Comparison of immune response in mice sensitized to an animal allergen, Can f 1, and to a food allergen, ovalbumin

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ABSTRACT

Can f 1 belongs to the lipocalin superfamily and is considered to be an animal allergen. The immune response induced by Can f 1 in mice was compared with that induced by ovalbumin (OVA), a typical food allergen. Female BALB/c and C57BL/6 mice (6 weeks of age) were subcutaneously injected with Can f 1 or OVA with or without aluminum hydroxide (Alum) three times with intervals of two weeks. Serum levels of total IgE or antigen-specific IgE and production of IL13 and IFNγ from splenocytes were analyzed. Immunization with Can f 1 or OVA increased serum levels of both total IgE and antigen-specific IgE significantly irrespective of Alum. These results indicate that Can f 1 and OVA were able to induce allergic sensitization in mice. Splenocyte production of IL13 in mice immunized with Can f 1 or OVA with and without Alum were significantly increased after stimulation with each antigen. However, IL13 levels in the mice immunized with Can f 1 with Alum were significantly lower than those immunized without Alum. Increases in IFNγ levels after stimulation with Can f 1 or OVA were not remarkable. No influence of genetic backgrounds of BALB/c and C57BL/6 mice was found. Although Can f 1 induced Th2 type immune responses as was also the case for immunization with OVA, an inhibitory effect of Alum on induction of IL13 was observed only in mice immunized with Can f 1. These results suggest that the immune mechanism for allergic sensitization with Can f 1 is different from that with OVA.

An allergy is defined as a condition showing immunological hypersensitivity to a specific object. A food allergy is a type of allergy caused by ingestion of food. Ovalbumin (OVA) and casein derived from eggs and milk, respectively, are the most common causes of food allergies. An animal allergy is a hypersensitivity caused by animal substances such as dander, saliva, and urine and is characterized by skin rashes, rhinitis, asthma, and occasionally anaphylaxis. In the development of an animal allergy, exposure to allergens mostly occurs through inhalation or through skin contact. Animal models, especially mice, have significantly contributed to the elucidation of onset mechanisms of allergies and the development of diagnosis and therapy for allergies (7). OVA has been used most widely as an immunogen to establish allergy models in mice which develop asthma, atopic dermatitis, allergic rhinitis, anaphylaxis, and food allergy. However, studies of mouse models using animal allergens have been limited.

Studies of mouse model sensitized to OVA have revealed that allergy is mediated by CD4+ T-helper type 2 (Th2) lymphocytes that, through the production of cytokines such as IL4, IL5 and IL13, orchestrate allergen-specific IgE synthesis and eventually eosinophilic inflammation in the target organs (16). Anaphylaxis can occur in OVA sensitized mice through two independent pathways; a pathway that...
involves IgE, FceRI, mast cells, platelet-activating factor and histamine, and a pathway that involves IgG, FcyRIII, macrophages, and platelet-activating factor and is promoted by endogenously produced IL4 and IL13 (4). The mouse models have also revealed that the genetic background of mouse strains has a striking and selective effect on the phenotype of murine allergic disease (8, 12, 23). For example, allergies were more strongly induced in BALB/c mice with a Th2-dominant genetic background than in C57BL/6 mice with a Th1-dominant genetic background. Therefore, aluminum hydroxide (Alum), which is a Th2 adjuvant, has been used extensively for allergy induction in mouse models of various strains (11).

Studies have shown that most of the animal allergens are members of the lipocalin superfamily, a large family of low-molecular-weight (~19 kDa) proteins, which are also known as major urinary proteins (1, 3, 6, 15). These proteins include mouse allergen Mus m 1 (1), dog allergen Can f 1 (9) and horse allergen Equ c 1 (5). Studies on diagnosis and therapy for animal allergies have been performed by using a mouse model sensitized to lipocalin allergens (15). However, the influence of the genetic backgrounds of inbred mouse strains, and the effect of Alum on immune responses in a mouse model have not been determined yet.

In this study, we analyzed the influence of genetic backgrounds among inbred mouse strains and the effect of Alum on production of IgE, IL13 (a Th2-type cytokine) and IFNγ (a Th1-type cytokine) in mice immunized with the dog allergen Can f 1 and compared them with those in mice immunized with OVA, a typical food allergen.

MATERIALS AND METHODS

Animals. All experimental procedures were approved by Hokkaido University Animal Experimentation Committee (NO.15-0074). Husbandry and care procedures followed recommendations within the Guide for the Care and Use of Laboratory Animals (13). Female BALB/c and C57BL/6 mice (5 weeks of age) were obtained from Charles River (Yokohama, Japan) and CLEA (Tokyo, Japan), respectively. The mice were acclimated for at least 1 week before immunization, and were housed with food (MF; ORIENTAL YEAST, Tokyo, Japan) and water ad libitum in individually ventilated cage system (Allentown, NJ, USA) under specific pathogen free conditions. The health status of animals in specific pathogen free conditions was controlled three times a year for the following pathogens: Citrobacter rodent, Corynebacterium kutscheri, Mycoplasma pulmonis, Salmonella spp., Clostridium piliforme, Ectromelia virus, Lymphocytic choriomeningitis virus, Mouse hepatitis virus, Sendai virus, ectoparasites, intestinal protozoa, and pinworm. The room was kept on a 12:12-h light:dark cycle, at 22.0 to 24.0°C, with 40 to 60% relative humidity and 15 air changes/h.

Immunization and sampling. Purified Can f 1 protein (endotoxin < 0.03 EU/μg; INDOOR Biotechnologies, VA, USA) and OVA (grade V; Sigma-Aldrich, MO, USA) were used as animal allergen and food allergen, respectively. Immunization of mice was performed as described previously (14). Briefly, 5 μg of each antigen with or without 1 mg of Alum (LSL, Tokyo, Japan) was dissolved in 100 μL of Dulbecco’s phosphate buffered saline (PBS). BALB/c and C57BL/6 mice were injected subcutaneously in the neck with each antigen with or without Alum on days 0, 14, and 28. PBS with or without Alum was used for a negative control. The numbers of mice in each group were 5 or 6. On day 35, the mice were euthanized with an intraperitoneal injection of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan). Whole blood obtained through cardiocentesis was centrifuged and serum was stored at −80°C until IgE measurement. Spleens were removed and placed in RPMI 1640 medium (Gibco, Illkirch, France) for splenocyte culture.

Measurement of total IgE antibodies in serum. Total IgE levels were quantified using Mouse IgE ELISA MAX™ Standard Set (BioLegend, CA, USA) according to the manufacturer’s instructions.

Measurement of antigen-specific IgE antibodies in serum. cDNA for Can f 1 subcloned in a pET20b expression vector was kindly provided by Dr. H. Grönlund (Karolinska University Hospital). The recombinant Can f 1 protein expressed in BL21(DE3) was purified as described previously (10). Can f 1 and OVA-specific IgE were analyzed by ELISA. Briefly, microtiter plates (96 wells) were coated with 5 μg/mL anti-mouse IgE antibody (Bethyl Laboratories, TX, USA), and diluted sera were applied and bound IgE was detected using biotinylated recombinant Can f 1 or OVA.

Analysis of cytokine production in antigen-stimulated splenocytes. Analysis of cytokine production in splenocytes was performed as described previously (20). Briefly, spleens were gently crushed, fil-
tered through a 70-μm nylon filter (FALCON, NY, USA), and rinsed in RPMI 1640 medium, and treated with 0.17 M ammonium chloride to remove red blood cells. Then the splenocytes were rinsed and resuspended in RPMI 1640 containing 0.3% 4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffer, 2 mM l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. The number of viable cells was determined using the trypan blue dye (0.25%) exclusion method. Splenocytes were cultured in a 24-well plate at a density of 2 × 10^6 cells per well with or without 5 μg/mL of each antigen at 37°C in a 5% CO₂ and 95% air atmosphere. Culture supernatants were collected after 48 h of culture and stored at −80°C until analysis.

Levels of IFNγ and IL13 in the culture supernatants were quantified by using Mouse IL13 ELISA Ready-SET-Go! (Affymetrix eBioscience, Vienna, Austria) and Mouse IFN gamma ELISA Ready-SET-Go! (Affymetrix eBioscience), respectively, according to the manufacturers’ instructions. Detection limits for ELISAs were 4 pg/mL for IL13 and 16 pg/mL for IFNγ.

Statistical analysis. A nonparametric Mann-Whitney U-test was used when two groups were compared. For all comparisons, P < 0.05 was considered significant.

RESULTS

IgE levels in serum
Total IgE levels in BALB/c and C57BL/6 mice after immunization with Can f 1 or OVA with and without Alum were significantly higher than those in controls (Fig. 1A and 1B) except for C57BL/6 mice immunized with Can f 1 with Alum, in which total IgE levels were not changed.

Similar results were obtained for the antigen-specific IgE (Fig. 2A and 2B).

IL13 production from splenocytes
Levels of IL13 were significantly increased in splenocytes from BALB/c and C57BL/6 mice immunized with Can f 1 or OVA with and without Alum when the splenocytes were stimulated with each antigen (Fig. 3A and 3B). No IL13 production was observed without the antigen stimulation except in BALB/c mice immunized with Can f 1 without Alum (arrow in Fig. 3A). The levels of IL13 in mice immunized with Can f 1 were higher without Alum than with Alum. No effect of Alum in OVA immunized mice was observed.

IFNγ production from splenocytes
Levels of IFNγ after stimulation with Can f 1 or OVA were significantly increased in splenocytes from the following mice that were BALB/c immunized with OVA with and without Alum, and C57BL/6 mice immunized with Can f 1 and OVA without Alum (Fig. 4A and 4B). However, those increases were not remarkable compared with the levels of IL13.
DISCUSSION

Comparative analysis of immune responses of mice immunized with the dog allergen Can f 1 as an animal allergen and OVA as a food allergen revealed that Can f 1 is able to induce allergic sensitization in mice as is also the case for immunization with OVA, and immunization with Can f 1 increased serum IgE levels compared to control mice. It is well known that Th2 type immune responses play an important role in induction of animal allergic sensitization. It was also reported that human CD4-positive naïve T cells differentiated into Th2 cells when activated by Can f 1 (17). In our study, it was shown that immunization with Can f 1 induced IL13, a Th2-type cytokine, rather than IFNγ, a Th1-type cytokine, in vitro. The results suggested that Can f 1 induced an animal allergy in mice by Th2 type immune responses as in humans. Typical symptoms of animal allergies are skin rashes, rhinitis, asthma, and occasionally anaphylaxis. It has been reported that serum levels of antigen-specific IgE in model mice subcutaneously sensitized to Can f 1 with Alum were significantly increased (14). Our results confirmed elevation of serum IgE and IL13 levels in mice sensitized with and without Alum by the same sensitization route in the same sensitization schedule; however, no typical animal allergy symptoms were observed. These results suggest that immunization with Can f 1 by the subcutaneous route was not adequate to induce the onset of animal allergy symptoms, although the immunization by the subcutaneous route could induce allergic sensitization in mice. It has been reported that direct allergen challenge to the target organ was necessary for maximal induction of pulmonary disease (23) and that IL13
Immune response to a dog allergen

Although further study is required to analyze immune responses in mice under other sensitized conditions of Can f 1, it was shown in the present study that IL13 is involved in the induction of Can f 1-specific IgE production because the levels of IL13 were remarkably increased by stimulation with Can f 1 in vitro and there was a significant positive correlation between IL13 and Can f 1-specific IgE.

Our experiments show that no influence of genetic backgrounds of BALB/c and C57BL/6 mice immunized subcutaneously with Can f 1 or OVA with and without Alum was found. On the other hand, analysis of the influence of Alum revealed a clear
The production of IgE in BALB/c mice injected subcutaneously with OVA was not affected by treatment with Alum (2, 22). These results are in agreement with our findings in mice immunized with OVA. Yamanishi R. et al. have described that Alum may have a biochemical activity other than its physico-chemical effect such as antigen adsorption and enhance IgE production at a suitable antigen dosage (22). The mechanism of the action of Alum has just started to be revealed. Initially, lipocalins were characterized as transport proteins of small hydrophobic molecules such as retinol, odorants, steroids, and pheromones. However, it is discussed in a recent review that they are also involved in a wide range of functions beyond their role in transportation.

Fig. 4  IFNγ production by cultured BALB/c (A) and C57BL/6 (B) splenocytes stimulated with or without Can f 1 or OVA. Mice were administered 5 μg of each antigen (Can f 1 (circle) or OVA (triangle)) or PBS (square) (negative control) with (closed) or without (open) 1 mg of Alum. Splenocytes were removed on day 35. The culture supernatants were collected 48 h after stimulation with Can f 1 or OVA in vitro. n = 5–6 per group. *P < 0.05, **P < 0.01 and N.S., not significant; Mann-Whitney U-test when compared with no stimulation in vitro. #P < 0.05 and N.S., not significant; Mann-Whitney U-test when compared with mice immunized to an antigen with Alum.

difference between Can f 1 immunization and OVA immunization. The levels of IL13 in both strains of mice immunized with Can f 1 together with Alum were lower than those in mice immunized only with Can f 1, which differ from the results for immunization with OVA (Fig. 3A and 3B). Alum is the most widely used adjuvant in human vaccines and in mouse models of allergies. It is well known that an antigen absorbed on Alum is captured by and taken into antigen-presenting cells and induces MHC II type antigen-specific memory responses. However, it was reported that macrophages carrying Alum exhibits distinct changes in their phenotype and function (18). Moreover, it was also reported that the production of IgE in BALB/c mice injected subcutaneously with OVA was not affected by treatment with Alum (2, 22). These results are in agreement with our findings in mice immunized with OVA. Yamanishi R. et al. have described that Alum may have a biochemical activity other than its physico-chemical effect such as antigen adsorption and enhance IgE production at a suitable antigen dosage (22). The mechanism of the action of Alum has just started to be revealed. Initially, lipocalins were characterized as transport proteins of small hydrophobic molecules such as retinol, odorants, steroids, and pheromones. However, it is discussed in a recent review that they are also involved in a wide range of functions beyond their role in transportation.
other biological functions. For example, Can f 1 binds to the C-type lectin-like carbohydrate recognition domains 4-7 of the mannose receptor (21). In the present study, the influence by Alum on the levels of IL13 in mice immunized with Can f 1 may be attributable to an unknown biological function of Can f 1 or Alum. Further work is required to clarify the control mechanisms of the production of IL13 in mice immunized with Can f 1 with and without Alum.

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