouse IgG2b (Cappel, Ohio, USA), goat anti-human IgE Ab (MBL, Nagoya, Japan) and human myeloma IgE (Bio-design, Me., USA). A mouse IgG2b anti-human FcεRI α-chain mAb (CRA-1 mAb) was used; this antibody can bind to the FcεRI α-chain regardless of whether or not it is occupied by IgE [9]. PIPES-A buffer contained 0.03% albumin. PIPES-ACM buffer contained 0.03% albumin, 2 mM Ca<sup>2+</sup> and 0.5 mM Mg<sup>2+</sup>.

### Preparation of Basophils

Venous blood was drawn from consenting volunteers with no history of atopic disease. For histamine release studies, basophils were prepared by a dextran sedimentation procedure. For leukotriene C<sub>4</sub> (LTC<sub>4</sub>) production analysis, basophils (purity >95%) were purified by Percoll density centrifugation and by negative selection with MACS beads (Miltenyi, Bergisch-Gladbach, Germany) [10].

### Preparation of Cultured Human Mast Cells

Cultured human cord blood-derived mast cells were prepared as previously described [11]. For some experiments, the mast cells were incubated for 4 days with 10 ng/ml IL-4 to augment their degranulation response [9].

### Pretreatment and Mediator Release by Basophils and Mast Cells

Basophils were resuspended in PIPES-A buffer and preincubated at 37°C for 1 h with either CRA-1 mAb or control IgG2b. Cells were then washed and resuspended in PIPES-ACM buffer. After pretreatment for 15 min with or without IL-3 or for 1 h with or without IL-33 or leptin, secretagogues were added to trigger degranulation. After incubation for 45 min, the supernatants were collected and stored for mediator assay. Histamine release was expressed as a percentage of the total cellular histamine after subtracting spontaneous release (usually <3%). LTC<sub>4</sub> was measured with an EIA kit (Cayman, Mich., USA). Spontaneous LTC<sub>4</sub> release was below the detectable limit. These experiments were performed in duplicate or triplicate.

Some experiments used basophils from mite-sensitive subjects, and the cells were preincubated with Der f 2 [12] in place of CRA-1 mAb.

Cultured human mast cells sensitized with human IgE at 1 μg/ml for 2 h were preincubated for 1 h with CRA-1 mAb or IgG2b mAb but without Ca<sup>2+</sup> or Mg<sup>2+</sup>. The cells were then washed, resuspended in PIPES-ACM and stimulated with various secretagogues for 45 min.

### Preparation of Fab Fragments of Antibodies

Fab fragments of CRA-1 and control IgG2b mAbs were prepared with a Fab preparation kit (Pierce, Ill., USA). Papain-digested Fab fragments were identified as a single 50-kDa band in nonreducing SDS-PAGE analysis. Under reducing conditions, fragments were seen as 25-kDa bands. No contamination by undigested intact IgG was observed.

### Statistics

All data are expressed as the mean ± SEM. Differences between values were analyzed by parametric ANOVA (Statcel Version 2,

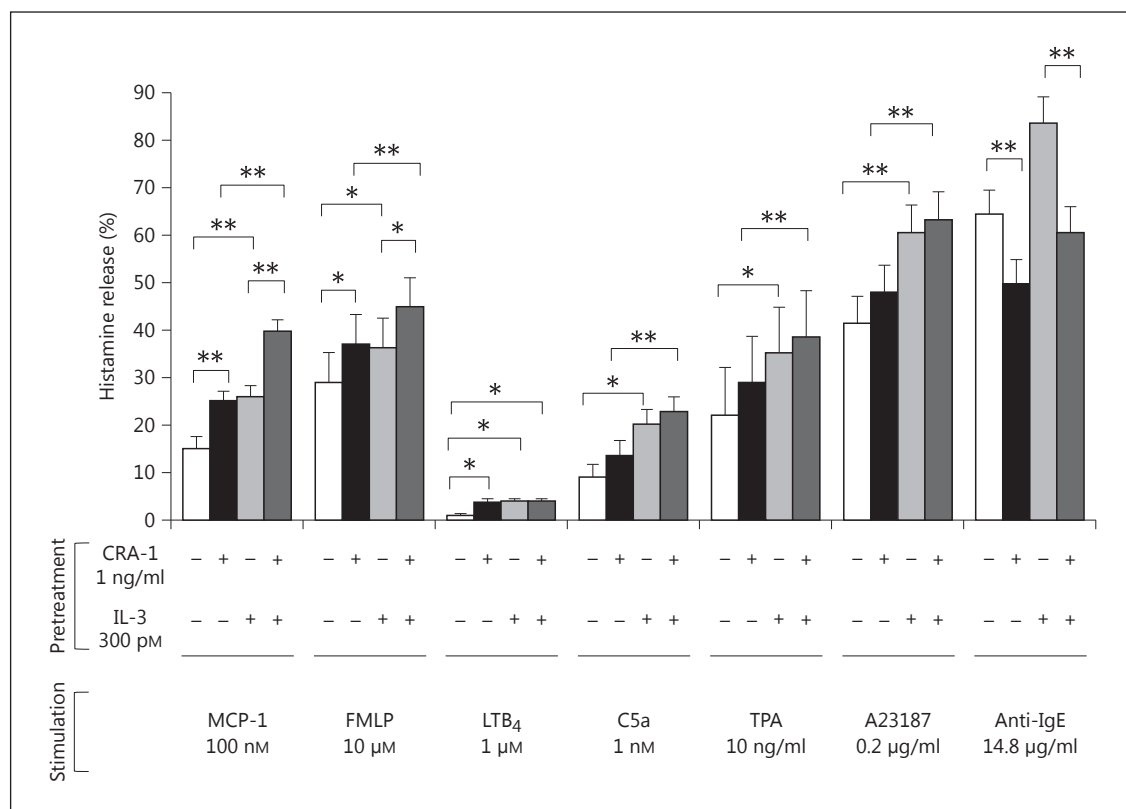


**Fig. 1.** Priming of basophils by preincubation with CRA-1 mAb. Basophils were preincubated with CRA-1 mAb or isotype control IgG2b at 1 ng/ml. After washing, the cells were pretreated with or without IL-3, and then stimulated. Data shown are the mean  $\pm$  SEM obtained from 5 experiments using basophils from 5 different donors. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus cells preincubated with control IgG2b or without IL-3.

OMS-publishing, Saitama, Japan) [10, 13]. When ANOVA indicated a significant difference, the Bonferroni/Dunn post hoc test was used to compare individual groups.

## Results

### *Modification of Basophil Activation by Preincubation with Low-Concentration CRA-1 mAb*

CRA-1 mAb at relatively high concentrations, i.e. 100–1,000 ng/ml, can potently elicit direct degranulation of basophils. On the other hand, at subthreshold levels as low as 1 ng/ml, CRA-1 mAb failed to induce detectable histamine release. Nevertheless, preincubation of basophils with 1 ng/ml CRA-1 for 1 h clearly potentiated histamine release in response to MCP-1, FMLP and LTB<sub>4</sub>, compared to preincubation with the same concentration of isotype-matched control IgG2b (fig. 1). Degranulation of basophils is known to be potentiated by IL-3 [14]. The above-noted potentiating effect of 1 ng/ml CRA-1 mAb

on basophil histamine release triggered by MCP-1 or FMLP was almost comparable to that of 300 pM IL-3. In addition, degranulation was further potentiated by pretreatment with combined CRA-1 and IL-3. These results indicate that a subthreshold (1 ng/ml) concentration of CRA-1 is able to prime basophils to respond to secretagogues such as MCP-1 and FMLP.

Degranulation of basophils by a lipid mediator, LTB<sub>4</sub>, was potentiated after preincubation with either IL-3 or CRA-1. However, this priming was somewhat different from that described above with MCP-1 and FMLP in that the combination of IL-3 and CRA-1 failed to show further enhancement of LTB<sub>4</sub>-induced histamine release. Degranulation induced by C5a, TPA and ionophore A23187 was slightly enhanced after pretreatment with 1 ng/ml CRA-1 mAb [15], although not with statistical significance. As expected, preincubation with CRA-1 induced basophil desensitization, and the response to subsequent potent anti-IgE Ab stimulation was suppressed [16, 17].

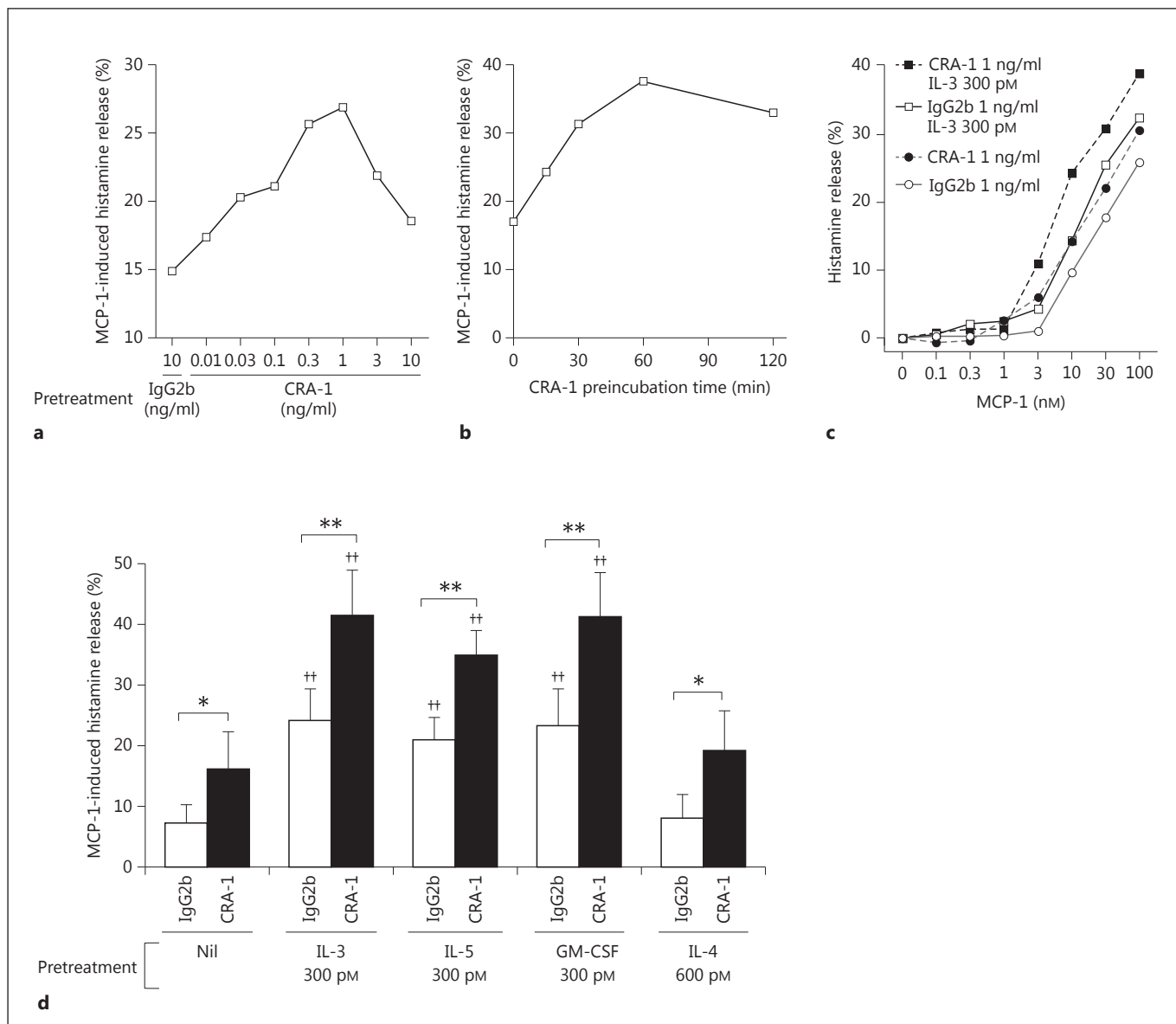


**Fig. 2.** Characteristics of basophil priming by preincubation with CRA-1 mAb. **a** CRA-1 mAb concentration-dependent priming of MCP-1-induced histamine release from basophils. Basophils were preincubated with various concentrations of CRA-1 or 10 ng/ml control IgG2b mAb. After washing, the cells were pretreated with 300 pM IL-3 and then challenged with MCP-1 at 100 nM. Data were generated using 3 different donors' basophils, and they exhibited essentially similar results. One representative result is shown. **b** Time-dependent priming of basophils by CRA-1 mAb. Basophils were preincubated with CRA-1 at 1 ng/ml for the indicated time periods, and then pretreated with IL-3 and stimulated with 100 nM MCP-1. Data shown are representative of 3 separate experiments using basophils from 3 different donors; other experi-

ments gave similar results. **c** Priming effects of CRA-1 mAb and IL-3 on basophil histamine release triggered by various doses of MCP-1. Basophils were preincubated with CRA-1 or control IgG2b, and then pretreated with or without IL-3. Data shown are representative of 3 separate experiments using basophils from 3 different donors; other experiments gave similar results. **d** Priming by IL-5 and GM-CSF. Basophils were preincubated with CRA-1 or control IgG2b, and then pretreated with or without the indicated cytokines before stimulation with MCP-1. Data shown are the mean  $\pm$  SEM obtained from 3 separate experiments using basophils from 3 different donors. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus corresponding cells preincubated with control IgG2b.  $\dagger\dagger$   $p < 0.01$  versus corresponding values of cells without cytokine pretreatment (Nil).

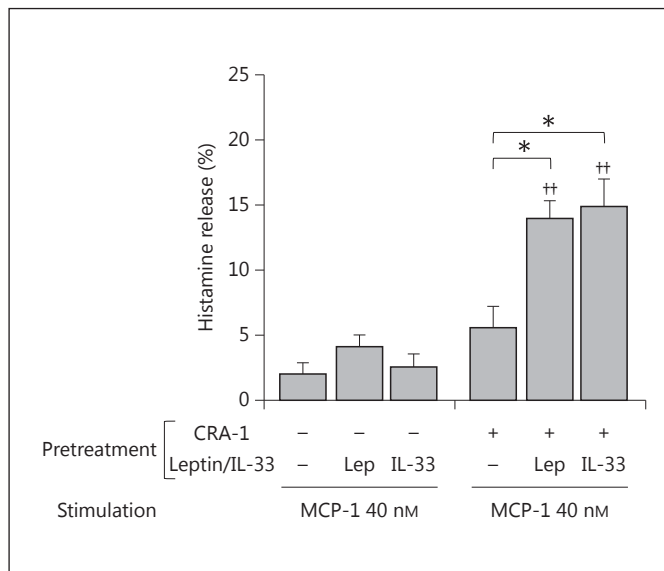


**Fig. 3.** Priming of basophils by CRA-1 mAb in cooperation with leptin or IL-33. Basophils were preincubated with CRA-1 mAb or control IgG2b at 1 ng/ml, washed and then pretreated with leptin or IL-33 for 1 h. Data shown are the mean  $\pm$  SEM of a representative experiment performed in triplicate. The two other experiments gave similar results. \*  $p < 0.05$  versus corresponding cells without leptin or IL-33 pretreatment. \*\*  $p < 0.01$  versus corresponding cells without CRA-1 mAb pretreatment.

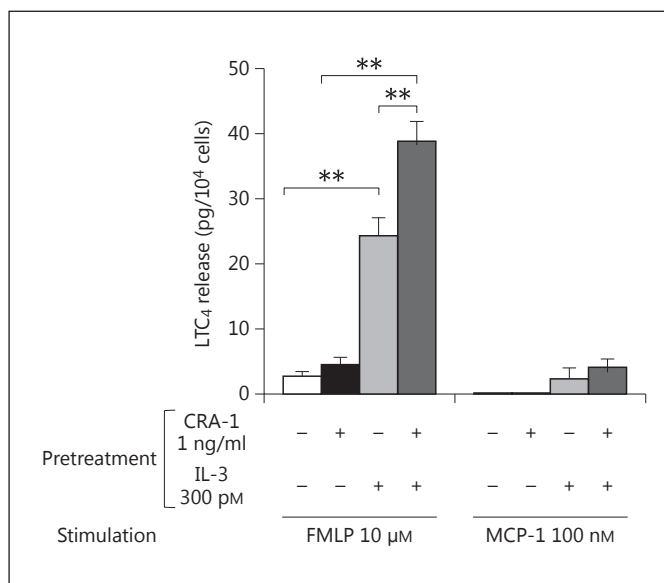


**Fig. 4.** Priming of basophil LTC<sub>4</sub> release by CRA-1 mAb. Basophils (purity >98%) were incubated with CRA-1 mAb or control IgG2b, pretreated with or without IL-3 and stimulated. Data shown are the mean  $\pm$  SEM ( $n = 3$ ).

Figure 2a shows representative results of CRA-1 mAb concentration-response studies. Weak histamine release enhancement was observed after pretreatment of basophils with CRA-1 at as low as 0.1 ng/ml. Maximum potentiation was achieved at 1 ng/ml of CRA-1. Figure 2b shows representative time-course results. Maximal enhancement of MCP-1-induced histamine release occurred with a 1-hour preincubation with CRA-1. Thus, basophil priming by low-concentration CRA-1 mAb appears to be a slow process. Actinomycin D and cycloheximide failed to affect degranulation enhancement in basophils preincubated with CRA-1 mAb, indicating that transcription or protein synthesis is not necessary for the functional changes in basophils.

We next investigated whether pretreatment of basophils with CRA-1 with and without IL-3 could enhance the cells' response to a wide range of MCP-1 doses. Compared to pretreatment with control IgG2b and no IL-3, pretreatment with CRA-1 at 1 ng/ml and/or IL-3 enhanced basophil histamine release evoked by MCP-1 concentrations between 3 and 100 nM (fig. 2c).

Figure 2d shows that each of IL-5 and GM-CSF, which are known to share a receptor subunit (common  $\beta$ -chain) with IL-3 [18], can cooperate with CRA-1 to prime basophils. Conversely, a Th2 cytokine, IL-4, showed no priming effect. IL-33 and leptin [10, 19] also enhanced MCP-1-induced basophil activation, in cooperation with CRA-1 at 1 ng/ml (fig. 3).

#### *CRA-1 mAb Pretreatment Augments LTC<sub>4</sub> Production by Basophils*

We investigated whether basophil LTC<sub>4</sub> production was modulated by pretreatment with CRA-1 mAb. As shown in figure 4, basophils (purity >98%) released a small amount of LTC<sub>4</sub> in response to FMLP stimulation. IL-3 obviously upregulated that release, which was even further enhanced by pretreatment with CRA-1 plus IL-3. On the other hand, LTC<sub>4</sub> release triggered by MCP-1 was rather low, as is already known; pretreatment of basophils with CRA-1 mAb failed to show apparent upregulation of MCP-1-induced LTC<sub>4</sub> production.

#### *Threshold Allergen Stimulation Can Prime Basophils for Enhanced Degranulation*

We assessed if allergens can prime basophils. Basophils from Der f 2-sensitive subjects ordinarily release histamine in response to direct stimulation with Der f 2 at concentrations of 100 pg/ml or higher, but not at 30 pg/ml or lower. When basophils were pretreated with either 30 or 100 pg/ml Der f 2, degranulation by MCP-1 and FMLP was slightly augmented (fig. 5). Pretreatment with

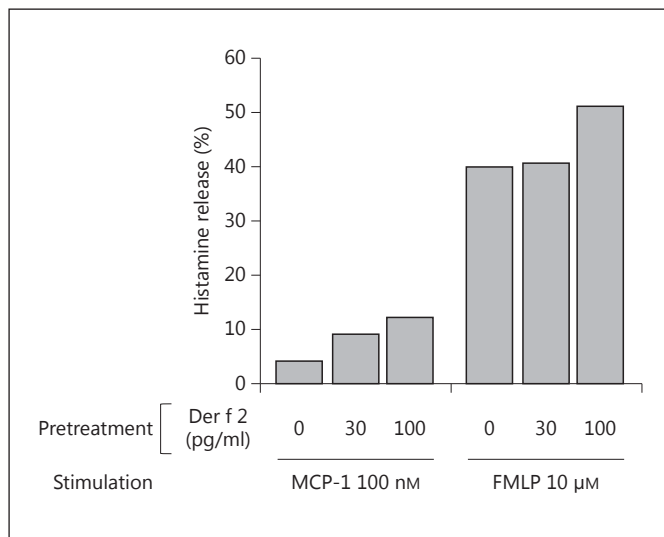


**Fig. 5.** Basophil priming by Der f 2. Basophils from mite-sensitive subjects were preincubated with or without low concentrations of Der f 2 for 1 h. Two separate experiments gave similar results; data from one representative donor are shown.

subthreshold levels of polyclonal anti-IgE Ab also augmented basophil degranulation triggered by MCP-1 or FMLP (data not shown).

We then assessed whether the Fab fragment of CRA-1 mAb could prime basophils. However, preincubation with CRA-1 Fab failed to enhance MCP-1-induced degranulation (data not shown).

#### *Analysis of Priming Effect of CRA-1 mAb on Cultured Human Mast Cells*

We next investigated whether pretreatment with CRA-1 mAb affected mast cell degranulation. In initial experiments mast cells released approximately 10% of their histamine when stimulated directly with optimal doses of CRA-1 (0.1 or 1 μg/ml) or anti-IgE (15 μg/ml). Preincubation with 1, 10 or 100 ng/ml CRA-1 failed to augment mast cell degranulation triggered by TPA or ionophore A23187 (data not shown). When mast cells cultured with IL-4 for 4 days were used [9], preincubation with CRA-1 at 100 ng/ml significantly increased histamine release initiated by 10 ng/ml TPA (fig. 6). Conversely, the preincubation with CRA-1 apparently induced desensitization, as the cells lost most of their degranulating response to subsequent high-dose CRA-1 or anti-IgE stimulation. These results indicate that preincubation with CRA-1 mAb at relatively high concentrations can mildly enhance mast cell degranulation in response to certain secretagogues.

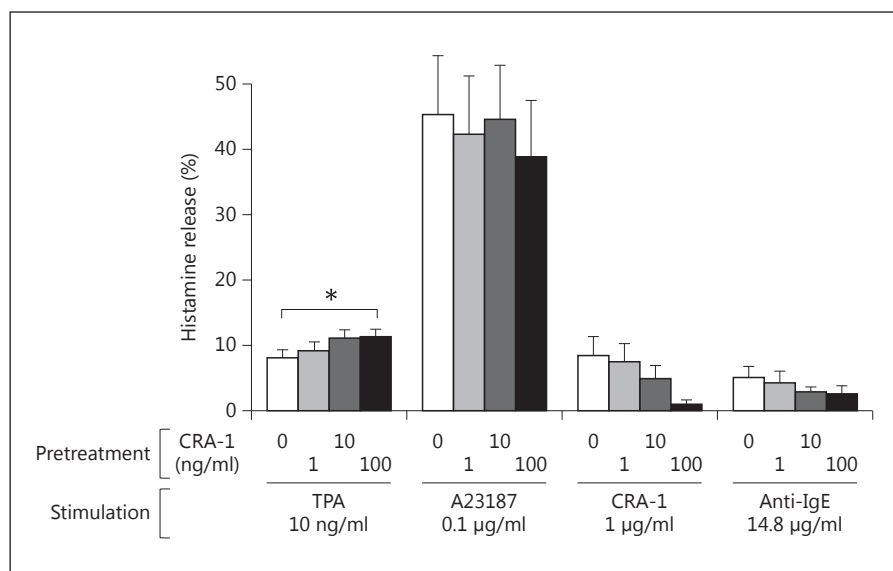
## **Discussion**

IgE-dependent activation of basophils and mast cells is a key event in type I allergic reactions [1]. Crosslinkage of surface IgE generates rapid activation signals, and the cells liberate histamine and lipid mediators such as LTC<sub>4</sub>.

In this study, we analyzed whether or not human basophils and mast cells show somewhat different activation profiles in the presence of low levels of IgE- or FcεRI-crosslinking substances. Basophils exposed to a low concentration of CRA-1 mAb for ≥1 h showed no changes in cell shape, intracellular histamine content or surface FcεRI expression levels [16, 20]. However, the cells showed higher levels of degranulation in response to non-IgE-mediated stimulation, such as by MCP-1. Enhancement of degranulation of basophils pretreated with CRA-1 was similar in potency to that with IL-3 pretreatment. Pretreatment of basophils with low-dose CRA-1 plus IL-3 further enhanced the cellular response to MCP-1 compared to pretreatment with either CRA-1 or IL-3 alone. In contrast, degranulation in response to LTB<sub>4</sub> [21] was observed when basophils were pretreated with either low-dose CRA-1 or IL-3, but combined CRA-1 and IL-3 pretreatment showed no synergism. Pretreated basophils also showed augmented secretion of a lipid mediator, LTC<sub>4</sub>. These results show that human basophils can recognize relatively low concentrations of CRA-1 and exhibit enhanced mediator release in response to IgE-independent secretagogues. Similar analyses were performed on degranulation of cultured mast cells, and pretreatment with a relatively high dose of CRA-1 mAb (100 ng/ml) resulted in enhanced degranulation in response to TPA.

Previous reports showed that pretreatment with IgE-crosslinking substances enhanced basophil degranulation induced by TPA and ionophore A23187 [15, 17]. However, in those reports, the concentrations of reagents used for pretreatment were rather high. In contrast, we found here that subthreshold CRA-1 or allergen pretreatment potently enhanced degranulation induced by IgE-independent stimulation. The Fab fragment of CRA-1 failed to enhance basophil degranulation triggered by MCP-1, indicating that cross-linkage of FcεRI is essential for the priming effect on basophils. In addition, preincubation of basophils with a low concentration of Der f 2 enhanced the histamine release response, suggesting that low allergen levels can prime basophils. It will be important to elucidate the intracellular changes in basophils treated with subthreshold CRA-1. As subthreshold CRA-1 pretreatment affects various aspects of basophils including degranulation, LTC<sub>4</sub> production, surface acti-

**Fig. 6.** Priming of cultured mast cells by CRA-1 mAb. Human mast cells were cultured for 4 days with 10 ng/ml IL-4. The cells were sensitized with human myeloma IgE for 2 h and then preincubated for 1 h with CRA-1 at the indicated concentrations. Data shown are the mean  $\pm$  SEM obtained from 5 separate experiments using mast cells from 4 different batches of cord blood. \*  $p < 0.05$  versus corresponding values of cells without CRA-1 mAb preincubation.



vation marker expression [8] and migration [12], we assume that some early signal(s) may account for CRA-1-induced functional changes. Very recent reports demonstrated that treatment of basophils with subthreshold anti-IgE for several days leads to loss of Fc $\epsilon$ RI and Syk [22]. Our analysis of nonreleaser basophils showed rather weak priming by 1-hour treatment with low-dose CRA-1 (data not shown), but we are not sure whether this weak effect was due to the low amount of Syk or the weak degranulating properties of those basophils [14, 23, 24]. The precise mechanisms induced by low-dose CRA-1 or allergens during 1-hour incubation need to be clarified.

We recently reported that basophil migration toward eotaxin was significantly upregulated in the presence of subthreshold CRA-1 at 1 ng/ml, although this mAb concentration failed to directly affect the chemokinetic activity of basophils. Surface expression of an activation marker, CD69, on basophils was also enhanced by CRA-1: IL-3-induced CD69 expression was strongly upregulated when CRA-1 at 1 ng/ml was included in overnight culture of basophils [8, 25]. These results indicate that subthreshold CRA-1 or allergens can alter various basophil biological activities, e.g. enhancement of degranulation and lipid mediator release in response to IgE-independent stimulation, eotaxin-directed migration [12] and IL-3-induced CD69 expression [8]. In contrast, subthreshold concentrations of CRA-1 mAb or allergens induced desensitization of basophils, as the subsequent response to IgE-mediated stimulation was clearly suppressed [17].

We think that these findings will contribute to our understanding of the complex interplay of cells with various molecules at sites of chronic allergic inflammation, involving continuous allergen exposure plus endogenous inflammatory mediators including IL-3, IL-33 and leptin [10, 19]. Importantly, IL-33 and leptin are known to enhance IgE-mediated basophil degranulation, and these cytokines also enhanced MCP-1-induced degranulation when basophils were additionally pretreated with CRA-1 (fig. 3). Activated basophils produced IL-3, which, in turn, affected the cellular functions [26]. This autocrine mechanism may be important in relation to some of our findings. However, basophil-derived IL-3 may not be able to account for all of the events, since *in vitro* basophil survival, which is highly sensitive to trace levels of IL-3 [18], was not affected by subthreshold concentrations of CRA-1 [8].

Recent findings suggest that basophils and mast cells may be important targets in therapeutic strategies for IgE-mediated allergic diseases, including asthma [1, 27, 28]. The priming of basophils by weak crosslinking of IgE or Fc $\epsilon$ RI, that we have documented as leading to exaggerated outcomes such as enhanced degranulation and LTC<sub>4</sub> production, can reasonably be presumed to occur in clinical settings. Low-level allergen exposure may thus be actively involved in the pathogenesis of allergic diseases as an exacerbating factor. This may partly explain why therapeutic targeting of IgE, using omalizumab, can show clinical efficacy on chronic allergic diseases such as asthma [29]. Although we do not think that basophils and

mast cells are the sole target cell types of anti-IgE therapy [30], our data contribute to our understanding of the basic underlying mechanisms of the effectiveness of anti-IgE.

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## Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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