Immunity
Article

Basophils Play a Pivotal Role in Immunoglobulin-G-Mediated but Not Immunoglobulin-E-Mediated Systemic Anaphylaxis

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DOI 10.1016/j.immuni.2008.02.008

SUMMARY

Anaphylaxis is an acute, severe, and potentially fatal systemic allergic reaction. Immunoglobulin E (IgE), mast cells, and histamine have long been associated with anaphylaxis, but an alternative pathway mediated by IgG has been suggested to be more important in the elicitation of anaphylaxis. Here, we showed that basophils, the least common blood cells, were dispensable for IgE-mediated anaphylaxis but played a critical role in IgG-mediated, passive and active systemic anaphylaxis in mice. In vivo depletion of basophils but not macrophages, neutrophils, or NK cells ameliorated IgG-mediated passive anaphylaxis and rescued mice from death in active anaphylaxis. Upon capture of IgG-allergen complexes, basophils released platelet-activating factor (PAF), leading to increased vascular permeability. These results highlight a pivotal role for basophils in vivo and contrast two major, distinct pathways leading to allergen-induced systemic anaphylaxis: one mediated by basophils, IgG, and PAF and the other "classical" pathway mediated by mast cells, IgE, and histamine.

INTRODUCTION

Anaphylaxis is a rapid-onset, life-threatening allergic reaction and is most commonly triggered by exposure to allergens such as insect venoms, foods, and medications (Sampson et al., 2006; Simons et al., 2007). Immunoglobulin E (IgE) and mast cells have long been associated with anaphylaxis (Bochner and Lichtenstein, 1991; Galli, 2005; Kemp and Lockey, 2002). In individuals sensitized with a given allergen, IgE antibodies are produced against the allergen and bind to high-affinity IgE receptor FcεRI expressed on mast cells (Kraft and Kinet, 2007). Upon binding of the allergen, IgE-FcεRI complexes on mast cells are crosslinked, thereby leading to mast cell activation. Activated mast cells undergo degranulation and release chemical mediators, such as histamine, prostaglandin D2, and leukotriene C4, that are responsible for the development of anaphylactic manifestations (Bochner and Lichtenstein, 1991; Galli, 2005; Kemp and Lockey, 2002). Histamine acts on endothelial cells to increase vascular permeability and also causes constriction of intestinal and bronchial smooth muscle (Winbery and Lieberman, 2002).

Various studies indicate that this classical pathway does not account for all anaphylactic responses. Active systemic anaphylaxis could be induced even in mice deficient for mast cells, IgE, or FcεRIα chain (Dombrowicz et al., 1997; Jacoby et al., 1984; Miyajima et al., 1997; Oettgen et al., 1994; Strait et al., 2002), demonstrating that neither mast cells nor IgE are essential for the development of allergen-induced anaphylaxis. In contrast, FcRγ-chain-deficient mice that lack the expression of FcεRI and stimulatory IgG receptors manifested no apparent sign of active systemic anaphylaxis (Miyajima et al., 1997), suggesting that IgG plays an important role in active anaphylaxis. Indeed, passive sensitization with allergen-specific IgG, particularly IgG1 subclass, conferred on mice the ability to develop systemic anaphylaxis upon exposure to the allergen (Dombrowicz et al., 1997; Miyajima et al., 1997). Pretreatment of mice with FcγRII-III mAb blocked active systemic anaphylaxis (Strait et al., 2002), indicating that the low-affinity IgG receptor FcγRIII is mainly involved in IgG-mediated systemic anaphylaxis. All these results strongly suggest that systemic anaphylaxis is predominantly mediated by nonmast cells, IgG, and FcγRIII, in contrast to the traditional thought (Finkelman, 2007). However, cells responsible for IgG-mediated systemic anaphylaxis have remained elusive, although a previous study suggested that macrophages are probably the major cell type contributing to IgG-mediated anaphylaxis (Strait et al., 2002).

Basophils represent less than 1% of peripheral blood leukocytes and share several features with tissue-resident mast cells, such as the surface expression of FcεRI and the release of chemical mediators upon stimulation (Falcone et al., 2000; Galli, 2000; Prussin and Metcalfe, 2003). Therefore, basophils are often considered to be minor and possibly redundant "circulating mast cells" and are analyzed as a surrogate of the less accessible tissue mast cells. The recent finding that basophils readily generate large quantities of T helper 2 (Th2) cytokines such as IL-4 and IL-13 has provided new insights into the possible roles for basophils in allergic diseases and immunity to pathogens (Min et al., 2004; Voehringer et al., 2004).

We previously demonstrated that basophils play a critical role in the development of IgE-mediated chronic allergic inflammation, independently of T cells and mast cells (Mukai et al., 2005; Obata et al., 2007). A single subcutaneous injection of multivalent allergens elicited not only immediate- and late-phase ear swelling but also delayed-onset ear swelling with massive eosinophil infiltration in mice that had been passively sensitized with antigen-specific IgE. Mast-cell-deficient mice could also develop the delayed-onset ear swelling, whereas treatment of mice with a newly developed basophil-depleting mAb Ba103 specific to CD200R3 (Kojima et al., 2007) prior to the allergen challenge completely abolished the IgE-mediated chronic allergic inflammation (Obata et al., 2007), even though basophils accounted for only ~2% of the infiltrates in the skin lesions (Mukai et al., 2005). The Ba103 treatment during the progression of the dermatitis showed a therapeutic effect on the inflammation and resulted in drastic reduction in numbers of infiltrating eosinophils and neutrophils, concomitantly with elimination of basophils from the skin lesions. Thus, basophils play a pivotal and nonredundant role in the development of IgE-mediated chronic allergic inflammation, as an initiator rather than as an effector (Obata et al., 2007). This finding prompted us to examine whether basophils also play a nonredundant role in immediate-type allergic responses, distinctively from mast cells.

In the present study, we explored the possible roles for basophils in IgE- and IgG-mediated passive systemic anaphylaxis, as well as in active systemic anaphylaxis, by treating mice with the basophil-depleting mAb. Our results demonstrated that basophils are dispensable for IgE-mediated anaphylaxis and that basophiles but not other cells, including macrophages, are the main contributor to IgG-mediated anaphylaxis through release of platelet-activating factor (PAF) instead of histamine.

RESULTS

Basophils Efficiently Capture Allergen-IgG1 Complexes

To clarify the pathogenesis of and cells responsible for IgG-mediated anaphylaxis, we have established a simple model of penicillin shock, in which mice were passively sensitized with penicillin V (PenV)-specific IgG1 mAbs and then challenged with intravenous injection of PenV-conjugated bovine-serum albumin (PenV-BSA). Both normal and mast-cell-deficient (*Kit*$^{W\text{-}sh/W\text{-}sh}$) mice developed systemic anaphylaxis, as judged by a drastic drop in rectal temperature, although the depression in temperature was slightly less in the latter than the former (Figure 1A). Thus, mast cells were dispensable for PenV-specific, IgG1-mediated anaphylaxis, although they appeared to make some contribution to it, in accord with previous reports on another mast-cell-deficient mouse, WBB6F1-*Kit*$^{W/W\text{-}v}$, sensitized with DNP-specific IgG1 (Dombrowicz et al., 1997; Miyajima et al., 1997).

For acute allergic reactions to be elicited, we expected that the cells responsible for IgG1-mediated anaphylaxis would capture the IgG1-allergen complexes immediately after the allergen challenge. Flow-cytometric analysis of samples from mice sensitized with PenV-specific IgG1 demonstrated that many cell types, including NK cells, macrophages, monocytes, dendritic cells, neutrophils, eosinophils, basophils, and mast cells (but not T cells and platelets), displayed PenV-BSA on their surface

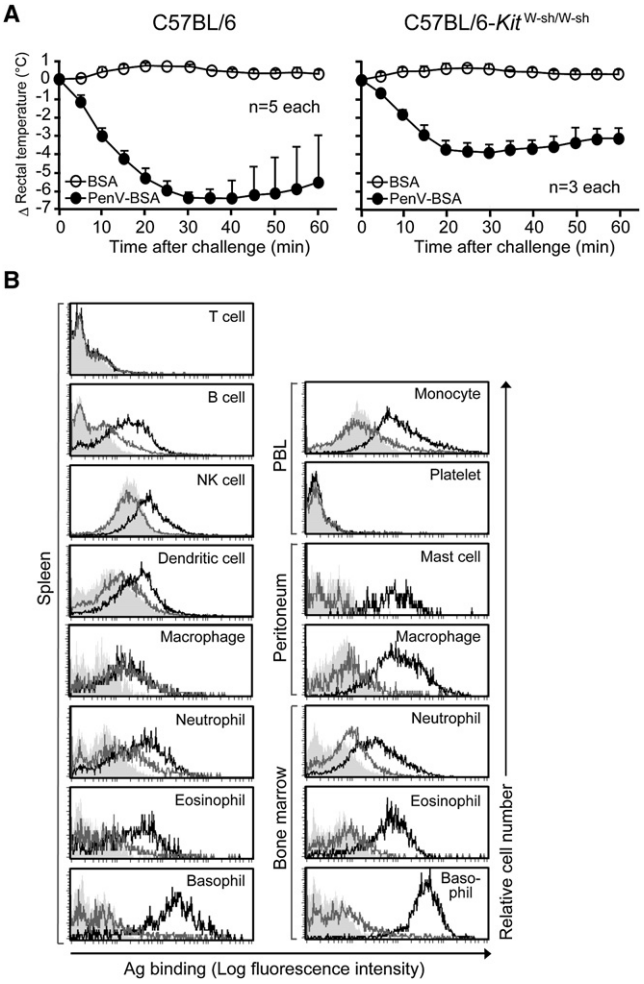

Figure 1. Basophils Capture the Highest Amount of Immune Complexes in PenV-Specific, IgG1-Mediated Systemic Anaphylaxis

(A) Wild-type and mast cell-deficient (*Kit*$^{W\text{-}sh/W\text{-}sh}$) C57BL/6 mice were passively sensitized with PenV-specific IgG1 mAbs and then challenged with PenV-BSA (closed circles) or control BSA (open circles). Change in rectal temperature over time after allergen challenge is shown. Data are expressed as the mean ± SD (n = 5 in wild-type, and n = 3 in *Kit*$^{W\text{-}sh/W\text{-}sh}$) and are representative of five independent experiments.

(B) C57BL/6 mice passively sensitized with anti-PenV IgG1 were challenged with biotinylated allergens (PenV-BSA or control BSA). Data shown are the binding of PenV-BSA (histograms with black line) and BSA (shaded histograms) to various types of cells isolated from mice immediately after the allergen challenge. Histograms with gray line show the binding of PenV-BSA when mice were treated with anti-FcγRII-III prior to the IgG1 sensitization. Data are representative of three independent experiments.

(Figure 1B). Among these cells, basophils bound the greatest amount of allergen per cell, and the binding was strongly inhibited in mice treated with FcγRII-III mAb prior to the IgG1 sensitization (Figure 1B, bottom panels, gray line). These results suggested that basophils can efficiently capture the IgG1-allergen complexes via FcγRII-III expressed on their surface, although we could not formally exclude the possibility that other cell types captured the complexes even more efficiently and then quickly internalized them within a minute after the allergen challenge.

In Vivo Depletion of Basophils Ameliorates IgG1-Mediated Anaphylaxis

We next examined the effect of in vivo depletion of a given cell lineage on the development of IgG1-mediated anaphylaxis to identify cells responsible for it. We recently established a CD200R3-specific mAb, Ba103 (Kojima et al., 2007; Obata et al., 2007), that selectively and reproducibly depletes 80%∼90% of the basophils from mouse peripheral blood and spleen after intravenous injection (Figure 2A). Treatment of mice with Ba103 1 day before the sensitization with anti-PenV IgG1 greatly suppressed allergen-induced anaphylaxis as judged by the extent of the rectal temperature drop (Figure 2A, and the titration of allergen dose is shown in Figure S1 available online). This was also true for mice that were sensitized with another IgG1 mAb specific to hapten 2,4,6-trinitrophenol (TNP) and then challenged with TNP-BSA (Figure S2). Of note, the same treatment with Ba103 had little or no effect on IgE-mediated, TNP-BSA-induced anaphylaxis (Figure 2A). The suppressive effect of Ba103 on IgG1-mediated anaphylaxis was also observed in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Figure 2A, bottom panels). These results indicated that basophils are dispensable for IgE-mediated anaphylaxis but play an essential role in IgG1-mediated anaphylaxis.

To examine the effect of macrophage depletion on IgG1-mediated anaphylaxis, we treated mice with clodronate-liposomes prior to the allergen challenge. The treatment did not have any substantial effect on the drop in body temperature in IgG1-mediated anaphylaxis, even though it efficiently eliminated F4/80$^+$ macrophages from the peritoneum and spleen (Figure 2B). Pretreatment of mice with anti-NK1.1 greatly reduced the number of CD49b$^+$IgE$^-$ NK cells but not of CD49b$^+$IgE$^+$ basophils and did not affect the IgG1-mediated anaphylaxis (Figure 2C). In accord with this, IgG1-mediated anaphylaxis could be induced even in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice that are deficient for NK, NKT, T, and B cells (data not shown). Furthermore, pretreatment with anti-Gr-1 had no marked effect on IgG1-mediated anaphylaxis, although it eliminated almost all the neutrophils (Gr-1^{hi}) and more than half of the eosinophils (Gr-1^{lo}Siglec-F$^+$) from spleen (Figure 2D), consistent with a previous observation in active systemic anaphylaxis (Strait et al., 2002). Thus, macrophages, NK cells, neutrophils, and perhaps eosinophils have little or no involvement in IgG1-mediated systemic anaphylaxis.

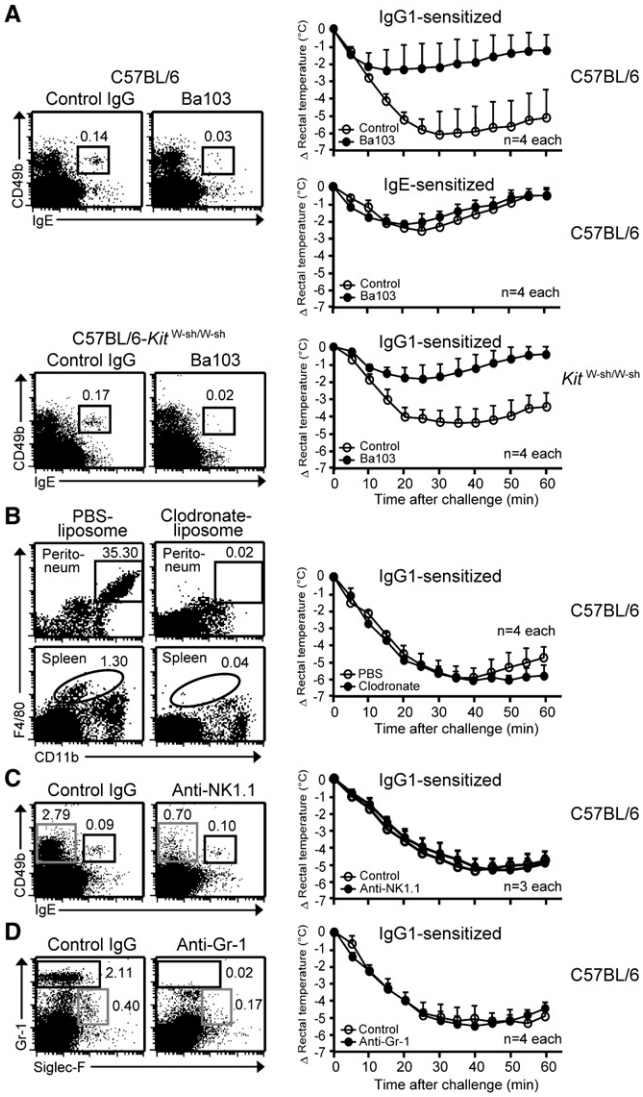

Figure 2. In Vivo Depletion of Basophils but Not Macrophages, NK Cells, or Neutrophils Ameliorates IgG1-Mediated Anaphylaxis

Wild-type (A–D) and mast-cell-deficient (*Kit*$^{W\text{-}sh/W\text{-}sh}$) C57BL/6 mice ([A], bottom) were treated with Ba103 (A), clodronate-liposomes (B), anti-NK1.1 (C), or anti-Gr-1 (D) for elimination of in vivo basophils, macrophages, NK cells, or neutrophils, respectively, prior to passive sensitization with anti-PenV IgG1 and challenge with PenV-BSA (A–D) or sensitization with anti-TNP IgE and challenge with TNP-BSA ([A], middle). Left panels show staining profiles of spleen cells (A–D) and peritoneal cells ([B], upper panels) from treated and control mice. Right panels show the time course of change in rectal temperature (mean ± SD, n = 3 or 4). Data are representative of three or four independent experiments.

Basophils Release PAF upon Stimulation with IgG1-Allergen Complexes

The treatment of wild-type and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice prior to allergen challenge with cyproheptadine, an antagonist of histamine and 5-HT, caused partial or no marked blockade of IgG1-mediated anaphylaxis, respectively (Figure 3A, upper panels), even though it greatly inhibited IgE-mediated anaphylaxis (Figure 3A, lower panel). In contrast, treatment with CV6209, a PAF antagonist, almost completely blocked IgG1-mediated anaphylaxis in both wild-type and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Figure 3A, upper panels), whereas it had much less effect on IgE-mediated anaphylaxis than did the cyproheptadine treatment (Figure 3A, lower panel). Thus, PAF rather than histamine is a major chemical mediator in IgG1-mediated anaphylaxis unlike in an IgE-mediated one.

PAF is one of the most potent and versatile mediators found in mammals and is produced by a variety of cell types including macrophages, neutrophils, eosinophils, basophils, platelets, and endothelial cells (Benveniste, 1974; Prescott et al., 2000). The finding that both basophils and PAF have major roles in IgG1-mediated anaphylaxis strongly suggested that basophils released PAF upon stimulation with IgG1-allergen complexes. Indeed, the basophil-containing CD49b$^+$ fraction of spleen cells, but not the basophil-deficient CD49b$^-$ fraction, released substantial amounts of PAF (∼60 pmol/2 × 10^6 cells/0.5 ml = 120 nM) when stimulated ex vivo with IgG1-allergen complexes (Figure 3B). The CD49b$^+$ fraction contained NK cells (>90%)

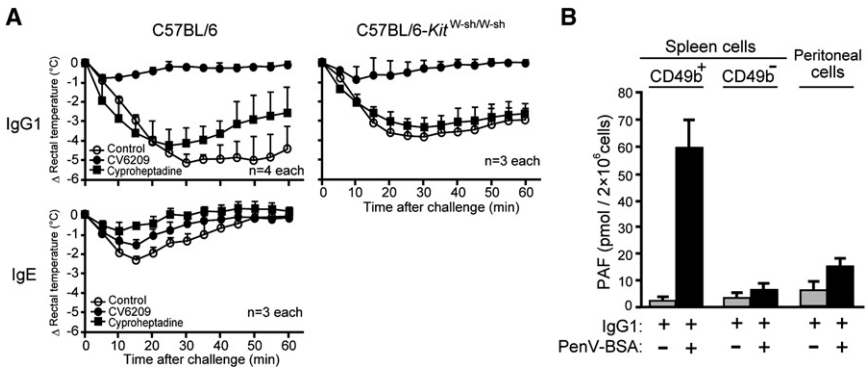

Figure 3. PAF Is a Major Mediator in IgG1-Mediated Anaphylaxis and Is Released from Basophils upon Stimulation with Immune Complexes

(A) PenV-specific, IgG1-mediated (upper panels), and TNP-specific, IgE-mediated (lower panel) anaphylaxis were induced in wild-type and $Kit^{W\text{-}sh/W\text{-}sh}$ C57BL/6 mice as for Figure 1. One half hour prior to allergen challenge, mice were given CV6209 (closed circles), cyproheptadine (closed squares), or control PBS (open circles). Time course of change in rectal temperature is shown (mean ± SD, n = 3 or 4), as a representative of three independent experiments.

(B) Basophil-containing $CD49b^+$ fraction and basophil-deficient $CD49b^-$ fraction of spleen cells and peritoneal cells were freshly isolated from C57BL/6 mice and incubated ex vivo with anti-PenV IgG1 alone or together with PenV-BSA at 37°C for 20 min. The quantity of PAF in culture supernatants was determined (mean ± SD, n = 3).

besides basophils (5%), but the in vivo depletion of NK cells showed no marked effect on IgG1-mediated anaphylaxis (Figure 2C), suggesting little or no contribution of NK cells to PAF production. Peritoneal cells, consisting of ~30% macrophages and 5% mast cells, released PAF ex vivo upon stimulation with immune complexes, but the amount of PAF was only 2- to 3-fold greater than the basal amount, in contrast to an ~30-fold increase in the $CD49b^+$ spleen cells (Figure 3B). Therefore, the PAF released in response to immune complexes is most probably from basophils.

We next examined the biological activity of PAF released from activated basophils, by using human umbilical vein endothelial cells (HUVECs) as an indicator (Bussolino et al., 1987). HUVECs contracted and lost reciprocal contact, and their stress fibers became less prominent, when they were incubated with supernatants from the IgG1-allergen-complex-stimulated $CD49b^+$ fraction but not the $CD49b^-$ fraction of spleen cells (Figure 4A). Such morphological changes were similar to those observed when the cells were stimulated with 10~100 nM PAF (Figure 4B), and they were inhibited by treatment with CV6209 (Figure 4A). No substantial morphological changes were observed when the HUVECs were incubated with supernatants from $CD49b^+$ or $CD49b^-$ spleen cells that were first cultured with IgE plus allergens (Figure 4C). Moreover, supernatants of peritoneal macrophages and mast cells incubated with the IgG1-allergen complexes induced few if any detectable changes in the HUVECs (Figure 4A). These results strongly suggest that upon stimulation with the IgG1-allergen complexes, basophils release PAF, which in turn stimulates endothelial cells to increase vascular permeability, thereby leading to systemic anaphylaxis. Indeed, the intravenous injection of 100 ng (200 pmol) PAF induced a drop in body temperature with the time course and magnitude comparable to that observed during IgG1-mediated anaphylaxis (Figure 4D).

Basophil Depletion Rescues Mast-Cell-Deficient Mice from Anaphylactic Death

We next examined the contribution of basophils to active systemic anaphylaxis in which wild-type or mast-cell-deficient ($Kit^{W\text{-}sh/W\text{-}sh}$ and $Kit^{W/W\text{-}v}$) mice were immunized with PenV-OVA and 14 days later challenged with intravenous injection of PenV-BSA (Figure 5 and summarized in Table 1). In contrast to IgE- or IgG1-mediated passive anaphylaxis, all the mice in any of the strains died from active anaphylaxis within 15 min. Treatment of mast-cell-deficient mice with Ba103 before the allergen challenge rescued them from death, even though some drop in body temperature was observed (Figure 5 and Table 1). This suppressive effect of Ba103 was also observed in another hapten TNP-specific, active systemic anaphylaxis, in which mast-cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice were immunized with TNP-OVA and then challenged with TNP-BSA (Figure S3). Thus, basophils play a pivotal role in active systemic anaphylaxis. Of note, the same treatment with Ba103 failed to rescue wild-type mice from death (Table 1). Therefore, in allergen-sensitized mice, both basophils and mast cells appear to contribute to the pathogenesis of anaphylaxis, most probably in an IgG- and IgE-dependent manner, respectively.

DISCUSSION

It has long been believed that mast cells and basophils are involved in classical IgE-mediated systemic anaphylaxis (Bochner and Lichtenstein, 1991; Galli, 2005; Kemp and Lockey, 2002). The present study clearly demonstrated that basophils are dispensable for IgE-mediated systemic anaphylaxis in contrast to mast cells and are instead responsible for IgG1-mediated systemic anaphylaxis. Thus, basophils and mast cells utilize distinct pathways leading to allergen-induced systemic anaphylaxis: Basophils mainly utilize the IgG-IgG receptor-mediated activating pathways, whereas mast cells predominantly utilize the IgE-FcεRI-mediated pathway. The difference between basophils and mast cells in the elicitation of systemic anaphylaxis was observed not only in Ig isotypes and Fc receptors they utilize but also in chemical mediators they release. Basophils release PAF rather than histamine upon stimulation with IgG1-allergen complexes, in contrast to mast cells stimulated with IgE and allergens.

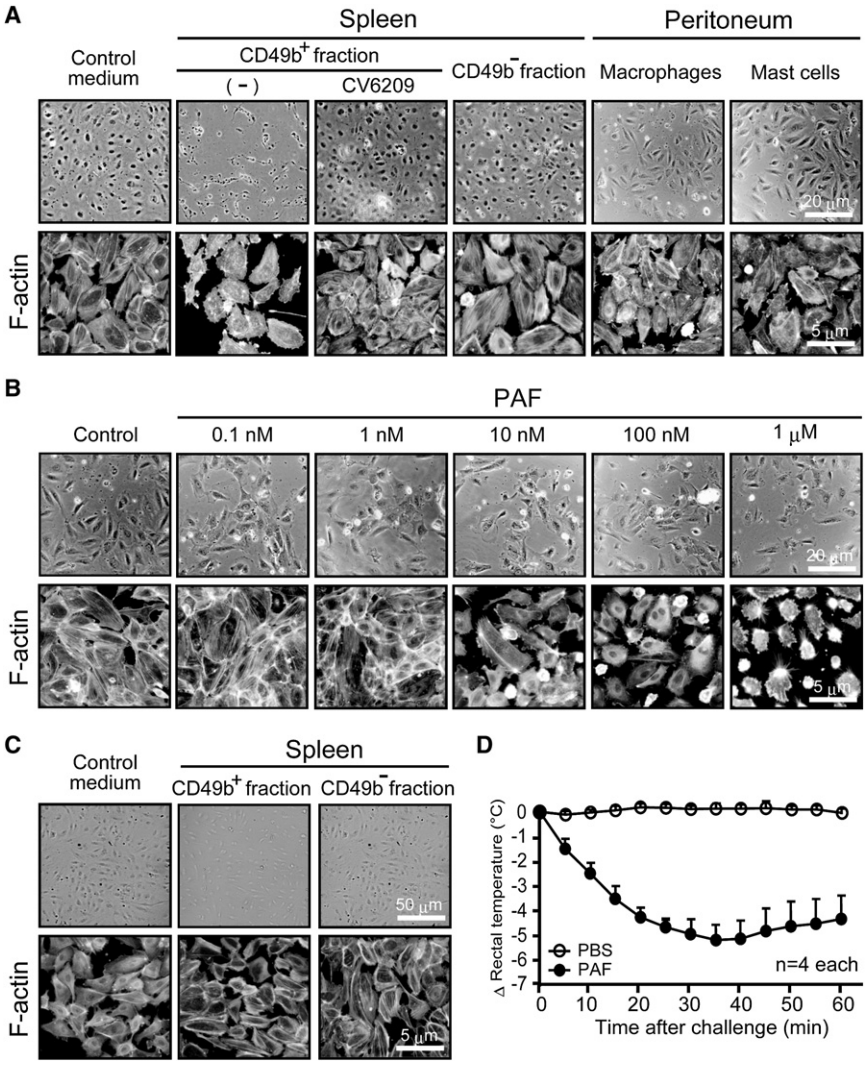

Figure 4. PAF Released by Activated Basophils Induces Morphological Changes in Endothelial Cells

(A) The CD49b$^+$ and CD49b$^-$ fractions of C57BL/6 spleen cells, macrophages, and mast cells enriched from peritoneal cells were incubated ex vivo with anti-PenV IgG1 and PenV-BSA as in Figure 3B. Their culture supernatants were collected and transferred to monolayer culture of HUVECs. Photographs show phase-contrast views (upper panels) and F-actin staining (lower panels) of HUVECs that were fixed and permeabilized after a half-hour incubation with the culture supernatants. In some experiments, HUVECs were treated with CV6209 prior to the addition of the culture supernatants.

(B) HUVECs in a monolayer culture were incubated with the indicated concentration of PAF at 37°C for 30 min.

(C) The CD49b$^+$ and CD49b$^-$ fractions of C57BL/6 spleen cells were incubated ex vivo with anti-TNP IgE and TNP-BSA at 37°C for 20 min. Their culture supernatants were subjected to the assay with HUVECs as described in (A).

(D) C57BL/6 mice were intravenously injected with 100 ng PAF (closed circles) in 0.2 ml PBS or PBS alone (open circles). Change in rectal temperature over time after allergen challenge is shown (mean ± SD, n = 4), as a representative of three independent experiments.

A previous study reported that mast cells were not essential for the development of IgE-dependent, active systemic anaphylaxis in a murine model of penicillin shock and suggested that basophils were most probably responsible for it (Choi et al., 1998). The authors showed that all mice examined, either sufficient or deficient for mast cells, died from severe anaphylaxis, as we observed in the present study. Administration of anti-IL-4 completely prevented the fatal reactions in those mice, in which amounts of PenV-specific IgE but not IgG1 in serum were drastically reduced. Taken together with their earlier study (Park et al., 1997), the authors concluded that PenV-induced systemic anaphylaxis totally depended on IgE but not IgG1 and therefore suggested that basophils instead of mast cells were responsible for IgE-mediated anaphylaxis. In sharp contrast, we demonstrated that the in vivo depletion of basophils had no significant impact on PenV-specific, IgE-mediated passive anaphylaxis, and others showed that mast cells were essential for IgE-mediated systemic anaphylaxis (Dombrowicz et al., 1997; Miyajima et al., 1997). Of note, two types of IgG1 were reported: one has anaphylactic activity, and its synthesis is IL-4 dependent, whereas the other lacks this activity, and its synthesis is stimulated by IL-12 and IFNγ (Faquim-Mauro et al., 1999). Furthermore, IL-4 has been shown to enhance the sensitivity to chemical mediators (Strait et al., 2003). These observations could well explain the anti-IL-4-induced suppression of active systemic anaphylaxis even in the presence of allergen-specific IgG1, and therefore, the IgE dependency of the anaphylaxis in this model need to be reexamined. However, we cannot formally exclude the possibility that the difference between polyclonal antibodies produced during sensitization for active anaphylaxis and monoclonal antibodies used for passive anaphylaxis might explain in part the discrepancy between our and previous studies.

Another study demonstrated an important role for macrophages in a unique model of active systemic anaphylaxis in that mice were immunized with goat anti-mouse IgD antiserum and then challenged with goat IgG (Strait et al., 2002). A single injection of the antiserum induced the secretion of IL-3 and IL-4 from T cells, and these cytokines in turn promoted the production of large amounts of IgE and IgG antibodies specific to goat IgG. Intravenous injection of gadolinium chloride (GdCl3) 1 day before the allergen challenge suppressed allergen-induced anaphylaxis that appeared to be mediated by FcγRIII and PAF. GdCl3 was used to deplete macrophages, even though no data were presented to confirm such effects. We found that the GdCl3 treatment with the same protocol as reported did not show any substantial change in numbers of F4/80$^+$ macrophages in the spleen and peritoneum of nonimmunized mice

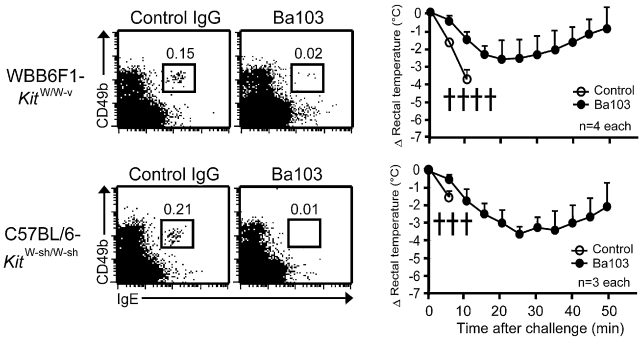

Figure 5. In Vivo Depletion of Basophils Rescues Mast-Cell-Deficient Mice from Death in Active Systemic Anaphylaxis

We immunized mast-cell-deficient ($Kit^{W/W-v}$ and $Kit^{W-sh/W-sh}$) mice with PenV-OVA and challenged 14 days later with PenV-BSA to induce anaphylaxis. One day before the allergen challenge, mice were treated with Ba103 (closed circles) or control IgG (open circles). Left panels show staining profiles of spleen cells from treated mice. Right panels show the change in rectal temperature over time after the allergen challenge (mean ± s.d., n = 3 or 4). The black cross indicates death.

Table 1. A Critical Role of Basophils in Active Systemic Anaphylaxis

Mice	Treatment	Mortality Rate
WBB6F1-$Kit^{W/W-v}$	control IgG	12/12
WBB6F1-$Kit^{W/W-v}$	Ba103	0/10
C57BL/6-$Kit^{W-sh/W-sh}$	control IgG	4/4
C57BL/6-$Kit^{W-sh/W-sh}$	Ba103	0/4
C57BL/6	control IgG	12/12
C57BL/6	Ba103	10/10

(data not shown), whereas the clodronate-liposome treatment efficiently eliminated them, in accord with a recent report (Nishioka et al., 2007). Importantly, neither clodronate-liposome nor GdCl3 treatment showed any detectable effect on IgG1-mediated passive systemic anaphylaxis. Furthermore, the basophil-containing fraction but not the macrophage-containing fraction of spleen cells released substantial amounts of PAF when it was incubated ex vivo with allergen-IgG1 immune complexes. Culture supernatants of the immune-complex-stimulated peritoneal macrophages showed little or no activity to change the morphology of HUVECs, in contrast to those of activated basophils. Therefore, we concluded that macrophages are not the major player in IgG1-mediated passive systemic anaphylaxis. On one hand, our results do not exclude the possible contribution of macrophages to active systemic anaphylaxis. On the other hand, our observation, in that in vivo depletion of basophils rescued mast-cell-deficient mice from death in the PenV-induced, active systemic anaphylaxis, clearly indicates that basophils play a critical role in active systemic anaphylaxis. FcεRIα-deficient mice reportedly manifested greater severity of IgG1-mediated anaphylaxis compared to wild-type mice (Dombrowicz et al., 1997; Miyajima et al., 1997). This phenomenon cannot be easily understood if IgG1-mediated anaphylaxis is caused by macrophages that are negative for FcεRI. Instead, it could be simply explained, in case of basophil-mediated anaphylaxis, by enhanced expression of basophil FcγRIII, which shares β and γ chains with FcεRI, in the absence of FcεRIα chains.

What makes it possible for basophils, the least common leukocytes, to induce systemic anaphylaxis upon exposure to allergens? First, basophils show much higher activity of capturing allergen-IgG1 complexes than other hematopoietic cells. This may be attributed to higher expression of the low-affinity IgG receptors on basophils (Mack et al., 2005). Second, basophils release PAF upon stimulation with allergen-IgG1 complexes. PAF increases vascular permeability with 1,000- to 10,000-fold more potency than histamine (Humphrey et al., 1982). We found that the intravenous injection of 100 ng (200 pmol) PAF induced a drop in body temperature with the time course and magnitude comparable to that observed during IgG1-mediated anaphylaxis. We estimated, on the basis of our in vitro experiment, that 200 pmol of PAF can be released from 3.3×10^5 basophils, which is close to the total number of basophils in peripheral blood, spleen, and bone marrow. Thus, basophils can elicit systemic anaphylaxis through the release of the potent vasoamine PAF upon stimulation with immune complexes, even though they account for less than 1% of leukocytes in the body. Basophils may release histamine upon stimulation through IgE or IgG receptors, but the total amounts of histamine released from basophils are probably too little to induce systemic anaphylaxis, compared to those released from large numbers of mast cells.

IgG1-mediated anaphylaxis can be induced locally in mice, by means of intradermal injection of IgG1 mAbs and then intravenous injection of corresponding allergens (Hirayama et al., 1982). This passive cutaneous anaphylaxis (PCA) is FcγRIII dependent (Hazenbos et al., 1996) and cannot be elicited in mast-cell-deficient mice (Arimura et al., 1990). We also confirmed the mast cell dependency of IgG1-mediated PCA in our system by using the PenV-specific IgG1 and PenV-BSA (data not shown). Thus, mast cells are mainly activated in IgG1-mediated PCA upon exposure to IgG1-allergen complexes. Indeed, peritoneal mast cells were shown to degranulate when incubated ex vivo with IgG-allergen complexes (Hazenbos et al., 1996). In contrast, little or no morphological evidence of degranulation of mast cells was detected in the ear skin, peribronchial tissues, or forestomach during IgG1-mediated passive systemic anaphylaxis unlike in IgE-mediated anaphylaxis (Miyajima et al., 1997). The different modes of action between the local and systemic anaphylaxes could be attributed to the difference in the route of antibody delivery and the anatomical localization of mast cells and basophils. In PCA, IgG1 antibodies are directly delivered into the skin tissue where mast cells but not basophils reside. Immune complexes are formed locally in the skin lesions after the allergen challenge and stimulate tissue-resident mast cells. In systemic anaphylaxis, the antibodies are delivered into the blood stream where basophils but not mast cells are circulating. Immune complexes are formed within blood vessels immediately after intravenous administration of allergens, quickly trapped by basophils and other cells in the circulation and spleen, and may not be delivered sufficiently into peripheral tissues where mast cells reside. Interestingly, the phenotypic difference was observed even between IgG1- and IgE-mediated systemic anaphylaxis, most probably reflecting the different anatomical localization of basophils and mast cells. Increased

vascular permeability during systemic anaphylaxis, as detected by dye extravasation, was more prominent in the trunk rather than the periphery such as ears and foot pads in IgG1-mediated anaphylaxis compared to IgE-mediated one (data not shown).

We previously showed that basophils play a critical role in the development of IgE-mediated chronic allergic inflammation in the skin (Mukai et al., 2005; Obata et al., 2007). Intradermal injection of allergens induced three waves of ear swelling, with a peak within 30 min, several hr, and 4 days, respectively, after the allergen challenge in mice passively sensitized with allergen-specific IgE or those with the IgE transgene. Although all the allergic responses are mediated by IgE and FcεRI, the first and second ones are mast cell dependent, whereas the third one with massive infiltration of eosinophils is basophil dependent. In the present study, we demonstrated that basophils play a pivotal role in IgG1-mediated but not IgE-mediated systemic anaphylaxis. Thus, basophils critically contribute to both the IgE-mediated chronic local allergic response and the IgG1-mediated systemic allergic response. On the basis of these findings, the following model can be proposed: When allergens (or pathogens in a physiological process) enter across the skin barrier in allergen-sensitized animals, mast cells in the skin first elicit immediate-type allergic responses against them. If these responses are not sufficient for their elimination, basophils infiltrate into the skin tissue and elicit a chronic inflammation with massive infiltration of other inflammatory cells such as eosinophils and neutrophils. When allergens (or pathogens) invade into the blood stream, mast cells and basophils induce massive and systemic responses against them in an IgE- and IgG-dependent manner, respectively. Larger amounts of allergens and antibodies are necessary to induce the IgG-mediated anaphylaxis than the IgE-mediated one (Strait et al., 2006).

Is the alternative pathway of systemic anaphylaxis mediated by basophils, IgG, and PAF operative in humans, beside the classical pathway? No definitive evidence for this has been provided. However, it is notable that several case reports mention human anaphylaxis, particularly in clinical settings such as medication, that occurred in the apparent absence of detectable allergen-specific IgE in serum or in the absence of increase in serum tryptase levels; this increased serum tryptase level is a reliable indicator of mast cell degranulation (Cheifetz et al., 2003; Dybendal et al., 2003). Allergen-specific IgG antibodies instead of IgE were detected in individuals who manifested systemic anaphylaxis against medicines such as protamine, dextran, and recombinant IgG (Adourian et al., 1993; Cheifetz et al., 2003; Kraft et al., 1982; Weiss et al., 1989). Human basophils were reported to release PAF upon activation with various stimuli, although IgG immune complexes were not analyzed for their ability to induce PAF release from human basophils (Lie et al., 2003). Therefore, it would be worthwhile to assess the possible involvement of basophils and PAF in human cases of anaphylaxis, particularly those with high levels of serum IgG but not IgE specific to a relevant allergen.

In conclusion, we have defined a previously unknown in vivo function of basophils, distinct from that played by mast cells, in the development of allergen-induced systemic anaphylaxis. Basophils but not macrophages are the prime player in IgG-mediated systemic anaphylaxis. This finding contrasts two major distinct pathways leading to systemic anaphylaxis: one is mediated by basophils, IgG, IgG receptor, and PAF and the other, classical pathway is mediated by mast cells, IgE, FcεRI, and histamine.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice and mast-cell-deficient WBB6F1-*Kit*$^{W/W\text{-}v}$ mice (Kitamura et al., 1978) were purchased from CLEA Japan and SLC, respectively. Mast-cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Grimbaldeston et al., 2005) were maintained in our animal facilities under specific pathogen-free conditions. All the experiments in this study were performed according to the Guidelines for Animal Use and Experimentation, as set out by Tokyo Medical and Dental University.

Reagents

PenV-conjugated OVA and BSA were prepared with $Na^+BO_3^{2-}$ buffer, as described (Park et al., 1997). PenV-specific IgG1-mAb-secreting hybridomas were established from splenocytes isolated from WBB6F1-*Kit*$^{W/W\text{-}v}$ mice that were immunized intraperitoneally with PenV-OVA together with alum and *B. pertussis* toxin and 2 weeks later boosted with intravenous injection of PenV-BSA. A basophil-depleting mAb Ba103 was established as described (Obata et al., 2007). FITC-conjugated mAbs specific for c-kit (2B8), CD11c (HL3), and CD3ε (145-2C11), PE-conjugated mAbs specific for Siglec F (E50-2440), CD19 (1D3), and CD11b (M1/70), biotinylated mAbs specific for CD49b (DX5), c-kit (2B8), and IgE (R35/72), and allophycocyanin (APC)-conjugated streptavidin were purchased from BD PharMingen. FITC-conjugated mAbs specific for CD49b (DX5), F4/80 (BM8), and Gr-1 (RB6-8C5), PE-conjugated anti-FcεRIα (MAR-1), and biotinylated anti-F4/80 (BM8) were purchased from eBioscience. Anti-FcγRII-III (2.4G2), anti-Gr-1 (RB6-8C5), and anti-NK1.1 (PK136) were prepared from hybridoma culture supernatants in our laboratories. CV6209, cyproheptadine, PAF, and purified rat IgG were purchased from Sigma-Aldrich. Liposomes containing dichloromethylene bisphosphonate (clodronate-liposomes) were prepared as described (Endo et al., 1995; Van Rooijen and Sanders, 1994).

Induction of Systemic Anaphylaxis

To induce passive anaphylaxis, mice (C57BL/6, C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$, and WBB6F1-*Kit*$^{W/W\text{-}v}$) were sensitized with an intravenous injection of 500 μg PenV-specific IgG1 mAb, TNP-specific IgG1 mAb (ATCC TIB-191), or 50 μg TNP-specific IgE mAb (IGELb4) (Rudolph et al., 1981) in 0.2 ml PBS. Three hours after IgG injection or 24 hr after IgE injection, mice were intravenously challenged with 1 mg PenV-BSA or 50 μg TNP_8-BSA in 0.2 ml PBS. In some experiments, mice were pretreated with i.p. injection of 100 μg CV6209 or 50 μg cyproheptadine. To induce active anaphylaxis, mice were primed by intraperitoneal injection of 0.5 mg PenV-OVA (or TNP-OVA) and 1 mg of alum together with subcutaneous injection of 300 ng of *B. pertussis* toxin. Fourteen days later, mice were challenged with intravenous injection of 100 μg PenV-BSA (or TNP-BSA) in 0.2 ml PBS. Their rectal temperature was measured with a digital thermometer (Shibaura Electronics).

In Vivo Depletion of Particular Cell Lineages

For basophil depletion, mice were given an intravenous injection of 100 μg Ba103 1 day before the antigen challenge (Obata et al., 2007). For neutrophil and eosinophil depletion, mice were given an intraperitoneal injection of 2 mg anti-Gr-1 2 days before the antigen challenge. For macrophage depletion, mice were given an intraperitoneal injection of 0.5 ml clodronate-liposomes 2 days before the antigen challenge and then given an intravenous injection of 0.2 ml clodronate-liposomes the next day (Endo et al., 1995; Van Rooijen and Sanders, 1994). For NK cell depletion, mice were given two intraperitoneal injections of 250 μg of anti-NK1.1: one 48 hr and one 8 hr before the antigen challenge.

Flow Cytometry

We preincubated cells with anti-FcγRII-III and normal rat serum on ice for 15 min prior to incubation with the indicated combination of Abs, to prevent the nonspecific binding of irrelevant Abs unless otherwise stated. Stained cells were analyzed with a FACSCalibur (BD Biosciences). To detect IgE receptors,

we incubated cells with IgE at 4°C for 30 min to saturate the IgE receptors and then stained them with anti-IgE. To detect IgG1-antigen complexes bound to the cell surface in IgG1-sensitized mice, we prepared single suspensions of cells from the indicated organs immediately after the intravenous injection of biotinylated antigens and stained them with APC-streptavidin and the indicated mAbs.

Fractionation of Spleen and Peritoneal Cells

For separation of the $CD49b^+$ and $CD49b^-$ fractions, spleen cells were reacted with biotinylated anti-CD49b at 4°C for 30 min, subsequently incubated with streptavidin-conjugated BD IMag magnetic particles (BD PharMingen) for 30 min, and subjected to cell separation with the BD IMag Cell Separation System. Macrophages and mast cells were isolated from peritoneal cells with the BD IMag System with biotinylated anti-F4/80 and anti-c-kit, respectively.

Measurement of PAF and Its Bioactivity

Cells (2×10^6 cells/0.5 ml) were incubated with 0.2 mg/ml of IgG1 alone or IgG1 plus 0.4 mg/ml of allergen at 37°C for 20 min in buffer containing 4.2 mM HEPES/NaOH (pH 7.4), 2.6 mM KCl, 137 mM NaCl, 5.6 mM glucose, 0.25% BSA, 1.3 mM $CaCl_2$, and 1 mM $MgCl_2$. Culture supernatants were collected and mixed with 1.875 ml of chloroform:methanol (1:2, v/v), and PAF-containing lipids were extracted as described (Bligh and Dyer, 1959). The quantity of PAF was determined by competitive binding assay (Shindou et al., 2000). To examine PAF bioactivity, we incubated cells with IgG1 plus antigen as above, but in PBS. Culture supernatants were collected and transferred to the culture wells containing a semiconfluent layer of HUVECs that were untreated or pretreated with 1 μM CV-6209 at 37°C for 5 min prior to the addition of the culture supernatants. After a half-hour culture at 37°C, the HUVECs were fixed with 3% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, and stained for F-actin with Alexa-594-labeled phalloidin. The specimens were examined by microscopy for that morphological changes in the cells could be identified.

SUPPLEMENTAL DATA

Three figures are available at http://www.immunity.com/cgi/content/full/28/4/581/DC1/.

ACKNOWLEDGMENTS

We thank S.J. Galli, S. Nakae, and H. Suto for providing C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. This work is supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Received: November 15, 2007
Revised: January 11, 2008
Accepted: February 7, 2008
Published online: March 13, 2008

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