

Short communication

In vitro kinetics of allergen- and microbe-induced IL-4 and IFN- γ mRNA expression in PBMC of pollen-allergic patients

Background: According to a hypothesis allergens induce Th2 responses in allergic patients, and microbes induce Th1 responses. We studied the kinetics of *in vitro* allergen-, tuberculin (PPD)- and tetanus toxin (TT)-induced IFN- γ and IL-4 mRNA expression in peripheral blood mononuclear cell (PBMC) cultures of pollen-allergic patients and healthy controls.

Methods: PBMC of 10 birch or timothy pollen-allergic patients and of 13 healthy controls were stimulated *in vitro* with allergen (birch or timothy), PPD or TT. Pellets and supernatants were collected at 24, 48, 72 and 96 h after stimulation. IFN- γ and IL-4 production was measured by enzyme linked immunosorbent assay and mRNA expression using RT-PCR and time-resolved fluorometry.

Results: Allergen induced IFN- γ production and mRNA expression in PBMC more in allergic patients than in healthy controls. Also allergen induced IL-4 mRNA expression more in allergic patients than in healthy controls. PPD induced IFN- γ mRNA expression both in allergic patients and healthy controls, whereas IFN- γ production was induced only in healthy controls and IL-4 was not induced at all. TT induced IFN- γ mRNA expression in both groups, IFN- γ production in allergic patients, and IL-4 mRNA expression in both allergic patients and healthy controls.

Conclusions: *In vitro* stimulation with allergen induced both IFN- γ and IL-4 mRNA expression of PBMC in allergic patients. These observations challenge the clearcut division of microbe-specific Th1 and allergen-specific Th2 responses in peripheral blood.

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Originally based on observations made in mice, T helper (Th) cells are subdivided into two major types on the basis of their cytokine production *in vitro*: Th1 and Th2 cells. Th1 cells produce IFN- γ and IL-2 and are involved primarily in cell-mediated immune responses against microbes. Th2 cells produce IL-4, IL-5, and IL-13 and are involved primarily in humoral immune responses (1–3). Because IL-4 promotes isotype switch to production of IgE in B cells and IL-5 promotes eosinophil growth and differentiation, Th2 cells play an important part in the pathogenesis of IgE-mediated allergic diseases (4–6). In fact, Th2 type cytokines have been demonstrated in human tissue biopsies of lesional atopic skin (7, 8), nasal mucosa in allergic rhinitis (9) and in asthmatic bronchi (10, 11). In peripheral blood mononuclear cells (PBMC) the role of Th1 and Th2 cells in IgE-mediated allergic diseases and in infections in man is more complex (12–17).

In the present study we made an attempt to mimic the response occurring *in vivo* after natural exposure of allergens or microbes in a small amount of *in vitro* stimulated PBMC. The aim of the study was to analyse the kinetics of IL-4 and IFN- γ mRNA expression and production after timothy (*Phleum pratense*) or birch (*Betula verrucosa*) pollen, tuberculin (PPD), or tetanus toxin (TT) stimulation in PBMC of patients having IgE-mediated allergy to timothy or birch pollen, and of healthy controls.

Material and methods

Subjects

Ten patients (aged 23–42; six males and four females) with skin prick test verified IgE-mediated pollen allergy (18) and a history of allergic symptoms were included in the study. Five of the patients

were allergic to timothy (*Phleum pratense*) and five to birch (*Betula verrucosa*). Thirteen subjects (aged 22–42; seven males and six females) with no history of allergic symptoms were studied as controls. All the subjects had been vaccinated against *M. tuberculosis* and *Clostridium tetani*.

Cell culture

PBMC were obtained by Ficoll–Isopaque centrifugation of heparinized peripheral blood from studied subjects. Cells were washed twice in HBS containing NaHCO_3 and suspended in RPMI 1640 medium (Gibco, Paisley, UK) containing 10% autologous serum, 2 mM L-glutamin (Fluka, Buchs, Switzerland) and 100 $\mu\text{g}/\text{ml}$ gentamycin (Biological Industries, Haemek, Israel) in the concentration of 1×10^6 cells/ml. PBMC were added to 96-well round-bottomed microtitre plate wells (Costar, USA) at a density of 100 000 cells per well and incubated at $+37^\circ\text{C}$ in a 5% CO_2 -incubator for 3 days. Duplicate wells were set up for each stimulus. After 3 days, timothy or birch pollen (50 $\mu\text{g}/\text{ml}$) (ALK, Horsholm, Denmark), tuberculin (PPD, 10 $\mu\text{g}/\text{ml}$) (Statens Seruminstitut, Copenhagen, Denmark) or tetanus toxin (TT, 1 $\mu\text{g}/\text{ml}$) were added to the plates. Unstimulated wells were used as controls. Cells from two adjacent wells were combined and collected in UltraspecTM-solution (Biotecx Laboratories Inc., Texas, USA) at 24, 48, 72 and 96 h after stimulation and stored at -70°C until mRNA extraction.

RNA extraction, RT-PCR and solution hybridization

RNA extraction, RT-PCR and solution hybridization were performed as earlier described (19). Briefly, cell pellets were collected, lysed and frozen at -70°C in 500 μl of Ultraspec solution for RNA extraction. RNA was isolated according to Ultraspec instructions with the exception that prior to isopropanol precipitation, 2 μl of glycogen was added into the reaction. Extracted RNA was suspended to 20 μl of diethylpyrocarbonate (DEPC)-treated water and stored at -70°C . The RT reaction was performed with a First Strand cDNA Synthesis Kit (Pharmacia, Sweden) using oligo (dT) primers, and cDNA was stored at -70°C until used for simultaneous amplification of IL-4, IFN- γ and β -actin. PCR was performed in 50 μl total volume overlaid with mineral oil, using respective biotinylated primers in the concentrations 3.5 pmol (0.07 μM) for IFN- γ and IL-4 and 1.5 pmol (0.03 μM) for β -actin and 1 unit of thermostable DNA-polymerase (Dynazyme, Finnzymes, Helsinki, Finland). PCR protocol included a pre-synthesis step (98°C ; 10 s, 95°C ; 2 min), the amplifying programme (95°C ; 1 min, 50°C ; 1 min, 72°C ; 1.25 min) with 31 cycles and the final extension step (95°C ; 1 min, 50°C ; 1 min, 72°C ; 7 min). PCR products were captured onto streptavidin-coated plates (Wallac) by adding 10 μl of each biotinylated PCR product to duplicate wells and incubated for 30 min. After denaturation with NaOH and washes, remaining single-strands were hybridized with 2.5 ng/well of IFN- γ and IL-4 and with 7.5 ng/well of β -actin probes labelled with lanthanide chelates of Europium, Terbium and Samarium, respectively, for 4 hours at 45°C . Post-hybridization wash was performed at 42°C . Finally, lanthanide labels were detached from the probes by incubation in enhancement solution and measured by time-resolved fluorometer. All the results were expressed as ratio to β -actin.

ELISA (enzyme linked immunosorbent assay)

Production of IL-4 and IFN- γ protein released by PBMC was measured by ELISA in 96-well, flat-bottomed microtitre plates (Nunc-Immuno Plate Maxisorp, Roskilde, Denmark) using

commercially available antibody pairs and standard kits for ELISA (Pharmingen, San Diego, CA, USA).

Statistics

The Wilcoxon signed-rank test was used for nonparametric paired comparisons, and the Mann–Whitney *U*-test for unpaired comparisons.

Results

In the preliminary experiments, PBMC from pollen-allergic patients were stimulated with different concentrations of allergens (0, 25, 50 and 100 $\mu\text{g}/\text{ml}$) to find out the optimal concentration. Allergen concentration of 50 $\mu\text{g}/\text{ml}$ induced both IFN- γ and IL-4 expression in a similar manner and was chosen for further experiments. There was a peak of unstimulated IFN- γ mRNA expression in cultures on days 2 and 3, which was not seen in IL-4 (Fig. 1). Therefore, the cells were allowed to rest for 3 days after PBMC isolation prior to antigen stimulation, and collected at 24, 48, 72 and 96 h after stimulation.

Unstimulated and antigen-induced IFN- γ and IL-4 production and mRNA expression measured by ELISA and RT-PCR in PBMC from 10 birch or timothy pollen

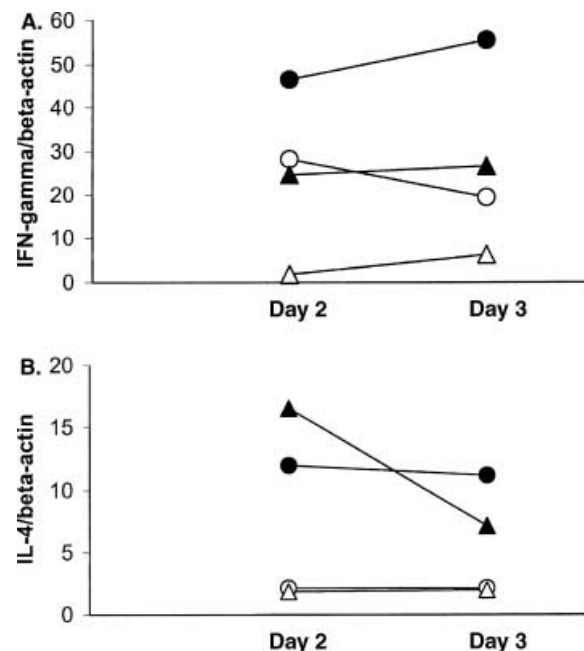


Figure 1. Effect of 3 days rest after PBMC isolation on (A) IFN- γ and (B) IL-4 mRNA expression in a timothy pollen-allergic patient. Cells were collected on day 2 or 3 after allergen stimulation. Triangles represent values with 3 days rest and dots without. Open triangles and dots are values for unstimulated cells and filled triangles and dots for allergen-stimulated (50 $\mu\text{g}/\text{ml}$) cells.

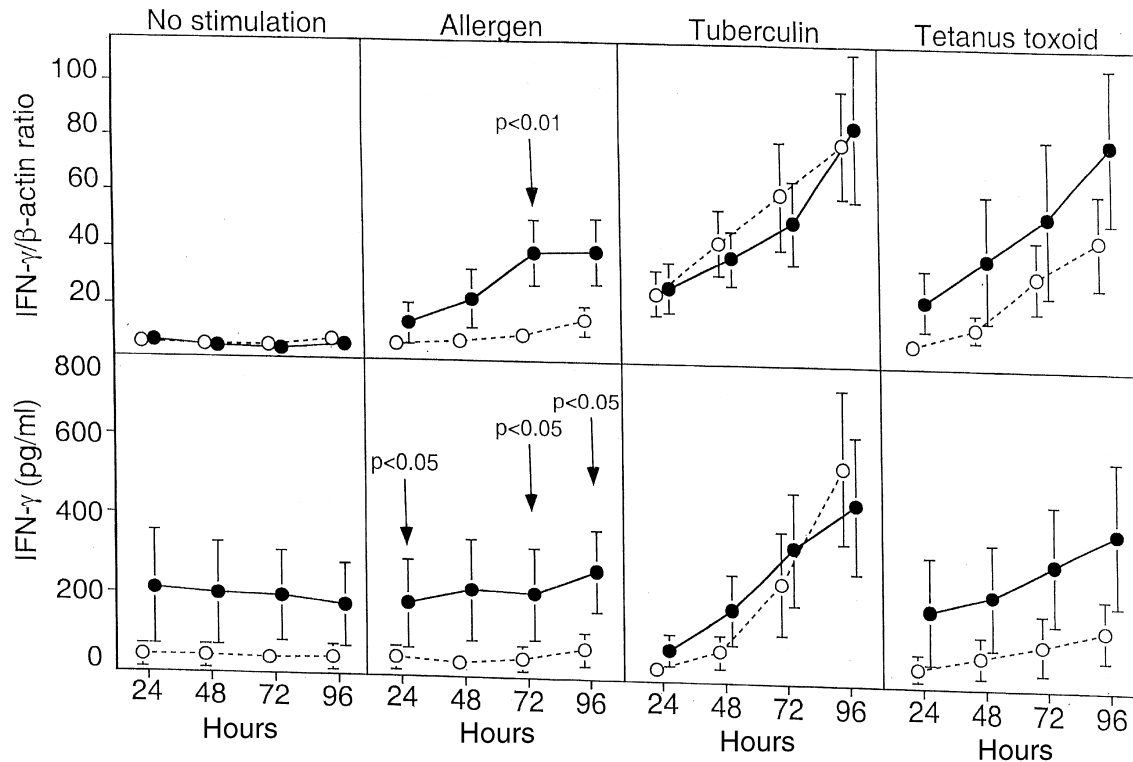


Figure 2. Kinetics of allergen-, PPD-, and TT-induced IFN- γ mRNA expression (IFN- γ / β -actin) and cytokine production (pg/ml) in allergic patients and in healthy controls measured at 24, 48, 72 and 96 h after stimulation. Filled dots represent the values for allergic patients and open dots for healthy controls. *P*-values refer to comparison between patients and controls (Wilcoxon signed rank test).

IgE-mediated allergic patients and 13 healthy subjects are presented in Figs 2 and 3 (mean + SEM).

The peaks of IFN- γ production and mRNA expression after allergen-, PPD- and TT-stimulation were at 96 h both in allergic patients and in healthy controls. Allergen stimulation induced significantly higher IFN- γ production ($P < 0.05$) and mRNA expression ($P < 0.01$) in allergic patients than in healthy controls. IFN- γ production and mRNA expression were elevated in allergic patients (268 pg/ml, $P < 0.05$, 39.4 IFN- γ / β -actin ratio, $P < 0.01$) and in healthy controls (72 pg/ml, $P < 0.05$, 14.6 IFN- γ / β -actin ratio, $P < 0.02$). PPD stimulation induced elevated IFN- γ mRNA expression both in allergic patients and in healthy controls (85.1 and 78.8 IFN- γ / β -actin ratio, $P < 0.01$). PPD increased IFN- γ production significantly in healthy controls (249 pg/ml, $P < 0.02$). TT stimulation induced elevated IFN- γ mRNA expression both in allergic patients (80.1 IFN- γ / β -actin ratio) and in healthy controls (46.2 IFN- γ / β -actin ratio), but IFN- γ production only in allergic patients.

Allergen induced elevated IL-4 mRNA expression at 48 and 72 h in allergic patients (7.4 and 12.9 IL-4/ β -actin ratio, $P < 0.05$), but in healthy controls only at 72 h (11.8 IL-4/ β -actin ratio, $P < 0.05$). PPD stimulation induced no IL-4 mRNA expression and diminished IL-4 production at every time point in both study groups ($P < 0.05$). TT caused increased IL-4 mRNA expression

with a peak at 96 h both in allergic patients (38.1 IL-4/ β -actin ratio) and in healthy controls (26.8 IL-4/ β -actin ratio). In general, ELISA had poor sensitivity to detect stimulation-induced IL-4 production.

Discussion

The aim of the present study was to study the kinetics of allergen-, PPD- and TT-induced IFN- γ and IL-4 mRNA expression and production in a small number of peripheral mononuclear cells using RT-PCR and ELISA in allergic patients and in healthy controls. The differences between allergic patients and healthy subjects were assessed.

There was a peak in IL-4 mRNA expression on day 3 and in IFN- γ mRNA expression on day 4 after stimulation. These data are in accordance with those of McHugh et al. (20). They found that cytokines are sequentially produced in the order IL-2, IL-4, IL-5, IL-3, IFN- γ , IL-10, IL-6, IL-12 and TNF- α , when cytokine production was measured in PHA-stimulated cultures by sensitive bioassays or ELISA.

According to our results, PBMC from both allergic and healthy subjects express IFN- γ mRNA after allergen stimulation. This response was also seen at the protein level. Allergen stimulation increased IL-4 mRNA expression both in allergic patients and, to some extent, in

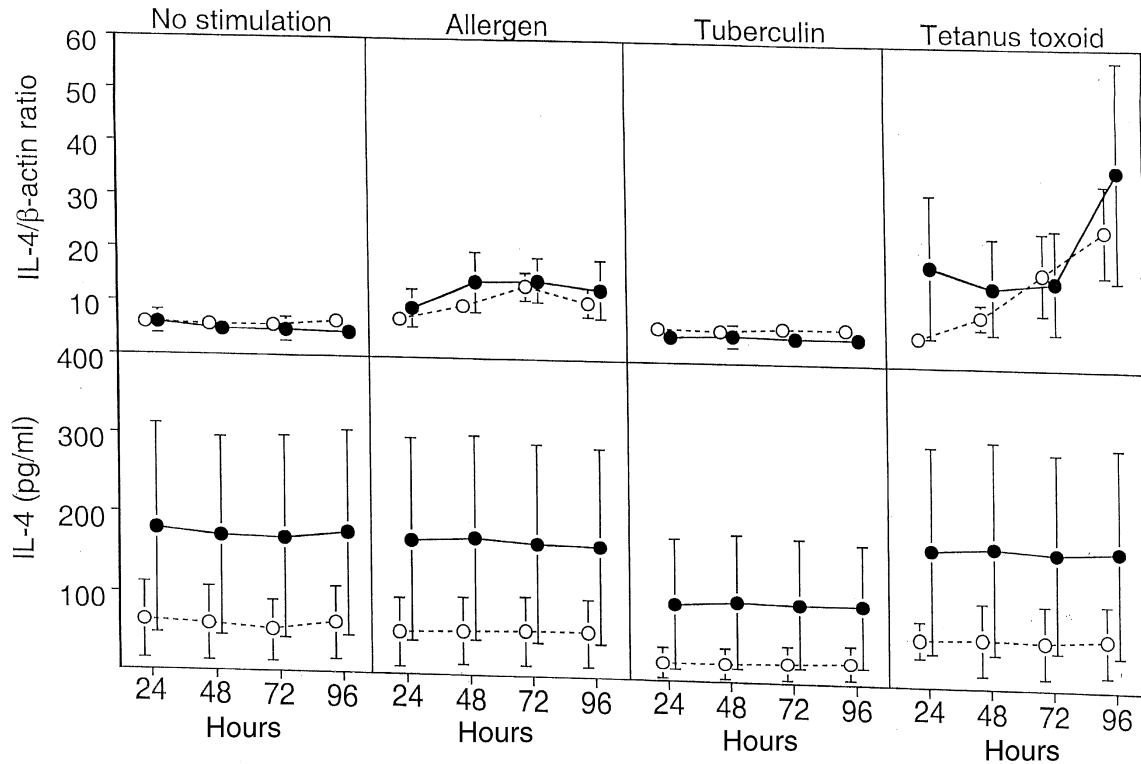


Figure 3. Kinetics of allergen-, PPD-, and TT-induced IL-4 mRNA expression (IL-4/ β -actin) and cytokine production (pg/ml) in allergic patients and in healthy controls measured at 24, 48, 72 and 96 h after stimulation. Filled dots represent the values for allergic subjects and open dots for non-allergic controls.

healthy subjects. When allergen-induced cytokine production from PBMC has been studied, the results have varied. Increased Th2-type responses have been reported in allergic subjects when compared with healthy controls (12–14). Both Th2 and Th1 cytokine production have been found (15), and allergen-specific IFN- γ production has been detected especially in CD8+ cells of allergic patients (16). A possible explanation for elevated IFN- γ levels in allergic patients is that with a chronic exposure to environmental allergens, the nature of Th-cell reactions becomes more complex. In the later stages of the response to allergen challenge, a significant Th1 component might be involved (7, 8, 17).

PPD did not induce IL-4 mRNA expression, but induced large amounts of IFN- γ (both mRNA and protein) in both allergic and in healthy subjects in a similar manner. TT induced both IL-4 and IFN- γ expression in both groups. These differences probably reflect different types of immunity that the antigens induce. *M. tuberculosis* preferentially induces a cell-mediated response as previously reported in a study with newborns. A memory Th1-type response to *Bacillus Calmette-Guérin* (BCG) vaccination was developed, although it is thought that neonatal immune responses are biased towards Th2-type cells (21). The results are also in agreement with a study in which IL-13, IL-5 and IFN- γ production was measured by ELISA in PBMC

obtained from allergic and healthy subjects in response to stimulation with allergens and PPD. Allergen stimulation resulted in elevated production of IL-13 and IL-5 in T cells from subjects with allergic rhinitis compared with healthy controls. When PBMC was stimulated with PPD, secretions of IL-13, IL-5 and IFN- γ were equal in both allergic patients and healthy controls with a relatively low ratio of IL-13 or IL-5 to IFN- γ production (22). Immunity to TT is more like an antibody-dependent response. Responses of infants to the tetanus component of the diphtheria, tetanus, a cellular pertussis (DtaP) vaccine were early and persistently of the Th2 type, whereas a more delayed and transient pattern was seen for IFN- γ production. IL-5, IL-10, IL-13 and IFN- γ were measured by ELISA and IL-9 and IL-4 by RT-PCR (23). In adults, both Th1 and Th2 cytokines have been implicated in vaccine-induced protection as a response to tetanus toxoid. PPD-stimulated PBMC cytokine production followed a Th1-like pattern. Both IL-4 and IFN- γ production were demonstrated as measured by ELISA in TT-stimulated cultures (24).

We observed an apparently spontaneous IFN- γ mRNA production on days 2 and 3 after PBMC isolation, but not anymore on days 5 and 6. To bypass this effect, the cells were allowed to rest for 3 days before allergen stimulation. In contrast, there was no difference in spontaneous IL-4 mRNA production on different

days. The possible explanation for our finding could be that when PBMC are isolated from peripheral blood, cells may be activated by the process to produce IFN- γ .

In conclusion, *in vitro* allergen stimulation induced both increased IFN- γ and IL-4 mRNA expression in allergic patients. In healthy controls, IFN- γ and IL-4 mRNA expressions increased less. *In vitro* PPD stimulation

induced IFN- γ but no IL-4 mRNA expression in both allergic and non-allergic subjects, whereas TT stimulation induced IFN- γ and IL-4 mRNA expression in both groups. Our results imply that the allergen-induced Th1/Th2 balance is different between allergic and healthy subjects and that the allergen-specific responses in peripheral blood are complex and can not be simplified as Th2 responses.

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