

INVESTIGATIVE REPORTS

Inhibitory Effect of Vasoactive Intestinal Peptide on the Challenge Phase of Allergic Contact Dermatitis in Humans

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There is increasing evidence that the nervous system has influence on the immune response. The effect of vasoactive intestinal peptide (VIP) and of serotonin and its antagonists on the challenge phase of allergic contact dermatitis in humans were tested. The substances were injected intracutaneously shortly before and 6 h after application of patch tests with nickel sulphate in nickel-allergic patients and the test areas were measured after a further 18 h. Biopsy specimens were also taken for immunohistochemistry. The diameter of the nickel sulphate-induced test reaction was significantly reduced after injection of VIP at 10^{-6} – 10^{-5} mol/l, but was not affected by serotonin or ketanserin. Also tested was the influence of the substances on the response of peripheral blood mononuclear cells from nickel-allergic subjects to nickel sulphate, when added at the same time as the antigen. No effect on the cell proliferative rate was seen, except for an inhibitory effect of serotonin and its antagonists at 10^{-5} – 10^{-4} mol/l. VIP, at 10^{-5} mol/l and serotonin at 10^{-4} mol/l stimulated the secretion of interferon gamma. The interleukin-2 soluble receptor secretion was slightly stimulated by 5-HT at 10^{-4} mol/l and by ketanserin at 10^{-6} mol/l. In conclusion, our results show that when injected intracutaneously in the challenge phase of allergic contact dermatitis, VIP has an inhibitory effect, which might be explained by enhanced leukocyte production of interferon gamma. **Key words:** 5-HT; nickel sulphate; immunohistochemistry; lymphocyte transformation; ELISA.

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There is increasing evidence that neuropeptides and monoamines modulate various kinds of inflammation and delayed-type hypersensitivity (1–3). In several studies using animal models the effects of neuroactive substances on the challenge phase of the delayed-type hypersensitivity reaction have been evaluated (4, 5). Vasoactive intestinal peptide (VIP) has been found to have an inhibitory effect on the delayed type hypersensitivity reaction in mice when infused subcutaneously, from 3 days before to 3 days after challenge (6). *In vivo* treatment of skin-sensitized mice with ketanserin (a serotonin (5-hydroxytryptamine, 5-HT) antagonist) inhibited their capacity to elicit a delayed-type hypersensitivity reaction and the ability of their lymphoid cells to transfer delayed-type hypersensitivity (7).

In a previous study (8) we observed that topical application of VIP at a concentration of 10^{-5} mol/l caused a reduction in the diameter of an established test reaction to nickel sulphate

in humans. In addition, CD4+ cells were reduced in number. Ketanserin at 10^{-4} mol/l also reduced the diameter of the test reaction. An increased production of interferon gamma was found when VIP, at 10^{-5} and 10^{-6} mol/l, was added to proliferating nickel-stimulated peripheral blood mononuclear cells from nickel-allergic patients.

To further investigate the modulatory effect of VIP, 5-HT and its antagonists on the challenge phase of allergic contact dermatitis in humans, these neuroactive substances were injected intracutaneously before and after application of nickel sulphate for 6 h in nickel-sensitive patients. In addition, peripheral blood mononuclear cells from nickel-allergic patients were cultured with nickel sulphate together with the substances, added at the same time as the antigen. The cell proliferation was measured and the supernatants were analysed by ELISA for their concentrations of interferon gamma, interleukin (IL)-2, IL-2 soluble receptor (IL-2sR) and IL-4.

MATERIALS AND METHODS

Patients

Thirty female patients (age range 22–55 years) with patch test-verified allergic contact dermatitis from nickel sulphate participated in this investigation. The study was approved by the local Medical Ethics Committee at the Karolinska Hospital, Stockholm, Sweden. All subjects had +++ positive patch test reactions (erythema, oedema, papules and vesicles), as according to the International Contact Dermatitis Research Group (ICDRG) (9).

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 3.8×10^{-5} mol/l, which had previously been found to be optimal (10).

Native VIP was purified from porcine upper intestine by sequential chromatography and countercurrent distribution, as described previously (11) and was found to be homogeneous by high performance liquid chromatography and capillary electrophoresis.

5-HT was obtained from Sigma (St Louis, MO, USA), ketanserin (a specific 5-HT₂ receptor blocker) from Janssen Pharmaceutica (Beerse, Belgium), methiotepin (a blocker of both 5-HT_{1c} and 5-HT₂ receptors) from Hoffman-La Roche (Basel, Switzerland) and tropisetron (ICS-205-930, a blocker of 5-HT₃) from Sandoz (Basel, Switzerland).

VIP, 5-HT, ketanserin, methiotepin and tropisetron were dissolved in saline and used for intracutaneous administrations 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 summarized in Fig. 1. VIP ($n=8$), 5-HT ($n=3$) and ketanserin ($n=7$) were injected intracutaneously (i.c.) in

normal skin on the upper part of the patients' backs. The volume injected was 50 µl. 5-HT was only tested in 3 patients and methiotepin and tropisetron were only tried in a pilot study (not shown). It was not possible to use these latter mediators as the patients felt a sharp pain when the solutions were injected. VIP was used at a concentration of 10^{-5} – 10^{-7} mol/l, and 5-HT and ketanserin at 10^{-4} – 10^{-6} mol/l. Saline was used as control. On the same site as the i.c. injection, 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 (Norgeplaster A/S, Oslo, Norway). After 6 h the Finn Chambers with nickel sulphate were removed and again the neuroactive substances or saline were injected with the same procedure as before. After an additional 18-h period the test reactions were measured. Two investigators independently measured the intensity of the test reactions, using the scoring system recommended by the ICDRG, and the maximal diameters of the test reactions, one of the investigators blindly.

Immunohistochemical studies

Biopsy specimens were taken from the test areas for immunohistochemistry after anaesthesia with lidocaine without adrenalin. Each specimen was immediately frozen in liquid nitrogen and stored at -70°C until used. Acetone-fixed cryostat sections, 6 µm thick, were stained with a two-stage monoclonal antibody peroxidase method (12). The panel of the mouse monoclonal antibodies (MAbs) contained Leu 2a (CD 8, T cytotoxic/suppressor cells), Leu 3a (CD 4, T helper/inducer cells), Leu 6 (CD 1a, Langerhans' cells), HLA-DR (activated T-cells, B cells, macrophages, monocytes and Langerhans' cells) and IL-2 receptor (CD 25). These antibodies were all from Becton Dickinson (San José, CA, USA) and the working dilution was 1:100. In addition, expression of intercellular adhesion molecule-1 (ICAM-1, CD 54) was examined with a mouse monoclonal antibody obtained from Serotec (Oxford, UK), used at 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 semi-quantitative scale (– no positive cells, + few, ++ moderate number, +++ many). The size of the infiltrates and the degree of spongiosis in the epidermis were also measured with a semi-quantitative scale, with evaluation of the infiltrates as small, medium or large, and the spongiosis as light, medium or heavy. Two investigators examined all the 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 6 of the 30 nickel-allergic patients. The cells were separated on Percoll gradients on the basis of buoyant density (13) (density of stock solution 1.129 g/ml; Pharmacia Fine Chemicals, Uppsala,

Experimental design

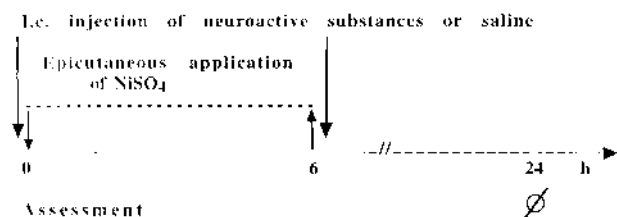


Fig. 1. VIP, serotonin and the serotonin antagonist ketanserin were tested in the challenge phase of allergic contact dermatitis in humans. The neuroactive substances were injected intracutaneously on the backs of patients allergic to nickel. On the same site nickel sulphate was applied under patch test conditions for 6 h. The patch tests were removed and the neuroactive substances and saline were injected again. The diameters of the test reactions were measured after 24 h.

Sweden). They were then cultured in RPMI 1640 medium (Life Technologies, Täby, Sweden) with addition of L-glutamine (2 µmol/ml), streptomycin (100 µg/ml), penicillin (100 IU/ml) and 10% heat-inactivated human AB serum at a concentration of 2×10^6 cells/ml. The cells were subsequently incubated in 100 µl cultures for 6 days in a Linbro microtitration plate at 37°C in an atmosphere of 5% CO_2 in air. After preincubation for 30 min, 20 µl of the nickel sulphate solution was added. After another 30 min, VIP, 5-HT and 5-HT antagonists were added in a volume of 20 µl and with saline as control. Six hours before interruption of the cultures, 0.5 µCi of ^3H -thymidine (5 Ci/mmol; Amersham International, Amersham, UK) in 10 µl saline was added to each well. The cells were then collected on glass fibre filters using a Skatron multiple-cell collector. Radioactivity was determined by counting in a Packard liquid scintillation spectrometer. The proliferative rate was measured in counts per minute (cpm). The cultures were performed in triplicate.

Interferon gamma and IL-4 assay

On the 6th day of incubation with nickel sulphate and the neuroactive substances, supernatants were obtained from the peripheral blood mononuclear cell cultures and immediately stored at -70°C . The concentrations of interferon gamma and IL-4 were determined by a sandwich ELISA (Pharmingen, San Diego, CA, USA), following the recommendation of the manufacturer. Briefly, flat bottom high binding plates (Costar, Cambridge, MA, USA) were coated, by incubation overnight at 4°C , with anticytokine MAbs diluted in binding solution, 50 µl per well. Sites for non-specific binding of protein were blocked with PBS/10% foetal calf serum (FCS), 200 µl per well, for 30 min (interferon gamma) or for 2 h (IL-4). Samples in duplicate and medium blanks were added, 100 µl per well, and a standard curve was constructed for each plate and incubated overnight. After washing, a biotinylated anticytokine detection MAb, diluted in PBS/10% FCS, was added in a volume of 100 µl per well. After incubation for 1 h at room temperature, 100 µl of avidin-peroxidase (Sigma, St Louis, MO, USA, 1:400 dilution of a 1 mg/ml stock solution) per well was added and the plates were incubated at room temperature for 30 min. For detection, the peroxidase substrate 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS) (Sigma) was used. The plates were read within 30 min at a wavelength of 405 nm by an ELISA reader (Vmax, Molecular Device Corporation, CA, USA).

IL-2 assay

After 48 h of incubation the concentrations of IL-2 were determined by a Duo Set ELISA (Genzyme Diagnostics, Cambridge, MA, USA), using the protocol supplied by the manufacturer. In brief, Costar plates were coated with capture antibody diluted in 0.1 M sodium carbonate, pH 9.5, 100 µl per well, and incubated overnight at 4°C . The plates were blocked with 4% bovine serum albumin in PBS, pH 7.3, 0.01 M, 250 µl per well and incubated for 2 h at 37°C . After washing diluted standards and samples in duplicate were added, 100 µl per well, and incubated for 1 h at 37°C . A volume of 100 µl of the diluted second antibody was then added and incubated for 1 h at 37°C . After this step 100 µl per well of the diluted detecting reagent was added and incubated for 15 min at 37°C . Finally tetramethyl benzidine and hydrogen peroxidase were added, 100 µl per well, and incubated for 10 min at room temperature. The plates were read at 450 nm within 30 min.

IL-2sR assay

At 2 days the concentrations of soluble IL-2sR were measured by ELISA using the human IL-2sR Immunoassay Quantikine kit (R&D Systems, Minneapolis, MN, USA), as recommended by the manufacturer. The plates were read at 450 nm.

Statistical analysis

Analyses of variance for repeated measurements (ANOVA) were used for statistical analyses. In order to determine the significance level in the pairwise comparisons, the method proposed by Dunnett was used (14).

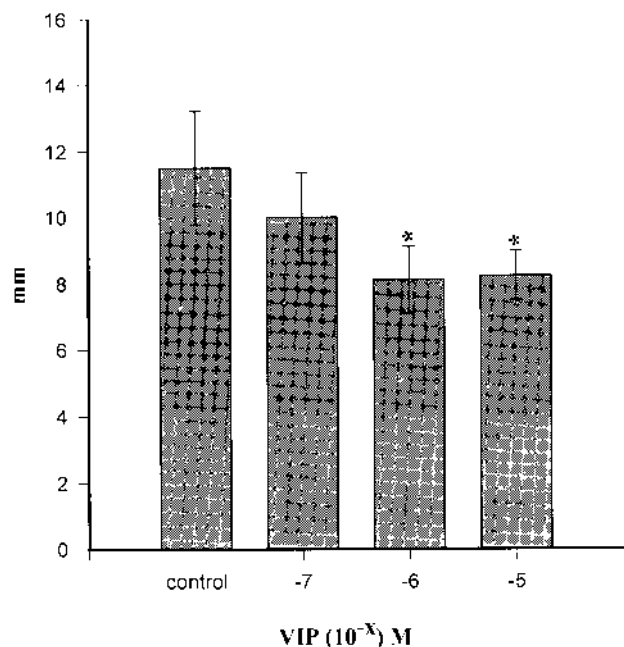


Fig. 2. The diameter of the nickel sulphate-induced test reactions modulated by intracutaneous injection of VIP at different concentrations and of saline (control). Each value represents the mean ($\pm$ SEM) of 8 experiments. Difference from control at $*p < 0.05$.

RESULTS

Patch testing

The diameter of the nickel sulphate-induced test reaction was significantly reduced ($p < 0.05$) after injection of VIP at concentrations of 10^{-5} and 10^{-6} mol/l (Fig. 2 and 3), however, no effect on the ICDRG scores was found. No effect was observed with 5-HT or ketanserin.

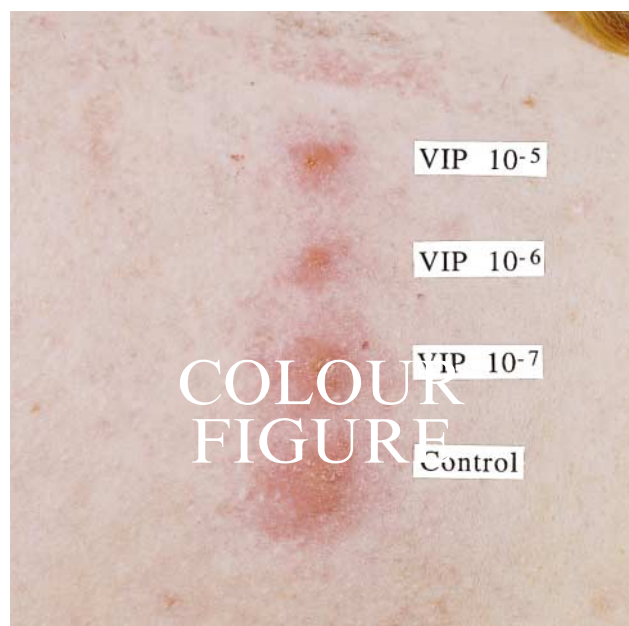


Fig. 3. One of the nickel-allergic patients with patch test-induced reactions modulated with different concentrations (mol/l) of VIP and saline (control).

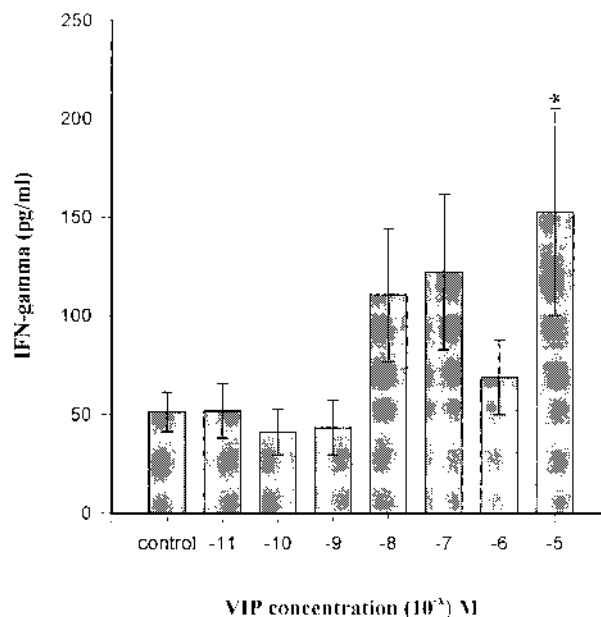


Fig. 4. The concentrations of interferon gamma (pg/ml) were measured by ELISA in the supernatants of lymphocyte cultures on day 6 of stimulation by nickel sulphate. VIP at different concentrations was added at the beginning of the culture. Each value represents the mean value ($\pm$ SEM) of 6 experiments. Difference from control at $*p < 0.05$.

Immunohistochemical studies

The histological picture was typical for inflammation in allergic contact eczema, with infiltrates of mononuclear cells in the dermis and spongiosis of the epidermis. In the infiltrates there was a predominance of CD4⁺ cells. HLA-DR was upregulated on the infiltrating cells, as well as ICAM-1, which was also found adjacent to the vessels and in the epidermis. Sometimes keratinocyte HLA-DR expression was also seen.

There were no differences in the number of different phenotypes of the infiltrating cells or in the size of the cellular infiltrate, or the degree of spongiosis, after injection of VIP, 5-HT and ketanserin. Neither did the number of cells with expression of IL-2 receptor or of HLA-DR show any difference and there was no difference in the epidermal level of ICAM-1 expression between neuroactive substance-treated and control skin (not shown).

DNA synthesis

Compared with saline, nickel sulphate had a distinct stimulatory effect on the proliferative response of the blood mononuclear cells, with a wide individual variation (1, 144–28, 200 cpm). VIP had no effect on the proliferative response, while 5-HT, ketanserin and tropisetron each had an inhibitory effect at 10^{-4} mol/l ($p < 0.05$). Methiotepin had an inhibitory effect at 10^{-4} and 10^{-5} mol/l ($p < 0.05$).

Interferon gamma secretion

There was a wide individual variation in the values for interferon gamma secretion when nickel sulphate was added together with saline, with a range of 85–668 pg/ml. VIP, at a

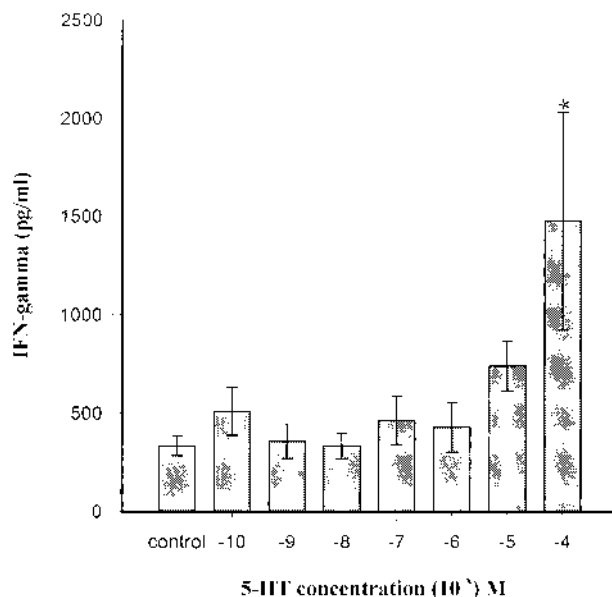


Fig. 5. The concentrations of interferon gamma (pg/ml) were measured by ELISA in the supernatants of lymphocyte cultures on day 6 of stimulation by nickel sulphate. 5-HT at different concentrations was added at the beginning of the culture. Each value represents the mean value ($\pm$ SEM) of 6 experiments. Difference from control at $*p < 0.05$.

concentration of 10^{-5} mol/l, and 5-HT, at 10^{-4} mol/l, had a significant ($p < 0.05$) stimulatory effect on the secretion of interferon gamma (Fig. 4 and 5). Ketanserin had no effect, while methiotepin and tropisetron showed an inhibitory effect at 10^{-4} mol/l ($p < 0.05$) (not shown).

IL-2 secretion

The IL-2 concentrations also varied considerably. When nickel sulphate and saline were added, the values ranged from 45 to 1,159 pg/ml. VIP, 5-HT, ketanserin and tropisetron had no effect on the secretion of IL-2. Methiotepin had an inhibitory effect at 10^{-4} mol/l ($p < 0.05$) (not shown).

IL-2sR secretion

The secretion of IL-2sR after addition of nickel sulphate and saline varied from 66 to 276 pg/ml. VIP had no effect on this secretion, while 5-HT (10^{-4} mol/l) and ketanserin (10^{-6} mol/l) showed a slight stimulatory effect. Methiotepin and tropisetron had an inhibitory effect at 10^{-4} mol/l ($p < 0.05$) (not shown).

IL-4 secretion

The concentrations of IL-4 were very low when nickel sulphate and saline were added, and all values were below 20 pg/ml. The neuroactive substances had no effects (not shown).

DISCUSSION

In the present study, VIP, at 10^{-5} – 10^{-6} mol/l, injected intracutaneously before and after the application of nickel sulphate by the patch testing procedure in nickel-allergic patients,

caused a significant reduction in the diameter of the test reaction. It had no effect on the size of the infiltrate, the grade of spongiosis or the phenotypes of the infiltrating mononuclear cells. The secretion of interferon gamma was increased when VIP was added at 10^{-5} mol/l. VIP had no effect on the secretion of IL-2, IL-2sR or IL-4, or on the proliferative response.

VIP has been found to have a protective effect against inflammatory injury or toxicity due to a variety of agents (15–17). It has also shown protective effects on lymphocytes against metal toxicity (18). Bandypadhyay et al. (19) concluded that VIP may play a significant protective role in the gastrointestinal tract against the damaging effects of nitric oxide induced by cytotoxic agents. In a murine model of inflammation in lung parenchyma, VIP exerts a late dampening effect on the pulmonary immune response (20).

VIP is known to have an inhibitory action on the proliferation of T lymphocytes and also on the IL-2 production in murine splenic or lymph node T lymphocytes stimulated with concanavalin A (Con A) (21–23). Few studies have used antigen-sensitized T-cells, as we have done in this investigation. Using antigen-sensitized T-cells, Nio et al. (24) did not find any significant effect of VIP, substance P (SP) or somatostatin upon the proliferation of AO40.1 cells. VIP stimulated the IL-2 release at low concentrations in this system, with a marked effect at 10^{-14} mol/l.

Interferon gamma is another cytokine responsible for the Th1 response, known to be involved in the delayed-type hypersensitivity reaction. VIP has been reported not to affect interferon gamma production by murine T lymphocytes stimulated with Con A, anti-CD3, or anti-CD3 plus phorbol 12-myristate-13-acetate (PMA) (23, 25). However, human peripheral blood lymphocytes stimulated with the polyclonal activator staphylococcal enterotoxin A showed a reduced production of interferon gamma with a maximum effect with VIP at 10^{-8} – 10^{-9} mol/l (26).

It has been suggested that interferon gamma may be important, either directly or indirectly, in both the initial resistance to and the resolution of infection with cryptosporiosis in mice (27). Interferon gamma also assists in the resolution of inflammation, partly by induction of apoptosis in lymphocytes (28). Scheynius & Skoglund (29) showed that interferon gamma may upregulate or downregulate the immune response depending on the pathological state of the recipient. In their study a pronounced reduction of the inflammatory response was observed when interferon gamma was given prior to elicitation. This might possibly explain our findings that VIP reduced the diameter of the test reaction, as an effect exerted through stimulation of the interferon gamma production by the leukocytes. Interferon gamma may have a dual role in the allergic contact reaction, upregulation of the immune response in the sensitization phase and downregulation in the elicitation or challenge phase.

The negative effect of VIP in the immunohistochemistry study might be explained by the fact that the patients only had patch tests with nickel sulphate for 6 h instead of 48 h. The number of infiltrating inflammatory cells producing cytokines does not reach a maximum before this latter time point (30).

We found that serotonin and its antagonists at a concentration of 10^{-4} mol/l (also methiotepine at 10^{-5} mol/l) had an inhibitory effect on the cell proliferation. This effect has previously been found to be toxic, as judged by the uptake of try-

pan blue (8). The most pronounced inhibition was observed with methiotepin and tropisetron.

In the present study, serotonin at a concentration of 10^{-4} mol/l significantly stimulated the secretion of interferon gamma and IL-2sR. Serotonin has been reported to promote the production of interferon gamma in natural killer cells in the presence of monocytes (31). Serotonin has also shown a stimulatory effect on murine splenic T-cells, with enhanced proliferation and IL-2 production (32). Production of Th1 cytokines, such as IL-2 and interferon gamma, by antigen-stimulated immune murine spleen cells is inhibited by 5-HT receptor antagonists *in vitro* (33). In the present investigation we noted that ketanserin caused a small increase in IL-2sR. This may be due to some agonistic effect of this serotonin antagonist.

In conclusion, our results indicate that VIP, when injected intracutaneously in the challenge phase of allergic contact dermatitis, has an inhibitory effect, possibly mediated by an enhanced leukocyte production of interferon gamma. This hypothesis for the mechanism of the immunomodulatory role of VIP needs further investigation.

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