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Galectin-9 Is a High Affinity IgE-binding Lectin with Anti-allergic Effect by Blocking IgE-Antigen Complex Formation

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Galectin (Gal)-9 was first described as an eosinophil chemoattractant. With the progress in research, Gal-9 has come to be known as a versatile immunomodulator that is involved in various aspects of immune regulations, and the entire picture of the function still remains elusive. To uncover as-yet unknown activity of Gal-9, we have been examining the effect of the protein in various disease animal models. Here we show that Gal-9 attenuated asthmatic reaction in guinea pigs and suppressed passive-cutaneous anaphylaxis in mice. These results indicate the mast cell stabilizing effect of Gal-9. *In vitro* studies of mast cell degranulation involving RBL-2H3 cells demonstrated that Gal-9 suppressed degranulation from the cells stimulated by IgE plus antigen and that the inhibitory effect was completely abrogated in the presence of lactose, indicating lectin activity of Gal-9 is critical. We found that Gal-9 strongly and specifically bound IgE, which is a heavily glycosylated immunoglobulin, and that the interaction prevented IgE-antigen complex formation, clarifying the mode of action of the anti-degranulation effect. Gal-9 is expressed by several mast cells including mouse mast cell line MC/9. The fact that immunological stimuli of MC/9 cells augmented Gal-9 secretion from the cells implies that Gal-9 is an autocrine regulator of mast cell function to suppress excessive degranulation. Collectively, these findings shed light on a novel function of Gal-9 in mast cells and suggest a beneficial utility of Gal-9 for the treatment of allergic disorders including asthma.

Galectin (Gal)[2] is a family of lectins characterized by a conserved carbohydrate recognition domain exhibiting binding specificity to β-galactoside (1). One of the members, Gal-9, has two carbohydrate recognition domains tethered by a linker peptide and is mainly expressed in the epithelium of the gastrointestinal tract and in immune cells (2–5). Gal-9, like other galectins, does not have a signal sequence and is localized in the cytoplasm. However, it is secreted into the extracellular milieu through poorly understood mechanisms and exerts biological functions by binding to the glycoproteins on the target cell surface via their carbohydrate chains.

Two target glycoproteins of Gal-9 have been identified, namely T-cell immunoglobulin and mucin containing-protein 3 (TIM3) and CD44. TIM3 is expressed by several populations of immune cells including terminally differentiated Th1 cells and $CD11b^+$ monocytes. Gal-9 stimulates cell death of $TIM3^+$ Th1 cells, leading to the termination of Th1-biased immunoreactions (6). On the other hand, Gal-9 promotes TNFα secretion from $CD11b^+$ $TIM3^+$ monocytes and enhances innate immunity (7). CD44 is an important adhesion molecule for migrating lymphocytes and eosinophils. Gal-9 interaction with CD44 prevents CD44 from binding to hyaluronic acid, which is a principal ligand for CD44 and for providing a foothold for migrating cells; hence, attenuates accumulation of activated lymphocytes and eosinophils to the inflamed lesion (8).

Other functions of Gal-9, such as in chemoattraction of eosinophils, suppression of Th17 cell differentiation, or promotion of regulatory T-cell differentiation (9, 10) cannot be explained either by TIM3 or CD44 with the limited knowledge we have present, which leaves the possibility of other target molecules of Gal-9. Because lectin binding is more promiscuous than protein to protein interactions, it is possible that Gal-9 has multiple target molecules to exert its various biological functions, as has been demonstrated in Gal-1 or Gal-3 (11–18).

Mast cells play an important defense role in the frontline of host immunity, whereas the excessive activation causes allergic or autoimmune disorders (19). Gal-9 expression has been shown in human cord blood-derived mast cells (20), but the function of Gal-9 in mast cells has not been elucidated yet, although an effect of TIM3 activation in mast cells was demonstrated to augment Th2 cytokine production using a polyclonal anti-TIM3 antibody, which is described as agonistic to TIM3 signaling (21).

In this report we demonstrate anti-allergic activity of Gal-9 administration in animal models. We also show that Gal-9 is an IgE-binding protein and suppresses IgE-antigen complex formation, which underlines the mode of action of the anti-allergic effect of Gal-9.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Gals—The expression and purification of recombinant Gal-1, Gal-3, Gal-7, Gal-9, stable-form Gal-9 (sGal-9), and mouse stable-form Gal-9

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[2] The abbreviations used are: Gal, galectin; sGal, stable-form galectin; TIM3, T-cell immunoglobulin and mucin domain containing-protein 3; PBS, Dulbecco's phosphate-buffered saline without calcium and magnesium; PBS-T, PBS containing 0.05% Tween 20; BSA, bovine serum albumin; HSA, human serum albumin; OVA, ovalbumin; sRAW, specific airway resistance; DNP, 2,4-dinitrophenol; β-HEX, β-hexosaminidase; LTC_4, leukotriene C_4; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbent assay.

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(msGal-9) were described previously (22–24). Human Gal-4 cDNA was amplified from first-strand cDNAs prepared from the poly(A)+ RNA fraction of human placenta (OriGene Technologies) using forward and reverse primers tagged with extra 5′ EcoRI (5′-cgtcctggattcccatggcctatgtccccgcaccg-3′) and XhoI (5′-cgaccgctcgagttagatctggacataggacaa-3′) sequences, respectively. The amplified cDNA was digested with EcoRI and XhoI, and the resulting cDNA fragment was inserted into the EcoRI-XhoI site of pGEX-4T-2. The glutathione *S*-transferase fusion protein of human Gal-4 was purified with a glutathione-Sepharose column (GE Healthcare). The purified protein was digested with thrombin (GE Healthcare), and then the glutathione *S*-transferase moiety was removed using a glutathione-Sepharose column. All the galectins were intensively dialyzed against Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS), and then endotoxin was eliminated using Cellufine ETclean L, a poly-ϵ-lysine-conjugated resin (Chisso), followed by sterilization through a 0.2-μm filter. All the preparations were >95% pure on SDS-PAGE with an endotoxin level of <0.001 endotoxin units/μg (<0.0001 ng of endotoxin/μg of protein) in limulus turbimetric kinetic assay with a Toxnometer ET-2000 (Wako). The protein concentration was determined using a bicinchoninic acid assay reagent (Pierce) with bovine serum albumin (BSA) as the standard. The molar concentration was calculated using the following molecular weights: Gal-1, 14,700; Gal-3, 26,200; Gal-4, 35,900; Gal-7, 15,100; Gal-9, 34,700; sGal-9, 33,100; smGal-9, 33,500.

Animal Studies—All the protocols for animal studies were approved by the animal care and biosafety committee of Kagawa University. To establish an asthmatic reaction, male guinea pigs (Hartley, 6 weeks; Kudou) were sensitized by means of daily inhalation of 1% ovalbumin (OVA) for 10 min using an ultrasonic nebulizer (Omron) for 8 days. After a 7-day interval, the animals were challenged by means of inhalation of 2% OVA for 5 min to induce an airway resistance increase, and the specific airway resistance (sRaw) was determined at the indicated time points using a respiratory analyzer Plumos-I (MIPS). To suppress the synthesis of endogenous glucocorticosteroids and facilitate the late-phase asthmatic reaction, the animals were injected with 10 mg/kg metyrapone (Sigma) intravenously 24 h and 1 h before the challenge, whereas 10 mg/kg pyrilamine (Sigma) was injected intraperitoneally 30 min before the challenge to avoid anaphylaxis shock. sGal-9 was administered intraperitoneally (1 mg/body) 30 min before each inhalation of OVA. The sRaw change was calculated by subtracting the initial sRaw value before the induction from the sRaws at the indicated time points and expressed as percentages of the initial sRaw values. After the last sRaw measurement, blood and bronchoalveolar-lavage fluid were taken for analysis. Total and OVA-specific IgE and IgG_1 in the serum were measured using ELISA kits from Dainippon-Sumitomo and Morinaga, respectively. Passive-cutaneous anaphylaxis was induced in female mice (BALB/c, 7 weeks, SLC) sensitized by intravenous administration of mouse IgE anti-2,4-dinitrophenol (DNP) antibody (clone Spe7; Sigma) at 5 μg/mouse a day before the assay. The following day sGal-9 or ketotifen (Sigma) was administered intraperitoneally at the indicated doses. After 30 min 30 μl of 0.15% 2,4-dinitrofluorobenzene (Sigma) dissolved in acetone/olive oil (4/1) was topically applied to the surface of the right ear of each mouse, whereas the solvent was applied to the left ear for control. The level of ear edema formation was determined by measuring the ear thickness using a calibrated thickness gauge (Mitsutoyo) and expressed as the ear thickness change according to the formula (R − L) − (R0 − L0), where R and L represent the thickness of the right and left ears at the indicated time points, and R0 and L0 represent the thicknesses just before the 2,4-dinitrofluorobenzene challenge. Statistical analysis of animal studies were carried out using GraphPad software Prism, assessed by two-way analysis of variance, and considered as significant at $p < 0.01$ (**) or $p < 0.001$ (***) compared with a PBS control.

Cell Culture—Rat basophilic leukemia RBL-2H3 cells and mouse mast cell line MC/9 were from RIKEN BioResource Center. Human mast cell line HMC-1 and human T-cell lines Jurkat and Molt-4 were from ATCC. RBL-2H3 cells were cultured in minimal essential medium (Sigma) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine. MC/9 was maintained in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, interleukin-2 culture supplement (BD Biosciences), and penicillin/streptomycin. HMC-1 was cultured in Iscove's modified Dulbecco's medium with 10% fetal bovine serum and penicillin/streptomycin. Jurkat and Molt-4 were maintained in RPMI 1640 medium with 10% fetal bovine serum and penicillin/streptomycin.

Mediator Release from RBL-2H3 Cells—20,000 cells of RBL-2H3 cells per well were seeded into 96-well plates a day before the assay and cultured at 37 °C in a humidified CO_2 incubator. Each cell layer was washed once with assay buffer (Hanks' balanced salt solution containing 20 mM Hepes and 1 mg/ml BSA) and then stimulated by the addition of the optimized concentrations of 0.1 μg/ml Spe7 and 0.016 μg/ml DNP-conjugated human serum albumin (DNP-HSA; Sigma) or 1 μg/ml rat IgE (clone IR162; Serotec) and 0.89 μg/ml anti-rat IgE antibody (clone MARE1; Zymed Laboratories Inc.). Galectins were added at the indicated time points and concentrations. For β-hexosaminidase (β-HEX) assay, supernatants were collected 60 min after the addition of DNP-HSA or MARE1, whereas the cell layers were lysed with 0.1% Triton X-100 and collected. The amount of β-HEX was determined by measuring the enzyme activity as described (25) with minor modifications. Briefly, 10 μl of each supernatant or cell lysate was mixed with 10 μl of 1.3 mg/ml 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma) dissolved in 100 mM citrate buffer (pH 4.5) and then incubated at 37 °C for 60 min. The reaction was terminated by the addition of 160 μl of 0.2 M glycine (pH 10.4), and the optical density was read at 405 nm. The amounts of histamine and leukotriene C_4 (LTC_4) were determined using specific ELISA kits from SPI Bio and Cayman, respectively, according to the manufacturer's instructions, except that 20 mM lactose was added to avoid interference with the ELISA by Gal-9 carried over from the assay.

Measurement of Intracellular Ca^{2+} Mobilization—RBL-2H3 cells seeded into an optical-bottomed black 96-well plate (Nunc) at 20,000 cells/well a day before the assay were incubated in 1 μM Fluo-3 AM (Molecular Probes) dissolved in cal-

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cium assay buffer (Hanks' balanced salt solution containing 20 mM Hepes, 1 mg/ml BSA, 1 mM probenecid) for 90 min at room temperature. The cell layer was washed twice with the calcium assay buffer, and then the kinetics of Ca^{2+} mobilization were monitored by measuring the fluorescence change (excitation, 485 nm; emission, 538 nm) using a microplate reader Fluoroskan Ascent (Thermo Electron).

Binding Assay—Human IgG_1 and IgE from myeloid were purchased from Calbiochem. IgG_1 and IgEs (1 μg/ml) were coated in 96-well plates (Nunc) by overnight incubation at 4 °C. After washing out the unbound antibodies with PBS containing 0.05% Tween 20 (PBS-T), 10 mg/ml polyethylene glycol 4000 (Merck) was added to the plates followed by incubation for 2 h at room temperature to reduce nonspecific binding. Biotinylated galectins prepared using an EZ-link NHS-biotinylation kit (Pierce) were diluted to 0.1 μM with 1 mg/ml BSA in PBS-T and then added to the plates followed by incubation for 1 h at room temperature. The average biotin incorporation rate was 1–2 biotins per galectin according to the results obtained with a biotin quantitation kit (Pierce), and the biotinylation did not affect the hemagglutination activity assessed with trypsin-treated fixed rabbit erythrocytes (26). After extensive washing with PBS-T, galectins bound to the plates were detected by the addition of streptavidin-conjugated horseradish peroxidase (Zymed Laboratories Inc.) and a colorimetric enzyme substrate, tetramethylbenzidine (KPL). The color development was stopped in its linear range by the addition of 1 M phosphate, and the optical density was read at 450 nm with a microplate reader Benchmark plus (Bio-Rad). The values were compensated for the number of biotins per galectin and then expressed as relative binding activity in comparison to that of Gal-1. Surface plasmon resonance analysis was carried out using a Biacore 3000 (Biacore). Galectins diluted to 1, 0.1, and 0.01 μM with the running buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20) were applied to biotinylated Spe7 immobilized on a SA-sensor chip (Biacore) at the flow rate of 10 μl/min at 20 °C. The kinetic constants of the interaction were calculated using BIAevaluation 4.1 software. The effect of galectins on IgE-antigen interaction was examined on the surface of 96-well plates as well as on RBL-2H3 cell surface. The assay in 96-well plates is carried out as follows. First, DNP-HSA (0.1 μg/ml) was coated in 96-well plates by overnight incubation at 4 °C. After blocking the plates with 10 mg/ml polyethylene glycol 4000, 0.1 μg/ml biotinylated Spe7 dissolved in PBS-T containing 1 mg/ml BSA was added followed by incubation for 1 h at room temperature to allow the interaction between the biotinylated Spe7 and DNP-HSA. Galectins of the indicated concentrations were added 10 min before the addition of the biotinylated Spe7. The plates were washed extensively with PBS-T, and then the remaining antibodies were detected with streptavidin-conjugated horseradish peroxidase and tetramethylbenzidine as described above. To examine dissociation of IgE from RBL-2H3 cells, RBL-2H3 cell layers prepared in 12-well plates by 1 day of culture from seeding at 80,000 cells/well were washed with FACS buffer (PBS containing 1 mg/ml BSA and 0.05% sodium azide), incubated with 0.1 μg/ml biotinylated Spe7 for 1 h on ice, and then washed with FACS buffer to eliminate unbound Spe7. Then the cells were incubated with 1 μM sGal-9 for 1 h on ice, washed with FACS buffer, and then incubated with 0.5 μg/ml streptavidin-conjugated phycoerythrin (Biolegend) for 30 min to label the remaining biotinylated Spe7 on RBL-2H3 cells. After washing with FACS buffer, the cells were dissociated from the plates by pipetting in FACS buffer containing 20 mM lactose and 2 mM EDTA, suspended in 1 μg/ml propidium iodide in FACS buffer, and examined with FACSCalibur (BD Biosciences). To examine the effect of Gal-9 on IgE-antigen interaction and IgE-anti-IgE antibody interaction on RBL-2H3 cells, the cell layers incubated with 0.1 μg/ml Spe7 or IR162 for 1 h on ice were washed with FACS buffer, incubated with 1 μM sGal-9 for 10 min on ice followed by the addition of 0.016 μg/ml DNP-conjugated biotinylated BSA (Biosearch Technologies) or 0.89 μg/ml biotinylated-MARE1 to the cell layers preincubated with Spe7 or IR162, respectively, and further incubated on ice for 30 min. The cell layers were washed with FACS buffer and incubated with 0.5 μg/ml streptavidin-conjugated phycoerythrin for 30 min. Then the cells were dissociated from the plates as described above, stained by propidium iodide, and examined with FACSCalibur.

Periodate Oxidation of IgE—The protocol for antibody was applied as described (27). Briefly, 1.5 μg of biotinylated Spe7 was incubated in 20 mM sodium *meta*-periodate/PBS (or PBS for control) at the concentration of 0.2 mg/ml for 25 h at 4 °C. The periodate reaction was terminated by the addition of the same volume of 50% glycerol and overnight incubation at 4 °C.

Gal-9 Measurement in the Cell Lines—MC/9, HMC-1, Jurkat, and Molt-4 cells were washed with PBS and suspended in lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, and Complete Protease Inhibitor Mixture (Roche Applied Science) and lysed by sonication. The amount of Gal-9 was measured with specific ELISA (28, 29), whereas the total protein amount was determined using bicinchoninic acid assay reagent with BSA as the standard.

Gal-9 Release from Activated Mouse Mast Cell Line MC/9—MC/9 cells were suspended in the culture medium containing 20 mM lactose, seeded into U-bottomed 96-well plates at 20,000 cells/well, and then stimulated by the addition of the indicated concentrations of Spe7 and DNP-HSA. Lactose was added to the medium to diminish binding of Gal-9 to the cell surface glycans. The culture supernatants were collected to measure the levels of LTC_4 and Gal-9, whereas the remaining cells were examined by trypan blue excretion to determine living cell numbers.

RESULTS

Gal-9 Suppresses Asthmatic Reaction and Passive Cutaneous Anaphylaxis in Animal Models—It has been shown that Gal-9 exerts eclectic functions in various types of immune cells, although entire picture of the immunomodulatory function of the protein remains elusive. As a part of our efforts to uncover as-yet unknown functions of Gal-9 and explore possible utilities of the protein for the treatment of immunological disorders other than autoimmune diseases in which therapeutic effect of Gal-9 is well established in animal models (6, 10, 29, 30), we asked the effect of Gal-9 in asthma model in guinea pigs (Fig. 1*A*). In this model animals sensitized with inhaled 1% OVA was challenged with 2% OVA to induce increased airway resistance.

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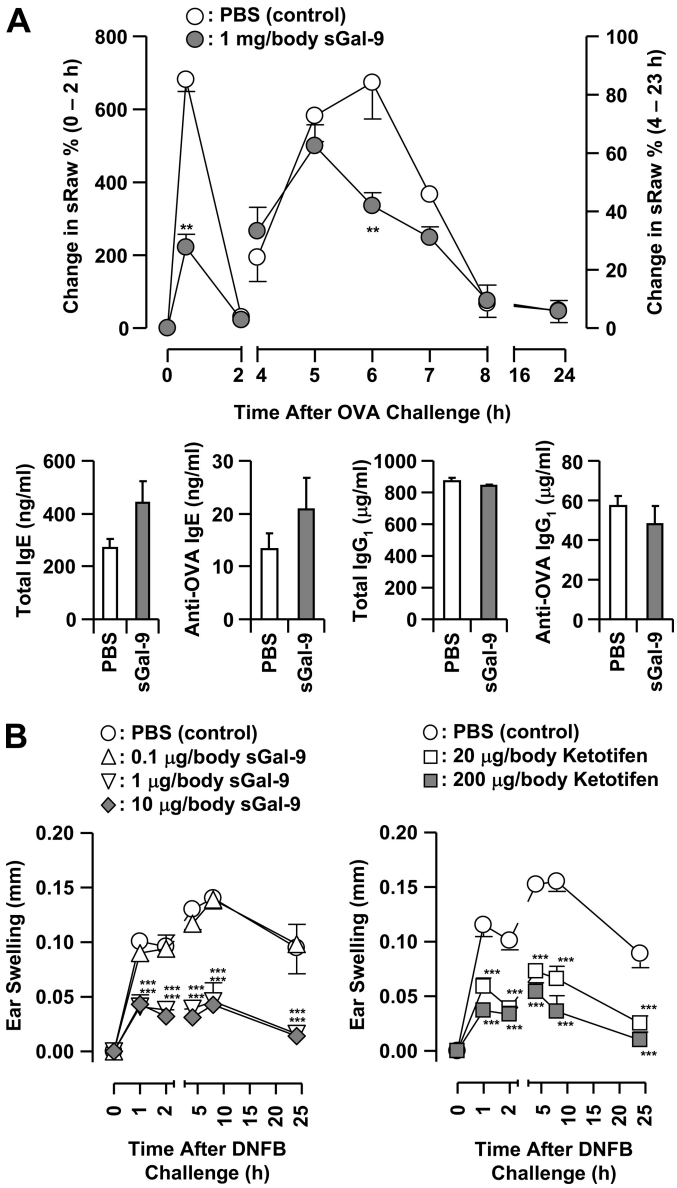

FIGURE 1. ***In vivo* effects of Gal-9 in asthma and allergy models.** *A*, to induce asthmatic reactions, guinea pigs were sensitized by inhaled 1% OVA for 8 days and challenged with 2% OVA to induce asthmatic reactions, and then sRaw was determined at the indicated time points. sGal-9 was administrated intraperitoneally (1 mg/body) 30 min before the OVA treatments in both the sensitization and challenge. The data are expressed by percent changes in sRaw. The amount of total and OVA-specific IgE and IgG_1 was measured by ELISA using serum obtained after the last sRaw measurement. Data shown are the mean ± S.E., $n = 7$ or 8; **, $p < 0.01$ compared with PBS control. *B*, effect on passive cutaneous anaphylaxis in mice. Ear edema was induced in mice by topical challenge with 2,4-dinitrofluorobenzene (*DNFB*) on the right ear of Spe7-sensitized mice. sGal-9 (*left*) or ketotifen (*right*) was administered intraperitoneally 30 min before the challenge. The thickness of the ears was measured and is expressed on the *vertical axes* as ear swelling (thickness of the right ear over the left; mean ± S.E., $n = 4$ or 5). ***, $p < 0.001$ compared with PBS control.

Gal-9 was administered before each OVA inhalation from the sensitization phase. In this study we used sGal-9 in which protease-sensitive linker peptide of Gal-9 is eliminated to stabilize the protein for long storage and for *in vivo* application but without compromising the activity (24). sGal-9 attenuated sRaw increase in both the immediate and late phases. The total and OVA-specific IgE and IgG_1 levels in serum determined after the last sRaw measurement were not statistically different between sGal-9 and control groups. The analysis of cells in bronchoalveolar-lavage fluid after the last sRaw measurement showed significant decrease of total and eosinophil numbers in sGal-9 treated group (total: PBS, 8584 ± 686/μl; sGal-9, 4679 ± 974/μl; $p < 0.01$; eosinophil: PBS, 3514 ± 427/μl; sGal-9, 1139 ± 310/μl; $p < 0.01$), whereas lymphocytes showed a decreasing tendency by sGal-9 treatment without statistical significance (lymphocyte: PBS, 312 ± 29/μl; sGal-9, 160 ± 71/μl).

The efficacy in the immediate asthmatic reaction implies that sGal-9 suppresses mast cell degranulation and prompted us to examine the effects of sGal-9 on passive cutaneous anaphylaxis model, a simpler *in vivo* model to examine mast cell degranulation. In this model sGal-9 was administered intraperitoneally to mice sensitized with anti-DNP antibody (Spe7), and then the animals were challenged with the antigen on an ear to induce ear edema. The administration of sGal-9 evidently suppressed the ear edema, 1 μg/mouse sGal-9 being equivalent to 20 μg/mouse or more of ketotifen (Fig. 1*B*). The administration of sGal-9 affected neither the general appearance nor the body weight increase of the animals throughout these two *in vivo* studies.

Gal-9 Suppresses Mediator Release from RBL-2H3 Cells—The effect of Gal-9 on mast cell degranulation was further assessed *in vitro* using RBL-2H3 cells, a commonly used model system to examine mast cell degranulation. In Fig. 2*A* RBL-2H3 cells pretreated with various concentration of Gal-9 were stimulated by the addition of Spe7 plus the antigen DNP-HSA, and then β-HEX release was measured. Gal-9 suppressed β-HEX release in a concentration-dependent manner; the efficacies of sGal-9 (Fig. 2*A*, *left*) and the natural form Gal-9 (Fig. 2*A*, *right*) being indistinguishable, whereas the effect was completely reversed in the presence of lactose. Table 1 summarizes the effects of various galectins as IC_{50} values. Gal-3, -4, and -9 exhibited inhibitory effects in the concentration range used, whereas Gal-1 and Gal-7 failed. The potency rank order of the inhibition was sGal-9 (both human and mouse type) = Gal-9 > Gal-3 > Gal-4. The effect of Gal-9 was much greater than that of ketotifen, a histamine receptor antagonist with a mast cell stabilizing effect, the IC_{50} values being 0.22 μM (7.3 μg/ml) and 385 μM (119 μg/ml) for sGal-9 and ketotifen, respectively. The inhibitory effect of Gal-9 was not limited to β-HEX release, but histamine and LTC_4 releases were also suppressed (Fig. 2, *B* and *C*).

Next, we examined the effects of Gal-9 when it was added before IgE, after IgE, or after IgE cross-linking (Fig. 2*D*). The inhibitory effect was observed even after the addition of IgE, indicating that Gal-9 is effective on IgE-primed mast cells, which is consistent with the efficacy of sGal-9 in our passive cutaneous anaphylaxis in mice. A similar effect of Gal-9 was observed when the cells were stimulated by rat IgE (IR162) plus anti-rat IgE antibody (MARE1) (Fig. 2*E*).

Gal-9 Suppresses the Intracellular Ca^{2+} Mobilization—Because Ca^{2+} influx is known to be a prerequisite step for the degranulation of RBL-2H3 cells (31, 32), we monitored the change in intracellular Ca^{2+} concentration using a calcium indicator, Fluo-3. The addition of Gal-9 by itself stimulated a

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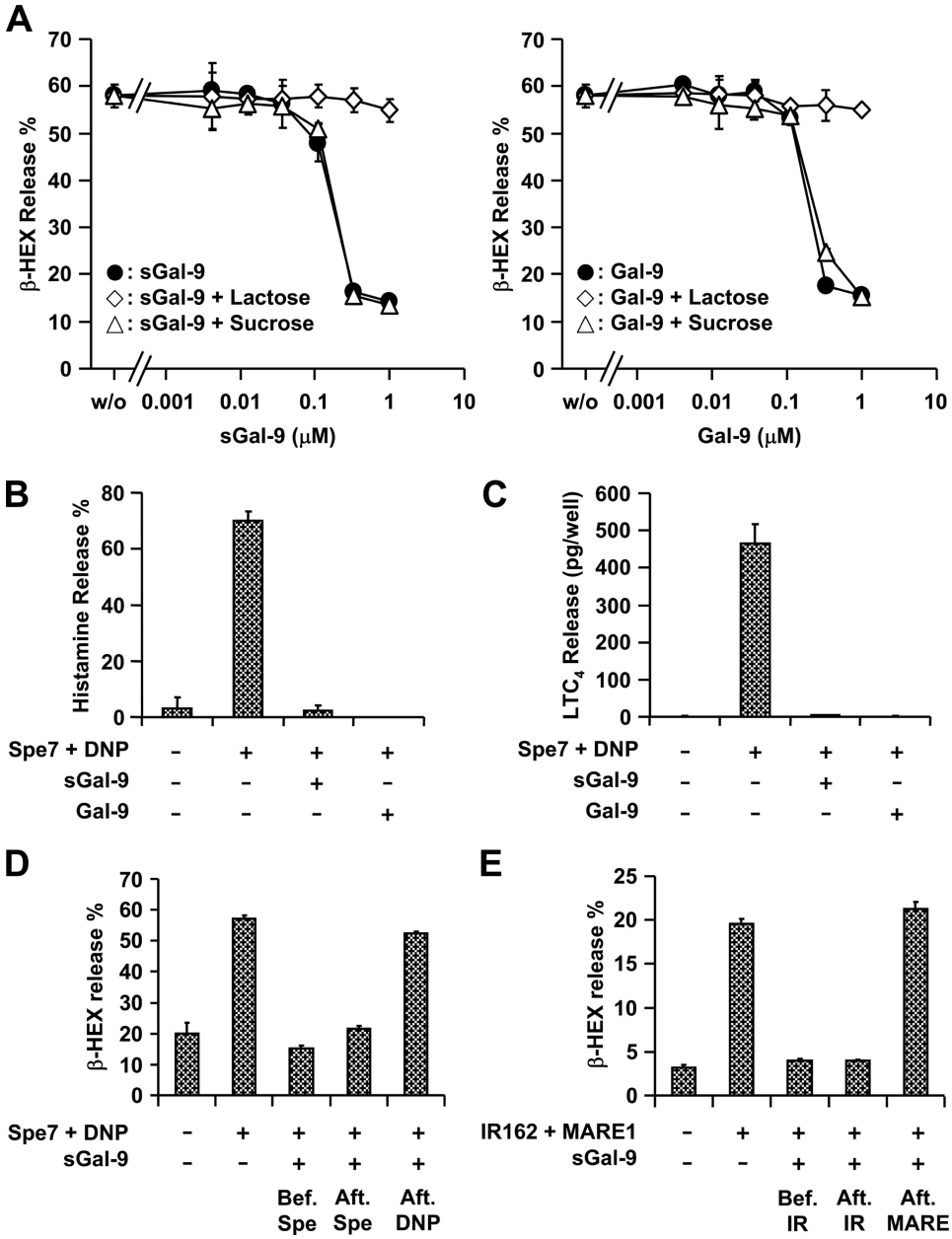

FIGURE 2. **Attenuation of mediator release from rat basophilic leukemia RBL-2H3 cells by Gal-9.** *A*, shown is the effect on β-HEX release by sGal-9 and natural-form Gal-9. RBL-2H3 cells were incubated with the indicated concentration of Gal-9 for 10 min in the presence or absence of 20 mM lactose or sucrose. Then the cells were sequentially added and incubated with Spe7 (0.1 μg/ml) for 30 min followed by DNP-HSA (0.016 μg/ml). The supernatants were collected 60 min after the stimulation for β-HEX assay, and the amount of the enzyme released into the supernatants was expressed as the percentage of total β-HEX in the cells. *w/o*, without. *B* and *C*, shown is the effect on histamine and LTC_4 release. RBL-2H3 cells were stimulated as above in the presence or absence of 1 μM Gal-9 or sGal-9. The supernatants were collected 5 and 20 min after the stimulation for histamine and LTC_4 assays, respectively. *D*, shown is suppression of β-HEX release from IgE-sensitized RBL-2H3 cells. Cells were stimulated as described in *A*, whereas sGal-9 was added 10 min before Spe7, 30 min after Spe7, or 30 min after the addition of DNP-HSA. *E*, the experimental procedure is basically the same to as in *D*, but RBL-2H3 cells were stimulated by rat IgE IR162 (1 μg/ml) and anti-rat IgE antibody MARE1 (0.89 μg/ml). All the data are derived from a single representative experiment of more than two and are shown as the mean ± S.D. ($n = 3$).

Ca^{2+} mobilization (Fig. 3*A*, *upper*), but nevertheless, this calcium signal did not stimulate degranulation as was shown in Fig. 2. The aggregation of FcεRI on the addition of IgE plus antigen stimulated a Ca^{2+} mobilization (Fig. 3*A*, *middle*); however, it was completely abolished in the presence of Gal-9 (Fig. 3*A*, *upper*). These effects of Gal-9 were abrogated in the presence of lactose (Fig. 3*A*, *lower*). We hypothesized that the Gal-9-dependent Ca^{2+} mobilization might be the signal that desensitizes cells as to degranulation. To test this hypothesis, lactose was added to the cells after termination of the Gal-9-induced Ca^{2+} mobilization followed by stimulation with IgE plus antigen to determine whether β-HEX is released (Fig. 3*B*). The addition of lactose reversed the inhibitory effect of Gal-9 on β-HEX release, indicating that Gal-9-dependent Ca^{2+} mobilization has no direct connection with the anti-degranulation effect.

Gal-9 Is an IgE-binding Protein and Deprives IgE of Binding to Antigen—Because IgE is a highly glycosylated protein, we examined if Gal-9 binds IgE by means of a solid-phase binding assay in which immobilized immunoglobulins on 96-well plates were bound with biotinylated-galectins (Fig. 4*A*). The average biotin incorporation rate was 1–2 per each galectin, and the biotinylation did not affect the lectin activity. Gal-9 exhibited prominent binding to IgEs from human (myeloid), mouse (Spe7), and rat (IR162), whereas the binding of Gal-1, -3, -4, and -7 to these IgEs was much weaker. The IgE binding was similarly strong between the natural form Gal-9 and the stable form, sGal-9. Gal-3 has been known as an IgE-binding protein, but the interaction was much weaker than that of Gal-9 even though our recombinant Gal-3 and the reported preparation seems to be similar at least in hemagglutination activity, *i.e.* the concentration for hemagglutination of our preparation and the reported one was 3 and 2 μg/ml, respectively, using the same referenced protocol involving trypsin-treated fixed rabbit erythrocytes (33). These interactions were completely abrogated in the presence of lactose. The binding between IgE and galectins was further studied by surface plasmon resonance analysis using Biacore. The dissociation constants (K_d) obtained for immobilized Spe7 against sGal-9 and Gal-3 were 3.6×10^{-8} and 2.4×10^{-6} M, respectively, which demonstrated the higher affinity of the Spe7-Gal-9 interaction over that of Spe7-Gal-3 in a different assay format.

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Not only binding to IgE, but Gal-9 suppresses the interaction between IgE and antigen. When added to the interaction assay between Spe7 and immobilized DNP-HSA, Gal-9 suppressed IgE-antigen complex formation in a concentration-dependent manner, whereas Gal-1 and Gal-3 were ineffective at 1 μM (Fig. 4*B*, *left*). The interaction between Gal-9 and DNP-HSA is at the background level in this assay. The effect of Gal-9 was also completely abolished in the presence of lactose (Fig. 4*B*, *right*), which supports the idea that the target of Gal-9 is carbohydrate chains on IgE. To further examine the relevance, Spe7 was subjected to periodate oxidation to degrade carbohydrate chains. Periodate oxidation decreased the Spe7 amount in the reaction (Fig. 4*C*, *left*) probably because of the increased adsorption of the carbohydrate-less Spe7 to the plastic tube. In accordance with the decreased amount, binding to DNP-HSA of periodate-treated Spe7 decreased compared with the mock treatment, but the binding was insensitive to Gal-9 (Fig. 4*C*, *right*). This observation clarifies that carbohydrate chains on IgE are the primary target of Gal-9.

TABLE 1

Effects of various galectins on RBL-2H3 degranulation

The same experimental procedure as in Fig. 2*A* was used to evaluate the effects of each galectin. The IC_{50} value was calculated as the concentration for 50% inhibition from the inhibition curve using GraphPad software Prism and is presented as the mean ± S.E. from at least three independent experiments. When 50% inhibition was not reached in the concentration range used, the IC_{50} values were assumed to be more than the highest concentration. msGal-9, mouse stable Gal-9.

Reagent	IC_{50} for β-HEX release
	μM
Gal-1	>9
Gal-3	2.39 ± 0.46
Gal-4	5.65 ± 0.39
Gal-7	>9
Gal-9	0.24 ± 0.04
sGal-9	0.22 ± 0.04
smGal-9	0.22 ± 0.01
Ketotifen	385±66

We further confirmed the inhibitory effect by Gal-9 on RBL-2H3 cell surface using the same amount of Spe7 and DNP-HSA for the degranulation assay. sGal-9 showed a limited effect on ripping pre-bound Spe7 out of RBL-2H3 cell surface (Fig. 4*D*) but suppressed DNP-HSA binding to Spe7 on the cell surface (Fig. 4*E*, *left*). We also examined if Gal-9 suppresses interaction between IR162 and MARE1 because Gal-9 suppressed degranulation from RBL-2H3 cells stimulated by these antibodies (Fig. 2*E*). Even in this case, Gal-9 significantly reduced MARE1 attachment by about 40% (Fig. 4*E*, *right*).

Induction of Gal-9 Release from Mast Cells—Gal-9 expression in mast cells is shown in human cord blood-derived mast cells (20) and human mast cell line HMC-1 (34). We have repeatedly seen strong signals of Gal-9 out of mast cells in immunohistochemical studies using mouse and human

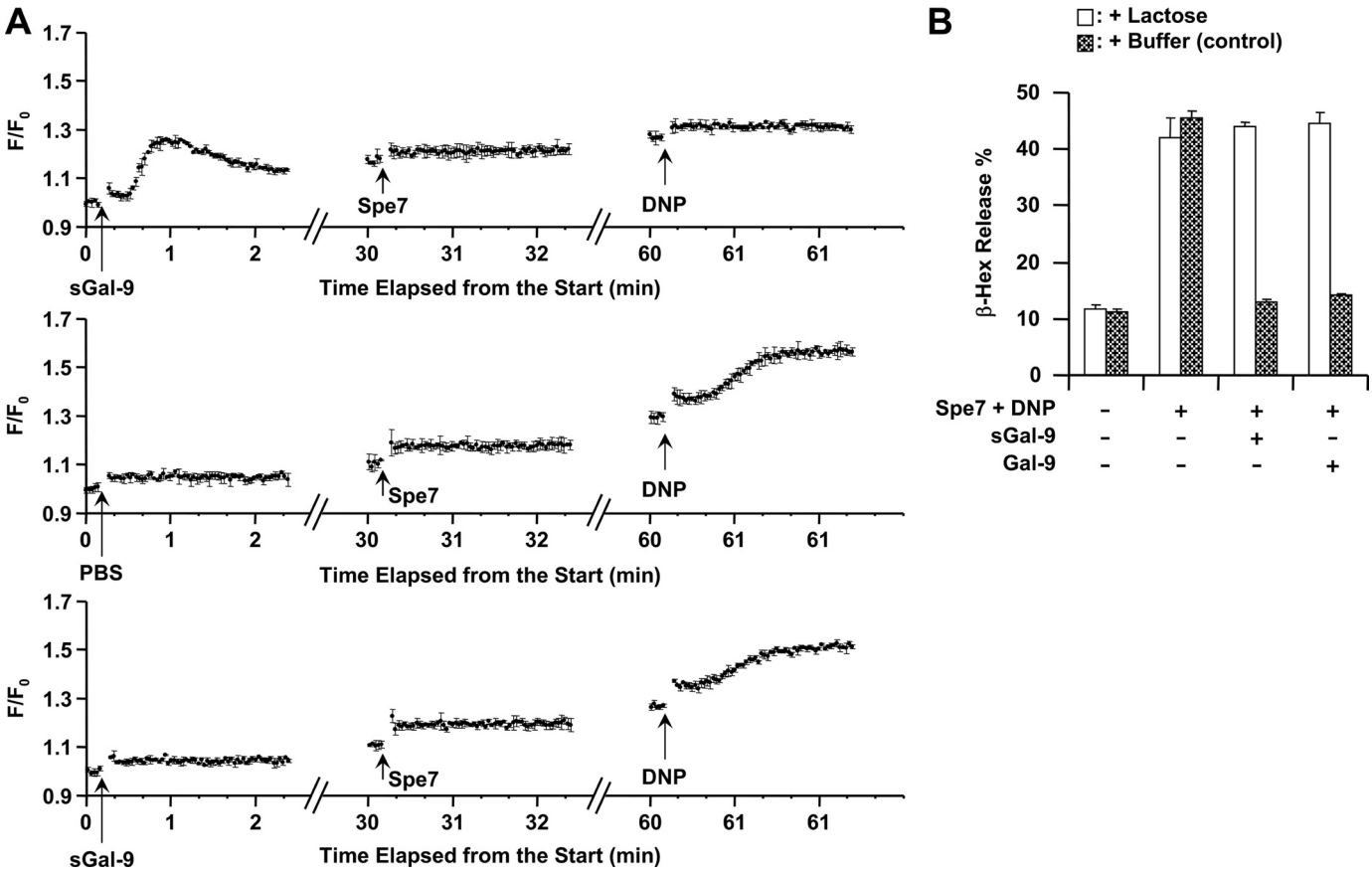

FIGURE 3. **Effect of Gal-9 on intracellular Ca^{2+} mobilization.** *A*, Fluo-3-AM-loaded RBL-2H3 cells were sequentially added with 1 μM sGal-9 (or PBS for control), 0.1 μg/ml Spe7, and then 0.016 μg/ml DNP-HSA at 30-min intervals as indicated by the *arrows*. The assay was carried out in the absence (*upper* and *middle*) or presence (*lower*) of 20 mM lactose. The fluorescence signal is expressed on the *vertical axes* as relative fluorescence change (F/F_0) from the initial signal value (Mean ± S.D., $n = 3$). *B*, no relations between Gal-9-dependent Ca^{2+} mobilization and the anti-degranulation effect of Gal-9 are shown. The assay procedure was basically the same as in Fig. 2*A*, except that 20 mM lactose or control buffer was added 10 min after the addition of 1 μM Gal-9, and then the cells were stimulated with Spe7 and DNP-HSA for β-HEX release (mean ± S.D., $n = 3$). Representative data of twice reproduced are shown.

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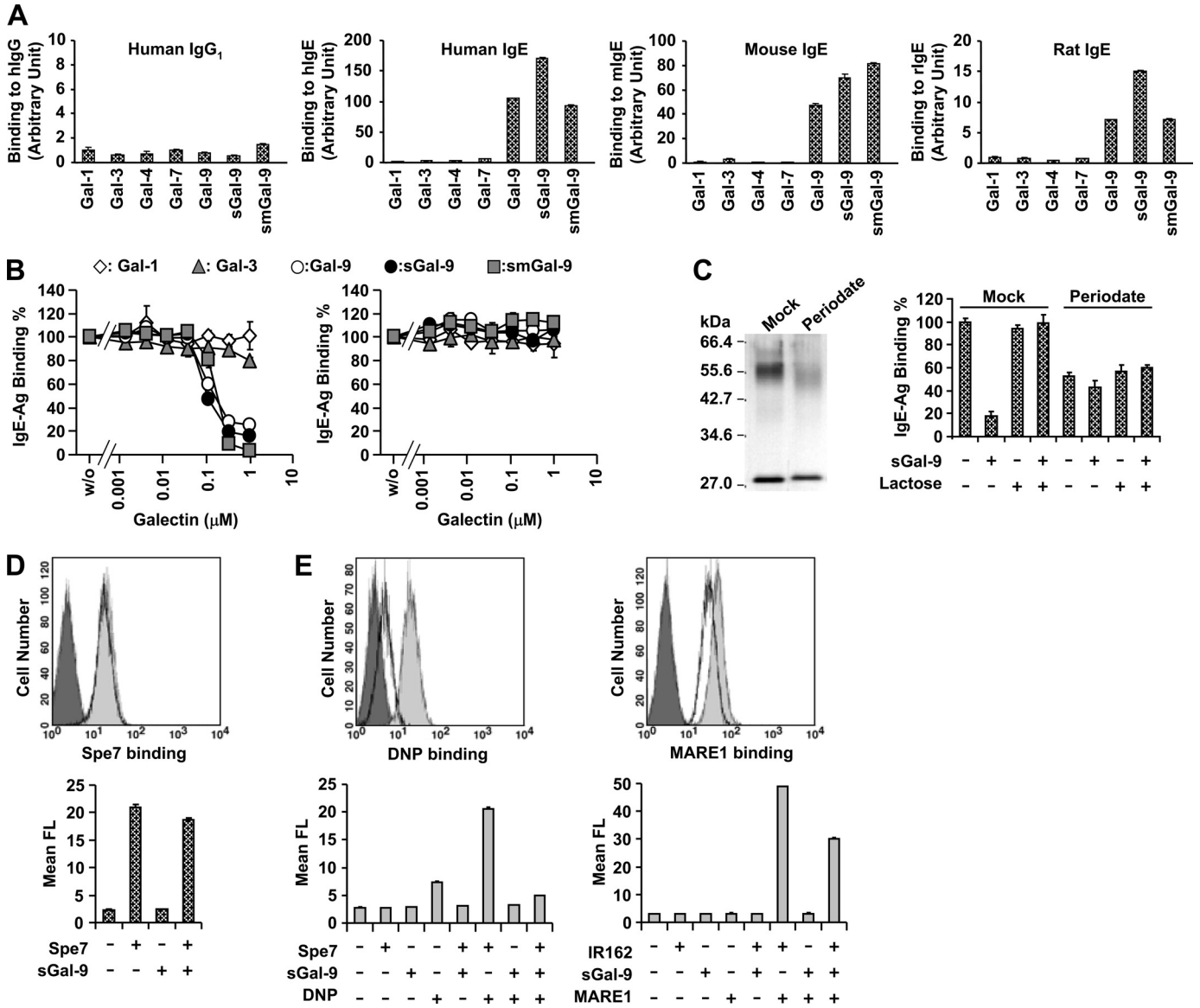

FIGURE 4. **Binding of Gal-9 to IgE prevent IgE-antigen complex formation.** *A*, human IgG_1 from myeloid, human IgE from myeloid, mouse monoclonal IgE Spe7, and rat monoclonal IgE IR162 were coated in 96-well plates and allowed to interact with biotinylated-galectins. Relative binding is expressed on the *vertical axes* in comparison with the binding of Gal-1 (mean ± S.D., $n = 3$). *B*, prevention of IgE-antigen complex formation by Gal-9 in 96-well plates is shown. DNP-HSA-coated 96-well plates were incubated with 0.1 μg/ml biotinylated Spe7 for 60 min in the indicated concentration of galectins in the presence (*right*) or absence (*left*) of 20 mM lactose. The data are expressed by the percentage of Spe7 binding as the binding without galectins is 100% (mean ± S.D., $n = 3$). *C*, no effect of Gal-9 on periodate-treated IgE is shown. Biotinylated Spe7 was subjected to periodate oxidation to disrupt carbohydrate chains. The samples were sieved by SDS-PAGE and silver-stained (*left*) and used for binding assay against DNP-HSA in the presence or absence of 1 μM sGal-9 and 20 mM lactose as indicated (*right*). The assay condition is basically the same as *B*. Periodate- and mock-treated Spe7 were used without compensating for the loss of protein during the treatment, and the binding of mock-treated Spe7 in the absence of sGal-9 and lactose was set as 100% (mean ± S.D., $n = 4$). *D*, RBL-2H3 cells pre-bound with 0.1 μg/ml biotinylated Spe7 were incubated with 1 μM sGal-9 when indicated, and Spe7, which stayed on the cell surface, was detected by labeling the cells with streptavidin-conjugated phycoerythrin and FACS analysis. The mean fluorescence values are shown in the *bar chart* (mean ± S.D., $n = 3$). *Dark gray histogram*, $Spe7^-sGal\text{-}9^-$; *pale gray histogram*, $Spe7^+sGal\text{-}9^-$; *open histogram*, $Spe7^+sGal\text{-}9^+$. *E*, RBL-2H3 cells pre-bound with 0.1 μg/ml Spe7 (*left*) or 1 μg/ml IR162 (*right*) were incubated with either 0.016 μg/ml DNP-conjugated biotinylated BSA or 0.89 μg/ml biotinylated-MARE1 in the presence or absence of 1 μM sGal-9 as indicated. The binding of DNP-BSA or MARE1 to the cells was determined by labeling the cells with streptavidin-conjugated phycoerythrin and FACS analysis. The mean fluorescent values are summarized in the *bar chart* (mean ± S.D., $n = 3$). *Dark gray histogram*, $Spe7^-sGal\text{-}9^-DNP^-$ or $IR162^-sGal\text{-}9^-MARE1^-$; *pale gray histogram*, $Spe7^+sGal\text{-}9^-DNP^+$ or $IR162^+sGal\text{-}9^-MARE1^+$; *open histogram*, $Spe7^+sGal\text{-}9^+DNP^+$ or $IR162^+sGal\text{-}9^+MARE1^+$. Representative data of at least twice-reproduced experiments are shown.

tissue sections (data not shown). ELISA analysis of Gal-9 expression in cell lines demonstrated overwhelmingly high amounts of Gal-9 in mast cell lines over T-cell lines (Fig. 5*A*). Because mouse mast cell line MC/9 retains FcεRI-dependent signaling for degranulation and proinflammatory mediator release, we investigated if Gal-9 is released from the cells with the activation. This cell line released Gal-9 constitutively under the culture conditions we used. The application of Spe7 plus DNP-HSA to the culture stimulated the release of LTC_4 at 30 min followed by the augmentation of Gal-9 release at 48 h (Fig. 5*B*), with the highest release in this experiment of 7.4 ng/10^6 cells, which corresponds to about 11% of total Gal-9 in the cells (63.6 ± 1.8 ng/10^6 cells). The viability of the cells monitored by means of trypan blue stain-

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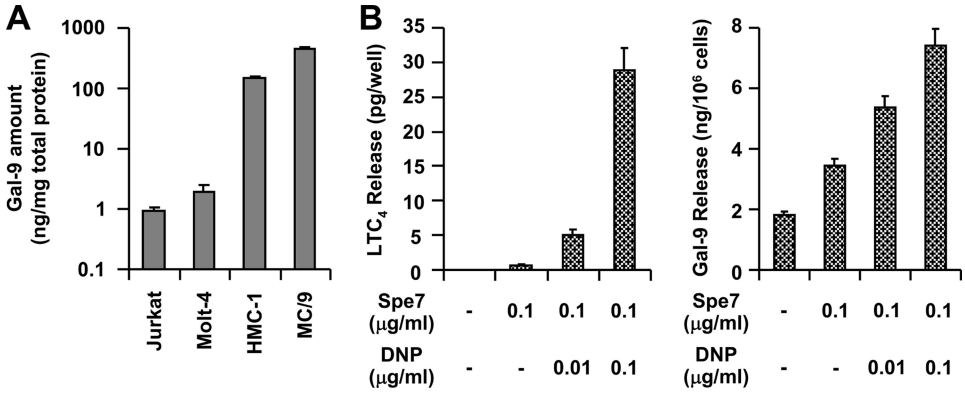

FIGURE 5. **Expression and release of Gal-9 from mast cells.** *A*, shown is ELISA analysis of Gal-9 expression in T-cell lines (Jurkat and Molt-4) and mast cell lines (HMC-1 and MC/9). *B*, release of Gal-9 from mast cells by the degranulation stimuli is shown. Mouse mast cell line MC/9 was incubated with the indicated concentrations of Spe7 and DNP-HSA for 30 min or 48 h to measure the released LTC_4 (*left*) or Gal-9 (*right*), respectively, using specific ELISA. After 48 h incubation, cell number was 5–30% higher in the presence of Spe7 and DNP-HSA compared with the control. Therefore, the amount of Gal-9 in the culture supernatant was compensated by the cell number and expressed on the *vertical axis* as release of Gal-9 per 10^6 cells. Data are representative of at least two experiments (mean ± S.D., $n = 3$).

ing was >95% under all the conditions throughout the experiment.

DISCUSSION

We demonstrated in a previous report that Gal-9 suppresses airway inflammation and Th2 cytokine release in a murine model of asthma by preventing CD44-hyaluronic acid interaction of activated lymphocytes and eosinophils (8). In the current study we examined Gal-9 administration in an asthma model in guinea pigs and observed a suppressing effect in both immediate- and late-phase asthmatic reactions. Gal-9 treatment attenuated eosinophil accumulation in the lung. This can be explained by the suppression of CD44-dependent migration of eosinophils by Gal-9, which is supposed to be the major mode of action in ameliorating the late-phase asthmatic reaction. The suppression at immediate-phase reaction, however, is presumed as an effect of Gal-9 on mast cell degranulation or on immediate reactions triggered by the degranulation, especially as Gal-9 administration did not decrease IgE level in the serum. The suppressive effect of Gal-9 on mast cell degranulation was further supported *in vivo* using a murine passive cutaneous anaphylaxis model and confirmed by *in vitro* studies involving RBL-2H3 cells.

It is interesting to note that Gal-3 also suppressed degranulation from RBL-2H3 cells at a 10-fold higher concentration than Gal-9, although Gal-3 was previously reported to stimulate the degranulation from the cells (35). These differences can be explained by the different assay formats, *i.e.* they added RBL-2H3 cells to Gal-3-coated plates, whereas we applied galectins directly to the cell culture layers. Because Gal-3 exhibits affinity to FcεRI as well, it can aggregate FcεRI to stimulate degranulation when immobilized at high density on a culture plate. However, soluble Gal-3 cannot cross-link FcεRI unless it forms a stable dimer or oligomer, and it probably prevents the interaction between IgE and FcεRI or between IgE and antigen.

We showed that Gal-9 prevented Spe7 from binding to DNP-HSA, whereas Gal-1 and Gal-3 were ineffective in the concentration ranges used, which correlates well to the binding affinity of these galectins as to IgE and the potency rank order of the inhibition in RBL-2H3 degranulation. These results support the hypothesis that the anti-degranulation effect of Gal-9 is largely because of interference with IgE-antigen complex formation. Gal-9 suppressed the interaction between rat IgE IR162 and anti-IgE antibody MARE1 on RBL-2H3 cells by 40%, although degranulation from the cells by these antibodies was completely suppressed by Gal-9. Probably, Gal-9 binding to IR162 is a steric hindrance for IR162-loaded FcεRIs to be in close proximity with each other by MARE1; hence, signal transduction and the following degranulation is suppressed. The exact binding site of Gal-9 on IgEs and how the binding affects IgE structure remain to be investigated.

Expression of TIM3 was reported in human cord blood-derived mast cells (20) and mouse bone marrow-derived mast cells (21). Activation of TIM3 on the bone marrow-derived mast cells by an agonistic polyclonal antibody was reported not to affect mast cell degranulation by immunological stimuli but to augment Th2 cytokine production and also to suppress mast cell apoptosis induced by interleukin-3 withdrawal (21). TIM3 expression in RBL-2H3 cells was detected only by reverse transcription-PCR, its level being less than the detection limit on Western blotting (data not shown), suggesting that the surface expression of TIM3, if present at all, must be low. The application of Gal-9 to RBL-2H3 cells stimulated a Ca^{2+} mobilization. This calcium signal might come from the Gal-9 interaction with TIM3 in RBL-2H3 cells because Gal-9 binding to TIM3 was reported to stimulate a Ca^{2+} mobilization in Th1 cells (6). Nevertheless, this calcium signal does not seem to play a role in the observed anti-degranulation effects of Gal-9 because the addition of lactose after the calcium signal reversed the effect of Gal-9.

Gal-9 expression was shown in T-cells, B-cells, macrophages, endothelial cells, and fibroblasts in synovial tissue samples from rheumatoid arthritis patients (29), and its release from T-cells was demonstrated using Jurkat T-cells (34). These cells are a plausible source of Gal-9 at the site of inflammation in modulating immune responses. Because mast cells express significantly high amount of Gal-9 over T-cells, we investigated if Gal-9 is secreted from mast cells with degranulation stimuli. Activation of mouse mast cell line MC/9 by Spe7 plus DNP-HSA augmented Gal-9 release into the supernatant. This result suggests that mast cells can be a source of Gal-9 and implies that Gal-9 acts as an autocrine regulator for mast cells to suppress excessive degranulation.

Mast cells release various proinflammatory mediators by activation and trigger allergic diseases such as asthma, atopic dermatitis, allergic rhinitis, and allergic conjunctivitis (36–40). A large number of antagonists against those proinflammatory

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mediators have been developed in an effort to cure uneasy and sometimes deadly symptoms of the disease. However, those antagonists offer limited benefit due to blocking only a part of the reactions triggered by many proinflammatory mediators released from activated mast cells. A humanized anti-IgE neutralizing antibody, Omalizumab, is a more radical approach for the treatment of allergic diseases. This antibody is directed against the binding site of IgE for FcεRI and prevents free IgE from attaching to mast cells, hence preventing IgE-mediated proinflammatory mediator release as a whole (41). Gal-9 also targets IgE and attenuates the activation of mast cells triggered by the cross-linking of FcεRI. Gal-9 blocks the access of antigen to the IgE, and the anti-degranulation effect was demonstrated even after the attachment of IgE to FcεRI on mast cells. This seems to be the beneficial characteristic of Gal-9 over the anti-IgE strategy. Mast cells of symptomatic patients are assumed to have bound IgE, and Gal-9, but not Omalizumab, probably exerts fast-acting efficacy under the conditions as well.

We found that Gal-9 is an IgE-binding protein suppresses IgE-antigen complex formation and, hence, prevents proinflammatory mediator release from mast cells. Immunological stimulation augments the secretion of Gal-9 from mast cells, implying that Gal-9 is an autocrine regulator of mast cell activity. Gal-9 administration suppresses immediate-phase asthmatic reaction in guinea pigs, which manifests the anti-degranulation effect of Gal-9 in complex pathophysiological conditions where various polyclonal IgEs are involved. Together with the previously reported function of Gal-9 in suppressing CD44-dependent migration of inflammatory cells to the lesions, the current study would add more value to Gal-9 as a potential candidate to allow the development of a novel protein drug for the treatment of asthma and allergic disorders.

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