patients with psoriasis, and indicated that estimating the impact of psoriasis on social life seemed to be difficult. Indeed, as the number of patients with psoriasis was low in this study, which may indicate that our data are not representative of the general population of patients with psoriasis.

Concerning WPAI-AS scores in patients with atopic dermatitis, the total loss of daily activities was relatively higher than for patients with other skin diseases (Table 6, Fig. 2). It has been said that the intensity of itch might be increased in a relaxed environment, such as coming home or at nighttime.[20] In support of this, daily activity in patients with atopic dermatitis or pruritus was severely impaired compared with the impairment in overall work productivity (Table 6). Thus, daily activity may be highly susceptible to impairment in patients with atopic dermatitis and pruritus.

The differences between patients taking non-sedative versus sedative antihistamines was also addressed. As previously reported, sedative antihistamines failed to reduce work productivity impairment despite decreasing itch VAS values and Skindex-16 measures.[12] Impaired performance as an adverse effect of sedative antihistamines may be a major factor in these divergent results. In fact, in patients treated with sedative antihistamines, the improvement ratio for itch VAS scores did not significantly correlate with either the Skindex-16 QOL measure, the reduced impairment in overall work productivity, or the reduced impairment in daily activity (Table 2). Additionally, the extent of impairment in overall work productivity can be predicted by the Skindex-16 measures (Table 1). Nevertheless, clinicians should keep in mind that they could overestimate the effect of sedative antihistamines to improve on work productivity by relying solely on patient itch-intensity and QOL values. For these reasons, non-sedative antihistamines have substantial value in the treatment of patients with pruritic skin diseases.

However, the criteria for selecting antihistamines differ from disease to disease and vary worldwide. It is well known that non-sedative antihistamines, but not sedative antihistamines, are recommended as first-line agents for urticaria treatment.[21-25] In contrast, many previous published reviews, guidelines, and position papers on the care of atopic dermatitis state that the antihistamines are no more than a supportive management for pruritus, and their sedative properties offer an advantage for reducing the magnitude of itch in atopic dermatitis.[14,15,26,27] Thus, there is a tendency worldwide to recommend sedative antihistamines for the treatment of atopic dermatitis with intense itch or sleep disturbance.[14,15,26] Our data challenge this trend, since non-sedative antihistamines reduced the impairments in daily activity in patients with atopic dermatitis, while sedative antihistamines were ineffective (Fig. 1A, 3). Accordingly, the criteria for selecting antihistamines in certain skin diseases should be reconsidered.

Limitations of this study include the number of patients in each group and the potential influences of the adverse global economic conditions. Nonetheless, this report may highlight a new goal in the treatment of pruritic skin diseases and provide a rationale for shifting the choice of treatment options to non-sedative antihistamines.

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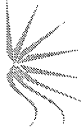

Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks

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Ticks are ectoparasitic arthropods that can transmit a variety of microorganisms to humans and animals during blood feeding, causing serious infectious disorders, including Lyme disease. Acaricides are pharmacologic agents that kill ticks. The emergence of acaricide-resistant ticks calls for alternative control strategies for ticks and tick-borne diseases. Many animals develop resistance to ticks after repeated infestations, but the nature of this acquired anti-tick immunity remains poorly understood. Here we investigated the cellular and molecular mechanisms underlying acquired resistance to *Haemaphysalis longicornis* ticks in mice and found that antibodies were required, as was IgFc receptor expression on basophils but not on mast cells. The infiltration of basophils at tick-feeding sites occurred during the second, but not the first, tick infestation. To assess the requirement for basophil infiltration to acquired tick resistance, mice expressing the human diphtheria toxin receptor under the control of the mast cell protease 8 (Mcpt8) promoter were generated. Diphtheria toxin administration to these mice selectively ablated basophils. Diphtheria toxin–mediated basophil depletion before the second tick infestation resulted in loss of acquired tick resistance. These data provide the first clear evidence, to our knowledge, that basophils play an essential and nonredundant role in antibody-mediated acquired immunity against ticks, which may suggest new strategies for controlling tick-borne diseases.

Introduction

Ticks, particularly members of the Ixodid family, are blood-feeding ectoparasites of vertebrates and are also important vectors of pathogens, including virus, bacteria, protozoa, and helminths, which can cause several serious infectious diseases in humans and animals (1, 2). Ticks suck a blood meal from hosts over a period of several days and increase their body weight up to 120-fold. Once they replete with blood meal (engorgement), ticks drop off from the host. While feeding, various substances of ticks are injected into the host to facilitate successful blood feeding, including a cement to anchor the mouth parts to the skin of the host, enzymes, vasodilators, and antiinflammatory, antihemostatic, and immunosuppressive substances (3–5). Pathogens can be transmitted from infected ticks to the host during salivation. Tick-transmitted diseases include viral encephalitis, Lyme disease caused by spirochetes of the *Borrelia burgdorferi*, Rocky Mountain spotted fever caused by *Rickettsia rickettsii*, monocytic human ehrlichiosis caused by *Ehrlichia chaffeensis*, and babesiosis caused by protozoa *Babesia* (1). The public health importance of ticks is not diminishing, and the emergence of acaricide-resistant ticks has shifted the anti-tick strategy toward the immunological control of ticks (6–8).

Several species of animals, including guinea pigs, rabbits, bovines, and mice, have been shown to develop resistance to tick feeding after a single or multiple infestation with ticks (6), which is characterized by reduced numbers and weights of engorged ticks or tick death in subsequent infestations. Acquired host resistance to tick infestation was first documented by Trager in 1938 (9) and extensively studied by using guinea pigs in the 1970s and 1980s. It can be transferred to naive animals with cells or sera isolated from infested animals, and therefore the resistance is considered to be a type of immunological reaction (9–11). Immune resistance to the ticks is an important element of protection from infestation with these ectoparasites and also contributes significantly to the reduction in pathogen transmission from infected ticks (12–15). This is the rationale for the development of tick antigen–based vaccines to prevent tick-borne diseases. However, the nature of naturally acquired protective immunity against ticks remains ill defined. The tick-feeding sites in guinea pigs that had already experienced tick infestation were characterized by large accumulation of basophils and eosinophils, and basophils compose up to 70% of the infiltrating cells (10, 11, 16). One study reported that the treatment of such guinea pigs with basophil-depleting antiserum abolished tick resistance, suggesting the importance of basophils for acquired tick resistance (17). However, it remains elusive whether this important finding can be generalized to other animal species including humans. In particular, mice were erroneously believed for a long time to lack basophils, due to the difficulty in identifying them (18–20), and no role in acquired tick resistance in mice has been shown for basophils. Instead, one research group suggests that mast cells in place of basophils are important for acquired immunity to ticks in mice, based on the observation that

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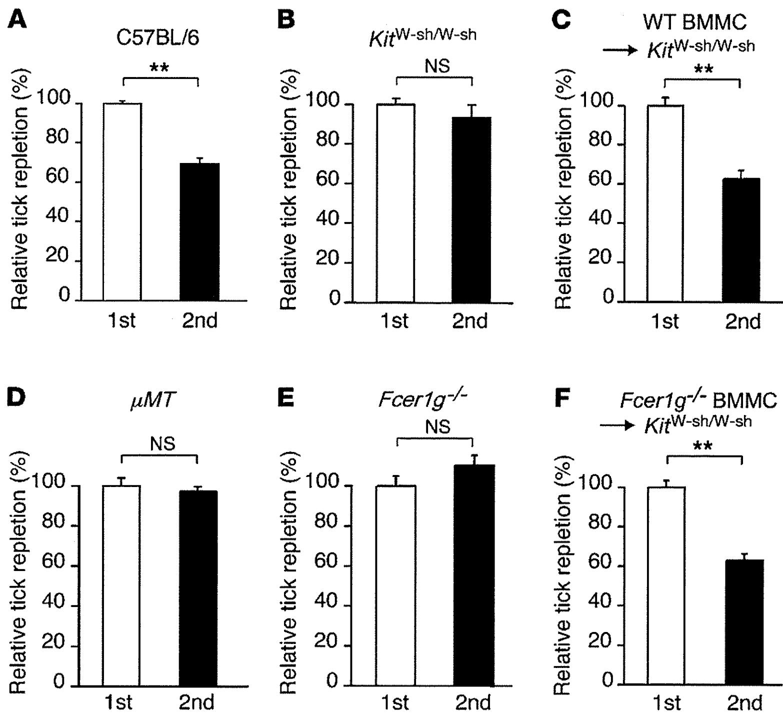


Figure 1
IgFc receptors on mast cells are dispensable for antibody-mediated, protective immunity against ticks. (**A**) C57BL/6 and (**B**) C57BL/6-*Kit*$^{W-sh/W-sh}$ mice were infested with larval *H. longicornis* ticks once or twice at an interval of 14 days. The relative tick repletion in each experimental group is shown, in that the value in the first infestation was defined as 100%. Data are shown as mean ± SEM, $n = 5$ (**A**); $n = 4$ (**B**). (**C**) BMMCs derived from wild-type mice were adoptively transferred to *Kit*$^{W-sh/W-sh}$ mice, and the recipient mice were infested and analyzed as in **A**. Data are shown as mean ± SEM, $n = 3$ each. (**D**) μ*MT* and (**E**) *Fcer1g*$^{-/-}$ C57BL/6 mice were infested with ticks as in **A**, and the relative tick repletion in each group is shown. Data are shown as mean ± SEM, $n = 5$ (**D**); $n = 4$ (**E**). (**F**) BMMCs derived from *Fcer1g*$^{-/-}$ mice were adoptively transferred to *Kit*$^{W-sh/W-sh}$ mice, and the recipient mice were infested and analyzed as in **A**. Data are shown as mean ± SEM, $n = 3$ each. Data shown in **A**–**F** are representative of at least 3 repeated experiments. $^{**}P < 0.01$.

mast cell–deficient mice failed to acquire tick resistance and that basophils were not detected in tick-feeding sites (21–23). On the other hand, another group showed that the same strain of mast cell–deficient mice developed resistance to another tick species (24, 25). Thus, the mechanism underlying acquired immunity to ticks, including the differential roles played by basophils and mast cells, and the influence of genetic background of both ticks and hosts remain to be clarified.

Studies on basophils, particularly their functions in vivo, have long been hampered by the lack of suitable animal models, including mice that are deficient only in basophils. We have recently established a CD200R3-specific mAb Ba103 (26, 27) that can deplete most basophils when administered into mice. MAR-1, a mAb specific to FcεRIα, also shows a similar basophil-depleting ability (28, 29). The use of these mAbs successfully identified previously unrecognized roles for basophils in allergic reactions and the regulation of acquired immunity (30–33), including IgE-mediated chronic cutaneous allergic inflammation (26, 34), IgG-mediated systemic anaphylaxis (35), and promotion of humoral memory responses (29) and Th2 cell differentiation (28, 36–38). Thus, the mAbs are very useful for analyses of in vivo functions of basophils. However, they might not be perfect in some experimental settings, especially those in which mast cells substantially contribute to a reaction of interest, since the mAbs react to both mast cells and basophils (26, 27, 29). Possible side effects of the treatment with the mAbs have to be considered, including activation, unresponsiveness after activation, or partial depletion of mast cells (29). This may make the interpretation of obtained data complicated, whether the phenotype of antibody-treated mice is due to either the basophil depletion or some deleterious effects on mast cells or both.

In the present study, to overcome the limitation of antibody-mediated basophil depletion, we utilized the technology of diphtheria toxin–mediated (DT-mediated) cell ablation (39) and generated engineered mice that allow selective ablation of basophils by means of basophil-specific expression of receptors for DT. Taking advantage of this mouse model, we examined the possible involvement of basophils in acquired tick resistance to *Haemaphysalis longicornis* in mice, for which mast cells are reportedly essential (21–23). *H. longicornis* is an important vector for human and animal pathogens, including those causing babesiosis, Q fever, and Russian encephalitis (40). We found that basophils but not mast cells directly contribute to the antibody/IgFc receptor–mediated manifestation of tick resistance, even though both types of cells are required for tick resistance.

Results

IgFc receptors on mast cells are dispensable for antibody-mediated, protective immunity against ticks. Acquired tick resistance is characterized by reduced numbers and weights of engorged ticks in the second infestation compared with the first. Therefore, to evaluate tick resistance, we used "relative tick repletion" (defined in Methods) as a quantitative measure of tick feeding that takes into account both the number and body weight of engorged ticks. The stronger the tick resistance, the lower the relative tick repletion in the second infestation. When C57BL/6 mice were infested with larval *H. longicornis* ticks twice at an interval of 14 days, the tick repletion in the second infestation typically decreased to approximately 60%–70% of that in the first one (Figure 1A and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI42680DS1). In contrast, mast cell–deficient C57BL/6-*Kit*$^{W-sh/W-sh}$ mice showed approximately 100% tick repletion in the second infestation as in the first one (Figure 1B). Adoptive transfer of bone marrow–derived mast cells (BMMCs) from wild-type C57BL/6 mice into the skin of C57BL/6-*Kit*$^{W-sh/W-sh}$ mice resulted in approximately 60% tick repletion in the second infestation (Figure 1C), as observed in wild-type C57BL/6 mice (Figure 1A). These results demonstrated that C57BL/6 mice acquire the resistance to tick feeding once they have experienced the tick infestation and

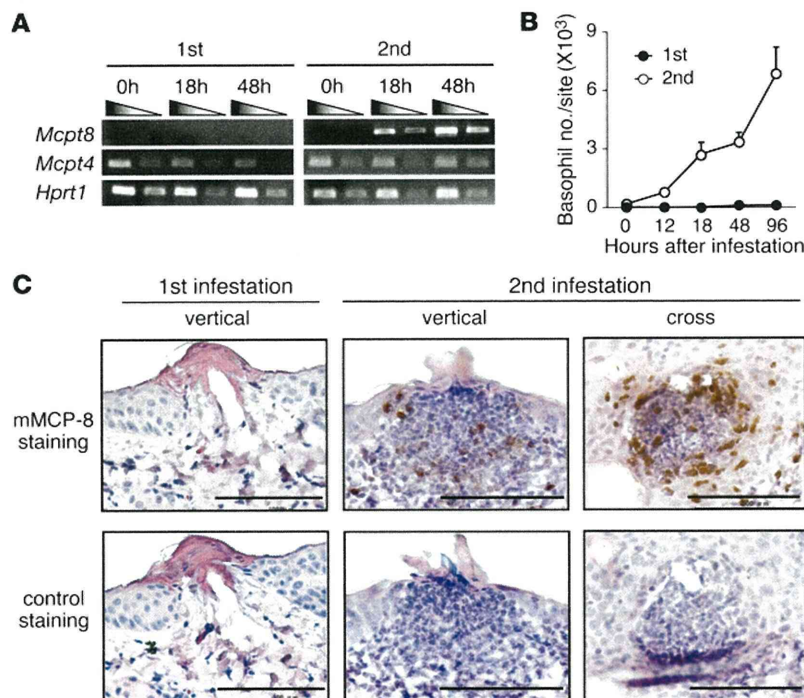


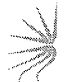
Figure 2
Recruitment of basophils to tick-feeding sites during the second, but rarely the first, infestation. (**A**) C57BL/6 mice were infested with ticks as in Figure 1. Tick-feeding–site skin lesions were isolated at the indicated time points during the first and second infestations and subjected to semiquantitative RT-PCR analysis (PCR templates were 5-fold serially diluted) for the expression of basophil-specific *Mcpt8*, mast cell–specific *Mcpt4*, and housekeeping *Hprt1* transcripts. (**B**) Skin lesions caused by tick feeding were isolated at the indicated time points during the first (black circles) and second (white circles) infestations and subjected to flow cytometric analysis. Changes in the number of basophils during the infestations are shown. Data are shown as mean ± SEM, $n = 3$ each. (**C**) Tick-feeding–site skin lesions were isolated 18 hours after the initiation of the first or second infestation and subjected to immunohistochemical examination using anti–mMCP-8 or an isotype-matched control antibody. mMCP-8–expressing basophils were stained in brown. Scale bars: 100 μm. Data shown in **A**–**C** are representative of at least 3 repeated experiments.

that mast cells play an important role in the acquisition of tick resistance, consistent with previous reports using other strains of mast cell–sufficient and -deficient mice, *WBB6F1*$^{+/+}$ and WBB6F1-*Kit*$^{W/W-v}$, respectively (21, 22).

Previous studies showed that transfer of serum from tick-infested but not uninfested mice conferred tick resistance on naive mice and that heat inactivation of the serum at 56°C for 2 hours abolished this activity, suggesting that antibodies made against ticks, particularly of the IgE class, are involved in the manifestation of tick resistance (23). In accordance with this, neither antibody-deficient *μMT* mice nor *Fcer1g*$^{-/-}$ mice, which lack the expression of IgE receptor (FcεRI) and stimulatory IgG receptors, displayed acquired tick resistance in the second infestation (Figure 1, D and E). Unexpectedly, however, mast cell–deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with BMMCs derived from *Fcer1g*$^{-/-}$ mice did develop tick resistance (Figure 1F), as did mice reconstituted with wild-type BMMCs (Figure 1C). This finding demonstrated that Ig receptors on mast cells are dispensable for the antibody-mediated acquired tick resistance and suggested a previously unrecognized mechanism for acquisition of tick resistance, distinct from the proposed scenario that tick antigens stimulate skin-resident mast cells through their binding to IgE/FcεRI on the cell surface, leading to an immediate-type hypersensitivity reaction against ticks (23). Given that the surface expression of FcεRI is restricted to mast cells and basophils in mice, basophils might be involved in antibody-mediated acquired tick resistance.

Recruitment of basophils to tick-feeding sites during the second, but rarely the first, infestation. Giemsa staining of skin tissue sections failed to detect basophils infiltrating the tick-feeding sites (data not shown), consistent with previous reports (21, 25, 41). Nevertheless, we readily detected *Mcpt8* transcripts, which encode the basophil-specific, granzyme B–like protease mMCP-8 (42–44) in tick-feeding–induced skin lesions 18 and 48 hours after the initiation of a second infestation; almost none were detected during the first infestation (Figure 2A). Flow-cytometric analysis of cells isolated from the tick-feeding sites revealed the presence of infiltrating basophils as early as 12 hours after the initiation of the second infestation, but such basophils were rare during the first one (Figure 2B). The number of basophils at the skin lesions increased approximately 40 times from the beginning to the 96-hour time point of the second infestation (Figure 2B). Finally, an immunohistochemical examination using a recently established anti-mMCP-8 mAb (TUG8) (44) demonstrated that mMCP-8–expressing basophils were recruited to and clustered around the tick mouth parts during the second, but rarely the first, infestation (Figure 2C). Other types of cells, including eosinophils and neutrophils, also increased their number in the tick-feeding sites during the second infestation compared with the first one (Supplemental Figure 1).

Treatment of mice with basophil-depleting antibodies abolishes acquired tick resistance. We next examined the possible role for basophils infiltrating the tick-feeding sites. The treatment of mice with the basophil-depleting anti-CD200R3 mAb Ba103 (26, 27) before the second infestation (Supplemental Figure 2) resulted in a drastically reduced number of infiltrating basophils (Figure 3A) and approximately 100% tick repletion in the second infestation (Figure 3B), while approximately 60% tick repletion (the same as in untreated mice) was seen when a control antibody was used (Figure 3B). Similar results were obtained when another basophil-depleting mAb, MAR-1, specific to FcεRIα (28, 29), was used instead of Ba103 (Figure 3C and Supplemental Figure 2). When used to treat mice prior to the first infestation, neither Ba103 nor MAR-1 had a significant effect on the tick repletion in the first infestation (Figure 3D and data not shown). These results supported the idea that basophils critically contribute to protection against ticks during the second infestation. However, the interpretation of these data was not so simple as compared with that in mast cell–independent reactions such as IgE-mediated chronic cutaneous allergic inflammation (26,



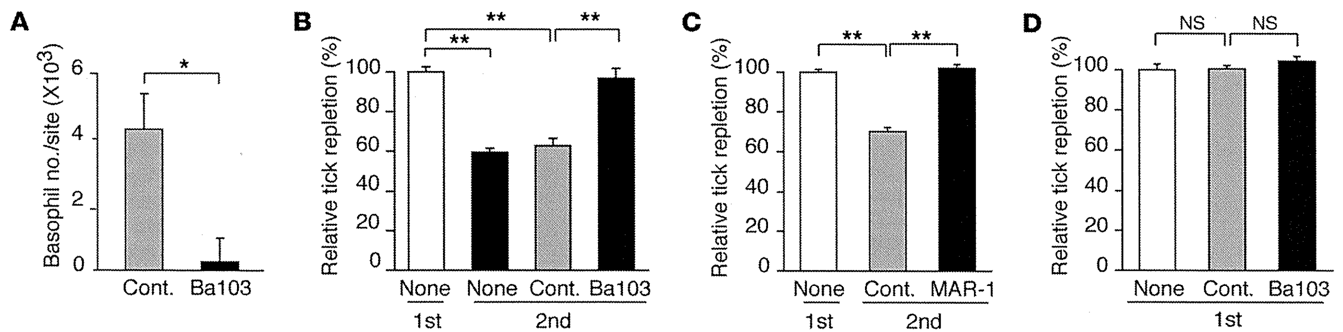


Figure 3
Treatment of mice with basophil-depleting antibodies abolishes acquired tick resistance. (**A**–**C**) C57BL/6 mice were infested once or twice and treated with Ba103 (**A** and **B**), MAR-1 (**C**), or an isotype-matched control (Cont) antibody or left untreated (none) before the second infestation. **A** shows the number of basophils infiltrating the tick-feeding site 2 days after the initiation of the second infestation. Data are shown as mean ± SEM, n = 3 each. The relative tick repletion in each experimental group is shown in **B** and **C**, in that the value in the first infestation was defined as 100%. Data are shown as mean ± SEM, n = 5 (**B**); n = 4 (**C**). (**D**) C57BL/6 mice were infested once with ticks after the treatment of Ba103 or control antibody or without any treatment (none). Data are shown as mean ± SEM, n = 4 each. Data shown in **A**–**D** are representative of at least 3 repeated experiments. **P < 0.01; *P < 0.05.

34), since both Ba103 and MAR-1 react with mast cells as well as basophils (26, 27, 29). Mast cell–deficient mice failed to acquire tick resistance (Figure 1B), and therefore we could not formally exclude the possibility that the treatment of mice with Ba103 or MAR-1 had a deleterious effect on mast cell functions that led to the loss of tick resistance.

Generation of Mcpt8^{DTR} mice for selective and inducible ablation of basophils. To overcome the limitation of antibody-mediated basophil depletion in discrimination of in vivo functions between basophils and mast cells, we generated *Mcpt8^{DTR}* mice that express the human DT receptor (DTR) only in basophils, which makes it possible to specifically and inducibly deplete basophils by treatment with DT. These mice harbor a cDNA-encoding DTR fused to GFP and an internal ribosome entry site (IRES) inserted into the 3′ untranslated region of the basophil-specific *Mcpt8* gene (Figure 4A). As expected, GFP was expressed only in the basophils of *Mcpt8^{DTR}* mice and not in other cell types, including mast cells, neutrophils, or eosinophils (Figure 4B). DT injection into the *Mcpt8^{DTR}* mice led to the transient depletion of basophils from the bone marrow, peripheral blood, and spleen (Figure 4, C and D, and Supplemental Figure 3) but did not affect other cell types, including peritoneal and dermal mast cells (Figure 4, C and E, and Supplemental Figure 3). IgE-mediated, passive cutaneous anaphylaxis was elicited normally in the DT-treated *Mcpt8^{DTR}* mice (Figure 4F), demonstrating that the function of the mast cells was also intact. However, tick resistance was abolished when the *Mcpt8^{DTR}* mice were treated with DT but not vehicle (PBS) before the second infestation (Figure 5A), concomitant with the depletion of basophils, but not mast cells, from the tick-feeding sites (Figure 5, B and C). The DT treatment showed no significant effect on the number of basophils or tick resistance in control littermates (Supplemental Figure 4), and the adoptive transfer of basophils from control littermates reconstituted tick resistance in the DT-treated *Mcpt8^{DTR}* mice (Supplemental Figure 5). These findings clearly demonstrated the importance of basophils in the manifestation of tick resistance.

IgFc receptors on basophils are essential for antibody-mediated, protective immunity against ticks. We next examined the possible contribution of basophils to the antibody dependency of acquired tick resistance. The adoptive transfer of basophil-enriched CD49b$^+$ spleen cells from wild-type mice that had been infested with ticks once, but not of those from uninfested mice, conferred tick resistance on naive mice (Figure 6A). This was also the case when basophil-enriched CD49b$^+$ spleen cells from infested *Mcpt8^{DTR}* mice were used as the donor cells, and DT-mediated basophil depletion of the donor mice before the transfer abolished the acquisition of tick resistance in the recipients (Figure 6B). These results strongly suggested that anti-tick antibody–armed basophils played a critical role in antibody-mediated tick resistance. Indeed, the adoptive transfer of wild-type, but not *Fcer1g$^{-/-}$* basophil-enriched, spleen cells from infested mice conferred tick resistance on naive mice (Figure 6C). Taken together with the result shown in Figure 1F, the expression of Ig receptors on basophils but not mast cells is required for the manifestation of tick resistance.

Discussion

Basophils account for less than 1% of peripheral blood leukocytes and share several characteristics with tissue-resident mast cells, including the surface expression of FcεRI. Therefore, basophils have long been considered to have minor, redundant roles in immune responses (45). Recent studies have illuminated previously unrecognized roles for basophils in both allergic responses and immune regulation (30–33). However, our understanding of the role of basophils in protective immunity to pathogens is still very limited compared with that of mast cells (46, 47). In the present study, we created a mouse strain suitable for analysis of basophil functions in vivo and uncovered the critical role for basophils in acquired resistance against blood-feeding tick *H. longicornis* in mice. Importantly, basophils but not mast cells are responsible for the antibody/IgFc-mediated acquisition of tick resistance, even though both types of cells are essential for the manifestation of resistance.

Mcpt8^{DTR}, a mouse model for selective and inducible ablation of basophils established in the present study, is exactly what many researchers studying basophils and mast cells have long been eager for (30–33). The discovery of mast cell–deficient mice, including *Kit$^{W/W-v}$* and *Kit$^{W-sh/W-sh}$* mice, has greatly advanced our understanding of mast cell functions in vivo (48). In contrast, neither natural nor genetically engineered mutant mice deficient only in basophils were available, which was a great obstacle in the basophil research.

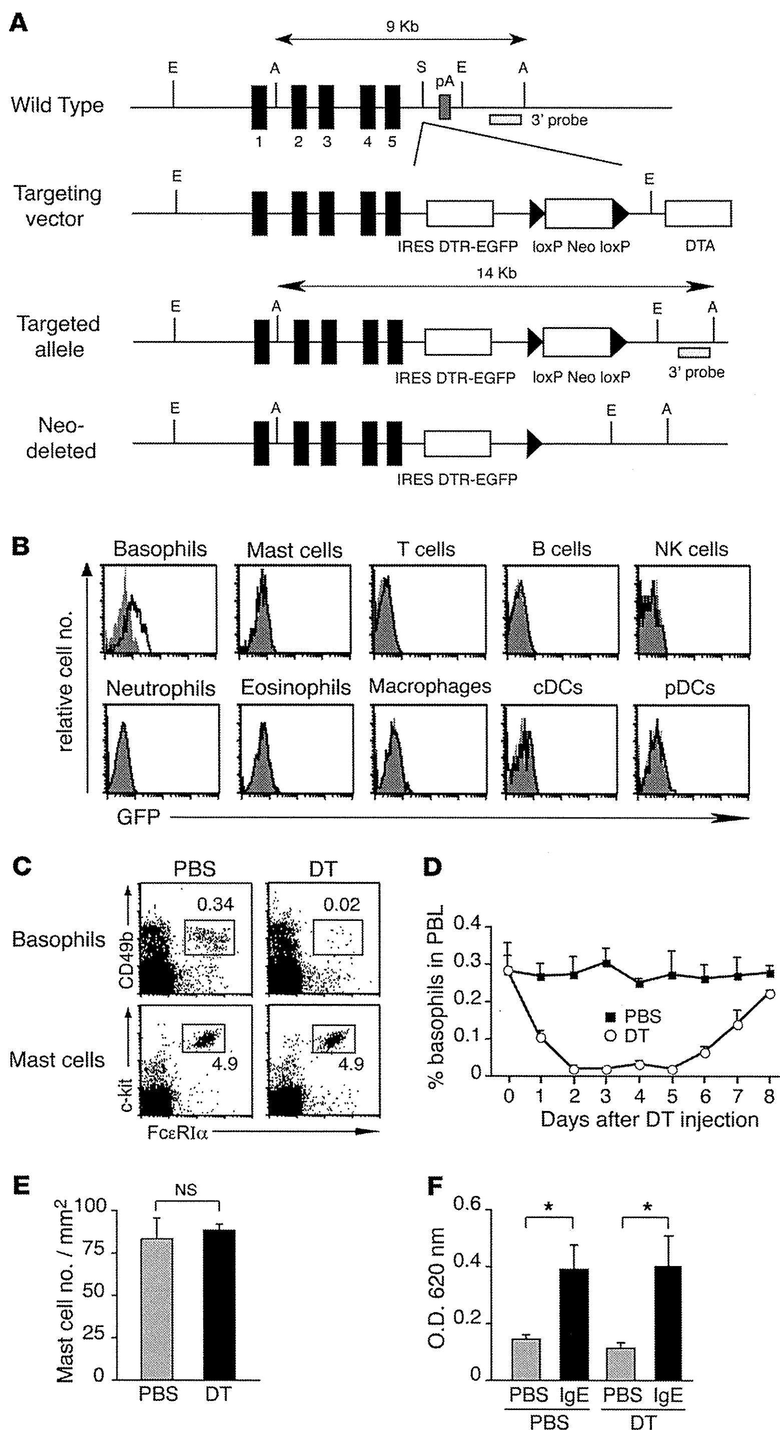


Figure 4

Generation of *Mcpt8*^{DTR} mice for selective and inducible ablation of basophils. (**A**) Schematic diagram of *Mcpt8*^{DTR} targeting construct. Exons are shown as black boxes. The restriction sites indicated are as follows: E, *EcoR*V; S, *Sal*I; A, *Afl*II. A *Sal*I site engineered in the 3' untranslated region of the *Mcpt8* gene was used to clone the IRES-DTR-EGFP-loxP-Neo-loxP cassettes. Neo cassette was removed by Cre-mediated recombination after mating with CAG-cre deleter mice. (**B**) Flow cytometric analysis of GFP expression in immune cells from *Mcpt8*^{DTR} (white histogram) or wild-type littermate (shaded histogram) mice. Data are shown for basophils, neutrophils, and eosinophils in the bone marrow; mast cells in the peritoneal cavity; and T cells, B cells, NK cells, macrophages, plasmacytoid DCs (pDCs), and conventional DCs (cDCs) in the spleen. (**C–F**) *Mcpt8*^{DTR} mice were treated with DT (750 ng/20 g body weight) or vehicle (PBS). The percentage of basophils (as indicated by the numbers in the panels) in the bone marrow and mast cells in the peritoneum 3 days after the injection are shown in **C**. The change in percentage of basophils in the peripheral blood after the injection is plotted in **D**. The number of mast cells in the flank skin 3 days after the injection is shown in **E**. Data are shown as mean ± SEM, n = 3 each. (**F**) Mice were subjected to passive cutaneous anaphylaxis 3 days after the DT injection. Data are shown as mean ± SEM, n = 4 each. Data shown in **B–F** are representative of at least 3 repeated experiments. *P < 0.05.

The establishment of *Mcpt8*^{DTR} mice has clearly solved the problem associated with antibody-mediated basophil depletion using Ba103 or MAR-1, which reacts to mast cells as well as basophils (26, 27, 29), and hence has potential to produce unwanted side effects on mast cells. The expression of DTR in *Mcpt8*^{DTR} mice is under the control of the *Mcpt8* promoter and regulatory elements, and the DT treatment depletes only basophils and leaves mast cells intact. Inducible depletion of basophils could also minimize possible compensation of basophil functions by other types of cells, compared with mice that are permanently deficient for basophils. Taking advantage of the selective and inducible ablation of basophils in *Mcpt8*^{DTR} mice, we definitely demonstrated that basophils play an essential role in the acquired protective immunity to *H. longicornis* in mice in contrast to the previously proposed scenario that mast cells substitute for basophils in the acquisition of tick resistance in mice, unlike in guinea pigs (23).

Previous studies showed that the number of mast cells increased at tick-feeding sites in mice with acquired tick resistance, whereas no infiltrating basophils were detected (21, 25, 41). In the present study, by using the recently established anti-mMCP-8 mAb TUG8, which is suitable for immunohistochemical staining of basophils in tissue sections (44), we clearly identified infiltrating basophils that made clusters surrounding tick mouth parts at *H. longicornis* feeding sites during the second but not first infestation. Thus, the absence of infiltrating basophils at the tick-feeding sites as reported in the previous studies (21, 25, 41) is probably due to the difficulty in identifying mouse basophils by conventional methods such as Giemsa staining (19, 20). We further demonstrated that the selective depletion of basophils abolished the acquired tick resistance. On the other hand, we confirmed the previous observation that the absence of mast cells resulted in the loss of acquired tick resistance (21–23). Thus, both basophils and mast cells critically contribute to the manifestation of tick resistance. However, their contribution does not seem additive, since the absence of either basophils or mast cells led to the com-

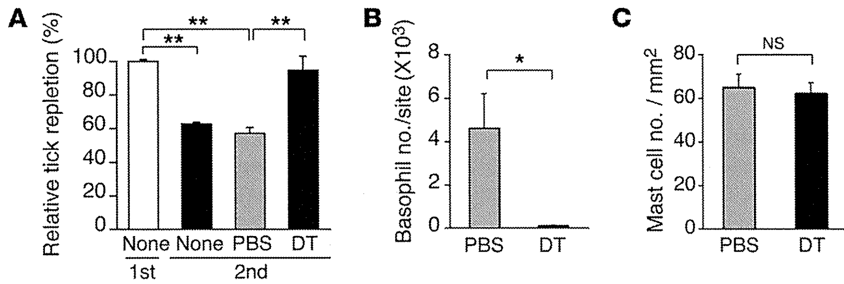


Figure 5
DT-mediated basophil ablation in *Mcpt8*^{DTR} mice abolishes acquired tick resistance. *Mcpt8*^{DTR} mice were infested once or twice with ticks as in Figure 1. DT or vehicle (PBS) was administered twice, 2 days before and 2 days after the initiation of the second infestation. The relative tick repletion for each group is shown in **A**. Data are shown as mean ± SEM, $n = 3$ each. (**B** and **C**) Numbers of basophils and mast cells in the tick-feeding sites 4 days after the initiation of the second infestation. Data are shown as mean ± SEM, $n = 3$ each. Data shown in **A**–**C** are representative of at least 3 experiments. **$P < 0.01$; *$P < 0.05$.

plete loss of acquired tick resistance, and the antibody-mediated depletion of basophils in mast cell–deficient mice showed no significant effect on tick repletion in the second infestation (data not shown). These results suggested that basophils and mast cells might have distinct roles in the acquisition of tick resistance. Indeed, the expression of IgFc receptors on basophils but not mast cells was required for it, indicating that the antibody-mediated acquisition of anti-tick immunity is ascribed to basophils rather than mast cells.

It remains to be determined how basophils and mast cells accomplish tick resistance in a cooperative or independent manner. Basophil infiltration in the tick-feeding sites was normally observed even in *Kit*$^{W\text{-}sh/W\text{-}sh}$ or *μMT* mice (Supplemental Figure 6, A and B), demonstrating that neither mast cells nor antibodies are essential for the recruitment of basophils, although both are required for the manifestation of acquired tick resistance. Moreover, the recruitment of eosinophils and neutrophils was also detected normally in mast cell–deficient or basophil-depleted mice (Supplemental Figures 6C and 7), while tick resistance was lost in these mice, suggesting that their recruitment is independent of basophils and mast cells and may be insufficient or dispensable for the manifestation of tick resistance. There are at least 2 possibilities in the interplay of basophils and mast cells: mast cells may help the priming, activation, or effector function of basophils in tick rejection. Alternatively, antigen/antibody-stimulated basophils may function as activators of mast cells that in turn produce effector molecules against ticks. A previous study using mice infested with another tick species, *Dermacentor variabilis*, demonstrated that mast cells were not essential for acquired tick resistance (24). To our knowledge, the contribution of mast cells to tick resistance has not been reported in other animal species, including guinea pigs. Thus, the extent of contribution of mast cells to acquired tick resistance may depend on the combination of host animal and tick species. Given that basophils cluster closer to tick mouth parts than mast cells, we assume that basophils rather than mast cells function as direct effectors of the anti-tick reaction mounted in response to tick antigens in our mouse model. The effector molecules involved in the manifestation of tick resistance remain to be identified. Immunohistochemical staining for mMCP-8 indicated that the basophils that were adjacent to tick mouth parts had degranulated to release mMCP-8 (Figure 2C), which is a basophil-specific, granzyme B–like serine protease that is stored in the secretory granules of basophils (42–44). We recently reported that a tryptase, mMCP-11, is also preferentially expressed by basophils and stored in their secretory granules (44). The mast cell protease mMCP-6 has been shown to play an immunoprotective role in bacterial and helminth infections (49, 50). Therefore, the

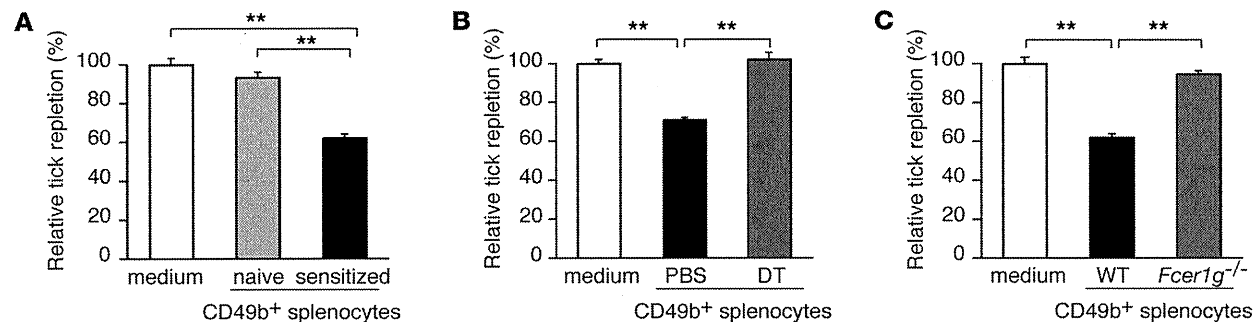
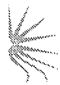


Figure 6
IgFc receptors on basophils are essential for antibody-mediated, protective immunity against ticks. (**A**) The CD49b^{+} basophil–enriched fraction of splenocytes from C57BL/6 mice infested only once (sensitized) or never (naive) was adoptively transferred into naive, uninfested C57BL/6 mice, and 2 hours later, the recipient mice were infested with ticks. The relative tick repletion for each group is shown. The value for the control, vehicle-treated mice was defined as 100%. Data are shown as mean ± SEM, $n = 3$ each. (**B**) The basophil-enriched fraction of splenocytes from DT- or PBS-treated *Mcpt8*^{DTR} mice that had been infested once was adoptively transferred into naive, uninfested littermate control mice. The recipient mice were then infested, and the relative tick repletion is shown as in **A**. Data are shown as mean ± SEM, $n = 3$ each. (**C**) The basophil-enriched fraction of splenocytes from wild-type or *Fcer1g*$^{-/-}$ mice that had been infested once was adoptively transferred into naive, uninfested C57BL/6 mice, which were then infested. The relative tick repletion is shown as in **A**. Data are shown as mean ± SEM, $n = 3$ each. Data shown in **A**–**C** are representative of at least 3 repeated experiments. **$P < 0.01$.



basophil-derived mMCP-8 and mMCP-11, among other factors, may contribute to tick resistance. Further studies, including the generation of mice deficient for these proteases, are needed to explore this possibility.

Recent studies demonstrated that basophils can enter lymph nodes, initiate Th2 responses, and enhance humoral memory responses in certain experimental settings (28, 29, 36–38). The infestation of mice with *H. longicornis* greatly increases serum IgE levels (23), suggesting that the tick infestation induces Th2-type immune responses. We detected the recruitment of basophils expressing MHC class II and CD86 to regional lymph nodes during the first infestation with ticks (Supplemental Figure 8A). However, the DT-mediated basophil depletion in the first infestation did not show any significant effect on increased levels of serum IgE (Supplemental Figure 8B) or the manifestation of acquired tick resistance in the second infestation (Supplemental Figure 9), although it abolished the basophil recruitment to the lymph nodes (Supplemental Figure 8A). The basophil depletion in the second infestation also showed no significant impact on serum IgE levels (Supplemental Figure 8B), even though it abolished acquired tick resistance (Figure 5A). These results imply that the loss of acquired tick resistance in the basophil-depleted mice may not be due to the failure in Th2-induced IgE production. Moreover, as shown in Figure 6, the adoptive transfer of basophils from infested mice conferred tick resistance on naive mice in that T cells and B cells had not been primed with tick antigens. This suggests that the enhancement of T and B cell memory responses mediated by basophils, if any, may not be essential for the manifestation of acquired tick resistance, even though our observations do not exclude roles for basophils in the promotion of Th2 response and the enhancement of memory response during tick infestation.

The present study reconciles the apparent discrepancy between guinea pigs and mice in the cellular components each requires for acquired tick resistance (17, 21–25) and illuminates a nonredundant role for basophils in protective immunity to ectoparasites. The acquisition of tick resistance is associated with reduced pathogen transmission from infected ticks (12–15), and therefore our findings may provide new avenues toward the development of novel control strategies for tick infestations and tick-borne diseases. Finally, since the study of basophils has long been hampered by the lack of basophil-deficient animal models, the establishment of $Mcpt8^{DTR}$ mice in the present study should facilitate our understanding of the in vivo roles played by basophils under physiological and pathological conditions.

Methods

Mice. C57BL/6 mice were purchased from Japan SLC. $Fcer1g^{-/-}$, μMT, and $Kit^{W\text{-}sh/W\text{-}sh}$ C57BL/6 mice were described previously (51–53). $Kit^{W\text{-}sh/W\text{-}sh}$ C57BL/6 mice were provided by S.J. Galli (Stanford University, Stanford, California, USA), S. Nakae (University of Tokyo, Tokyo, Japan), and K. Sudo (Tokyo Medical University, Tokyo, Japan). CAG-cre transgenic mice were provided by J. Miyazaki (Osaka University, Osaka, Japan). Mice were maintained under specific pathogen-free conditions in our animal facilities. All animal studies were approved by the Animal Research Committee of Tokyo Medical and Dental University and the Animal Care Committee of the Jikei University School of Medicine.

Generation of $Mcpt8^{DTR}$ mice. The targeting construct used to generate the $Mcpt8^{DTR}$ mice was made by subcloning a 10-kbp *EcoRV* fragment of *Mcpt8* containing exons 1–5 from a C57BL/6 mouse genomic DNA BAC library (BPRC) into the pBluescript II (Stratagene) vector. During this step, the *SalI* site was destroyed, and a pMC1-DTA negative-selection cassette was then inserted into the *SmaI* site. A *SalI* restriction site was engineered into the 3′ untranslated region of *Mcpt8*, upstream of the polyadenylation signal. The PCR-amplified IRES-huDTR cDNA as an *EcoRI/BamHI* fragment was cloned into pEGFP-N3 (Stratagene) to generate the gene encoding the IRES-huDTR-EGFP fusion protein, which was then cloned into the MCS-BGHpA-loxP-Neo-loxP shuttle vector. The IRES-huDTR-EGFP-BGHpA-loxP-Neo-loxP fragment was cloned into the above-mentioned *SalI* site. The linearized targeting vector was electroporated into embryonic stem cells. G418-resistant clones were screened for homologous recombination by Southern blotting analysis. Clones carrying the correctly targeted locus were injected into BDF2 blastocysts to generate chimeric mice. These chimeras were subsequently crossed with C57BL/6 females. Chimeric offspring were mated to CAG-cre transgenic mice (54) to excise the PGK-Neo cassette. Mice were backcrossed to C57BL/6 at least 6 times before use.

Ticks and tick infestation. H. longicornis of the Okayama strain was provided by K. Fujisaki (Kagoshima University, Kagoshima, Japan). *H. longicornis* larvae were used at 4–10 weeks of age for the cutaneous infestation of mice as described (21). A patch of the mouse's flank was shaved, and a short piece of acrylic pipe with 40 larvae in it was attached to the skin. The open end of the pipe was covered with nylon gauze to prevent the ticks from escaping. Of the 40 larvae applied initially, those that detached over a period lasting up to 8 days were defined as being engorged. Under our experimental conditions, approximately 85% of the ticks became engorged during the first infestation, regardless of the mouse strain examined, and the rest of them failed to feed and died. The mean body weight of the engorged ticks in the first infestation was typically approximately 750 μg, regardless of the mouse strain, whereas that of unengorged ticks was only approximately 1–2 μg. To assess the acquisition of tick resistance, mice were infested with tick larvae at 2 different locations. The initial infestation was on the left flank. Two weeks later, the second infestation was started on the right flank. In mice that had become resistant to ticks, both the number and body weight of the engorged ticks was reduced compared with naive mice (Supplemental Table 1). For evaluation of tick resistance, we summed up the body weight of all engorged ticks in each mouse as our measure of tick feeding and calculated "relative tick repletion." Relative tick repletion (%) = 100 × the sum of the body weights of all engorged ticks in the test experiment/the sum of the body weights of all engorged ticks in the reference experiment. When the tick repletion in the first infestation was defined as 100%, that in the second infestation was typically approximately 60%–70% in C57BL/6 mice. Because the body weight of unengorged ticks is negligible compared with that of engorged ones, the relative tick repletion in the second infestation compared with the first one actually represents the ratio of the total weight of all ticks in the second infestation to that in the first infestation.

Antibodies. PE-conjugated mAbs specific to Siglec-F (E50-2440), c-kit (2B8), NK1.1 (PK136), CD11b (M1/70), CD19 (1D3), and B220 (RA3-6B2) and biotinylated mAbs specific to mouse IgE (R35-72), CD49b (DX5), CD11c (HL3), c-kit (2B8), CD40 (3/23), and CD86 (GL-1) were purchased from BD Biosciences – Pharmingen. Unlabeled and PE-conjugated anti-mouse FcεRIα (MAR-1) and biotinylated anti–Gr-1 (RB6-8C5), anti-F4/80 (BM8), anti-CD3 (145-2C11), MHC class II (M5/114.15.2), and CD80 (16-10A1) were from eBioscience. The HRP-conjugated goat anti-rat IgG was obtained from Santa Cruz Biotechnology Inc. The basophil-depleting, anti-CD200R3 mAb (Ba103) and the anti–mMCP-8 mAb (TUG8) were established as reported previously (26, 27, 44).

Semiquantitative RT-PCR analysis. Total RNA was prepared from pieces of skin taken from tick-feeding sites and subjected to first-strand cDNA synthesis with reverse transcription using oligo(dT)primers. Semiquantitative PCR was performed with 5-fold serially diluted cDNA templates using the following prim-

ers: for *Mcpt8*, forward, 5′-CCGGAATTCATGTTCCTGCTCCTGGTCC-3′ and reverse 5′-CGCGGATCCCTAGGGTTGTTGCAGGAGTTTCATTG-3′; for *Mcpt4*, forward 5′-GGGCTGGAGCTGAGGAGATT-3′ and reverse 5′-CTTCTGCTGTGTGGATTCTGTC-3′; and for *Hprt1*, forward, 5′-GCTGGTGAAAAGGACCTCT-3′ and reverse 5′-CACAGGACTAGAACACCTGC-3′.

Flow cytometric and histological analyses. Pieces of skin from the tick-feeding sites were treated with collagenase (130 U/ml, Wako Pure Chemical) in RPMI complete medium at 37°C for 2 hours to obtain single-cell suspensions. Cells prepared from skin, bone marrow, the peritoneal cavity, and peripheral blood were depleted of rbcs by lysis with hypotonic buffer and preincubated with anti-CD16/32 (2.4G2) mAb (BD Biosciences — Pharmingen) and normal rat serum on ice for 10 minutes to prevent the nonspecific binding of other Abs. Cells were stained with the indicated combinations of Abs and analyzed by FACSCalibur (BD Biosciences): basophils (IgE$^+$c-kit$^-$ or FcεRIα$^+$CD49b$^+$), mast cells (IgE$^+$c-kit$^+$ or FcεRIα$^+$c-kit$^+$), eosinophils (Gr-1^{int}siglec-F$^+$), neutrophils (Gr-1^{hi}), T cells (CD3$^+$), B cells (CD19$^+$), NK cells (NK1.1$^+$CD49b$^+$), monocytes/macrophages (Gr-1^{int}Siglec-F$^-$), macrophages (CD11b$^+$F4/80$^+$), plasmacytoid DCs (pDCs, CD11c^{int}B220$^+$), and conventional DCs (cDCs, CD11c^{hi}B220$^-$). For immunohistochemical examination, paraffin-embedded skin specimens were prepared and pretreated as described (44), and tissue sections were incubated with anti-mMCP-8 (1 μg/ml) or an isotype-matched control antibody at 4°C overnight, followed by HRP-conjugated goat anti-rat IgG (diluted 1:500). The sections were subsequently incubated in a DAB solution (Dako) and counterstained with H&E. Mast cells in the skin tissue sections were identified by toluidine blue staining (pH 2.5).

Basophil depletion. For the antibody-mediated depletion of basophils, mice were treated with either Ba103 or MAR-1. One intravenous injection of 50 μg Ba103 or control rat IgG was given 1 day before the tick infestation. A total of 30 μg MAR-1 or control hamster IgG was administered intraperitoneally twice a day (5 μg each time) for 3 consecutive days, starting 4 days before the tick infestation. For the DT-mediated depletion, *Mcpt8^{DTR}* mice were given an intravenous injection of DT (750 ng/20 g body weight; Sigma-Aldrich).

Adoptive transfer of cells. Mouse bone marrow cells were cultured with 3.5 ng/ml rIL-3 and 15 ng/ml rSCF for approximately 4–6 weeks to obtain BMMCs. A total of 10^6 BMMCs was injected in 10 separate intradermal injections to form a circle (16 mm in diameter) on the right flank of *Kit$^{W-sh/W-sh}$* mice. Four weeks later, the mice were infested with tick larvae, first on the left flank and then, 2 weeks later, by a second infestation in which the larvae were placed within the BMMC-injected site. For the adoptive transfer of basophils, spleen cells were isolated from mice that were uninfested or infested with ticks 2 weeks previously, and the basophil-enriched CD49b$^+$ fraction was prepared by using the BD IMag Cell Separation System (BD Biosciences — Pharmingen). Mice were infested with ticks 2 hours after the adoptive transfer of the CD49b$^+$ fraction (1.5×10^6 cells).

Passive cutaneous anaphylaxis. Mice were passively sensitized with an intradermal injection of TNP-specific IgE or control PBS into the ear and challenged 24 hours later with an intravenous injection of TNP-OVA plus 0.5% Evans blue dye as described. The absorbance of Evans blue in the ear extract was measured with a spectrophotometer at 620 nm.

Statistics. Statistical significance ($P < 0.05$) was determined by 2-tailed Student's *t* test or ANOVA followed by Ryan's test.

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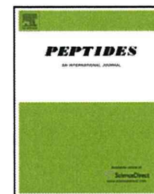

Gastrin-releasing peptide induces itch-related responses through mast cell degranulation in mice

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ABSTRACT

Gastrin-releasing peptide (GRP), secreted from the central terminals of primary afferents, is involved in the transmission of itch signals in the spinal dorsal horn. Although primary afferents containing GRP are distributed throughout the skin, the role of peripherally released GRP in the itch response is unknown. We investigated whether GRP acts on the skin to induce an itch response in mice. Intradermal injections of GRP_{18-27} (1–300 nmol/site) elicited scratching. GRP_{18-27}-induced scratching was inhibited by the μ-opioid receptor antagonist naltrexone hydrochloride, the BB_2 bombesin receptor antagonist RC-3095, the H_1 histamine receptor antagonists fexofenadine hydrochloride and chlorpheniramine maleate, and the PAR_2 proteinase-activated receptor antagonist FSLLRY-NH_2. Mast cell deficiency significantly, but not completely, reduced the GRP_{18-27}-induced scratching. BB_2 bombesin receptors are present in mast cells in the skin, and intradermal injection of GRP_{18-27}, not only induced scratching, but also led to mast cell degranulation. GRP_{18-27}-induced mast cell degranulation was inhibited by the BB_2 bombesin receptor antagonist RC-3095. These results suggest that peripherally released GRP can induce an itch response, at least partly, through activation of BB_2 receptors present in the mast cells, triggering their degradation and the release of histamine and the serine proteinase, tryptase.
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1. Introduction

Gastrin-releasing peptide (GRP) is a bombesin family peptide consisting of 27 amino acids [25]. It is widely distributed in the central [9] and peripheral nervous systems [15,16,35,39]. GRP is cleaved to form the decapeptide GRP_{18-27}, previously named neuromedin C. GRP and GRP_{18-27} act on the BB_2 bombesin receptor (also known as the GRP receptor) with similar binding affinity and selectivity [17] and have many physiological functions, including the release of gastric acid [11,17]. BB_2 bombesin receptors are expressed in the central nervous system [9] and peripheral tissues [17].

Itching is often a distressing symptom associated with a variety of dermatologic and systemic disorders [12]. This sensation provokes a desire to scratch, thereby making the cutaneous symptoms worse. Unfortunately, the underlying mechanisms and endogenous mediators of itching in most pruritic diseases are unclear. Recently, it has been shown that the BB_2 bombesin receptor is involved in the transmission of itch signals in the spinal dorsal horn [39,40]. GRP is contained in the primary sensory neurons [15,35,39], the terminals of which are distributed in, not only the spinal dorsal horn, but also peripheral tissues. In the skin of mice with itching chronic dermatitis, GRP-immunoreactive nerve fibers are increased in the epidermis and dermis [41]. These findings suggest that GRP is released from the peripheral terminals of primary sensory neurons into the skin. To investigate the role of GRP in the skin, we determined whether GRP could act on the skin to induce an itch response.

2. Materials and methods

2.1. Animals

Male ICR mice (Japan SLC, Shizuoka, Japan) were generally used at 7–10 weeks of age. In a series of experiments, male mast cell-deficient mice (WBB6F1-W/W^v, Japan SLC) and controls (WBB6F1-+/+, Japan SLC) were used at 5 weeks of age. Mice were housed under controlled temperature (22 ± 1 °C), humidity ($55 \pm 10\%$), and light (lights on from 7:00 AM to 7:00 PM). Food and water were freely available. The study protocol was approved by the Committee for Animal Experiments at the University of Toyama and was conducted in accordance with the guidelines of the Japanese Pharmacological Society.

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2.2. Agents and administration

GRP_{18-27} (Gly–Asn–His–Trp–Ala–Val–Gly–His–Leu–Met–NH_2) and the PAR_2 proteinase-activated receptor antagonist Phe–Ser–Leu–Leu–Arg–Tyr–NH_2 (FSLLRY-NH_2) were synthesized and identified by using a peptide synthesizer PSSM-8 (Shimazu Co., Kyoto, Japan) and a matrix assisted laser desorption/ionization time-of-flight mass spectrometry Autoflex T1 (Bruker Daltonics, Bullerica, MA, USA), respectively. GRP_{18-27}, FSLLRY-NH_2, the BB_2 receptor antagonist RC-3095 (Sigma, St. Louis, MO, USA), histamine (Wako Pure. Chem. Ind., Osaka, Japan), and serotonin (Sigma) were dissolved in physiological saline. In behavioral experiments, they were injected intradermally in a volume of 50 μl into the rostral back of the mice. RC-3095 and FSLLRY-NH_2 were injected 10 and 30 min before GRP_{18-27} injection, respectively. For the experiments measuring mast cell participation, GRP_{18-27} and RC-3095 were injected subcutaneously to observe the degranulation of mast cells. Fexofenadine hydrochloride (LTK Laboratories, St Paul, MN, USA) and chlorpheniramine maleate (Sigma) were suspended in 0.5% sodium carboxymethylcellulose and administered orally 30 min before pruritogen injection. Naltrexone hydrochloride (Sigma) was dissolved in physiological saline and injected subcutaneously 15 min prior to GRP_{18-27} injection.

2.3. Behavioral experiments

The day before behavioral experiments, the animals' hair was clipped over the rostral part of the back. For acclimation, mice were put individually in an acrylic cage composed of four cells (13 cm × 9 cm × 35 cm) for at least 1 h prior to testing. Immediately following intradermal injection, mice were returned to the same cells, and their behaviors were videotaped for 1 h. No personnel were present in the observation room during this time. Using the videotape, mice were observed and bouts of scratching of the injected site by the hind paws were counted [19]. The mice stretched either hind paw toward the injection site, leaned the head toward it, and rapidly scratched several times for about 1 s. A series of these movements was counted as one bout of scratching [42].

2.4. Toluidine blue staining

GRP_{18-27} was injected subcutaneously under deep anesthesia induced with pentobarbital sodium (80 mg/kg, i.p.; Sigma) and 10 min later, the subcutaneous connective tissue was isolated. This tissue was attached to the microscope slides, dried, and fixed with Carnoy's fixative composed of ethanol, chloroform, and glacial acetic acid (6:3:1) for 5 h. The slides were then immersed sequentially in 100% and 80% ethanol for 1 min each and fully rinsed in tap water for 5 min. The preparations were stained with 0.1% toluidine blue and washed with tap water. For the rehydration, the slides were immersed sequentially in 60%, 80%, 90%, and 95% ethanol for 1 min each and then in 100% ethanol and xylene for 10 min each. The preparations were mounted with Canada balsam and observed using a light microscope (BX-61, Olympus, Oasaka, Japan) with a CCD camera (DP70, Olympus).

2.5. Immunohistochemical staining

Subcutaneous connective tissue samples were attached to microscope slides and fixed with 4% paraformaldehyde at 4 °C for 1 h. After being washed three times with 0.1 M phosphate-buffered saline, the tissues were treated with 0.3% Triton X-100 for 30 min, blocked with 1% fetal bovine serum, and incubated with anti-BB_2 bombesin receptor antibody (1/500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C over night. The slides were then washed and incubated with Alexa Fluor 488-conjugated anti-goat IgG antibody (Molecular Probes Inc., Eugene, OR, USA) for 1 h. Fluorescence signals were observed using a confocal laser scanning microscope (TCS-SP5, Leica, Mannheim, Germany). After scanning, the slide glass was washed with phosphate-buffered saline. The tissue sample was stained with toluidine blue and observed using a light microscope (BX-61, Olympus, Osaka, Japan) with a CCD camera (DP70, Olympus).

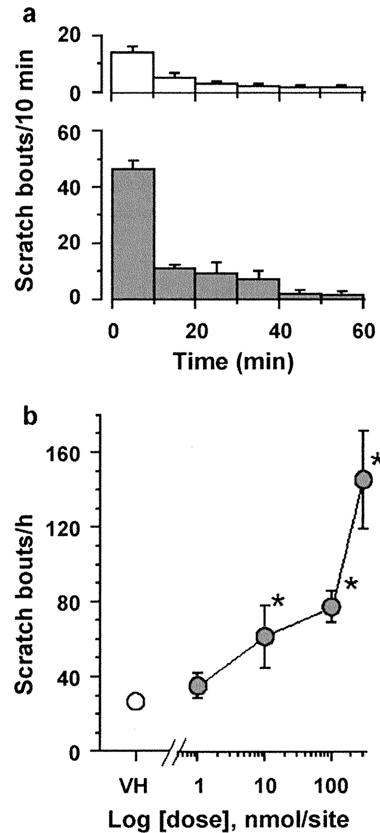

Fig. 1. Scratching after intradermal injections of GRP_{18-27}. Mice were given an intradermal injection of GRP_{18-27} or vehicle and scratch bouts were counted for 1 h. (a) Time course of scratching after the vehicle (VH, upper panel) and GRP_{18-27} (100 nmol/site, lower panel) injections. (b) Dose–response curve for the scratch-inducing effects of GRP_{18-27}. Values represent the mean ± SEM for 8 animals. *$P < 0.05$ when compared with VH (Dunnett's multiple comparisons).

2.6. Data processing

Data are presented as means ± standard error of the mean (SEM). The time course data sets were statistically analyzed with two-way repeated measures analysis of variance. Statistical significance between groups was analyzed with Dunnett's multiple comparisons, Bonferroni's multiple comparisons, or Student's t-test. $P < 0.05$ was considered significant.

3. Results

3.1. Behavioral effect of intradermal GRP_{18-27}

An intradermal injection of GRP_{18-27} (100 nmol/site) elicited marked scratching of the injected site by the hind paws compared with the vehicle. The effect peaked in the initial 10 min period and almost completely subsided within 60 min (Fig. 1a). Plotting the number of scratching bouts for 60 min post-GRP_{18-27} injection against the dose (1–300 nmol/site) revealed a dose-dependent effect on scratching behavior, with a significant increase observed at a dose of 10 nmol/site or more (Fig. 1b).

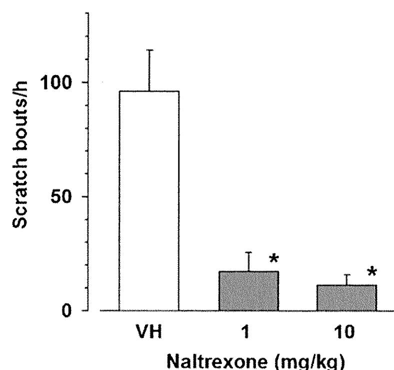

Fig. 2. Effects of naltrexone on GRP_{18-27}-induced scratching. Mice were given an intradermal injection of GRP_{18-27} (100 nmol/site) and scratch bouts were counted for 1 h. Naltrexone hydrochloride or vehicle (VH, saline) was injected subcutaneously 15 min before intradermal injection. Values represent the mean ± SEM for 8 animals. $^{*}P < 0.05$ when compared with VH (Dunnett's multiple comparisons).

3.2. Characterization of GRP_{18-27}-induced scratching

To characterize GRP-induced scratching, several agents were examined for their ability to modulate scratching behavior caused by GRP_{18-27} (100 nmol/site, intradermal). Subcutaneous pretreatment with the μ-opioid receptor antagonist naltrexone hydrochloride (1 and 10 mg/kg) significantly inhibited GRP_{18-27}-induced scratching (Fig. 2). GRP_{18-27}-induced scratching was also significantly inhibited by local pretreatment with the BB_2 bombesin receptor antagonist RC-3095 (50 nmol/site) (Fig. 3). Oral pretreatment with the H_1 histamine receptor antagonists chlorpheniramine maleate (10 and 30 mg/kg) and fexofenadine hydrochloride (10 and 30 mg/kg) produced a dose-dependent inhibition in GRP_{18-27}-induced scratching, with significant inhibition at a dose of 30 mg/kg (Fig. 4a and b). The 30 mg/kg dose of fexofenadine hydrochloride significantly inhibited scratching induced by histamine (100 nmol/site), but not serotonin (100 nmol/site) (Fig. 4c and d). The PAR_2 proteinase-activated receptor antagonist FSLLRY-NH_2 (12.5–250 nmol/site) also produced a dose-dependent inhibition in GRP_{18-27}-induced scratching (Fig. 5).

3.3. Effect of mast cell deficiency on GRP_{18-27}-induced scratching

Intradermal injections of GRP_{18-27} (100 nmol/site) caused a significant increase in scratching in both mast cell-deficient

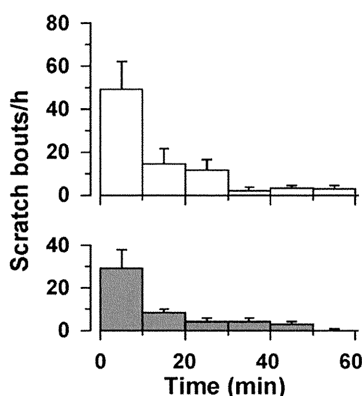

Fig. 3. Effect of BB_2 bombesin receptor antagonist on GRP_{18-27}-induced scratching. Mice were given an intradermal injection of GRP_{18-27} (100 nmol/site) and scratch bouts were counted for 1 h. The vehicle (saline, upper panel) and RC-3095 (50 nmol/site, lower panel) were injected intradermally 10 min before the injection. Values represent the mean ± SEM for 6 animals. Group × time interaction, $F_{(5, 50)} = 15.94$, $P < 0.0001$ (two-way repeated measures analysis of variance).

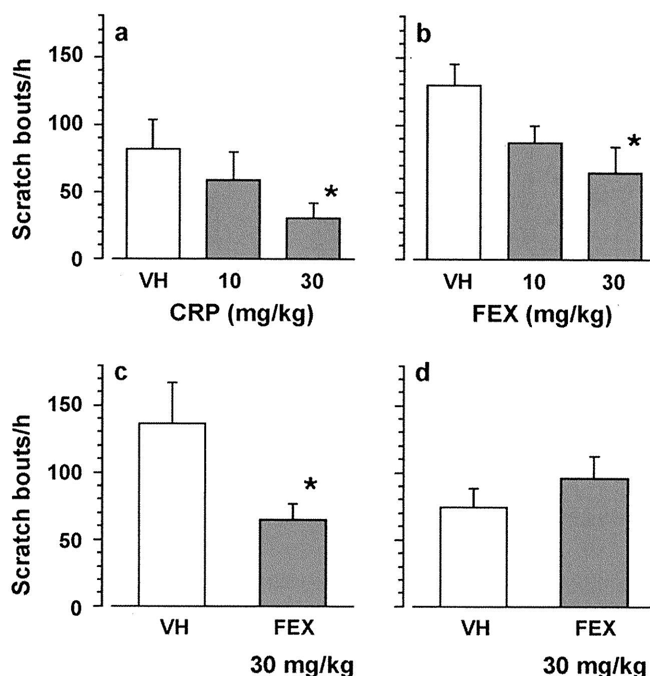

Fig. 4. Effects of H_1 histamine receptor antagonists on scratching induced by intradermal injections of GRP_{18-27}, histamine, and serotonin. Mice were given an intradermal injection of (a and b) GRP_{18-27} (100 nmol/site), (c) histamine (100 nmol/site), or (d) serotonin (100 nmol/site) and scratch bouts were counted for 1 h. (a) Chlorphenilamine maleate (CRP), (b–d) fexofenadine hydrochloride (FEX), or vehicle (VH, 0.5% sodium carboxymethylcellulose) was administered orally 30 min before pruritogen injection. Values represent the mean ± SEM for 6–8 animals. $^{*}P < 0.05$ when compared with VH (Student's t-test or Dunnett's multiple comparisons).

(WBB6F1-W/W^{v}) and control (WBB6F1-+/+) mice. However, the number of scratching bouts was significantly lower in the mast cell-deficient mice compared with the controls (Fig. 6).

3.4. Mast cell degranulation induced by GRP_{18-27} and expression of BB_2 receptor in mast cells

Since mast cells are densely and sparesely distributed in the subcutaneous connective tissue and dermis, respectively, and have the same biochemical and pharmacological characteristics in these regions [26], we investigated the action of GRP_{18-27} on the mast cells in the subcutaneous connective tissue. GRP_{18-27}

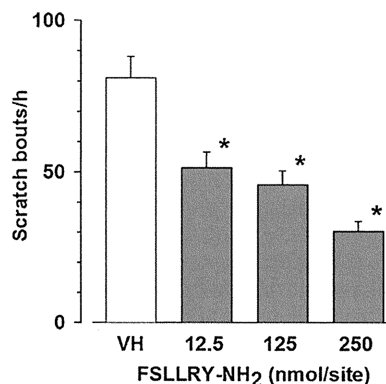

Fig. 5. Effects of PAR_2 antagonist on GRP_{18-27}-induced scratching. Mice were given an intradermal injection of GRP_{18-27} (100 nmol/site) and scratch bouts were counted for 1 h. The PAR_2 antagonist FSLLRY-NH_2 or vehicle (VH, saline) was injected intradermally 30 min before the injection. Values represent the mean ± SEM for 8 animals. $^{*}P < 0.05$ when compared with VH (Dunnett's multiple comparisons).

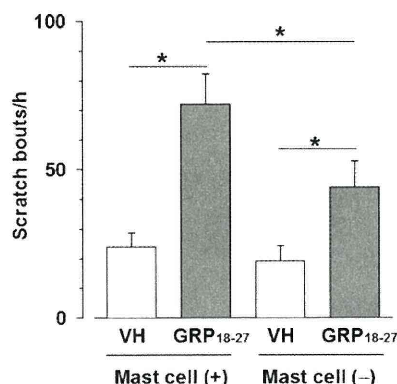

Fig. 6. Effect of mast-cell deficiency on GRP_{18-27}-induced scratching. GRP_{18-27} (100 nmol/site) or vehicle (VH, saline) was injected intrademally into WBB6F1-W/W^v (mast cell-deficient) and WBB6F1-+/+ (control) mice. Scratch bouts were counted for 1 h. Values represent the mean ± SEM for eight animals. $^*P < 0.05$ when compared with VH or control mice (Bonferoni's multiple comparisons).

(100 nmol/site) injection induced a marked and significant mast cell degranulation, whereas only a few degranulated mast cells were observed in mice given a subcutaneous injection of saline (Fig. 7a–c). GRP_{18-27}-induced mast cell degranulation was significantly inhibited by local pretreatment with the BB_2 bombesin receptor antagonist, RC-3095 (50 nmol/site) (Fig. 7c). In addition, BB_2 bombesin receptor-like immunoreactivity was found in mast cells (Fig. 7d).

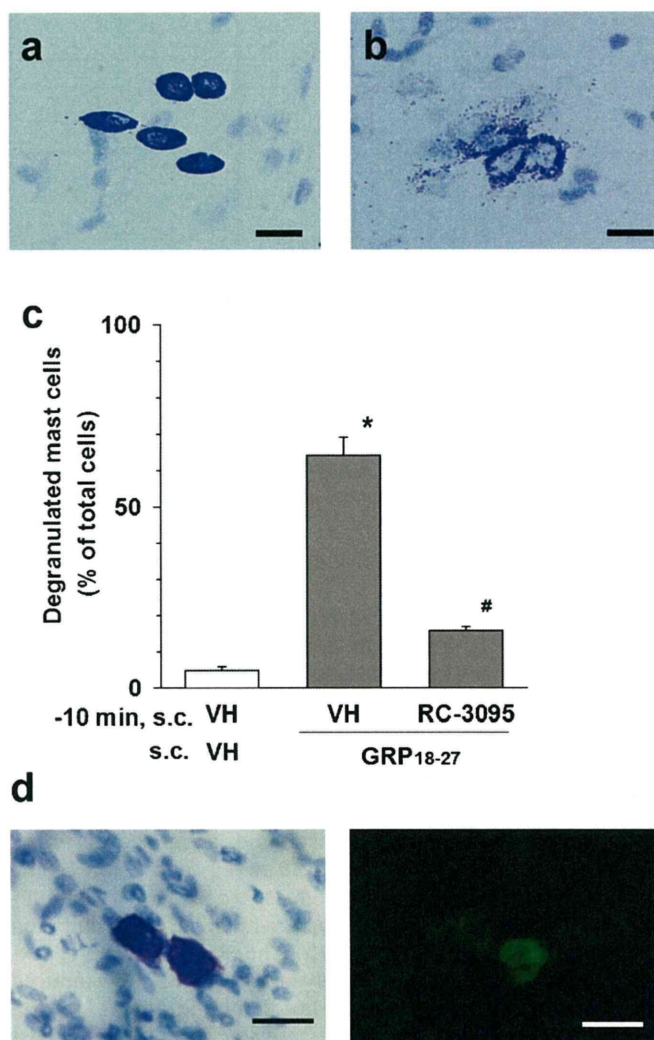

Fig. 7. GRP_{18-27}-induced degranulation and BB_2 bombesin receptor expression in mast cells. (a–c) Mast cell degranulation. Mice were given a subcutaneous injection of vehicle (VH, saline) or GRP_{18-27} (100 nmol/site), and 10 min later the subcutaneous connective tissue was isolated for toluidine blue staining. The BB_2 receptor antagonist RC-3095 (50 nmol/site) was injected 10 min prior to GRP_{18-27} injection. (a) VH and (b) GRP_{18-27}. (c) The proportion of degranulated mast cells. Values represent the mean ± SEM for five samples. $^*P < 0.05$ vs. VH alone, $^\#P < 0.05$ vs. VH + GRP_{18-27} (Bonferoni's multiple comparisons). (d) BB_2 bombesin receptor in a mast cell. The subcutaneous connective tissue was isolated, immunostained for BB_2 receptor, and then stained with toluidine blue. BB_2 receptor-like immunoreactivity (right) and mast cell (left) are shown. Scale bars represent 20 μm.

4. Discussion

Intradermal injections of GRP_{18-27} (1–300 nmol/site) elicited dose dependent scratching of the injection site in mice. The minimum effective concentration (10 nmol/50 μl = 0.2 mM) of GRP_{18-27} was the same as its effective intrathecal concentration in mice [39]. Additionally, the effective dose range was similar to those of intradermal histamine, serotonin, and substance P [4,23]. GRP_{18-27}-induced scratching was inhibited by the μ-opioid receptor antagonist naltrexone. Blockade of the μ-opioid receptor results in the inhibition of scratching induced by several pruritogens [4,6,45] and dermatoses in rodents [27,33,46], and itching/scratching in humans with pruritic diseases [10,29]. μ-Opioid receptor antagonists suppress itch-associated behaviors without inhibiting pain-related behaviors [1,14]. They may inhibit itch-related responses by acting on μ-opioid receptors in the central nervous system [24,32], especially in the lower brainstem [20]. Thus, intradermal injection of GRP_{18-27} caused an itch-associated response, scratching, in a similar manner to the other endogenous pruritogenic compounds.

The BB_2 bombesin receptor antagonist RC-3095 suppressed scratching induced by intradermal GRP_{18-27}. This suggests that GRP_{18-27} acts on the BB_2 receptors in the skin to cause itching. Moreover, GRP_{18-27}-induced scratching was inhibited by the H_1 histamine receptor antagonists fexofenadine and chlorpheniramine. The 30 mg/kg dose of fexofenadine caused similar inhibition of scratching elicited by GRP_{18-27} and histamine, but did not inhibit serotonin-induced scratching. Thus, the H_1 histamine receptor antagonists might suppress the action of GRP_{18-27} through the inhibition of histamine within the skin. In addition to H_1 histamine receptor antagonists, deficiency in mast cells also produced a marked inhibition in GRP_{18-27}-induced scratching. GRP_{18-27} triggered an RC-3095-reversible degranulation of the mast cells, which express BB_2 bombesin receptors. The BB_2 bombesin receptor is a $G_{q/11}$-coupled receptor [17], and its activation leads to an increase in intracellular Ca^{2+} concentration [30], which then induces mast cell degranulation through the activation of phospholipase C and mobilization of Ca^{2+} from intracellular Ca^{2+} stores [28]. Thus, mast cell degranulation and the release of mediator(s) such as histamine may be a peripheral mechanism for the pruritogenic action of GRP.

Like H_1 receptor antagonists, PAR_2 receptor antagonists were also capable of suppressing GRP_{18-27}-induced scratching. Similar to GRP_{18-27}, scratching induced by compound 48/80 which produces mast cell degranulation is suppressed by a serine proteinase inhibitor and an H_1 receptor antagonist [44]. PAR_2 receptors are activated by serine proteinases [34]. Although connective tissue mast cells contain several kinds of proteases, including tryptase and chymase [26], only tryptase elicits scratching behaviors following intradermal injection in mice [44]. This protease has also been implicated in itching/scratching in chronic dermatitis [38,43]. Scratching elicited by tryptase and compound 48/80 is suppressed by a PAR_2 antagonist and anti-PAR_2 receptor antibody [44]. Collectively, these findings suggest that histamine

and serine proteinases, mainly tryptase released from mast cells, are involved in the itching response induced by the cutaneous action of GRP. Transient receptor potential vanilloid 1 (TRPV1) receptor-positive primary afferents have been postulated to play an important role in itch singnaling [4,18,31,45,47]. PAR_2 receptors are co-expressed with TRPV1 receptors in a subset of primary sensory neurons and the latter may mediate PAR_2 receptor-induced signaling [13]. Thus, it is possible that TRPV1 receptors play a role in GRP-induced scratching. Mas-related G-protein-coupled receptor (Mrgpr) has been implicated in histamine-independent itch. Chloroquine and bovine adrenal medulla 8–22 peptide, activators of Mrgpr, elicit scratching and itching in rodents and humans, respectively [21,36]. However, since deficiency in Mrgpr does not affect scratching induced by trypsin, PAR_2-activating protease [22], the involvement of Mrgpr in GRP-induced scratching is not clear.

Although mast cells clearly play a key role in GRP_{18-27}-induced scratching, the behavioral response to GRP_{18-27} was not completely abolished in mast cell-deficient mice, suggesting that other cutaneous cells are also responsible for the pruritogenic action of GRP. BB_2 bombesin receptors are expressed in the epidermal keratinocytes [37]. Keratinocytes release itch mediators and enhancers, such as leukotriene B_4 [2,6], thromboxane A_2 [5], nociceptin [8], and nitric oxide [3]. Future research is needed to determine whether keratinocytes and their pruritogenic factors are involved in scratching induced by intradermal GRP_{18-27}.

In the skin, GRP immunoreactivity is present mainly in primary afferents and GRP-positive nerve fibers are positive for nociceptive nerve markers such as substance P, calcitonin-gene related peptide, transient receptor potential vanilloid 1 receptor and PAR_2 receptor [41]. GRP-positive nerve fibers are increased in the skin of mice with chronic dermatitis [41]. Therefore, in patients with dermatitis, primary afferents may increase their secretion of GRP into the lesional skin. The increased GRP present in the skin may then enhance the itch response through its action on the mast cells (and keratinocytes), in addition to its role in itch signal transmission within the dorsal horn [39,40].

Acknowledgements

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ORIGINAL ARTICLE — SKIN AND EYE DISEASES

Basophil recruitment and activation in inflammatory skin diseases

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Abstract

Background: Basophils are blood leukocytes constituting less than 1% of leukocytes. They share morphological and functional similarities with mast cells, but recent studies indicate that basophils play non-redundant roles via the release of several cytokines and lipid mediators, as well as functioning as antigen presenting cells. However, basophil infiltration into the tissues in human skin diseases remains to be addressed.
Methods: The infiltration of basophils in 24 skin diseases (136 samples) was immunohistochemically analyzed using basophil-specific BB1 antibody. In addition, activation of blood basophils was examined by assessing CD203c expression with flow cytometry.
Results: Basophils were detected in skin lesions of atopic dermatitis, prurigo, urticaria, bullous pemphigoid, drug eruptions, eosinophilic pustular folliculitis, insect bites, scabies, Henoch–Schönlein purpura and dermatomyositis. While cell densities in urticaria, bullous pemphigoid and eosinophilic pustular folliculitis were prominent, much lower numbers of basophils were seen in lesional skin of atopic dermatitis. Basophils were entirely absent in psoriasis vulgaris, mastocytosis, tumoral lesions, systemic sclerosis, and systemic lupus erythematosus. Levels of CD203c expression on blood basophils from prurigo and urticaria patients were higher than those from healthy donors.
Conclusions: Basophils infiltrate into skin lesions more commonly than previously thought, and thus they may play important roles in a variety of inflammatory skin diseases.

Basophils account for less than 1.0% of blood leukocytes. In the 1970s, Dvorak et al. observed marked basophil infiltration in a guinea pig model of skin inflammation induced by protein antigens with incomplete Freund's adjuvant (1). This delayed-onset, but transient skin reaction (Jones–Mote-type), was designated as 'cutaneous basophil hypersensitivity' (CBH) (1, 2). Contact hypersensitivity reactions in guinea pigs were also recognized as a type of CBH (3, 4). The immunological nature of guinea pig CBH has since been extensively analyzed in a number of studies (5–7). In contrast, our understanding of tissue basophils in human skin diseases is limited because basophils in human skin tissues, unlike guinea pig tissues, cannot be stained in routinely processed histological tissue specimens. An older study by Juhlin et al. in 1964 observed basophils in artificial cantharidin blisters or spontaneous bullous lesions in atopic dermatitis (AD) and other eczematous diseases (8). Other studies detected basophils in contact allergies to urushiol, metals (9, 10) and on patch-tested sites for house dust mite allergens (11).

Basophils share morphological and functional similarities with tissue-resident mast cells. Both basophils and mast cells contain basophilic granules and express the $\alpha\beta\gamma2$ form of $Fc\varepsilon RI$. They contain chondroitin sulfate and tryptase, and secrete histamine and leukotriene (LT) C4 and its peptidolytic products, LTD4 and LTE4 upon activation (12, 13). Thus,

Abbreviations
AD, atopic dermatitis; CAI, chronic allergic skin inflammation; CBH, cutaneous basophil hypersensitivity; LT, leukotriene; PGD2, prostaglandin D2; TNP, trinitrophenyl.

basophils have long been considered to be redundant 'circulating mast cells' (14) and minor (probably negligible) players in allergic inflammation. However, recent studies in mice have revealed that mast cells are dispensable in IgG-, but not IgE-mediated systemic anaphylaxis, which is probably mediated by basophil-derived platelet-activating factor (15). Basophils play non-redundant roles from mast cells in protective immunity against tick bites (16), and function as a source of Th2-type cytokines, such as IL-4 and IL-13, and chemokines, including CCL17 and CCL22, thereby contributing to Th2-type immunity (17–19). They are also capable of presenting antigens, leading to the induction of Th2 cells (20). A particularly striking finding is that basophils play a critical role in the development of IgE-mediated chronic allergic reactions (IgE-CAI) by functioning as initiator cells (21, 22). Depletion of basophilis with a specific antibody resulted in the almost complete abrogation of skin inflammation of IgE-CAI. Thus, murine basophils are an important player in innate and/or acquired immunity. In humans, they may also contribute to the pathogenesis of a variety of allergic skin diseases, and it is therefore important to clarify whether basophils infiltrate skin lesions in human diseases.

In this study, we comprehensively analyzed tissue basophils in a variety of skin diseases by immunohistochemical staining with basophil-specific antibody (BB1) (23). The activation status of blood basophils *in vivo* was also assessed.

Materials and methods

Skin samples

Biopsied skin specimens obtained for histological diagnosis with informed consent from 136 subjects (24 skin diseases) were analyzed. This study was approved by the Ethics Committee of Tokyo Medical and Dental University.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were treated with 0.1% trypsin in 50 mM Tris–HCl (pH 7.5) containing 0.1% $CaCl_2$ for 37°C at 30 min, followed by additional treatment with 0.1% saponin in TBS buffer (pH 7.6) for 30 min at room temperature. They were then stained with human basophil-specific antibody (BB1, anti-basogranulin Ab, mouse IgG2a) (23) following 10-min incubation with Protein block serum free® (Dako Denmark A/S, Glostrup, Denmark). Reaction steps using alkaline phosphatase-conjugated polymers were processed with Histofine® Simple SatinAP (M) kit (Nichirei Biosciences Inc., Tokyo, Japan). Reaction products were visualized by Fuchsin + Substrate-Chromogen (Dako Denmark A/S). Mouse IgG2a (Dako Denmark A/S) was used as a negative control. In some experiments, frozen tissue sections were stained without trypsin treatment.

Image analysis

Density of positively stained basophils in the tissues was analyzed using Image Pro®Plus (Media Cybernetics, Inc., Maryland, Silver Spring, MD, USA) software. At least three fields were examined by light microscopy. For detecting eosinophils, formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E).

Flow cytometry

Peripheral blood anti-coagulated with 20 mM EDTA was obtained with informed consent and stained with FITC-conjugated CRTH2 Ab, PC7-conjugated CD3 Ab and phycoerythrin-conjugated CD203c Ab. Blood basophils were gated by side scatter and CRTH2 positivity, followed by identification as CD3 (-) cells. Activation of blood basophils was assessed based on expression levels of CD203c. Cell staining was processed with reagents in an Allergenisity kit (Immunotech, a Beckman Coulter Company, Marseille, France). Flow cytometric analysis was performed by FACS Caliber (Becton, Dickinson and Company, New Jersey, USA).

Statistical analyses

Mann-Whitney's U-test with Bonferroni correction following Kruskal–Wallis H-test was used to assess the statistical significance of differences between mean values.

Results

Detection of basophils in skin diseases

Profiles of the 136 samples (24 skin diseases) are summarized in Table 1. Skin tissue specimens of urticaria in this study were from patients with long-lasting urticaria, in which wheals persisted for more than 24 h. Immunohistochemical staining with BB1 antibody revealed that basophils were frequently observed in AD, prurigo and urticaria. It was notable that skin lesions of bullous pemphigoid, classical eosinophilic pustular folliculitis (Ofuji's disease) and Henoch–Schönlein purpura also frequently showed tissue basophilia.

Dermal basophils in the lesional skin of AD are shown in Fig. 1A. Although basophils were detected in 62% of samples (13 out of 21 specimens), cell accumulation was not prominent. In contrast, patch-test skin sites for house dust mite allergen in AD at 24 h showed marked basophil accumulation. Basophils were even localized within the epidermis (Fig. 1B). Basophils were found around perivascular areas and intercollagenous spaces of the dermis in prurigo and urticarial lesions (Fig. 1C, D). In bullous pemphigoid, basophils accumulated underneath the subepidermal clefts (Fig. 1F). Basophils in Henoch–Schönlein purpura were found in perivascular areas intermingled with neutrophils and lymphocytes (Fig. 1G). Marked basophil recruitment into periappendageal regions was seen together with eosinophils in eosinophilic pustular folliculitis (Fig. 2A), as presented in our recent report (Satoh et al., Acta Derm-Venereol, in press, doi: 10.2340/00015555-1052). In dermatomyositis, basophil accumulation occurred in skin lesions of itchy erythema on the trunk, but they were absent in Gottron's papules (not depicted). On the contrary, no basophils were detected in

Table 1 Sample profiles and basophil staining results

Disease	Number of samples	Number of basophil (+) samples
Atopic dermatitis		
Lesional skin	21	13
Patch tested site	2	2
Prurigo		
Prurigo chronica	17	13
Prurigo subacuta	8	4
Urticaria	10	6
Psoriasis vulgaris	5	0
Mastocytosis	2	0
Pemphigus vulgaris	8	1
Bullous pemphigoid	10	9
Drug eruption		
Erythema multiforme type	5	2
Stevens-Johnson syndrome	6	4
Toxic epidermal necrosis	2	0
Classical-type of eosinophilic pustular folliculitis (Ofuji's disease)	3	3
Henoch-Schönlein purpura	6	5
Varicella/Herpes zoser	3	1
Insect bites (*Cimex lectularius*)	1	1
Tick bites (*Ixodes persulcatus*)	1	1
Scabies (*Sarcoptes scabiei var. hominis*)	2	2
Tinea corporis	1	1
Seborrheic keratosis	3	0
Nevus cell nevus	4	0
Basal cell carcinoma	3	0
Eosinophilic fascilitis	1	0
Connective tissue disease		
Systemic sclerosis	4	0
Systemic lupus erythematosus	3	0
Dermatomyositis	5	2
Total	136	–

skin lesions of psoriasis vulgaris (Fig. 2B), mastocytosis (Fig. 2C), toxic epidermal necrolysis, benign and/or malignant tumors, systemic sclerosis. Similarly, basophils were absent in skin lesions of systemic lupus erythematosus, although a recent study suggested the involvement of basophils in its pathological etiology (24). Notably, basophils were scarcely detectable around tick mouthparts of *Ixodes persulcatus* (Fig. 2E), whereas papular skin lesions of scabies showed tissue basophilia (Fig. 2F).

Basophil density

Dermal densities of basophils in skin diseases were highly variable (Fig. 3). In general, urticarial lesions, bullous pemphigoid and eosinophilic pustular folliculitis showed high basophil densities, whereas those in prurigo and Henoch–Schönlein purpura were moderate. Skin lesions in AD showed the lowest tissue basophil densities among diseases where basophils were detected.

Skin diseases with basophil recruitment were commonly accompanied by tissue eosinophilia. Thus, we attempted to assess the relative accumulation of basophils to eosinophils. The mean number of basophilis in prurigo, long-lasting urticaria, bullous pemphigoid, eosinophilic pustular folliculitis was comparable to that of eosinophils; the ratios of basophils/eosinophils were approximately 1. In contrast, the ratio of basophils/eosinophils was extremely low in AD lesions (Fig. 4). In Henoch–Schönlein purpura, basophil was predominant when compared with eosinophil recruitment.

Basophils are activated in blood of inflammatory skin diseases

Given the evidence of basophil recruitment in some skin diseases, we next aimed to verify whether these diseases were accompanied by blood basophil activation. To this end, we assessed basal levels of CD203c expression (25) by flow cytometry (Fig. 5A). In this set of experiments, blood samples from urticaria patients were obtained during the onset of urticarial rash. As expected, levels of CD203c expression of blood basophils were higher in prurigo and urticaria than in healthy donors (Fig. 5B). Activation levels of blood basophils in psoriasis patients were comparable to those in healthy donors. Basophils from AD showed varying levels of CD203c, but it was noted that some patients had high basophil activation levels in their blood. Fifteen of 17 AD patients had the extrinsic type (IgE: mean, 13 296.5 IU/ml; range 413–31 728 IU/ml), but there was no correlation between IgE levels and CD203c expression (data not shown). We also analyzed a correlation between basophil CD203c and numbers of blood Th2 cells (CRTH2+/CD3+ cells), but a statistically significant correlation did not occur (n = 55, Spearman's correlation) (data not shown).

Discussion

While little attention has been paid to the pathophysiological roles of basophils in allergic reactions because of their redundant functions with mast cells, recent studies have revealed critical roles for basophils in innate and acquired immunity in mice (15, 16, 20–22). Thus, we were interested in basophil recruitment in human skin diseases, which has not been studied in detail. To this end, we performed immunohistochemical analysis with basophil-specific BB1 antibody and demonstrated that basophils more frequently infiltrate in a number of skin diseases than previously thought.

In AD patients, basophils were detected in lesional skin, but the cell density was sparse. In contrast, marked basophil accumulation was detected in patch-tested sites for house dust mite allergen, which was consistent with results from an older study (11). The low basophil density in lesional skin could be due to the fact that the AD tissue samples in this study were generally from chronic skin lesions where local cytokine profiles were biased toward Th1 (26).

In mouse models, IgE induces urticarial and late-phase responses followed by the long-lasting, third phase inflammation with significant eosinophil infiltration and occasional