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—Original—

Novel phenotype in beagle dogs characterized by skin response to compound 48/80 focusing on skin mast cell degranulation

Mitsuhiro UCHIDA[1, 2], Fumi ITO[1], Toshiyuki TSUCHIYA[1], Yoko SHOJI[1], and Toru KUROSAWA[1]

[1] *Toxicology Laboratory, Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd., Kanagawa, Japan*
[2] *Laboratory of Veterinary Pharmacology, School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan*

Abstract: Beagle dogs have long been employed in toxicology studies and as skin disease models. Compared with other experimental animal species, they are known to be susceptible to skin responses, such as rashes, from exposure to various chemical compounds. Here, a unique dog phenotype was identified that showed no skin response to compound 48/80, a mast cell degranulating agent. Although the skin responses to intradermal injection of polyoxyethylene castor oil derivative (HCO-60, a nonionic detergent), histamine dihydrochloride, concanavalin A (IgE receptor-mediated stimuli), or calcium ionophore A23187 were comparable in wild-type (WT) dogs and these nonresponder (NR) dogs, only the response to compound 48/80 was entirely absent from NR dogs. The skin mast cell density and histamine content per mast cell were histologically comparable between WT and NR dogs. By checking for skin responses to compound 48/80, NR dogs were found to exist at the proportion of 17–20% among four animal breeders. From retrospective analysis of in-house breeding histories, the NR phenotype appears to conform to the Mendelian pattern of recessive inheritance. The standard skin response in WT dogs developed at 2–4 months of age. In conclusion, this unique phenotype, typified by insensitivity in the compound 48/80-induced degranulation pathway in mast cells, has been widely retained by recessive inheritance in beagle dogs among general experimental animal breeders. The knowledge concerning this phenotype could lead to better utilization of dogs in studies and aid in model development.

Key words: immunotoxicity, phenotype, rash, skin reaction

Introduction

Drug-induced skin rash (DISR) is one of the critical adverse effects of pharmaceuticals that cause discontinuation of development of many pharmaceutical candidates. The severity of DISR broadly varies from mild to life-threatening [1] and is known to occur through immune- and/or nonimmune-mediated (anaphylactoid) mechanisms in response to parent drugs and their metabolites [12]. In general, it is difficult to predict the potential for a DISR in preclinical studies. It is sometimes first recognized only during human studies, but screening of anaphylactoid reactions in preclinical studies were performed using mast cell assays *in vitro,* and in laboratory animals, especially dogs.

Dog skin response and dog-derived primary mast cells have often been employed for studies of anaphylactoid reactions, as dogs exhibit higher sensitivity to a wide

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Address corresponding: M. Uchida, Toxicology Laboratory, Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd., 760 Morooka, Kohoku, Yokohama, Kanagawa 222-8567, Japan

range of chemicals than other species, such as mice, rats, guinea pigs, and monkeys [5, 7, 9]. Indeed, in our laboratory, several drug candidates comprising small molecules have shown anaphylactoid reactions only in dogs, and dogs are sometimes not chosen as an experimental animal because of their hypersensitivity in studies of skin, gastrointestinal, and respiratory systems. It is thus important to investigate the characteristics of these dogs in view of the species utility in DISR assessment.

Investigations of anaphylactoid reactions have focused on the peptidergic pathway of mast cell activation, in addition to the other allergic pathway through the cross-linking of IgE bound to FcεRI receptors. The pathway to anaphylactoid reactions is activated by cationic secretagogues, including positively charged neuropeptides (e.g., substance P and neuropeptide Y), amines (e.g., compound 48/80 and natural polyamines), or cationic complement factors through nonspecific trimetric GTP-binding proteins (G_i proteins) via a receptor-independent but membrane-assisted process [10]. Inositol phospholipid breakdown by phosphatidylinositol 3-kinase or phospholipase C leads to intracellular Ca^{2+} elevation and subsequent mast cell degranulation [3]. This process is known to play an important role in neurogenic inflammation, tissue injury by venom peptides or pharmaceuticals, and diseases involving extensive activation of the blood complement cascade.

Beagle dogs have also been widely used for nonclinical toxicology studies, and in the course of long-term use, historical data have been accumulated and standardized at each dog-breeding facility. However, characteristics of these dogs, such as body weight, size, aggression, nervousness, and adaptability, have been seen to vary according to breeder, with large individual differences in susceptibility to test compounds observed even among littermates. The number of animals per test group in dog studies is usually less than the number in rodent studies, and thus, it is sometimes difficult in practice to evaluate test results due to these variations and small study sizes. Revealing the phenotype and its characteristics in beagle dogs is important from the perspective of understanding the range of variation and its effects on studies.

In this study, a novel subpopulation of nonresponder (NR) dogs, produced by selective breeding, was identified and investigated regarding their special phenotypic characteristics by morphological and pharmacological approaches using several stimulants of mast cell degranulation pathways. We also proposed a method for distinguishing wild-type (WT) and NR dogs through skin evaluation.

Materials and Methods

Chemicals

Compound 48/80 was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), HCO-60 was purchased from Nihon Surfactant Kogyo K.K. (Tokyo, Japan), histamine dihydrochloride was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), Concanavalin A was purchased from Seikagaku Corp. (Tokyo, Japan), and A23187 was purchased from Alomone Labs, Ltd.. (Jerusalem, Israel).

Animals

Beagle dogs of both sexes weighing approximately 8.5–12.5 kg were purchased from Covance Inc. (Cumberland, VA, USA), Marshall BioResources (North Rose, NY, USA), Ridglan Farms, Inc. (Mt. Horeb, WI, USA), and Narc Corp. (Narita, Japan); some of the Covance dogs were bred in-house. As for the beagle strains, HRA beagles from Covance and Nosan beagles from Narc were used. In totally 220 adult dogs and 88 pups were employed. Adult animals were housed individually in cages in an air conditioned facility with a temperature of 23 ± 2°C, relative humidity of 55 ± 10%, and light cycle of 7:00–19:00 and given standard chow (Labo D Stock, Nosan Corporation, Yokohama, Japan) at approximately 250 g/day and tap water *ad libitum*; the animal rooms and cages were cleaned daily. Skin diseases, such as atopic or infective dermatitis, were properly controlled and had not occurred before this study. Dogs were used for experiments after a habituation period of more than 2 wk. All experimental protocols were approved by the Meiji Seika Pharma Institutional Ethics Committee for Use of Laboratory Animals in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals prepared by Japanese Association for Laboratory Animal Science.

Vascular permeability to intradermal (ID)-injected compounds

Compound 48/80, HCO-60, histamine dihydrochloride, concanavalin A, or A23187 was dissolved in saline at 0.01–100 μg/ml and applied by ID injection at 0.05 ml/site into shaved thoracic skin under anesthesia with

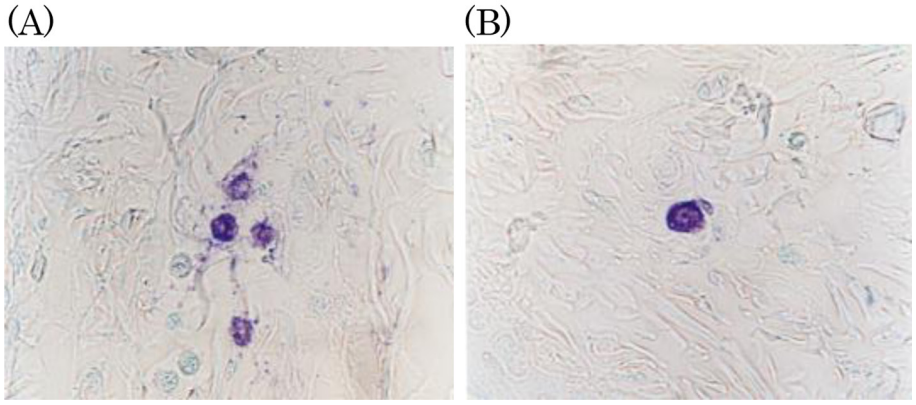

Fig. 1. Representative photos of degranulated (A) or intact (B) skin mast cells from wild-type (WT) dogs IV treated with compound 48/80 at 1 mg/kg.

25 mg/kg of pentobarbital. Just prior to ID injections, Evans blue (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) dissolved in saline was injected IV at 1 mg/kg. Approximately 10 min after IV injection, skin biopsies of injection sites were collected for pigment leakage determination, in which each skin sample was placed into a 1 N NaOH solution and incubated at 37°C overnight for pigment release. The optical density at 620 nm was measured for determination of pigment concentration per skin segment.

Determination of skin mast cells and the degranulation ratio

Skin mast cells and the histamine contained in them were visualized by toluidine blue staining and immunohistochemistry, respectively [5]. Dogs were anesthetized by intramuscular injection of 2 mg/kg xylazine hydrochloride followed by IV injection of 25 mg/kg sodium pentobarbital, and their lateral thorax was shaved. At 10–15 min after ID injection at 0.05 ml/site of either 10 μg/ml of compound 48/80 in saline or saline alone, punch biopsies were performed at the injection sites. Skin samples were treated sequentially for 2 h in Carnoy's fixative and then for 1–2 h in 100% ethanol, and they were then embedded in paraffin and sectioned for subsequent histology. Mast cells were counted by microscope (×200) in 10 fields of toluidine blue (pH 0.5)-stained sections, and the ratio of degranulated to total mast cells was calculated. Degranulated mast cells were defined and judged by a fuzzy cell membrane and blue-stained granules scattered around the cells (Fig. 1A). The microscopic image/shape of mast cells did not differ between WT and NR dogs.

Immunohistochemistry for histamine was performed using skin samples from WT and NR dogs according to the manufacturer's instructions for applying rabbit anti-histamine polyclonal antibody (#AB5885, Millipore Corporation, Billerica, MA, USA). Briefly, skin samples were fixed sequentially in 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 4% paraformaldehyde (by vol) in a 250 W microwave for 20 min each and then immersed in 80% ethanol for 1 wk. Fixed samples were then embedded in paraffin and sectioned at a thickness of 4 μm. The sections were incubated in normal swine sera for blocking and sequential staining with primary antibody (#AB5885) at room temperature (r.t.) for 1 h and biotinylated anti-rabbit IgG at r.t. for 30 min, and they were then visualized using avidin-biotinylated horseradish peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate from a DAKO detection kit (Dako North America, Inc., Carpinteria, CA, USA) with washing steps using phosphate-buffered saline.

Determination of NR dogs

Gross skin responses in dogs were observed 10 min after ID or IV injection of compound 48/80. ID injection involved 0.01 mg of test chemical/ml saline and 0.05 ml/site, and IV injection involved 0.1 mg of test chemical/ml saline and 0.1 ml/kg body wt. Saline served as the negative control, and ID injections were made into shaved ventral neck skin. Dogs not showing any typical skin reactions from compound 48/80 applications were designated as NR dogs.

Table 1. Acute response to intravenous bolus injection of compound 48/80 in dogs

Dose (mg/kg)	Phenotype	
	WT	NR
0.05	No remarks	No data
0.15	Flare (ear), swelling (face), urination, lacrimation	No remarks
0.5	Flare (ear), swelling (face), urination, lacrimation, prone position, convulsion	No remarks
1.5	No data	Convulsion, limping, no skin response

WT: wild-type. NR: nonresponder. n=1–3.

Acute response to compound 48/80 in WT and NR dogs

Compound 48/80 dissolved in saline was administered at 0.1 ml/kg to 3 WT dogs and 3 NR dogs by intravenous (IV) bolus injection. The animals used here were originally purchased from Covance, Inc. and bred in-house. Skin and systemic responses were observed for 1 h after injection, and if the initial dog injected showed a marked response, the remaining 2 dogs were not treated. Injections to the same dogs were administered sequentially at intervals of at least one week.

Retrospective analysis of breeding histories

Breeding histories were retrospectively traced using available records and summarized. A working hypothesis was formulated in which the desired characteristics were transmitted in a recessive manner. The dominant/recessive gene of a dog was determined via analysis of skin test results from intradermal injection of compound 48/80 at 0.01 mg/ml.

Data analysis

Quantitative data are expressed as the mean ± standard error of the mean (SEM). Variance analysis between WT and NR dogs was conducted by F-test, and then the significance of the differences in Evans blue leakage between WT and NR dogs was estimated by Student's *t*-test.

Results

Acute systemic and skin response to compound 48/80

When WT and NR dogs were IV injected with compound 48/80, WT dogs injected at 0.05–0.5 mg/kg showed typical skin and behavioral responses that were dose dependent, such as flares and swelling in abdominal skin and the face, mainly in the ear, eyelid, and corner of the mouth, and systemic signs that presented as scratching by the hind limbs, head shaking, and convulsions (Table 1). These responses were clearly seen 5–10 min after injection and disappeared within 30 min. In contrast, NR dogs did not show any skin response to compound 48/80, even at 1.5 mg/kg, but systemic responses were observed in NR dogs at 1.5 mg/kg. The systemic response might be induced by the off-target toxicity of compound 48/80.

Vascular permeability to stimulants of mast cell degranulation

Compound 48/80, HCO-60 (a surfactant), histamine dihydrochloride, concanavalin A (a chemical stimulant to IgE FcεR), and A23187 (a calcium ionophore) were ID administered to WT and NR dogs, and vascular permeability at the injection site was determined by pigment leakage. Of these chemicals, compound 48/80 showed poor dose-dependent pigment leakage in NR dogs (Fig. 2).

Morphological analysis for skin mast cells in NR dogs

Histological determination of mast cell numbers in normal skin showed that the density and numbers of mast cells were comparable between WT and NR dogs (123 ± 11 vs. 105 ± 10, respectively, Figs. 3A–C). In skin treated with compound 48/80 at 10 μg/ml, almost all mast cells in WT dogs were degranulated, while those in NR dogs were degranulated to a lesser extent (Fig. 3D). Also, the histamine content per mast cell in NR dogs was visually comparable to that in WT dogs (Fig. 4).

Proportion of NR dogs

Beagle dogs were purchased for experimental use from four commercial breeders, and the proportion of NR dogs was determined by skin response to ID or IV injection of compound 48/80. The proportion of NR dogs in each population was about 17–20% among the four breeder sources (Tables 2 A–D).

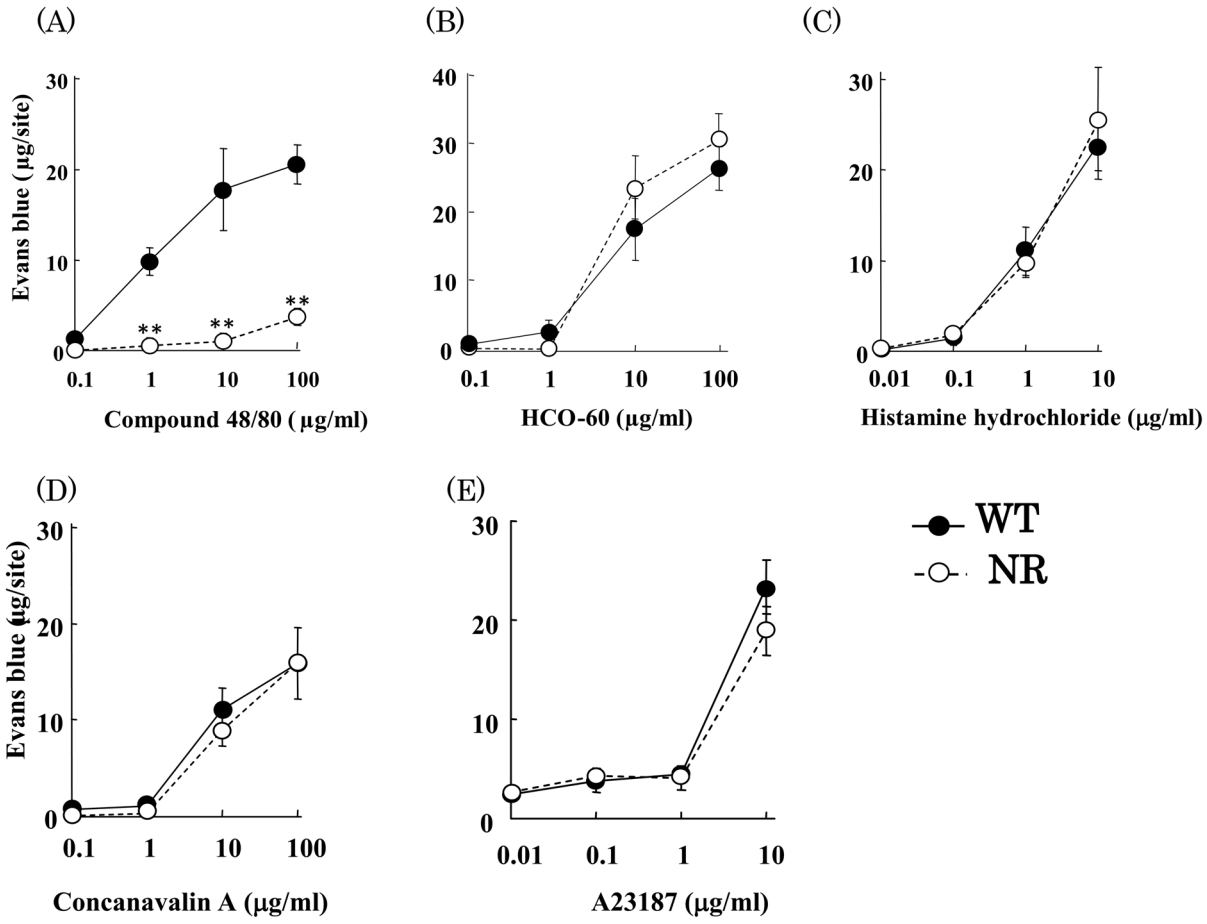

Fig. 2. Vascular permeability to various compounds. Compound 48/80 (A), HCO-60 (B), histamine dihydrochloride (C), concanavalin A (D), and A23187 (E) at 0.01–100 mg/ml ID injected at 0.05 ml/site into the shaved thorax of anesthetized wild-type (WT) and nonresponder (NR) dogs; Evans blue (1 mg/kg) dissolved in saline IV injected just prior to ID injections; and 10 min later, ID injection sites collected for determination of pigment leakage. The significance of the differences between WT and NR groups was determined by Student's *t*-test. $^{**}P<0.01$.

Breeding history

The in-house breeding history is retrospectively summarized in Table 3, using "*A*" and "*a*" to indicate the dominant and recessive gene, respectively, and "*Aa*" and "*AA*" to indicate WT and "*aa*" for NR dogs. The proportions of WT and NR phenotype dogs were 65.0 and 35.0%, respectively, from heterozygous × heterozygous mating. All pups born by mating NR (homozygous recessive) dogs were determined to be NR dogs.

Age dependency of skin response in pups

Pups from three litters born from mating heterozygous *Aa* with homozygous recessive *aa* dogs were determined to be WT offspring by monthly testing of their skin response to ID or IV injection of compound 48/80. None of the pups at 1 month of age showed any response to ID injection of compound 48/80, and typical skin responses appeared at around 2–4 months of age in both sexes (Table 4). In clear contrast, NR dogs from the same litters did not show any skin response at any age (data not shown).

Discussion

A unique beagle phenotype was identified that showed no skin response to ID or IV injection of compound 48/80. The target pathway that defined the characteristics of the NR dogs studied here was concluded to be the Gi protein-mediated pathway to mast cell degranulation (Fig. 5). Mast cell reactivity to compound 48/80, not to other stimulants, was completely different between WT and NR dogs despite comparable populations and hista-

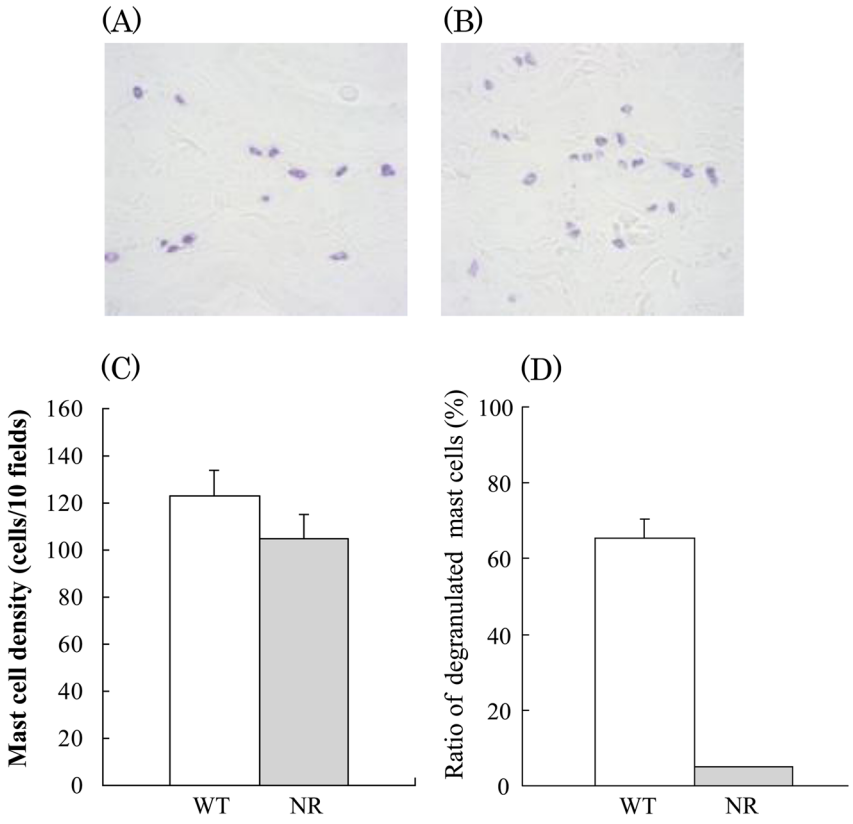

Fig. 3. Determination of mast cells in dog skin. Representative photos of punched skin sections stained with toluidine blue from wild-type (WT) (A) and nonresponder (NR) (B) dogs (n=3); (C) mast cell density (cells/field, mean ± SEM) calculated by cell count in 10 fields (200×); (D) proportion of degranulated skin mast cells after treatment with compound 48/80 at 10 μg/ml; compound 48/80 at 10 μg/ml ID injected into lateral thorax skin under anesthesia; injection sites biopsied 10 min after injection; and calculated proportion (%, mean ± SEM) of degranulated mast cells to total mast cells.

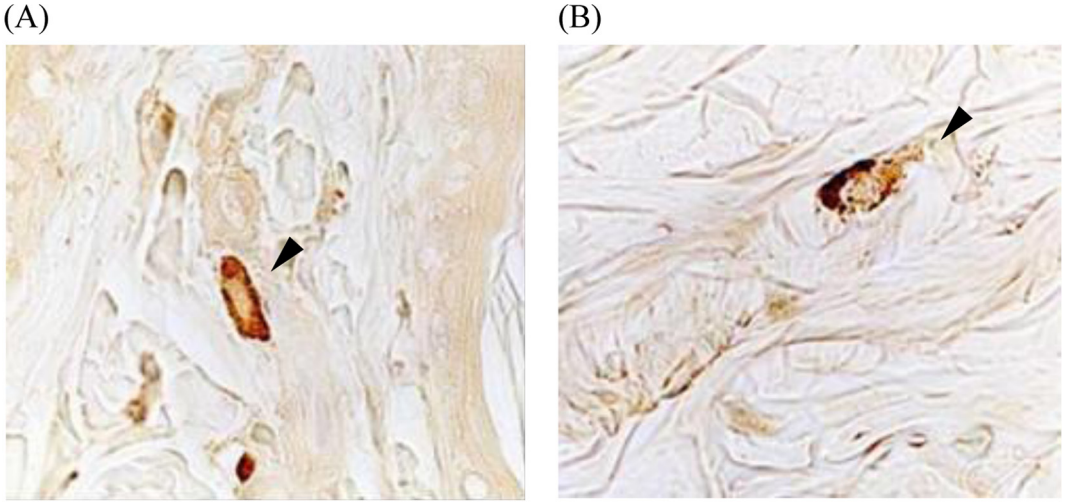

Fig. 4. Representative photos of immunohistochemical analysis for histamine targeting in skin mast cells from wild-type (WT) (A) and nonresponder (NR) (B) dogs. Arrow heads, mast cells; histamine, dark brown.

Table 2. Numbers of dogs tested and proportions of NR dogs from four breeders

	Breeder			
	A	B	C	D
Total (n)	22	113	46	29
WT (n)	18	93	13	24
NR (n)	4	20	9	5
NR	18%	18%	20%	17%

WT: wild-type. NR: nonresponder.

mine contents of their mast cells as well as similar reactivity to ID histamine injection. Compound 48/80, a synthetic polyamine, has been used for many years as a potent endogenous histamine releaser and is thought to partially penetrate the mast cell plasma membrane, interact with membrane trimetric G proteins, activate phospholipase C, increase intracellular calcium stores, and activate protein kinase C, culminating in degranula-

Table 3. Retrospective analysis of the breeding histories of the WT and NR dogs

Cross	WT (*AA*/*Aa*)			NR (*aa*)		
	Male	Female	Total no. (%)	Male	Female	Total no. (%)
Hetero × Homo (*Aa* × *aa*)	12	8	20 (43.5)	14	12	26 (56.5)
Hetero × Hetero (*Aa* × *Aa*)	6	7	13 (65.0)	4	3	7 (35.0)
Homo × Homo (*aa* × *aa*)	0	0	0 (0)	15	7	22 (100)

WT: wild-type. NR: nonresponder.

Table 4. Skin or systemic responses to compound 48/80 in pups up to 5 months of age

Pup No.	Sex	1 mo, ID	2 mo, ID	3 mo, ID	4 mo, IV	5 mo, IV
A1	M	–	–	–	+	+
A2	M	–	–	+	+	+
A3	F	–	–	+	+	+
B1	M	–	–	+	+	+
B2	M	–	+	+	+	+
B3	F	–	+	+	+	+
C1	M	–	–	+	+	+
C2	F	–	–	+	+	+

ID, Intradermal injection; IV, Intravenous injection; -, no skin or systemic response; +, positive skin response described in Table 1. Pup No.: pups indicated by same letters are littermates.

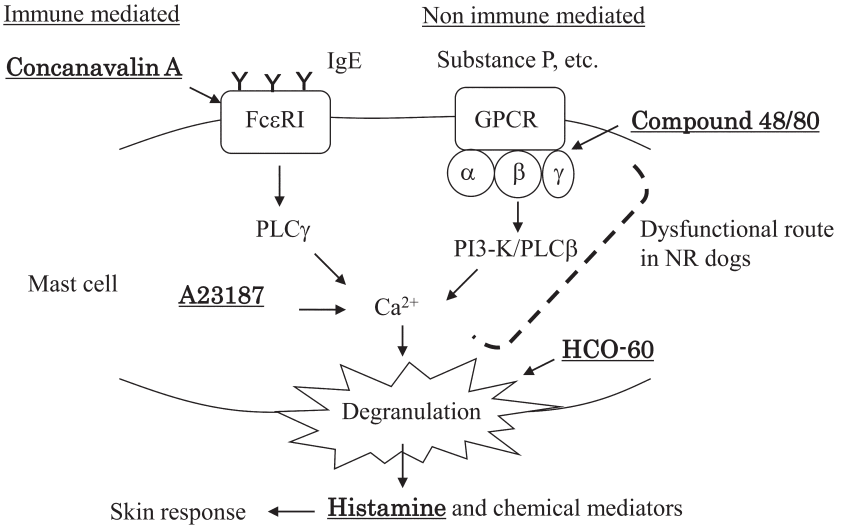

Fig. 5. Illustration of main pathways to skin response via mast cells based on results shown in Fig. 4. Nonresponder dogs showed skin responses to HCO-60, histamine dihydrochloride, concanavalin A, and A23187 comparable to those of wild-type dogs but no response to compound 48/80; the dysfunctional route in NR dogs would be downstream of the GPCR.

tion [4]. Although the interactions with G protein and specific receptors are unclear, compared with the mode of action of degranulation stimulators, the gene target that defines the characteristics observed in NR dogs would be a G protein-related molecule. A canine GeneChip® analysis performed using skin from WT and NR dogs did not detect strongly or weakly expressed pro/anti-inflammatory genes in either group (data not shown). The gene responsible for skin refractoriness in NR dogs has not yet been identified.

The presence of NR dogs was confirmed in the beagle populations from several general animal breeders (Table 2). In a few papers, a similar phenotype has been reported. One out of eight dogs was determined as NR, showing no histamine elevation in plasma or skin response to fluoroquinolones, which are known to induce anaphylactoid reactions [6]. Of 82 suspected atopic mongrel dogs, three showed poor skin responses to compound 48/80 despite having strong responses to histamine. The authors reported that the three NR dogs had a history of recent anti-inflammatory use that may have inhibited responses, but the responses were sustained for at least 9 months [13]. No histamine release as a result of ID injection of compound 48/80 at 10 μg/ml was detected in 5 out of 24 mongrel dogs [2]. Although they were not a focus in these papers, NR dogs appear to have previously existed.

At all breeders that were checked, NR dogs were seen at a constant proportion of ~20%, and selective breeding easily produced a pure subpopulation, in which NR dogs were produced by mating homozygous *aa* individuals. Mating two heterozygous *Aa* WT dogs produced homozygous *AA* and heterozygous *Aa* WT offspring and homozygous *aa* NR offspring, with a littermate ratio of the WT to NR phenotype dogs of almost 2:1, not the theoretical 3:1 ratio. No differences, including skin responses between the WT genotypes *AA* and *Aa*, have been found thus far. According to retrospective breeding histories, this phenotype was considered to conform to Mendelian inheritance patterns of dominant/recessive alleles and to be regulated by a specific gene.

NR dogs do not show any skin response even at the high dose of compound 48/80. Beagle dogs are particularly known to show susceptible skin responses to test compounds compared with other species of experimental animals. Thus, these dogs have been sometimes employed for dermal disease models [6, 8]. In toxicity studies, NR dogs have shown no remarkable differences in regularly monitored parameters (e.g., body weight, clinical observation, and clinical pathology and histopathology) compared with WT dogs. However, this difference, depending on polymorphism, might be overlooked as an individual difference, particularly as the usual number of dogs used for a study is relatively small, at 4–6 dogs/group. At least in terms of skin response, it would be very useful to know the potency of their skin reactivity by pre/post monitoring of their skin response to compound 48/80 injection, which would allow appropriate determination of their drug-induced skin responses. Also, application of this approach, for understanding individual characteristics, might lead to smaller study groups and a reduction of animal usage numbers.

The typical anaphylactoid skin response to compound 48/80 developed beginning at 2–4 months old in both sexes (Table 4), and reactivity was sustained at least up to 8 years of age. The number of mast cells and their functions have been reported to gradually mature in the postnatal infant period [14]. Regarding the appropriate body parts for monitoring skin responses, the front of the neck was more susceptible than other parts, such as the abdomen, chest, and backside, in both ID and IV injections (data not shown). Reactivity partly corresponded with mast cell density in body surface areas [11], and the heterogeneity of skin mast cell numbers is based on histological sections of skin [4]. Premonitoring of NR dogs should be done after 4 months of age, at which point the reactivity would be well developed.

NR dogs have a variety of potential applications. For example, in repeated-dose toxicology studies, researchers sometimes encounter skin reactions that are not distinguished by either immune-mediated allergic reactions or anaphylactoid skin responses. If the reaction disappeared when applied to NR dogs, the mechanism would be determined to be an anaphylactoid reaction. Moreover, if an allergic or infective skin disease model in dogs could be established, the pathophysiological functions of the compound 48/80-mediated pathway in mast cells might be clearly illuminated. As the observations here indicated that the dysfunctional pathway in NR dogs was that from the G protein-coupled receptor leading to intracellular Ca^{2+} elevation, mast cells isolated from NR dogs might be useful in studying the mechanism of GPCR-related degranulation.

In summary, this unique phenotype, typified by insensitivity in the compound 48/80-induced degranulation

pathway in mast cells, has been widely retained in beagle dogs by general experimental animal breeders through recessive inheritance. This knowledge concerning this phenotype could lead to better utilization of dogs in studies and aid in model development.

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