Allergy 1998: 53: 420–425
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ALLERGY
ISSN 0105-4538

Short communication

Effect of seasonal exposure to pollen on nonspecific interleukin-4, interleukin-5, and interferon-gamma *in vitro* release by peripheral blood mononuclear cells from subjects with pollinosis

Muñoz-Bellido FJ, Monteseirín FJ, Escribano MM, Delgado J, Velázquez E, Conde J. Effect of seasonal exposure to pollen on nonspecific interleukin-4, interleukin-5, and interferon-gamma *in vitro* release by peripheral blood mononuclear cells from subjects with pollinosis.
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The immune response to environmental allergens depends on both genetic and environmental factors. Allergen exposure triggers the activation of allergen-specific Th2 cells in allergic patients, as well as increased Th2-type cytokine mRNA expression and eosinophil recruitment. Nevertheless, different patterns of release of cytokines could explain the heterogeneity of atopic response. In our study, 25 patients with pollinosis and 15 healthy donors were selected to characterize their release of Th2 (interleukin [IL]-4 and IL-5) and Th1 (interferon-gamma [IFN-γ]) cytokines, both during and outside the pollen season. Peripheral blood mononuclear cells from patients and controls were isolated, cultured in the presence of phorbol-12-myristate-13-acetate plus ionomycine, and phytohemagglutinin (PHA), and cytokine release was assessed by titration in the supernatants. Both IL-4 and IL-5 showed higher levels during than outside the pollen season in pollinic patients ($P<0.05$) after nonspecific stimuli, whereas IFN-γ levels were significantly lower during than outside the pollen season only after culture with PHA. Significant differences were not observed in the control group. Our results are consistent with the hypothesis that release of cytokines by peripheral blood mononuclear cells from patients with pollinosis depends on environmental exposure to sensitizing pollens, and that influence can be revealed by *in vitro* nonspecific stimulation. Nevertheless, the heterogeneity in results suggests that the use of mitogens to assess Th1/Th2 dominance may need careful evaluation.

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Key words: allergy; cytokines; environmental exposure; interferon-gamma; interleukin-4; interleukin-5; ionomycine; phorbol ester; phytohemagglutinin; pollinosis.

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Accepted for publication 25 November 1997

Induction of IgE synthesis is based on two signals, one delivered mainly by the cytokine interleukin-4 (IL-4) (1) and the other by contact-dependent T- and B-cell interaction (2). *In vitro* studies examining the responses of allergic patients have been previously carried out (3, 4). Recently, several aspects of control mechanisms of IgE production have been identified and others are under study (5–10). It is clear that much needs to be learned about cytokine networks, pathways of signal transduction, and whether or not cytokines are differentially activated in a given context.

Our study aimed to determine whether natural environmental exposure to sensitizing pollens influences the *in vitro* secretion of Th2-type (IL-4 and IL-5) and Th1-type (interferon-gamma [IFN-γ]) cytokines in sensitized patients, even after culture with nonspecific stimuli.

Material and methods

Subjects

Twenty-five atopic patients diagnosed with allergic seasonal rhinoconjunctivitis and asthma, and multisensitized to common pollens in our area, were selected from among the outpatients of our service. The selected subjects showed negative skin tests with other inhalant allergens (mites, molds, or danders), did not present signs of infection, and did not receive immunotherapy or systemic corticosteroids during the study. Fifteen healthy, age-matched volunteers, without either personal or family history of atopy, served as a control group.

Cell isolation and culture

Blood extractions (heparinized blood in 10-ml aliquots) were performed at two different times, both in patients and in controls: during the pollen season (t_1), and at least 6 weeks after pollen counts fell below reactive levels (t_2). Peripheral blood mononuclear cells (PBMC) were isolated by conventional Ficoll-Paque (Pharmacia) sedimentation, washed three times with cold phosphate-buffered saline (Sigma), resuspended in culture medium, and seeded at 10^6/ml in flat-bottomed plates (Nunclon). The culture medium was RPMI 1640 (Gibco), supplemented with 10% heat-inactivated fetal calf serum, 1 mM L-glutamine, 1% HEPES buffer, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

Stimulation with mitogens

PBMC were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycine by addition of stock solutions (in dimethyl sulfoxide) to the same wells to final concentrations of 0.1 µM and 1 µM, respectively. PBMC were also stimulated with phytohemagglutinin (PHA) by addition of stock solution to other, different wells to a final concentration of 1 µg/ml. The plates were incubated at 37°C in an incubator with 5% CO_2 in air. Culture supernatants were harvested after 24 and 48 h, and stored at –20°C until used. Nontreated PBMC were included in each experiment as negative control.

IL-4, IL-5, and IFN-γ assays

Supernatants were assayed for IL-4, IL-5, and IFN-γ by the enzyme-amplified sensitivity immunoassay (EASIA) (Medgenix Diagnostics, Belgium), according to the manufacturer's instructions. The minimum detectable concentration is estimated to be 2.0 pg/ml for IL-4, 1.5 pg/ml for IL-5, and 0.03 IU/ml for IFN-γ.

Statistical analysis

All data were expressed as arithmetic mean ± standard error of the mean (SEM). Comparison between t_1 and t_2 values, and between pollinic subjects and healthy donors was done with the Wilcoxon signed rank test for paired observations, and the Mann–Whitney test, respectively. A difference of $P<0.05$ was considered to be statistically significant.

Results

IL-4 production (Fig. 1)

IL-4 secretion reached its maximum level in supernatants harvested after 24 h. The mean PMA-plus-ionomycine-induced IL-4 production by PBMC from patients with pollinosis (t_1=158.48 pg/ml ± 39.30; t_2=81.12 pg/ml ± 11.54; P=0.0043) and the mean concentration detected when PBMC were stimulated with PHA (t_1=36.40 pg/ml ± 6.47; t_2=20.0 pg/ml ± 4.74; P=0.0403) were significantly higher during than outside the pollen season. In contrast, IL-4 secretion by nonstimulated PBMC from atopic patients did not prove to be statistically higher in t_1 (1.44 pg/ml ± 1.02) than in t_2 (1.24 pg/ml ± 0.74; $P>0.05$). On the other hand, IL-4 released by PBMC from the control group, after *in vitro* stimulation with PMA-plus-ionomycine (t_1=115.60 pg/ml ± 11.59; t_2=98.80 pg/ml ± 13.74; $P>0.05$), and with PHA (t_1=6.87 pg/ml ± 3.38; t_2=1.47 pg/ml ± 1.47; $P>0.05$), and in nonstimulated PBMC ($t_1<2$ pg/ml; $t_2<2$ pg/ml) was not statistically higher during than outside the pollen season.

IL-5 production (Fig. 2)

Maximum differences were detected after 48-h culture. The mean PMA-plus-ionomycine-induced IL-5 secretion by PBMC from patients with pollinosis (t_1=103.32 pg/ml ± 22.01; t_2=65.52 pg/ml ± 19.22) was statistically higher during than outside the pollen season (P=0.0057). When stimulated with PHA, the mean induced IL-5 release by PBMC from patients was statistically higher at t_1 (65.76 pg/ml ± 12.34) than t_2 (45.60 pg/ml ± 10.29; P=0.0288). The difference between the mean IL-5 concentrations detected in the supernatants of nonstimulated PBMC (t_1=1.08 pg/ml ± 0.46; t_2=0.32 pg/ml ± 0.25) did not appear to be statistically significant ($P>0.05$). In the control group, the mean IL-5 production by both nonstimulated and mitogen-stimulated PBMC was not statistically higher during than outside the pollen season ($P>0.05$).

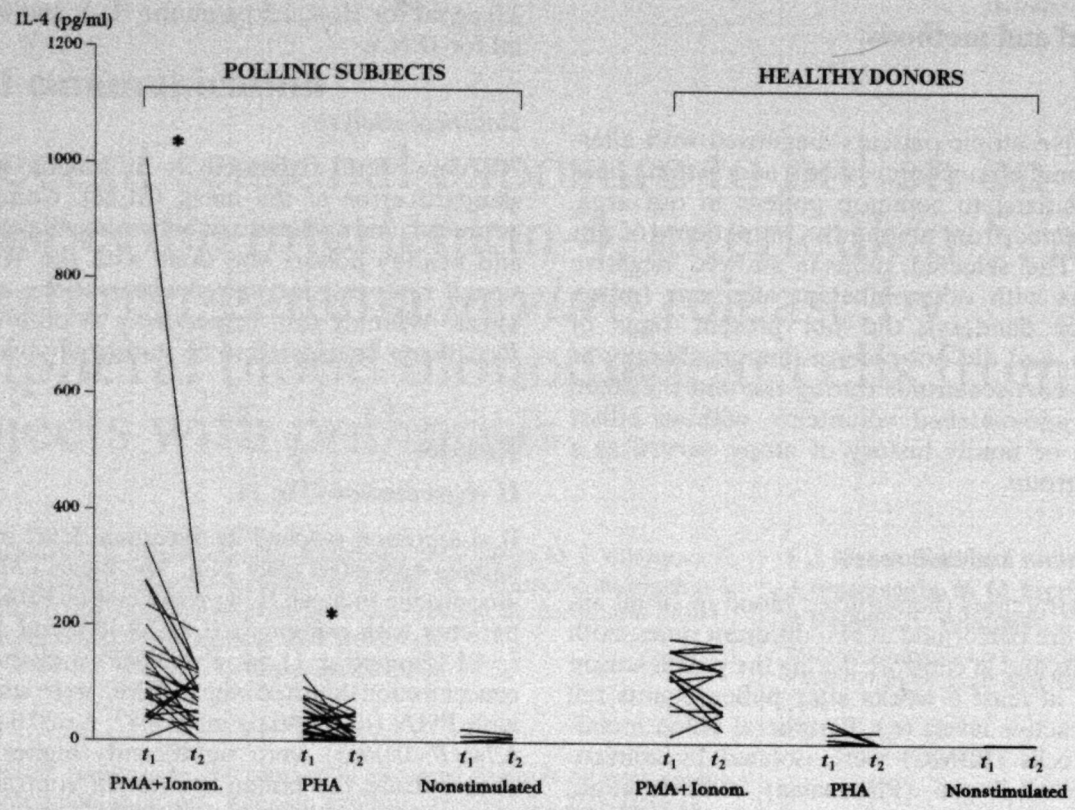


Fig. 1. IL-4 production (pg/ml) by PBMC in response to nonspecific stimuli, during (t_1) and outside (t_2) pollen season, in both pollinic subjects and control group. *Significant difference.

IFN-γ production (Fig. 3)

The mean PHA-induced IFN-γ production by PBMC from atopic patients at t_1 (36.85 IU/ml ± 10.93) was significantly lower than at t_2 (65.05 IU/ml ± 14.41; P=0.0138). Nevertheless, when PBMC were not stimulated (t_1=3.33 IU/ml ± 1.36; t_2=6.28 IU/ml ± 2.18; P>0.05), and when they were stimulated with PMA plus ionomycine (t_1=970.06 IU/ml ± 203.41; t_2=975.18 IU/ml ± 237.92; P>0.05), IFN-γ production did not show a significant difference. In cultures with PBMC from the control group, the mean IFN-γ production by both nonstimulated and mitogen-stimulated PBMC was not statistically different during and outside the pollen season (P>0.05).

Comparison between pollinic subjects and healthy donors

IL-4 and IL-5 production by PBMC from atopic patients was significantly higher than that from healthy donors when PBMC were stimulated with PHA at t_1 and t_2, or when they were stimulated with PMA plus ionomycine at t_1. Significant differences were not found when PBMC were stimulated with PMA plus ionomycine at t_2, and compared with nonstimulated PBMC from those groups. IFN-γ production by PBMC was significantly higher in the pollinic group than the control group when nonstimulated and when stimulated with PHA, at both t_1 and t_2. Statistically significant differences were not found when we compared IFN-γ production by PBMC when stimulated with PMA plus ionomycine.

Discussion

In recent years, much information from studies carried out *in vivo* and *in vitro* supports the idea that Th2-type cytokines are important not only in IgE-antibody synthesis, but also in the induction and maintenance of allergic inflammation (11, 12). Moreover, various authors (13–18) have studied the heterogeneity of the response of pollinic subjects, and have confirmed the existence of the priming effect caused by pollens appearing at an earlier season, suggesting that the IgE response of patients allergic to only one pollen species differs from that of patients allergic to many pollen species. The cause may lie in different patterns of release of cytokines that play a major role in regulating IgE synthesis (15–19).

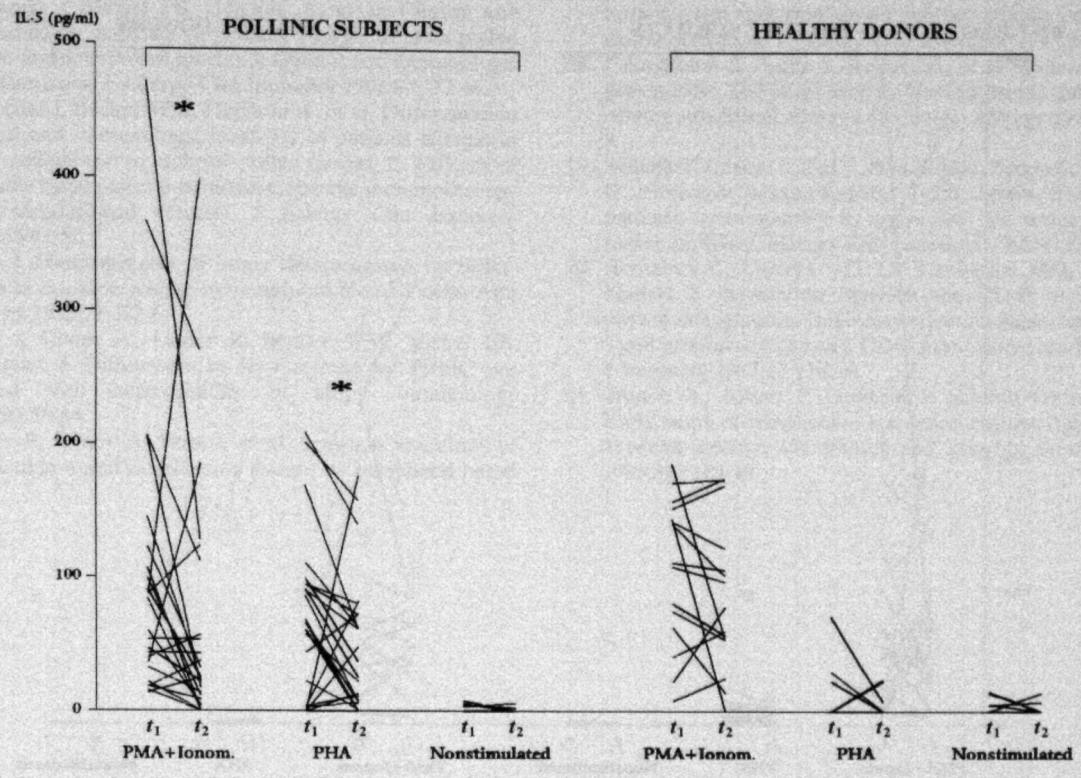


Fig. 2. IL-5 production (pg/ml) by PBMC in response to nonspecific stimuli, during (t_1) and outside (t_2) pollen season, in both from pollinic subjects and control group. *Significant difference.

In our study, we attempted to investigate changes in the production of Th2-type cytokines during and outside the pollen season, and whether such changes can be shown by stimulation with nonspecific mitogens. Our data are consistent with the hypothesis of the priming effect. In our study, PBMC from patients with pollinosis released higher amounts of IL-4 and IL-5 during than outside the pollen season. Such increased secretion of Th2-type cytokines was detected after stimulation with nonspecific mitogens. Our data lead us to suggest that significant contact with sensitizing pollens enables the immune cells of pollinic patients to provide the necessary signals for developing the allergic response; but a second sensitizing pollen seems not to be necessary to reveal the increased ability of patients with pollinosis to develop an IgE-mediated response. Regarding IFN-γ *in vitro* release by PBMC from atopic subjects, other studies reveal that IFN-γ production is not affected by the pollen season (17), and the amount of IFN-γ produced by T-cell clones is comparable after induction by PMA plus anti-CD3 and after induction by specific antigen (19). We did not find seasonal differences in IFN-γ production by PBMC from pollinic patients either when nonstimulated or when cultured in the presence of PMA plus ionomycine. Nevertheless, seasonal differences did appear when PBMC were stimulated with PHA, showing lower levels of IFN-γ *in vitro* release during than outside the pollen season. Our data lead us to suggest that environmental exposure to sensitizing allergens changes the immune response in sensitized patients into a proallergenic response, even in response to nonspecific stimuli, as shown by the enhanced release of Th2-type cytokines *in vitro*.

It has been suggested that stimulation of cells, by agents such as mitogens, is not sufficient to provide those signals (10). However, correlation between the heterogeneity of atopy and the deregulation of the cytokines by PBMC *in vitro* has been demonstrated by stimulation with polyclonal activators (such as PHA or PMA) (13, 16). Activation of T cells by PHA results from its interaction with membrane glycoproteins, such as the CD45 glycoprotein family (20). PMA induces calcium-dependent activation of protein-kinase C, and its combination with calcium ionophores bypasses the requirement for antigen- or lectin-induced signal at the onset of lymphocyte activation (21). Our findings suggest that the decrease in IFN-γ *in vitro* release may reflect a defect in T-cell transmembrane signaling. But such a conclusion may be flawed: the decreased IFN-γ production may simply reflect a lower number of Th1 cells after allergen

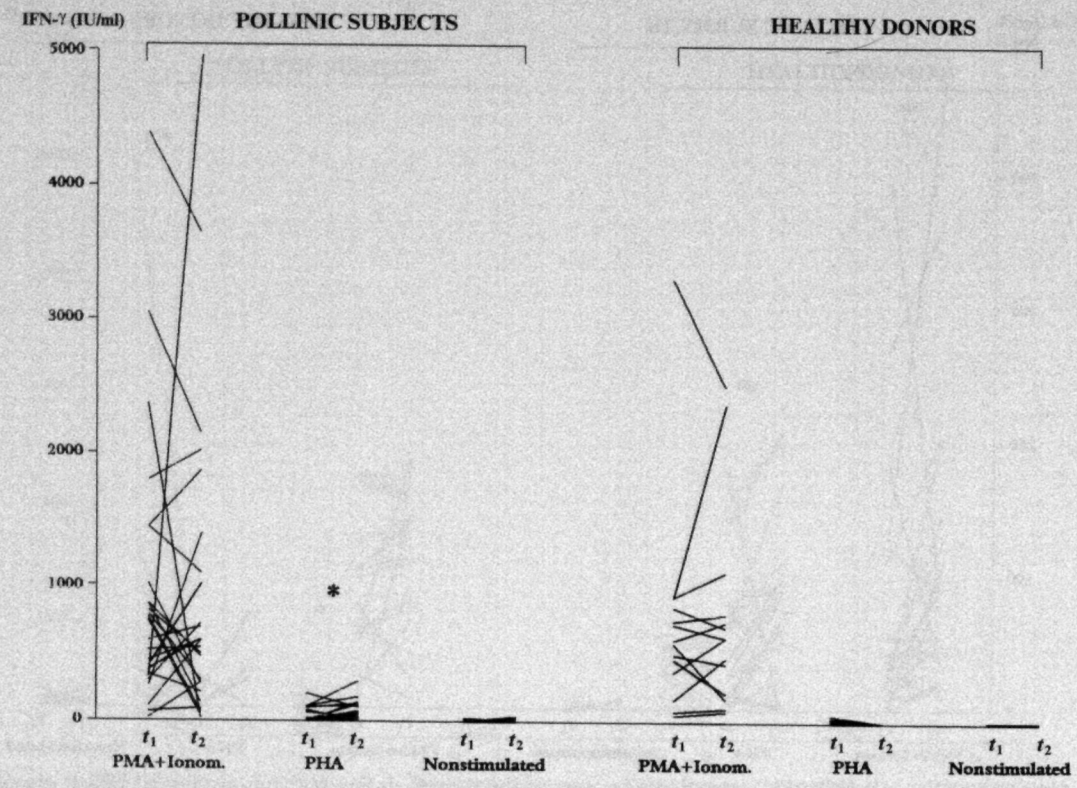


Fig. 3. IFN-γ production (IU/ml) by PBMC in response to nonspecific stimuli, during (t_1) and outside (t_2) pollen season, in both pollinic subjects and control group. *Significant difference.

exposure, and the increased IL-4 and IL-5 may simply reflect an increased frequency of Th2 cells rather than an intrinsic abnormality in signaling.

It has been stated that the TcR is not the critical element determining the type of immune response (Th1 or Th2) (10). In our study, the use of two different polyclonal activators which act by different mechanisms in the activation of PBMC allows us to suggest that nonspecific mitogens, bypassing the TcR activation, can reveal the tendency of PBMC from pollinic patients to show an enhanced production of Th2-type cytokines when they are exposed to environmental sensitizing pollens. But the heterogeneity of the results of stimulation by PHA and PMA, when we compared t_1 with t_2, and when we compared atopic subjects with healthy donors, lead us to conclude that the use of mitogens to assess Th1/Th2 dominance may need careful evaluation.

Acknowledgments

We thank the nursing and laboratory staff for their contribution to this study.

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