

The influence of substance P on the proliferation of peripheral blood lymphocytes from normal individuals and birch pollen-allergic patients

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We have studied the influence of substance P (SP) on the proliferative response of concanavalin A (ConA)-activated peripheral blood lymphocytes from 16 birch pollen-allergic patients, sampled before and during the pollen season, and from 15 normal individuals. The median response to ConA 3 µg/ml in the presence of SP 10^{-11} – 10^{-6} M, was in most instances within $\pm 10\%$ of the control value for cells from both healthy and atopic individuals. However, the individual differences were considerable. Analysis of the proliferative responses to ConA of the cells from the allergic patients sampled before and during season, revealed higher responses in the presence of 10^{-6} than of 10^{-7} M SP. This was in contrast to the findings in the normal individuals: only half of their cells showed such increased responses. This difference in response frequency was statistically significant between allergic patients before season and normal individuals ($P < 0.05$) and between allergic patients during season and normal individuals ($P < 0.01$). The difference in proliferation rate in the SP concentration interval, 10^{-6} to 10^{-7} M, for the cells from allergic patients, sampled both before and during season, was significantly different from the cells from healthy individuals ($P < 0.03$ and $P < 0.001$ respectively). The cells sampled from four allergic subjects during the birch pollen season showed a more profoundly decreased response to ConA in the presence of SP 10^{-8} M, compared with their cells sampled before season. Such responses were never seen with cells sampled before season and with cells from normal individuals. The results suggest an involvement of SP in the immunoregulation, particularly in patients exhibiting allergic reactions to birch pollen.

Key words: allergy; lymphocyte proliferation; neuropeptides; substance P.

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CLINICAL ASPECTS

Patients suffering from allergy to birch pollen have different kinds of immunoregulatory dysfunction. Various components act in concert resulting in an allergic inflammation and hyperreactivity. Neuropeptides, e.g. substance P (SP), have been proposed as participating in the tissue response evoking hyperreactivity. Besides the effect on tissue, SP also has an effect on the immune system, e.g. lymphocytes. In the present study we show different lymphoproliferative responses to SP of cells from birch pollen-allergic patients and healthy individuals. This indicates that SP may be active in lymphocyte activation, particularly during the allergic reaction.

Several functions of the cholinergic, adrenergic and peptidergic autonomic systems are involved in the development of hyperreactivity in allergic individuals (1, 3). Such hyperreactivity is seen

particularly in conjunction with exposure to a variety of agents, including allergen, irritating vapours such as SO₂, NO₂, O₃, and cold air (22). During recent years, interest has focused

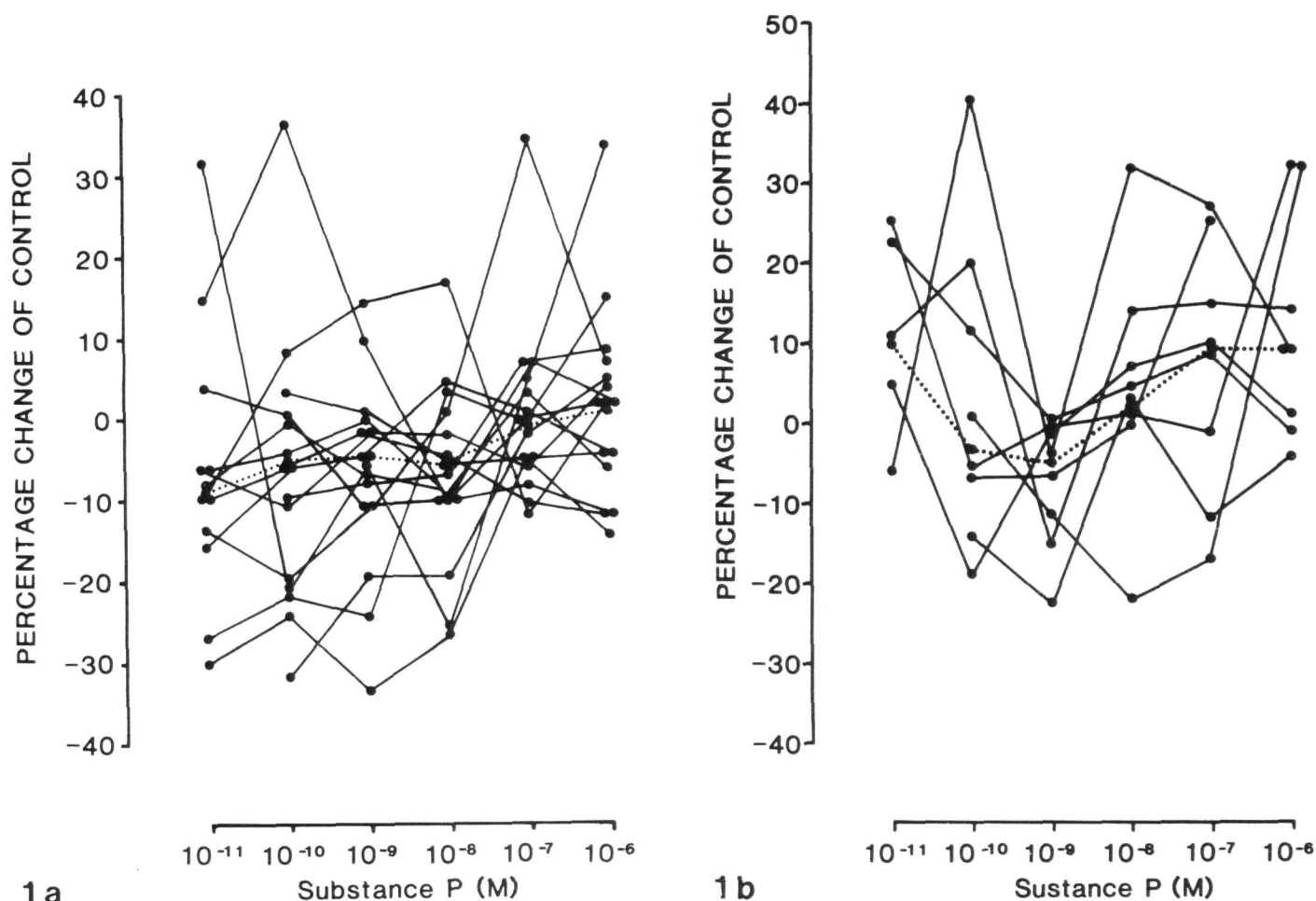


Fig. 1. Proliferation of cells from normal individuals separated on Ficoll-Paque (a) and by E-rosetting (b) after stimulation with 3 $\mu\text{g/ml}$ of ConA and different concentrations of SP. Data are presented as a percentage change in ^3H -thymide incorporation compared to control. The dotted line represents the median values. Cpm value (mean $\pm$ SEM) for ConA stimulation of PBLs was $33\,956 \pm 6026$ and of E-rosetting cells $25\,974 \pm 7300$.

on the constituents of the peptidergic system, e.g. the neuropeptides, such as substance P (SP), which are present in the terminals of primary sensory neurones. These are distributed in the mucosal membrane, adjacent to the mediator-releasing cells, e.g. mast cells and lymphocytes (9). Thus, a firm link has been proposed between the sensory nerves in the mucosa and the immune system in evoking allergic inflammatory disorders (2, 20).

Data from several research groups imply that neuropeptides influence various immune functions *in vitro* through binding to lymphoid cells. Such binding to and/or activation of lymphocytes has been demonstrated for vasoactive intestinal peptide (VIP) (4, 15), somatostatin (19) and SP (18). The binding to lymphocytes stimulates proliferation of human peripheral

blood T cells (17) and mouse lymphoid cells (23). Not all investigators have been able to confirm such an effect (8). In contrast to the activity of SP, those of VIP and somatostatin have been found to be suppressive in similar systems (16, 19, 23).

In mice the effects on immunoglobulin synthesis of SP, VIP, and somatostatin, can be both stimulatory and suppressive, depending on the origin of the cells (5, 23). Whether these differently responding cells can be present in the circulation after migration in response to immunological stimuli is not known.

In the present study we analysed the effect of SP on the proliferation of peripheral blood lymphocytes from normal individuals and from patients suffering from atopic disease and hyperreactivity to birch pollen. The allergic

patients were studied both before and during season. The observations demonstrate that exposure to SP, especially at high concentrations, 10^{-7} – 10^{-6} M, results in altered proliferation of human peripheral blood lymphocytes. This effect is more pronounced in cells from allergic patients, although great heterogeneity was seen among the individuals.

MATERIAL AND METHODS

Cell preparation

Peripheral blood lymphocytes (PBLs) were prepared from venous blood from 15 healthy persons (median age 28, range 21–50 years) and 16 atopic individuals (median age 32, range 19–49) described in a recent study (21). The allergic patients with birch pollen-induced rhinitis were documented by skin prick test and nasal provocation test. The tests were performed before and 1 year after the investigation. The blood was collected in heparinized vacutainer tubes and diluted 1:3 in Hank's balanced salt solution (HBSS) (Flow Svenska AB, Solna, Sweden) and separated on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). The PBLs were recovered from the interface and washed three times in HBSS and resuspended in RPMI-1640 Dutch modification (Flow) supplemented with 10% fetal calf serum 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine and 100 µg/ml Garamycin (Flow).

Cell fractionations

The PBLs were separated into an E-rosette-positive fraction with AET-treated sheep erythrocytes (10). The rosetted mixture was separated on Ficoll-Paque. The recovered sediment was treated with 0.83% NH_4Cl to lyse erythrocytes. Viability of the enriched T cells exceeded 95%, as determined by the exclusion of trypan blue. The fraction contained > 85% E-rosette forming cells. No further characterization of the cells was performed. Because of insufficient blood samples from some of the patients, both PBLs and E-rosetting cells could not be obtained.

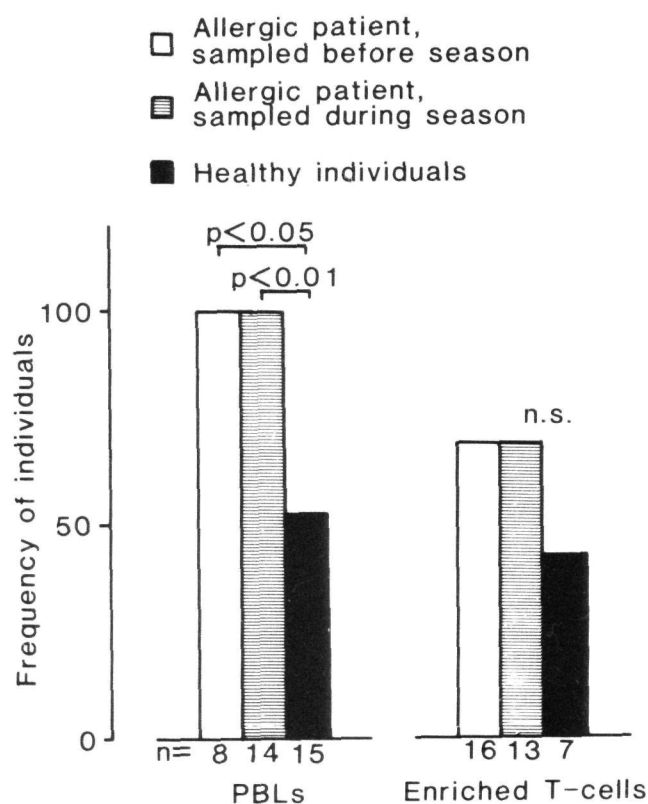
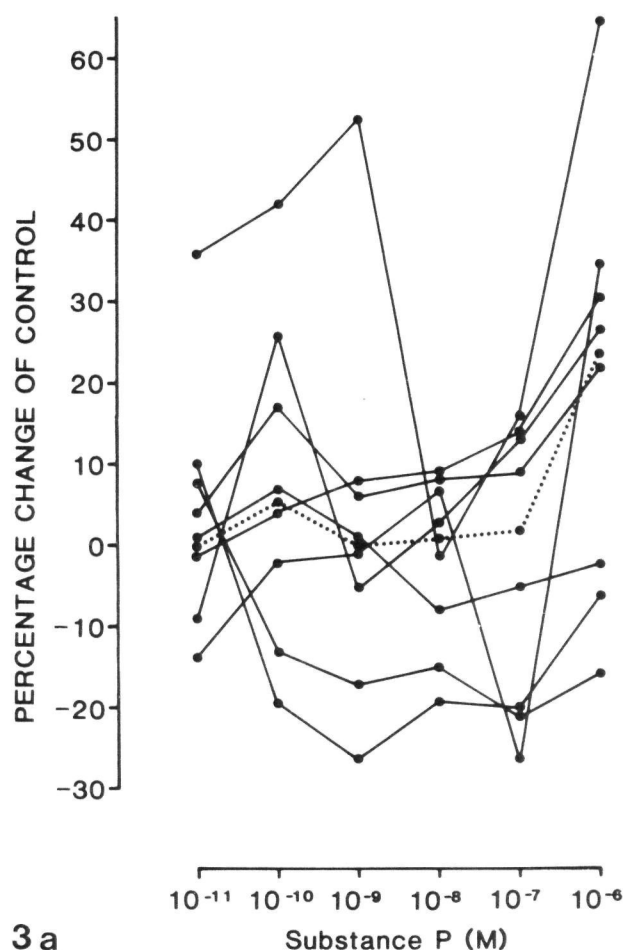


Fig. 2. Frequency of allergic and healthy individuals with increased proliferation rates of PBLs and enriched T cells from the dose interval of SP 10^{-7} to 10^{-6} M (n = number of individuals).

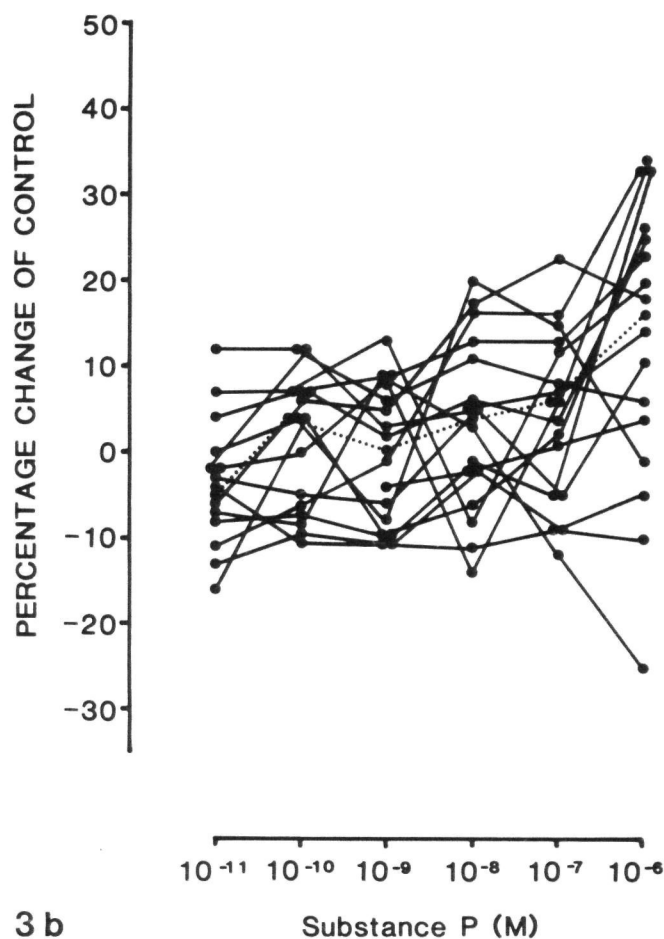
The PBLs were further fractionated on nylon-wool columns. The nylon-wool (Travenol AB, Bromma, Sweden) was washed and packed into columns consisting of syringes, 0.3 g nylon wool/3 ml column, and autoclaved. Prior to use, the columns were rinsed with 10 ml medium and incubated for 30 min at 37°C. The resuspended cells, $2-3 \times 10^7$ in 1 ml, were run into the column and were incubated at 37°C, 7% CO_2 , 95% humidity for 60 min. The column was washed with 10 ml of medium prewarmed to 37°C, and the effluent containing the non-adherent cells, mainly T lymphocytes, was collected.

Culture conditions

The cells, 1×10^6 /ml, were cultured in quadruplicate for 3 days in a total volume of 200 µl in a flat-bottomed microtiter plate (Costar Laboratorie Design, Lidingö, Sweden) at 37°C in an atmosphere of 7% CO_2 and 95% humidity. Substance P (SIGMA Co., St. Louis, Mo),



3 a



3 b

Fig. 3. Proliferation of cells from birch pollen patients before season, separated on Ficoll-Paque (a) and by E-rosetting (b) after stimulation with 3 $\mu\text{g/ml}$ of ConA and different concentrations of SP. Data are presented as a percentage change in ^3H -thymidine incorporation compared with control. Dotted line: median value. Cpm value (mean $\pm$ SEM) for ConA stimulation of PBLs was $50\,446 \pm 6208$ and of E-rosetting cells $65\,402 \pm 5272$.

freshly diluted in medium to 10^{-11} – 10^{-6} M, was added, alone or together with concanavalin A (ConA) (Pharmacia) to a final concentration of 3 $\mu\text{g/ml}$.

The proliferative responses were determined by the addition of 1 $\mu\text{Ci/well}$ of ^3H -thymidine (specific activity 6.7 Ci/mmol) (New England Nuclear, Boston, Mass) 6 h before termination of the cultures. The cells were harvested onto glass fibre filter papers (Titertek Cell Harvester, Flow Laboratories). The ^3H -thymidine uptake was determined by liquid scintillation counting and expressed in cpm. The coefficient of variation (CV) for the method depended on the degree of incorporation of ^3H -thymidine. For stimulations giving more than 50 000 cpm the CV was 10 %, for 30 000–50 000 cpm the CV was 16 %; and for responses below 30 000 the CV was 20 %.

The proliferative response of cells incubated with SP was expressed as a percentage of change from the control cells incubated without SP.

Statistical significance was assessed using the Chi-square test and the Student's *t*-test.

RESULTS

Effect of SP on cells from non-allergic individuals

The cell responses to SP alone was weak. The counts determined were around 600–1200 cpm, which was too low to calculate the influence of SP on the blastogenesis. This was seen both with PBLs and enriched T cells (data not shown). We therefore stimulated the cells with a suboptimal concentration of ConA, 3 $\mu\text{g/ml}$ to obtain

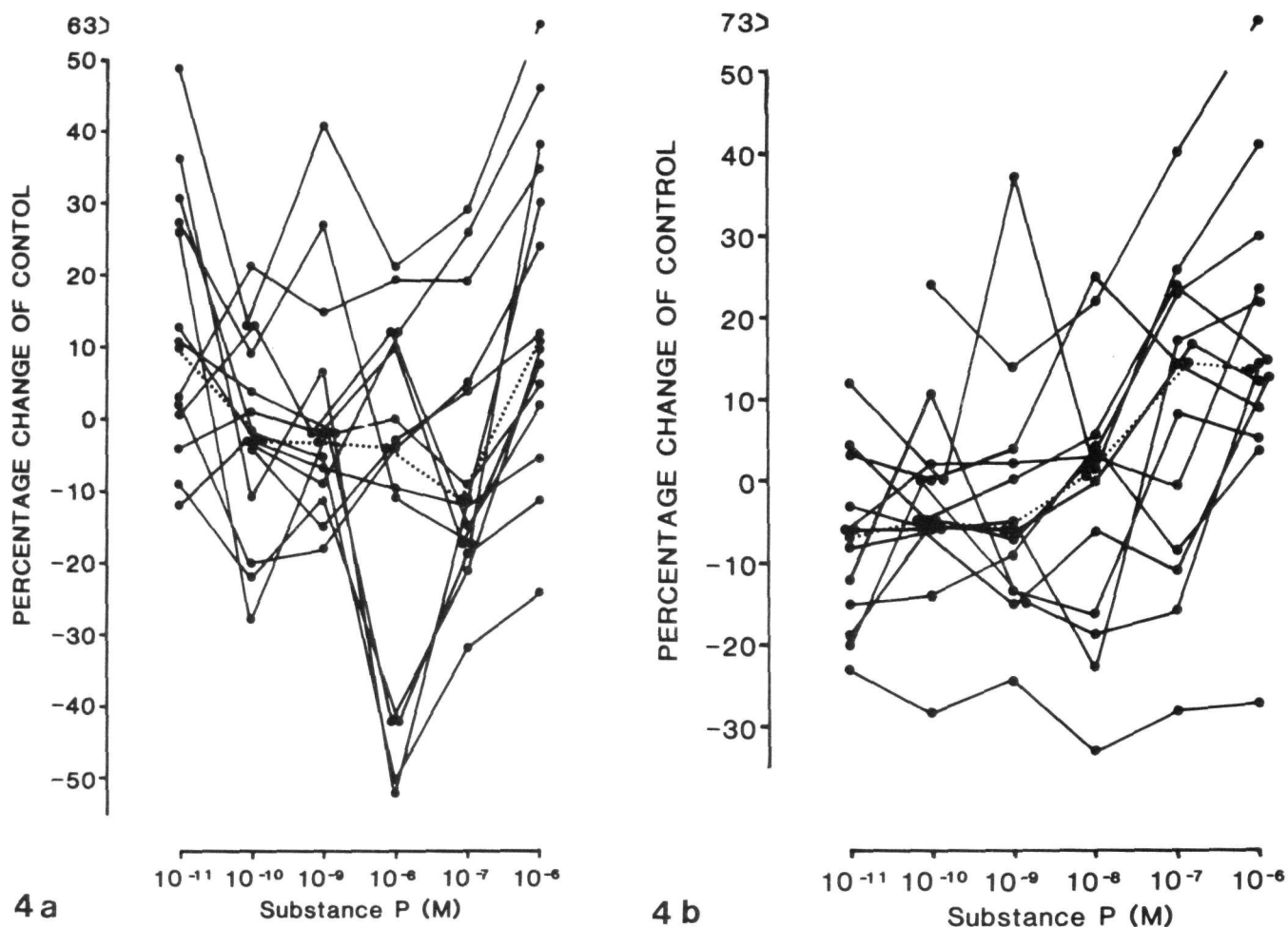


Fig. 4. Proliferation of cells from birch pollen patients sampled during season, separated on Ficoll-Paque (a) and by E-rosetting (b) after stimulation with 3 μ g/ml ConA and different SP concentrations. Data are presented as a percentage change in ^{3} H-thymidine incorporation compared with control. Dotted line: median value. Cpm value (mean $\pm$ SEM) for ConA stimulation of PBLs was 39 744 $\pm$ 5772 and of E-rosetting cells 23 733 $\pm$ 3078.

a stimulation of 30 000–50 000 cpm. The responses of PBLs and enriched T cells from most individuals stimulated with 3 μ g/ml ConA were only slightly affected in the presence of SP (Fig. 1a and 1b). The proliferation level of SP 10^{-11} M compared with 10^{-6} M was unchanged. However, at some SP concentrations there was a fall in the proliferation level, differing from individual to individual. The cell response from six of 15 individuals showed a more pronounced alteration on exposure to different concentrations of SP. Both increased (4 individuals) and decreased (4 individuals) responses were seen.

The stimulation with ConA combined with SP 10^{-6} M, compared with ConA and SP 10^{-7} M, resulted in a higher proliferation rate of cells

from eight of 15 individuals (53 %) (Fig. 2). For enriched T cells, such a pattern was shown in three of seven (43 %) (Fig. 2).

Peripheral blood lymphocytes separated by nylon-wool showed patterns of reactivity to ConA and SP similar to those described above (results not shown).

Stimulation of cells from allergic patients before birch pollen season

In view of the poor cell responses from normal individuals to SP alone, the cells responses from atopic individuals were not analysed. The combined effect of SP and ConA on PBLs from atopic individuals sampled before the birch pollen season (Fig. 3a) was similar to that seen on the PBLs

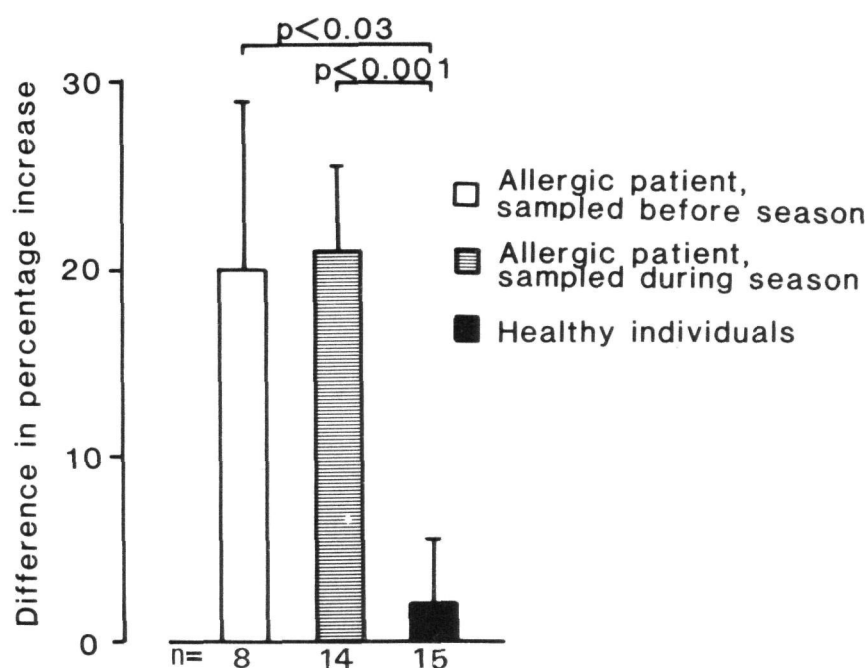


Fig. 5. Percentage increase in proliferation of PBLs in the concentration interval 10^{-7} to 10^{-6} of SP. (n = number of individuals).

from normal individuals (Fig. 1a). The only exception was that in the interval 10^{-7} to 10^{-6} M SP the PBLs from the eight birch pollen patients showed increased proliferative responses. This difference between atopics and normals was statistically significant ($P < 0.05$) (Fig. 2).

Enriched T cells, stimulated with SP and ConA, showed proliferative response similar to those seen with samples taken from normal individuals. The enriched T cells showed increased proliferative response when the SP concentration was increased from 10^{-7} M to 10^{-6} M (Fig. 3b) in 11 out of 16 (69%) allergic patients sampled before birch pollen season compared with three out of seven (43%) normal individuals. However, this difference was not significant (Fig. 2).

Both PBLs and enriched T cells showed an increased proliferation level (approx. 22%) when they were stimulated with SP 10^{-6} M compared with SP 10^{-11} M.

Stimulation of cells from allergic patients during birch pollen season

SP had a greater effect on ConA-activated PBLs sampled during the birch pollen season than on those sampled before season (Fig. 4).

During the birch pollen season the PBL responses from four of 14 of the patients were profoundly decreased by SP at a concentration of 10^{-8} M. At the SP concentration interval from 10^{-7} M to 10^{-6} M the PBLs from all 14 patients showed increased proliferative responses (Fig. 4a). These enhanced responses differed significantly from those of normal individuals ($P < 0.01$) (Fig. 2).

The enriched T cells from the patients sampled during the birch pollen season showed almost the same pattern as the cells sampled before season. During the birch pollen season nine of 13 (69%) patients investigated showed increased response when the SP concentration was increased from 10^{-7} to 10^{-6} M (Fig. 4b). The difference noted between allergic patients and control individuals was not significant (Fig. 2).

Comparison between the groups

In the dose interval 10^{-7} to 10^{-6} M of SP, the mean increase was 20% (SE 8%) and 22% (SE 4%) for PBLs sampled from allergic patients before and during season, respectively. The increase in PBL responses from healthy individuals was 2% (SE 4%). This difference

in PBL response between allergic patients sampled before and during season and that of healthy individuals was statistically significant ($P < 0.03$ and $P < 0.001$, respectively) (Fig. 5).

DISCUSSION

The results of the present study show the effect of SP on blood lymphocytes to be much weaker than previously reported by Payan et al. (17). The cell response from normal individuals, both to SP alone and to a combination of substance P and ConA, showed patterns more similar to those reported by Liping et al. (8). The reason for this is not obvious, although differences in culture conditions and the selection of patients cannot be excluded. Another discrepancy between our findings and those Payan et al. (17) was that enrichment of T cells by E rosetting did not result in higher responses than those of the whole cell population. There is no obvious explanation for this, since reported numbers of helper-inducer T cells and suppressor-cytotoxic T cells in patients with respiratory allergy or allergic rhinitis were almost the same as those in healthy controls (7). However, patients suffering from atopic dermatitis were reported to have a lower number of suppressor-cytotoxic T cells together with a normal number of helper-inducer T cells. Consequently, we do not know whether the discrepancy between normal individuals and atopics is due to different susceptibility of T helper cells, which are reported to be the predominant responder cells to SP (18). The observations may also be due to different receptor affinity to SP or to a different density of receptors on the cells from allergic patients during pollen season compared with normal individuals.

It should be mentioned that even lower concentrations of SP had an influence on the proliferation level. Enriched T-cells from healthy individuals and PBLs from allergic patients sampled during season showed a reduction in their proliferative response when the SP concentration was changed from 10^{-11} M to 10^{-10} M.

The stronger influence of SP, particularly at high concentrations, on cells from birch pollen-allergic individuals may reflect the importance of an ongoing immune response to render the cells susceptible to SP. We have obtained similar results in studies on rats (12). However, an ongoing immune response may also alter the cell distribution and induce responsive cells in various lymphoid organs to recirculate. In addition, the cell distribution can be influenced by released neurotransmitter substances (11, 14).

This study was performed on cells separated from the peripheral blood. However, it would be desirable to study the activity of SP on lymphocytes from the shock organ, in this instance the lung, since the mucosal membranes are innervated with sensory nerves using SP as neurotransmitter. These nerves appear to be closely associated with cells in the mucosal immune system (6, 9). This in turn might lead to a high concentration of the peptide in the microenvironment corresponding to 10^{-6} M. Levels of SP in human plasma have been recorded at concentrations between 100 and 300 pmol/l (13).

The findings presented in this study are complementary to earlier findings concerning the interaction between neurological and immune systems. The significant difference between the allergic and control groups may cast some light on the complicated interactions and regulation of allergic responses. The new aspect in this study is the difference found between atopic and non-atopic individuals in their lymphocyte sensitivity to SP. This may reflect that SP is involved in the mechanism of hyperreactivity in allergic patients, not only as a bronchoconstrictor but also as an immunologically active mediator.

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