

ORIGINAL ARTICLE

## IL-4-dependent induction of IgE<sup>+</sup> basophils in peripheral blood and IgE<sup>+</sup> B cells in spleen as respective indicators of allergen sensitization and a precursor of cells secreting allergen-specific IgE antibody

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### ABSTRACT

It was recently reported by us that either primary i.n. or i.p. injection of cedar pollen extract into BALB/c mice, or a second s.c. injection of the allergen into i.v. or s.c. sensitized mice, causes an IL-4-dependent increase in total IgE serum antibody to produce allergen-specific IgE antibody upon further s.c. sensitization. To determine the biology of total IgE antibody, in the present study IgE<sup>+</sup> cells in peripheral blood or lymphoid tissues of allergen-sensitized BALB/c mice have been characterized. In peripheral blood, mice sensitized one to three times with the allergen produced a 2.5- to 4-fold increase in the number of IgE<sup>+</sup> cells, with a time-course similar to that of the concentration of total IgE antibody in serum. These IgE<sup>+</sup> cells were basophils. On the other hand, the number of IgE<sup>+</sup> cells in the lymphoid tissues did not change significantly after an i.n., i.p., i.v. or s.c. injection of allergen into the mice, whereas a second s.c. injection of the allergen into the i.v., but not into the i.n., i.p. or s.c., sensitized mice induced a small number of IgE<sup>+</sup>/IgM<sup>+</sup>/B220<sup>+</sup> B cells in the spleen. In contrast, IgE<sup>+</sup> cells were not seen in the blood or spleen of IL-4<sup>-/-</sup> mice after sensitization with the allergen.

These results suggest that IgE<sup>+</sup> basophils in the peripheral blood, and IgE<sup>+</sup> B cells in the spleen, might be IL-4-dependently induced as an indicator of sensitization with allergen, and a precursor of cells secreting allergen-specific IgE antibody, respectively.

**Key words** IgE, pollen, rhinitis, rodent.

Ig<sup>3</sup> class expression is strikingly influenced by the action of T lymphocytes. The *in vitro* stimulation of resting B lymphocytes with LPS has provided a particularly valuable model for the analysis of T cell regulation of the isotype of Ig produced: IL-4 promotes the secretion of IgG1 and IgE (1–6); while IFN- $\gamma$  and IL-5 or TGF- $\beta$  cause LPS-stimulated cells to secrete IgG2a and IgA, respectively

(7–10), indicating that lymphokines can have selective regulatory effects on Ig class switching.

IgE Abs play a key role in allergic diseases. Binding of allergen-specific IgE Abs to surface receptors on a wide variety of cells, mainly mast cells and eosinophils (11), and cross-linking of the receptor-bound IgE with antigen, result in the release of inflammatory mediators that lead

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**List of Abbreviations:** Ab, antibody; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HE, hematoxylin-eosin; Ig, immunoglobulin; i.n., intranasal; i.p., intraperitoneal; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NALT, nasal-associated lymphoid tissue; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; s.c., subcutaneous.

to symptoms of allergic diseases (12). Allergic rhinitis is one typical atopic disease, the prevalence of which seems to have been accelerating worldwide recently. In a sensitized patient, the production of specific IgE Abs directed toward an allergen is a prerequisite for the immunologic response leading to allergic rhinitis (13). Furthermore, allergenic activity as an increase in total IgE Ab is induced by primary i.n. or i.p., (but not i.v. or s.c.), injection of cedar pollen extract; and one or two more s.c. injections of the same allergen into i.n., i.p., i.v. or s.c.-allergen-sensitized mice induce specific IgE Ab production (14, 15). These results indicate that production of total IgE Ab without a change in the IgA, IgG or IgM concentration is a prerequisite for the production of allergen-specific IgE Abs. Therefore, finding the cells that produce total IgE Ab during the initial sensitization stage is one of the major goals of allergy research.

Despite the importance of the IgE response, little is known about the location of switching to IgE, the biology of IgE<sup>+</sup> cells, and even whether memory IgE<sup>+</sup> cells exist. In the present study, since injections of allergen with adjuvant obscure the characteristics of the injection sites, we injected i.n., i.p., s.c. or i.v. cedar pollen without adjuvant once or twice into wild-type, IFN- $\gamma$  -/- or IL-4 -/- BALB/c mice to explore the cellular mechanism of the production of total IgE Ab in the serum. After primary (i.p., i.n., i.v. or s.c.) and/or secondary (s.c.) injection of cedar pollen, we obtained peripheral blood leukocytes, PBMC, lymph node cells, and spleen cells and stained them with fluorescein-labeled anti-IgE Ab. The results revealed that time-dependent changes in the number of IgE<sup>+</sup> cells (basophils) in the peripheral blood correlated well with the concentration of total IgE Ab in the serum, and that a second (s.c.) injection of allergen into mice sensitized once i.v., (but not i.p., i.n. or s.c.-) caused a small increase in the number of IgE<sup>+</sup> B cells in the spleen.

## MATERIALS AND METHODS

### Animals

There are many gene knockout lines in BALB/c and C57BL/6 strains of mice, and their gene mapping has already been established. However, it has been reported that serum allergen-specific IgE levels 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 (16, 17). We have also found that, after treatment with Japanese cedar pollen allergen, BALB/c mice produce higher titers of serum IgE Ab than C57BL/6 mice (14, 15). Thus, in the present study, we used BALB/c mice as the experimental animals. Specific pathogen-free male BALB/c mice (6–7 weeks of age) were purchased from

Japan SLC (Hamamatsu, Japan). BALB/c IL-4 -/- mice were donated by Dr. S. Sakaguchi (Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan). After i.n., i.p., s.c., or i.v. injection of 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  $\approx 50\%$  humidity for 1–3 weeks. All 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 (Notification No.6 of the Prime Minister's Office); and the experimental protocol was approved by The Review Committee for Animal Experiments of Osaka Medical College.

### Antigen

A crude extract-cj of pollen from Japanese cedar (*Cryptomeria japonica*) was purchased from Cosmo Bio, Tokyo, Japan.

### Abs

Abs used in this study were the following: FITC-labeled anti-mouse IgE (R35-72) and CD3 (145-2C11) Abs, PE-conjugated anti-mouse CD45R/B220 (RA3-6B2), CD11b (M1/70), IgM (R6-60.2), and Ly-6G (1A8) Abs, and purified anti-mouse CD16/CD32 (2.4G2) and CD23 (B3B4) Abs, all from PharMingen (San Diego, CA, USA). PE-labeled anti-mouse CCR3 (83101) Ab was a product of R&D Systems (Minneapolis, MN, USA).

### Preparation of peripheral blood leukocytes, PBMC, and lymphoid cells

Blood samples were taken by cardiac puncture under chloroform anesthesia before treatment, or at various time intervals after i.n., i.p., s.c., or i.v. injection of 100  $\mu\text{g}$  cedar pollen crude extract-cj in 0.2 ml of PBS/mouse. **Peripheral blood leukocytes:** Peripheral blood was diluted with an equal volume of PBS containing 5 mM EDTA. Two times-diluted peripheral blood was centrifuged at  $2260 \times g$  for 30 min at  $20^\circ\text{C}$  to obtain the buffy coat (leukocyte-rich) fraction, and peripheral blood leukocytes were obtained by removing contaminating red blood cells from the buffy coat by treatment with buffered ammonium chloride solution. **PBMC:** Peripheral blood was diluted with an equal volume of PBS containing 5 mM EDTA; and red blood cells and most of the granulocytes were removed by PBS/sodium metrizoate Ficoll (Japan Immunoresearch Laboratories, Takasaki, Japan) gradient centrifugation ( $1420 \times g$  for 15 min at  $20^\circ\text{C}$ ). The cells at the interface (PBMCs) were washed with PBS containing 5 mM EDTA. **Spleen cells and cells from other lymphoid tissues:** After mice had been killed by cervical

dislocation, their spleens and other lymphoid tissues [e.g. NALT, mandibular or mesenteric lymph nodes, and Peyer's Patches] were removed aseptically; and single-cell suspensions were prepared from them as described earlier (18).

### Removal of cytophilic Ab

Cytophilic Ab was removed by treatment of cells with dilute acid: The cells in a pellet were mixed with 1 ml of acid solution (equal volumes of cold 0.1 M acetic acid, pH 4.0 and a solution containing 0.17 M NaCl and 0.01 M KCl) for 1 min. The pH was then adjusted to 7.4 by the addition of cold Tris buffer (0.03 M Tris-HCl, pH 7.4, containing  $0.8 \times$  PBS and 2% FCS) and, after the cells had been washed with PBS containing 2% FCS, they were suspended in the same medium containing fluorescein-labeled Ab.

### Flow cytometric analysis

Cells ( $5 \times 10^5$  cells) suspended in 25  $\mu$ l of cold PBS containing 2% FCS and 2  $\mu$ l of rat control serum in the presence (for staining) or absence (for staining and sorting) of 0.1% sodium azide were stained with 1  $\mu$ g of fluorescein-labeled Ab in the presence of 1  $\mu$ l of control rat serum and 1  $\mu$ g of anti-mouse Fc $\gamma$ III/IIR (CD16/32) Ab at 4°C for 20 min, washed, and analyzed by flow cytometry (FACSaria; Becton Dickinson, Mountain View, CA, USA). Dead cells and erythrocytes were gated out on the basis of light scattering as the data were being collected. The antigen<sup>+</sup> cells were isolated by sorting and viewed under a fluorescence microscope (Biozero, BZ-8000; Keyence, Osaka, Japan); and the cell types assessed morphologically after HE staining. The % of IgE<sup>+</sup> cells was determined by FACS after staining of PBMCs or a buffy coat-rich population of leukocytes with FITC-labeled anti-mouse IgE Ab.

### Cell number and viability

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

## RESULTS

### Time-dependent changes in the number of IgE<sup>+</sup> cells after injection of cedar pollen into BALB/c mice

Figure 1a shows time-dependent changes in the number of IgE<sup>+</sup> cells in the peripheral blood of BALB/c mice after an i.n., i.p., s.c. or i.v. injection of cedar pollen. The basal level of IgE<sup>+</sup> cells in the peripheral blood of these animals was  $0.43 \pm 0.18\%$  ( $n = 3$ ). I.n. or i.p. injection of the allergen

resulted in a significant increase in the number of IgE<sup>+</sup> cells on days 10 and 14 (1.7- to 2.7-fold as compared to control) or on days 7, 10 or 14 (2.4- to 2.9-fold as compared to the control), respectively. Also, an s.c. or i.v. injection of allergen induced IgE<sup>+</sup> cells significantly on day 10 (2.1- or 2.5-fold as compared to the control), respectively. Whereas total IgE Ab was not enhanced significantly in the serum after an s.c. or i.v. injection of cedar pollen (14, 15), there was a significant increase in the number of IgE<sup>+</sup> cells in the peripheral blood, suggesting that the percentage of IgE<sup>+</sup> cells in the peripheral blood is a sensitive indicator of allergen sensitization.

A second (s.c.) injection of the same allergen on day 14 into i.n., i.p., s.c. or i.v. allergen-sensitized animals caused a significant increase (2.5- to 3.9-fold as compared to the control) in the number of IgE<sup>+</sup> cells in the peripheral blood of these animals on day 7 after this second injection (i.e. on day 21). Similarly, an i.n. injection of the allergen resulted in a 1.2-fold increase in the number of IgE<sup>+</sup> cells in IFN- $\gamma$  -/- BALB/c mice on day 10. A second (s.c.) injection of the same allergen on day 14 into i.n. allergen-sensitized animals caused an increase (1.5-fold as compared to control) in the number of IgE<sup>+</sup> cells in the peripheral blood of these animals on day 7 after this second injection (i.e. on day 21). In contrast, in the case of IL-4 -/- BALB/c mice, IgE<sup>+</sup> cells were not detectable in the peripheral blood in the basal state; and their numbers did not change significantly after i.n., i.p., s.c. or i.v. injections of cedar pollen 1 to 3 times.

Figure 1b shows that sensitization of wild-type BALB/c mice with allergen causes an increase (right shift of relative fluorescence on flow cytometry) in the amount of IgE on the IgE<sup>+</sup> cells: there was no change (MFI: 3158 [Fig. 1bA]  $\rightarrow$  3465) in the amount of IgE on the IgE<sup>+</sup> cells from mice sensitized s.c. once (Fig. 1b-B); a 4-fold increase (MFI: 3158  $\rightarrow$  12747) in that from mice sensitized s.c. twice (Fig. 1b-C); and a 6.3-fold increase (MFI: 3158  $\rightarrow$  19827) in that from mice sensitized s.c. 3 times (Fig. 1b-D).

These results reveal that the effects of 1–3 injections of cedar pollen on the number of IgE<sup>+</sup> cells in the peripheral blood of wild-type, IFN- $\gamma$  -/-, and IL-4 -/- BALB/c mice are similar to those on the concentration of serum IgE Ab (14, 15), and suggest that the number of IgE<sup>+</sup> cells (or the amount of IgE on IgE<sup>+</sup> cells) might be a sensitive indicator of sensitization with allergen.

### Identification of IgE<sup>+</sup> cells in peripheral blood as basophils

Since the numbers of IgE<sup>+</sup> cells ( $6.4 \times 10^5$  cells/ml of blood) in the buffy coat-rich population of leukocytes from wild-type BALB/c mice allergen-sensitized s.c. 3 times, (which induced allergen-specific IgE in the

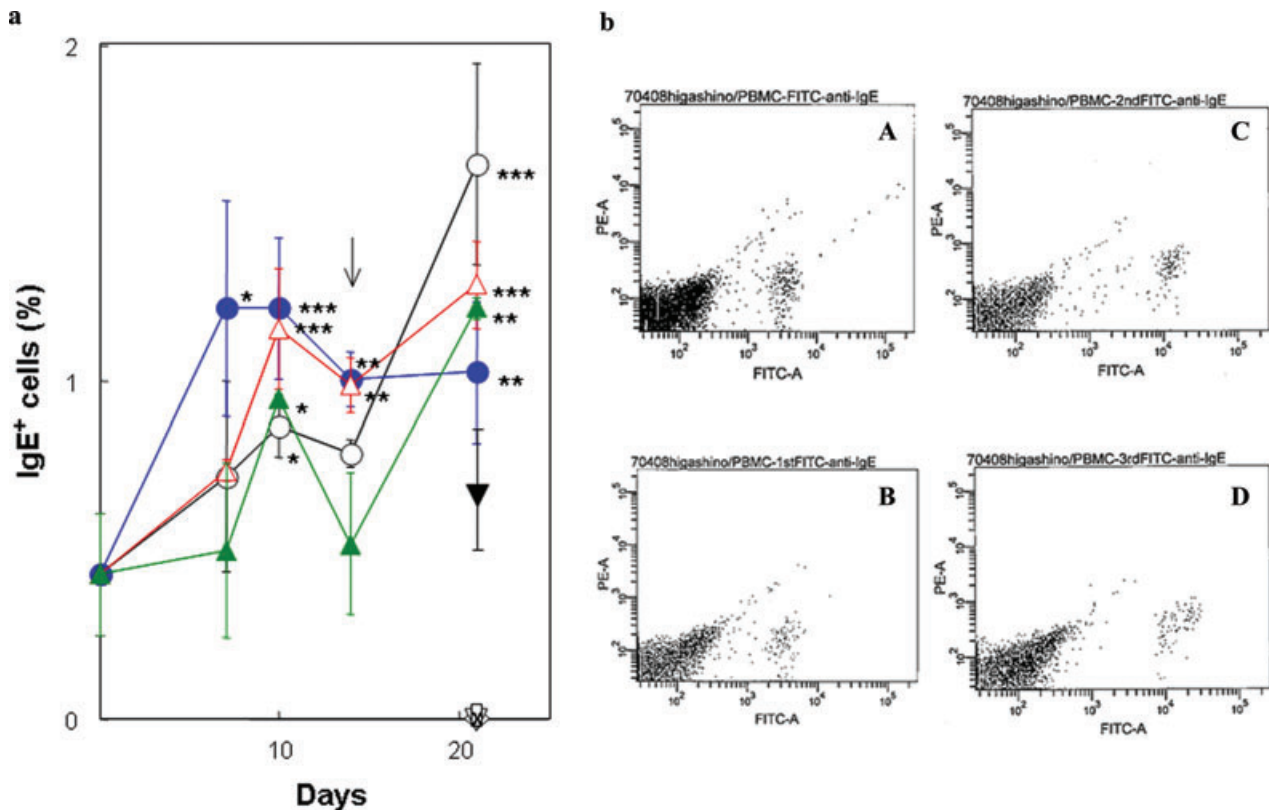


**Fig. 1.** (a) Effects of first (i.n., i.p., i.v. or s.c.) and a second (s.c.) injection of cedar pollen on the numbers of IgE<sup>+</sup> cells in peripheral blood of BALB/c mice. The percentage of IgE<sup>+</sup> cells in the blood was determined either on days 7, 10, and 14 after the first injection, or on day 7 after the second injection (arrow) given on day 14 (day 21 after first injection), by flow cytometry using anti-mouse IgE Ab. Each point represents the mean  $\pm$  SD ( $n = 3$ ) from three different experiments. The difference between sample and control (day 0) is significant (\* $P < 0.05$ ; \*\* $P < 0.02$ ; \*\*\* $P < 0.01$ ) according to Student's  $t$  test. s.c. (○), i.p. (●),

i.n. (△) or i.v. (▲) injection into wild-type BALB/c mice; i.n. (▼) injection into IFN- $\gamma$   $-/-$  mice; s.c. (▽), i.p. (□), i.n. (◇) or i.v. (×) injection into IL-4  $-/-$  mice. (b) The effects of 1–3 s.c. injections of the allergen on the amounts of IgE on the IgE<sup>+</sup> cells in peripheral blood of BALB/c mice. Amount of IgE on the IgE<sup>+</sup> cells was assessed as relative fluorescence on flow cytometry by using FITC-labeled anti-mouse IgE Ab. (b-A) untreated; (b-B) sensitized s.c. once; (b-C) sensitized s.c. twice; (b-D) sensitized s.c. three times.

serum), were similar to those in PBMCs from these mice ( $5.1 \times 10^5$  cells/ml of blood), we expected the IgE<sup>+</sup> cells to be a type of B lymphocyte. Unexpectedly however, the IgE<sup>+</sup> cells were phenotypically CD3<sup>−</sup>/IgM<sup>−</sup>/B220<sup>−</sup>/Mac-1<sup>+</sup>/CCR3<sup>−</sup>/Ly-6G<sup>−</sup>, flow cytometrically medium-sized, less-granular cells, and morphologically polymorphonuclear cells (Fig. 2), suggesting that the IgE<sup>+</sup> cells might be basophils but not eosinophils (CCR3<sup>+</sup>) or neutrophils (Ly-6G<sup>+</sup>), as described previously (19). Similarly, either two s.c. injections of cedar pollen, or a second (s.c.) injection of the same allergen on day 14 into i.v. allergen-sensitized animals, (both of which induced total IgE in the serum), caused a 3- to 4.7-fold increase in the number of IgE<sup>+</sup> cells in the peripheral blood of these animals on day 7 after this second injection (i.e. on day 21). These IgE<sup>+</sup> cells were phenotypically

(CD3<sup>−</sup>/IgM<sup>−</sup>/B220<sup>−</sup>/Mac-1<sup>+</sup>/CCR3<sup>−</sup>/Ly-6G<sup>−</sup>), flow cytometrically (medium-sized, less-granular), and morphologically (polymorphonuclear) basophils (data not shown).

These results suggest that the IgE<sup>+</sup> cells induced in the peripheral blood of mice sensitized i.n., i.p., s.c. or i.v. 1 to 3 times were basophils with allergen-specific or nonspecific IgE bound on their surface.

### Time-dependent changes in the number of IgE<sup>+</sup> cells in spleen after injection of cedar pollen into BALB/c mice

When peripheral blood leukocytes from untreated or allergen-sensitized wild-type BALB/c mice were incubated with FITC-labeled anti-mouse IgE Ab, IgE<sup>+</sup> cells were

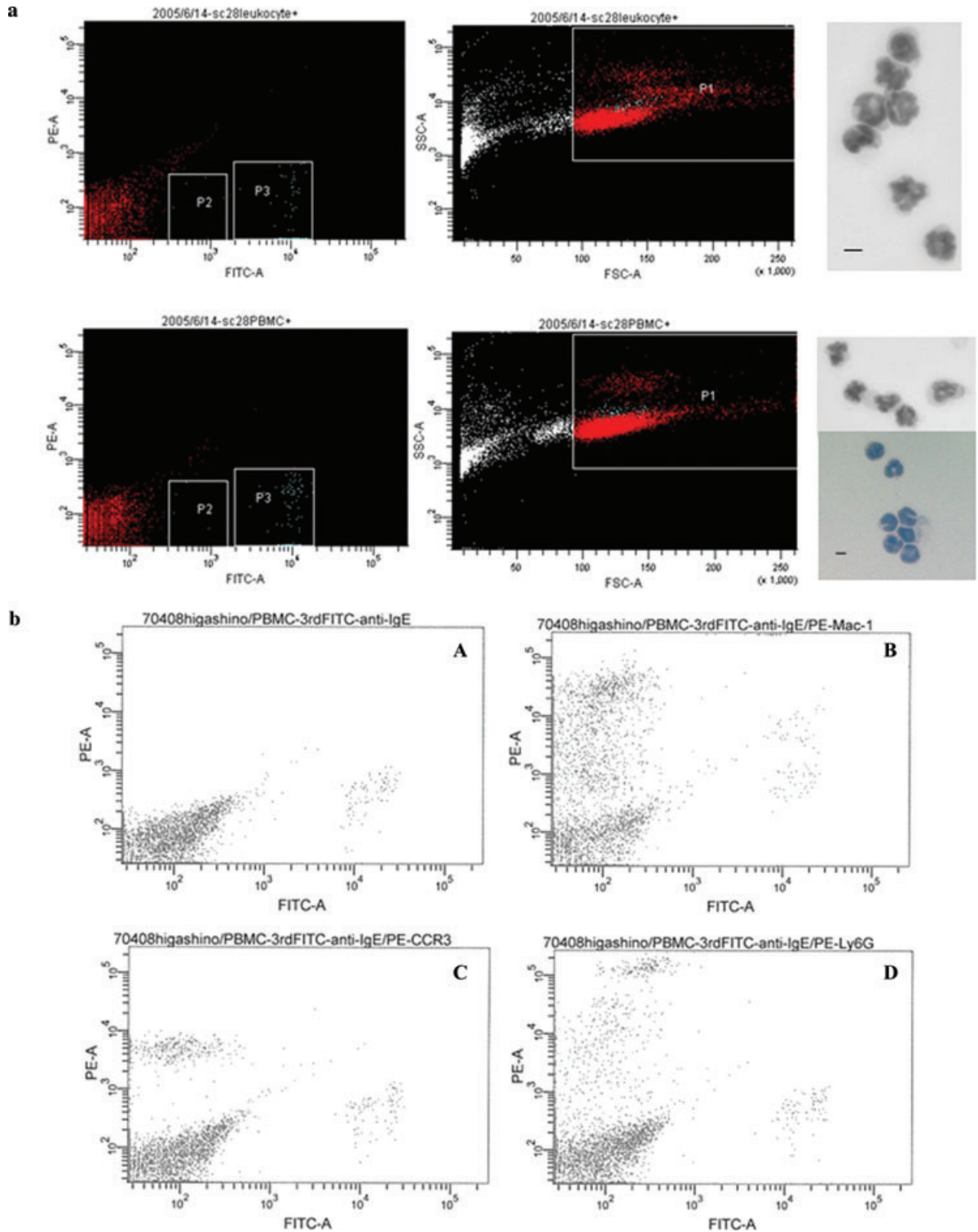


**Fig. 2.** Flow cytometric properties of IgE<sup>+</sup> cells in peripheral blood of BALB/c mice sensitized s.c. 3 times. (a) White blood cells either in buffy coat (top) or mononuclear (bottom) fraction of peripheral blood were stained with FITC-labeled anti-mouse IgE Ab. IgE<sup>+</sup> cells were analyzed by flow cytometry in a mode of forward scattering/side scattering (middle) or fluorescence 1/fluorescence 2 (left). HE-stained IgE<sup>+</sup> cells are

also shown (right). Scale bar = 10  $\mu$ m. (b) Phenotype of IgE<sup>+</sup> cells. (b-A), PBMC + FITC- labeled anti-mouse IgE Ab; (b-B), PBMC + FITC- labeled anti-mouse IgE Ab + PE-labeled anti-mouse Mac-1 Ab; (b-C), PBMC + FITC- labeled anti-mouse IgE Ab + PE-labeled anti-mouse CCR3 Ab; (b-D), PBMC + FITC- labeled anti-mouse IgE Ab + PE-labeled anti-mouse Ly-6G Ab.

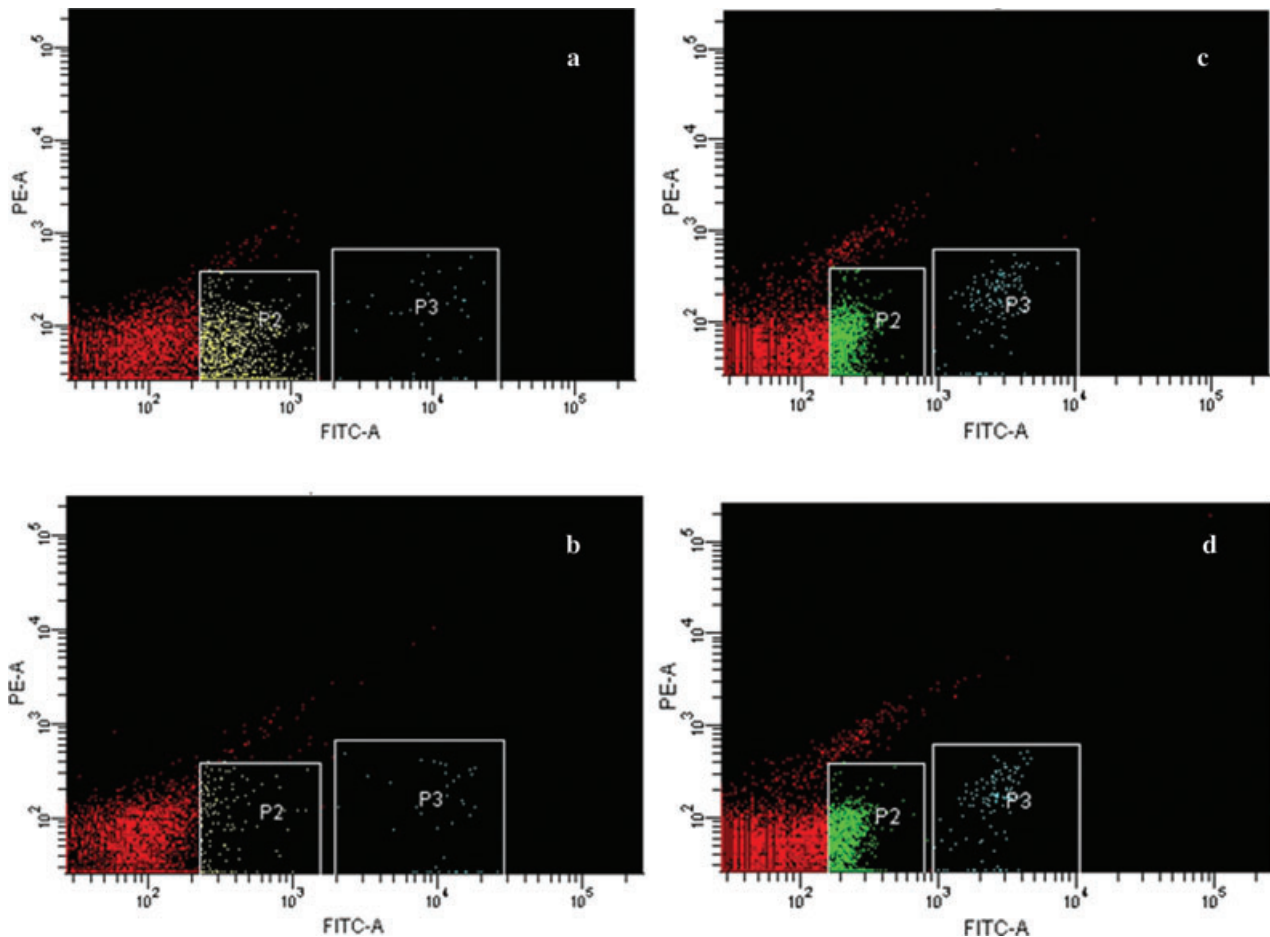


**Fig. 3. Effects of acid or anti-Fc $\epsilon$ RII Ab treatment of IgE<sup>+</sup> cells among spleen cells.** Spleen cells were obtained from mice after a second (s.c.) injection of cedar pollen into mice sensitized i.v. once. The spleen cells were incubated with FITC-labeled anti-mouse IgE Ab and the results (a and c) were compared with those after acid treatment (b) or in the presence of anti-mouse Fc $\epsilon$ RII Ab (d). Most of

the dimly stained cell-surface IgE in the spleen (P2) disappeared after treatment with acid solution, whereas brightly stained ones (P3) were unaffected by acid treatment or by the presence of anti-Fc $\epsilon$ RII Ab. Meaning of Y-axis: The spleen cells were stained with FITC-labeled anti-mouse IgE Ab; and the results are presented in a FL1 (FITC)/FL2 (PE) mode.

recovered in one population (all brightly stained) in terms of relative fluorescence. However, when spleen cells from wild-type BALB/c mice allergen-sensitized twice (i.v. and then s.c.) were stained with FITC-labeled anti-mouse IgE Ab, we obtained two (both dimly and brightly) stained populations (Fig. 3a). Exposure of the cells to a pH of 4 can elute any cell-bound IgE passively adsorbed to the lymphoid cells (20). Most of the dimly stained cells (24.7%) disappeared (a drop to 1.8%) after treatment with acid solution, whereas the brightly stained ones ( $1.23 \pm 0.06\%$ ,  $n = 3$ ) remained ( $1.29 \pm 0.10\%$ ,  $n = 3$ ) after this treatment (Fig. 3b). Moreover, the low affinity receptor for IgE (Fc $\epsilon$ RII) binds monomeric IgE with significantly greater affinity than that of the Fc $\gamma$ R for monomeric IgG and,

as such, represents a particular problem in evaluating the numbers of true membrane IgE<sup>+</sup> B cells (21). The percentage of brightly stained IgE<sup>+</sup> cells in the spleen (Fig. 3c) was also unaffected ( $1.31 \pm 0.08\%$ ,  $n = 3$ ) by the presence of anti-Fc $\epsilon$ RII Ab (Fig. 3d), suggesting that the brightly stained cell-surface IgE in the spleen might not be cytophilic IgE or IgE bound to Fc $\epsilon$ RII.

Figure 4 shows time-dependent changes in the number of brightly stained IgE<sup>+</sup> cells in the spleen of wild-type BALB/c mice after a primary (i.n., i.p., s.c. or i.v.) injection of cedar pollen or after a second (s.c.) injection of the same allergen into i.n.-, i.p.- i.v.- or s.c.- allergen-sensitized animals. The basal concentration of IgE<sup>+</sup> cells in the peripheral blood of these animals was  $0.3 \pm 0.2\%$

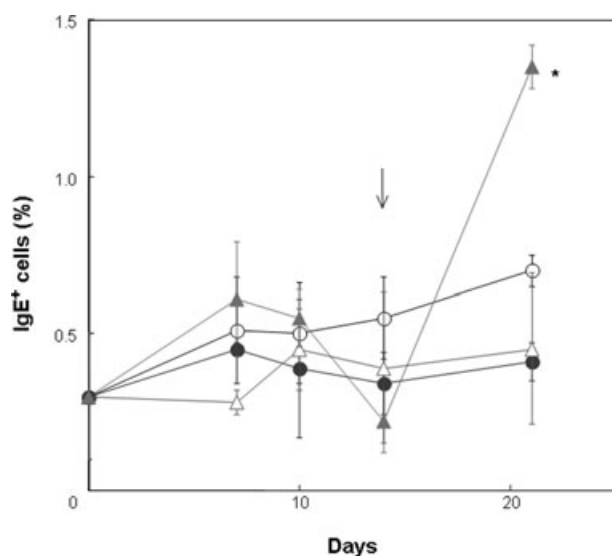


**Fig. 4.** Effects of 1st (i.n., i.p., i.v. or s.c.) and a second (s.c.) injection of cedar pollen on the numbers of IgE<sup>+</sup> cells in the spleen of BALB/c mice. The percentage of IgE<sup>+</sup> cells in the spleen was determined either on days 7, 10, and 14 after the first injection, or on day 7 after the second injection (arrow) given on day 14 (day 21 after first injection). Each point represents the mean  $\pm$  SD ( $n = 3$ ) from three different experiments. The difference between sample and control (day 0) is significant (\* $P < 0.01$ ) according to Student's *t* test. ○, s.c.; ●, i.p.; △, i.n.; ▲, i.v.

( $n = 3$ ); and the number of IgE<sup>+</sup> cells in the spleen did not change significantly after i.n., i.p. or s.c. injections of the allergen once or twice. Similarly, an i.v. injection of the allergen did not enhance the number of IgE<sup>+</sup> cells in the spleen significantly on days 7–14 after sensitization; whereas a second (s.c.) injection induced a significant increase in the number of IgE<sup>+</sup> cells ( $1.35 \pm 0.08\%$ ,  $n = 3$ ). In contrast, the numbers of IgE<sup>+</sup> cells in Peyer's Patches and mesenteric lymph nodes were very low after a second (s.c.) injection into s.c.- or i.n.- ( $0.11 \pm 0.16\%$ ,  $n = 3$ ) (or i.p.- or i.v.- [undetectable,  $n = 3$ ]) sensitized animals; and those in NALT were also negligible after one (i.n.;  $0.02 \pm 0\%$ ,  $n = 3$ ) or two (i.n. + s.c.;  $0.01 \pm 0\%$ ,  $n = 3$ ) injections of the allergen, implying that these organs might not be the place for presentation of allergic antigen. These results suggest that time-dependent changes in IgE<sup>+</sup> cells in the spleen correlate with those of the concentrations of total IgE Ab in the serum (14, 15), and that the spleen might be specifically responsive to i.v.- injected allergen. In IL-4  $\alpha$ -BALB/c mice, IgE<sup>+</sup> cells in the blood and spleen were not detectable in the basal state; and all routes of injection of allergen both once and twice had no effect on the number of IgE<sup>+</sup> cells in the spleen, indicating the essential role of IL-4 in the induction of IgE<sup>+</sup> cells in the spleen.

IgE<sup>+</sup> cells in the spleens from allergen-sensitized mice were isolated by FACS and stained with fluorescein-

conjugated anti-mouse CD3, Mac-1, B220 or IgM Ab. These IgE<sup>+</sup> cells consisted of two populations (Fig. 5): one (major population:  $0.85 \pm 0.1\%$ ) was phenotypically CD3<sup>+</sup>/IgM<sup>+</sup>/B220<sup>+</sup>/Mac-1<sup>+</sup>/CCR3<sup>+</sup>/Ly-6G<sup>+</sup>, flow cytometrically medium-sized, less-granular cells, and morphologically basophil in appearance (data not shown); and the other one (minor population:  $0.25 \pm 0.1\%$ ) was phenotypically CD3<sup>+</sup>/IgM<sup>+</sup>/B220<sup>+</sup>/Mac-1<sup>+</sup>/CCR3<sup>+</sup>/Ly-6G<sup>+</sup> (Fig. 5a,b), flow cytometrically medium-sized, non-granular cells, and morphologically B lymphocyte in nature (data not shown) (Fig. 5c).

## DISCUSSION

Previously, we have shown that an increase in the production of total IgE Ab without a change in the IgA, IgG or IgM concentration is a prerequisite for the production of allergen-specific IgE Abs (14) and that Mac-1<sup>+</sup> cells are essential for IL-4 and total IgE Ab production by lymphocytes (15). In the present study, we reveal that time-dependent changes in the number of IgE<sup>+</sup> cells (i.e. basophils) in the peripheral blood correlate well with those in the concentration of total IgE Ab in the serum (Figs 1, 2), that the number of IgE<sup>+</sup> cells is a sensitive indicator of sensitization with allergen (Fig. 1), and that a second (s.c.) injection of allergen into mice i.v.-, but not i.p.-, i.n.- or s.c.-, sensitized once causes an increase in the number of IgE<sup>+</sup> B cells in the blood and in basophils in the spleen (Figs 3–5). The IgE<sup>+</sup> cells in the blood and spleen were found to consist of one (i.e. basophils) and two (i.e. B cells and basophils) populations, respectively. Although time-dependent changes in the number of IgE<sup>+</sup> basophils (or in the amounts of IgE on them) (Figs 1, 2) correlated well with those in the concentration of total IgE Ab in the serum, there was a lack of association between allergen-specific IgE levels and basophil degranulation (22), implying that IgE<sup>+</sup> cells (i.e. basophils) in the peripheral blood might trap these IgE Abs to prevent allergic reactions in the blood. On the other hand, primary i.v. injection of cedar pollen into BALB/c mice followed by a second (s.c.) injection of the allergen IL-4-dependently induced total IgE Ab in their serum, (a prerequisite for the production of allergen-specific IgE Ab) (14), and a small number of IgE<sup>+</sup> B cell (Figs 3–5).

The B cells that express specific Ig isotypes on their surface have been shown to be precursors of cells secreting those particular isotypes (23). Furthermore, preliminary experiments from our laboratory have demonstrated that IgE<sup>+</sup> B cells isolated by FACS produce a small amount ( $0.000171$ : relative to the OD<sub>450nm</sub> of high-titer IgE Ab, which was assessed as 1.0) of allergen-specific IgE *in vitro*. These results suggest that the spleen may be where total IgE Ab against i.v.- and s.c.- injected allergen is produced, and

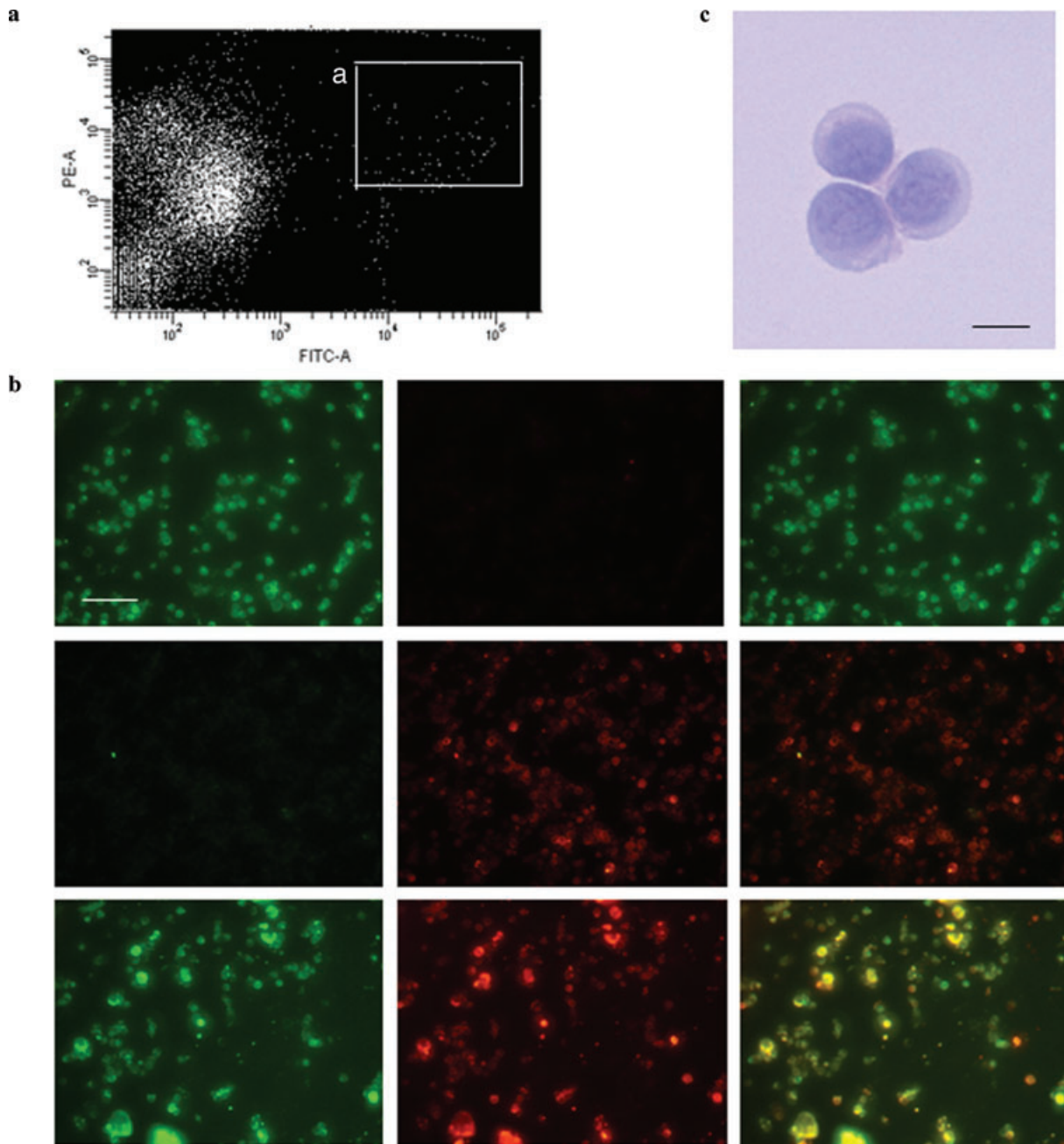


**Fig. 5.** IgE<sup>+</sup>, IgM<sup>+</sup> or IgE<sup>+</sup>/IgM<sup>+</sup> cells in the spleen of BALB/c mice sensitized twice (i.v. and s.c.). Spleen cells were stained with FITC-labeled anti-mouse IgE and PE-labeled anti-mouse IgM Abs. (a) a flow cytometric pattern of spleen cells. The population enclosed by rectangle "a" contains IgE<sup>+</sup>/IgM<sup>+</sup> cells. (b) IgE<sup>+</sup>, IgM<sup>+</sup> and IgE<sup>+</sup>/IgM<sup>+</sup> cells were

isolated by flow cytometry. *Top row*, IgE<sup>+</sup> cells; *middle row*, IgM<sup>+</sup> cells; *bottom row*, IgE<sup>+</sup>/IgM<sup>+</sup> cells. *Left column*, FITC; *middle column*, PE; *right column*, merged view. Scale bar = 50  $\mu$ m. (c) HE-stained IgE<sup>+</sup>/IgM<sup>+</sup> cells. Scale bar = 10  $\mu$ m.

that B cells in the spleen bearing membrane-bound IgE may play an important role in the production of allergen-specific IgE antibody.

It is well-known that stimulation of mouse B cells with LPS results in differentiation of B cells and the formation

of a variety of Ig isotypes excluding IgE (24). However, when the same B cells are cultured for 5 days with LPS together with 100 to 500 units/ml of IL-4, the result is the formation of IgE and selective enhancement of IgG1 formation, which is accompanied by a decrease in IgG2b

and IgG3 formation (2). It is also known that the effect of IL-4 on B cells is counteracted by IFN- $\gamma$  (1, 25, 26). To estimate the role of IFN- $\gamma$  and IL-4 in the allergic reaction, we here assessed the numbers of IgE<sup>+</sup> cells in peripheral blood or spleen of IFN- $\gamma$  -/- or IL-4 -/- mice that had been allergen-sensitized once or twice. Our results reveal that IL-4 is essential for specific increase in the number of IgE<sup>+</sup> cells; whereas IFN- $\gamma$  seems not to be inhibitory. Stern *et al.* have also reported that IFN- $\gamma$  appears to contribute to the pathogenesis of murine allergic conjunctivitis at the effector phase, but not during the initial sensitization stage (27).

When an immune response is induced, the Ab response to the antigen has certain defined characteristics that are common to all immune responses (28). The first exposure of an individual to a particular antigen is referred to as the priming event and, in this primary response, the first class of Ab detected is generally IgM, which in some instances is the only class of Ig that is made. If production of IgG Ab ensues, its appearance is generally accompanied by rapid cessation of production of IgM. Although production of Ab after a priming contact with antigen may cease entirely within a few weeks, the immunized individual is left with a cellular memory of this contact. This memory becomes apparent when a response is induced by a second injection of antigen. After the second injection, the lag phase is considerably shorter and Ab may appear in less half the time required for the primary response. Furthermore, the production of Ab is much greater, and higher concentrations of Ab are detectable in the serum. The production of Ab may also continue for a longer period, with persistent amounts remaining in the serum months or even years later.

Similarly, animals that have been treated once i.n. with allergen produce a significant amount of total IgE Ab in their serum; but the serum IgM level does not change significantly (10, 21). Upon a second (s.c.) injection of the same allergen to the i.n.-allergen-sensitized animals they produce a large amount of IgE Ab, and most of them react to the allergen (14, 15), suggesting an increase in allergen-specific IgE Ab in the serum. However, the serum IgG concentrations do not change significantly or are only somewhat decreased, the IgM concentrations remain constant, and the IgA concentrations actually decrease (14). Thus, we have concluded that increased production of total IgE Ab, without a change in IgA, IgG or IgM concentrations, is a prerequisite for production of allergen-specific IgE Abs. In addition, increased production of total IgE Ab, without a change in IgA, IgG or IgM concentrations, implies unique mechanisms for IgE Ab production, including the presence of total IgE-producing B cells as precursors of cells secreting allergen-specific IgE (Figs 3–5).

Studies on both mice and rats indicate that infection with *Nippostrongylus brasiliensis* induces an increase in IgE Ab production in response to non-parasite antigens (29–31). The cellular basis for the increase in IgE synthesis after parasite infection has been studied primarily in the rat. These studies demonstrate a substantial increase in the number of IgE-bearing B lymphocytes in the lymph nodes that drain the site of infection, as well as a concomitant increase in the number of IgE-containing plasma cells in these lymph nodes (32, 33). Katona *et al.* have demonstrated that when BALB/c mice are inoculated s.c. with third-stage infective larvae of *Nippostrongylus brasiliensis*, the adult worms are cleared from the intestine of infected mice by 10 days after inoculation. They have also shown that this infection in the mouse, as in the rat, induces large numerical increases in lymphoid cells, as well as in B lymphocytes with surface or intracytoplasmic IgE. In addition they have shown that all or most of the detectable surface IgE<sup>+</sup> lymphocytes in *Nippostrongylus brasiliensis*-infected mice are B cells, and that more than 90% of the surface IgE<sup>+</sup> cells in these mice bear cytophilic rather than intrinsic surface IgE (20). The finding that the IgE on the surface of most mesenteric lymph node B cells of *Nippostrongylus brasiliensis*-infected mice is cytophilic rather than intrinsic is important, because the existence of “triple isotype-bearing” B lymphocytes with intrinsic surface IgD, surface IgM, and surface IgE would appear to be incompatible with theories of molecular mechanisms of isotype switching (34, 35).

In contrast, Snapper *et al.* have reported that IL-4 promotes IgG<sub>1</sub> and IgE secretion by murine B cells stimulated with bacterial LPS (36). In fact, cells that synthesize  $\gamma 2b$  without having deleted a  $\mu$ -chain gene have been described (37). Moreover, a report describes the association, during the process of Ig isotype switching *in vivo*, of a transgenic VDJ-C<sub>H</sub> $\mu$  gene with a native C<sub>H</sub> $\gamma$ -specific gene to form an IgG molecule expressing the VDJ transgene-specific protein (38). Similarly, our data also indicate that acid treatment or pretreatment with anti-mouse Fc $\epsilon$ R2 Ab fails to affect surface IgE<sup>+</sup> basophils or IgE<sup>+</sup>/IgM<sup>+</sup> (or B220<sup>+</sup>) B cells (Figs 3, 4). These studies suggest the possibility of an additional, although not mutually exclusive, non-deletional mechanism of Ig isotype switching.

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