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## Technical Note

# A new, rapid in vivo method to evaluate allergic responses through distinctive distribution of a fluorescent-labeled immune complex: Potential to investigate anti-allergic effects of compounds administered either systemically or topically to the skin

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## ABSTRACT

We herein established a new method to evaluate allergic responses in mice rapidly and easily with ethical improvement by reducing the number of animals used. A single intravenous injection of a mixture of anti-OVA monoclonal IgE and fluorescein-ovalbumin (FITC-OVA) induced the distinctive spotted distribution of FITC-OVA in skin, named “ASDIS (Anaphylaxis-dependent Spotted Distribution of a fluorescent-labeled Immune complex in Skin)”, and this was easily detected by in vivo imaging. The parallel induction of hypothermia, scratching, serum histamine increases, and ASDIS as well as the inhibition of ASDIS by either the systemic administration of a histamine H<sub>1</sub> receptor antagonist or mast cell-depleting antibody suggested that our method, which only required 15 min, induced these allergic responses including ASDIS. Relatively mild but significant ASDIS was induced also in mice with passive systemic anaphylaxis by the method, requiring 2 separate days. The painting of anti-histamines on the skin markedly reduced ASDIS in the painted area only, suggesting the potential of this model to simultaneously compare the anti-allergic effects of several candidate compounds with control drugs in the same mice. ASDIS was suggested to originate from extravasated FITC-OVA/OE-1 immune complexes from blood to skin tissues other than mast cells. Our new method has the advantages of rapidity, easy method, and lower animal numbers to evaluate anti-allergic compounds as well as the characteristics of the used antibody, antigen, labeling molecules, additives, and other formulations. Our model for inducing ASDIS may contribute to the development of anti-allergic drugs, especially those intended for application to the skin.

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## 1. Introduction

Repeated exposure to antigens has been reported to cause sensitization to antigens and occasionally induces allergic reactions. These reactions are commonly divided into four types, named types I to IV, according to the symptoms observed. Of these, type I allergies, including food allergy, asthma, and allergic rhinitis, induce acute symptoms such as itching, diarrhea, and, in some cases, lethal anaphylaxis associated with bronchoconstriction and hypotension. Since the secretion of mediators from mast cells activated by IgE and recognized antigens is considered to trigger these acute symptoms, many anti-allergic drugs, namely histamine receptor antagonists, mast cell-stabilizing

drugs, and cysteinyl leukotriene receptor antagonists, have been developed and effectively used to prevent and treat type I allergic diseases (Bischoff, 2007).

Various allergic models using in vivo as well as in vitro mast cell cultures have been established to investigate novel anti-allergic drugs with improved properties (Bischoff, 2007). The indicators of type I allergic reactions measurable in passive cutaneous anaphylaxis, passive and active systemic anaphylaxis, and asthma and food allergy mouse models are mast cell degranulation (histological analysis), mediator release, increased vascular permeability, allergic behaviors (scratching, loss of activities, and death), and physical functions (airway constriction and decreases in body temperature), ordered by time. However, the identification of other indicators of acute allergic symptoms that can be detected more easily and rapidly, are more specific for allergic reactions, do not require additional procedures for determination, and reduce the number of used animals, may improve experimental conditions for the screening of anti-allergic drugs.

In vivo imaging is a suitable method for use toward this aim because of its ability to detect signals from labeled molecules optically in the living body in real time. Previous studies using this method revealed

**Abbreviations:** ARE, average radiant efficiency; ASDIS, Anaphylaxis-dependent Spotted Distribution of a fluorescent-labeled Immune complex in Skin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; OVA, ovalbumin; PBS, phosphate-buffered saline; PE, phycoerythrin; PSA, passive systemic anaphylaxis.

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that inflamed areas in the lungs of asthmatic mice can be rapidly and easily distinguished by the accumulation of dendritic polyglycerolsulfate near infrared fluorescent dye conjugate (Biffi et al., 2013). The migration of transferred labeled T cells into the dermis with irritant contact dermatitis indicated their contribution to the pathogenesis of this disease (Egawa et al., 2011). We also observed using this method that anti-ovalbumin (OVA) IgA increased fluorescein (FITC)-OVA in the gut through hepatobiliary transport with a compensative decrease in its levels in the urine, as evidenced by a decrease in its accumulation in the urinary bladder (Yamaki et al., Unpublished results).

In the present study, we applied this method to investigate the distribution of an immune complex constructed with IgE and fluorescent-labeled antigens in mice. We found that the immune complex of IgE and a fluorescent-labeled antigen was rapidly distributed in the skin in a distinct spotted manner, named “Anaphylaxis-dependent Spotted Distribution of a fluorescent-labeled Immune complex in Skin (ASDIS)”. In contrast to conventional methods to induce passive systemic anaphylaxis (PSA) that require 24 h for sensitization with IgE (Doyle et al., 2013), our method with a single injection of the mixture of IgE and the labeled antigen only required 15 min to induce severe anaphylaxis and ASDIS. ASDIS was an allergic reaction-dependent phenomenon, as evidenced by its inhibition by histamine H<sub>1</sub> antagonists and the mast cell-depleting antibody MAR-1. We also discussed the meaning and reliability of ASDIS as a representative phenomenon of anaphylactic symptoms and its possible applications to the screening of anti-allergic drugs administered systemically and topically to the skin based on our results obtained using histamine H<sub>1</sub> receptor antagonist-containing ointments. Additionally, the mechanisms generating the spots of ASDIS were investigated by measuring the distribution of the injected FITC-OVA/OE-1 immune complex within skin, focusing on mast cells and blood vessels.

## 2. Materials and methods

### 2.1. Hybridomas and antibodies

Hybridomas producing anti-OVA monoclonal IgE (OE-1, OE-2, and OE-3) and anti-OVA monoclonal IgG1 (O1-10) were established using PEG1500 (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. These anti-OVA monoclonal antibodies were purified using OVA-conjugated resin chromatography as described previously (Yamaki et al., 2014). Anti-mouse Fc $\epsilon$ RI (MAR-1) was purchased from eBioscience, Inc., San Diego, CA, USA.

### 2.2. Animals

HR-1 mice (6–10 weeks old; Japan SLC Inc., Shizuoka, Japan) and DBA/1J mice (6–8 weeks old; Charles River Laboratories Japan Inc., Yokohama, Japan) were maintained in a temperature-controlled facility and given chow and water ad libitum. The animal procedures used in this study were approved by the Animal Ethics Committee of Kobe Pharmaceutical University.

### 2.3. Conventional PSA model and new anaphylaxis model inducing ASDIS

Mice were divided into two groups: one was set as a conventional PSA group and the other as a new anaphylaxis model group. Mice in the PSA group were passively sensitized with an intravenous injection of OE-1 (200  $\mu$ g) and challenged with an intravenous injection of 50  $\mu$ g FITC-OVA on the next day. Mice in the new anaphylaxis model group were intravenously injected with OE-1, OE-2, OE-3, or O1-10 (200  $\mu$ g) preincubated with FITC-OVA (50  $\mu$ g) for 30 min. In some experiments, a mixture of FITC-OE-1 (200  $\mu$ g) and OVA (50  $\mu$ g) was injected. Rectal temperatures for 60 min, scratching behaviors for 15 min, and serum histamine concentrations 60 min after the antigen challenge were evaluated using a BAT-12 thermometer (Physitemp Instruments, Inc., Clifton, NJ, USA), visual monitoring, and a histamine

EIA kit (Cayman Chemical, Ann Arbor, MI, USA), respectively. Images of FITC-derived fluorescence in mice were obtained by IVIS Lumina XRMS Series III (PerkinElmer, Inc., Waltham, MA, USA). For quantification of ASDIS severity, average radiance efficiency (ARE) within 1 cm  $\times$  1 cm areas of interest was calculated with Living Image software (Perkin Elmer, Inc.).

### 2.4. Drug treatment

The mast cell-depleting antibody MAR-1 (0.15 mg) was intravenously injected into mice 48 h before the FITC-OVA injection. Chlorpheniramine, cyproheptadine, and diphenhydramine (Sigma-Aldrich, Co., St. Louis, MO, USA) were systemically and topically administered by an intraperitoneal injection and painting as a 2% solution in PBS containing 3% methylcellulose 30 min before the FITC-OVA injection, respectively.

### 2.5. Immunohistochemistry

Skin of mice was fixed with 10% formalin neutral buffer solution for 1 week or more and embedded in paraffin. Four-micrometer sections were stained with 0.05% toluidine blue (pH 2.5, Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 3 min. Alternatively, 11- $\mu$ m frozen sections of skin embedded in O.C.T. compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) were fixed with 4% paraformaldehyde-PBS or cold-acetone for 10 min. The sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min and then with PBS containing 2% rabbit serum and 2% rat serum for 1 h at room temperature. After washing with PBS containing 0.5% Tween 20, the sections were incubated with horseradish peroxidase-labeled rabbit anti-Fluorescein antibody (Abcam plc., Cambridge, UK) overnight at 4 °C followed by DAB staining (Vector Laboratories, Inc., Burlingame, CA, USA). The stained sections were further incubated with toluidine blue for 3 min for detecting mast cells or with phycoerythrin (PE)-labeled rat anti-mouse CD31 (BD Bioscience, San Jose, CA, USA) for 1 h at room temperature for visualizing blood vessels.

### 2.6. Statistical analysis

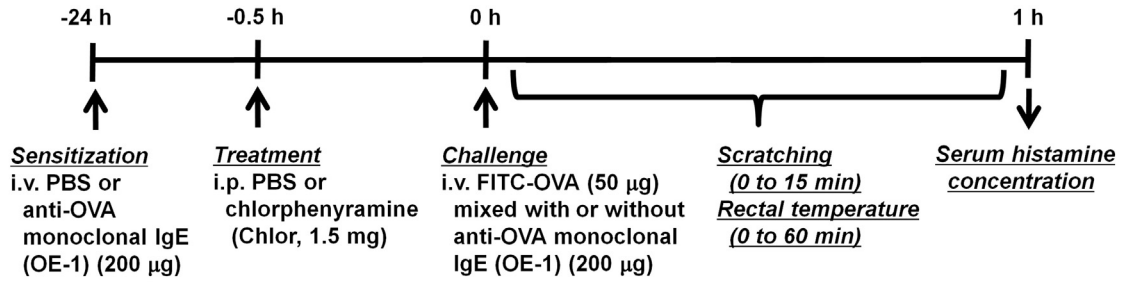
Tukey's post hoc test was only performed for multiple-group comparisons when the p value for a one-way analysis of variance (ANOVA) was less than 0.05. A p value of less than 0.05 with Tukey's post hoc test was considered significant.

## 3. Results and discussion

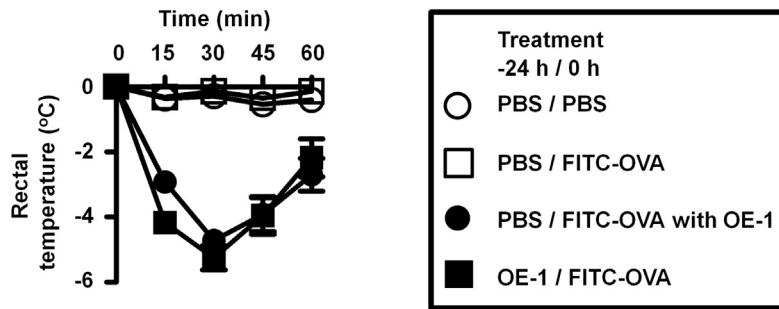
### 3.1. Intravenous injection of the immune complex constructed with FITC-OVA and OE-1 induced severe anaphylaxis, similar to conventional PSA triggered by OE-1 sensitization and a subsequent FITC-OVA challenge on the next day

When mice received the FITC-OVA challenge 24 h after OE-1 sensitization following the conventional protocol to induce PSA (Fig. 1A), they exhibited anaphylactic symptoms such as hypothermia (Fig. 1B) and some scratching (Fig. 1C). The low count of scratching in PSA-induced mice was considered to be due to the induction of severe systemic anaphylaxis. Those mice rapidly entered a more severe anaphylactic phase, exhibiting reduced activity, loss of consciousness and convulsions, within 10 min, and thus no longer demonstrated heavy scratching. Mice intravenously injected with the mixture of FITC-OVA and OE-1 (Fig. 1A) also exhibited anaphylactic reactions including hypothermia (Fig. 1B) and scratching (Fig. 1C), as observed in mice with traditional PSA (Fig. 1B and C). The inhibition of scratching in FITC-OVA/OE-1 injected mice by the pretreatment with histamine H<sub>1</sub> antagonist chlorpheniramine (Fig. 1C) and similar increases in serum histamine concentrations were observed in mice injected with the FITC-OVA/OE-1 mixture and conventional PSA mice (Fig. 1D), suggesting that both treatments induced anaphylaxis through mast cell activation.

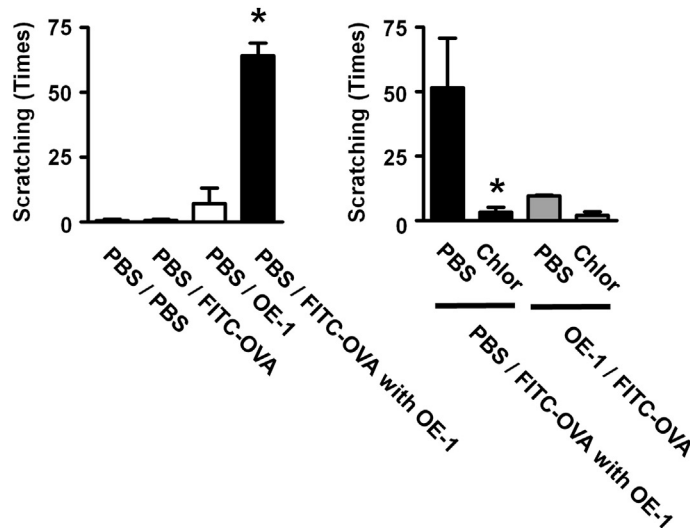
## (A) Experimental protocol



## (B) Anaphylaxis



## (C) Scratching behavior



## (D) Serum histamine concentration

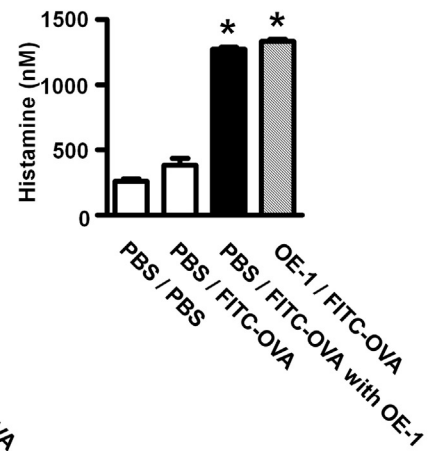


**Fig. 1.** Induction of severe anaphylaxis by a single injection of OE-1 mixed with FITC-OVA. HR-1 hairless mice were intravenously injected with OE-1 mixed with FITC-OVA (A). Alternatively, mice were challenged with intravenous FITC-OVA 24 h after sensitization with intravenous OE-1. In some cases, chlorpheniramine (Chlor, 1.5 mg) was intraperitoneally injected 30 min before the antigen injection. Rectal temperatures (B) and scratching behaviors (C) were measured with a thermometer and visually monitored, respectively. Serum histamine concentrations 60 min after the antigen injection were determined with a commercially available EIA kit (D). Symbols indicate the means  $\pm$  SEM (B) and bars indicate the means  $\pm$  SEM (C and D) of 5 or more mice. Data are representative of two independent experiments. \* $p < 0.05$  versus the PBS/FITC-OVA group (Tukey's post hoc test after a one-way ANOVA).

OE-1 is a useful antibody for inducing allergic reactions in vivo and in vitro. However, it currently remains unclear why the monoclonal antibody induces mast cell activation through Fc $\epsilon$ RI crosslinking with the monovalent antigen OVA. The existence of dimeric and polymeric OVA in commercially available OVA (Yamaki et al., Unpublished results) may partially explain its activating ability on mast cells sensitized by the monoclonal anti-OVA IgE OE-1. The complex of monomeric OVA with monomeric OE-1 may also attach and induce an activating signal from Fc $\epsilon$ RI on mast cells, because the activation mechanism by IgE

through Fc $\epsilon$ RI remains controversial, for example, monomeric IgE SPE-7 has been shown to start signals, even under conditions lacking antigens (Bax et al., 2012).

### 3.2. Intravenous injection of the immune complex constructed with FITC-OVA and OE-1 induced ASDIS in histamine- and mast cell-dependent manners

Injected FITC-OVA-dependent fluorescence was monitored in vivo to investigate the distribution of the antigen alone and the immune

complex in mice (Fig. 2A). As shown in Fig. 2B, the ASDIS distinctive spotted distribution of FITC-OVA and/or the immune complex of FITC-OVA and OE-1 was observed on the skin 15 min after a single injection of the mixture of the antigen and IgE antibody. The measurement of ARE allowed us to quantify the severity of ASDIS (Fig. 2C). ARE of ASDIS-induced skin (Area 2 in Fig. 2B and C) was markedly higher than that of skin of control, FITC-OVA-injected mice (Area 1 in Fig. 2B and C). ASDIS was almost equally induced in skin throughout the body (Fig. 2D). The same distinctive distribution of fluorescence was observed in mice injected with the immune complex of FITC-OE-1 and OVA (Fig. 2E). The low background and low variation of fluorescence was the determinant of our decision to use abdominal skin for ASDIS measurement. Measurement of ASDIS in the back is acceptable for quantifying purposes, but is rather recommended for qualifying purposes because of the relatively high variation in basal fluorescence. It should be also noted that the spot diffused gradually in a time-dependent manner (Fig. 2B), meaning that measurement within 15 to 30 min after the injection is recommended. A decrease in fluorescence in the urinary bladder in compensation for its increase in the skin was observed in FITC-OVA and OE-1 co-injected mice 60 min after the injection (Fig. 2B, white arrows). The inhibited secretion of FITC-OVA in the urine by OE-1 was attributed to an increase in the apparent molecular size of FITC-OVA due to the binding of OE-1, as caused by anti-OVA IgA, which we observed previously (Yamaki et al., Unpublished results), in addition to an increase in its distribution to the skin (Fig. 2B and C). This was supported by the stronger and weaker fluorescence of FITC-OVA in the serum and urine, respectively, in FITC-OVA/OE-1 co-injected DBA/1J mice than in mice injected with FITC-OVA alone (data not shown). High-resolution photos of the skin of mice injected with OE-1 and OVA mixture indicated the preferential distribution of the immune complex to the hair follicles (Fig. 2F, white and black arrowheads). An investigation of the relationships between scratching behavior and the distribution of the immune complex to hair follicles in the skin using our new method may also be meaningful to reveal a novel mechanism associated with the induction of itching by IgE and antigens. From this point of view, the lack of obvious modification of ASDIS within the areas spontaneously scratched in non-anesthetized mice indicated that scratching did not, or only scarcely affected ASDIS induction. Next, it should be clarified whether spot formation triggers massive scratching and/or is associated with other pathological changes in the skin.

Similar to OE-1, another anti-OVA IgE OE-3, was also capable of inducing ASDIS (Fig. 2G) and anaphylactic symptoms (Yamaki and Yoshino, 2012) when co-administered with FITC-OVA. On the other hand, the mixture of the non-allergenic anti-OVA IgE OE-2 and FITC-OVA did not induce ASDIS (Fig. 2G) or anaphylactic symptoms (Yamaki and Yoshino, in press). Similarly, the injection of anti-OVA IgG1 O1-10 with FITC-OVA did not induce ASDIS (Fig. 2G) or anaphylactic symptoms (data not shown). The observed decrease in fluorescence in the urinary bladder of OE-2 or O1-10 co-injected mice (Fig. 2G, white arrows) indicated that OE-2 and O1-10 bound to FITC-OVA and changed the distribution of FITC-OVA, similar to OA-4 (Yamaki et al., Unpublished results). Relatively mild and diffused but detectable ASDIS was induced by the FITC-OVA injection to mice sensitized with OE-1 on the previous day following the protocol for conventional PSA (Fig. 2H). Moreover, ASDIS induced by OE-1 and FITC-OVA was diminished by systemic pretreatments, as shown in Fig. 3A, with the H<sub>1</sub> receptor antagonist chlorpheniramine (Fig. 3B), H<sub>1</sub> and 5-HT<sub>2</sub> receptor antagonist cyproheptadine (Fig. 3C), or mast cell-depleting anti-FcεRI antibody MAR-1 (Fig. 3D). Increased ARE by FITC-OVA/OE-1 injection (Area 1 to Area 2 in Fig. 3D) was obviously reduced by MAR-1 (Area 3 in Fig. 3D). These results suggested that ASDIS is a histamine-dependent phenomenon released from mast cells, similar to conventional PSA. Taken together, our model with ASDIS is a new, distinctive anaphylaxis model and is as reliable as conventional PSA. Moreover, the rapidity of the induction and detection of ASDIS with *in vivo* imaging by IVIS is an advantage of its use for screening anti-allergic drugs.

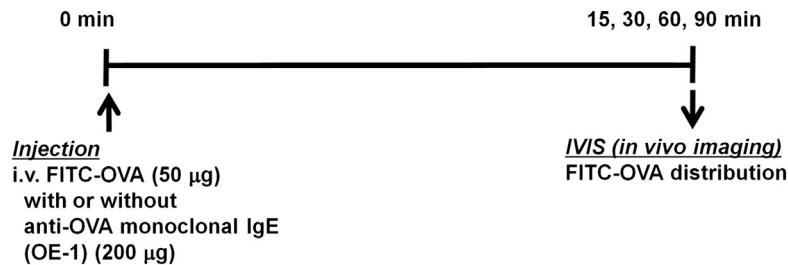
The topical painting of the histamine H<sub>1</sub> antagonist chlorpheniramine or diphenhydramine in 3% methylcellulose-PBS on abdominal skin moderately and strongly reduced ASDIS only in the painted areas (Fig. 3E). As well as the comparison of AREs between vehicle-treated mice (Area 1 in Fig. 3E) and anti-histamine-treated mice (Areas 2 and 3 in Fig. 3E), the comparison of AREs between areas treated with and without diphenhydramine within the same mouse (Areas 3 and 4 in Fig. 3E) indicated the stronger inhibition of ASDIS by diphenhydramine treatment. Similar to our *in vivo* results, the stronger inhibition by diphenhydramine than by chlorpheniramine on the histamine-induced intracellular Ca<sup>2+</sup> increase in HL-60 *in vitro* has been reported (Seifert et al., 1992). This finding suggested that ASDIS is a useful phenomenon for evaluating the efficacy of anti-allergic drugs applied as ointments. It should also be noted that ASDIS was induced without any operation to the skin, which is required by other methods such as passive cutaneous anaphylaxis, namely intradermal injections. The lack of direct injury to the skin when inducing ASDIS may reduce the possibility of artifacts, which is a major concern with other methods that require damage to the tissue. Furthermore, the topical inhibition of ASDIS by painting enabled us to investigate the effects of several drugs within the same mouse. Thus, reductions in the numbers of mice used ("Reduction" in 3Rs animal ethics) may be sufficient for screening of the tested drugs for this model when drugs are administered to the skin, in relation to other methods requiring systemic drug administration for the same purpose. Moreover, comparisons of ASDIS induced using various antibodies, antigens, labeling molecules such as fluorescent and luminescent, and vehicles will provide valuable information on their characteristics for basic research, industrial usage, and clinical application. The re-inductivity of ASDIS in the same mice is also favorable to achieve the aim of "reduction" in 3R. ASDIS was induced on day 0 and re-induced on day 7, but was not re-induced clearly on day 14, when the same HR-1 mice were injected with FITC-OVA/OE-1 on days 0, 7, and 14 (data not shown). The failure of ASDIS induction on day 14 may be due to the establishment of sensitization against FITC-OVA, namely induction of *de novo* anti-FITC-OVA antibody production. Injection of an immune complex composed of IgE was reported to induce primary antibody responses strongly against the composed antigen (Getahun et al., 2005). The antibody level in serum began to increase at 7 days and reached to a maximum 14 days after the injection. The abundance of *de novo* synthesized antibodies on day 14 may have distributed throughout the body and affected the distribution of injected immune complex, disturbing ASDIS induction. Although validation of re-induced ASDIS is needed before its application to research experiments, it may be useful at least for trials and survey examinations before proceeding to final examinations.

Anesthesia with isoflurane throughout the period from pretreatment with drugs and injection of an immune complex to *in vivo* imaging by IVIS did not interfere with the induction of ASDIS (data not shown). This is a different characteristic of ASDIS from other indicators of anaphylaxis including anaphylactic symptoms and hypothermia, which are masked and disturbed in anesthetized mice (data not shown). Intravenous histamine-induced bronchoconstriction is also known to be diminished by anesthetics (Kato and Ikeda, 1994). These findings indicate that the model with ASDIS has an exclusive advantage in the detection of allergic responses within mice that must be operated on under anesthesia. The inductivity of ASDIS in mice under anesthesia allows easy handling and minimizes pain with experiments, achieving "Refinement" in 3Rs.

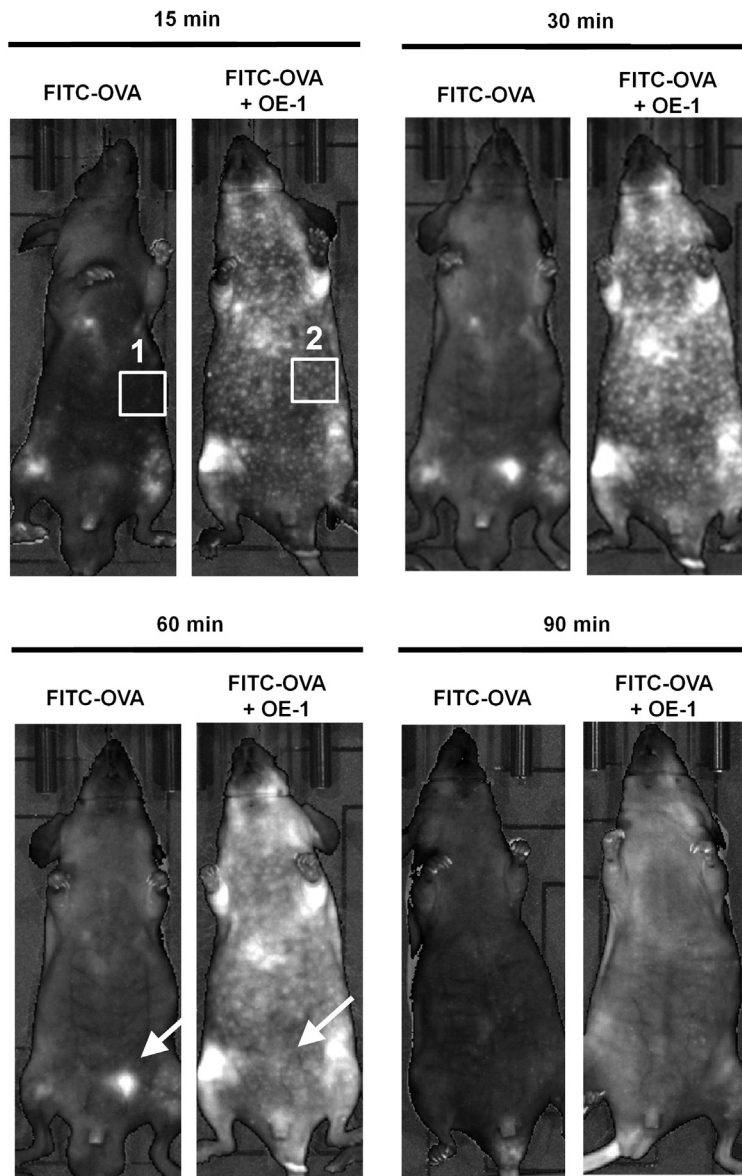
### 3.3. Mast cells may not be the direct deposition target of FITC-OVA/OE-1-immune complex

ASDIS originated from a fluorescent-labeled immune complex, and gradually diffused in a time-dependent manner as shown in Fig. 2B. Therefore, the immune complex may not be rigidly retained on the cells including mast cells or tissues. In order to clarify the mechanisms

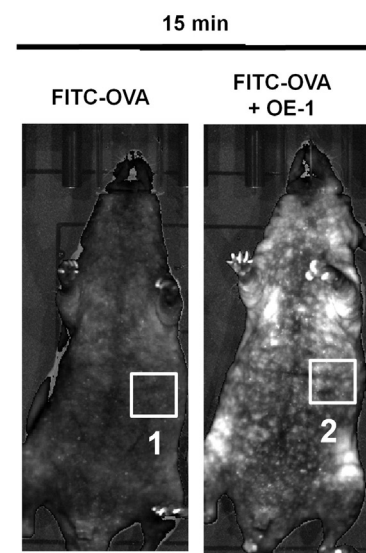
## (A) Experimental protocol



## (B) Time changes in the distribution of FITC-OVA complexed with OE-1 (ASDIS)



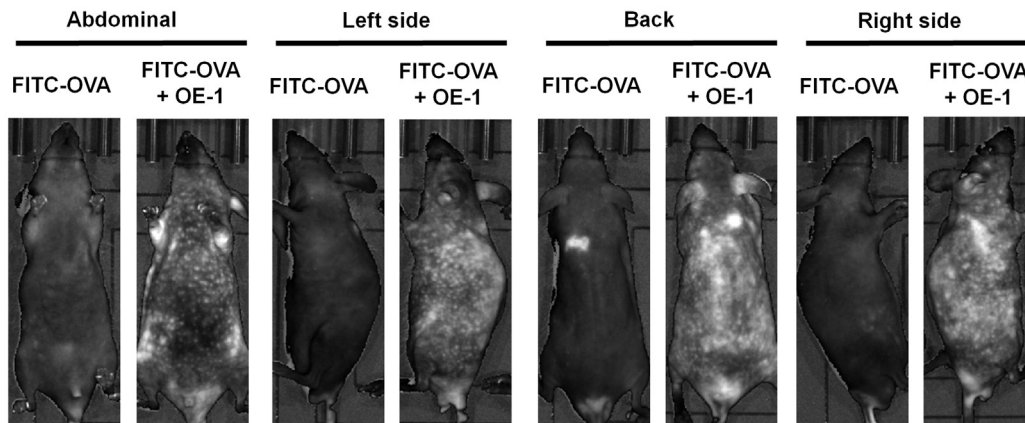
## (C) Quantification of the severity of ASDIS (15 min)



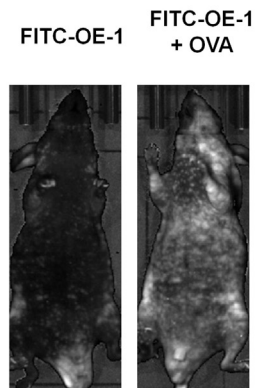
|                                         | Average Radiant Efficiency (ARE)<br>( $\times 10^8$ ) |                             |
|-----------------------------------------|-------------------------------------------------------|-----------------------------|
|                                         | Area 1<br>(FITC-OVA)                                  | Area 2<br>(FITC-OVA + OE-1) |
| Exp. 1<br>(Photos in (B))               | 2.33                                                  | 3.77                        |
| Exp. 2<br>(Photos in (C))               | 2.71                                                  | 3.62                        |
| Mean<br>$\pm$ SEM<br>of Exp. 1<br>and 2 | 2.52 $\pm$<br>0.190                                   | 3.70 $\pm$<br>0.075         |

**Fig. 2.** Anaphylaxis-dependent Spotted Distribution of a fluorescent-labeled Immune complex in Skin (ASDIS) induced by a single injection of anti-OVA IgE OE-1 mixed with FITC-OVA into HR-1 hairless mice. As shown in (A), in vivo images of fluorescence in the abdominal skin at the time indicated after the injection of the mixture of OE-1 and FITC-OVA were obtained (B) and average radiant efficiency (ARE) of the indicated areas was measured (C). ASDIS in the back, left and right sides, and abdominal skin were compared in (D). The same distinctive distribution of the immune complex constructed with FITC-OE-1 and OVA was observed (E). The preferential distribution of the immune complex to hair follicles was observed in magnified photos and images of fluorescence (F). In vivo images of fluorescence in the abdominal skin obtained from mice injected with a mixture of OE-2 and FITC-OVA, a mixture of OE-3 and FITC-OVA, and a mixture of O1-10 and FITC-OVA (G). Alternatively, mice were challenged with FITC-OVA 24 h after sensitization with or without OE-1 and fluorescence was monitored in the abdominal skin (H). White arrows indicate the position of the urinary bladder. Black arrowheads indicate the position of hair follicles and white arrowheads indicate fluorescence detected on the follicles. Data are representative of two independent experiments.

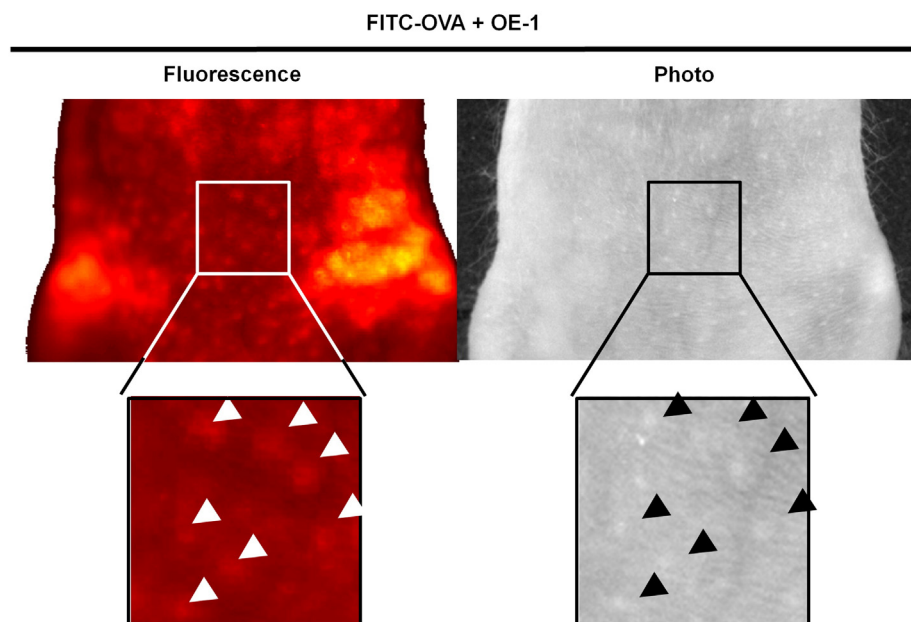
## (D) Distribution of FITC-OVA complexed with OE-1 in abdominal, back, and side skins (30 min)



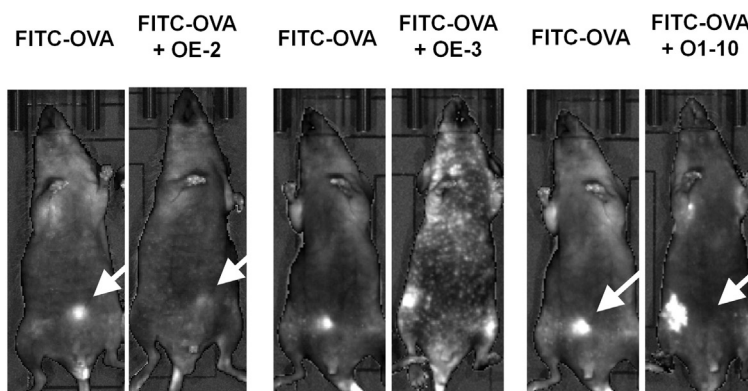
## (E) Distribution of FITC-OE-1 complexed with OVA (15 min)



## (F) Distribution of FITC-OVA complexed with OE-1 in hair follicles (30 min)



## (G) Distribution of FITC-OVA complexed with OE-2, OE-3 or O1-10 (30 min)



## (H) Distribution of FITC-OVA in OE-1-sensitized mice (30 min)

Sensitized with OE-1 (-24 h)

FITC-OVA

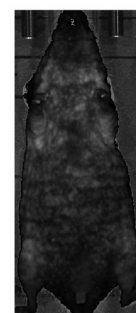
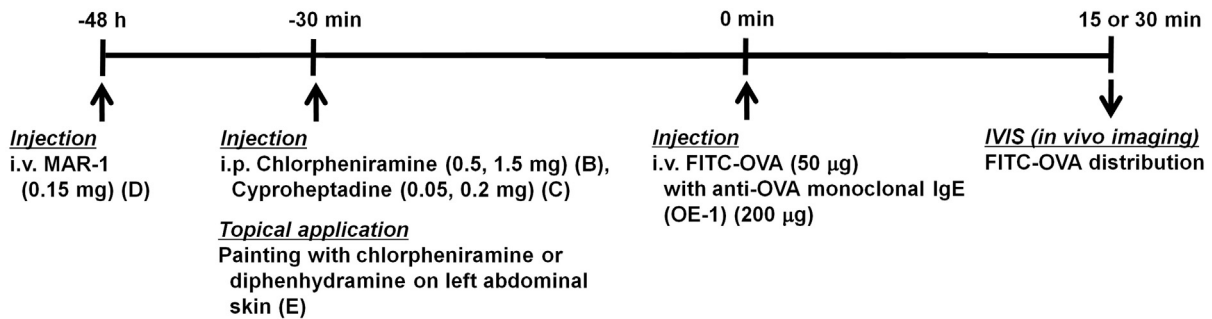


Fig. 2 (continued).

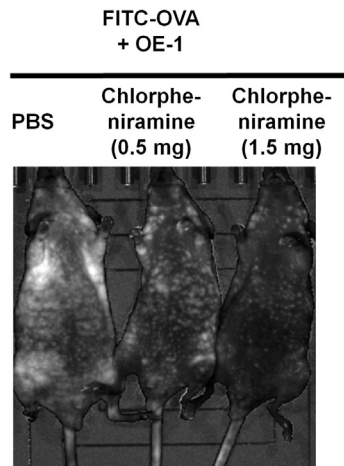
underlying the generation of spotted signals in ASDIS, we examined the phenomenon histologically. In HRS/J hairless (*hr/hr*) mice, the genetic mutation of which is responsible for the hairless phenotype, as in HR-1, have atypical skin tissues containing many utriculi and dermal cysts

(Panteleyev et al., 1998; Panteleyev et al., 1999). The changes were supposed to start from the disintegration of various cells associated with hair follicle cycling. We also observed the similar histological features such as utriculi and dermal cysts in HR-1 (hairless) mice but not in

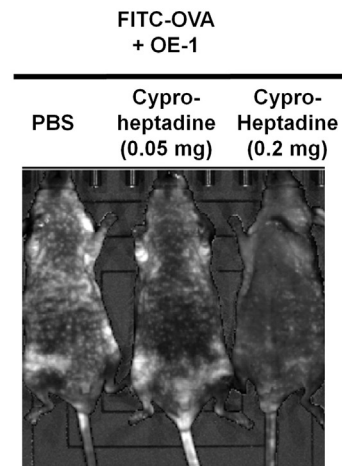
#### (A) Experimental protocol



#### (B) Effects of the histamine H<sub>1</sub> receptor antagonist on the distribution of FITC-OVA complexed with OE-1 (30 min)



#### (C) Effects of the histamine H<sub>1</sub> and 5-HT<sub>2</sub> receptor antagonist on the distribution of FITC-OVA complexed with OE-1 (30 min)



#### (D) Effects of MAR-1 on the distribution of FITC-OVA complexed with OE-1 (15 min)

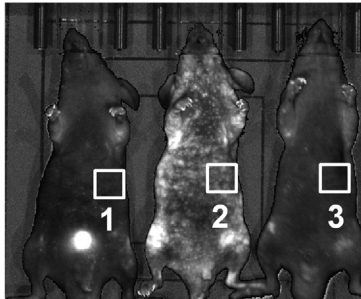
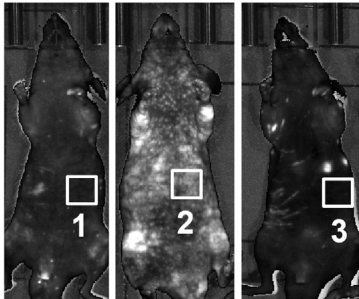
| Exp. 1                                                                              |     |                    | Exp. 2                                                                              |     |                    | Average Radiant Efficiency<br>(ARE) (x 10 <sup>8</sup> ) |                    |                   |                 |
|-------------------------------------------------------------------------------------|-----|--------------------|-------------------------------------------------------------------------------------|-----|--------------------|----------------------------------------------------------|--------------------|-------------------|-----------------|
| FITC-OVA                                                                            |     | FITC-OVA<br>+ OE-1 | FITC-OVA                                                                            |     | FITC-OVA<br>+ OE-1 | FITC-OVA                                                 | FITC-OVA<br>+ OE-1 |                   |                 |
| PBS                                                                                 | PBS | MAR-1              | PBS                                                                                 | PBS | MAR-1              | PBS<br>(Area 1)                                          | PBS<br>(Area 2)    | MAR-1<br>(Area 3) |                 |
|  |     |                    |  |     |                    | Exp. 1                                                   | 1.47               | 3.39              | 1.90            |
|                                                                                     |     |                    |                                                                                     |     |                    | Exp. 2                                                   | 1.65               | 3.07              | 1.57            |
|                                                                                     |     |                    |                                                                                     |     |                    | Mean<br>± SEM<br>of Exp. 1<br>and 2                      | 1.56 ±<br>0.090    | 3.23 ±<br>0.160   | 1.74 ±<br>0.165 |

**Fig. 3.** Inhibition of ASDIS by intraperitoneal administration of a histamine H<sub>1</sub> receptor antagonist and a histamine H<sub>1</sub>/serotonin 5-HT<sub>2</sub> receptor antagonist, intravenous administration of the mast cell-depleting antibody MAR-1, or topical painting of histamine H<sub>1</sub> receptor antagonists. As shown in (A), HR-1 hairless mice were administered the histamine H<sub>1</sub> receptor antagonist chlorpheniramine (B), histamine H<sub>1</sub> and serotonin 5-HT<sub>2</sub> receptor antagonist cyproheptadine (C) intraperitoneally, or mast cell-depleting antibody MAR-1 (D) intravenously. Alternatively, mice were painted with the histamine H<sub>1</sub> receptor antagonist chlorpheniramine or diphenhydramine topically on the left area of the abdominal skin (E). The vehicle 3% methylcellulose-PBS was painted on the right area of the abdominal skin in the same mice. In vivo images of fluorescence in the abdominal skin of these mice induced by an injection of the OE-1/FITC-OVA mixture were then obtained. Data are representative of two independent experiments. Average radiant efficiency (ARE) of the indicated areas was measured in (D) and (E).

## (E) Effects of the topical application of chlorpheniramine and diphenhydramine on the distribution of FITC-OVA complexed with OE-1 (15 min)

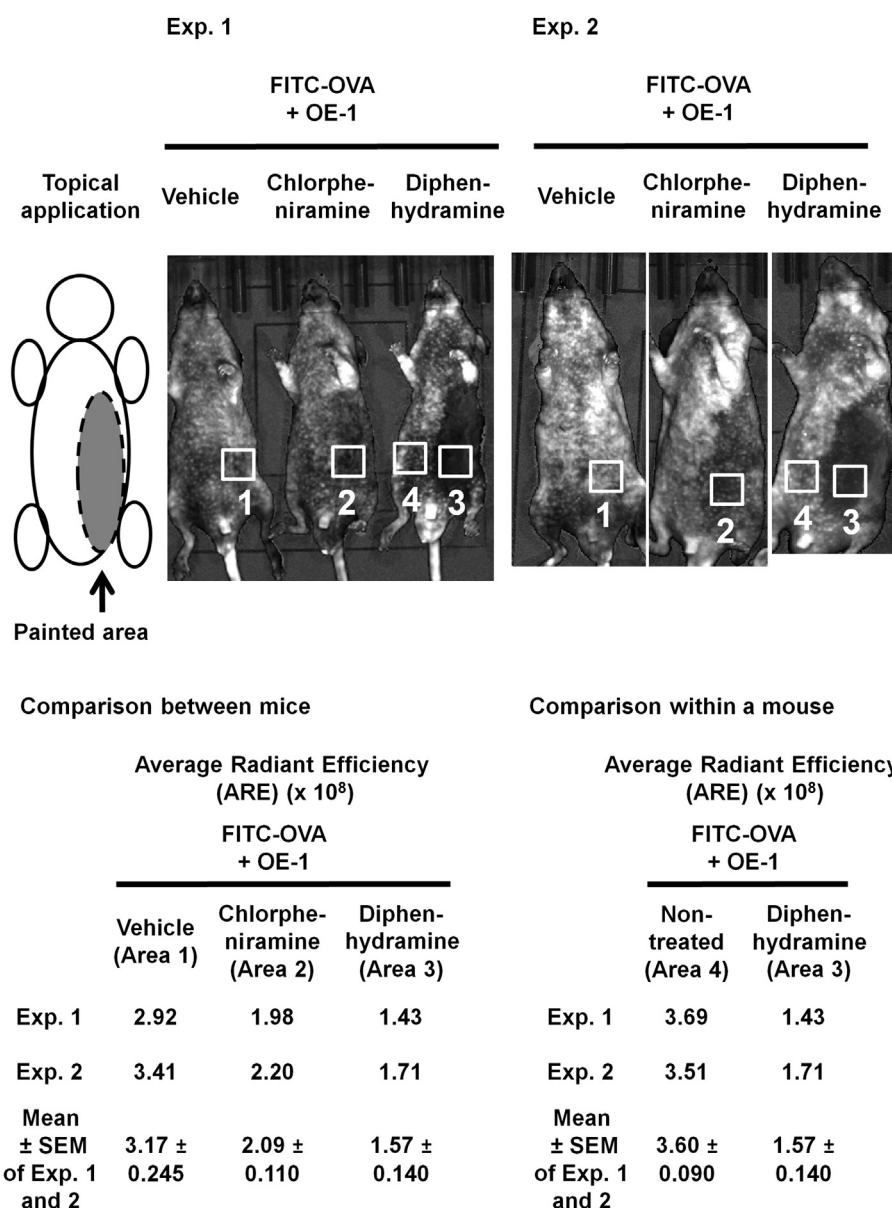


Fig. 3 (continued).

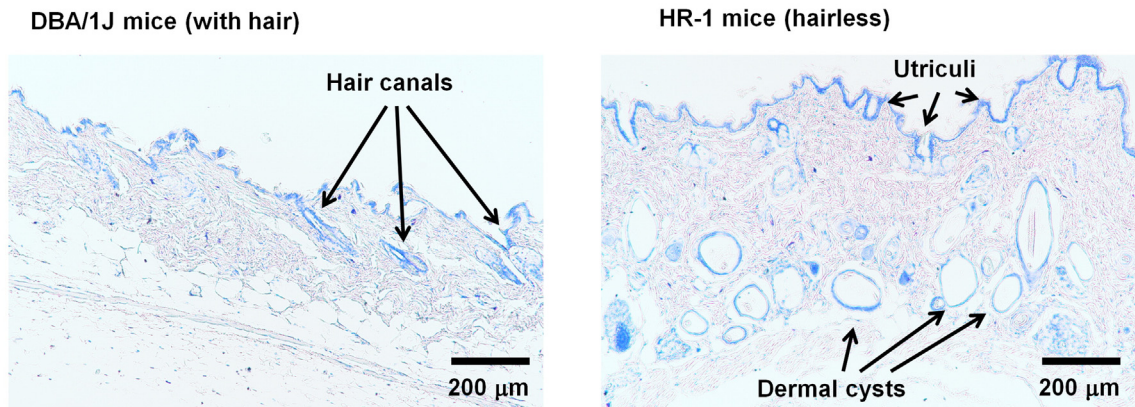
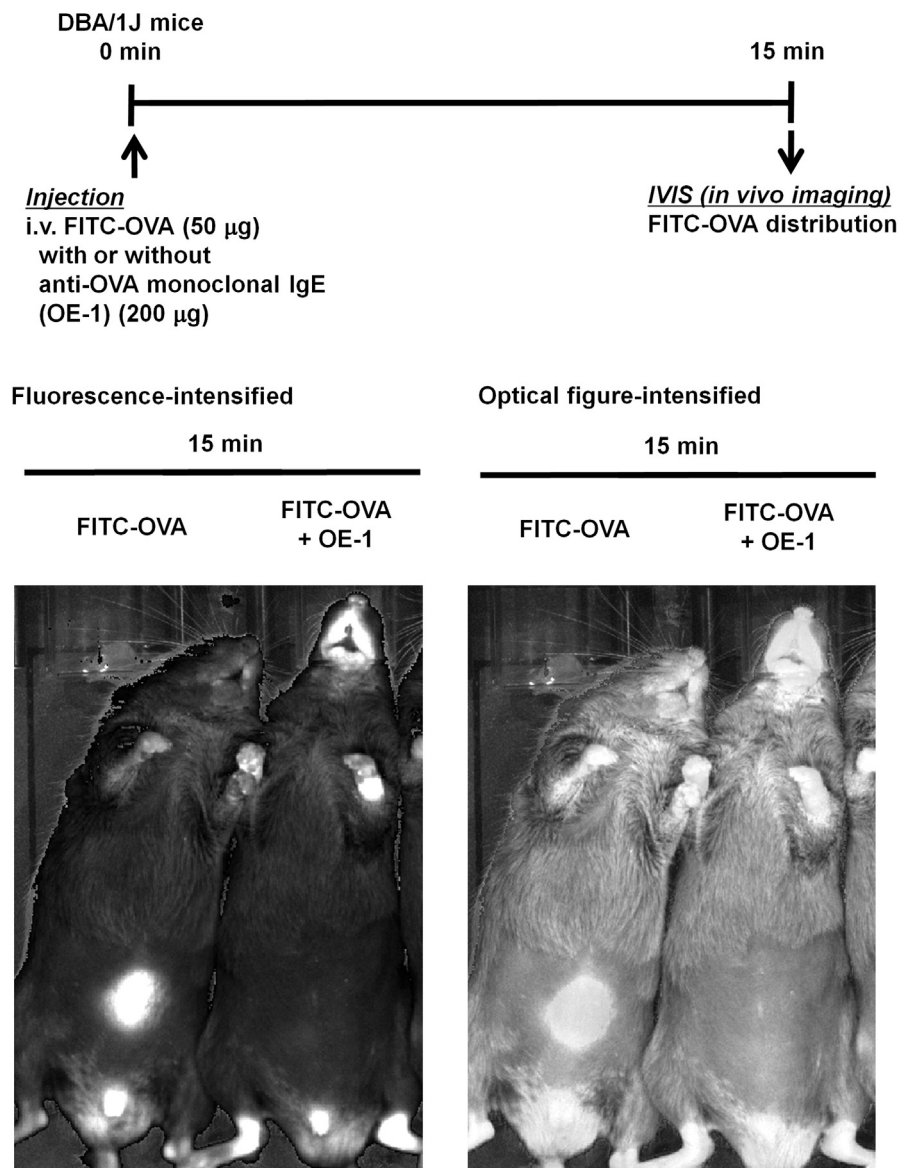
DBA/1J (with hairs) mice (Fig. 4A). Moreover, ASDIS was not observed in DBA/1J mice (Fig. 4B), although we could not eliminate the possibility that the shaving or application of depilatory cream to remove the hair for DBA/1J mice artificially suppressed ASDIS. The presence and absence of ASDIS in HR-1 and DBA/1J mice respectively, made us think that the exclusive features of skin in HR-1 such as dermal cysts and utriculi (Fig. 4A) may be candidates for the FITC-OVA/OE-1-accumulating tissues. Immunohistochemical analysis with paraformaldehyde-fixed, frozen sections revealed that each area of accumulated FITC (Fig. 5A,

DAB stains indicated by arrow) was located separately, similar to the spotted signals in ASDIS. However, the analysis also indicated that the tissues of skin in HR-1 mice may not be the deposition targets of FITC-OVA/OE-1 (Fig. 5A). Utricle and dermal cysts were located on the proximal skin in contrast to FITC-deposited sites which were located on the distal skin.

Since mast cells have been detected in and/or around hair follicles in humans (Christoph et al., 2000) and contribute to managing the hair cycle in mice (Maurer et al., 1995), these cells may be an acceptor of

**Fig. 4.** The distinctive features observed in skin of HR-1 mice compared with DBA/1J mice which did not develop ASDIS. (A) The 4- $\mu$ m sections of 10% formalin-fixed skin of DBA/1J or HR-1 mice were stained with 0.05% toluidine blue solution (pH 2.5). DBA/1J mice, but not HR-1 mice had hair canals. Utricle and dermal cysts were observed only in skin of HR-1 mice. (B) DBA/1J mice with shaved abdominal hair were injected with FITC-OVA or FITC-OVA/OE-1 intravenously and whole body image was gained with following the indicated protocol. Images of the same mice with highlighted fluorescence signals (left photo) and optical image (right photo) are shown. In contrast to HR-1 mice, DBA/1J mice did not develop ASDIS in abdominal skin shaved in order to facilitate to detect the signals by intravenous injection of FITC-OVA/OE-1 mixture. Detection of the accumulated FITC signals in the urinary bladder in FITC-OVA-injected mice confirmed that the shaved skin was adequate for examining in vivo imaging with the fluorescence. Data are representative of two independent experiments.

## (A) Formalin-fixed paraffin-embedded sections stained with toluidine blue

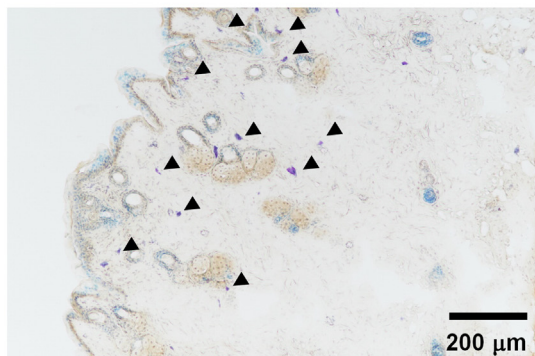
(B) *In vivo* imaging of FITC-OVA/OE-1 distribution in DBA/1J mice

the immune complex and, thus, hair follicles were detected as high fluorescence spots in ASDIS (Fig. 2F). However, toluidine blue staining of paraformaldehyde-fixed, frozen sections showed that mast cells

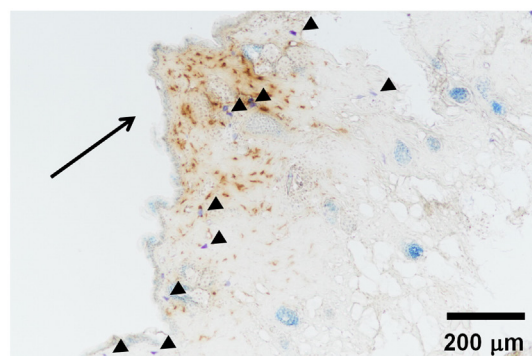
were distributed equally in skin (Fig. 5A, arrowheads). Moreover, FITC (Fig. 5A, DAB stains indicated by arrow) appeared not to co-localize specifically to mast cells (Fig. 5A, arrowheads). These results suggested that

**(A) FITC distribution (DAB staining) in frozen-section fixed with paraformaldehyde (counterstained with toluidine blue)**

**FITC-OVA-injected HR-1 mice**

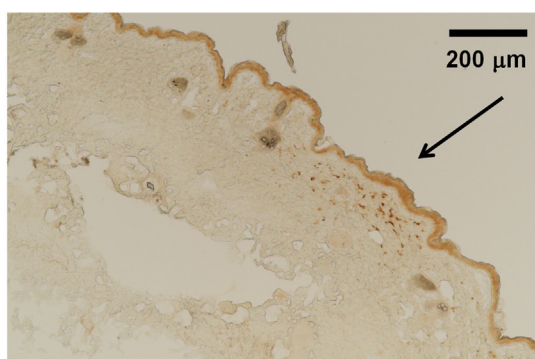


**FITC-OVA/OE-1-injected HR-1 mice**

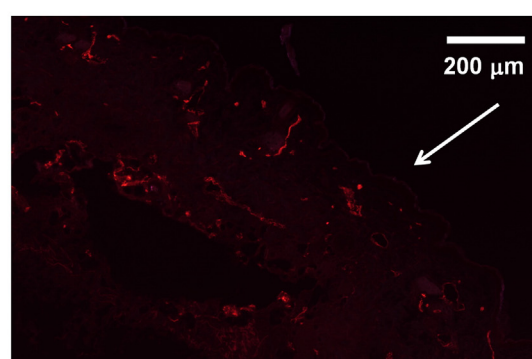


**(B) FITC and CD31 distribution in frozen-section of FITC-OVA/OE-1-injected mice fixed with acetone**

**DAB staining (FITC)**



**PE fluorescence (CD31)**



**Merge**

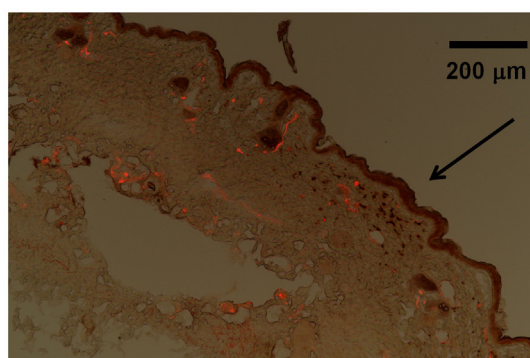


**Fig. 5.** Immunohistochemical detection of spotted distribution of areas accumulated with FITC in skin of HR-1 mice injected with FITC-OVA/OE-1. (A) FITC-OVA distribution in the 11-μm frozen sections of HR-1 mice injected with FITC-OVA or FITC-OVA/OE-1 were detected by peroxidase-labeled anti-FITC antibody followed by DAB staining. Arrows indicate the areas accumulated with the stains. Mast cells counterstained by toluidine blue (arrowheads) appeared to distribute equally in both DAB-stained and DAB-non-stained areas. (B) With simultaneous detection of FITC distribution (upper-left photo), CD31 was visualized by phycoerythrin (PE)-labeled anti-CD31 (upper-right photo). Merged photo (lower photo) as well as the upper individuals indicated some co-localization of PE and DAB stains (arrows). Data are representative of two independent experiments.

mast cells may not be the direct deposition target of the IgE-immune complex, although mediators released from those cells must contribute to the phenomenon.

In frozen sections with acetone fixation, only some but not the majority of the microvessels visualized by PE-labeled anti-CD31 were co-localized with FITC detected with DAB stains (Fig. 5B). Therefore, the spotted signals of FITC in ASDIS was thought to be due to the extravasated FITC-containing immune complex in skin rather than those in blood vessels. Further studies are required to address the direct target of FITC-OVA/OE-1 immune complex. In future, we intend to clarify in more detail the mechanisms responsible for generating the spots in ASDIS-induced mice, in various ways.

### 3.4. Conclusions

ASDIS induced by our new method is a new, distinctive phenomenon representing anaphylactic reactions. The advantages of using this anaphylaxis model to screen anti-allergic compounds are its rapidity, easiness to judge, specificity to anaphylaxis, practicability to evaluate transdermal applications, comparability of multiple drugs in one mouse, and, thus, reductions in the number of mice used. This model may contribute to investigations of a new mechanism inducing anaphylaxis associated with ASDIS and the development of an efficacious strategy to investigate anti-allergic drugs.

### Author contributions

Kouya Yamaki planned and performed all the experiments described herein and drafted the manuscript. Shin Yoshino acted as the laboratory supervisor for Kouya Yamaki and assisted in the preparation and proofing of this manuscript.

### Declaration of interests

This work was not supported by any funding. Dr. Yamaki and Prof. Yoshino are co-inventors on pending ASDIS-related patents described in this manuscript.

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