

Essential Role of Monocytes in the *In Vitro* Production of IL-4 and Nonspecific IgE Antibody by Peripheral Blood Lymphocytes from Mice Sensitized s.c. Once with Cedar Pollen

YUMIKO YAMAMOTO,^{1,2} JUNKO TASHIRO-YAMAJI,¹ KANJI SAKURAI,¹
MASAKO MIYOSHI-HIGASHINO,¹ HAYAHITO NOMI,¹ SAYAKO MIURA-TAKEDA,¹
MASASHI OKADA,¹ SATOKO YAMAGUCHI,¹ HIROSHI TAKENAKA,²
TAKAHIRO KUBOTA,¹ and RYOTARO YOSHIDA¹

ABSTRACT

To explore which cytokine or cell is essential for the production of antibodies (Abs) of the IgE class in allergic diseases, we injected cedar pollen into wild-type, interferon- $\gamma^{-/-}$ (IFN- $\gamma^{-/-}$), or interleukin-4 $^{-/-}$ (IL-4 $^{-/-}$) BALB/c mice through four (i.n., i.p., s.c., and i.v.) different routes without adjuvant. Wild-type or IFN- $\gamma^{-/-}$, but not IL-4 $^{-/-}$, mice sensitized once or twice showed a significant increase in total IgE Ab in their serum, revealing the essential role of IL-4 in the production of total IgE Ab. We separated peripheral blood mononuclear cells (PBMCs) from untreated or sensitized mice into monocyte-rich, lymphocyte-rich, and granulocyte-rich populations by Percoll density-gradient centrifugation or into specific antigen cells by flow cytometry, cultured the cells in various combinations, and examined the levels of cytokines and IgE Ab released into the medium. The PBMCs from mice sensitized s.c. once, but not those from untreated animals, produced significant amounts of IL-4 and total IgE Ab, whereas the lymphocyte-rich population alone did not. Unexpectedly, IL-4 and IgE Ab production was restored by the addition of Mac-1⁺ cells in the monocyte-rich fraction to the lymphocyte-rich fraction. These results indicate the essential role of monocytes in the production of IL-4 and total IgE Ab by lymphocytes during the initial stage of sensitization.

INTRODUCTION

IG E ANTIBODIES (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,¹ and cross-linking of the receptor-bound IgE with antigen cause the release of inflammatory mediators that lead to the symptoms of allergic diseases.² Allergic rhinitis is one of the typical atopic diseases, and the prevalence of this disease seems to be accelerating worldwide. 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.³ Furthermore, primary i.v. or s.c. followed by s.c. injections of allergen into mice produced a significant increase in the amount of total IgE but not in that of IgA, IgG, or IgM, Ab, and pri-

mary i.v. or s.c. followed by two s.c. injections of allergen into the immunized mice produced a large amount of allergen-specific IgE Ab in the serum.⁴ These results indicate 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. Therefore, finding a way to control total IgE Ab production during the initial sensitization stage is one of the major goals of allergy research. Several questions about the mechanisms involved have still not been fully answered: What kinds of immune cells can recognize allergenic molecules as nonself? Are allergens recognized as nonself nonspecifically or specifically by the defense system? What kinds of antigen-presenting cells (APCs) (e.g., macrophages, dendritic cells [DCs], and B blasts) can present a processed antigen to T cells? What kinds of cytokines can be released from

Departments of ¹Physiology and ²Otorhinolaryngology, Osaka Medical College, Takatsuki 569-8686, Japan.

APCs or T cells? Why are IgE Abs rather than IgA, IgM, or IgG Abs produced toward an allergen?

In the present study, because injections of allergen with adjuvant obscure the characteristics of the injection sites, we intranasally (i.n.), i.p., s.c., or i.v. injected cedar pollen without adjuvant once or twice into wild-type, interferon- $\gamma^{-/-}$ (IFN- $\gamma^{-/-}$) or interleukin-4 $^{-/-}$ (IL-4 $^{-/-}$) BALB/c mice to explore which cytokine was essential for the production of total IgE Ab in the serum. After separation of peripheral blood mononuclear cells (PBMCs) into three cell populations (monocyte-rich, lymphocyte-rich, and granulocyte-rich) by Percoll density-gradient centrifugation or into specific antigen $^{+}$ cells by flow cytometry, we examined which population of peripheral blood leukocytes was involved in the *in vitro* production of nonspecific IgE Ab in response to the allergen. The results revealed that IL-4 was a cytokine essential for the production of total IgE Ab in the serum. The PBMCs from mice sensitized s.c. once produced IL-4 and total IgE Ab in the presence of the allergen *in vitro*. Unexpectedly, the lymphocyte-rich fraction alone was ineffective in the production of IL-4 and total IgE Ab, and the addition of Mac-1 $^{+}$ cells in the monocyte-rich fraction to the lymphocyte fraction was essential for production. The cellular sources of IFN- γ and IL-4 and their levels in the culture medium of PBMCs were also examined.

MATERIALS AND METHODS

Animals

There are many gene (IFN- γ and IL-4) knockout lines in the BALB/c and C57BL/6 strains of mice, and their gene mapping has already been established. It has been reported, however that serum allergen-specific IgE levels were much higher in BALB/c mice than in C57BL/6 mice after allergen (ovalbumin and *Schistosoma mansoni* egg antigen) sensitization and exposure,^{5,6} and we also found that BALB/c mice produced higher titers of serum IgE Ab than C57BL/6 mice after treatment with Japanese cedar pollen allergen (data not shown). 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). BALB/c IFN- $\gamma^{-/-}$ mice were obtained from the breeding colony of Jackson Laboratory (Bar Harbor, ME). After i.n., i.p., s.c., or i.v. injection 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 50% humidity for 1–3 weeks. The experiment was 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). The experimental protocol was approved by The Review Committee for Animal Experiments of Osaka Medical College.

Antigen

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

Antibodies (Abs)

Abs used in this study were the following: mouse IgE, IgG, and IgA from Zymed (San Francisco, CA); mouse IgM from Bethyl (Montgomery, TX); rat antimouse IgE, IgG, IgM, and IgA from Biosource (Camarillo, CA); horseradish peroxidase (HRP)-labeled goat antimouse IgE, IgG, IgM, and IgA Abs from Nordic (Tilburg, the Netherlands), Cappel (Aurora, OH), and Southern Biotech (Birmingham, AL), respectively; phycoerythrin (PE)-labeled antimouse CD4 (RM4-4) and CD45R/B220 (RA3-6B2) Abs and fluorescein isothiocyanate (FITC)-labeled CD8 (53-6.7) and CD11b/Mac-1 (M1/70) Abs from PharMingen (San Diego, CA).

Preparation of high-titer IgE Ab toward cedar pollen

To determine if the serum sample contained total IgE or specific IgE Abs directed toward cedar pollen, we prepared mouse cedar pollen-specific IgE Ab, as described previously.⁴ Titers in samples relative to the high-titer cedar pollen-specific IgE Ab (= 1.0) were determined based on a standard curve.

Serum preparation

Blood samples were taken by cardiac puncture under chloroform anesthesia before treatment or at various times after i.n., i.p., s.c., or i.v. injection of 100 μg cedar pollen crude extract-cj in 0.2 mL phosphate-buffered saline (PBS)/mouse. The whole blood was incubated in a CO₂ incubator at 37°C for 1 h, left overnight at 4°C, and then centrifuged at 440g for 20 min. The supernatant fraction was stored in microtubes at -80°C prior to use.

Enzyme-linked immunosorbent assay (ELISA) for total IgE, IgG, IgM, or IgA Ab

EIA/RIA plates (Costar 3591, Corning, Corning, NY) were coated with 100 μL rat antimouse IgE Ab, anti-IgG, anti-IgM, or anti-IgA Ab (each 2 $\mu\text{g}/\text{mL}$) at 4°C overnight. After three washes with PBS/Tween (PBS + 0.5% Tween 20), the wells were filled with 400 μL of 1% bovine serum albumin (BSA)/PBS and incubated for 2 h at ambient temperature to block unsaturated protein binding sites. The plates were next washed three times with PBS/Tween, and 100 μL of appropriately diluted serum samples in 1% BSA/PBS was added to each of triplicate wells, after which the plates were incubated for 2 h at ambient temperature. After three more washes with PBS/Tween, 100 μL HRP-labeled goat antimouse IgE, IgG, IgM, or IgA Ab in 1% BSA/PBS was dispensed into each well, and the preparation was allowed to react for 1 h at ambient temperature. The wells were then washed thoroughly with PBS/Tween. Next, the antigen-Ab complex was incubated with 100 μL tetramethylbenzidine (Sigma, St. Louis, MO) for 30 min at ambient temperature, and the reaction was stopped by the addition of 100 μL of 1 M H₂SO₄. Thereafter OD_{450nm} was read by a microplate reader M-Emax (Molecular Devices Co., Sunnyvale, CA). For preparation of standard curves for Igs, the concentrations used in this study were as follow: IgG: 0.01 $\mu\text{g}/\text{mL}$, 0.02 $\mu\text{g}/\text{mL}$, 0.05 $\mu\text{g}/\text{mL}$, 0.1 $\mu\text{g}/\text{mL}$, and 0.2 $\mu\text{g}/\text{mL}$; IgE: 0.005 $\mu\text{g}/\text{mL}$, 0.01 $\mu\text{g}/\text{mL}$, 0.02 $\mu\text{g}/\text{mL}$, 0.05 $\mu\text{g}/\text{mL}$, and 0.1 $\mu\text{g}/\text{mL}$; IgM: 0.02 $\mu\text{g}/\text{mL}$, 0.05 $\mu\text{g}/\text{mL}$, 0.1 $\mu\text{g}/\text{mL}$, 0.2 $\mu\text{g}/\text{mL}$, and 0.5 $\mu\text{g}/\text{mL}$; IgA: 0.05 $\mu\text{g}/\text{mL}$, 0.1 $\mu\text{g}/\text{mL}$, 0.2

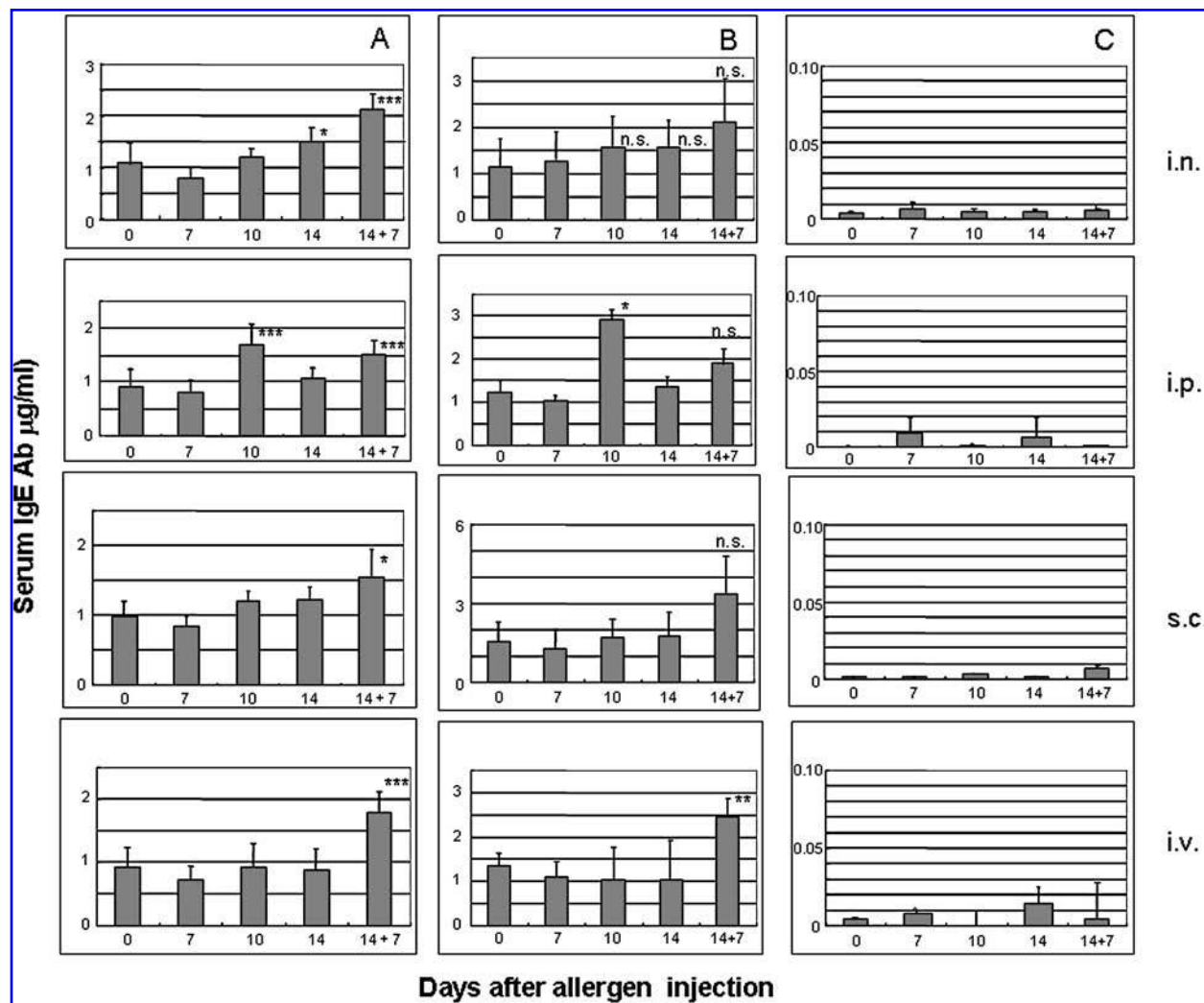


FIG. 1. Effects of first (i.n., i.p., i.v. or s.c.) and second (s.c.) injections of cedar pollen on the levels of serum IgE Ab in wild-type (A), IFN- $\gamma^{-/-}$ (B), and IL-4 $^{-/-}$ (C) BALB/c mice. The levels of IgE Ab in the serum were determined either on days 0, 7, 10, and 14 after the first injection or on day 7 after the second injection (14 + 7 days). Each value represents the mean $\pm$ SD of IgE Ab concentrations ($\mu\text{g/mL}$) in six serum samples from two different experiments. The difference between sample and control (day 0) is significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$) according to Student's *t*-test; n.s., not significant.

$\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, and 1 $\mu\text{g/mL}$. To measure the total Ig concentrations, we diluted serum samples 20, 50, and 100 times for IgE and 5,000, 10,000, and 20,000 times for IgG, IgM, and IgA, respectively. To measure the IgE concentrations in the culture medium, we diluted samples 1 and 2.5 times. Based on the standard curve, determination of the amount of Ig was carried out using Microsoft Excel software. The lower limit of the serum Ab assayed in this system was 10–30 ng/mL. The SD of the triplicate assay was <30% at approximately 50 ng/mL and <15% at approximately 100 ng/mL.

ELISA for specific IgE Ab

The procedure was the same as that for the measurement of the total IgE, IgG, IgM, or IgA Ab concentration 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 the cedar pollen in 100 μL PBS. For preparation of the standard curve for specific IgE Ab, 80–10,240 times diluted, high-titer, cedar pollen-specific IgE Ab in 100 μL of 1% BSA/PBS was added to each of triplicate wells. Samples (culture medium) were diluted 1 and 2.5 times, and the titers relative to the high-titer, cedar pollen-specific IgE Ab (= 1.0) were determined based on the standard curve.

Percoll density-gradient centrifugation

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 (1420g, 15 min at 20°C). The cells at the interface (PBMCs) were washed with PBS containing 5 mM EDTA, resuspended in 60% Percoll, and separated into lym-

phocyte-rich, monocyte-rich, and granulocyte-rich populations by discontinuous Percoll density-gradient (PBS/40%/50%/60% Percoll) centrifugation (1420g, 15 min at 20°C).^{7,8} The cell types were assessed morphologically after hematoxylin-eosin staining.

Flow cytometric analysis and sorting

For analysis, cells (10^6 cells) suspended in 50 μ L cold PBS containing 2% fetal bovine serum (FBS) were stained with a fluorescein-labeled Ab (1 μ g) in the presence of 1.5 μ L rat whole serum and 1 μ g Fc γ RIII/II mAb at 4°C for 20 min, washed, and analyzed by fluorescence-activated cell sorter (FACS). For sorting, the cells were isolated by FACS in the forward scattering/side scattering or FITC/PE mode, as described previously.⁹

Cell number and viability

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

Cell culture

Cells in various combinations of lymphocyte-rich, monocyte-rich, and granulocyte-rich populations and specific antigen⁺ cells were cultured for 6 days with cedar pollen (0–60 μ g/2 mL) in a 24-well plate with RPMI 1640 medium containing 10% FBS. The culture media were stored in microtubes at -80°C prior to use.

ELISA for IFN- γ and IL-4

The amounts of IFN- γ and IL-4 in the culture medium were assessed using Cytoscreen ELISA kits (Biosource International Inc.): 50 μ L biotin-conjugated mAb specific for IFN- γ or IL-4 and 50 μ L standard, control, or experimental sample were added to each of several anti-IFN- γ or IL-4 Ab-coated wells. Incubation was carried out at 37°C for 2 h. After four washes with washing buffer, 100 μ L streptavidin-HRP solution was added to each well, and incubation was continued at ambient temperature for 30 min. After four more washes with washing buffer and the addition of 100 μ L stop solution, the absorbance of each well was read at 450 nm. The standard curves for IFN- γ and IL-4 were prepared over the ranges of 0–500 and 0–1000 pg/mL, respectively.

RESULTS

Time-dependent changes in serum IgE Ab after i.n., i.p., s.c., or i.v. injection of cedar pollen into IFN- $\gamma^{-/-}$ BALB/c mice

Figure 1 shows the time-dependent changes in the serum IgE Ab in wild-type, IFN- $\gamma^{-/-}$, and IL-4 $^{-/-}$ mice after an i.n., i.p., s.c., or i.v. injection of cedar pollen, compared with the level before treatment. Either the i.n. or i.p. injection of the allergen caused an increase (1.4–2.3-fold of control) in the serum IgE Ab in wild-type mice on day 10 or 14 and in the IFN- $\gamma^{-/-}$ mice on day 10 after the i.p. injection, whereas the serum IgG, IgM,

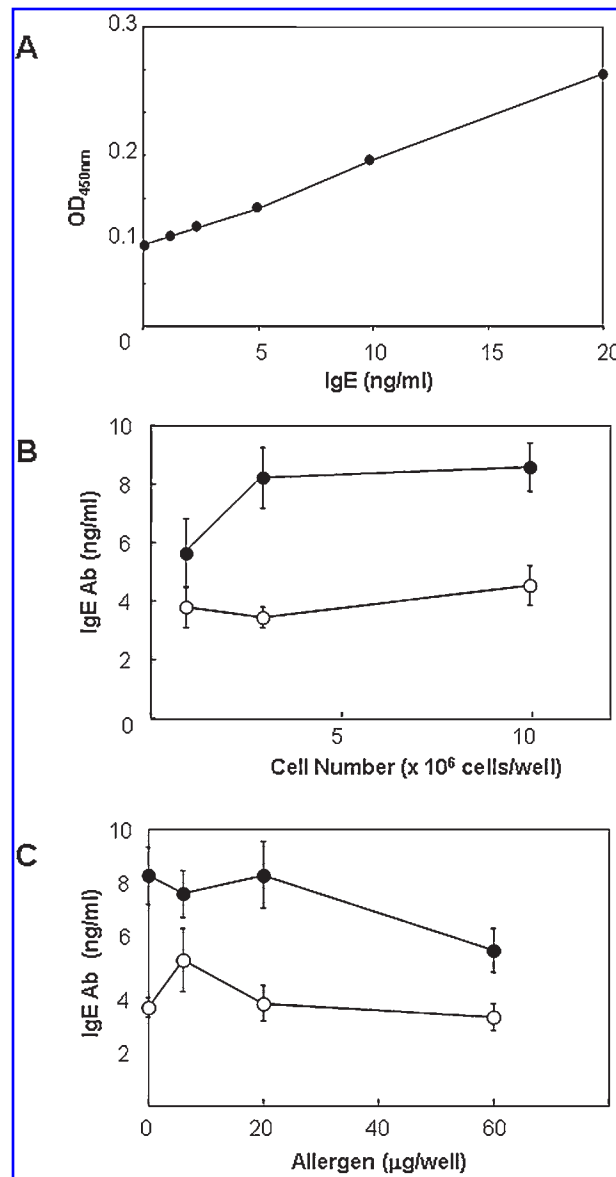


FIG. 2. IgE Ab concentrations in the culture medium. (A) A representative standard curve of the ELISA assay for IgE Ab (0–20 ng/mL). Each value represents the mean $\pm$ SD ($n = 3$), and all SD bars are hidden by the symbols. (B) Cell number-dependent IgE Ab production. Various numbers of PBMCs ($1\text{--}10 \times 10^6$ cells) were cultured with cedar pollen (20 μ g/mL) in a 24-well plate. The levels of IgE Ab in the culture medium were determined in 6-day cultures. Each value represents the mean $\pm$ SD of the IgE Ab (ng/mL) in three samples. Open circles, before sensitization; closed circles, after s.c. sensitization twice. (C) Dose-dependency of allergen for IgE Ab production. PBMCs (3×10^6 cells) were cultured in the presence of various concentrations (0–60 μ g/2 mL/well) of allergen. The levels of IgE Ab in the culture medium were determined in 6-day cultures. Each value represents the mean $\pm$ SD of the IgE Ab concentration (ng/mL) in three samples. Open circles, before sensitization; closed circles, after s.c. sensitization twice.

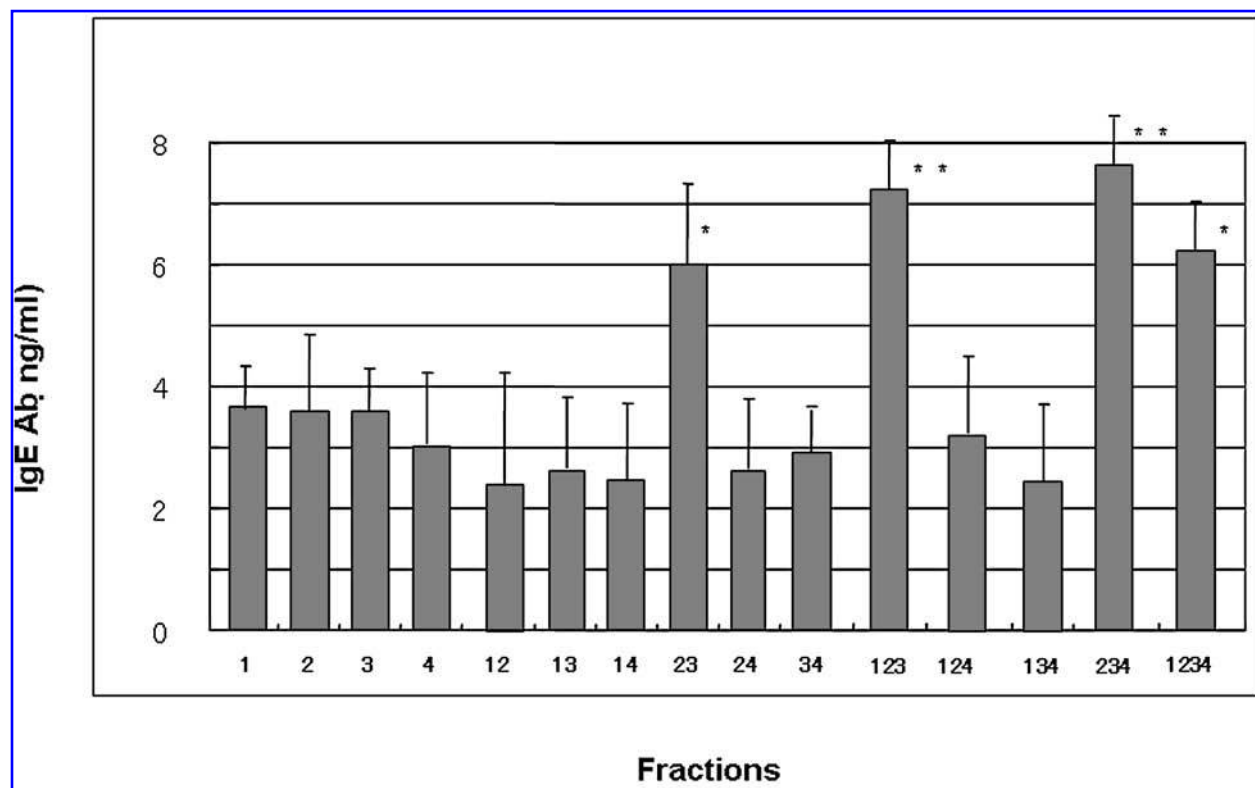


FIG. 3. IgE Ab released from the cells in various combinations of fractions of PBMCs from mice s.c. sensitized once. The levels of IgE Ab in the culture medium were determined after the cells had been cultured for 6 days with cedar pollen (20 $\mu\text{g}/\text{well}$). Each value represents the mean $\pm$ SD of IgE Ab concentration (ng/mL) in the six samples from two different experiments. The difference between sample and control (fraction 3 alone) is significant (* $p < 0.05$; ** $p < 0.01$) according to Student's *t*-test. The average number of cells added to the cocultures: 7.4×10^3 cells/well (fraction 1); 5.2×10^4 cells/well (fraction 2); 2.1×10^6 cells/well (fraction 3); 8.3×10^5 cells/well (fraction 4).

and IgA concentrations did not change significantly or actually decreased (data not shown). In contrast, an s.c. or i.v. injection of the allergen did not induce an increase in serum IgE Ab production on days 7, 10, and 14 after treatment, and the concentrations of other Ig isotypes in the serum were also unchanged or somewhat decreased (data not shown). A second (s.c.) injection of the same allergen on day 14 into i.n., i.p., s.c., or i.v. allergen-sensitized wild-type animals and into IFN- $\gamma^{-/-}$ mice via the i.v. route caused an increase in the levels of serum IgE Ab on day 7 after this second injection (day 21). In contrast, the serum IgG, IgM, and IgA concentrations did not change significantly or somewhat decreased after this second injection (data not shown). Thus, the effects of one or two injections of cedar pollen on the levels of serum IgE Ab in these IFN- $\gamma^{-/-}$ mice were similar to those in their control (wild-type) mice. In contrast, the basal level of serum IgE in IL-4 $^{-/-}$ mice was a trace amount ($\leq 0.005 \mu\text{g}/\text{mL}$), and injections of the allergen once or twice did not cause an increase in the serum IgE Ab. The basal levels of serum IgG, IgM, and IgA concentrations were similar to those of the control mice, and they did not change significantly after one or two injections of the allergen (data not shown).

These results indicate little inhibitory effect of IFN- γ on the initial production of IgE Ab against the allergen and the es-

sential role of IL-4 in the production of total IgE Ab in the serum.

Separation of PBMCs into monocyte-rich, lymphocyte-rich, and granulocyte-rich populations by Percoll density-gradient centrifugation

An s.c. injection of allergen into normal BALB/c mice was inactive as an inducer of total IgE Ab in their serum, whereas a second (s.c.) injection of the allergen caused a ≈ 2 -fold increase in the total IgE Ab (Fig. 1). To explore which population of PBMCs was involved in the production of nonspecific IgE Ab in response to the allergen, we separated various amounts of PBMCs into monocyte-rich, lymphocyte-rich, and granulocyte-rich populations by Percoll density-gradient centrifugation. The yield was generally good (75%–86%; $n = 3$). Fraction 3 (rich in lymphocytes) was the major ($70.0\% \pm 14.3\%$; mean $\pm$ SD, $n = 3$) population, followed by fraction 4 (rich in granulocytes; $28.0\% \pm 14.6\%$; mean $\pm$ SD, $n = 3$) and fraction 2 (rich in monocytes; $1.6\% \pm 1.3\%$; mean $\pm$ SD, $n = 3$) in that order. Fraction 1 (rich in somewhat damaged cells) contained a low number of cells.

Figure 2A is a representative standard curve of ELISA assay for very low levels (1–20 ng/mL) of IgE. Most of the re-

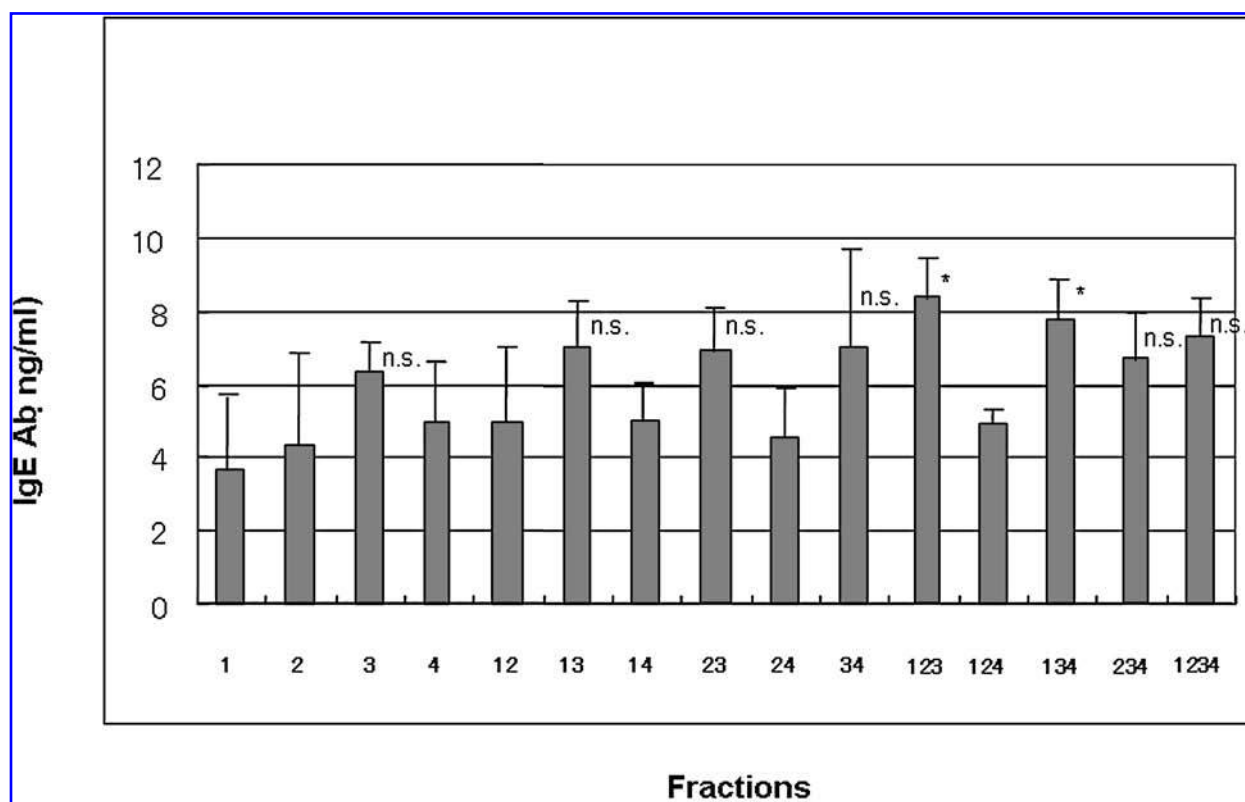


FIG. 4. IgE Ab released from the cells in various combinations of fractions of PBMCs from mice s.c. sensitized twice. The levels of IgE Ab in the culture medium were determined after the cells had been cultured for 6 days with cedar pollen (20 μ g/well). Each value represents the mean $\pm$ SD of IgE Ab concentration (ng/mL) in six samples from two different experiments. The difference between sample and control (fraction 2 alone) is significant ($*p < 0.05$) according to Student's *t*-test. n.s., not significant. The average number of cells added to the cocultures: 2.9×10^4 cells/well (fraction 1); 3.1×10^4 cells/well (fraction 2); 2.8×10^6 cells/well (fraction 3); 1.5×10^5 cells/well (fraction 4).

peated standard curves were linear up to 20 ng/mL. As shown in Figure 2B, the bulk of PBMCs from mice sensitized s.c. twice released a significant amount of IgE Ab into the culture medium after 6 days in culture in a cell number-dependent manner, reaching a submaximal level at 3×10^6 cells/well. The levels of IgE Ab in the culture medium of bulk PBMCs from untreated mice, however, were almost constant (≈ 4 ng/mL), suggesting that the basal level in this *in vitro* system might be ≈ 4 ng/mL. The levels of IgE Ab in the culture medium containing PBMCs from mice sensitized s.c. once or twice sometimes varied from sample to sample in the absence of allergen. Thus, we added 20 μ g/well of cedar pollen to the culture medium, although there was no clear allergen amount-dependent IgE Ab production (Fig. 2C).

In vitro IgE Ab production obtained by mixing lymphocyte-rich fraction with monocyte-rich fraction of PBMCs from BALB/c mice allergen-sensitized s.c. once

As we obtained monocyte-rich, lymphocyte-rich, and granulocyte-rich fractions, we incubated various combinations of these cells with allergen and assessed the levels of IgE Ab in the culture medium (Fig. 3). Bulk PBMCs from mice that had been s.c. treated once with allergen produced a significant

amount of IgE Ab (8.17 ± 1.02 ng/mL), whereas the lymphocyte-rich fraction (fraction 3) of the PBMCs did not. Unexpectedly, the IgE Ab production was restored by the addition of the monocyte-rich fraction (fraction 2) but not by that of the granulocyte-rich fraction (fraction 4) to the lymphocyte-rich one. As expected, these IgE Abs hardly reacted with the antigen, suggesting the increase to be due to nonspecific IgE Ab in the culture medium. In contrast, bulk PBMCs from untreated mice did not produce IgE Ab significantly because the levels (3.66 ± 0.64 ng/mL) were around the basal level. These results suggest that cedar pollen might be recognized as an allergen by the mixture of lymphocyte-rich and monocyte-rich fractions to release nonspecific IgE Ab from lymphocytes into the culture medium.

In vitro IgE Ab production by lymphocyte-rich population of PBMCs from BALB/c mice allergen-sensitized s.c. twice

A second (s.c.) injection of the allergen resulted in a ~ 2 -fold increase in nonspecific IgE Ab (Fig. 1), whereas the third (s.c.) sensitization with the same allergen (cedar pollen) produced a large amount of allergen-specific IgE Ab in the serum.⁴ Therefore, we explored whether the PBMCs from BALB/c mice allergen-sensitized s.c. twice produced allergen-specific IgE Ab

TABLE 1. PRODUCTION OF IFN- γ AND IL-4 BY CELLS IN VARIOUS FRACTIONS OF PBMCs FROM MICE UNTREATED OR SENSITIZED S.C. ONCE OR TWICE

Fraction ^a	Cytokine (pg/mL) ^b	
	IFN- γ	IL-4
Untreated		
2	3.1 $\pm$ 0.2	144.8 $\pm$ 17.9
3	295.1 $\pm$ 25.5	597.3 $\pm$ 10.5
2 + 3	275.6 $\pm$ 3.6	625.1 $\pm$ 2.7
Bulk	276.7 $\pm$ 17.6	635.0 $\pm$ 11.0
Sensitized s.c. once		
2	0.0 $\pm$ 0.0	42.3 $\pm$ 58.2
3	0.6 $\pm$ 0.6	345.9 $\pm$ 84.3
2 + 3	28.3 $\pm$ 4.3	1105.6 $\pm$ 64.3**
Bulk	38.6 $\pm$ 3.7	1125.1 $\pm$ 54.5**
Sensitized s.c. twice		
2	0.0 $\pm$ 0.0	1.0 $\pm$ 1.4
3	0.1 $\pm$ 0.1	345.9 $\pm$ 7.8
2 + 3	0.4 $\pm$ 0.4	485.1 $\pm$ 31.6
Bulk	2.8 $\pm$ 1.2	772.0 $\pm$ 59.7*

^aFraction 2 or fraction 3 and bulk PBMCs were obtained as described in Materials and Methods. The average number of cells added to the coculture: untreated, 4.2×10^4 cells/well (fraction 2), 2.6×10^6 cells/well (fraction 3), 3.0×10^6 cells/well (bulk); sensitized s.c. once, 5.0×10^4 cells/well (fraction 2), 2.5×10^6 cells/well (fraction 3), 3.0×10^6 cells/well (bulk); sensitized s.c. twice, 4.1×10^4 cells/well (fraction 2), 2.8×10^6 cells/well (fraction 3), 3.0×10^6 cells/well (bulk).

^bThe levels of IFN- γ and IL-4 in the culture medium were determined after the cells had been cultured for 6 days with cedar pollen (20 mg/well). Each value represents the mean $\pm$ SD ($n = 6$) of two different experiments. The difference between sample and control (untreated) is significant (* $p < 0.05$; ** $p < 0.01$) according to Student's *t*-test.

in the presence of the allergen *in vitro*. As expected, the cells in various combinations of fractions containing the lymphocyte-rich population (fraction 3) of PBMCs produced a significant amount of IgE Ab (6–8 ng/mL) (Fig. 4); however, the titers of the specific IgE relative to high-titer pollen-specific IgE Ab (= 1.0) were <0.1 , suggesting the increase in the IgE Ab titer to be due to nonspecific IgE Ab in the culture medium. In contrast, the cells in the monocyte-rich or granulocyte-rich fraction (fraction 2 or 4) of PBMCs from the same animal did not produce IgE Ab significantly and had no additive or crucial effects on the IgE Ab production by lymphocytes. Furthermore, the cells in fractions 2 and 3 from mice that had been five times i.p. treated with the mixture of allergen and adjuvant produced 19.6 ± 6.32 ng/mL medium after 6 days of being cultured with allergen. This level of IgE Ab was 2–3-fold higher than that (Figs. 3 and 4) released from cells in fractions 2 and 3 from mice that had been injected once or twice s.c. with allergen.

Production of IFN- γ and IL-4 by cells in various fractions of PBMCs from mice untreated or sensitized s.c. once or twice

Next, we assessed the cellular source of IFN- γ and IL-4 and their levels in the culture medium (Table 1). IFN- γ was released

mainly from the lymphocyte-rich fraction of untreated mice, whereas the levels were markedly reduced in the animals sensitized s.c. once and became negligible in those sensitized s.c. twice. Bulk PBMCs from mice that had been injected 1 time s.c. with allergen produced a large amount of IL-4, whereas the lymphocyte-rich fraction of the PBMCs did not. Surprisingly, mixed cultures of the lymphocyte-rich fraction and monocyte-rich fraction produced a large amount of IL-4 that was released into the culture medium. In the case of twice-sensitized animals, the levels of IL-4 returned to almost those in untreated animals.

Essential role of Mac-1⁺ cells in *in vitro* production of IL-4 and nonspecific IgE Ab by peripheral blood lymphocytes from mice sensitized s.c. once with cedar pollen

The results described suggested that production of IL-4, which was essential for IgE Ab production, might have been dependent on monocytes. To test this possibility, we stained cells in fractions 2 and 3 with fluorescein-labeled antimouse antigen antibodies. As expected, fraction 3, the lymphocyte-rich population, contained CD4⁺ (58.6%), B220⁺ (21.4%), and CD8⁺ (15.9%) cells. Although antibody binding to cells sometimes activates or inhibits their biologic functions,¹⁰ the amounts of IL-4 and IgE Ab, both of which were released into the culture medium containing cells in fractions 2 and 3, did not change significantly after antibody labeling and cell sorting (Fig. 5). The cells in combined fractions 2 and 3 from mice that had been injected once s.c. with allergen produced a large amount of IL-4 and IgE Ab, whereas fraction 2 or 3 alone did not. When CD4⁺, Mac-1⁺, or CD4⁺/Mac-1⁺ cells in fraction 2 were isolated by FACS and cultured with fraction 3, however, the combination of Mac-1⁺, but not CD4⁺ or CD4⁺/Mac-1⁺, cells with fraction 3 produced large amounts of IL-4 and IgE Ab comparable to those released by the combined fractions 2 and 3.

Cellular source of IL-4

Figure 1 showed the essential role of IL-4 in the production of total IgE Ab in the serum, and Figure 5 demonstrated that the combination of Mac-1⁺, but not CD4⁺ or CD4⁺/Mac-1⁺, cells in fraction 2 with fraction 3 produced large amounts of IL-4 and IgE Ab comparable to those released by combined fractions 2 and 3. We next examined if the requirement for IL-4 was in the monocyte, lymphocyte, or both populations by using wild-type and IL-4^{-/-} mice. We obtained fractions 2 and 3 from wild-type and IL-4^{-/-} mice that had been sensitized s.c. once, incubated various combinations of these fractions with allergen, and assessed the levels of IL-4 and IgE Ab in the culture medium (Fig. 6). Either combined fractions 2 and 3 (normal mice) or combined fraction 2 (IL-4 knockout mouse) and fraction 3 (normal mouse) produced a large amount of IL-4 and IgE Ab, whereas either combined fraction 2 (normal mouse) and fraction 3 (IL-4 knockout mouse) or combined fractions 2 and 3 (IL-4 knockout mouse) did not. These results taken together indicate that IL-4 was essential for total IgE Ab production in this *in vitro* culture system and that monocytes were crucial for IL-4 and IgE Ab production in lymphocytes.

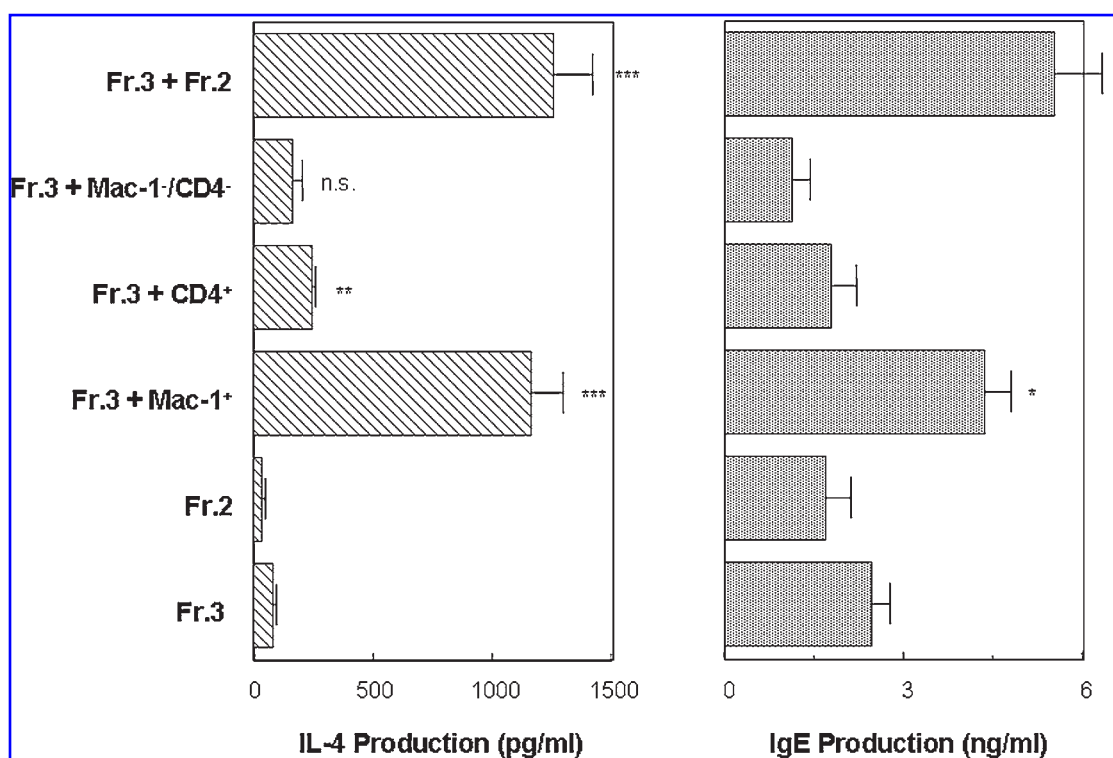


FIG. 5. Production of IL-4 and IgE Ab by the cells in various combinations of fractions of PBMCs from mice s.c. sensitized one time. After $CD4^+$, $Mac-1^+$, or $CD4^-/Mac-1^-$ cells in fraction 2 had been isolated by FACS, the specific antigen⁺ or antigen⁻ cells were cultured with fraction 3. The levels of IL-4 and IgE Ab in the culture medium were determined after the cells had been cultured for 6 days with cedar pollen (20 μ g/well). Each value represents the mean $\pm$ SD ($n = 6$) of two different experiments. The difference between sample and control (untreated) is significant (* $p < 0.05$; ** $p < 0.02$; *** $p < 0.001$) according to Student's *t*-test. The average number of cells added to the cocultures: 8.5×10^4 cells/well (fraction 2); 2.8×10^6 cells/well (fraction 3); 3.1×10^4 cells/well ($CD4^+$ cells in fraction 2); 1.4×10^4 cells/well ($Mac-1^+$ cells in fraction 2); 1.4×10^4 cells/well ($Mac-1^-/CD4^-$ cells in fraction 2). Fr., fraction; n.s., not significant.

DISCUSSION

In most previous animal models of pollen-induced allergic rhinitis, the allergic reaction was induced by repetitive pollen inhalation challenges to animals that had been sensitized by repeated instillation of the pollen extract plus adjuvant, including aluminum hydroxide gel into their nostrils.¹¹⁻¹³ Under these conditions, leukocytes, especially eosinophils, migrate into the cavity and induce edema in the nasal mucosa, and it is unlikely that the inhalation or the instillation of the pollen extract is quantitative. To understand precisely the first reaction of the immune system to the allergen, in the present study we i.n., i.p. i.v., or s.c. injected cedar pollen without adjuvant once or twice into normal, $IFN-\gamma^{-/-}$ or $IL-4^{-/-}$ BALB/c mice and found IL-4 to play an essential role in the production of total IgE Ab in the serum (Fig. 1). Although an s.c. injection of allergen into normal BALB/c mice was inactive as an inducer of nonspecific IgE Ab in their serum, a second (s.c.) injection of the allergen resulted in a ≈ 2 -fold increase in the IgE Ab.⁴ Therefore, we separated PBMCs from mice sensitized s.c. once into three cell populations (monocytes, lymphocytes, and granulocytes) by Percoll density-gradient centrifugation, and examined which population of PBMCs was involved in the *in vitro* production of nonspecific IgE Ab. As expected, the PBMCs from mice sen-

sitized s.c. once produced IL-4 and total IgE Ab. Unexpectedly, the lymphocyte-rich fraction alone was ineffective in the production of IL-4 and IgE Ab, and addition of the monocyte-rich fraction to the lymphocyte fraction was needed for production (Fig. 3 and Table 1). The cells required for IL-4 and IgE Ab production were found to be $Mac-1^+$ cells in the monocyte-rich population (Fig. 5). By using combined fractions 2 and 3 from wild-type and $IL-4^{-/-}$ mice, we demonstrated that the $Mac-1^+$ cells were essential for IL-4 and IgE Ab production in lymphocytes.

The animals that had been once i.n. treated with allergen produced a significant amount of nonspecific IgE Ab in their serum (Fig. 1). Upon the second (s.c.) injection of the same allergen to the i.n. allergen-sensitized animals, they produced a large amount of IgE Ab, and most of them reacted with the allergen,⁴ suggesting an increase in the allergen-specific IgE Ab in the serum. In contrast, a primary i.v. or s.c. injection of the allergen did not enhance IgE Ab production (Fig. 1), the second (s.c.) injection of the allergen into the i.v. or s.c. allergen-sensitized mice induced nonspecific IgE Ab (Fig. 1), and the third (s.c.) sensitization with the same allergen produced a large amount of allergen-specific IgE Ab in the serum.⁴ Under these conditions, serum IgA, IgM, and IgG levels did not change significantly.⁴ Thus, we concluded that increased production of to-

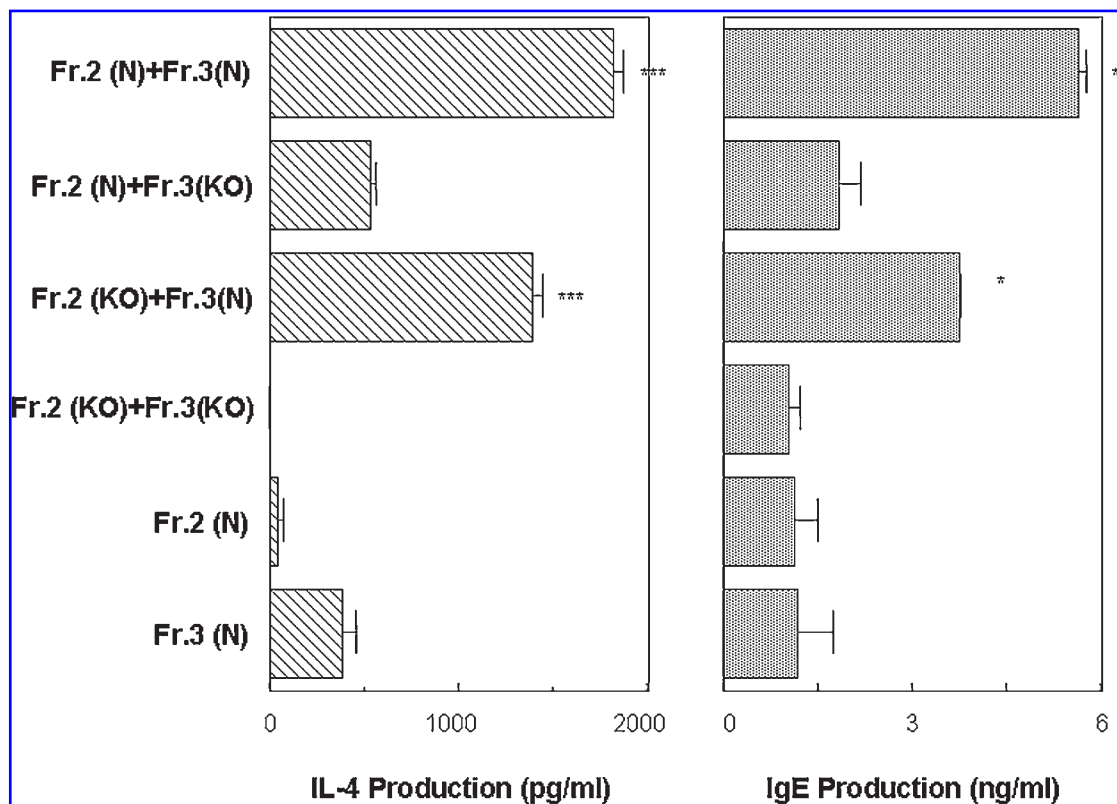


FIG. 6. Production of IL-4 and IgE Ab by the cells in various combinations of fractions of PBMCs from normal or IL-4^{-/-} mice s.c. sensitized once. The cells in fractions 2 and 3 were obtained by Percoll density-gradient centrifugation. The levels of IL-4 and IgE Ab in the culture medium were determined after the cells had been cultured for 6 days with cedar pollen (20 μ g/well). Each value represents the mean $\pm$ SD ($n = 6$) of two different experiments. The difference between sample and control (untreated) is significant (* $p < 0.02$; ** $p < 0.005$; *** $p < 0.002$) according to Student's *t*-test. The average number of cells added to the cocultures: IL-4 knockout, 3.3×10^4 cells/well (fraction 2); 2.8×10^6 cells/well (fraction 3); normal, 3.2×10^4 cells/well (fraction 2); 2.8×10^6 cells/well (fraction 3). Fr., fraction; N, normal mouse; KO, knockout mouse.

tal IgE Ab without a change in the IgA, IgG, or IgM concentration was a prerequisite for the production of allergen-specific IgE Abs. The cells in the combined fractions 2 and 3 from mice that had been treated i.p. five times with the mixture of allergen and adjuvant produced 19.6 ± 6.32 ng IgE Ab/mL of medium after 6 days of being cultured with allergen. This level of IgE Ab was 2–3-fold higher than that of IgE released from cells in the combined fractions 2 and 3 from mice that had been injected once or twice s.c. with allergen (Figs. 2, 3, and 4). In the present study, we also found Mac-1⁺ cells to have an essential part in the *in vitro* production of IL-4 and nonspecific IgE Ab by peripheral blood lymphocytes from mice sensitized s.c. once to cedar pollen (Fig. 5) and confirmed our previous finding that resident cells (e.g., macrophages) at the i.n. or i.p. injection site might play a crucial role in the efficient production of nonspecific IgE Ab in response to the allergen. Moreover, various combinations of fractions 2 and 3 from wild-type and IL-4^{-/-} mice that had been sensitized s.c. once showed the essential role of IL-4 in lymphocytes in the production of total IgE Ab in this *in vitro* culture system (Fig. 6), suggesting that the magnitude of the IgE response as measured might reflect the IgE Ab response that occurs *in vivo*.

When cedar pollen allergen was primarily injected i.n. or i.p.

into mice, the total IgE Ab level was increased in the serum of normal BALB/c mice, whereas an s.c. or i.v. injection of the same antigen did not increase the level.⁴ In a previous study,⁴ we speculated that resident cells at the i.n. or i.p. injection site might play a crucial role in the efficient production of nonspecific IgE Ab in response to the allergen. In fact, 6-day cultures of the combination of the lymphocyte-rich fraction with the monocyte-rich fraction of PBMCs from mice sensitized s.c. once showed the essential role of Mac-1⁺ but not of CD4⁺, CD8⁺, B220⁺, or polymorphonuclear cells in the production of IL-4 and nonspecific IgE Ab by lymphocytes (Figs. 3, 5, and 6 and Table 1). As the IgE Ab was nonspecific but not specific toward the allergen, it is unlikely that the monocytes/macrophages simply took up and processed protein antigens, a step that is accompanied by cellular activation.¹⁴ Rather, these results imply that the monocytic cells may be involved in either antigen recognition or class switch of IgM to IgE.

Pouliot et al.¹⁵ reported that IL-4 mRNA was expressed and IL-4 protein was produced and released by human alveolar macrophages. In our mouse model system, however, the lymphocyte-rich population of PBMCs from untreated mice or those sensitized once or twice seemed to be the main producer of IL-4. IL-4, essential for either conversion of Th0 to Th2¹⁶ or

class switch of IgM to IgE,¹⁷ was released from PBMCs from normal mice sensitized s.c. once, whereas the lymphocyte-rich fraction alone of the PBMCs released little IL-4 (Table 1). Unexpectedly, the IL-4 production was restored by the addition of Mac-1⁺ cells of the PBMCs to the lymphocyte culture medium (Table 1), suggesting that the Mac-1⁺ cells might release some cytokine(s) to stimulate IL-4 release from the cells in the lymphocyte-rich population.

It is well known that stimulation of mouse B cells with LPS causes differentiation of B cells and the formation of a variety of Ig isotypes, except IgE.¹⁸ However, when the same B cells were cultured for 5 days with LPS together with 100–500 U/mL IL-4, the result was the formation of IgE and selective enhancement of IgG1 formation,¹⁹ which was accompanied by a decrease in IgG2b and IgG3 formation. It is also known that the effect of IL-4 on B cells was counteracted by IFN- γ .^{20–22} To estimate the role of IFN- γ and IL-4 in the allergic reaction, we here assessed the levels of serum IgE Ab in IFN- γ ^{-/-} or IL-4^{-/-} mice that had been allergen sensitized s.c. once or twice. The results revealed that IL-4 was essential for the specific increase in the serum IgE but had no effect on IgA, IgG, or IgM Ab levels, whereas IFN- γ seemed not to be inhibitory (Fig. 1). Stern et al.²³ also reported that IFN- γ appeared to contribute to the pathogenesis of murine allergic conjunctivitis at the effector phase but not during the initial sensitization stage.

Increased production of IL-4 and total IgE Ab without a change in the IgA, IgG, or IgM concentration was a prerequisite for the production of allergen-specific IgE Abs in the serum⁴ (Fig. 1). Consistently, bulk PBMCs or the combination of Mac-1⁺ cells with lymphocytes from wild-type mice that had been s.c. treated once with allergen produced IL-4 (Table 1 and Fig. 5) and total IgE Ab (Figs. 3 and 5), whereas those from IL-4^{-/-} mice sensitized s.c. once did not (Fig. 6). Although cells in the lymphocyte-rich population of PBMCs from wild-type mice that had been sensitized s.c. twice with allergen alone produced a significant amount of IgE Ab ($\approx$ 2-fold increase) (Fig. 4), the increase in the IgE Ab titer was due to total IgE Ab in the culture medium (Fig. 4), and the levels of IL-4 produced by the PBMCs returned to almost those in untreated animals (Table 1). These results suggest that IL-4 might play an essential role in the production of total IgE Ab in lymphocytes and that IL-4 or some other lymphokines might be necessary for the *in vitro* production of allergen-specific IgE Ab. In this regard, studies on atopic families have implicated loci on chromosomes 11q and 5q that might be important in determining the presence of atopy. The candidate gene on chromosome 11 encodes the β -subunit of the high-affinity IgE receptor; whereas on chromosome 5, there is a cluster of tightly linked genes, including those for IL-3, IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF),²⁴ implying IL-3, IL-5, IL-9, IL-13, and GM-CSF as candidate proteins for involvement in allergen-specific IgE Ab production *in vitro*. Further investigations are underway in our laboratory.

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Address reprint requests or correspondence to:

Dr. Ryotaro Yoshida
Department of Physiology
Osaka Medical College
2-7 Daigaku-machi
Takatsuki 569-8686
Japan

Tel: 81-72-683-1221 ex. 3400

Fax: 81-72-684-6525

E-mail: ryoshida@art.osaka-med.ac.jp

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