

Allergen-specific IgE production of committed B cells from allergic patients in vitro

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The allergen-specific in vitro IgE synthesis in blood leukocytes from patients with allergy was monitored outside the pollen season with recombinant and natural pollen allergens and was compared with the total IgE production. The addition of interleukin-4 (IL-4) and antibody to CD40 increased the amount of total IgE by up to 20-fold in the culture supernatants of peripheral blood leukocytes from patients with allergy that could be antagonized by a neutralizing anti-IL-4 antibody in a dose-dependent manner. In contrast to total IgE, the amount of allergen-specific IgE was not affected by IL-4, and anti-CD40 or anti-IL-4, treatment. With oligonucleotides specific for IgE, complementary DNA from the amino terminal of the IgE heavy chain could be reversely transcribed and amplified by polymerase chain reaction from RNA of patients' unstimulated blood leukocytes, indicating that the IgE secretion in the cultures is due to a de novo IgE synthesis. It is concluded that the peripheral blood of patients with allergy contains long-lived allergen-specific B cells, which are not responsive to IL-4-mediated signals. These results may have implications for attempts to modulate specific IgE production in allergic patients with cytokines or cytokine antagonists. (J ALLERGY CLIN IMMUNOL 1995;96:209-18.)

Key words: Allergy, specific IgE, interleukin-4, anti-CD40, recombinant allergens

Type I allergy is a major health problem in industrialized countries, where up to 20% of the population have rhinitis, conjunctivitis, or bronchial asthma. It is well established that type I allergic symptoms are caused by allergen-specific IgE,¹ which is bound to high-affinity Fcε receptors on mast cells and basophils, and which on cross-linking with allergens triggers release of histamine and other inflammatory mediators.² Extremely low amounts of specific IgE produced by a small number of IgE-secreting B cells³ are required to elicit allergic effector mechanisms.

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Abbreviations used

Ig-Fd:	Amino-terminal portion of the IgG heavy chain
IL:	Interleukin
mAb:	Monoclonal antibody
PBMCs:	Peripheral blood mononuclear cells
PCR:	Polymerase chain reaction
rhu:	Recombinant human

Considerable attention was therefore given in the investigation to how IgE synthesis is regulated. Soluble factors^{4,5} such as interleukin (IL)-4, which is mainly secreted by T cells, function as potent inducers of IgE synthesis.⁶⁻⁸ In addition to soluble factors, a cognate interaction of T cells with the CD40 structure on B cells is required for B-cell growth and antibody synthesis.^{9,10} Antibodies raised against CD40 were discovered as potent B-cell growth factors and enhancers of IgE synthesis.¹¹⁻¹³

The fact that B cells of patients with allergy

produce IgE spontaneously in vitro^{14, 15} and that in murine systems an ongoing spontaneous IgE synthesis could be demonstrated^{16, 17} prompted us to compare the total IgE synthesis with the production of allergen-specific IgE.

With IL-4 and anti-CD40 antibodies and a neutralizing anti-human IL-4 antibody, the IL-4 dependence of the specific in vitro IgE synthesis of peripheral blood mononuclear cells (PBMCs) from patients with allergy was investigated. PBMCs were obtained outside the pollen season.

Defined natural and recombinant allergens¹⁸⁻²² were used for the determination of specific IgE. In contrast to the total IgE synthesis, the specific IgE production was neither influenced by IL-4 and anti-CD40 treatment nor by addition of a neutralizing anti-IL-4 antibody. The spontaneous synthesis of IgE by unstimulated blood leukocytes from patients with allergy was also demonstrated at the mRNA level by reverse transcription and polymerase chain reaction (PCR) amplification with oligonucleotides specific for human IgE.

METHODS

Production of a neutralizing antibody to human IL-4

Anti-IL4 monoclonal antibodies (mAbs) were produced as described.²³ In brief, BALB/c mice (4 to 6 weeks old) were immunized subcutaneously with 50 µg recombinant human (rhu) IL-4 (rhu IL-4 was purified from IL-4-secreting Chinese hamster ovary cells; Sandoz Pharma, Basel, Switzerland) in complete Freund's adjuvant at the base of the tail and in the neck. Mice were then given booster injections subcutaneously two times, 4 weeks apart, with rhu IL-4 (50 µg/mouse) in incomplete Freund's adjuvant. The mice with the highest anti-IL-4 antibody titer were given booster injections intravenously with rhu IL-4 (50 µg/mouse) 4 days before the fusion. Spleen cells of mice were fused with a non-immunoglobulin-secreting murine myeloma cell line as described.^{24, 25} Culture supernatants were screened by ELISA for anti-IL-4 mAbs with microtiter plates coated with rhu IL-4 (1 µg/ml). Cloning of hybridomas was performed with BALB/c peritoneal cells as feeder cells. Anti-IL-4 mAbs were tested for neutralization of IL-4 effects on activated PBMCs. PBMCs (1×10^6 cells/ml) were cultured in RPMI 1640 (Gibco, Basel, Switzerland), 10% fetal calf serum (Gibco, Basel, Switzerland) in the presence of phytohemagglutinin (1 µg/ml), and 50 ng/ml rhu IL-2 (Sandoz Pharma). After 5 days activated T cells were isolated by Ficoll density-gradient centrifugation and cultured for 3 additional days (5×10^4 cells/ml) in the presence of 3 ng/ml of rhu IL-4 and in the presence or absence of anti-IL-4 mAbs. The anti-IL-4 mAb 1-41-1, which at a concentration of

30 ng/ml inhibited the IL-4-dependent (3 ng/ml) proliferation of T-cell blasts by 50%, was selected.

Characterization of patients with allergy

Patients with tree and grass pollen allergy and nonallergic control subjects were characterized by case history and skin prick test results. In addition, sera were characterized by RAST and by IgE immunoblotting with natural and recombinant pollen allergens as described.^{20, 22} For each patient, the specific profile of IgE reactivity was established with recombinant tree pollen allergens such as rBet v I²¹ and birch profilin (rBet v II)^{18, 19} and recombinant timothy grass pollen (*Phleum pratense*) allergens, such as rPhl p I,²⁰ rPhl p V,^{20, 22} and rPhl p II.²⁰ Culture supernatants analyzed by immunoblot were from PBMCs obtained in October, more than 4 months after the grass pollen season, whereas culture supernatants tested by a RAST-based assay were from PBMCs obtained 4 weeks after the grass pollen season and 3 months after the tree pollen season, respectively.

Preparation and culture of PBMCs from patients with allergy

After informed consent was obtained from the donors, heparinized blood samples were taken. PBMCs were prepared by Ficoll density-gradient centrifugation, washed twice in phosphate-buffered saline solution and cultured in RPMI 1640 (Flow Laboratories, Meckenheim, Germany) containing 10% fetal calf serum (JRH Biosciences, Lenexa, Kan.), 50 µg/ml gentamycin, and 150 µg/ml L-glutamine at a density of 1.5×10^6 cells/ml for 8 days. Cells were cultured without addition of agonists, with rhu IL-4 (purified from Chinese hamster ovary cells; Sandoz Pharma) (100 U/ml) and with a combination of rhu IL-4 (100 U/ml) and anti-CD40 antibody (300 ng/ml).¹¹ Neutralizing anti-IL-4 antibody 1-41-1 was added as indicated in Figs. 1 to 6.

Determination of total and specific IgE in culture supernatants

Total IgE in the culture supernatants was measured by radioimmunoassay (IgE RIA Ultra 50; Pharmacia, Uppsala, Sweden). Results reflect means of duplicate determinations. Values below the detection level (0.2 U/ml) are displayed at the baseline.

Natural timothy grass (*P. pratense*) pollen proteins were prepared from pollen purchased from Allergon, Vålänge, Sweden, as described.²⁰ Recombinant Phl p V²² was expressed as a β-galactosidase fusion protein in *Escherichia coli* Y 1089; recombinant birch pollen profilin was expressed as a nonfusion protein in *E. coli* JM 105 and purified by affinity to poly-(L-proline)-Sepharose.¹⁹ Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with 1:2 diluted supernatants from cultures of PBMCs from patients with allergy or 1:10 diluted sera (Figs. 1 to 5 and Fig. 7). To ensure that allergens were present on the membrane in excess of specific IgE, serial

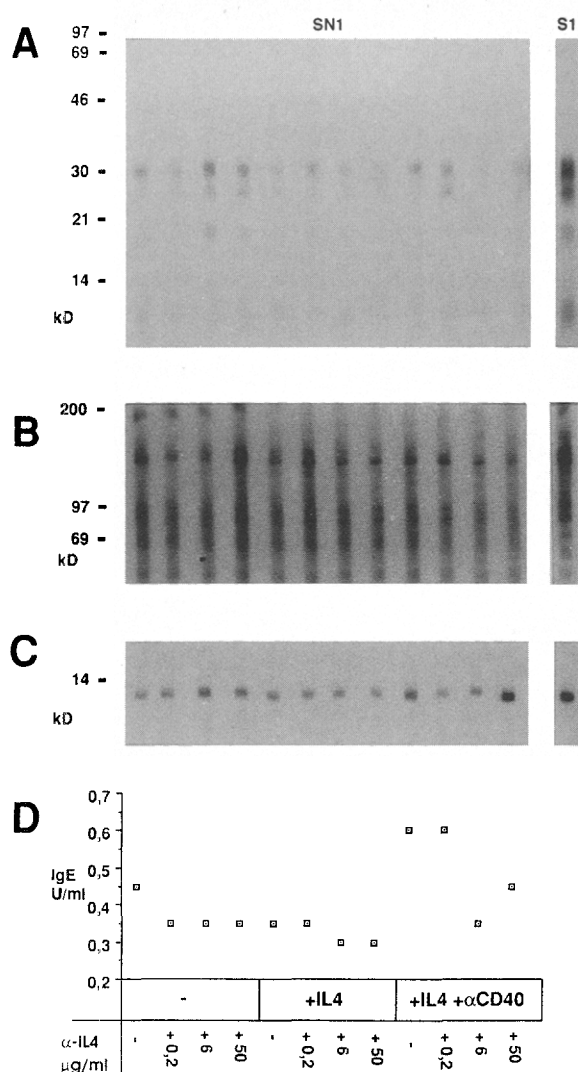


FIG. 1. Donor 1. Figs. 1 to 6 show comparison of specific and total IgE in culture supernatants (SN) of PBMCs from patients with allergy and from subjects without allergy. PBMCs from patients with allergy (donors 1 to 3) and from subjects without allergy (donors 4 and 5) were cultured without agonists (-), in the presence of rhu IL-4 (+IL-4) or with a combination of rhu IL-4 and anti-CD40 antibody (+IL-4 + αCD40). A neutralizing anti-IL-4 antibody was added as displayed at the bottom (-, without addition of anti-IL-4 antibody; or addition of 0.2, 6, and 50 μg/ml). IgE reactivity of culture supernatants with nitrocellulose-blotted natural timothy grass pollen allergens (A) and recombinant allergens (B and C) corresponds to determination of total IgE (D) in each lane. Group I and group V allergens migrate at 28 to 32 kd molecular weight, whereas profilin and group II and III allergens show molecular weight of 10 to 14 kd (A). Recombinant *Phl p V* was expressed in *E. coli* as a 140 kd β-galactosidase fusion protein (B), and purified recombinant birch profilin has a molecular weight of 14 kd (C). Allergens were in excess of IgE antibodies. Nitrocellulose strips were incubated with culture supernatants (SN) of unstimulated or stimulated donor PBMCs. IgE reactivity of corresponding serum (S) with blotted allergens is shown

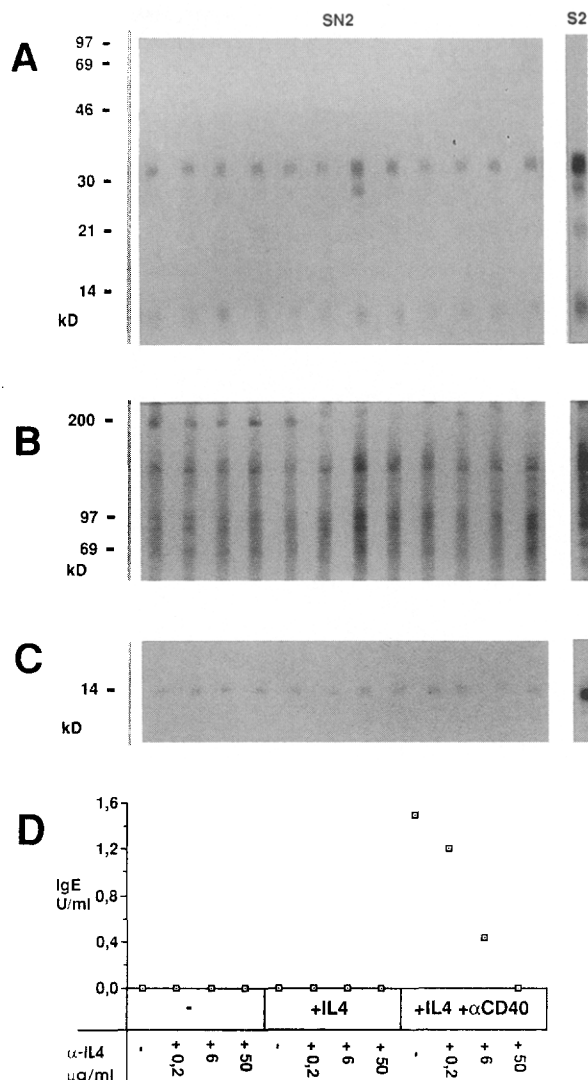


FIG. 2. Donor 2. PBMCs from patient with allergy. A, IgE reactivity of culture supernatants with nitrocellulose-blotted natural timothy grass pollen allergens. B and C, IgE reactivity of culture supernatants with recombinant allergens. D, Determination of total IgE. See legend to Fig. 1.

dilutions of sera were previously done. Bound IgE was detected with iodine 125-labeled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden). Incubation of blots and dilution of antibodies was done in buffer A (50 mmol/L sodium phosphate (pH 7.5), 0.5% Tween-20, 0.5% bovine serum albumin, 0.05% NaN₃). Bound IgE was visualized by autoradiography with Kodak X-Omat S films and intensifying screens (Kodak, Heidelberg, Germany).

at right. IgE binding beneath *Phl p V* β-galactosidase fusion allergen results from partial degradation of the fusion protein. No IgE reactivity to natural or recombinant allergens could be detected with supernatants or sera from nonallergic donors (donors 4 and 5).

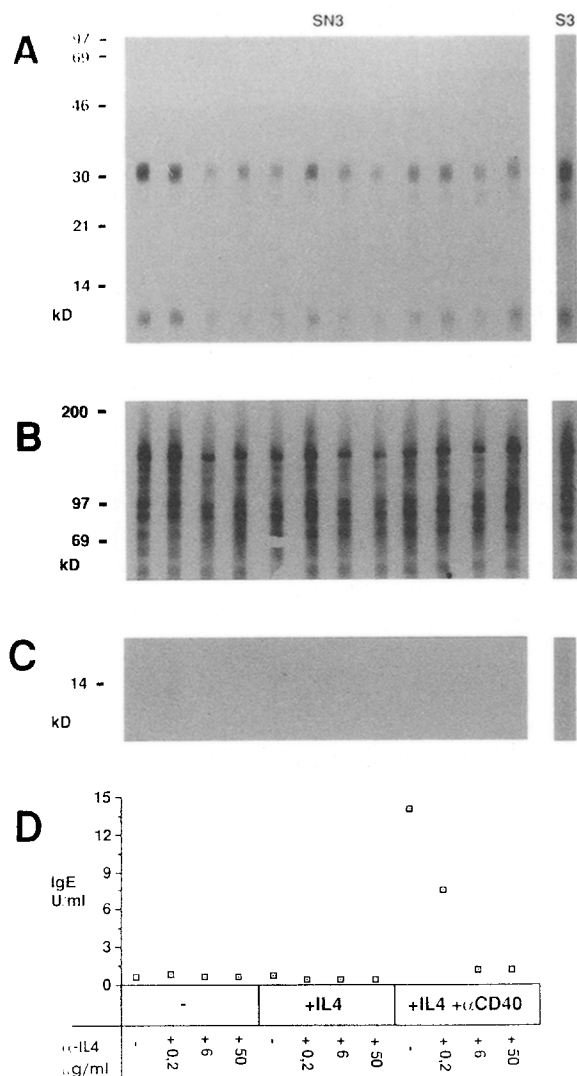


FIG. 3. Donor 3. PBMCs from patient with allergy. **A**, IgE reactivity of culture supernatants with nitrocellulose-blotted natural timothy grass pollen allergens. **B and C**, IgE reactivity of culture supernatants with recombinant allergens. **D**, Determination of total IgE. See legend to Fig. 1.

Determination of specific IgE in culture supernatants by RAST with purified recombinant pollen allergens

Recombinant birch profilin (*Bet v* II) was purified as described previously.¹⁹ Recombinant *Phl p* V²² was expressed in *E. coli* BL21 (DE3) with the pET expression system (Novagene, Madison, Wis.). The recombinant nonfusion protein was purified by ion-exchange chromatography (M. Susani et al. Unpublished data). Ten micrograms of more than 95% pure recombinant allergens and human serum albumin (negative control) were dot blotted to nitrocellulose sectors (10 × 6 mm). Nitrocellulose sectors were then incubated with 500 µl of culture supernatants, and bound IgE was

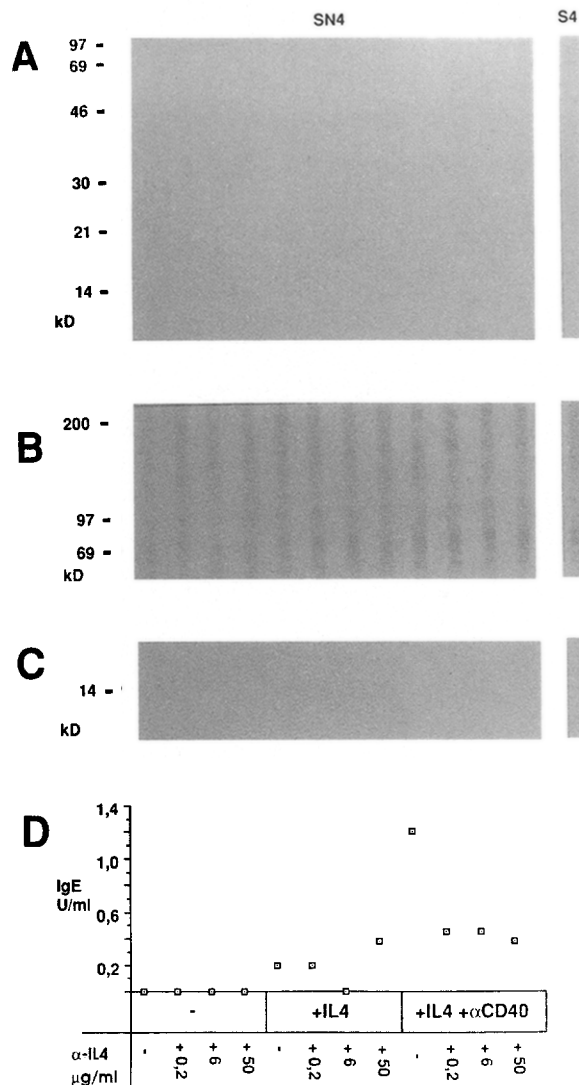


FIG. 4. Donor 4. PBMCs from subject without allergy. **A**, IgE reactivity of culture supernatants with nitrocellulose-blotted natural timothy grass pollen allergens. **B and C**, IgE reactivity of culture supernatants with recombinant allergens. **D**, Determination of total IgE. See legend to Fig. 1.

detected with ^{125}I -labeled anti-human IgE (Pharmacia), which was diluted 1:5 in buffer A. Sectors were washed in buffer A as described for the immunoblots, and bound IgE was measured in a gamma-counter (LKB, Uppsala, Sweden).

IgE and IgG-Fd cDNA preparation from PBMCs of patients with allergy

Total RNA was prepared from patients' PBMCs according to the method of Chirgwin et al.²⁶ Reverse transcription of mRNA coding for IgE or IgG was done with specific oligonucleotide primers (Biomedica, Vienna, Austria) (Fig. 8) and an RNA-PCR kit (Perkin Elmer Cetus, Norwalk, Conn.). The PCR product was

analyzed with a 1.5% agarose gel and ethidium bromide staining. DNA was transferred to nitrocellulose and hybridized with oligonucleotides specific for IgE or IgG mRNA that had been 5'-end labeled with polynucleotide kinase (Boehringer, Mannheim, Germany) and γ - ^{32} P-labeled adenosine triphosphate (NEN, Stevenage, U.K.). All molecular biologic manipulations were performed as described.²⁷

RESULTS

Allergen-specific IgE secretion by PBMCs from patients with allergy outside the pollen season is IL-4-independent

PBMCs from patients with allergy and from subjects without allergy were obtained after the pollen season and kept in culture without addition of allergens. Allergen-specific IgE synthesis and total IgE production were compared by probing culture supernatants with natural and recombinant allergens and a parallel determination of total IgE. The addition of IL-4 only weakly increased the amount of total IgE in donor 4 (Fig. 4), whereas IL-4 and antibody to CD40¹¹ increased the amount of total IgE in the culture supernatants up to 20 times; this amount could be reduced to values obtained from non-stimulated cultures by addition of a neutralizing anti-IL-4 antibody (Figs. 1 to 6). In contrast to total IgE levels, the amount of allergen-specific IgE was not affected either by IL-4 and anti-CD40 or by anti-IL-4 treatment. Only little variation regarding the specific in vitro IgE production, which showed no correlation to any treatment and rather reflected the distribution of specific B cells in the different culture wells (Figs. 1 to 6), was observed. No binding to natural or recombinant allergens was observed with supernatants from persons without allergy (Figs. 4 and 5), whereas, as in patients with allergy (Figs. 1, 2, and 3) a combination of IL-4 and anti-CD40 antibody increased the total IgE production. The increase in total IgE could be antagonized in a dose-dependent manner by addition of the neutralizing anti-IL-4 antibody. None of the supernatants showed IgE reactivity with *E. coli*-phage proteins (Fig. 7). To confirm the immunoblot results, RAST with purified recombinant allergens (rBet v II, rPhl p V) dotted to nitrocellulose sectors were used. Fig. 6 shows that results comparable to those of the immunoblots, namely, that allergen-specific IgE synthesis was not influenced by IL-4 or IL-4 antagonists, could be obtained with PBMCs from three different allergic donors (Fig. 6) and with two different recombinant allergens.

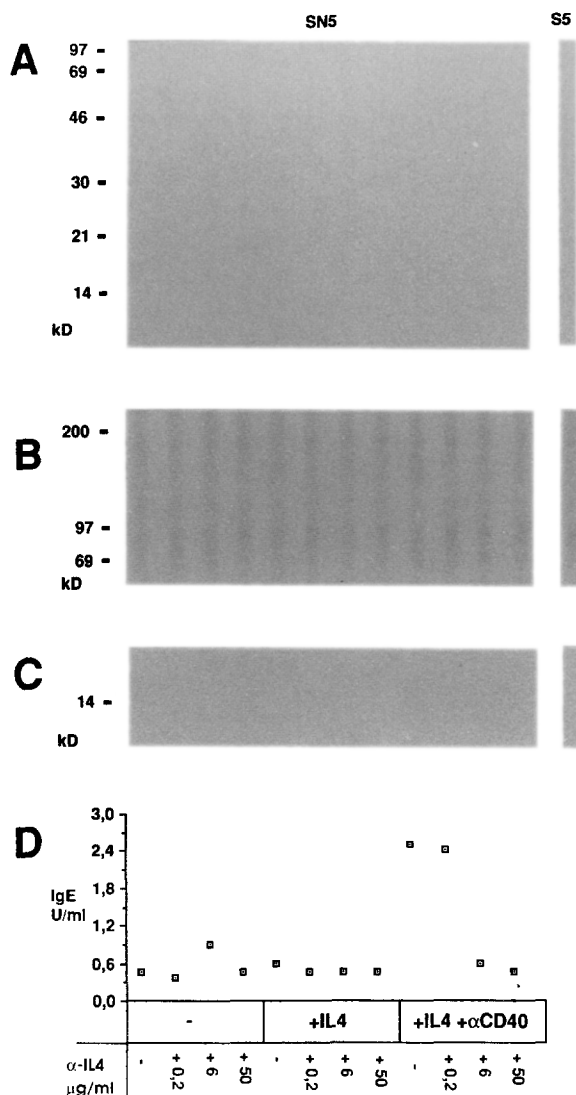


FIG. 5. Donor 5. PBMCs from subject without allergy. **A**, IgE reactivity of culture supernatants with nitrocellulose-blotted natural timothy grass pollen allergens. **B and C**, IgE reactivity of culture supernatants with recombinant allergens. **D**, Determination of total IgE. See legend to Fig. 1.

PCR amplification of IgE-Fd cDNA from PBMCs from patients with allergy

To provide evidence as to whether the spontaneously secreted IgE results from preformed IgE or from a de novo synthesis involving transcription, IgE-specific mRNA was reversely transcribed and PCR amplified from PBMCs derived from donors with allergy. A combination of oligonucleotide primers specific for human IgE and the variable regions (Fig. 8) was used to obtain the amino-terminal portion of the IgE heavy chain (IgE-Fd) cDNA from PBMCs. As a control, IgG-Fd cDNA was also amplified, and both PCR products were

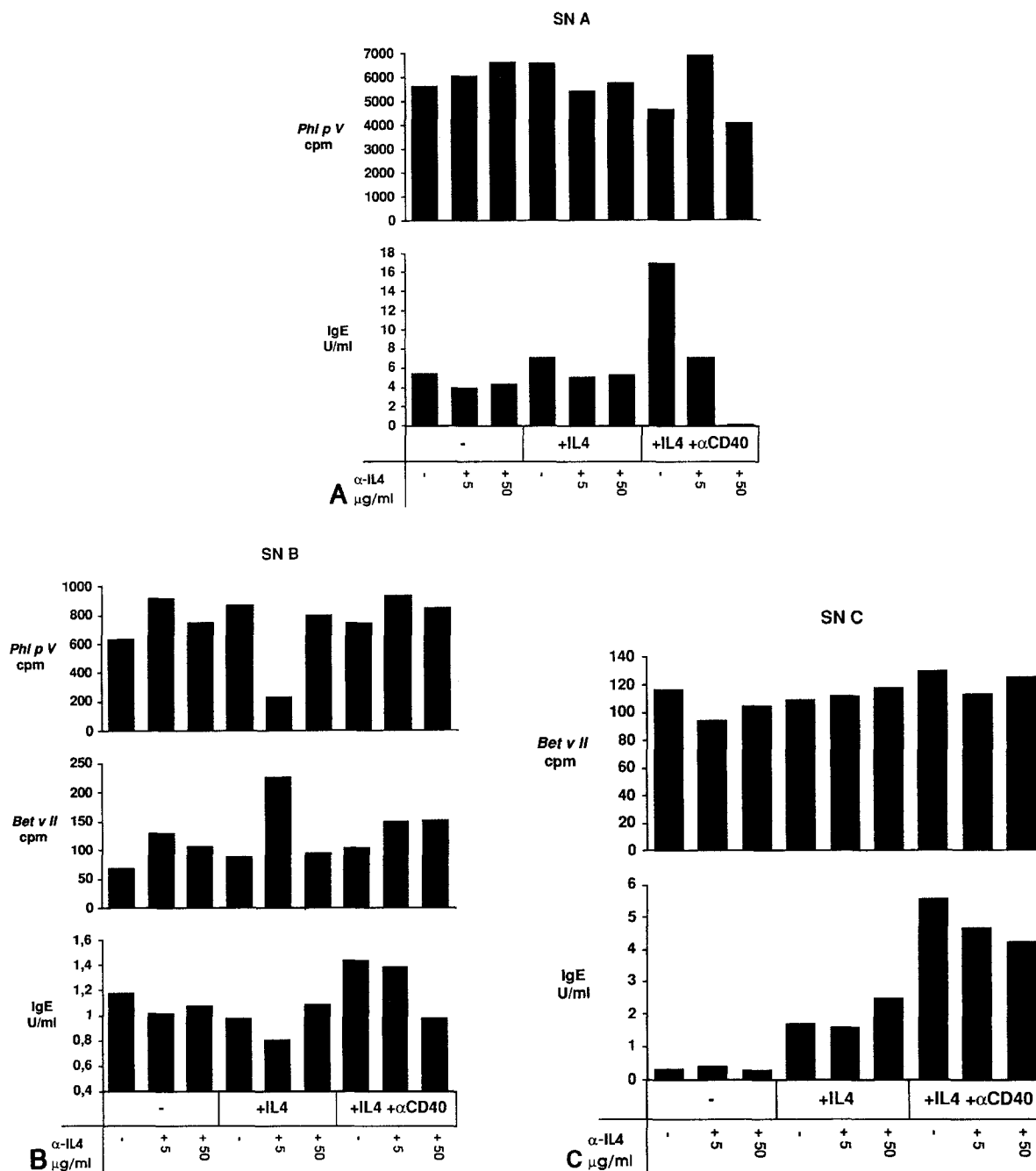


FIG. 6. Comparison of total and allergen-specific IgE synthesis in culture supernatants determined by radioimmunoassay. PBMCs from three patients (**A to C**) were cultured without and in presence of different concentrations of agonists. Total IgE in cultures was determined as in Figs. 1 to 5, whereas allergen-specific IgE synthesis was measured with nitrocellulose-dotted purified recombinant allergens (*rBet v II*, *rPhl p V*) followed by detection of bound IgE with ^{125} I-labeled anti-human IgE (Pharmacia). Bound allergen-specific IgE was measured in counts per minute. Counts per minute with human serum albumin (negative control) representing background levels were subtracted from values obtained with recombinant allergens.

hybridized with oligonucleotides specific for IgE and IgG. IgE-Fd cDNA could be amplified from unstimulated PBMCs of patients with allergy, which indicates that the specific IgE measured in

the culture supernatants may result from de novo transcribed IgE mRNA (Fig. 9).

Fig. 10 shows a comparison of the amounts of IgE-Fd cDNA product that was amplified from

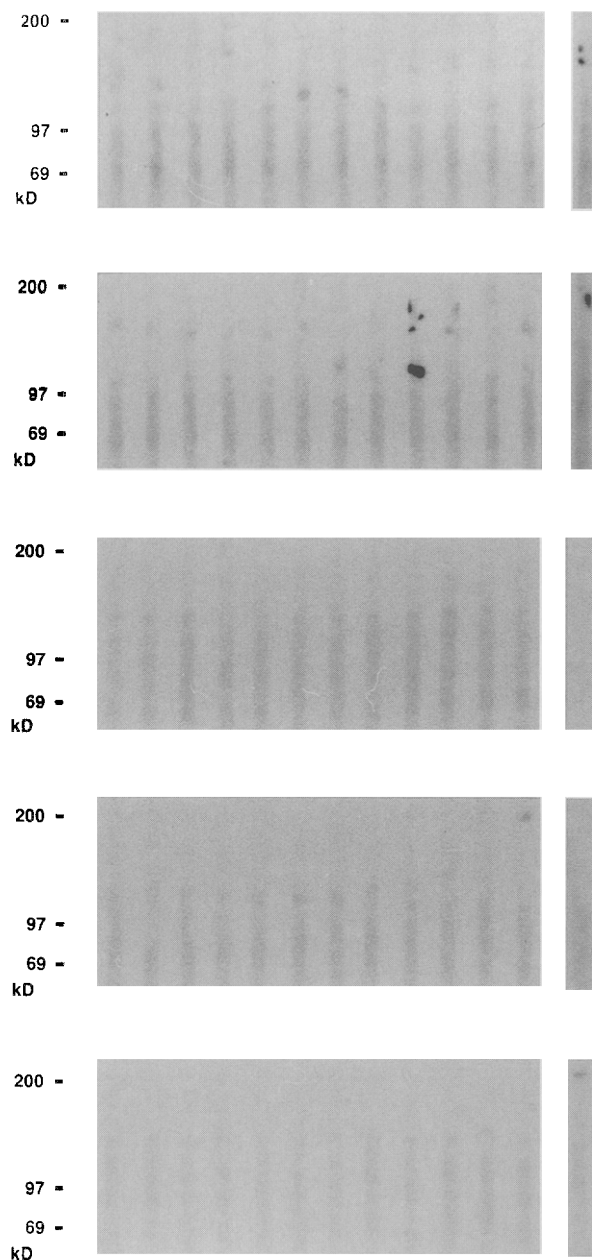


FIG. 7. Reactivity of supernatants from patients with allergy and from subjects without allergy with *E. coli*-phage proteins. The same supernatants from patients with allergy (donors 1 to 3) and from subjects without allergy (donors 4 and 5) and sera (right panel) as in Figs. 1 to 5 were probed with proteins from *E. coli* Y1089 infected with λ gt11 phage without insert. No specific IgE binding was observed with any sample.

fresh PBMCs from an allergic donor without stimulation and from the same number of PBMCs of the same donor after 8 days of culture without stimulation, with addition of IL-4, or with a combination of IL-4 and anti-CD40 antibody. Approximately 10 ng of IgE-Fd cDNA was obtained from

IgG heavy chain primer:

5' GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG 3'

IgE-heavy chain primer:

5' GCT ACT AGT TTT GTT GTC GAC CCA GTC 3'

Variable region primers:

5' CAG GTG CAG CTC GAG CAG TCT GGG 3'

5' GAG GTG CAG CTC GAG GAG TCT GGG 3'

Internal IgE-specific oligonucleotide:

5' GCC CAC GCA CCC GAG ACG GTC AGC AA 3'

Internal IgG-specific oligonucleotide:

5' TCC ACC AAG GGC CCA TCG 3'

FIG. 8. DNA sequences of oligonucleotides. Oligonucleotides that were used for amplification of IgE-Fd and IgG-Fd cDNA are heavy-chain primers and corresponding variable-chain primers. Internal oligonucleotides specific for IgE and IgG were used to identify PCR products by hybridization.

3×10^6 freshly isolated PBMCs and from cells that were kept in culture for 8 days with addition of IL-4. When the cells were cultured for 8 days without any stimulation, no IgE-Fd cDNA could be stained with ethidium bromide, whereas the addition of IL-4 and anti-CD40 increased the amount of IgE-Fd cDNA product approximately 20 times to 200 ng. The amounts of PCR-amplified IgE-Fd cDNA reflected the pattern obtained by the determination of total IgE in the culture supernatants.

DISCUSSION

It is well established that IL-4 and anti-CD40 antibodies are potent enhancers of human IgE synthesis.¹¹⁻¹³ It may hence be expected that antagonists of the IL-4 and anti-CD40 effects might be useful tools to reduce elevated IgE levels in patients with allergy. We were therefore interested to measure the effects of IL-4 and IL-4 antagonists on the production of allergen-specific IgE in patients with allergy. A considerable percentage of type I allergic disease is caused by sensitization of patients to pollen allergens. Among these allergens, proteins from grass pollen, tree pollen, and ragweed pollen are the most important elicitors of allergic symptoms. In the case of pollen allergy persons are exposed only at certain times of the

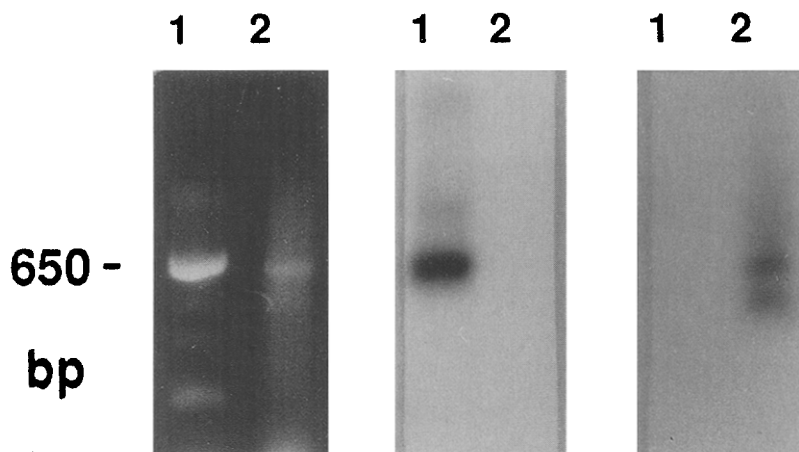


FIG. 9. Amplification of IgG and IgE-Fd-cDNA from peripheral blood leukocytes derived from a patient with grass pollen allergy. RNA from fresh PBMCs was prepared, reversely transcribed, and PCR amplified with primers specific for IgG (*lane 1*) or IgE (*lane 2*) and equimolar mixture of two ν region-specific primers. DNA was subjected to 1.5% agarose gel electrophoresis and ethidium bromide staining (*left panel*). Bands of approximately 650 base pairs were obtained, which, after transfer to nitrocellulose, could be hybridized with oligonucleotides specific for IgG (*middle panel*) and IgE (*right panel*), respectively.

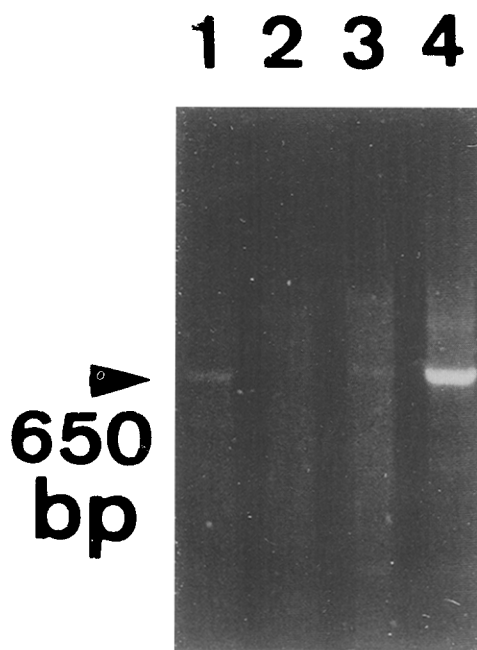


FIG. 10. Influence of IL-4 and anti-CD40 treatment on amount of IgE-Fd cDNA amplified from blood leukocytes of patients with allergy. IgE-Fd cDNA was reversely transcribed and PCR-amplified from 3×10^6 peripheral blood leukocytes from a donor with allergy. Cells were isolated freshly by Ficoll density-gradient centrifugation (*lane 1*) and were then kept in culture for 8 days without stimulation (*lane 2*) or were stimulated by IL-4 (*lane 3*) or by combination of IL-4 and anti-CD40 antibodies (*lane 4*) for 8 days. Arrowhead indicates position of IgE-Fd cDNA at approximately 650 base pairs.

year to the allergens, and levels of IgE against most pollen allergens decrease considerably outside the pollen season (unpublished data). Serum IgE specific for seasonal allergens, such as the major birch pollen allergen *Bet v I*,²⁸ or for *Phl p V*,²² a major timothy grass allergen, is reduced markedly outside the pollen season but is still sufficient to elicit positive skin prick test responses and histamine release from basophils at that time.^{22, 29} PBMCs from patients with pollen allergy were obtained 4 months and shortly after the pollen season and were cultured for 8 days with rhu IL-4, anti-CD40 antibodies, and a neutralizing anti-human IL-4 antibody.

The effects of IL-4 and IL-4 antagonists on the synthesis of allergen-specific IgE were measured with immunoblotted natural and recombinant pollen allergens, subsequent autoradiography and densitometric analysis (data not shown), and a RAST based on purified recombinant allergens. Total IgE synthesis was increased by addition of IL-4 and anti-CD40 antibody and could be inhibited in a dose-dependent way by the addition of a neutralizing anti-IL-4 antibody. IL-4 alone increased the total IgE secretion only moderately, and the effects were much more pronounced with PBMCs obtained during the pollen season (data not shown). The changes in the total IgE production could be found at both the protein and the mRNA level with reverse transcription and PCR with IgE-specific oligonucleotide primers. In

marked contrast to the total IgE synthesis, allergen-specific IgE was secreted spontaneously and was neither increased by addition of IL-4 and anti-CD40 nor reduced by treatment with a neutralizing anti-IL-4 antibody. Differences in the amount of specific IgE were due to the distribution of specific B cells in the culture wells rather than the stimuli (Figs. 1 to 6). Although IL-4 and stimulation via the CD40 structure are essentially involved in the onset of IgE production, the synthesis of allergen-specific IgE in the peripheral blood of patients with allergy was not affected by these substances. Because these data were obtained long after allergen exposure, it appears that either the peripheral blood of patients with allergy contains long-lived B cells that are committed to a continuous production of allergen-specific IgE, or already IgE-switched specific B-cell clones remain activated long after the allergen exposure. A prolonged presence of allergens in the patients appears to be extremely unlikely because most allergens are soluble proteins of a low molecular weight.³⁰

These in vitro experimental data provide an explanation for the clinical observation that, despite the rather short half-life of serum IgE, allergic symptoms can be elicited in persons with pollen allergy outside the pollen season. The results may also be considered to be of clinical interest because even low amounts of specific IgE are sufficient to trigger allergic effector mechanisms. It might be therefore expected that therapeutic approaches on the basis of cytokines or cytokine inhibitors applied outside the pollen season might not contribute to a sufficient reduction of specific IgE. It may therefore be important to develop therapeutic strategies that are not based only on a general downregulation of the IgE production. The B cells that are responsible for the continuous production of specific IgE were certainly primed initially by an IL-4-mediated signal, and it is possible that cytokines other than IL-4 such as IL-13³¹⁻³³ or cytokine inhibitors may influence the ongoing production of allergen-specific IgE. It is also possible that during the allergy season anti-IL-4 may help to control allergen-specific IgE levels more efficiently.

However, our observation may lead to the consideration of allergen-specific strategies as a therapy for type I allergy, particularly in view of the recent progress made in the field of allergen characterization by recombinant techniques.³⁴ Allergen-targeted lysis of specific B cells or the modulation of a specific IgE response in vivo, as demonstrated in a mouse model,³⁵ might be useful

therapeutic approaches in addition to a use of cytokines or cytokine antagonists.

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