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Does β -Hexosaminidase Function Only as a Degranulation Indicator in Mast Cells? The Primary Role of β -Hexosaminidase in Mast Cell Granules

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β -Hexosaminidase, which is generally present in the lysosome, is essential for glycoprotein metabolism in the maintenance of cell homeostasis. In mast cells (MCs), large amounts of β -hexosaminidase are present in the granules as opposed to the lysosome, and the biological role of MC β -hexosaminidase has yet to be fully elucidated. Therefore, we investigated the biological role of β -hexosaminidase in MC granules. Bone marrow-derived MCs from C57BL/6 (BL/6-BMMC) or β -hexosaminidase gene-deficient (*hexb*^{-/-}-BMMC) mice were transplanted into MC-deficient (WBB6F1/J-*Kit*^{W/Kit}^{W-v} [*W/W*^v]) mice to generate MC-reconstituted models. In asthma model experiments, no differences were observed in the symptoms of BL/6, *W/W*^v, BL/6-BMMC-reconstituted *W/W*^v, or *hexb*^{-/-}-BMMC-reconstituted *W/W*^v mice. In *Staphylococcus epidermidis* experimental infection model experiments, the severity of symptoms and frequency of death were markedly higher in *W/W*^v and *hexb*^{-/-}-BMMC-reconstituted *W/W*^v mice than in BL/6 and BL/6-BMMC-reconstituted *W/W*^v mice. The growth of *S. epidermidis* in an in vitro study was clearly inhibited by addition of BL/6-BMMC lysate, but not by addition of *hexb*^{-/-}-BMMC lysate. Moreover, suppression of bacterial proliferation was completely recovered when bacteria were incubated with *hexb*^{-/-}-BMMC lysate plus β -hexosaminidase. Transmission electron microscopy indicated that the cell wall of *S. epidermidis* was heavily degraded following coinubation of bacteria with BL/6-BMMC lysate, but not following coinubation with *hexb*^{-/-}-BMMC lysate. These findings strongly suggest that MC granule β -hexosaminidase is crucial for defense against bacterial invasion, but is not involved in the allergic response. Our results also suggest that the bactericidal mechanism of β -hexosaminidase involves degradation of bacterial cell wall peptidoglycan. *The Journal of Immunology*, 2014, 193: 000–000.

The enzyme β -hexosaminidase (Enzyme Commission No. 3.2.1.52) cleaves the terminal linked *N*-acetylhexosamine residues in *N*-acetyl- β -hexosaminides (1, 2). In the lysosome, removal of the terminal linked *N*-acetyl- β -D-galactosamine from GM2 ganglioside is catalyzed by β -hexosaminidase (1). Three β -hexosaminidase isozymes composed of a combination of α and β subunits have been identified and are designated β -hexosaminidase A (HexA), β -hexosaminidase B (HexB), and β -hexosaminidase S (HexS) (3). HexA is a heterodimer composed of an α and β subunit, whereas HexB and HexS consist of β and α subunit homodimers, respectively (4). In mammalian cells, HexA and HexB are the major forms of β -hexosaminidase, whereas

HexS is a minor form and is physiologically labile (5). Mutations in *hexb*, the gene encoding the β subunit, disrupt HexA and HexB activity, resulting in massive accumulation of GM2 ganglioside, especially in neuronal cells of the CNS (6). This type of gangliosidosis is termed Sandhoff disease and presents with clinically serious symptoms, including motor function deterioration, blindness, seizures, hepatosplenomegaly, and psychosis (6–8). Thus, lysosomal β -hexosaminidase is important for ganglioside metabolism and cell homeostasis. In mast cells, most of the β -hexosaminidase is localized in the granules rather than in the lysosome. After the exposure by harmless Ag such as ragweed pollen for sensitization, Ag-specific IgE-bound Fc ϵ RI are cross-linked, and then mast cell granules are released (9, 10). As well, invasion by harmful microbes or exposure to the components of bacteria (11–13), hyphae (14), or toxins generated by infecting microbes (15–17) also lead to mast cell degranulation. As a result, many of the mediators in the granules, including β -hexosaminidase, spread through the tissues, triggering typical allergic reactions for exclusion of harmless Ag or the inflammatory response designed to eliminate harmful substances (9). However, although β -hexosaminidase is used as a typical marker of mast cell degranulation, the physiological or pathophysiological roles of β -hexosaminidase in mast cells remain unclear. To reveal the roles of β -hexosaminidase in the granules of mast cells, in this study we examined its function with respect to both the elimination of harmless substances and harmful intruders. We generated bone marrow-derived mast cells (BMMCs) from C57BL/6 (BL/6) mice (BL/6-BMMCs), from *hexb*^{-/-} BL/6 mice (*hexb*^{-/-}-BMMCs), and from mast cell-deficient WBB6F1/J-*Kit*^{W/Kit}^{W-v} (*W/W*^v) mice and employed experimental asthma and infection models using these mice.

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Abbreviations used in this article: alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; BL/6, C57BL/6; BMMC, bone marrow-derived mast cell; HexA, β -hexosaminidase A; HexB, β -hexosaminidase B; HexS, β -hexosaminidase S; HSA, human serum albumin; iNKT, invariant NKT; PGN, peptidoglycan; *W/W*^v, WBB6F1/J-*Kit*^{W/Kit}^{W-v}.

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All of the asthma models we generated exhibited very similar behaviors and symptoms. Infection with *Staphylococcus epidermidis* was substantially more lethal to W/W^v and $hexb^{-/-}$ -BMMC- W/W^v mice than BL/6 and BL/6-BMMC- W/W^v mice, and the symptoms were more severe in W/W^v and $hexb^{-/-}$ -BMMC- W/W^v mice than in BL/6 and BL/6-BMMC- W/W^v mice. We also studied the roles of mast cell β -hexosaminidase in the exclusion of bacteria. In vitro studies revealed that although incubating *Bacillus cereus* or *S. epidermidis* with BL/6-BMMC lysate caused marked inhibition of bacterial growth, $hexb^{-/-}$ -BMMC lysate did not inhibit proliferation of these bacteria. Furthermore, transmission electron microscopic findings showed that incubating *S. epidermidis* with BL/6-BMMC lysate caused marked disruption of the cell wall, although incubation with $hexb^{-/-}$ -BMMC lysate did not destroy the bacterial cell wall. Moreover, when incubated with peptidoglycan (PGN), BL/6-BMMC lysate, but not $hexb^{-/-}$ -BMMC lysate, generated massive amounts of *N*-acetylglucosamine, a primary component of cell wall PGN. These findings indicate that β -hexosaminidase contained in mast cell granules is crucial for the self-defense responses but is not relevant to allergic reactions.

Materials and Methods

Animals

BL/6 and W/W^v mice (Japan SLC, Shizuoka, Japan) were used for all experiments. The $hexb^{-/-}$ mice (BL/6 $\times$ 129sv) were provided by Dr. S. Yamanaka (18). Animal studies were approved by the Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences at Tokushima Bunri University.

Reagents

Mouse IL-3 and FcR blocking reagent were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Ab against IL-4 (11B11) conjugated with allophycocyanin was purchased from BioLegend (San Diego, CA). Ab against Fc ϵ RI α (MAR-1) conjugated with FITC was purchased from eBioscience (San Diego, CA). Abs against CD117 (2B8) conjugated with PE and against IFN- γ conjugated with PE-Cy7 were purchased from BD Pharmingen (Franklin Lakes, NJ). Aluminum hydroxide (alum) was purchased from Thermo Scientific (Rockford, IL). L-Glutamine, sodium pyruvate, nonessential amino acids, and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). 2-ME was purchased from Kanto Chemical (Tokyo, Japan). FBS was purchased from Equitech-Bio (Kerrville, TX). RPMI 1640, OVA, β -*N*-acetylhexosaminidase, anti-DNP IgE (SPE-7), and DNP-human serum albumin (HSA) were obtained from Sigma-Aldrich (St. Louis, MO).

BMMC culture

BM cells were isolated from 5- to 8-wk-old BL/6 and $hexb^{-/-}$ mice, which were maintained and used in accordance with guidelines of Tokushima Bunri University. BM cells were maintained as described previously (19, 20). Briefly, primary BM cells were maintained for 2 wk in RPMI 1640 medium containing 10% heat-inactivated FCS, 50 pM 2-ME, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, penicillin/streptomycin, and 10% (v/v) PWM-conditioned medium. The cells were then continuously maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 pM 2-ME, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, penicillin/streptomycin, and 5 ng/ml IL-3 for an additional 2 wk. Cultures were monitored using an Epics Altra (Beckman Coulter, Brea, CA) for surface expression of Fc ϵ RI and CD117 and used for experiments when >95% of the cells were Fc ϵ RI-CD117 double positive. Cultures of 4–7 wk were used in these experiments.

β -Hexosaminidase release assay

BMMCs (1×10^5 cells/ml) were incubated for 16 h with 1 μ g/ml DNP-IgE. Surface-bound IgE was cross-linked for 30 min at 37°C using DNP-HSA. Reactions were stopped by placing the cells on ice. β -Hexosaminidase contained in the supernatant and cell lysate was incubated with 26 mM citrate buffer (pH 4.5) and 2.3 mg/ml *p*-nitrophenyl *N*-acetyl- α -D-glucosaminide for 60 min at 37°C. The reaction was developed by adding 0.4 M glycine (pH 10.7), and the absorbance was measured at 405 and 570 nm using a multiplate reader. The percentage of β -hexosaminidase release was calculated as a percentage of the total β -hexosaminidase content.

Histamine assay

BMMCs (5×10^5 cells/ml) were incubated for 16 h with 1 μ g/ml DNP-IgE. Surface-bound IgE was cross-linked for 30 min at 37°C using DNP-HSA. Reactions were stopped by placing the cells on ice. The samples were centrifuged at 1500 rpm for 5 min at 4°C. The supernatants were separated from the pellets, and saline containing 1% Triton X-100 (Sigma-Aldrich) was added to the pellet to extract the residual histamine from the cells. Hydrochloric acid was then added to the supernatants and the pellet lysates and the histamine content was determined using a precolumn HPLC method, as described previously (20–22).

Experimental asthma model

OVA (100 μ g/100 μ l saline) with 100 μ l alum was injected into the peritoneal cavity of mice on days 0 and 5, and then mice were subjected to inhalation of 3% OVA solution or saline for 30 min on days 12, 13, and 14. Twenty-four hours after the last inhalation, mice were sacrificed under anesthesia and blood and bronchoalveolar lavage fluid (BALF) were collected. Serum was prepared for measurement of serum anti-OVA IgE.

Measurement of anti-OVA IgE

Serum anti-OVA IgE was measured by ELISA. Briefly, affinity-purified anti-mouse IgE (Bethyl Laboratories, Montgomery, TX) was coated onto a 96-well plate. After blocking, serum from sensitized or nonsensitized mice was applied and the plate was incubated for 1 h at room temperature. After washing, HRP-conjugated OVA (AbD Serotec, Raleigh, NC) was added and the plate was incubated for 1 h at room temperature. After washing, 3,3',5,5'-tetramethylbenzidine solution (Thermo Scientific) was added and the plate was incubated for 45 min at room temperature, after which H_2SO_4 was added to stop the reaction. The absorbance at 405 nm was measured using a multiplate reader.

Flow cytometry

BALF was centrifuged at 1500 rpm for 5 min at room temperature, after which the supernatant was discarded and cells were washed with RPMI 1640 medium. The BALF cells were resuspended in RPMI 1640 and incubated with FcR blocking reagent for 30 min on ice. After washing, resuspended BALF cells were stained with Abs for 30 min at room temperature in the dark. Stained cells were analyzed using an Epics Altra (Beckman Coulter).

Preparation of tissue sections

Mice were anesthetized and sacrificed and the organs were quickly removed and immersed in fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer. After washing with water, the fixed organs were cut into half blocks. The blocks were dehydrated in graded ethanol and embedded in paraffin using xylene as an intermediate. The half blocks were cut into 10- μ m coronal sections. The paraffin sections were stained with hematoxylin and counterstained with eosin or toluidine blue.

Bacterial culture

Cultures of *Bacillus subtilis*, *Escherichia coli*, *B. cereus*, *Serratia marcescens*, *Staphylococcus aureus*, and *S. epidermidis* were gifts from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University. Bacteria were precultured in lactose broth medium (Nissui Pharmaceutical, Tokyo, Japan) for 12 h at 37°C with shaking at 170 rpm. A 100- μ l aliquot of precultured bacteria was added to 1 ml lactose broth with or without BMMC lysate or supernatant of degranulated BMMCs. The absorbance at 620 nm was measured using a spectrophotometer.

Infection experiments

S. epidermidis was preincubated in lactose broth medium and then centrifuged at 5000 rpm for 15 min at room temperature, after which the supernatant was discarded. The pellet was washed twice with 20 ml saline, resuspended in saline, and adjusted to a concentration of 8×10^8 particles/200 μ l. The bacterial suspension was injected into the peritoneal cavity of BL/6, W/W^v , BL/6-BMMC- W/W^v , and $hexb^{-/-}$ - W/W^v mice. The survival rate and behavior were observed for up to 80 h.

Evaluation of live *S. epidermidis* in the peritoneal fluid from infected mice

After infection, the peritoneal fluid was collected from infected mice (at 80 h or upon death) and the fluid was appropriately diluted with sterile saline and inoculated onto lactose broth agar plates. Bacterial colonies were counted after 24 h of incubation at 37°C to evaluate the number of live *S. epidermidis* in the peritoneal cavity.

Evaluation of immune cells in the peritoneal fluid from uninfected or infected mice

Each group of mice was inoculated i.p. with *S. epidermidis*. After 24 h of infection, the peritoneal fluid was collected and the total cell number was assessed using a hemacytometer. The collected cells were then stained by May–Grünwald–Giemsa staining, and the cell numbers of neutrophils, monocytes, lymphocytes, and eosinophils were separately counted under the microscope.

Transmission electron microscopy

After incubation for 6 h with or without cell lysate and/or enzymes, *S. epidermidis* was fixed and processed for transmission electron microscopy as previously described (23). Briefly, bacteria were harvested and washed twice with PBS, pelleted, and fixed with 2.5% glutaraldehyde followed by 1% OsO₄. The specimens were dehydrated by passage through an ethanol series and then embedded in Spurr's/Epon resin. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate and examined at 80 kV using a JEOL JEM-2000EXII transmission electron microscope (JEOL, Tokyo, Japan).

Purification of PGN and liquid chromatography–electrospray mass spectrometry analysis of N-acetylglucosamine

S. epidermidis was cultured as described above. The bacterial cell wall and PGN were purified and prepared as previously described (24). Briefly, bacteria were washed with distilled water three times and then resuspended in PBS (pH 7.4) and sonicated for 45 min to degrade the cell wall. This suspension was centrifuged at 5000 × *g* for 30 min at room temperature to remove undegraded cell walls and whole bacteria. The supernatant was collected and centrifuged at 18,000 × *g* for 30 min, and the pellet was resuspended in 4% SDS solution. The suspension was incubated for 40 min at 100°C, centrifuged at 18,000 × *g* for 30 min, and the resulting pellet was washed twice with distilled water. The final pellet was used as partially purified cell wall. The cell wall material was extracted from the pellet using 5% trichloroacetic acid and chloroform. The aqueous phase was collected and PBS, ethanol, and diethyl ether were added and the mixture was centrifuged at 12,000 × *g* for 30 min. The collected pellet was used as PGN. PGN was then incubated with or without 1600 U lysozyme or 10 mU β-hexosaminidase at 37°C for 16 h. The suspension was then centrifuged at 14,000 × *g* for 10 min and the supernatant was collected. Using commercially purchased N-acetylglucosamine (Sigma-Aldrich) as a standard, the concentration of N-acetylglucosamine in the supernatant was determined by

liquid chromatography–electrospray mass spectrometry (series 1100MSD; Hewlett-Packard, Palo Alto, CA) using a TSK-GEL amide-80 column (4.6 × 250 mm; Tosoh, Tokyo, Japan).

Statistical analysis

Statistical significance was determined using a two-tailed unpaired Student *t* test for comparison of differences between two groups or the Student–Newman–Keuls test for comparison of differences between three or more groups. Differences were considered significant at *p* < 0.05. Data are expressed as the means ± SEM.

Results

Basic properties of *hexb*^{−/−}-BMMCs are similar to those of BL/6-BMMCs

We first examined the surface expression of FcεRI and c-Kit, β-hexosaminidase activity, histamine content, Safranin O and Alcian blue staining properties, and the degranulation ratio of BL/6-BMMCs and *hexb*^{−/−}-BMMCs. In BL/6-BMMCs, FcεRI expression was observed after 7 d and was complete by 21 d. Expression of c-Kit (CD117) began by 14 d and was detected on most of the cells by 28 d. The trend in surface expression of FcεRI and c-Kit was exactly the same in *hexb*^{−/−}-BMMCs (Fig. 1A). β-Hexosaminidase activity was determined by spectrophotometry. Although a correlation between β-hexosaminidase activity and cell number was apparent with BL/6-BMMCs, no β-hexosaminidase activity was detected in *hexb*^{−/−}-BMMCs (Fig. 1B). There was no significant difference in histamine content (0.835 ± 0.130 pg/cell in BL/6-BMMCs and 1.007 ± 0.063 pg/cell in *hexb*^{−/−}-BMMCs) (Fig. 1C). The granules of both BMMC types were Safranin O[−] and Alcian blue⁺ (Fig. 1D). The degranulation ratio was assessed by histamine release instead of β-hexosaminidase measurement. Degranulation caused by IgE cross-linking reached its submaximal peak with stimulation by 250 ng/ml Ab. No significant difference in degranulation resulting from Ag stimulation was observed between the BMMC types (Fig. 1E).

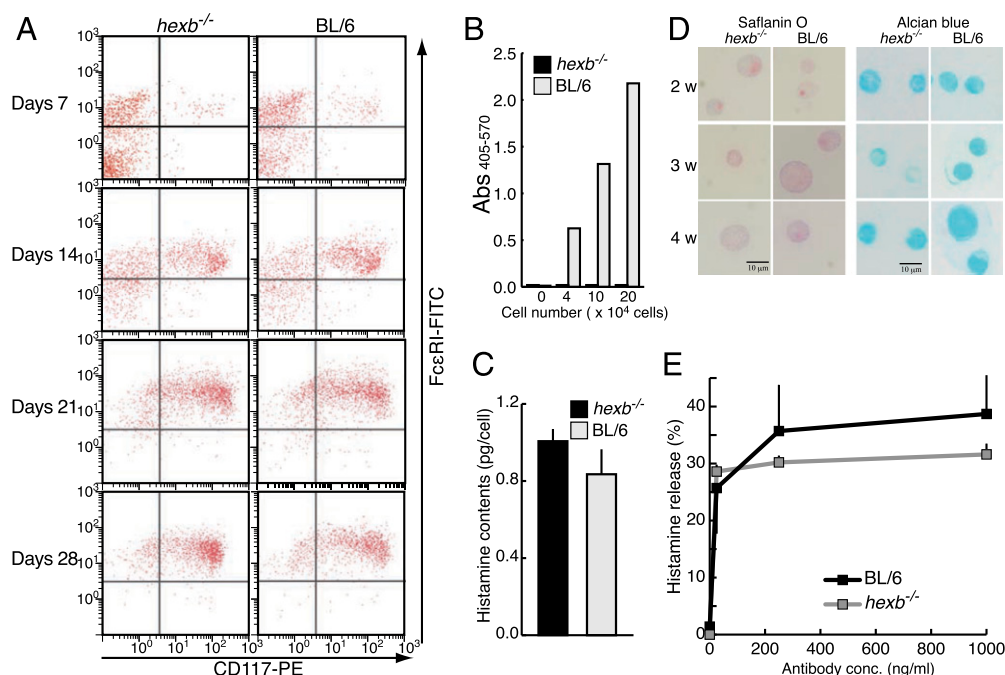


FIGURE 1. Comparison of the profiles of BL/6-BMMCs and *hexb*^{−/−}-BMMCs. **(A)** Expression of FcεRI and CD117 on bone marrow (BM) cells from β-hexosaminidase gene-deficient (*hexb*^{−/−}) mice and wild-type (BL/6) mice. Representative histograms of three independent experiments are shown. **(B)** Content of β-hexosaminidase in BMMCs generated from *hexb*^{−/−} or BL/6 mice (*n* = 3). **(C)** Histamine content in *hexb*^{−/−}-BMMCs and BL/6-BMMCs (*n* = 3). **(D)** Staining properties of BMMCs. Representative micrographs of three independent experiments are shown. **(E)** Degranulation ratio in *hexb*^{−/−}-BMMCs and BL/6-BMMCs.

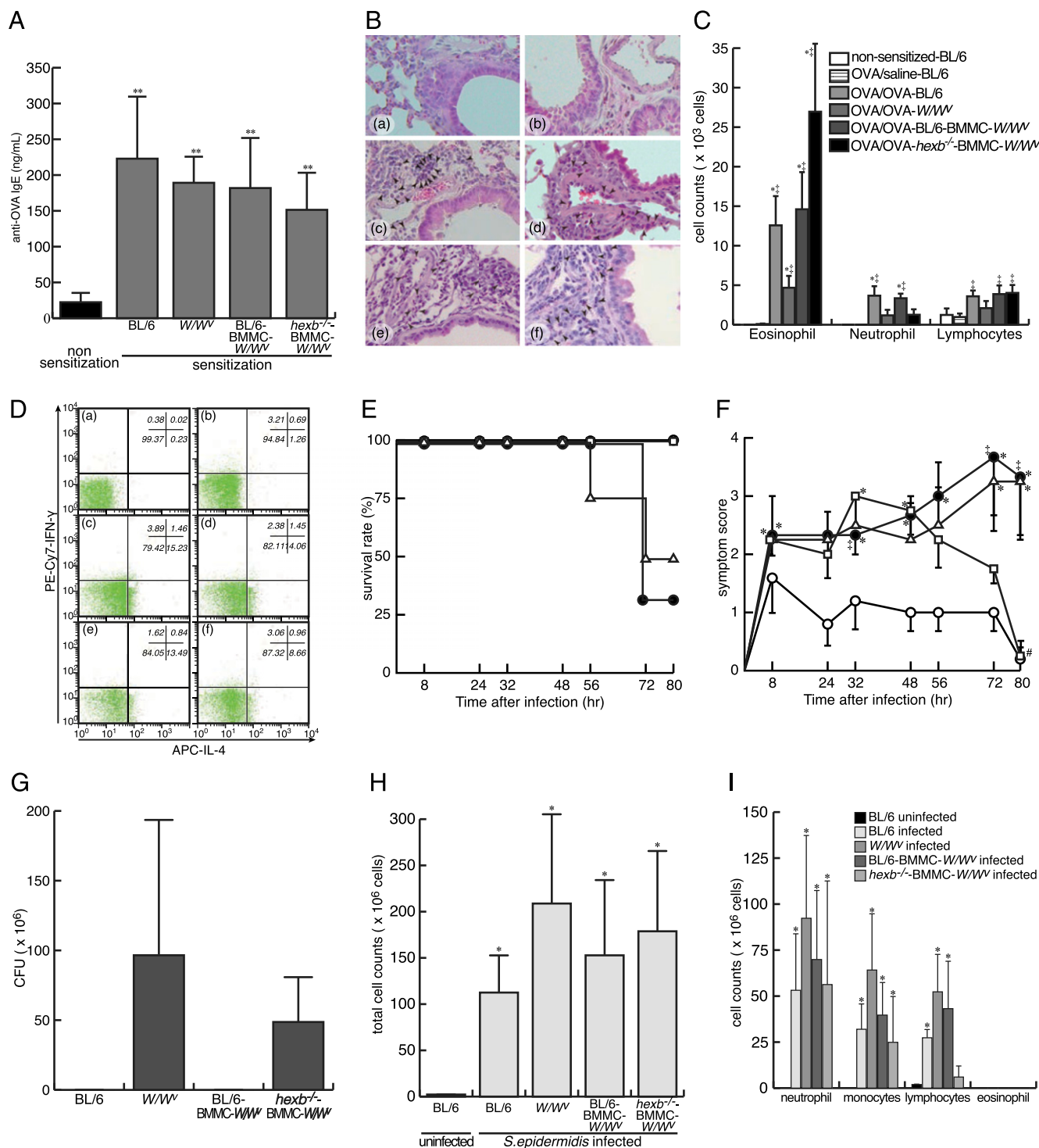
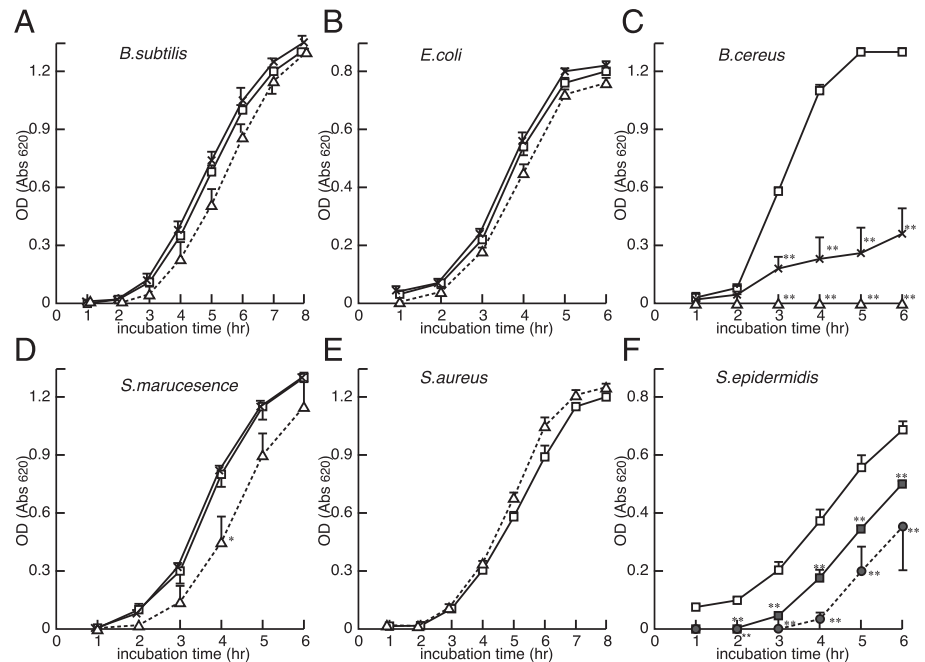


FIGURE 2. Properties of BL/6, W/W^v, hexb^{-/-}-BMMC-W/W^v, and BL/6-BMMC-W/W^v asthma and infection models. BL/6 mice, W/W^v mice, hexb^{-/-}-BMMC-reconstituted mice (hexb^{-/-}-BMMC-W/W^v), and BL/6-BMMC-reconstituted mice (BL/6-BMMC-W/W^v) were sensitized with OVA and alum. After 14 d, the mice were subjected to inhalation of aerosolized Ag three times during 3 d. Changes in the level of OVA-specific IgE in serum (**A**), accumulation of inflammatory cells in the lung assessed by H&E staining at 200-fold magnification. ***p* < 0.01 versus nonsensitization; *n* = 4–8. (**B**), number of inflammatory cells in BALF (**C**), and IL-4/IFN-γ production in T lymphocytes prepared from BALF (**D**) were assessed. (**Ba**) Nonsensitized BL/6 mice. (**Bb**) OVA-sensitized and saline-challenged BL/6 (OVA/saline-BL/6) mice. (**Bc**) OVA-sensitized and OVA-challenged BL/6 (OVA/OVA-BL/6) mice. (**Bd**) OVA-sensitized and OVA-challenged W/W^v (OVA/OVA-W/W^v) mice. (**Be**) OVA-sensitized and OVA-challenged BL/6-BMMC-W/W^v (OVA/OVA-BL/6-BMMC-W/W^v) mice. (**Bf**) OVA-sensitized and OVA-challenged hexb^{-/-}-BMMC-W/W^v (OVA/OVA-hexb^{-/-}-BMMC-W/W^v) mice. (**Da**) Nonsensitized BL/6 mice stained by isotypic control. (**Db**) Nonsensitized BL/6 mice stained by specific Abs. (**Dc**) OVA/OVA-BL/6 mice stained by specific Abs. (**Dd**) OVA/OVA-W/W^v mice stained by specific Abs. (**De**) OVA/OVA-BL/6-BMMC-W/W^v mice stained by specific Abs. (**Df**) OVA/OVA-hexb^{-/-}-BMMC-W/W^v mice stained by specific Abs. Representative micrographs of three independent experiments are shown in (**B**), and representative histograms of three independent experiments are shown in (**D**). (**E**) A total of 8 × 10⁸ CFUs *S. epidermidis* were injected into the abdominal cavity of BL/6 (○), W/W^v (△), BL/6-BMMC-W/W^v (□), and hexb^{-/-}-BMMC-W/W^v (●) mice and the survival rates were assessed; *n* = 4–6. (**F**) Symptom scores (1, mild; 2, moderate; 3, severe; 4, serious) were assessed. **p* < 0.05 versus BL/6 mice; #*p* < 0.05 versus W/W^v mice; †*p* < 0.05 versus BL/6-BMMC-W/W^v mice; *n* = 4–8. (**G**) Peritoneal fluid was collected from infected mice, and the diluted (Figure legend continues)

FIGURE 3. Effect of BL/6-BMMC lysate on bacterial growth. The effect of BMMC lysate on the growth of *B. subtilis* (A), *E. coli* (B), *B. cereus* (C), *S. marcescens* (D), *S. aureus* (E), and *S. epidermidis* (F) was examined. These bacteria were incubated without (—□—) or with the lysate of 5×10^4 (—■—), 1×10^5 (—●—), 2×10^5 (—x—), or 1×10^6 (—△—) BMMCs. Although growth of *S. epidermidis* and *B. cereus* was markedly inhibited by BMMC lysate, growth of the other bacteria examined was not affected by addition of lysate. * $p < 0.05$ versus bacteria alone; ** $p < 0.01$ versus bacteria alone; $n = 3$.



Mast cell granule β -hexosaminidase contributes to host defenses against infection but is not involved in allergic asthma

We generated BL/6-BMMC-reconstituted mice (BL/6-BMMC- W/W^v) and *hexb*^{-/-}-BMMC-reconstituted mice (*hexb*^{-/-}-BMMC- W/W^v) using in vitro-differentiated BL/6-BMMCs and *hexb*^{-/-}-BMMCs with mast cell-deficient W/W^v mice. No substantial changes in the organ distribution of BMMCs were observed in the reconstituted mice (Supplemental Fig. 1). Asthma model mice were generated using C57BL/6, W/W^v , BL/6-BMMC- W/W^v , and *hexb*^{-/-}-BMMC- W/W^v mice. OVA-specific IgE in sera was markedly elevated following sensitization and/or challenge, and airway hypersensitivity increased after challenge (Supplemental Fig. 2). Fig. 2A shows the concentration of OVA-specific IgE in serum. The specific IgE level was markedly increased in sensitized mice compared with nonsensitized mice, and there was no significant difference in the specific IgE level between the four groups of sensitized mice. Pathologic analyses showed a marked accumulation of inflammatory cells in the lungs of Ag-challenged BL/6 (OVA/OVA-BL/6) (Fig. 2Bc), W/W^v (OVA/OVA- W/W^v) (Fig. 2Bd), BL/6-BMMC- W/W^v (OVA/OVA-BL/6-BMMC- W/W^v) (Fig. 2Be), and *hexb*^{-/-}-BMMC- W/W^v (OVA/OVA-*hexb*^{-/-}-BMMC- W/W^v) (Fig. 2Bf) mice. In contrast, almost no infiltration was observed in the lungs of nonsensitized BL/6 (Fig. 2Ba) and saline-challenged BL/6 (OVA/saline-BL/6) (Fig. 2Bb) mice. The number of infiltrating cells appeared to be slightly lower in the lungs of OVA/OVA- W/W^v mice (Fig. 2Bd) compared with the other Ag-challenged mice (Fig. 2Bc, 2Be, and 2Bf). Flow cytometry analysis showed that the number of eosinophils in BALF was significantly higher in all of the OVA/OVA mice compared with the nonsensitized and OVA/saline mice (Fig. 2C). Eosinophil, neutrophil, and lymphocyte numbers tended to be slightly lower in OVA/OVA- W/W^v mice compared with the other groups of OVA/OVA mice (Fig. 2C). The production of IL-4 and IFN- γ by lymphocytes in BALF was examined using flow cytometry. The IL-4-generating

lymphocytes from nonsensitized and OVA/saline mice composed 0.25% (0.02 + 0.23%) and 1.95% (0.69 + 1.26%) of the lymphocyte population, respectively (Fig. 2Da, 2Db). Although the percentage of IFN- γ -producing lymphocytes in BALF from the OVA/OVA groups of mice was very similar to that in BALF from OVA/saline mice, the percentage of IL-4-producing cells increased markedly in the OVA/OVA groups of mice (Fig. 2Dc-f). No significant change in the percentage of IL-4-producing lymphocytes was observed among the OVA/OVA groups.

To evaluate the role of mast cell β -hexosaminidase in the self-defense system, *S. epidermidis* cells were injected into the peritoneal cavities of BL/6, W/W^v , BL/6-BMMC- W/W^v , and *hexb*^{-/-}-BMMC- W/W^v mice. Although none of the BL/6 mice died, two of the W/W^v mice died 56 h after being infected, and two more mice died 72 h after being infected. Transplantation of BL/6-BMMCs into W/W^v mice prior to infection resulted in a 100% survival rate. A total of four of the *hexb*^{-/-}-BMMC- W/W^v mice died after being infected, resulting in a cumulative survival rate of 33% at 80 h (Fig. 2E). The symptoms of infected mice were monitored until 80 h. The symptoms associated with the inoculation went into a transient decline at 8 h in BL/6 mice, and these mice had a relatively stable course of infection in which the symptoms did not become exacerbated (Fig. 2F). In W/W^v mice, however, the symptoms resulting from *S. epidermidis* infection were more severe between 8 and 80 h (Fig. 2F). Although the progression of infection up until 48 h was almost the same in both BL/6-BMMC- W/W^v and W/W^v mice, the severity of symptoms diminished in the BL/6-BMMC- W/W^v mice after 48 h. The course of infection in *hexb*^{-/-}-BMMC- W/W^v mice was exactly the same as that of W/W^v mice (Fig. 2F). Although no CFUs were observed on plates inoculated with peritoneal fluid from BL/6 and BL/6-BMMC- W/W^v mice, many CFUs were observed on plates inoculated with fluid from W/W^v and *hexb*^{-/-}-BMMC- W/W^v mice (Fig. 2G). Because some W/W^v and *hexb*^{-/-}-BMMC- W/W^v mice

fluid was inoculated onto agar plates. The bacterial colonies were counted to evaluate the number of live *S. epidermidis* in the peritoneal cavity; $n = 4$. (H) After 24 h of infection, the peritoneal fluid from each group of mice was collected and the total immune cell number was assessed; $n = 4$. (I) The collected cell numbers of neutrophils, monocytes, lymphocytes, and eosinophils were separately counted; $n = 4$. * $p < 0.05$ versus BL/6 uninfected mice.

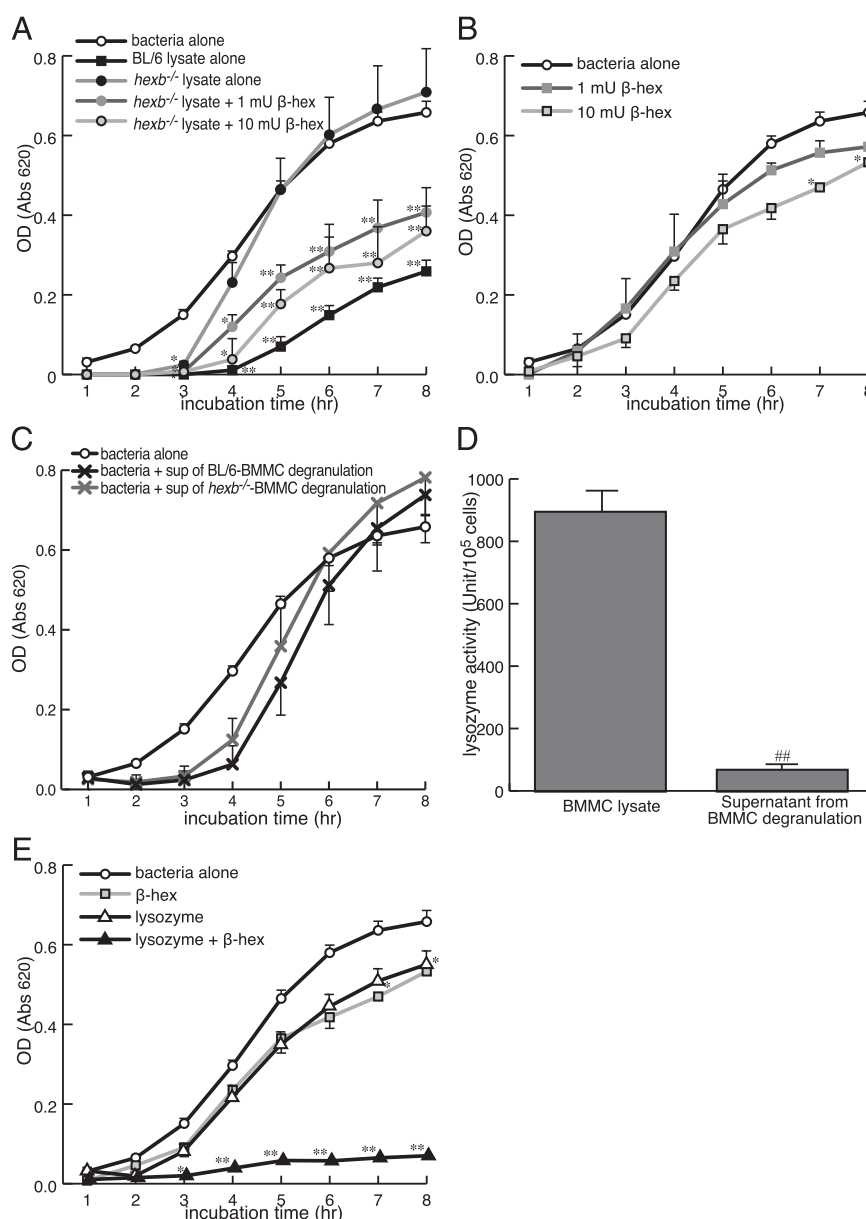
died during the experiment, the number of *S. epidermidis* CFUs in the peritoneal fluid was determined separately in living and dead mice (Supplemental Fig. 3A). Few CFUs were observed in samples from living mice, whereas a large number of CFUs were observed in samples from the dead mice (Supplemental Fig. 3A). The change of cell numbers in the peritoneal cavity by inoculation of *S. epidermidis* into the peritoneal cavity was evaluated. After 24 h of infection, the peritoneal fluid was collected and the total cell number was assessed using a hemacytometer (Fig. 2H). The collected cells were then stained by May-Grünwald-Giemsa staining, and the cell numbers of neutrophils, monocytes, lymphocytes, and eosinophils were separately counted under the microscope (Fig. 2I). Although the cell number was markedly increased following the infection, the difference was not observed among *S. epidermidis*-infected BL/6, W/W^v, BL/6-BMMC-W/W^v, and *hexb*^{-/-}-BMMC-W/W^v mice.

*BL/6-BMMC lysate, but not *hexb*^{-/-}-BMMC lysate, exhibits bactericidal activity against a kind of Gram-positive bacteria*

Various bacteria were cultured with and without BMMC lysate to investigate the effect of mast cell components on bacterial growth. The growth of *B. subtilis* (Gram-positive), *E. coli* (Gram-negative),

S. marcescens (Gram-negative), and *S. aureus* (Gram-positive) was unaffected by BL/6-BMMC lysate (Fig. 3A, 3B, 3D, 3E). Proliferation of both *B. cereus* (Gram-positive) and *S. epidermidis* (Gram-positive) was markedly inhibited by the lysate in a concentration-dependent fashion (Fig. 3C, 3F). We also examined the effect of *hexb*^{-/-}-BMMC lysate on bacterial growth. In contrast to the case of BL/6-BMMC lysate, growth of *S. epidermidis* was not inhibited by *hexb*^{-/-}-BMMC lysate. However, growth of *S. epidermidis* was significantly inhibited by simultaneous exposure to both *hexb*^{-/-}-BMMC lysate and β -hexosaminidase, and the inhibition was β -hexosaminidase dose-dependent (Fig. 4A). Interestingly, incubation with β -hexosaminidase alone or with culture supernatant of degranulated BMMCs alone failed to inhibit the proliferation of *S. epidermidis* (Fig. 4B, 4C). We found that the level of lysozyme activity in the supernatant of degranulated BMMCs was much less than that in the BMMC lysate (Fig. 4D), and therefore we examined the effect of incubation in the presence of both lysozyme and β -hexosaminidase on the growth of *S. epidermidis*. Although lysozyme alone or β -hexosaminidase alone had a minimal inhibitory effect on growth, the combination of lysozyme and β -hexosaminidase significantly inhibited the growth of *S. epidermidis* (Fig. 4E).

FIGURE 4. Effect of *hexb*^{-/-}-BMMC lysate, β -hexosaminidase, and lysozyme on bacterial growth. (A) Effects of BL/6-BMMC lysate, *hexb*^{-/-}-BMMC lysate, *hexb*^{-/-}-BMMC lysate plus 1 mU β -hexosaminidase, or *hexb*^{-/-}-BMMC lysate plus 10 mU β -hexosaminidase on the growth of *S. epidermidis*. (B) Effects of 1 or 10 mU β -hexosaminidase on the growth of *S. epidermidis*. (C) Effects of the supernatant from degranulated BL/6-BMMCs or *hexb*^{-/-}-BMMCs on the growth of *S. epidermidis*. (D) β -Hexosaminidase activity in the BMMC lysate was 9-fold higher than that in the supernatant of degranulated BMMCs. The effect of the combination of lysozyme and β -hexosaminidase on the growth of *S. epidermidis* is shown. (E) Effects of β -Hexosaminidase, lysozyme, or the combination of lysozyme and β -hexosaminidase on growth of *S. epidermidis*. **p* < 0.05 versus bacteria alone, ***p* < 0.01 versus bacteria alone, ##*p* < 0.01 versus BMMC lysate; *n* = 3.



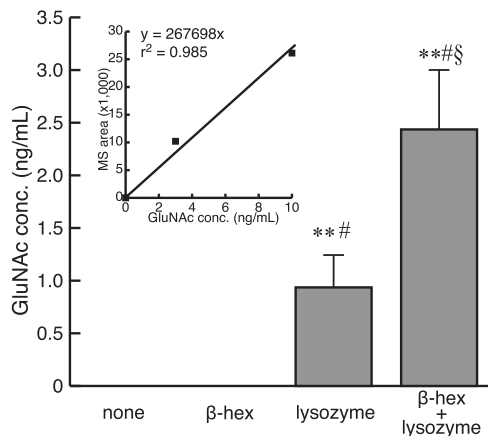


FIGURE 5. Measurement of *N*-acetylglucosamine (GlcNAc) in bacterial culture supernatant. The concentration of GlcNAc in the bacterial culture supernatant was measured by liquid chromatography/mass spectrometry. The inserted line plot indicates the standard curve. $**p < 0.01$ versus none, $^{\#}p < 0.05$ versus β -hexosaminidase, $^{\S}p < 0.05$ versus lysozyme; $n = 4$.

Incubation of *S. epidermidis* with lysozyme and β -hexosaminidase results in the release of *N*-acetylglucosamine

PGN is the primary component of the bacterial cell wall and is composed of *N*-acetylglucosamine, *N*-acetylmuramic acid, and peptides. Using liquid chromatography–electrospray mass spectrometry, we analyzed the concentration of *N*-acetylglucosamine in the supernatants of PGN prepared from *S. epidermidis* incubated in the presence of lysozyme and/or β -hexosaminidase. Although no *N*-acetylglucosamine was detected when the PGNs were incubated with β -hexosaminidase alone, *N*-acetylglucosamine was detected when the PGNs were incubated with lysozyme alone. Incubation of the PGNs with both lysozyme and β -hexosaminidase resulted in the release of a massive amount of *N*-acetylglucosamine (Fig. 5).

Transmission electron microscopy confirms disruption of the bacterial cell wall

S. epidermidis was cultured for 6 h with or without BL/6-BMMC lysate or *hexb*^{−/−}-BMMC lysate, and bacteria were then observed using transmission electron microscopy. Although the cell wall was not affected by the 6-h incubation in the absence of lysate (Fig. 6B), significant disruption of the cell wall and leakage of cytoplasmic components were observed after incubation with BL/6-BMMC lysate (Fig. 6C). Neither disruption of the cell wall nor leakage of cytoplasmic contents was observed following incubation with *hexb*^{−/−}-BMMC lysate (Fig. 6D).

Discussion

Mast cells contain a large amount of enzymes in the granules (25, 26), particularly proteases (27). These enzymes are known to be involved in inflammation (28–30) and host defense against bacteria (31, 32). Glycolytic enzymes, such as β -hexosaminidase, are also found in abundance in mast cells. About 85% of the β -hexosaminidase contained in mast cells is localized in the granules (33). Although β -hexosaminidase is commonly used as a biomarker of mast cell degranulation caused by various stimuli, the distinct physiological role of this enzyme in mast cells remains unclear and was thus the focus of this study.

We first examined the role of β -hexosaminidase in asthma to determine whether there is an association between this enzyme and allergic inflammatory diseases. The number of eosinophils in

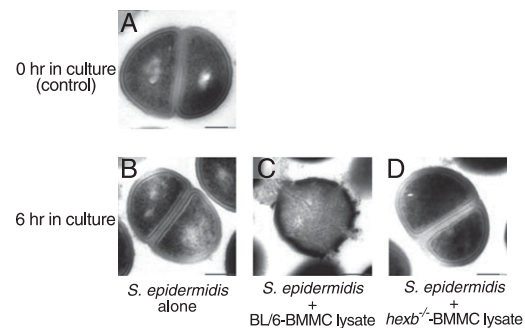


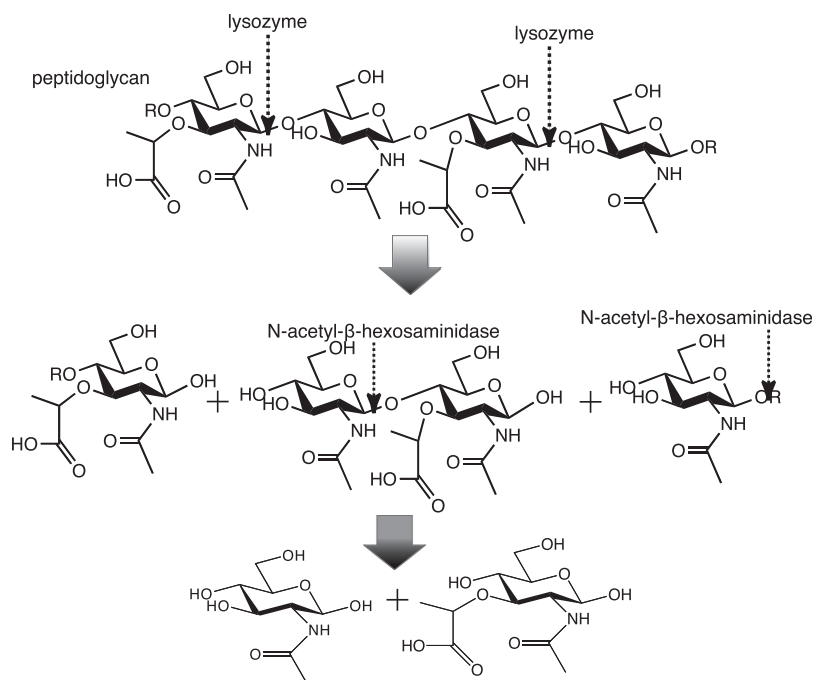
FIGURE 6. Transmission electron microscopy analysis of *S. epidermidis* after incubation with BL/6-BMMC or *hexb*^{−/−}-BMMC lysates. Transmission electron microscopy analysis of *S. epidermidis* at 0 h (A) and after 6 h of culture without (B) or with the lysates of BL/6-BMMCs (C) or *hexb*^{−/−}-BMMCs (D). Representative micrographs of three independent experiments are shown, original magnification $\times 10,000$.

BALF and the accumulation of eosinophils in the lung were similar in OVA-sensitized/challenged BL/6, BL/6-BMMC-*W/W*^v, and *hexb*^{−/−}-BMMC-*W/W*^v mice. There was also no difference in the pattern of cytokine production by lymphocytes in these mice. Furthermore, although airway resistance increased in OVA-sensitized/challenged mice compared with nonsensitized mice, this tendency was similar with sensitized/challenged BL/6, BL/6-BMMC-*W/W*^v, and *hexb*^{−/−}-BMMC-*W/W*^v mice.

Invariant NKT (iNKT) cells are known to play a crucial role in the pathogenesis of bronchial asthma (34, 35), and large numbers of iNKT cells are found in the lungs of asthmatic patients and asthmatic animal models (36, 37). Zhou and colleagues (38, 39) showed that mice lacking *hexb* exhibit a 95% reduction in the number of iNKT cells and suggested that isoglobotrihexosylceramide generated from isoglobotetrahexosylceramide by β -hexosaminidase is essential for the differentiation and development of iNKT cells. In our findings, however, although the number of iNKT cells and generation of cytokines by iNKT cells in BALF were augmented significantly by OVA sensitization and inhalation in both BL/6 and *W/W*^v mice, there were no significant differences in these parameters between groups (data not shown). These findings indicate that mast cell granule β -hexosaminidase plays little or no role in the pathophysiology of asthma.

The role of mast cells in the self-defense system has been well established (31, 32, 40, 41). For instance, mast cells sense the type 1 fimbriae of *E. coli* through surface-expressed CD48, resulting in release of the potent neutrophil chemoattractant TNF- α in a process that is pivotal for elimination of the bacteria (42). Additionally, mast cell tryptase β I reportedly plays a central role in the host defenses of the lung by recruiting neutrophils (43), and cecal ligation and puncture models demonstrated that mast cell chymase is essential for survival in cases of septic peritonitis (44). We revealed in the present study, to our knowledge for the first time, that although reconstitution of *W/W*^v mice with BL/6-BMMCs reduced the lethality of *S. epidermidis* infection, grafting of *hexb*^{−/−}-BMMCs did not reduce mortality. Additionally, although none of the live bacteria in the peritoneal cavity was observed in BL/6 and BL/6-BMMC-*W/W*^v mice, many live bacteria were observed in *W/W*^v and *hexb*^{−/−}-BMMC-*W/W*^v mice. Moreover, we evaluated the numbers of neutrophils, monocytes, macrophages, lymphocytes, and eosinophils in the abdominal cavities of *S. epidermidis*-infected BL/6, *W/W*^v, BL/6-BMMC-*W/W*^v, and *hexb*^{−/−}-BMMC-*W/W*^v mice at 24 h of infection. Although a marked increase in the number of neutrophils, monocytes, macrophages, and lymphocytes was observed in *S. epidermidis*-infected mice compared with uninfected mice, there

FIGURE 7. Proposed scheme of the cleavage of PGN by lysozyme and β -hexosaminidase. Lysozyme first nicks the endogenous β -1,4 bond between *N*-acetylglucosamine and *N*-acetylmuramic acid. At this stage, β -hexosaminidase cannot attack the β -1,4 bond because it is an exo-type enzyme. Next, the generated disaccharide consisting of *N*-acetylmuramic acid and *N*-acetylglucosamine is digested by β -hexosaminidase. As a consequence, these monomeric sugars are generated and PGN is completely digested.



were no differences among the infected groups. These observations indicate that peritoneally injected bacteria were completely eliminated from BL/6 and BL/6-BMMC-*W/W^v* mice but not from *W/W^v* and *hexb^{-/-}*-BMMC-*W/W^v* mice, although almost the same numbers of migrated immune cells were observed in infected groups. In vitro experiments demonstrated that BL/6-BMMC lysate inhibits the growth of some bacteria, but *hexb^{-/-}*-BMMC lysate does not have any bactericidal effect. As well, addition of β -hexosaminidase to the *hexb^{-/-}*-BMMC lysate completely recovered the bactericidal activity. The supernatant from degranulated BMMCs, unlike BMMC lysate, had no antibacterial activity, and the lysozyme activity in the supernatant was considerably lower than that in the lysate. However, the presence of both β -hexosaminidase and lysozyme completely suppressed bacterial growth. These findings suggest that mast cell degranulation causes the release of β -hexosaminidase but not lysozyme, and although released β -hexosaminidase in itself has little bactericidal activity, the enzyme may begin to exert antibacterial activity when lysozyme is released from the other immune cells around the site of infection. Mast cell granule β -hexosaminidase appears to exert its host-protective effect through degradation of PGN. In this study, we found that 1) mast cell β -hexosaminidase seems to effectively kill *B. cereus* and *S. epidermidis* but is ineffective against both *B. subtilis* and *S. aureus*, and 2) Gram-negative bacteria such as *E. coli* and *S. marcescens* are not affected by the components of BMMC lysate. Striker et al. (45) suggested that degradation of gonococcal PGN is mediated by β -hexosaminidase released from neutrophil granules. Additionally, Koo et al. (46) reported that β -hexosaminidase exerts mycobactericidal activity in the lysosome of macrophages. Bacterial cell wall PGN is a polymer composed of sugars and amino acids. The sugar chain is elaborated with repeating *N*-acetylglucosamine and *N*-acetylmuramic acid units, and *N*-acetylmuramic acid is cross-linked by peptides to produce a dense three-dimensional network (47, 48). β -Hexosaminidase affects the *N*-acetylglucosamine or *N*-acetylgalactosamine residues in the termini of glycoproteins or glycolipids (49–51). Lysozyme, which is also known as muramidase, is found in considerable quantities in the granules of sentinel cells such as neutrophils (52), and this enzyme cleaves bacterial PGN by hydrolyzing the 1,4- β bond between *N*-acetylmuramic acid and *N*-acetylglucosamine (53). Because

β -hexosaminidase affects only the terminus of the sugar chain, the enzyme cannot degrade the β -1,4 linkage of the *N*-acetylglucosamine moieties inside PGN. However, β -hexosaminidase can attack *N*-acetylglucosamine residues if lysozyme first nicks the PGN (Fig. 7). We thus hypothesize that the microbicidal effect observed against some bacteria might be a result of sequential digestion of cell wall PGN by lysozyme and β -hexosaminidase. We observed in this study that *S. aureus* was not killed by β -hexosaminidase even though *S. epidermidis* was. As well, the cultivation of *S. aureus* with BL/6-BMMC lysate did not cause any degradation or disruption of bacterial cell wall (Supplemental Fig. 3D) although the cell wall of *S. epidermidis* was disrupted (Fig. 6C). Labischinski and Maidhof (54) reported that ~100 PGN types have been identified, including a few containing modifications within the *N*-acetylglucosamyl/*N*-acetylmuramic acid sugar unit, although the basic chemical structure of these PGNs is the same. Therefore, we hypothesize that the detailed structures of the PGNs of *S. aureus* and *S. epidermidis* may differ. More detailed data are needed to clarify this point.

Other enzymes, such as proteases, are known to exert microbicidal effects through cytokine production (42–44). Our findings in this study imply that proteases may not be the primary antimicrobial component in mast cells. There was little difference between BL/6, BL/6-BMMC-*W/W^v*, and *hexb^{-/-}*-BMMC-*W/W^v* mice with respect to the level of TNF- α in the peritoneal fluid following infection (data not shown), suggesting that the host defense mechanisms involving mast cell granule β -hexosaminidase do not correlate with the production of cytokines. Presumably, the β -hexosaminidase in mast cell granules cannot serve as a broadly acting “aegis”-like defensive agent against a wide range of invaders but rather acts as a “spear” against specific bacteria.

In conclusion, we demonstrate in this study for the first time, to our knowledge, that mast cell granule β -hexosaminidase is crucial for defense against bacterial infection but is not relevant to allergic reactions.

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Disclosures

The authors have no financial conflicts of interest.

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