Large particulate allergens can elicit mast cell-mediated anaphylaxis without exit from blood vessels as efficiently as do small soluble allergens

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Abstract

Anaphylaxis is a rapid-onset, life-threatening allergic reaction in that IgE, mast cells and histamine are commonly involved. It can be experimentally induced in IgE-sensitized animals by intravenous injection of corresponding allergens, and the sign of anaphylactic reaction can be detected within minutes after allergen challenge. However, it remains puzzling why the anaphylactic reaction can be initiated *in vivo* so quickly, considering that allergens are delivered into the blood circulation while mast cells reside within peripheral tissues but not in the blood circulation. To address this issue, we compared two different forms of the same allergen, small soluble and large particulate ones, in their ability to induce anaphylaxis in IgE-sensitized mice. In contrast to our expectation, particulate allergens could induce anaphylaxis as quickly and efficiently as did soluble allergens, even though they remained inside of blood vessels. *In vivo* imaging analysis suggested the direct interaction of intravascular particulate allergens and perivascular mast cells across the capillary wall. Taken together with previous report that perivascular mast cells can capture IgE in the blood circulation by extending cell processes across the vessel wall, our findings imply that blood-circulating allergens, regardless of their size, can stimulate mast cells without exit from blood vessels, by means of cross-linking IgE on mast cell processes inserted into the vessel lumen, and hence initiate anaphylactic reaction so quickly.

Key words

anaphylaxis, basophil, IgE, mast cell, particulate allergen

Abbreviations

OVA, ovalbumin; BSA, bovine serum albumin; TNP, 2,4,6-trinitrophenol; Immunoglobulin E, IgE; Mcpt8, mouse mast cell protease-8; DTR, diphtheria toxin receptor; PCA, passive cutaneous anaphylaxis; ESAM-1, endothelial cell-selective adhesion molecule-1

1. Introduction

Anaphylaxis is a rapid-onset and potentially fatal allergic reaction. It is well documented that mast cells and IgE are critically involved in anaphylaxis [1-3]. IgE binds to the cell surface of mast cells through the high-affinity IgE receptor FcεRI [4]. When corresponding allergens bind to and cross-link IgE on mast cells, chemical mediators such as histamine are released from activated mast cells, leading to anaphylaxis with increased vascular permeability [4-6]. Two major protocols are available for inducing systemic anaphylaxis in animals, active and passive ones in terms of sensitization [7]. In both models, soluble proteins such as bovine serum albumin (BSA) and ovalbumin (OVA) are commonly used as allergens, and the sign of anaphylaxis, including hypothermia, becomes evident within minutes after the challenge of allergens to sensitized animals [7].

It remains poorly documented why anaphylaxis can be elicited within a short period of time after intravenous administration of allergens. In contrast to blood-circulating basophils, mast cells reside in peripheral tissues, particularly in perivascular regions [4]. One may assume that intravenously administered allergens diffuse quickly out of vessels and stimulate IgE-sensitized mast cells located nearby outside of vessels. However, this scenario does not seem to be consistent with the observation of Evans blue dye extravasation. Evans blue dye is widely used to assess the vascular permeability in animal models [8-10]. This dye binds albumin when delivered into the blood circulation [11]. Under physiological conditions, the endothelium is impermeable to albumin, and therefore the dye bound to albumin remains restricted within blood vessels. When the vascular permeability increased under pathological conditions such as anaphylaxis, the extravasation of albumin-bound dye occurs, resulting in blue colorization of surrounding tissues [8-10]. Thus, the above-mentioned scenario may become operative once anaphylaxis is elicited, but does not seem to account for the initial step triggering anaphylaxis. Intriguingly, Cheng et al. recently reported that perivascular mast cells can capture IgE in the

blood circulation by extending cell processes across the vessel wall [12]. This prompted us to examine the possibility that allergens delivered into the blood circulation may stimulate IgE-sensitized mast cells even without exit from blood vessels, leading to acute-onset of anaphylactic reaction.

In the present study, we examined this possibility by using soluble allergen-conjugated polystyrene microspheres (3 μm in diameter) as particulate allergens that were expected too large to leak out from blood vessels, and examined their ability to induce anaphylaxis in IgE-sensitized mice.

2. MATERIALS AND METHODS

2.1. Mice

C57BL/6 wild-type mice (7-8 weeks old) were purchased from the SLC (Shizuoka, Japan). Mast cell-deficient *Kit*^{W-sh/W-sh} [13], basophil-deficient *Mcp1*^{8^{DTR}} [14] and histidine decarboxylase (HDC)-deficient [15] C57BL/6 mice were as described previously and maintained under specific-pathogen-free conditions in our animal facilities. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

2.2. Preparation of particulate allergens

Plain or fluorescent Polybead[®] Carboxylate Microspheres (3.0 μ m in diameter, Ploysciences, Inc) were treated with activation solution (20 mM EDAC, 50 mM 2-[N-morpholino] ethanesulfonic acid (MES), 100 mM N-hydroxysuccinimide (Sigma-Aldrich) in deionized water) at room temperature for 30 min. After washing with deionized water, activated beads (1 % w/v) were mixed with equal volume of 1 mg/ml hapten 2,4,6-trinitrophenol-conjugated OVA (TNP₁₂-OVA) or OVA containing 50 mM MES, and incubated overnight, followed by addition of 41.4 μ M of ethanolamine to stop the reaction. Allergen-conjugated beads (TNP-OVA-beads and OVA-beads) were washed ten times with 1% BSA in PBS to remove unbound allergens and ethanolamine, and stored at 4 °C.

2.3. Bone marrow-derived mast cells and degranulation assay

Bone marrow-derived mast cells (BMMC) were prepared and cultured for 4 weeks as described previously [16]. Degranulation of BMMCs cells were determined by measurement of β -hexosaminidase release as previously described [17] with some modifications. Briefly, BMMCs (2.0 x 10⁶ cells/0.5 ml/well) were sensitized with TNP-specific IgE (IGELb4, 1 μ g/ml)

for overnight, and then stimulated with 300 ng/ml soluble allergens or 1.0×10^6 of allergen-conjugated beads. The activity of β -hexosaminidase in culture supernatants and cell lysates was measured, and the % release of β -hexosaminidase was calculated as described [17]. In experiments shown in Fig. 1B, soluble or particulate antigens were added to the upper chamber of an inserted membrane filter (Greiner) while BMMCs were placed in the lower chamber. Culture media were collected from both upper and lower chambers after 1h-culture, and analyzed for β -hexosaminidase activity.

2.4. Basophil depletion *in vivo*

Mcpt8^{DTR} mice were treated with intravenous administration of diphtheria toxin (DT, 25 μ g/kg body weight) or control PBS one day before experiments.

2.5. Passive systemic anaphylaxis

Mice were systemically sensitized with intravenous administration of 50 μ g TNP-specific IgE. One day after IgE injection, mice were intravenously challenged with soluble allergens (50 μ g) or particulate allergens (1×10^7). Their rectal temperatures were monitored by digital thermometer (Shibaura Electronics Co., Ltd. Saitama, Japan).

2.6. Passive cutaneous anaphylaxis (PCA)

Mice were locally sensitized with an intradermal injection of 20 ng of TNP-specific IgE into the right ear, and the same volume of PBS was injected into the right ear as a control. One day later, they were intravenously challenged with soluble allergens (50 μ g) or particulate allergens (1×10^7) in 100 μ l of PBS containing 0.5% Evans blue dye. Thirty minutes later, the dye was extracted from each dissected ear in 0.7 ml of formamide at 63 °C for overnight. The amount of dye extracted was determined spectrophotometrically at 620 nm and calculated from a

standard curve established with known amounts of dye.

2.7. Fluorescent intravital imaging

Mice were anesthetized with isoflurane gas (5% induction and 1.5% maintenance via a nose cone), and their body temperature was maintained at 37°C with a heating blanket. To visualize mast cells and blood vessels, mice were treated with allophycocyanin (APC)-conjugated anti-c-kit (8 µg) and phycoerythrin (PE)-conjugated anti-ESAM-1 (8 µg). Intravital images of ear skin during PCA were obtained with an inverted laser scanning microscope (A1R⁺; Nikon, Tokyo, Japan) using CFI Plan Apochromat $\lambda \times 10$ or $\times 20$ objective lens. NIS-elements and Volocity software were used for acquisition and analysis of images.

2.8. Statistical analysis

The statistical significance of differences between groups was evaluated with a two-tailed Student t test. *P*-value <0.05 was considered statistically significant.

3. RESULTS

3.1. Polystyrene microspheres can be used as particulate allergens when conjugated with haptened proteins

Polystyrene microspheres (3 μm in diameter) were chemically conjugated with hapten TNP-conjugated ovalbumin (TNP-OVA) and control ovalbumin (OVA) to obtain TNP-OVA-beads and OVA-beads, respectively. To evaluate the functional activity of these beads as allergens, bone marrow-derived mast cells (BMMCs) were sensitized with TNP-specific IgE and then incubated with these beads. TNP-OVA-beads were able to stimulate mast cells to an extent comparable to that by soluble TNP-OVA proteins, as judged by β -hexosaminidase release assay (Fig. 1A). Control OVA-beads failed to stimulate mast cells as in the case of soluble OVA proteins. Notably, when mast cells and TNP-OVA-beads were separated by a membrane filter with 0.4 μm pores, no significant activation of mast cells was detected (Fig. 1B). In contrast, soluble TNP-OVA proteins (0.005~0.006 μm in diameter) could activate mast cells even when separated by a membrane filter. These results indicated that TNP-OVA-beads can be used as particulate allergens which are able to stimulate IgE-sensitized mast cells directly but not through a membrane filter.

3.2. Particulate allergens can induce IgE-mediated systemic anaphylaxis *in vivo* in a mast cell-dependent manner

We next examined whether particulate allergens can induce anaphylaxis. Mice were passively sensitized with TNP-specific IgE and then challenged with intravenous administration of soluble TNP-OVA proteins (Fig. 2A) or TNP-OVA-beads (Fig. 2B). The rectal temperature dropped by 3~4 degrees within 30 min after the allergen injection, regardless of the form of allergens, either soluble or particulate, indicating that particulate allergens as large as 3 μm in

diameter can induce IgE-mediated systemic anaphylaxis, as quickly as and to a comparable extent as soluble allergens.

The expression of FcεRI is confined to mast cells and basophils in mice [18]. Previous studies demonstrated that mast cells but not basophils are essential for the development of IgE-mediated systemic anaphylaxis [19] while recent study reported that basophils contribute to it under certain experimental conditions [20]. In all these experiments, soluble haptenated proteins were used as allergens, and it remains to be determined what type of cells are involved in particulate allergen-elicited systemic anaphylaxis. Mast cells and basophils differ in their anatomical localization, and basophils but not mast cells circulate in the peripheral blood [4, 21]. We thought that because of their large size, particulate allergens might have difficulty in passing through the vessel wall to reach tissue-resident mast cells in a short period, and therefore suspected that basophils could be responsible for the development of particulate allergen-elicited anaphylaxis. Intriguingly, however, diphtheria toxin-mediated basophil depletion in *Mcpt8^{DTR}* mice before allergen challenge showed no significant impact on the TNP-OVA-bead-elicited systemic anaphylaxis, as judged by the change of rectal temperature (Fig. 2C). In contrast, no apparent drop in rectal temperature was observed in mast cell deficient *Kit^{W-sh/W-sh}* mice that were similarly treated for induction of TNP-OVA-bead-elicited systemic anaphylaxis (Fig. 2D). Thus, mast cells but not basophils play a key role in the development of particulate allergen-induced systemic anaphylaxis.

3.3. Particulate allergens can also induce passive cutaneous anaphylaxis (PCA) *in vivo* in a mast cell-dependent manner

The above findings raised an important question where particulate allergens and mast cells meet with each other to induce anaphylaxis, either inside or outside of blood vessels. It has been shown that in the liver, spleen, bone marrow and peritoneum, some capillaries have fenestrae

and interendothelial slits, up to a few μm in size [22, 23]. Therefore, it is possible that intravascular particulate allergens might pass through such gaps to meet and activate mast cells outside of capillaries, resulting in systemic anaphylaxis. Alternatively, particulate allergens can somehow interact with tissue-resident mast cells without exit from blood vessels. To address this issue, we drew attention to the fact that most of capillaries in the skin contain few or no intracellular gaps or fenestrae [22], and examined whether particulate allergens can induce an anaphylactic reaction locally in the skin. To this end, mice were passively sensitized with intradermal administration of TNP-specific IgE, and then challenged with intravenous injection of soluble TNP-OVA proteins (Fig. 3A) or TNP-OVA-beads (Fig. 3B and 3C). The extent of anaphylactic reaction was evaluated by vascular permeability assay using Evans blue dye. Notably, particulate allergens induced PCA reaction to an extent comparable to that by soluble allergens. Mast cell-deficient mice failed to elicit the reaction (Fig. 3D), indicating that mast cells are essential for the development of particulate allergen-induced PCA as in case of particulate allergen-induced systemic anaphylaxis. These observations suggested that particulate allergens may be able to interact with mast cells even without leaking out from blood vessels.

3.4. Particulate allergens interact with perivascular mast cells across the capillary wall without exit from blood vessels

To visualize the interaction of particulate allergens and mast cells in PCA, we used fluorescent polystyrene microspheres to prepare TNP-OVA-beads, and tissue-mast cells were labeled *in vivo* with other fluorescent-conjugated anti-c-kit antibody. *In vivo* imaging analysis under confocal microscopy demonstrated that TNP-OVA-beads were indeed detected inside but not outside of capillaries in the skin lesion of PCA (Fig. 4A). Many of them were localized in close proximity to c-kit⁺ perivascular mast cells, and appeared to contact with mast cells across the

capillary wall (Fig. 4B). When control OVA-beads were intravenously injected, they moved inside capillaries along the blood stream, and few or no beads remained immobile in the skin lesion (data not shown).

We next analyzed the allergen-mast cell interaction in mice deficient for histidine decarboxylase (HDC). Even though they cannot produce histamine and therefore fail to manifest anaphylaxis [24], the co-localization of mast cells with TNP-OVA-beads was observed in the PCA skin lesion (Fig. 4C) as observed in HDC-sufficient mice (Fig. 4B). Therefore, it seems unlikely that histamine-mediated gap formation in the endothelium resulted in contact between particulate allergens and perivascular mast cells through open window in the capillary wall. Co-localization of mast cells with TNP-OVA-beads was hardly observed when mice had not been sensitized with TNP-specific IgE (data not shown). Taken together, mast cells appeared to trap blood-circulating particulate allergens via allergen-specific IgE.

4. Discussion

It was generally thought that intravenously administered allergens may diffuse out of blood vessels and stimulate IgE-sensitized mast cells located nearby outside of vessels, resulting in anaphylaxis. If this is the case, one may expect that allergens of a larger size need more time to reach mast cells and produce less severe anaphylaxis. However, in the present study we demonstrated no significant delay in the onset of particulate allergen-induced anaphylaxis when compared with that of soluble antigen-induced one, and the extent of anaphylactic reaction was comparable between them. Importantly, no apparent extravasation of particulate allergens was detected in the skin. The cell depletion experiment revealed that mast cells but not blood-circulating basophils are essential for the development of anaphylaxis. This raised a critical question how intravascular allergens can stimulate tissue-resident mast cells without exit from blood vessels.

The *in vivo* imaging analysis of fluorescent-labeled allergens and mast cells suggested that particulate allergens contacted with perivascular mast cells across the capillary wall during anaphylaxis. Cheng et al. recently reported that perivascular mast cells can capture IgE in the blood circulation by extending cell processes across the vessel wall [12]. Taking this into consideration, our findings imply that particulate allergens in the circulation interact with mast cell projections that are inserted into the capillary lumen, and that the cross-link of IgE-FcεRI complexes on the mast cell projections by particulate allergens is sufficient for mast cell activation leading to anaphylaxis. In other words, the extravasation of allergens is not essential for the induction of anaphylaxis. Thus, perivascular mast cells appear to perform not only IgE sampling from blood but also capture of blood-circulating allergens, triggering immediate allergic responses. This could be applied to not only large particulate allergens but also small soluble allergens, and may explain well why anaphylaxis can be elicited within a short period of time after intravenous administration of allergens.

In conclusion, we have demonstrated that large particulate allergens circulating in the peripheral blood can activate perivascular mast cells without exit from the blood vessels, eliciting anaphylaxis as quickly and efficiently as soluble allergens. Direct interaction of intravascular allergens and perivascular mast cells across the capillary wall appears to account for the rapid onset of anaphylaxis elicited by allergens, regardless of their size or ability of extravasation.

Acknowledgments

We thank Dr. S.J. Galli, Dr. S. Nakae, and Dr. K. Sudo for providing KitW-sh/W-sh mice, Hideto Nishikado and Ryosuke Ishikawa for technical advice for induction of anaphylaxis, the members of the Karasuyama laboratory for helpful discussions, Rina Matsunaga for technical support and Michiko Miki for secretary assistance. This work was supported by research Grants from JST, CREST, and the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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FIGURE LEGENDS

FIG. 1. Particulate allergens can stimulate IgE-sensitized mast cells directly but not through a membrane filter, unlike soluble allergens

BMMC_s sensitized with TNP-specific IgE were incubated for 1 hr with soluble allergens (TNP-OVA or control OVA) or allergen-conjugated beads (TNP-OVA-beads or control OVA-beads) in the same chamber (A) or in different chambers separated by a membrane filter with 0.4 μ m pores (B), followed by β -hexosaminidase release assay (mean $\pm$ SEM, n=3). Data shown are representative of three independent experiments. N.S., not significant. *** p <0.001.

FIG. 2. Particulate allergens can induce IgE-mediated, mast cell-dependent systemic anaphylaxis *in vivo* as efficiently as do soluble allergens

(A and B) C57BL/6 mice sensitized with TNP-specific IgE were challenged with intravenous administration of soluble allergens TNP-OVA (closed circles) or control OVA (open circles) in A, or particulate allergens TNP-OVA-beads (closed circles) or control OVA-beads (open circles) in B. (C) *Mcpt8*^{DT^R} mice were passively sensitized with TNP-specific IgE and treated with diphtheria toxin (closed circles) or control PBS (open circles). One day later, they were challenged with intravenous administration of TNP-OVA-beads. (D) Wild-type (closed circles) and mast cell-deficient *Kit*^{W-sh/W-sh} (open circles) mice were passively sensitized with TNP-specific IgE and then challenged with TNP-OVA-beads as in A. In A-D, changes in the rectal temperature over time after allergen challenge are shown (mean $\pm$ SEM, n=3). Data shown are representative of at least three independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

FIG. 3. Particulate allergens can induce IgE-mediated, passive cutaneous anaphylaxis (PCA) *in vivo* as efficiently as do soluble allergens

(A-C) C57BL/6 mice were locally sensitized by intradermal administration of TNP-specific

IgE or control PBS in the ear skin and then challenged with soluble allergens TNP-OVA (closed bars) or control OVA (open bars) in A, or particulate allergens TNP-OVA-beads (closed bars) or control OVA-beads (open bars) in B and C. In all cases, Evans blue dye was included in the allergen-containing solutions. The extent of PCA reaction was evaluated by measuring the amount of dye leaked into the skin tissue (mean $\pm$ SEM, n=3). Representative photos of the ear skin lesions taken 30 min after the administration are shown in C. (D) Mast cell-deficient *Kit*^{W-sh/W-sh} mice were treated and analyzed as in B. Data shown in A-D are representative of three independent experiments. N.S., not significant. ****p*<0.001.

FIG. 4. Particulate allergens interact with perivascular mast cells across the capillary wall without exit from blood vessels

Particulate allergen-mediated PCA reactions were elicited in wild-type (A and B) and HDC-deficient mice (C) as in Figure 3B. In all experiments here, fluorescent microspheres were utilized for visualization of beads *in vivo*, and endothelial cells were labeled with PE-conjugated anti-ESAM-1 antibody. In B and C, mast cells were labeled with APC-conjugated anti-c-kit antibody. Ear skin lesions were subjected to intravital imaging, and photos shown were taken 0.5~1 hr after allergen challenge. Beads, endothelial cells, and mast cells are marked in white, red, and green, respectively, using pseudo colors. White arrows indicate beads. Scale bars represent 100 μ m in A, and 10 μ m in B and C. Data shown in A-C are representative of at least three independent experiments.

Figure 1

A

B

Figure 2

A

B

C

D

Figure 3

Figure 4

A

B

C