

ORIGINAL ARTICLE

The 1st step initiation essential for allergen-specific IgE antibody production upon the 2nd step: Induction of non-specific IgE⁺ small B cells containing secondly-sensitized allergen-specific ones in mice firstly-sensitized with an allergen

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ABSTRACT

There was a significant amount of non-specific, but not of allergen (e.g., papain, mite feces and four kinds of pollen)-specific, IgE antibodies (Abs) in the sera of normal mice. An i.n. injection of each allergen without adjuvant into mice caused an increase in total IgE Ab titers with a similar time course in the serum. However, the stage of initiation of allergy varied from allergen to allergen. Submandibular lymph node cells from normal mice contained papain-, but not mite feces- or pollen-specific IgE⁺ cells and an i.n. injection of papain induced papain-specific IgE Abs in the serum. In contrast, one (i.n.) or two (i.n. and s.c.) injections of mite feces induced neither mite feces-specific IgE⁺ cells in the lymph nodes nor mite feces-specific IgE Abs in the serum. I.n. sensitization with cedar pollen induced cedar pollen-specific IgE⁺ small B cells in the lymph nodes on Day 10, when non-specific IgE Ab titers reached a peak in the serum, implying induction of related allergen-specific IgE⁺ small cells as well. In fact, a second (s.c.) injection of ragweed (or cedar) pollen into mice sensitized i.n. once with cedar (or ragweed) pollen, but not with mite feces, induced a large amount of ragweed (or cedar) pollen-specific IgE Abs in the serum. These results indicate that when firstly-sensitized non-specific IgE⁺ small B cells in mouse lymph nodes include some secondly-sensitized allergen-specific ones, mice produce IgE Abs specific for the secondly-injected allergen.

Key words allergy initiation, allergen-specific IgE⁺ cell, allergen-specific IgE antibody.

Allergic rhinitis (seasonal or perennial) is a typical atopic disease, the prevalence of which has recently been accelerating worldwide (1). Production of specific IgE antibodies (Abs) directed toward an allergen is a prerequisite for an immunologic response leading to allergic rhinitis in repeatedly sensitized individuals (2, 3). It has been assumed that allergens in tissues encounter DCs that take up

protein antigens of these allergens, process them, and promote T_H2-driven production of allergen-specific IgE Abs by B cells (4). Also, in most previous animal models of pollen-induced allergic rhinitis, allergic reactions have been induced by administering repeated pollen inhalation challenges to animals that have been sensitized by repeated instillation of the pollen extract plus adjuvant into their

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Received 28 July 2017; revised 1 November 2017; accepted 24 November 2017.

List of Abbreviations: Ab, antibody; FACS, fluorescence-activated cell sorter; i.n., intranasally; i.p., intraperitoneally; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin.

nostrils (5–7), the repeated instillation and addition of adjuvant presumably being required because instillation into the nostrils has limitations (e.g., volume [$\leq 25 \mu\text{L}/\text{day}/\text{mouse}$], concentration [$\leq 16.6 \mu\text{g}/25 \mu\text{L}$] and loss of allergen because of drinking or sneezing) (our unpublished data).

The animal models described above are clinically relevant; however, repeated sensitization with allergen plus adjuvant obscures the mechanism of allergen-specific IgE Ab production. To precisely characterize the first reaction of the immune system to the allergen, we i.n. injected cedar pollen without adjuvant once into BALB/c mice. We have previously shown that submandibular lymph nodes are the main site of reaction to an i.n. injection of cedar pollen into mice (8) and that macrophages induce T cells to produce large amounts of IL-4 and induce IL-4-dependent IgE class switching on B cells, which then secrete non-specific IgE Abs (8–11). We have also shown that, after a second s.c. injection of cedar pollen, abundant cedar pollen-specific IgE Abs can be found in the serum (9, 10). However, there is no increase in non-specific IgE or IgM Abs in the sera of IL-4 $-/-$ mice sensitized i.n. once with cedar pollen (10), suggesting that induction of non-specific IgE Abs is IL-4-dependent and not caused by contamination with inflammatory agents (e.g., lipopolysaccharide and other toll-like receptor ligands) in cedar pollen extract. Thus, our previous data indicate that an increase in IL-4-dependent non-specific IgE Ab production after a first (i.n.) injection of cedar pollen is a prerequisite for production of cedar pollen-specific IgE Abs after a second (s.c.) injection of the same allergen.

Small (or dense [density ≥ 1.080]) and large (or less dense [density < 1.080]) B cells respond differently to T cell-derived B cell growth and differentiation factors (12) and Ig $^+$ B cells are precursors of the cells that secrete isotype Abs (13). Therefore, we here used a variety of allergens (namely papain, mite feces and four kinds of pollen) to explore when and where small or large B cells containing allergen-specific IgE $^+$ B cells are induced in lymph nodes. We found that submandibular lymph node cells from mice (Days 0–14) sensitized once with an allergen contain two different sizes of lymphocytes (i.e., small [$\sim 20\%$] and large [$\sim 80\%$] cells) and that IL-4 from large T cells on Days 5–10 cause polyclonal activation of B cells (i.e., induction of related allergen-specific IgE $^+$ small B cells) to produce non-specific IgE Abs. We also showed that when IgE $^+$ small B cells in mice firstly-sensitized with an allergen include some secondly-sensitized allergen-specific ones, the mice produce IgE Abs specific for the secondly-injected allergen. These results indicate that non-specific (or related allergen-

specific) IgE $^+$ small B cells are IL-4-dependently induced by the first sensitization with an allergen and that secondly-sensitized allergenic antigen-presenting small B cells and their cognate T cells may be induced by the second sensitization to produce secondly-sensitized allergen-specific IgE Abs.

MATERIALS AND METHODS

Animals

It has been reported that serum allergen-specific IgE titers are much higher in BALB/c mice than in C57BL/6 mice after allergen (e.g., ovalbumin and *Schistosoma mansoni* egg antigen) sensitization and exposure (14, 15). We have also found that BALB/c mice produce higher titers of serum IgE Abs than C57BL/6 mice after treatment with Japanese cedar pollen allergen (10). We therefore used BALB/c mice as the experimental animals in the present study. Specific pathogen-free male BALB/c mice (7 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). After one (i.n.) or two (i.n. and 14 days later s.c.) injections of the allergen, the mice were housed in our animal facility under specific pathogen-free conditions in an air-conditioned room at $23 \pm 2^\circ\text{C}$ and $\sim 50\%$ humidity for 3 days to 3 months. The experiments were carried out in accordance with the Guidelines on Animal Experiments of Osaka Medical College and the Japanese Government Notification on Feeding and Safekeeping of Animals, and the experimental protocol was approved by the Review Committee for Animal Experiments of Osaka Medical College.

Allergic sensitization

Japanese cedar (*Cryptomeria japonica*, to which we are exposed in February–April in Japan), hinoki (*Chamaecyparis obtuse*, with exposure in March–May in Japan), short ragweed (*Ambrosia artemisiifolia*, to which we are exposed in July–October in Japan), and wormwood (*Artemisia absinthium*, with exposure in August–October in Japan) pollen extracts, and house dust mites, *Dermatophagoides pteronyssinus*, feces AG (Catalog No. LG-2444), which belong to the papain-like cysteine protease family (a 30% primary structure homology with papain), were purchased from Cosmo Bio (Tokyo, Japan). Papain (*Carica papaya*) was purchased from Calbiochem (La Jolla, CA, USA). Pollen or mite feces were suspended in borate-buffered saline and papain in PBS. A suspension (100 μg of papain, pollen [cedar, hinoki or wormwood] or mite feces/0.15 mL/mouse or 100–500 μg of ragweed pollen/0.15 mL/mouse) was slowly injected to a depth of 5–7 mm by a needle (26G; 13 mm-long) attached to a 1 mL tuberculin syringe (SS-01T2613S; Terumo, Tokyo,

Japan) into the area surrounding the nostrils of mice anesthetized i.p. with 0.25 mL of pentobarbital/ethanol/PBS (0.8 mL/2 mL/8 mL, respectively). None of the animals died within the several months after sensitization.

Abs

Abs used in this study were as follows: mouse IgE Ab from Zymed (San Francisco, CA, USA); rat anti-mouse IgE Ab from Biosource (Camarillo, CA, USA); HRP-labeled goat anti-mouse IgE Ab from Nordic (Tilburg, the Netherlands); PE-labeled rat anti-mouse CD45R/B220 (RA3-6B2) Ab, FITC-labeled rat anti-mouse CD3 (145-2C11), IgE (R35-72) and CD11b/Mac-1 (M1/70) Abs from PharMingen (San Diego, CA, USA).

Serum preparation

Blood samples were taken by cardiac puncture under chloroform anesthesia at various time intervals after the i.n. or s.c. injections of allergen. The whole blood was incubated in a CO₂ incubator at 37°C for 1 hr, stood overnight at 4°C, and then centrifuged at 440 g for 20 min. The supernatant fractions were stored in microtubes at -20°C prior to use.

Preparation of lymphocytes from lymphoid organs

Mice were anesthetized with chloroform and then bled from the inferior vena cava. After exsanguination, lymphoid tissues such as submandibular, axillar, inguinal or mesenteric lymph nodes and Peyer's patches were removed and single-cell suspensions prepared from them as described earlier (8).

PBMCs

Peripheral blood was diluted with an equal volume of PBS containing 5 mM EDTA and red blood cells and most of the granulocytes removed by PBS/sodium metrizoate Ficoll (Japan Immunoresearch Laboratories, Takasaki, Japan) gradient centrifugation (1420 g, 15 min at 20°C). The cells at the interface (PBMCs) were washed with PBS containing 5 mM EDTA.

Flow cytometric analysis and sorting

For analysis, 10⁶ cells suspended in 50 µL of cold PBS containing 2% FCS were stained with a fluorescein-labeled rat anti-mouse Ab (0.5–1 µg) in the presence of 1.5 µL of rat whole serum and 1 µg of purified rat anti-mouse FcγRIII/II Ab (PharMingen) at 4°C for 20 min, washed, and analyzed by use of a FACS (FACSaria;

Becton Dickinson, Mountain View, CA, USA). For sorting, fluorescein-labeled or unlabeled cells were isolated by FACS in the modes of forward scattering/side scattering and/or FITC/PE, as described previously (16, 17).

Cell number and viability

Cell numbers were determined by counting the cells in Turk's solution with a hemocytometer. The viability of cells was assessed by the trypan blue exclusion method.

Cell culture

Bulk cells, specific antigen⁺ cells or small or large cells of submandibular lymph nodes (1.5 × 10⁶ cells/0.5 mL/48-well plate or 3 × 10⁶ cells/mL/24-well plate) that had been isolated by FACS on Days 0–14 after sensitization were cultured for 6 days with or without allergen in a 24- or 48-well plate, each well containing RPMI 1640 medium supplemented with 10% FCS. The culture medium was stored in microtubes at -20°C prior to use.

ELISA for total IgE Abs

The wells of EIA/RIA plates (Costar #2592; Corning, Corning, NY, USA) were coated with 100 µL of rat anti-mouse IgE Ab (2 µg/mL) at 4°C overnight. After three washes with PBS/Tween (PBS + 0.5% Tween 20), the wells were filled with 400 µL of 1% BSA/PBS and then incubated for 2 hr at room temperature to block unsaturated protein binding sites. The plates were next washed three times with PBS/Tween and 100 µL of appropriately diluted samples (serum or culture medium) in 1% BSA/PBS added to each of triplicate wells, after which the plates were incubated for 2 hr at room temperature. After three more washes with PBS/Tween, 100 µL of HRP-labeled goat anti-mouse IgE Ab in 1% BSA/PBS was dispensed into each well and the preparation allowed to react for 1 hr at room temperature. The wells were then washed thoroughly with PBS/Tween. Next, the antigen-Ab complex was incubated with 100 µL of tetramethylbenzidine (Sigma-Aldrich) for 30 min at room temperature, after which the reaction was stopped by the addition of 100 µL of 1 M H₂SO₄. Thereafter the OD_{450 nm} of the solution in each well was read by a microplate reader SH-1000 (Corona Electric, Hitachinaka, Japan). The concentrations used in this study for preparation of standard curves for serum IgE were as follows: 0.00156 µg/mL, 0.00313 µg/mL, 0.00625 µg/mL, 0.0125 µg/mL and 0.025 µg/mL for IgE. To measure IgE concentrations, serum samples were diluted 20-, 50-, and 100-fold, whereas to measure

them in the culture medium they were diluted 1- and 2.5-fold. Using a standard curve, the amount of IgE was determined using Microsoft Excel software. The lower limit for the Abs assayed in this system was 0.5 to 1 ng/mL. The SD of the triplicate assay was less than 4% at 12.5 ng/mL and less than 2% at 25 ng/mL.

ELISA for allergen-specific IgE Abs

The procedure was the same as that used to measure the total IgE concentrations except that the wells of Immulon 4 HBX flat-bottom microtiter plates (Thermo Labsystems, Franklin, MA) were used after having been precoated with 10 µg of papain, pollen or mite feces in 100 µL PBS. The OD_{450 nm} of the solution in each well was read by a microplate reader SH-1000.

Binding assay

Twenty-four-well plates (Costar #3526; Corning) were coated with 60 µg of the allergen (10 µg/0.1 mL) at 4°C overnight. After three washes with 2.7 mL of PBS/Tween (PBS + 0.05% Tween 20), the wells were filled with 3.6 mL of 1% BSA/PBS and then incubated for 3 hr at room temperature to block unsaturated protein binding sites. The plates were next washed three times with 2.7 mL of PBS/Tween and 0.6 mL of cell suspensions (10⁷ cells/mL of PBS) added to each of triplicate wells, after which the plates were incubated for 15 min at room temperature. After two washes with 2.7 mL of PBS/Tween, 0.2 mL of EDTA/trypsin solution was added. The solution was immediately aspirated and the plates incubated in a CO₂ incubator (95% air/5% CO₂) at 37°C for 2 min. To terminate the reaction, RPMI 1640 medium containing 0.1 mL of 10% FCS was added, after which the cells were counted with a hemocytometer.

RT-PCR

Total RNAs were isolated from various kinds of cells by using Isogen (Nippongene, Toyama, Japan) and then reverse transcribed to synthesize first-stranded cDNA by using SuperScriptII reverse transcriptase (Gibco-BRL, Cleveland, OH, USA). A mouse primer set for *IL-4* cDNA (F, 5'-ACG GAG ATG GAT GTG CCA AAC GTC-3'; R, 5'-CGA GTA ATC CAT TTG CAT GAT GC-3'; Kurabo, Osaka, Japan) was used to amplify a 279 bp fragment; and 40 cycles of PCR were conducted in a GeneAmp PCR System apparatus (9700; PE Applied Biosystems, Foster City, CA, USA). A mouse *β-actin* primer set (F, 5'-TGT GAT GGT GGG AAT GGG TCA G-3'; R, 5'-TTT GAT GTC ACG CAC GAT TTC C-3'; Kurabo) was used to

amplify a 514 bp fragment by 30 cycles of PCR. The PCR products were electrophoresed on 2% agarose gels (Funakoshi, Tokyo, Japan) and analyzed after ethidium bromide staining. Experimental schedules are shown in Figure 1.

RESULTS

Total IgE Ab production by one (i.n.) or two (i.n. and s.c.) injections of allergens

After an i.n. injection of the allergen (cedar pollen, papain or mite feces; 100 µg/mouse), the total number of submandibular lymph node cells started to increase on Day 3, reached a peak (~three-fold of PBS-injected control) on Day 10, and then declined (ref. 8 for cedar pollen; data not shown for others). Similarly, total serum IgE Ab titers started to increase on Day 7, reached a peak (seven- to 18-fold of control [Day 0]) on Day 10, and then declined (Fig. 2a). On Day 10 after an i.n. injection of hinoki pollen, total serum IgE Ab titers also increased significantly, whereas they did not change significantly in the sera of mice sensitized i.n. once with wormwood or ragweed pollen (Fig. 2b).

High total serum IgE Ab titers were maintained after a second (s.c.) injection of cedar pollen, papain or mite feces (Fig. 2c). Figure 2c also shows that after the second (s.c.) injection of hinoki or wormwood pollen, serum total IgE Abs titers increased much more, whereas there was no significant increase in serum total IgE Abs in mice sensitized twice (i.n. and s.c.) with ragweed pollen (100 µg/mouse). Dose-dependent experiments using 100–500 µg/mouse of ragweed pollen showed that one or two injections of ragweed pollen (500 µg/mouse) caused a significant increase in total IgE Abs in the serum (once: 82.2 ± 9.7 ng/mL [$n = 4$]; twice: 199.6 ± 82.9 ng/mL [$n = 3$]).

These results demonstrate that total IgE Abs are induced with a similar time course by one or two injections of various kinds of allergens and that the inducibility of total IgE Abs by pollen in BALB/c mice is cedar > hinoki > wormwood > ragweed, in that order.

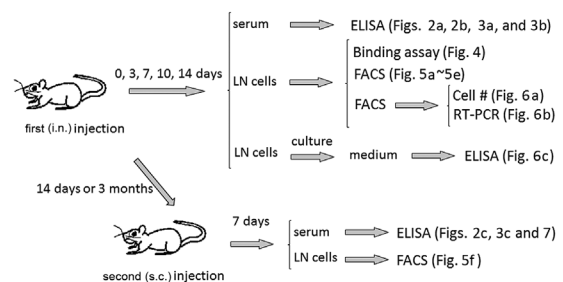


Fig. 1. Experimental schedules.

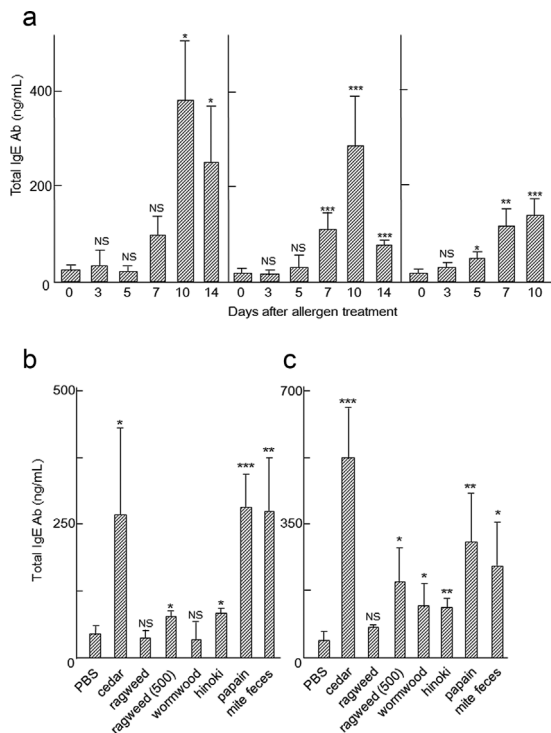


Fig. 2. (a) Time-dependent increases in total IgE Abs in mouse serum after i.n. injection of cedar pollen (left panel), papain (middle panel) or mite feces (right panel) into mice (100 μ g/mouse). Each value represents the mean $\pm$ SD ($n=3-7$) of total IgE Ab serum titers, which were measured (b) on Day 10 after an i.n. injection or (c) 7 days after a second (s.c.) injection of various kinds of allergens (ragweed: 100 μ g and 500 μ g; others: 100 μ g) into mice. Data are expressed as the mean $\pm$ SD ($n=3$). The differences between sample and control (Day 0 for [a] or PBS alone for [b] and [c]) are significant (*, $P<0.05$; **, $P<0.02$; ***, $P<0.005$) according to Student's *t*-test. NS, not significant.

Allergen-specific IgE production after one (i.n.; papain) or two (i.n. and s.c.; pollen), but not two (i.n. and s.c.; mite feces), injections of allergens

Next, whether total IgE Abs that had been induced in the sera of mice sensitized once (i.n.) or twice (i.n. and s.c.) with allergens were non-specific or allergen-specific IgE Abs was explored. As expected, non-specific IgE Abs were induced in the serum of mice sensitized i.n. once with pollen (i.e., cedar, hinoki, wormwood or ragweed) or mite feces. In contrast, an i.n. injection of papain into mice induced papain-specific IgE Abs in the serum (Fig. 3a). Time-dependent changes in the titers of these IgE Abs in the serum were exactly the same as those of total IgE Abs (Fig. 3b), suggesting non-specific IgE Ab production at an earlier time or inherently. Figure 3b also shows that there were no papain-specific IgE Abs in the sera of normal mice (Day 0).

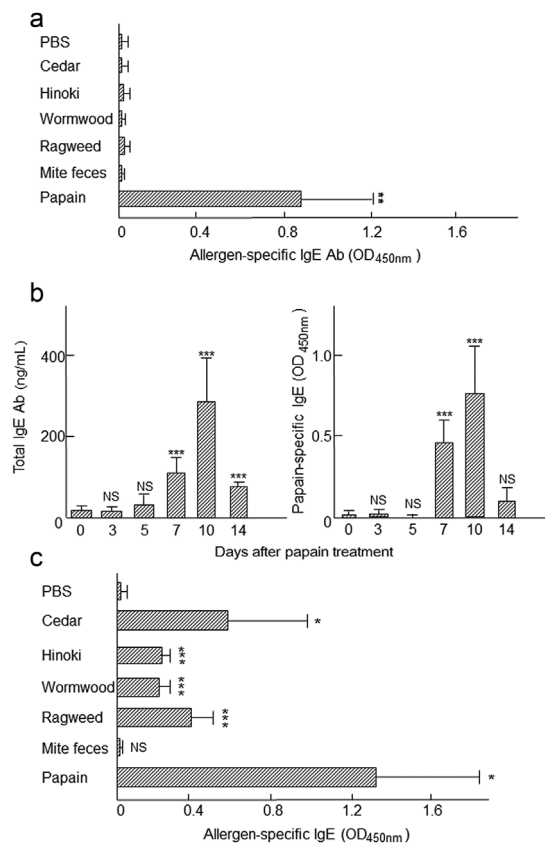


Fig. 3. Allergen-specific IgE Ab production in response to one (i.n.) or two (i.n. and s.c.) injections of various allergens. (a) Allergen-specific IgE Ab production on Day 10 after i.n. injection of various kinds of allergens (ragweed: 500 μ g; others: 100 μ g) into mice. (b) Time-dependent changes in total (left panel) or papain-specific (right panel) IgE Abs in the serum of mice sensitized i.n. once with papain. (c) Allergen-specific IgE Ab production on day 7 after 2 (i.n. and s.c.) injections of various kinds of allergens (ragweed: 500 μ g; others: 100 μ g) into mice. Each value represents the mean $\pm$ SD ($n=3-4$). The differences between sample and control (PBS alone for [a] and [c] and Day 0 for [b]) are significant (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$) according to Student's *t* test. NS, not significant.

Upon a second (s.c.) injection of the same allergen into mice sensitized i.n. once with papain, pollen or mite feces, a large amount of papain- or pollen-, but not of mite feces-, specific IgE Abs was found in the serum, whereas one or two injections of PBS into mice did not induce total or allergen-specific IgE Abs in the serum (Fig. 3c).

Induction of allergen-specific IgE⁺ B cells in submandibular lymph nodes from normal mice (e.g., papain) or from mice sensitized i.n. once (e.g., cedar pollen)

IgE⁺ B cells are precursors of the cells that secrete isotypic Abs (13). Significant amounts of non-specific IgE Abs

(21.1 ± 3.3 ng/mL, $n = 5$ [Fig. 2a]) were present in the sera of normal mice. Papain-specific IgE Ab titers in the sera of mice sensitized i.n. once with papain started to increase on Day 7, reached a peak on Day 10, and then decreased (Fig. 3b), suggesting that the population of non-specific IgE⁺ B cells in the lymph nodes of untreated mice contains papain-, but not pollen- or mite feces-, specific IgE⁺ B cells. To test this possibility, an assay method in which allergen-specific IgE⁺ B cells in lymph node cells from untreated or allergen-sensitized mice were bound to an allergen-coated plate was developed. Submandibular lymph node cells from untreated mice clearly bound to a papain-coated, but not to a cedar (or ragweed) pollen- or mite feces-coated, plate (Fig. 4). Figure 4 also shows that submandibular lymph node cells from mice sensitized i.n. once with cedar pollen bound to a cedar pollen-coated plate. Similarly, the numbers of bulk lymph node cells and IgE⁺ cells from mice sensitized i.n. once with mite feces increased on Day 3 and peaked on Day 7 (4.9- and 10.8-fold [each $n = 3$], respectively; data not shown), whereas the lymph node cells did not bind significantly to mite feces-coated plates (Fig. 4).

In vitro production of papain-specific IgE Abs by submandibular lymph node cells from mice sensitized i.n. once with papain

Papain-specific IgE⁺ cells were present in the submandibular lymph nodes from normal mice that had previously been sensitized or inherently (Fig. 4), whereas

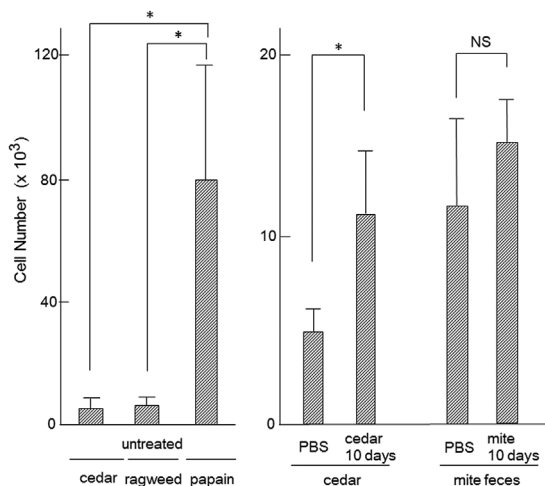


Fig. 4. Specific binding of submandibular lymph node cells from untreated mice or from mice (Day 10) sensitized i.n. once with cedar pollen or mite feces (100 µg/mouse) to papain- or cedar pollen-, but not to mite feces-, coated plates. Data are expressed as the mean $\pm$ SD ($n = 3$). The differences between sample and control (other allergens or PBS alone) are significant (*, $P < 0.05$) according to Student's *t*-test. NS, not significant.

there was a significant amount of non-specific, but not of papain-specific, IgE Abs in the serum (Fig. 3b). Moreover, papain-specific IgE Abs were not produced after 6-day culturing of bulk lymph node cells from normal or untreated mice in the presence of 0, 2, 10 or 30 µg of papain (Table 1). However, when bulk lymph node cells (Day 10) that had been obtained from mice sensitized i.n. once with papain were cultured for 6 days in the absence of papain, they produced a large amount of papain-specific IgE Abs (Table 1), implying induction of papain-derived antigen-presenting B cells and cognate T cells upon second sensitization.

These results (Figs. 2–4 and Table 1) suggest that allergen-specific IgE⁺ B cells may be induced in mice sensitized once with papain or cedar pollen, but not with mite feces.

Two different sizes of cells in submandibular lymph nodes from mice sensitized i.n. once with cedar pollen

To examine the capacity of T cells to activate B cells, other investigators have used small, dense B cells, including G0 B cells (density ≥ 1.080 ; ~ 5.5 µm in diameter) and a population of large, less dense B cells containing activated cells (density < 1.080 ; ~ 10 µm in diameter) from spleens of mice (12, 18, 19). In the present study, submandibular lymph node cells (Days 0, 3, 7, 10, 14, and 14 + 7) from mice sensitized i.n. once with cedar pollen also consisted of two different sizes of cells ($\sim 20\%$ small cells [at the position of erythrocytes: ~ 6 µm in diameter] and $\sim 80\%$ large cells [at the position of mononuclear cells: ~ 10 µm in diameter]) (Fig. 5). The FACS pattern did not change significantly even after removal of red blood cells from bulk lymph node cells by hypotonic treatment and the number of small cells in the trypan blue solution was exactly the

Table 1. *In vitro* production of papain-specific IgE Ab after 6-day culturing of bulk lymph node cells from normal, untreated mice or from mice sensitized i.n. once with papain

Mice	Addition of papain	OD _{450 nm} [†]
Untreated	0 µg	0.000, 0.002, 0.005
Untreated	2	0.000, 0.000, 0.002
Untreated	10	0.000, 0.002, 0.008
Untreated	30	0.000, 0.003, 0.003
i.n. once (day 10)	0	0.346, 0.401, 0.901

[†]Amounts of papain-specific IgE Abs in the medium were determined by ELISA after 6-day culturing of bulk lymph node cells from normal, untreated mice or from mice sensitized i.n. once with papain in the presence or absence of papain. The OD_{450 nm} of the solution in each well was read by a microplate reader SH-1000.

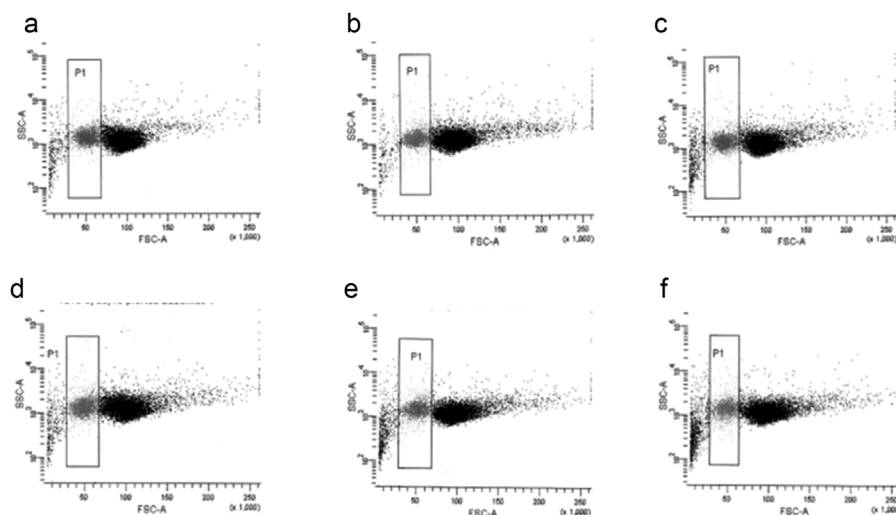


Fig. 5. Two different sizes of cells in submandibular lymph nodes from cedar pollen-sensitized mice (Days 0, 3, 7, 10, 14 or 14 + 7 [i.n. + s.c.]). (a) Day 0; (b) Day 3; (c) Day 7; (d) Day 10; (e) Day 14; (f) Days 14 + 7. Percentage of P₁ (i.e., small cell) fraction (mean $\pm$ SD) on Day 0: $21.7 \pm 2.3\%$ ($n=4$); Day 3: $22.5 \pm 1.9\%$ ($n=6$); Day 7: 20.6 ± 2.5 ($n=17$); Day 10: 20.5 ± 1.7 ($n=5$); Day 14: 19.4 ± 1.8 ($n=5$); Day 14 + 7: 20.1 ± 1.2 ($n=4$).

same as that in Turk's solution (data not shown). The FACS pattern for the submandibular lymph nodes was essentially the same as that for the other lymph nodes (e.g., axillary lymph nodes, mesenteric lymph nodes, Peyer's patch, and inguinal lymph nodes) on Days 0 or 10. However, the presence of small, dense cells was unclear in the case of PBMCs (data not shown), presumably because the dense cells had been separated from the less dense PBMCs by PBS/Ficoll gradient centrifugation.

Time-dependent changes in the numbers of Mac-1⁺ macrophages, CD3⁺ T cells, and B220⁺ and B220⁺⁺ B cells in submandibular lymph nodes after an i.n. injection of cedar pollen into BALB/c mice

When bulk cells from submandibular lymph nodes from normal mice were stained either with FITC-labeled anti-CD3/PE-labeled anti-B220 Abs or with FITC-labeled anti-Mac-1/PE-labeled anti-B220 Abs, they were largely separated into six populations by FACS (i.e., Mac-1⁻/CD3⁻/B220⁺⁺, Mac-1⁻/CD3⁻/B220⁺, Mac-1⁻/CD3⁺/B220⁻, Mac-1⁺/CD3⁻/B220⁻, Mac-1⁻/CD3⁺/B220⁺ and Mac-1⁺/CD3⁺/B220⁺⁺). Time-dependent changes in the numbers of these cells in the submandibular lymph nodes from mice allergen-sensitized i.n. once were assessed by FACS after incubation of the bulk cells with fluorescein-labeled Abs (Fig. 6a). The number of Mac-1⁺/CD3⁻/B220⁻ cells, which are morphologically mononuclear cells that are essential both for IL-4 or

non-specific Ig production and for class switching of Ig in lymphocytes (8), increased within several days (~ 3.5 -fold on Day 3). The increase in the number of macrophages was followed by increases in Mac-1⁻/CD3⁺/B220⁻, Mac-1⁻/B220⁺/CD3⁺, Mac-1⁻/CD3⁻/B220⁺⁺ and Mac-1⁺/CD3⁻/B220⁺ cells, with a peak (1.7- to 5-fold) on Day 7, and by an increase in Mac-1⁻/CD3⁻/B220⁺ cells, with a peak (~ 3.5 -fold) on Day 10.

It has been reported that IL-4 is produced by T cells, mast cells, basophils, eosinophils and macrophages (20–23). In our mouse model system, CD3⁺ cells in the submandibular lymph nodes from mice that had been i.n. sensitized once with the allergen alone seemed to be the main producers of IL-4 (~ 100 pg/mL; ref. 8). To explore whether small (1.4–2.6%) or large (32.4–47.9%) CD3⁺ cells are involved in production of IL-4 in response to the allergen, bulk, small CD3⁺ and large CD3⁺ cells were obtained by FACS on Days 0–14 after i.n. sensitization and their IL-4 expression assessed by performing RT-PCR (Fig. 6b). Both the large CD3⁺ cells and bulk lymph node cells expressed IL-4 on Days 5, 7 and 10, with a peak on Day 5, whereas there was no expression in the small CD3⁺ cells on Days 5–10. In contrast, there was no expression of IL-4 in bulk or the large CD3⁺ population on Days 0, 3 and 14 (data not shown).

Total IgE Ab titers were also assessed in 6-day cultures of bulk submandibular lymph node cells that had been obtained on Days 0–14 after an i.n. injection of cedar pollen into mice (Fig. 6c). The bulk lymph node cells (3×10^6 cells/mL) produced a significant amount of total IgE Abs on Day 7 (2.8 ± 1.5 ng/mL; mean $\pm$ SD

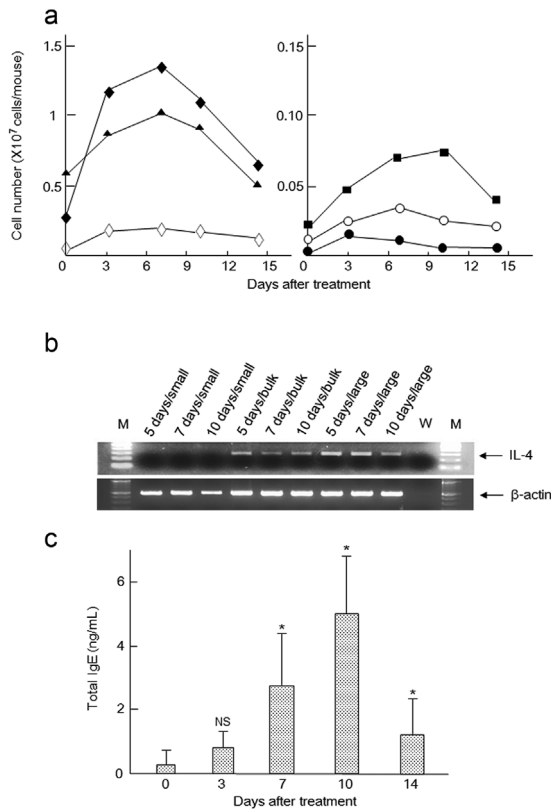


Fig. 6. (a) Time-dependent changes in the numbers of Mac-1⁺ macrophages, CD3⁺ T cells or B220⁺ or ⁺⁺ B cells in submandibular lymph nodes after an i.n. injection of cedar pollen into BALB/c mice. ◆, B220⁺⁺ cells; ■, B220⁺ cells; ▲, CD3⁺ cells; ●, Mac-1⁺ cells; ◇, CD3⁺/B220⁺ cells; ○, Mac-1⁺/B220⁺ cells. Each point represents the mean ($n=4-7$). (b) PCR analysis of IL-4 cDNA in bulk cells, small CD3⁺ cells, and large CD3⁺ cells in submandibular lymph nodes on Days 5-10 after an i.n. injection of cedar pollen into mice. M, marker; W, water. (c) *In vitro* IgE Ab production in 6-day cultures of bulk submandibular lymph node cells that had been obtained on Days 0-14 after an i.n. injection of cedar pollen into mice. Each value represents the mean $\pm$ SD ($n=4-7$). *The difference between sample and control (day 0) is significant ($P < 0.05$) according to Student's *t*-test. NS, not significant.

[$n=10$]); the titers peaking on Day 10 (5.1 ± 1.9 ng/mL; mean $\pm$ SD [$n=10$]) and then decreasing on Day 14 (0.9 ± 1.1 ng/mL; mean $\pm$ SD [$n=10$]). The control concentration of total IgE Abs in the culture medium was 0.2 ± 0.5 ng/mL (mean $\pm$ SD; $n=5$) and there was no increase in allergen-specific IgE Abs on Days 7-14 after sensitization ($OD_{450\text{ nm}} = <0.001$). On Day 10, small cells and large cells were isolated by FACS. IgE Ab titers in the media of 6-day cultures of large cells (5.7 ± 0.8 ng/ 3×10^6 cells/mL; mean $\pm$ SD [$n=5$]) were almost the same as those in cultures of small cells (5.1 ± 0.9 ng/ 3×10^6 cells/mL; mean $\pm$ SD [$n=5$]) (data not shown).

On Day 10, when total IgE Ab titers in the serum reached a peak (Fig. 2a), the percentage of small cells in the lymph node cells was 20.5% and >95% of the IgE⁺ cells (19.5%) were recovered in the small-cell fraction. This IgE⁺ cell population consisted of B220⁺ (54.4%) and B220⁺⁺ (45.6%) cells. In contrast, the large cell population (76.7%) consisted of IgE⁻/B220⁺⁺ (51.5%) and IgE⁻/B220⁻ (46.0%) cells (Table 2).

Taken together, these results indicate that a significant number of papain-specific IgE⁺ B cells were present in the lymph nodes of untreated mice, were induced on Days 7-14 with a peak on Day 10 (*when*) in the small-cell fraction of lymph node cells (*where*) from mice sensitized once with pollen, and were not induced by the first sensitization with mite feces (Table 3).

Increase in concentrations of ragweed (or cedar) pollen-specific IgE Abs in the serum upon a second (s.c.) injection of ragweed (or cedar) pollen into mice that had been sensitized i.n. once with cedar (or ragweed) pollen

Ig⁺ B cells are known to be the precursors of cells that secrete isotypic Abs (13). Non-specific IgE Abs were produced by both IgE⁻ large and IgE⁺ small cells in mice sensitized i.n. once with cedar pollen (Fig. 6c), suggesting non-specific (or related allergen-specific) IgE are expressed on the membrane of small cells. In addition, Figures 2 and 3 show that cedar (or ragweed) pollen is the

Table 2. Composition of antigen⁺ or ⁻ small or large cells in submandibular lymph nodes from mice (Day 10) sensitized i.n. once with cedar pollen

Fraction	% [†]
Total	100
Small cells	20.5 $\pm$ 1.7
IgE ⁺ cells	19.5 $\pm$ 1.3
B220 ⁺⁺ ‡	8.9 $\pm$ 1.9
B220 ⁺ ‡	10.6 $\pm$ 1.7
Others	1.0 $\pm$ 1.2
Large cells	76.7 $\pm$ 3.0
B220 ⁺⁺ ‡	39.5 $\pm$ 1.8
B220 ⁻ ‡	35.3 $\pm$ 1.1
Others	1.9 $\pm$ 1.0
Others	2.8 $\pm$ 0.8

[†]Percentages of antigen⁺ or ⁻ cells among the bulk cells were assessed by FACS in the modes of forward scattering/side scattering and FITC/PE after staining with FITC-conjugated anti-mouse IgE/PE-labeled anti-mouse B220 Abs. Each value represents the mean $\pm$ SD ($n=3$). [‡]Mean fluorescence intensities of PE-labeled anti-mouse B220 Ab. Small B220⁺⁺ cells: 18526.5 ± 682.2 ; small B220⁺ cells: 1374.0 ± 283.4 ; large B220⁺⁺ cells: 30939.5 ± 403.8 ; large B220⁻ cells: 188.3 ± 42.5 . Data are expressed as the mean $\pm$ SD ($n=3$).

Table 3. Expression of allergen-specific IgE on submandibular lymph node cells in response to first sensitization to produce allergen-specific IgE Abs after second sensitization

Stage of activation		Untreated	Once (i.n.)	Twice (i.n. + s.c.)
Papain	Allergen-specific IgE ⁺ cells	+		
	Total IgE Ab production	— [†]	+	
	Allergen-specific IgE Ab production	—	+	
Pollen	Allergen-specific IgE ⁺ cells	—	+	
	Total IgE Ab production	— [†]	+	+
	Allergen-specific IgE Ab production	—	—	+
Mite feces	Allergen-specific IgE ⁺ cells	—	—	
	Total IgE Ab production	— [†]	+	+
	Allergen-specific IgE Ab production	—	—	—

[†]Total IgE Abs were produced on Days 7–14 after an (i.n. or s.c.) injection of allergen, as shown in Figs. 2a, 3b and 6c.

best (or worst) inducer of non-specific IgE Abs. Next, on the one hand, cedar pollen (or mite feces) or PBS was i.n. injected into mice and 14 days later ragweed pollen was s.c. injected. On the other hand, ragweed pollen (or mite feces) or PBS was i.n. injected into mice and 2 weeks later cedar pollen was s.c. injected. As expected, after an s.c. injection of ragweed (or cedar) pollen into mice treated i.n. once with PBS, sera did not bind to a ragweed (or cedar) pollen-coated plate, whereas sera from mice sensitized twice (i.n. and s.c.) with ragweed (or cedar) pollen bound to a ragweed (or cedar), but not to a cedar (or ragweed) pollen-coated plate, indicating that ragweed (or cedar) pollen-specific IgE Abs does not cross-react with cedar (or ragweed) pollen. Of particular interest, after a second (s.c.) injection of ragweed pollen into mice (on Day 14) sensitized i.n. once with cedar pollen, a large amount of ragweed pollen-specific IgE Ab was found in the serum. Additionally, a second (s.c.) sensitization with cedar pollen 2 weeks after an i.n. injection of ragweed pollen induced a significant amount of cedar pollen-specific IgE Abs in the serum of 2/4 mice (Fig. 7a). Similarly, a second (s.c.) injection of wormwood pollen into mice sensitized i.n. once with cedar pollen induced a considerable, but not significant, amount of wormwood pollen-specific IgE Abs ($OD_{450} = 0.044 \pm 0.022$; $n = 3$) in the serum. The control (i.n. [PBS] and s.c. [wormwood]) was 0.016 ± 0.006 ng/mL ($n = 3$) (data not shown). Figure 7 also shows that a combination of ragweed (or cedar) pollen and mite feces induced neither ragweed pollen-specific nor cedar pollen-specific IgE Abs, whereas a second (s.c.) injection of mite feces into mice sensitized i.n. once with ragweed or cedar pollen caused no induction of mite feces-specific IgE Abs (data not shown).

In Japan, we are exposed to cedar pollen in February–April and to ragweed pollen in July–October. Upon a second (s.c.) injection of ragweed pollen 3 months after

an i.n. sensitization with cedar pollen, a large amount of ragweed-specific IgE Abs ($OD_{450} = 0.176 \pm 0.084$, $n = 3$) was found in the serum, similar in amount to that ($OD_{450} = 0.179 \pm 0.123$, $n = 3$) observed in mice sensitized s.c. with ragweed pollen 2 weeks after an i.n. injection of cedar pollen (Fig. 7b).

DISCUSSION

Introduction of proteinaceous foreign antigens into an animal generally results in proliferation and differentiation of B cells, the B-cell response largely being controlled by soluble growth and differentiation factors from T cells (24, 25). The interaction of CD40 ligand with CD40 in the presence of IL-4 is sufficient to cause B cell proliferation and IgE secretion (26, 27), and recombinant human CD40 ligand alone can stimulate proliferation and IgE secretion (28). Under these conditions, T cells can induce polyclonal activation of normal B cells in an entirely antigen and MHC non-restricted manner (29, 30). Additionally, addition of crude or purified cytokines to resting B cells in the absence of antigen-specific helper T cells fails to induce antigen-specific B cells (31–34), implying that specific T cell–B cell interactions may also be required for antigen-specific IgE Ab production. Therefore, a major problem in B cell biology is differentiation of the roles played by T cells versus cytokines in activation, replication and differentiation of B cells. In addition, some of the B cells that proliferate early in the immune response take a circuitous route before they become plasma cells in the spleen or lymph nodes (35). In lymph nodes, both T cells and B cells proliferate to form primary foci in the medullary cords for several days. Some of these proliferating B cells differentiate into Ab-synthesizing, Ig class-switching, MHC class II⁺ plasmablasts in the primary focus, some of which then migrate across the

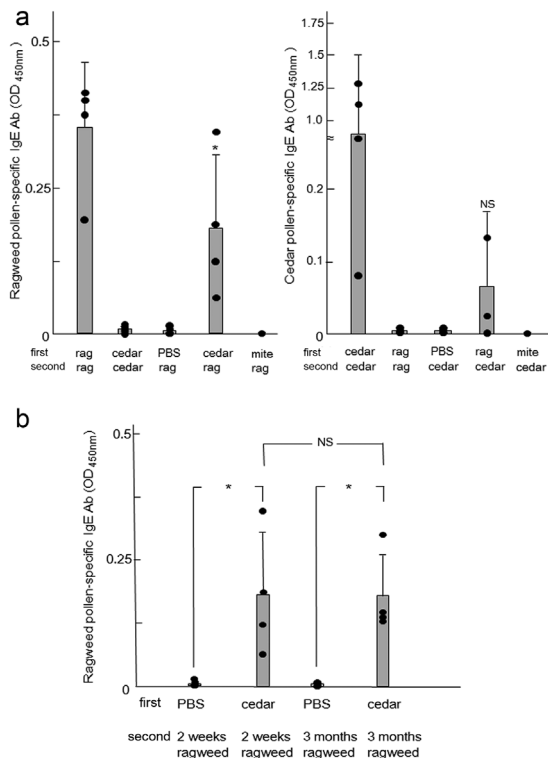


Fig. 7. (a) Serum concentrations (OD_{450nm}) of ragweed (left panel) or cedar (right panel) pollen-specific IgE Abs on Day 7 after a second (s.c.) injection of ragweed or cedar pollen into the mice (on Day 14) that had been i.n. injected with PBS, cedar or ragweed pollen or mite feces. Ragweed (or cedar) pollen-specific IgE Ab levels after two (i.n. and s.c.) injections of cedar (or ragweed) pollen are also shown as a control. Data are expressed as the mean $\pm$ SD ($n=4$). The differences between sample and control (i.n. [PBS] and s.c. [ragweed or cedar]) are significant ($*P<0.05$) according to Student's *t*-test. NS, not significant. (b) Serum concentrations of ragweed-specific IgE Ab on Day 7 after a second (s.c.) injection of ragweed into the mice 2 weeks or 3 months after an i.n. injection of cedar pollen. Each value represents the mean $\pm$ SD ($n=4$). The difference between sample and control (first [i.n.; PBS] and second [s.c.; ragweed]) is significant ($*P<0.05$) according to Student's *t*-test. In contrast, the difference between the two samples is not significant (NS).

T-cell zone to the primary follicle, forming a germinal center. In the present study, we showed that the number of IgE⁺ small B cells in submandibular lymph nodes from mice sensitized once with an allergen started to increase on Day 3 and peaked on Day 10. Additionally, on Days 5–10, IL-4 from large T cells induced polyclonal activation of normal B cells (i.e., induction of non-specific IgE⁺ small B cells) to produce non-specific IgE Abs, implying that IgE⁺ small B cells may be a type of plasmablast. Thus, we designate this IL-4-dependent polyclonal activation of normal IgM⁺ small B cells into non-specific (or related allergen-specific) IgE⁺ ones,

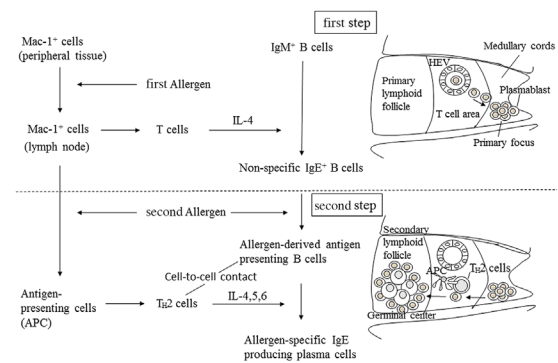


Fig. 8. Possible mechanisms of allergen-specific IgE Ab production.

possibly in the primary focus, as the first step in initiation of allergy (Fig. 8, upper panel).

If T cells encounter an allergen-derived antigen on the surface of an antigen-presenting cell in the T-cell zone, they become activated, some differentiating into helper T cells (7). In addition, if some plasmablasts specific for the same antigen encounter it in the primary foci, they bind, internalize and degrade it to display allergen-derived peptides on their MHC class II molecules. Antigen-presenting B cells traveling from there across the T-cell zone to a primary lymphoid follicle can interact with cognate helper T cells in the T-cell zone; thus, activated B cells migrate to the primary follicle, forming a germinal center to produce antigen-specific IgE Abs. In the present study, papain-specific IgE Abs were not produced after 6-day culturing of bulk lymph node cells containing papain-specific IgE⁺ B cells even in the presence of papain, whereas bulk lymph node cells from mice secondly-sensitized with papain produced a large amount of papain-specific IgE Abs in the absence of papain (Table 1), suggesting there are no cognate helper T cells in lymph nodes from mice sensitized once with an allergen. On the one hand, it is therefore conceivable that a second sensitization results in allergen-specific IgE⁺ small B cells that bind, internalize and degrade the secondly-sensitized allergenic antigens in the primary foci to display allergen-derived peptides on their MHC class II molecules. It is also conceivable that, on the other hand, it also results in antigen-presenting cells that possibly express a receptor for a secondly-sensitized allergen to present the allergenic antigen to T cells (Fig. 8, lower panel). Under these conditions, small B cells that bind to antigen-specific helper T cells and face the site of cytokine production in the helper T cells can selectively proliferate to produce allergen-specific Abs, as reported previously by others (19, 36, 37). Thus, we designate the antigen-restricted activation of allergen-specific

IgE⁺ small B cells by cognate T cells to produce allergen-specific IgE Abs as the second step in initiation of allergy.

Perennial allergic rhinitis is known to be a season-independent chronic disorder induced by allergens found inside the home (38). In Japan, the allergy season for cedar pollen (February–April) precedes that for ragweed pollen (July–October) by 3 months. Ragweed (or cedar) pollen-specific IgE Abs did not cross-react with cedar (or ragweed) pollen; whereas after a second (s.c.) injection of ragweed (or cedar) pollen 2 weeks or 3 months after i.n. sensitization of mice with cedar (or ragweed) pollen, a large amount of ragweed (or cedar) pollen-specific IgE Abs was induced in the serum (Fig. 7). In contrast, a combination of ragweed (or cedar) pollen with mite feces induced neither mite feces-specific nor ragweed (or cedar) pollen-specific IgE Abs (Fig. 7), indicating that allergen-specific IgE Ab production is not attributable to non-specific, by-stander inflammatory responses. We are exposed to various kinds of plant allergens throughout the year. Therefore, when the population of IgE⁺ small B cells in the lymph nodes of an individual person firstly-sensitized with a plant allergen contain secondly-sensitized plant allergen-specific IgE⁺ ones, we can produce IgE Abs specific for various kinds of plant allergenic antigens throughout the year, suggesting a possible mechanism for perennial plant allergic rhinitis.

ACKNOWLEDGMENTS

We are indebted to T. Ueno for his technical assistance. This work was supported in part by a grant (No. 21791652) from the program Grants-in-aid for Young Scientists (B) of the Ministry of Education, Science, and Culture, Japan.

DISCLOSURE

The authors have no financial conflict of interest.

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