

## Inhibitory Effect of Vasoactive Intestinal Polypeptide and Ketanserin on Established Allergic Contact Dermatitis in Man

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Neuromediators may influence the immune response. To investigate their potential immunomodulating role in established allergic contact dermatitis in man, the following neuromediators were tested: vasoactive intestinal polypeptide (VIP), serotonin, and the serotonin antagonists ketanserin, methiotepine and ICS-205-930. Positive patch test reactions were elicited by application of nickel sulphate for 48 h. The neuromediators were applied under patch test conditions after another 24 h. The test areas were measured before and 24 h after application of the neuromediators and biopsy specimens were taken for immunohistochemistry. After application of VIP at a concentration of  $10^{-5}$  mol/l, and of ketanserin at a concentration of  $10^{-4}$  mol/l, there was a significant reduction in the diameter of the test reaction. In addition, with VIP there was a reduction in the number of Leu 3a+ cells. Also tested was the influence of the neuromediators on the proliferative response of peripheral blood mononuclear cells from nickel-allergic subjects to nickel sulphate. The cells were cultured for 6 days and the neuromediators were added after 3 days. There was no effect on the proliferative response, except for slight inhibition by serotonin and by ketanserin at  $10^{-4}$  mol/l. More interferon gamma was found in the supernatants when VIP was added at  $10^{-5}$  and  $10^{-6}$  mol/l than in the control cultures. Thus, VIP and ketanserin may have an inhibitory effect on established allergic contact dermatitis. The effect of VIP is possibly mediated by an increased production of interferon gamma. **Key word:** VIP.

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There is increasing evidence to indicate that the nervous system has an influence on the immune response (1). The peripheral nervous system may play a role in the modulation of the inflammatory reaction (2). Previous studies have shown that depletion of the neuropeptide contents of primary sensory neurons with capsaicin enhances the cutaneous inflammatory response to various chemical and physical agents (3, 4). Quantitative variations in cutaneous levels of some neuropeptides have been found in lesional skin in a number of inflammatory dermatoses (5).

VIP is a 28 amino acid neuropeptide, which has been described as an inhibitor of several immunological functions, both in vitro and in vivo (6–8). In the skin, VIP is mainly contained in autonomic nerve fibres. Monoamines may also alter the immune function (for ref. see 9). Serotonin, or 5-hydroxytryptamine (5-HT), is a neuromediator of the central and peripheral nervous systems and a vasoactive amine, which is stored in the blood by platelets and released at sites of inflammation in response to various stimuli. It has been known for several years that pharmacological enhancement of 5-HT production suppresses the immune response both in vivo and

in vitro, giving a decrease in antibody production, delayed hypersensitivity and transplantation immunity (10).

In order to investigate the possible modulatory effects of VIP, 5-HT and the 5-HT antagonists ketanserin, methiotepine and ICS-205-930 on allergic contact reactions, we used an in vivo model with positive patch test reactions to nickel sulphate as indicators. In addition, the effects on peripheral blood mononuclear cell proliferation and secretion of interferon gamma, IL-2 and soluble IL-2 receptor were measured in vitro.

### MATERIALS AND METHODS

#### Patients

Thirty-four female patients with patch test-verified allergic contact dermatitis from nickel sulphate, 5.0% in petrolatum, gave their informed consent to participate in this study. Approval had been given by the local Medical Ethics Committee. All subjects had +++ positive test reactions (erythema, oedema, papules and vesicles), evaluated by the scoring system recommended by the International Contact Dermatitis Research Group (11).

#### Test substances

Nickel sulphate was obtained from Merck (Darmstadt, Germany). When used for patch testing it was dissolved in distilled water to a concentration of 5.0%. For the lymphocyte proliferation test it was dissolved in 0.9% saline to a concentration of  $3.8 \times 10^{-5}$  mol/l, which has previously been found to be optimal (12).

Native VIP was purified from porcine upper intestine by sequential chromatography and countercurrent distribution and found to be homogeneous by high performance liquid chromatography and capillary electrophoresis, essentially as described (13).

5-HT was obtained from Sigma (St. Louis, MO, USA), ketanserin (a specific 5-HT<sub>2</sub> receptor blocker) from Janssen Pharmaceutica (Beerse, Belgium), methiotepine (a blocker of both 5-HT<sub>1c</sub> and 5-HT<sub>2</sub> receptors) from Hoffman-La Roche (Basel, Switzerland) and ICS-205-930 (a blocker of 5-HT<sub>3</sub>) from Sandoz (Basel, Switzerland).

VIP, 5-HT, ketanserin, methiotepine and ICS-205-930 were dissolved in saline and used for patch testing at concentrations of  $10^{-4}$ – $10^{-7}$  mol/l and in the cell cultures at final concentrations of  $10^{-4}$ – $10^{-11}$  mol/l.

#### Patch testing procedure

The experimental design is summarised in Fig. 1. Patch tests with 5% nickel sulphate in distilled water, in a volume of 15 µl, were performed with Finn chambers (Epitest, Helsinki, Finland), using polypropylene-coated aluminium discs with filters on Scanpor tape (Norgesplaster A/S, Norway). The patch tests were applied on normal skin of the back for 48 h and were read after an additional 24-h period. The diameters of the test reactions were recorded. VIP ( $n=7$ ), 5-HT ( $n=9$ ), ketanserin ( $n=8$ ), methiotepine ( $n=6$ ) and ICS-205-930 ( $n=4$ ) were then applied topically on the test reaction areas, using the same types of aluminium discs and filters as above, and concentrations of VIP of  $10^{-5}$ – $10^{-7}$  mol/l and of the other neuromodulators of  $10^{-4}$ – $10^{-6}$  mol/l, in a volume of 15 µl. Saline was used as control. After an additional 24-h period the test reactions were measured. Two investigators (LB, SL) independently measured the intensity scored by the scoring system recommended by the

## Experimental design

### Epicutaneous application of test substances

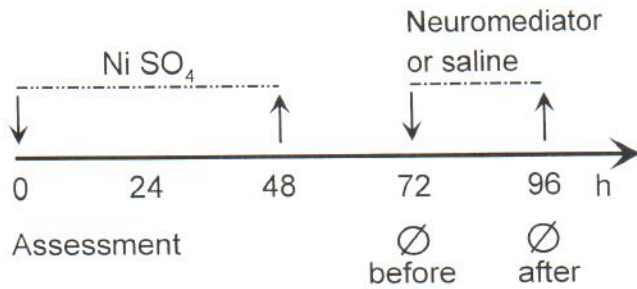


Fig. 1. The neuromediators VIP, serotonin and the serotonin antagonists ketanserin, methiotepine and ICS-205-930 were tested on established allergic contact dermatitis in man. Positive patch test reactions were elicited in patients allergic to nickel by application of nickel sulphate for 48 h. The neuromediators or saline (control) were applied under patch test conditions after another 24 h. The diameters of the test areas were measured before and after application of the neuromediators for 24 h.

ICDRG and the maximal diameters of the test reactions, one of the investigators blindly. In the cases of difference, the opinion of the investigator judging the reactions blindly was accepted.

#### Immunohistochemical studies

Biopsy specimens were taken from the test areas for immunohistochemistry after anaesthesia with lidocaine without epinephrine. Each specimen was immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until used. Acetone-fixed cryostat sections, 6  $\mu\text{m}$  thick, were stained with a two-stage monoclonal antibody peroxidase method (14). The panel of mouse monoclonal antibodies contained Leu 2a (CD8, T cytotoxic/suppressor cells), Leu 3a (CD4, T helper/inducer cells), Leu 6 (CD1a, Langerhans' cells), HLA-DR (activated T cells, B cells, macrophages, monocytes and Langerhans' cells) and interleukin-2 receptor (CD25). These antibodies were all from Becton Dickinson (San José, CA, USA), and the working dilution was 1:100. In addition, ICAM-1 (CD54) expression was examined with a mouse monoclonal antibody obtained from Serotec (Oxford, UK), used in a working dilution of 1:60. Three sections per antibody and biopsy specimen were examined. The estimation of the total number of cells per section reactive to the different monoclonal antibodies was based on a semiquantitative scale: -, no positive cells; +, few; ++, moderate; and +++, many. The size of the infiltrates and any spongiosis in the epidermis were also measured with a semiquantitative scale. Two investigators (LB,KN) examined all slides independently of each other, and the mean values were recorded. The slides were coded to avoid observer bias.

#### Lymphocyte transformation test

Peripheral blood mononuclear cells were obtained from heparinized blood from 8 of the 34 nickel-allergic patients. The cells were separated on Percoll gradients on the basis of buoyant density (15) (density of stock solution 1.129 g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden). They were then cultured in RPMI 1640 medium with addition of L-glutamine (2  $\mu\text{mol}/\text{ml}$ ), streptomycin (100  $\mu\text{g}/\text{ml}$ ), penicillin (100 IU/ml) and 10% heat-inactivated human AB serum at a concentration of  $2 \times 10^6$  cells/ml. The cells were subsequently incubated in 100- $\mu\text{l}$  cultures for 6 days in a Linbro microtitration plate at  $37^{\circ}\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  in air. After preincubation for 30 min, 20  $\mu\text{l}$  of nickel sulphate solution was added. After 72 h, VIP, 5-HT and 5-HT antagonists were added in a volume of 20  $\mu\text{l}$  and with saline

as control. Six hours before interruption of the cultures, 0.5  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (5 Ci/mmol; Amersham International, Amersham, UK) in 10  $\mu\text{l}$  saline was added to each well. The cells were then collected on glass fibre filters by using a Skatron multiple-cell collector. Radioactivity was determined by counting in a Packard liquid scintillation spectrometer. The cultures were performed in triplicate.

#### Dye exclusion test

The peripheral blood mononuclear cells were cultured by the same procedure as described above. 5-HT, ketanserin, methiotepine and ICS-205-930 at the highest concentrations, and with saline as control, were added at 3 days. VIP was not tested. At 6 days, 200  $\mu\text{l}$  of the trypan blue (0.1%) solution was added to each microculture. After 2 min the suspension was poured onto a slide and covered with a coverslip, and the frequency of stained cells was determined in a light microscope (1,000 $\times$ ). Two hundred cells of each microculture were counted.

#### Interferon gamma assay

On the 6th day of incubation with nickel sulphate, supernatants were obtained from the peripheral blood mononuclear cell cultures of the above-mentioned 8 patients. The supernatants were analysed for interferon gamma content by using an ELISA kit (Biosource International, Camarillo, CA, USA), with a detection limit of 20 pg/ml of interferon gamma and following the recommendations of the manufacturer.

#### IL-2 assay

At 6 days, supernatants were obtained from cell cultures of 5 patients. The supernatants were analysed for IL-2 content by ELISA, using the human IL-2 ELISA system (Amersham, UK).

#### IL-2 receptor assay

After 6 days, supernatants were obtained from cell cultures of 8 patients. The concentration of soluble IL-2 receptors was measured by ELISA using the IL-2R test kit (T Cell Diagnostics, Cambridge, MA, USA).

#### Statistics

The Wilcoxon rank sum test and the Wilcoxon signed rank test were used for statistical analyses.

## RESULTS

### Patch testing

The results are exemplified in Fig. 2. The diameter of the nickel sulphate-induced test reaction was significantly reduced ( $p < 0.05$ ) after 24 h exposure to VIP at a concentration of  $10^{-5}$  mol/l, and to ketanserin at  $10^{-4}$  mol/l. No effect on the diameters was seen with 5-HT, methiotepine or ICS-205-930. The test response to nickel decreased from +++ (erythema, oedema, papules and vesicles) to ++ (erythema, oedema and papules) in 3 of 7 cases when VIP was added at  $10^{-5}$  mol/l, in 1 of 8 cases when ketanserin was added at  $10^{-4}$  mol/l, and in 1 of 6 cases when methiotepine was added at  $10^{-4}$  mol/l.

### Immunohistochemical studies

When VIP was added at a concentration of  $10^{-5}$  mol/l, there was a statistically significant ( $p < 0.05$ ) reduction of the number of Leu 3a<sup>+</sup> cells. Except for this finding there were no differences in the phenotypes of the infiltrating cells or in the density of the cellular infiltrate, when the allergic test reaction was treated with the different neuromediators. Neither the number of cells reactive to IL-2 receptor nor HLA-DR anti-

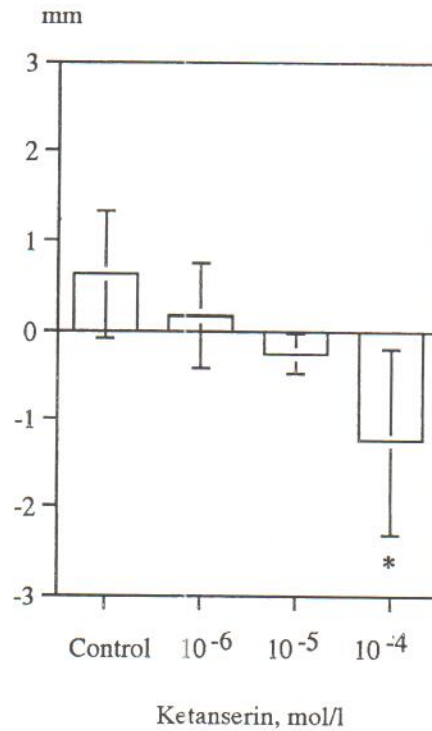
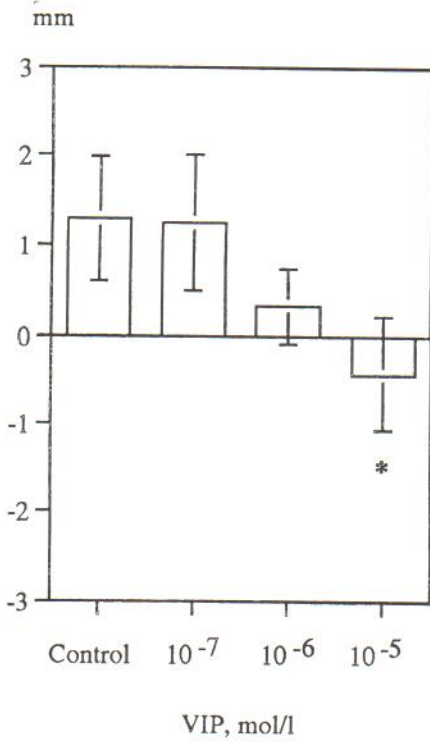


Fig. 2 Changes in the diameter of the nickel sulphate-induced test reactions produced by VIP, ketanserin and saline (control). Each value represents the mean ( $\pm$ SD) of 7 experiments (VIP) or 9 experiments (ketanserin). Differs from control at  $*p < 0.05$ .

bodies showed any difference, and there was no change of the ICAM-1 expression on keratinocytes.

*DNA synthesis*

VIP had no effect on the proliferative response of the blood mononuclear cells, but both 5-HT and the 5-HT antagonists had an inhibitory effect at  $10^{-4}$  mol/l ( $p < 0.01-0.001$ ).

*Dye exclusion test*

The results are summarised in Table I. The number of cells with an ability to exclude trypan blue decreased evidently after exposure to methiotepine and ICS-205-930 at a concentration of  $10^{-4}$  mol/l. In addition, there was a slight decrease after exposure to 5-HT and ketanserin.

*Interferon gamma secretion*

The results are summarised in Fig. 3. VIP at  $10^{-5}$  mol/l significantly ( $p < 0.05$ ) increased the interferon gamma secre-

tion in the cell cultures. There was a tendency ( $p < 0.08$ ) towards stimulation with VIP at  $10^{-6}$  mol/l. Neither serotonin nor the serotonin antagonists had any effect on this secretion.

*Interferon gamma*

pg/ml

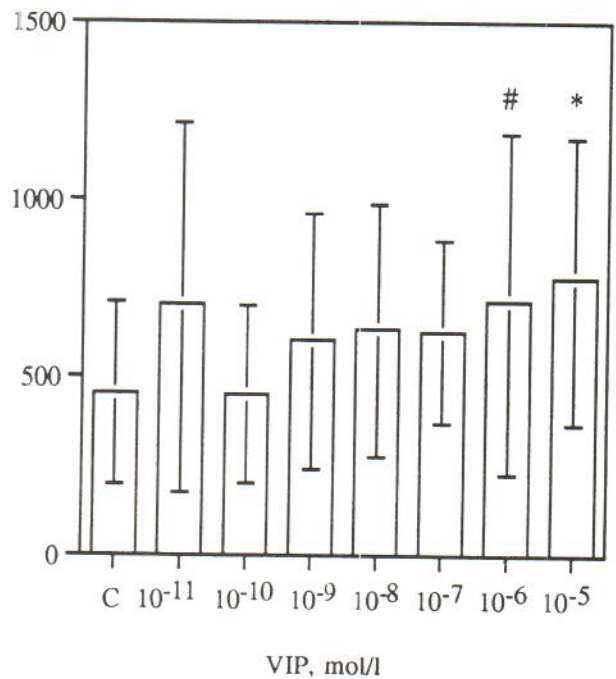


Fig. 3. The levels of interferon gamma (pg/ml) were measured by ELISA in the supernatants of lymphocyte cultures on day 6 of stimulation by nickel sulphate. VIP at different concentrations was added at 3 days. Each value represents the median value (and quartile deviation) of ten experiments. Differs from control (C) at  $*p < 0.05$ ,  $\#p < 0.08$ .

Table I. Effects of 5-HT and 5-HT antagonists on the variability of peripheral blood mononuclear cells stimulated with nickel sulphate, as judged by a dye exclusion test

The number of cells with an ability to exclude trypan blue is given in per cent of the total number of cells at 6 days. 5-HT and the antagonists were added at 3 days. Each value represents the mean ( $\pm$ SD) of 4 experiments.

mol/l	5-HT	Ketanserin	Methiotepine	ICS-205-930
$10^{-4}$	69 $\pm$ 7	62 $\pm$ 3	0 $\pm$ 0	45 $\pm$ 5
$10^{-5}$	79 $\pm$ 3	76 $\pm$ 2	52 $\pm$ 2	77 $\pm$ 2
Control	81 $\pm$ 1	81 $\pm$ 1	81 $\pm$ 1	81 $\pm$ 1

Table II. Soluble interleukin-2 receptor secretion in peripheral blood mononuclear cells stimulated with nickel sulphate and different concentrations of VIP, 5-HT, ketanserin, methiotepine and ICS-205-930

The concentrations of interleukin-2 receptor (U/ml) were measured by ELISA in the supernatants of cells stimulated by nickel sulphate for 6 days. The neuromediators were added after 3 days. Each value represents the median value (and quartile deviation) of 8 experiments. Differs from control at \* $p < 0.05$ .

mol/l	VIP	5-HT	Ketanserin	Methiotepine	ICS-205-930
$10^{-4}$		224 (44)	205 (86)	111 (56)*	238 (138)
$10^{-5}$	235 (65)				
$10^{-6}$		211 (48)	247 (42)	230 (63)	216 (95)
$10^{-7}$	213 (81)				
$10^{-8}$		226 (105)	230 (82)	269 (91)	236 (38)
$10^{-9}$	230 (89)				
$10^{-10}$		255 (155)	150 (100)	188 (98)	186 (79)
$10^{-11}$	241 (65)				
Control	194 (78)	194 (78)	194 (78)	194 (78)	194 (78)

#### Interleukin-2 secretion

The levels of interleukin-2 were very low, and no effects of the neuromediators were observed (not shown).

#### Soluble IL-2 receptor secretion

The results are summarised in Table II. The neuromediators had no apparent effect, except for methiotepine at a concentration of  $10^{-4}$  mol/l, which had an inhibitory action.

#### DISCUSSION

In the present study, topical application of VIP at a concentration of  $10^{-5}$  mol/l caused a reduction of the diameter of an established test reaction to nickel sulphate. In addition, Leu  $3a^+$  cells were reduced in number. Ketanserin, at  $10^{-4}$  mol/l, also reduced the diameter of the test reaction.

To evaluate the modulatory effects of the neuromediators by studying the diameters of the test reactions, is a crude method. However, it was not possible to use the ICDRG score evaluation because there was no great difference in such a score from day 3 to day 4 and moreover, there was most often still a +++ test reaction at day 4.

It is known from earlier studies that neuropeptides may penetrate murine skin. In one study a murine model of contact dermatitis was used and the neuropeptides were applied to the site of allergen contact. The ear-swelling response, which represented the intensity of inflammation, was measured. Substance P (SP), calcitonin-gene related peptide (CGRP) and somatostatin increased the inflammatory response, and in addition, CGRP enhanced the sensitization process (16). In vivo treatment of sensitized mice with ketanserin inhibited both their capacity to develop delayed-type hypersensitivity and the ability of their lymphoid cells to transfer delayed-type hypersensitivity (17).

In human studies, it has been shown by radioimmunoassay that a shorter analogue (29 amino acids) of human growth hormone releasing factor (GRF) can penetrate epidermis in vitro (18). In addition, Dawson et al. (19) found that a melanotropic peptide could be delivered transdermally through human skin in vitro.

VIP is known to have immunomodulatory properties. If VIP can penetrate human skin it might be able to modulate

the delayed-type hypersensitivity reaction. In the present study only the highest concentrations of VIP had an effect, even though the skin barrier was damaged by the allergic contact dermatitis. Wallengren et al. (20) injected SP, VIP and spantide (SP antagonist) into the same skin sites as where the allergen was injected in patients with contact allergy to nickel. SP and VIP did not influence the nickel reaction, while spantide inhibited this reaction significantly.

In the present study, in the investigation of the influence of the neuropeptides on the proliferative response of peripheral blood mononuclear cells of nickel-allergic subjects to nickel sulphate, no effect was observed when VIP at different concentrations was added after 72 h.

Slight inhibition of the cell proliferation was noted when 5-HT and ketanserin were added at  $10^{-4}$  mol/l. The more profound inhibiting effect on the cell proliferation by methiotepine and ICS-205-930, at  $10^{-4}$  mol/l, was probably due to toxicity, as judged by the uptake of trypan blue. As judged by this assay, it cannot be totally excluded that the inhibiting effects of 5-HT and ketanserin might also be due to toxicity.

In earlier studies we have found an inhibition of the migration of mononuclear leukocytes both with 5-HT and ketanserin. In the present study the antagonist had an inhibiting potency similar to 5-HT. Thus, if there is a non-toxic inhibitory effect of 5-HT and ketanserin on cellular proliferation, a common mechanism other than through 5-HT receptors is possible (21).

The secretion of interferon gamma was increased when VIP was added at  $10^{-5}$  mol/l. VIP at a concentration of  $10^{-5}$  mol/l was also found to have an inhibitory effect on the test reaction when applied topically. This is in accordance with the finding by Said (22) that VIP could protect tissues against inflammatory injury or toxicity due to a variety of agents. VIP has also shown protective effects on lymphocytes against metal toxicity (23).

In conclusion, our results may indicate that VIP and ketanserin could have a protective effect against tissue damage in the established delayed-type hypersensitivity reaction. In the case of VIP, the enhancement of interferon gamma secretion might have a role in this respect, but the exact mechanism is not known.

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