

Effects of IL-4 and IL-13 on total and allergen specific IgE production by cultured PBMC from allergic patients determined with recombinant pollen allergens

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Summary

Background Interleukin (IL)-4 and IL-13 have been shown to be potent switch factors for IgE synthesis in human B cells.

Objective In this study we investigated the effects of recombinant human IL-4 and IL-13 on total and allergen specific IgE synthesis by peripheral blood mononuclear cells (PBMC) from pollen allergic patients and healthy control individuals.

Methods Peripheral blood mononuclear cells (PBMC) from allergic patients were investigated for their capacity to produce allergen specific IgE *in vitro*. Total protein extracts from birch pollen and timothy grass pollen as well as purified recombinant birch pollen allergens, *Bet v I*, birch profilin (*Bet v II*) and recombinant timothy grass pollen allergens, *Phl p I*, *Phl p II*, and *Phl p V* were used to measure specific IgE-antibody synthesis in cell culture supernatants by IgE-immunoblot and ELISA.

Results PBMC obtained from allergic patients spontaneously secreted allergen specific IgE in the culture supernatants. Addition of Interleukin 4, Interleukin 13 and anti-CD40 antibody to the cultures alone or in combinations significantly induced total IgE production whereas allergen specific IgE production was not affected.

Conclusion Our results indicate that the peripheral blood of allergic individuals contains long lived allergen specific B cells which have already switched to IgE production and which are not sensitive to IL-4 and IL-13 treatment. These results may have implications on attempts to use cytokines or cytokine antagonists in therapy of Type I allergy.

Keywords: Type I allergy, recombinant tree and grass pollen allergens, Interleukin 4, Interleukin 13, anti-CD40 antibody, *in vitro* IgE synthesis

Clinical and Experimental Allergy, Vol. 25, pp. 879–889. Submitted 4 July 1994; revised 16 October 1994; accepted 5 April 1995.

Introduction

It is well established that IL-4, a T cell-derived cytokine is capable of increasing IgE synthesis in B cells [1–4] which even more efficiently occurs in the presence of CD40 stimulation [5]. In addition to IL-4 another T cell-derived cytokine designated IL-13 [6], was recently described as a potent *in vitro* modulator of human

monocyte and B cell function. IL-13 shares biological functions with IL-4, such as upregulation of MHC class II and CD23 expression on monocytes and B cells [7,8], growth promotion of human B cells preactivated by anti-IgM antibodies or through ligation of surface CD40 [8] and induction of highly purified naive human sIgD⁺ B cells to switch to production of IgG4 and IgE [7,9], when costimulated by activated CD4⁺ T-helper clones or anti-CD40 MoAbs [10]. Despite the similar effects of IL-4 and IL-13 and the use of a common component in

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their receptor complexes [11,12] evidence was provided that IL-13 acts independently of IL-4 [7,9]. On the other hand, IL-13 which is two- to fivefold less potent than IL-4 in inducing IgE production, shows no additive or synergistic effects with IL-4 on the induction of IgG4 or IgE synthesis, suggesting that IL-4 and IL-13 use common signalling pathways [7,9].

Earlier studies have demonstrated that the peripheral blood of allergic patients contains long lived B cells capable of spontaneously secreting allergen-specific IgE [13–15]. Considering the potential application of IL-4 or IL-13 antagonists in therapy of Type I allergy it was of considerable interest to investigate whether the production of specific IgE by peripheral B cells can be influenced by these cytokines.

In this study we compared the effects of recombinant human IL-4 and IL-13 on total and allergen specific IgE synthesis by PBMC from pollen allergic patients and healthy control individuals. An assay system was developed which allowed the sensitive measurement of allergen specific *in vitro* IgE production using immunoblotted natural and recombinant tree and grass pollen allergens. It could be demonstrated that although both cytokines were able to significantly upregulate the total IgE production of cultured PBMC from allergic patients, no effects on the synthesis of allergen specific IgE could be observed. These results are interpreted to mean that peripheral blood of allergic patients contains long lived B cells which have already switched to specific IgE production and therefore cannot be influenced directly by cytokines.

Materials and methods

Allergic patients and non-allergic control individuals

Tree and grass pollen allergic individuals were selected by case history and positive skin-prick test. Sera were further characterized by RAST and IgE-immunoblotting with natural and recombinant pollen allergens as described [16,17]. For each patient a specific profile of IgE-reactivity was established using recombinant tree pollen allergens (rBet v I [18], recombinant birch profilin, Bet v II) [19,20] and recombinant timothy grass (*Phleum pratense*) pollen allergens (rPhl p I, rPhl p II and rPhl p V) [21–23]. Heparinized blood samples from four allergic and two non-allergic (negative case history, negative skin-prick tests, negative RAST) donors were obtained out of the pollen season after informed consent was obtained.

Cytokines and antibodies

Recombinant human IL-4 purified from CHO cells, and

anti-CD40 antibody [24] was kindly provided by Dr E. Liehl, Sandoz, Vienna, Austria. *E. coli* derived recombinant human IL-13 and anti-IL-13 antibody was provided by Dr R. de Waal Malefyt, DNAX, Palo Alto, CA, USA.

Natural and recombinant pollen allergens

Natural pollen proteins of birch pollen and timothy grass pollen were prepared by incubating 2 g of pollen for 1 h in 50 mL of distilled water at room temperature under continuous shaking. Extracts were then centrifuged at $20\,000 \times g$ at 4°C to remove insoluble particles. Supernatants containing the aqueous pollen protein extracts were then dialysed overnight against distilled water, lyophilized and stored at –20°C until use [25].

The cDNAs coding for the major birch pollen allergen Bet v I [18], birch profilin [19], major timothy grass pollen allergens Phl p I [21], Phl p II [22] and Phl p V [23] were expressed as non-fusion proteins in plasmid pMW175 using *E. coli* strain BL21 (Novagene, Madison, WI, USA). Cells were grown in Lurea Broth to an OD₆₀₀ = 1.0 and induced with 0.4 mM IPTG (Isopropyl-β-thiogalactoside) for 6 h. Cells were then harvested and pellets stored at –20°C until protein purification. Recombinant birch pollen profilin was purified by affinity to poly (L)-proline Sepharose [20]. Recombinant Bet v I was purified by chromatofocusing and subsequent HPLC runs as described [26] whereas recombinant Phl p I, Phl p II and Phl p V were purified by chromatography (M. Susani, unpublished data).

Culture conditions for *in vitro* IgE production

PBMC were isolated from heparinized blood samples by Ficoll-density gradient centrifugation. Cells were washed two times in phosphate buffered saline and resuspended in RPMI 1640 (Flow Laboratories, Meckenheim, Germany), containing 10% fetal calf serum (JRH Biosciences, Lenexa, USA), 50 µg/mL gentamycin (Sigma, St Louis, USA) and 150 µg/mL L-glutamine (Sigma, St Louis, USA). Cells were cultured in duplicates, at a density of 1.5×10^6 /mL in flat-bottomed, 24-well plates (Costar, Cambridge, MA, USA) in a final volume of 2 mL at 37°C in a fully humidified 5% CO₂ atmosphere. PBMC were stimulated with different concentrations of IL-4 (100U and 500U/mL); IL-13 (100 and 500U/mL) with or without anti-CD40 antibody (200 ng/mL) and anti-IL-13 (1.2 µg/mL). LPS was used at 100 µg/mL which represents a concentration that far exceeds any possible endotoxin contamination of the IL-13 preparation. After 8 days, culture supernatants were harvested by centrifugation, and stored at –20° until

measurement of total IgE levels by RIA and specific IgE by ELISA and immunoblot.

FACS analysis of PBMC

Routinely, 5×10^5 freshly prepared PBMC were pre-incubated 30 min on ice with 20% human AB-serum. Cells were stained with mouse anti-human CD19, CD3 and CD14-FITC conjugated antibodies (DAKO, Glostrup, Denmark) and mouse anti-Fc γ RIII FITC-conjugated antibody (Medarex, West Lebanon, NH, USA) in PBS/0.5% BSA (USB, Cleveland, Ohio, USA)/ 0.02% sodium azide (Merck, Darmstadt, Germany) for 30 min on ice.

Appropriate isotype-matched control antibodies were used. Cells were washed three times in PBS/0.02% sodium azide and analysed on a FACScan using Lysis II software (Becton Dickinson, San José, CA, USA). The average amount of CD19+ B cells in the sample was 9.3% (ranging from 7% to 12%).

Determination of total IgE and specific IgE antibodies in sera and culture supernatants

Total IgE levels in the culture supernatants were determined by radioimmunoassay (RIA) (IgE RIA Ultra 50, Pharmacia, Uppsala, Sweden), with a detection limit less than 0.04 kU/L. Results reflect the means of duplicate determinations. For a sensitive determination of allergen specific IgE antibodies a sandwich-ELISA system based on recombinant allergens was compared with an IgE-immunoblotting procedure using radiolabelled anti-human IgE antibodies.

IgE-immunoblots Nitrocellulose strips containing blotted natural and recombinant allergens were incubated with cell culture supernatants diluted 1:2 in buffer A (50 mM sodium phosphate pH 7.5, 0.5% Tween 20, 0.5% BSA, 0.05% NaN₃) or sera diluted 1:10 in buffer A. Bound IgE-antibodies were detected with ¹²⁵I labelled anti-human IgE (Pharmacia, Uppsala, Sweden) and visualized by autoradiography using X-OMAT S films and intensifying screens (Kodak, Heidelberg, Germany).

ELISA 96-well-plates (Nunc-Maxisorb, Roskilde, Denmark) were coated with 100 μ L of 2 μ g/mL purified recombinant *Bet v* I, recombinant *Phl p* I, *Phl p* II or *Phl p* V in 0.1 M sodium carbonate buffer, pH 9.6 overnight at 4°C. Pure culture supernatants and sera 1:10 diluted in PBS/0.05% Tween 20/0.5% BSA were incubated overnight at 4°C. Bound IgE-antibodies were detected with a mouse monoclonal anti-human IgE antibody (Pharmin-gen, San Diego, CA, USA) and a peroxidase-coupled

sheep anti-mouse Ig (Amersham, Buckinghamshire, UK). Both IgE-detection systems allowed the measurement of specific serum IgE. Specific IgE in the culture supernatants was assayed by immunoblotting and subsequent densitometrical analysis (Hirschmann, Unterhaching, Germany) of the autoradiographs. The ELISA system was not sensitive enough to discriminate specific signals from background levels in the culture supernatants.

Results

Interleukin-4, interleukin-13 and anti-CD40 antibody induce total IgE synthesis by cultured PBMC from normal and allergic individuals

Total IgE was determined in supernatants from PBMC of allergic patients (Fig. 1a) and non-allergic individuals (Fig. 1b) to investigate the effects of different stimuli on total IgE production. Figure 1 shows that total IgE synthesis could be most effectively induced by addition of a combination of anti-CD40 antibody plus IL-4 or anti-CD40 antibody plus IL-4 and IL-13. In two patients (patient 1 and patient 4) the total IgE production was significantly increased by stimulation with anti-CD40 antibody alone as previously described [27]. Addition of IL-4 increased IgE synthesis in all experiments. The addition of IL-13 alone also led to significant increases in total IgE synthesis but was less effective than IL-4. All results are displayed in kU/L IgE and represent means of duplicate determinations. The differences between the single determinations were always less than 10%.

In vitro cultured PBMC from allergic individuals spontaneously secrete specific IgE antibodies

In earlier studies [13–15] it has been demonstrated that *in vitro* cultured PBMC from allergic patients are capable of spontaneously secreting specific IgE. Culture supernatants from PBMC of four representative pollen allergic individuals were tested for IgE-reactivity with natural and recombinant allergens. The patterns of specific IgE-reactivity of culture supernatants from non-stimulated PBMC (Fig. 2, lane 1) corresponded with the IgE-reactivity pattern observed with the diluted sera (Fig. 2, lane S). The intensity of IgE-reactivity found in the supernatants was more than 10 magnitudes lower than in the sera and in some supernatants specificities for certain allergens could not be detected.

Allergen specific IgE synthesis by cultured PBMC is not affected by addition of IL-4 and IL-13.

To compare the effects of IL-4, IL-13 and/or anti-CD40

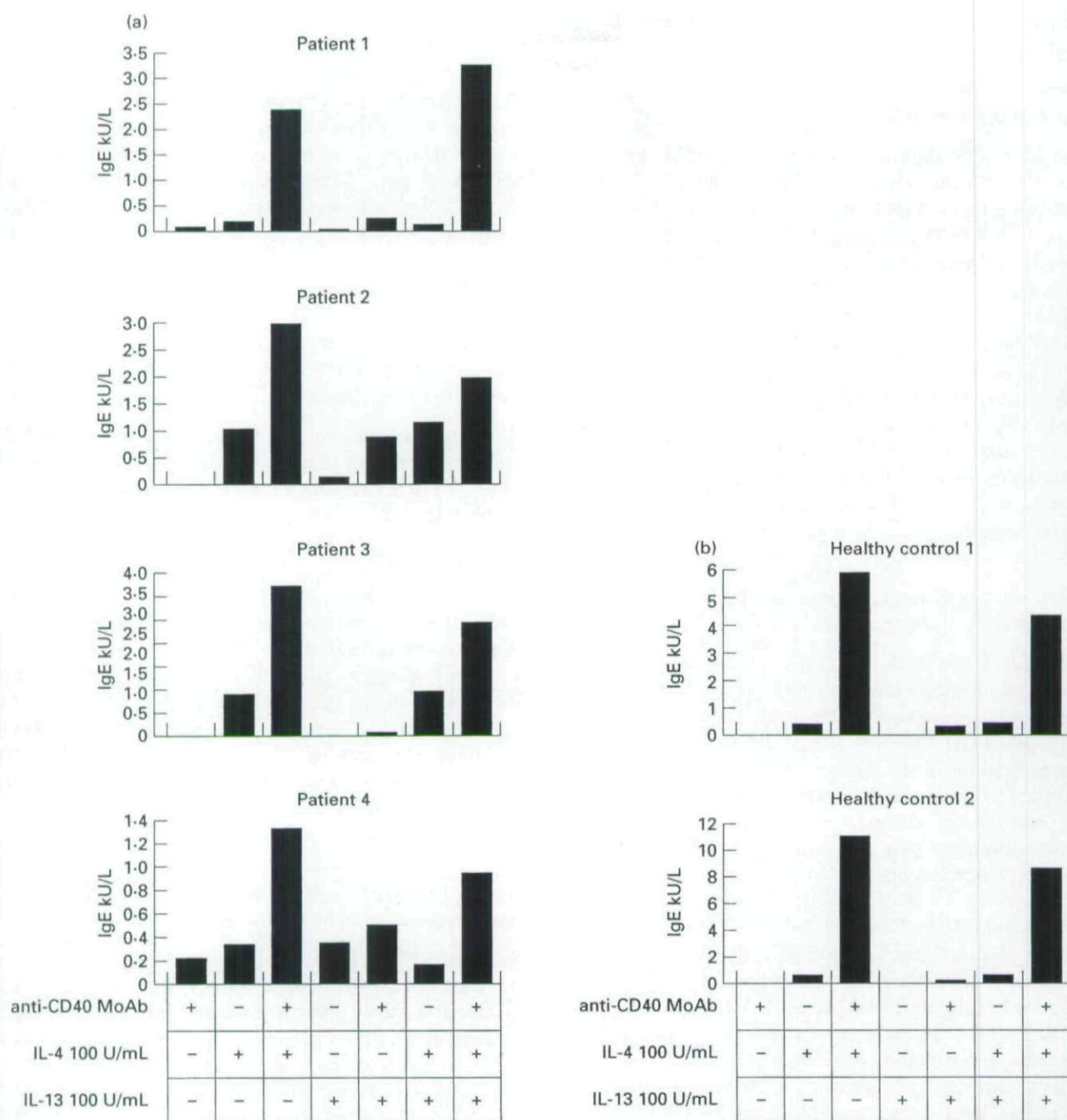


Fig. 1. Influence of IL-4, IL-13 and anti-CD40 antibody on the synthesis of total IgE by unfractionated PBMC from allergic patients (a) and non-allergic individuals (b). IgE levels in the culture supernatants were measured after a culture period of 8 days by RIA. IL-4 and IL-13 were added as indicated; anti-CD40 antibody was used at 200 ng/mL. The results represent means of duplicate determinations from two independent cultures and the single determinations did not differ more than 10%.

antibody on total and specific IgE production, PBMC from allergic individuals were cultured and supernatants analysed in parallel for total IgE-content (Fig. 1) and amount of allergen specific IgE (Fig. 2). The densitometrical analysis of the allergen specific IgE production is displayed for some selected patients and allergens.

Compared with total IgE production which depended on the addition of the different agonists (Fig. 1) the amounts of specific IgE did not correlate with cytokine treatment and/or addition of anti-CD40 antibody. Densitometrical analysis of the autoradiographs documenting the allergen specific IgE-production showed that

(a)

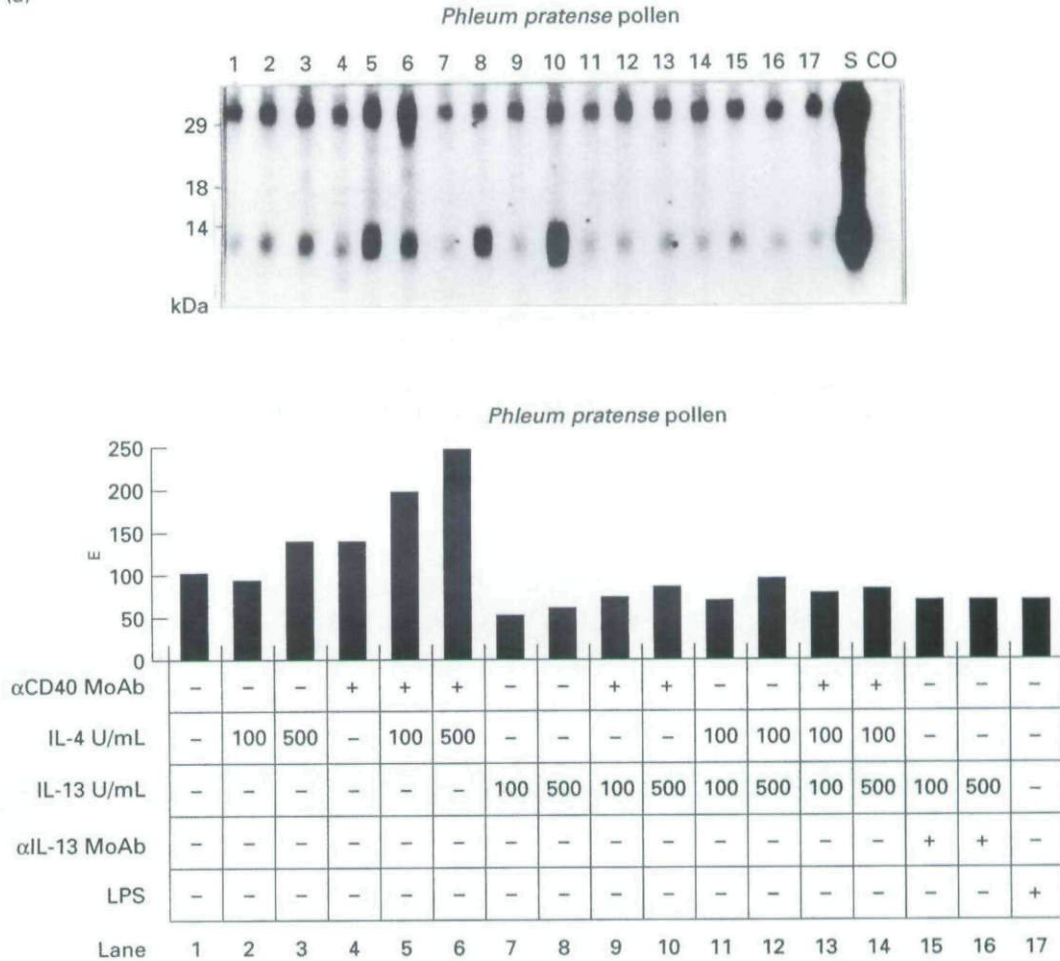


Fig. 2. Influence of IL-4, IL-13 and anti-CD40 antibody on the synthesis of allergen specific IgE by PBMC from allergic patients (patient 7: Fig. 2a; patient 2: Fig. 2b; patient 3: Fig. 2c; patient 4: Fig. 2d) and a non-allergic individual (control 1: Fig. 2e). Supernatants from cultured PBMC were diluted 1:2 in phosphate buffer and tested for IgE-reactivity with nitrocellulose blotted natural allergens (timothy grass pollen, birch pollen) and recombinant allergens (recombinant *Phl p* I, *Phl p* II, *Phl p* V, *Bet v* I). Culture supernatants tested in the individual lanes were obtained from PBMC cultivated under different conditions: lane 1, medium; lane 2, IL-4 100U/mL; lane 3, IL-4 500U/mL; lane 4, anti-CD40 MoAb at 200 ng/mL; lane 5, IL-4 100U/mL + anti-CD40 MoAb; lane 6, IL-4 500U/mL + anti-CD40 MoAb; lane 7, IL-13 100U/mL; lane 8, IL-13 500U/mL; lane 9, IL-13 100U/mL + anti-CD40 MoAb; lane 10, IL-13 500U/mL + anti-CD40 MoAb; lane 11, IL-4 100U/mL + IL-13 100U/mL; lane 12, IL-4 100U/mL + IL-13 500U/mL; lane 13, IL-4 100U/mL + IL-13 100U/mL + anti-CD40 MoAb; lane 14, IL-4 100U/mL + IL-13 500U/mL + anti-CD40 MoAb; lane 15, IL-13 100U/mL + anti-IL-13 (1.2 μ g/mL); lane 16, IL-13 500U/mL + anti-IL-13 (1.2 μ g/mL); lane 17, LPS (100 μ g/mL). In lane S the positive control using patient's serum diluted 1:10 in phosphate buffer is shown and lane CO represents the buffer control without addition of serum. For patients 1, 2, 3 and 4 representative densitometrical analyses of the specific IgE reactivity against selected natural and recombinant allergens are displayed.

specific IgE levels were in many cases even higher without addition of agonists, e.g. in patients 2 and 3 a significant increase in total IgE synthesis was observed with IL-4 (Fig. 1a) whereas the *Phl p* V-specific reactivity of patient 3 was higher without any stimulation (Fig. 2c, lane 1, 2) and patient 2 produced more *Phl p* II specific IgE (Fig. 2b, lane 1, 2). IL-13 significantly increased the total IgE production of patient 4 (Fig. 1a) whereas the allergen specific IgE production was not

affected (Fig. 2d, lane 7). The observed differences in allergen specific IgE production reflected different clonal distributions in the culture wells rather than the effects of stimulation; this is corroborated by the observation that in some wells IgE with different specificities could be detected, e.g. Fig. 2b; patient 2, lane 7, *Phleum pratense* pollen; Fig. 2d; patient 4, lanes 1, 2 and 10, *Phleum pratense* pollen.

Culture supernatants and sera from non-allergic

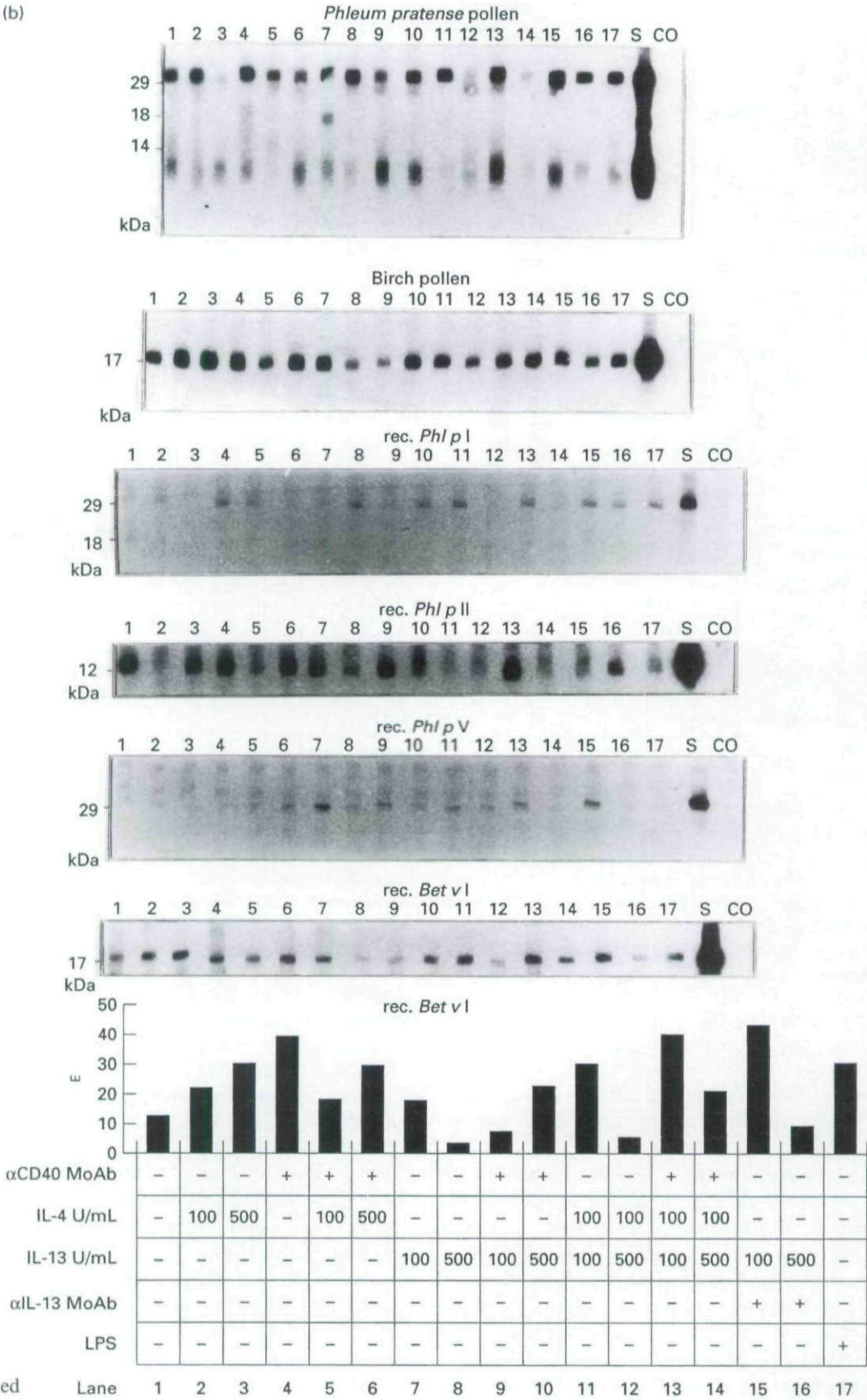


Fig. 2 continued

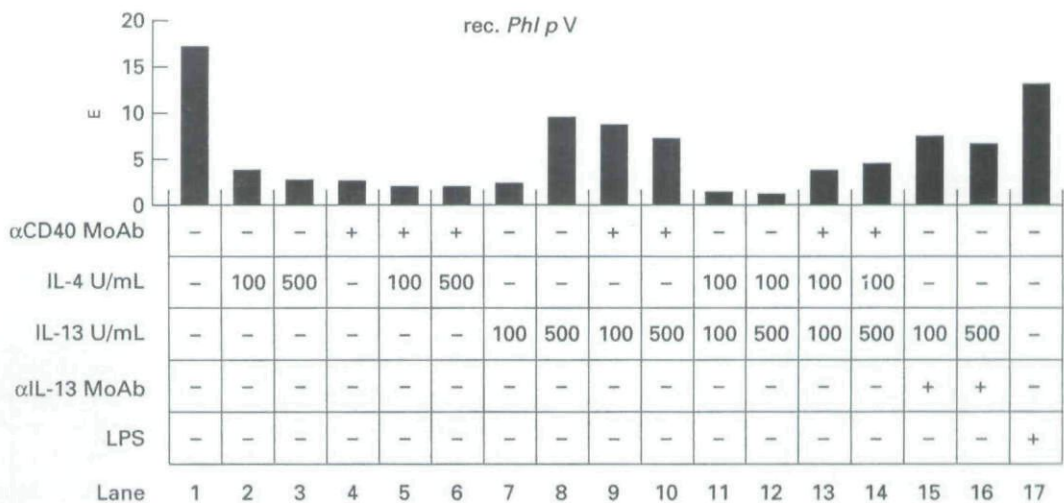
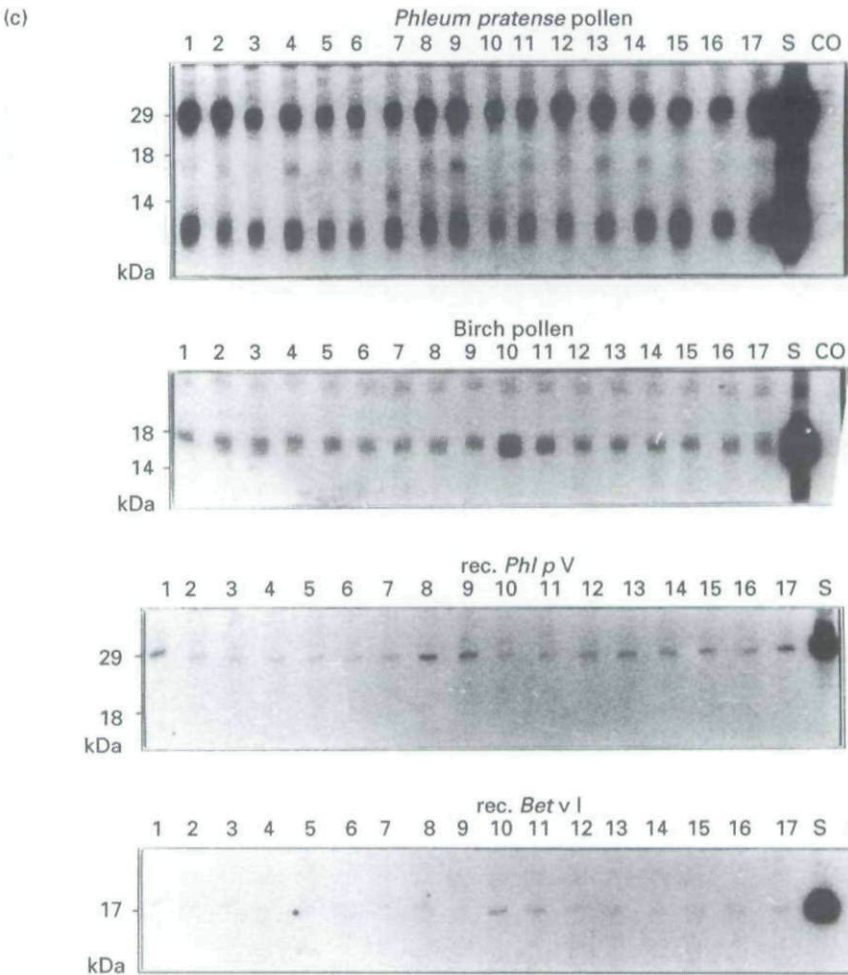


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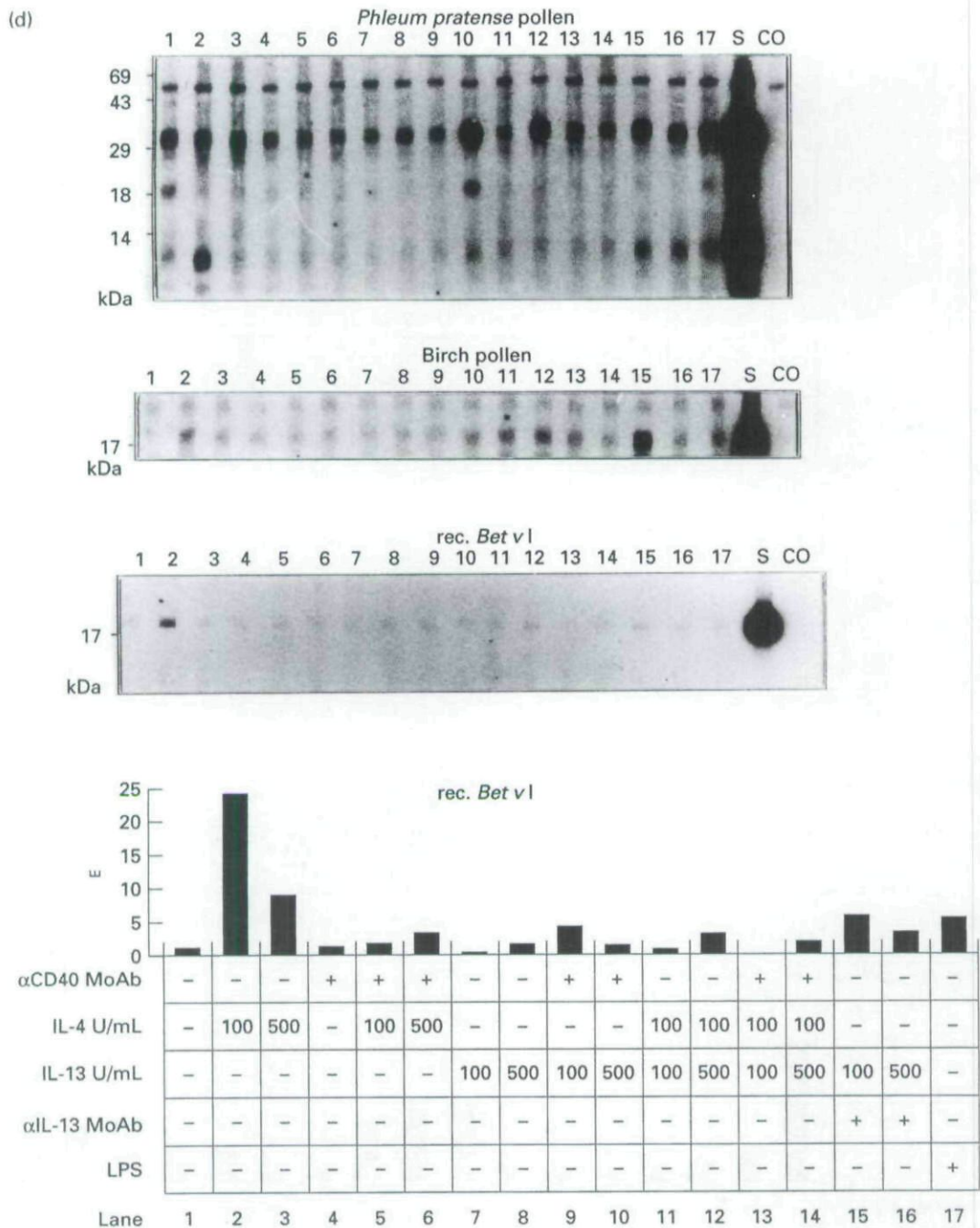


Fig. 2 continued

control individuals such as control 1 (Fig. 2e) showed no IgE-reactivity with natural or recombinant pollen allergens.

Discussion

In addition to IL-4, IL-13 has been described as a cytokine capable of inducing human IgE synthesis by switching the isotype of secreted antibody from μ to ϵ .

Both cytokines and their receptors therefore represent potential targets for strategies intervening with an up-regulated IgE response in allergic and atopic diseases [28]. IL-4 and IL-13 based strategies for therapy of Type I allergic diseases might involve anti-cytokine antibodies or ligands competing with the respective cytokine receptors. It can be assumed that such therapeutical concepts might focus on the inhibition of switching of IgM

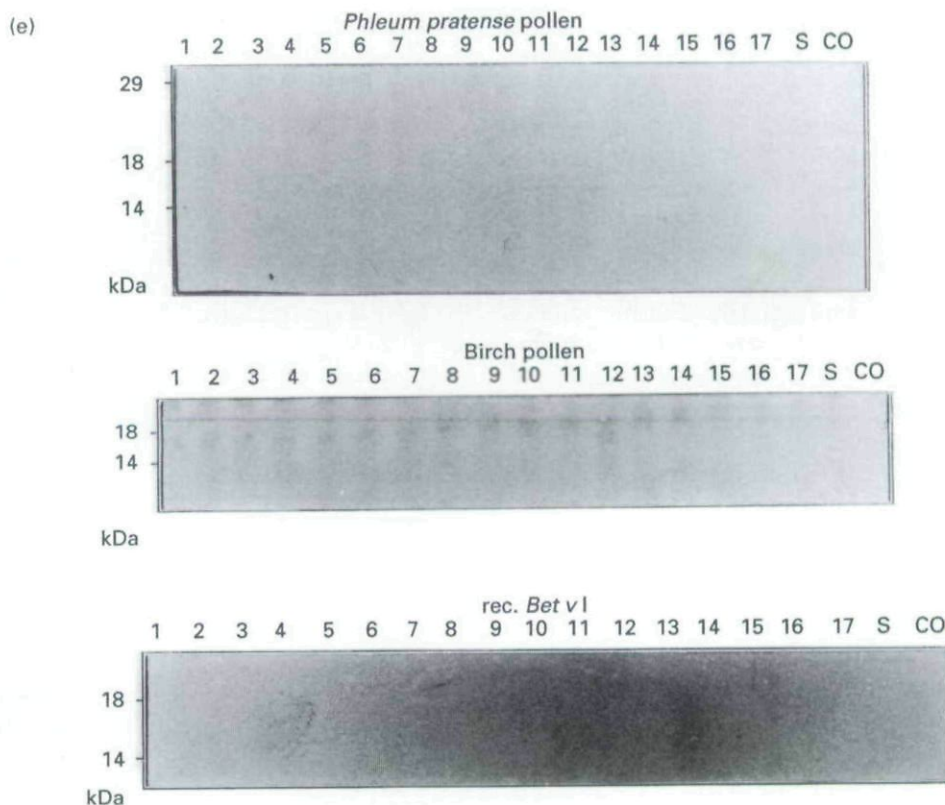


Fig. 2 continued

secreting B cells to IgE synthesis. However, several authors have described the occurrence of long-lived B cells in the peripheral blood of allergic patients which are capable of spontaneously producing IgE [13–15].

In the present study, the influence of IL-4 and IL-13 with and without addition of anti-CD40 antibodies on the total and allergen specific IgE synthesis of *in vitro* cultured PBMC from allergic patients was investigated. For a sensitive monitoring of allergen specific IgE production in the culture supernatants an immunoblot detection system based on natural and recombinant pollen allergens was used. Compared with an ELISA system using recombinant allergens coated to ELISA plates and monoclonal anti-IgE antibodies detected by a peroxidase conjugated sheep anti-mouse antiserum the method of probing nitrocellulose blotted recombinant allergens with culture supernatants and detecting bound IgE with radiolabelled anti-human IgE antibodies proved to be more sensitive and also allowed to obtain quantification of data by subsequent densitometrical analysis of the autoradiographs. When compared with total IgE levels which were substantially increased in 8 day culture supernatants after treatment with IL-4 and IL-13 the synthesis of allergen specific IgE could not be enhanced. The production of allergen specific IgE was

found to occur spontaneously and differences in the amount of specific IgE rather reflected different clonal distributions of B cells in the individual wells. Since IL-4 and IL-13 switch IgM precursor B cells to IgE production the observed increase of total IgE production is most likely to be due to activation of IgM B cells with other specificities. Allergen specific B cells circulating in the peripheral blood which apparently have already switched to IgE production, seemed to be insensitive to cytokine treatment. This was demonstrated in our study for IL-4 and IL-13. Other cytokines which are known as potent downregulators of IgE production such as interferon γ and interferon α showed also no significant downregulation of the allergen specific IgE synthesis (data not shown). In a previous study we have shown that a neutralizing anti-IL-4 antibody does not influence allergen specific IgE synthesis [29].

Neither IL-4 nor IL-13 alone or in combinations with anti-CD40 antibodies increased allergen specific IgE production when PBMC were obtained out of the pollen season. Since as yet recombinant allergens were not available, the effects of cytokines on the allergen specific IgE production *in vitro* could not be investigated which might explain the lack of data concerning the effects of IL-4 and IL-13 on the allergen specific IgE

production. Although neither IL-4, IL-13, nor combinations with anti-CD40 antibody were able to increase allergen specific IgE-production *in vitro* it is possible that other substances, cellular contact or allergens might be able to influence *in vivo* specific IgE-production and IgE-memory.

In conclusion, our results corroborate earlier observations that the peripheral blood of allergic patients contains long-lived B cells which are capable of producing specific IgE antibodies spontaneously. Further studies might investigate the effects of IL-4 and/or IL-13 antagonists on allergen specific IgE synthesis to evaluate the potential usefulness of these cytokine antagonists as therapeutic tools to decrease levels of specific serum IgE in patients.

Acknowledgements

This study was supported by grants S06708 and S06703 of the 'Fonds zur Förderung der wissenschaftlichen Forschung Österreichs'.

We wish to thank Trevor Lukas for critically reading the manuscript.

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