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Disturbed immunoregulatory properties of the neuropeptide substance P on lymphocyte proliferation in HIV infection

M. J. COVAS, L. A. PINTO & R. M. M. VICTORINO *Faculty of Medicine of Lisbon, University Hospital of Santa Maria, Medicine 2 and Clinical Immunology, Lisbon, Portugal*

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SUMMARY

The neuropeptide substance P (SP) is known to increase cell-mediated immune responses in animal models and healthy subjects. Several studies have suggested an involvement of neuropeptides in the immunopathogenesis of some diseases. The study of the immunomodulatory effects of neuropeptides, namely SP, may represent a model for the analysis of immunoregulatory defects in HIV infection at the level of the interaction between the immune and nervous systems, both of which are known to be affected by the virus. In the present study, we investigate the possibility of a disturbance in the immunomodulatory properties of SP in HIV infection by analysing the effects of SP (10^{-10}–10^{-6} M) on the lymphocyte proliferative responses to concanavalin A (Con A) and phytohaemagglutinin (PHA) assessed by ^{3}H-thymidine incorporation in peripheral blood lymphocytes from 34 HIV-infected patients (16 asymptomtic (ASY)/persistent generalized lymphadenopathy (PGL); 18 ARC/AIDS) and in 37 healthy subjects. In ASY/PGL HIV-infected patients, SP 10^{-7} M was identified as the concentration inducing the maximal increase in the lymphocyte responses to Con A and PHA, similarly to what was observed in healthy subjects. In ARC/AIDS patients, SP appeared to inhibit the mitogenic responses, particularly those induced by Con A, in contrast to the effects found either in healthy subjects or in ASY/PGL patients. These results suggest the existence of an alteration in the *in vitro* immunomodulatory properties of SP in ARC/AIDS patients compared with healthy subjects and ASY/PGL patients. In conclusion, the unexpected finding of an inhibitory effect of SP on lymphocyte proliferation from ARC/AIDS patients justifies further investigation of the neuropeptide-dependent immunoregulatory systems in HIV infection.

Keywords substance P neuropeptides lymphocyte proliferation HIV infection AIDS

INTRODUCTION

There is increasing evidence for a functional link between the immune and nervous systems [1]. Neuropeptides have been considered important mediators of this interaction since it has been reported that they can modulate several immune functions [2] and may be involved in the pathogenesis of some immunological diseases, both in experimental models and in humans [3–5].

HIV infection is characterized by a severe progressive immunosuppression as a result of a quantitative depletion of $CD4^{+}$ T lymphocytes as well as several functional abnormalities [6]. Thus, the study of immunoregulatory properties of neuropeptides could represent a model for the analysis of immunoregulatory defects in HIV infection at the level of the interaction between the immune and nervous systems, both of which are known to be affected by the HIV [7].

Substance P (SP) is an 11 amino acid neuropeptide widely distributed in the central nervous system (CNS) and peripheral sensory nerves [8]; it has been described as an immunostimulatory peptide which could modulate the function of several immune cells, including T and B lymphocytes, macrophages/monocytes and polymorphonuclear leucocytes [2]. Recent studies have shown that SP can enhance the T cell production of IL-2 in mitogen-activated peripheral blood T lymphocytes as well as in human and murine T cell lines responding to mitogen or specific antigens [9–11]. In addition, several authors have described an enhancing effect of SP on mitogen-induced proliferation [12–14] and, although others did not detect such an effect [15,16], this neuropeptide is generally considered as a stimulatory immunomodulator [2].

The present study was therefore performed to evaluate the

Correspondence: Professor Rui M.M. Victorino, Faculty of Medicine of Lisbon, Medicine 2, Av. Prof. Egas Moniz, 1600 Lisbon, Portugal.

possibility of an alteration in the immunomodulatory properties of SP in HIV infection by analysing the effect of this neuropeptide on mitogen-induced lymphocyte proliferation in patients in different stages of disease.

PATIENTS AND METHODS

Patients and controls

Thirty-four patients seropositive for HIV infection (23 male, 11 female) by ELISA testing and Western blot confirmation, with a median age of 39 years (range 21–65 years), were studied. Thirty-seven seronegative healthy controls with a median age of 36 years (range 22–65 years) were also evaluated. HIV patients were stratified in two subgroups based on clinical data and the Centre for Disease Control (CDC) classification criteria. There were 16 seropositive asymptomatic (ASY)/persistent generalized lymphadenopathy (PGL) patients (eight male, eight female) and 18 ARC/AIDS patients (15 male, three female).

Reagents

The neuropeptide SP (Sigma, St Louis, MO) was dissolved in degassed sterile water, aliquoted at 10^{-3} M and stored at −70°C until use. SP was diluted with RPMI 1640 (Gibco, Grand Island, NY) to working concentrations ranging from 10^{-10} to 10^{-6} M immediately before addition to lymphocyte cultures.

The T cell mitogens phytohaemagglutinin (PHA-P) and concanavalin A (Con A) (Sigma) were used at final culture concentrations of 28 μg/ml and 10 μg/ml, respectively.

Isolation of mononuclear cells

Heparinized venous blood was collected from patients and controls. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll–Hypaque (Gibco) density gradient. Cells were washed three times in PBS (Biomérieux, Marcy L'Etoile, France) and resuspended in RPMI 1640 (Gibco) supplemented with 10% inactivated fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Flow, McLean, VA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco).

Lymphocyte proliferation assays

The assessment of lymphocyte proliferation was done as previously described [17]. Briefly, PBMC (1×10^5/well) were cultured in quadruplicate in round-bottomed 96-well microtitre plates (Costar, Cambridge, MA) and stimulated with optimal doses of PHA-P or Con A in the presence or absence of SP ranging from 10^{-10} to 10^{-6} M. Cultures were incubated at 37°C in 5% CO_2 humidified atmosphere for 3 days. Six hours before the end of the cultures, 1 μCi of ^{3}H-thymidine (specific activity 5 Ci/mmol; RCC, Amersham, UK) was added to each well. Cells were harvested with an automatic harvester (Skatron, Lier, Norway) and ^{3}H-thymidine incorporation was quantified in a liquid scintillation counter (Beckman, Fullerton, CA). Cell viability in the presence of SP, as determined by trypan blue exclusion, was above 90% in all cultures tested.

The effect of SP on mitogen-induced proliferation was expressed as the mean percentage of change in the ^{3}H-thymidine incorporation ± s.e.m.:

$$\text{Per cent change} = \frac{\text{ct/min in cultures with SP} - \text{ct/min in cultures without SP}}{\text{ct/min in cultures without SP}} \times 100$$

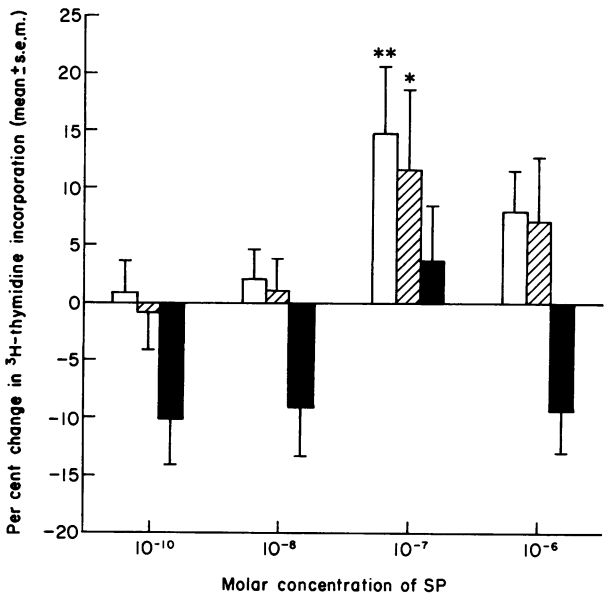

Fig. 1. Effects of substance P (SP) on concanavalin A (Con A)-induced lymphocyte proliferation in healthy controls and HIV-infected subjects. Peripheral blood mononuclear cells (PBMC) (1×10^5/well) were cultured with various concentrations of SP and stimulated with 10 μg/ml Con A. Proliferative responses were determined at day 3 by ^{3}H-thymidine incorporation. Data are expressed as percentage of change from control cultures (mean ± s.e.m.). $^*P < 0.05$; $^{**}P < 0.02$ compared with the control cultures incubated with medium. □, Healthy controls; ▨, asymptomatic (ASY)/persistent generalized lymphadenopathy (PGL); ■, ARC/AIDS.

Flow cytometric analysis

T cell subsets were determined by flow cytometry in an EPICS Profile flow cytometer (Coulter, Hialeah, FL) using directly labelled anti-CD4 and anti-CD8 MoAbs (Dako, Dakopatts, Denmark). Briefly, PBMC (2×10^5 cells) were incubated for 30 min at 4°C with anti-CD4 or anti-CD8 labelled with FITC. After gating for the lymphocytes on forward and right angle light scatter parameters, at least 10000 cells/sample were analysed.

Statistical analysis

Statistical significance was assessed by paired *t*-test and statistical comparisons were made by Student's *t*-test. Correlations were calculated by linear regression analysis. $P < 0.05$ was considered significant.

RESULTS

Effects of SP on lymphocyte proliferation

In order to determine the immunomodulatory properties of SP in HIV infection we analysed the effect of this neuropeptide on spontaneous and mitogen-induced proliferation.

Figure 1 shows that SP (ranging from 10^{-10} to 10^{-6} M) enhanced in a dose-dependent manner the Con A-induced PBMC proliferation both in ASY/PGL patients and in healthy controls. This increase was maximal and statistically significant at the concentration of 10^{-7} M (11·6 ± 6·6%, $P < 0.05$ for

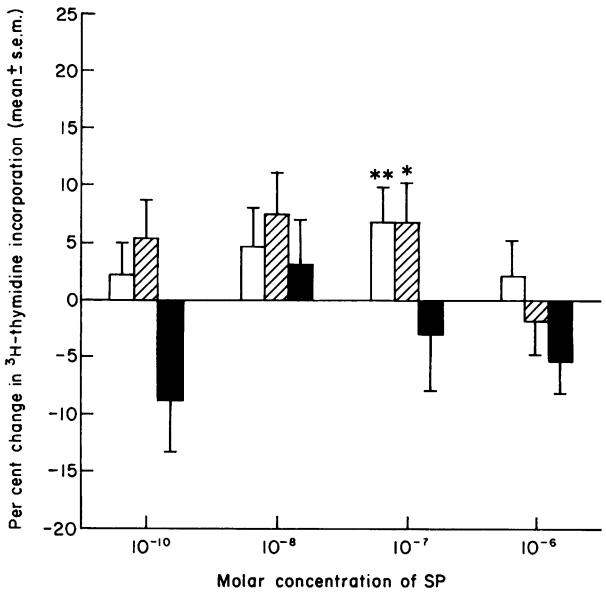

Fig. 2. Effects of substance P (SP) on phytohaemagglutinin (PHA)-induced lymphocyte proliferation in healthy controls and HIV-infected subjects. Peripheral blood mononuclear cells (PBMC) (1×10^5/well) were cultured with various concentrations of SP and stimulated with 28 μg/ml PHA. Proliferative responses were determined at day 3 by ^{3}H-thymidine incorporation. Data are expressed as percentage of change from control cultures (mean ± s.e.m.). $^*P < 0.05$; $^{**}P < 0.01$ compared with the control cultures incubated with medium. □, healthy controls; ▨, asymptomatic (ASY)/persistent generalized lymphadenopathy (PGL); ■, ARC/AIDS.

ASY/PGL; 14·8 ± 5·9%, $P < 0.02$ for controls) and, although there was a considerable individual variability, an enhancing effect prevailed in most concentrations tested. In contrast, in ARC/AIDS patients the addition of SP to the cultures inhibited the Con A-induced proliferation in three of the four concentrations tested (Fig. 1), reaching a statistically significant decrease at SP 10^{-8} M (−9·1 ± 4·6%, $P < 0.05$). Incubation of SP 10^{-7} M resulted in a slight enhancement of proliferation. Moreover, the comparison of the immunomodulatory effects of SP between HIV-infected patients and healthy controls showed that the mean effect of the neuropeptide was significantly different for SP 10^{-10} M ($P < 0.05$), 10^{-8} M ($P < 0.05$) and SP 10^{-6} M ($P < 0.001$) in ARC/AIDS patients *versus* controls.

The effect of SP on PHA-induced proliferation of ASY/PGL and normal PBMC (Fig. 2) was quite similar to those seen in Con A-induced proliferation; SP 10^{-7} M was also identified as the concentration inducing a statistically significant increase in the lymphocyte response to PHA (6·8 ± 3·5%, $P < 0.05$ for ASY/PGL; 6·8 ± 2·8%, $P < 0.01$ for controls). In ARC/AIDS patients SP exhibited an inhibitory effect at SP concentrations of 10^{-10}, 10^{-7} and 10^{-6} M which did not reach statistical significance (Fig. 2). SP 10^{-8} M induced a marginal enhancing effect. The comparison of the immunomodulatory effects of SP in HIV-infected patients *versus* healthy controls revealed that the mean effect of SP is significantly different for SP 10^{-10} M ($P < 0.05$) in the ARC/AIDS subgroup of patients.

The inhibitory effect of SP could not be explained by loss of cellular viability, as this was not affected by concentrations of SP up to 10^{-6} M. In addition, we observed that SP in all of the concentrations tested had no effect on spontaneous proliferation both in HIV-infected patients and healthy controls (data not shown). The mean basal ^{3}H-thymidine incorporation found was 2358 ct/min (range 683–6189 ct/min) in healthy controls, 1349 ct/min (940–3243 ct/min) in ASY/PGL patients, and 720 ct/min (341–1243 ct/min) in ARC/AIDS patients.

Correlation between the effects of SP and T cell subsets

In order to characterize the T cell composition of PBMC from HIV-infected patients we determined the $CD4^+$ and $CD8^+$ T cell subsets in all patients studied. As expected, patients with ARC/AIDS had a marked depletion of CD4 T lymphocytes (12·8 ± 10·9% (mean ± s.d.)), as well as increases in CD8 levels (48.5 ± 14·7%), while ASY/PGL subjects showed a less pronounced alteration of these T cell subsets (29·9 ± 7·4% for CD4; 37·4 ± 11·4% for CD8).

Analysis of correlation between the percentage of $CD4^+$ T cell subset and the immunomodulatory effects of SP in all HIV^+ subjects showed a positive correlation between the percentage of CD4 and the effects of SP 10^{-8} M ($r = 0.384, P < 0.05$) and SP 10^{-7} M ($r = 0.394, P < 0.05$) on Con A- and PHA-induced proliferation, respectively. Correlation between percentage of CD8 lymphocytes and the effect of different concentrations of SP on mitogenic proliferative responses was not statistically significant, except for SP 10^{-7} M on PHA-stimulated cultures, which revealed a negative correlation ($r = -0.388, P < 0.05$).

DISCUSSION

Since neuropeptides have been shown to regulate immune responses it is important to investigate whether disturbances in neuropeptide-dependent immunoregulatory systems are present in patients with HIV infection, particularly in view of the fact that both immune and nervous systems are affected by the virus [7].

The main finding of this study was the demonstration of an inhibitory effect of the neuropeptide SP on the mitogen-induced lymphocyte proliferation in ARC/AIDS patients, which was significantly different from controls. In contrast, in ASY/PGL-infected subjects SP enhanced in a dose-dependent manner the proliferative responses to PHA and Con A similarly to what was observed in healthy controls.

Most authors have reported enhancing effects of SP in mitogen- and antigen-induced lymphocyte proliferation [12–14] or IL-2 production [9–11], although some have not confirmed such results [15,16]. Our data in healthy subjects show a slight enhancing effect of SP, as well as a marked variability of this effect among individuals, and this agrees with data from other workers who have documented a pronounced interindividual variability in the effects of several neuropeptides [18–20]. However, in spite of this variability in the effect of SP both in controls and in HIV-infected patients, a clearly significant difference was found between ARC/AIDS patients and healthy subjects. Although our experiments were not designed to elucidate the mechanisms underlying the inhibitory effect of SP on mitogen-induced proliferation in ARC/AIDS patients, several explanations can be considered.

First, it has been well recognized that the final effect of a neuropeptide on a given immune effector response can be the

result of distinct effects of the peptide on different cell types, particularly on T lymphocytes and monocytes [20–22]. This has been documented with β-endorphin (β-end), where Peterson *et al.* showed that the suppression of the lymphocyte activation and interferon-gamma (IFN-γ) production was due to opioid-induced generation of inhibitory monocyte metabolites such as prostaglandin E_2 (PGE_2) [21]. Furthermore, Millar *et al.* reported variable effects (positive or negative) of β-end on lymphocyte proliferation, which could depend in part on which modulatory effect was dominant, namely a positive effect in lymphocytes *versus* a negative one on monocytes [20]. Finally, in a murine system, Stanisz and co-workers suggested that the inhibitory effects of SP on lymphocyte proliferation observed in certain experimental conditions could be due to the release of inhibitory factors from monocytes [22]. Thus, it is possible to speculate that the inhibitory effect observed in our ARC/AIDS patients could be the consequence of a predominant negative effect on proliferation via monocytes. Interestingly, recent studies in HIV infection indicate that increased amounts of PGE_2 are released by monocytes, and that this mediator could be implicated in the inhibition of antigen or mitogen-induced lymphocyte proliferation [23,24]. On the other hand, since Pascual & Bost [25] have shown that SP can be produced by some macrophage cell lines, and thus could act in an autocrine fashion to modulate macrophage function, we can hypothesize that in our system monocyte-derived SP might also contribute to the inhibition of lymphocyte proliferation observed in HIV-infected patients in advanced stages of the disease.

Second, as the percentage of $CD4^+$ T cells is markedly reduced in ARC/AIDS patients, it is possible to speculate that if the positive effects of SP were essentially on $CD4^+$ lymphocytes, this could explain a predominant inhibitory effect as a consequence of disturbed T cell subset distribution in HIV infection. In this respect it is interesting that in healthy subjects the percentage of $CD4^+$ T cells expressing specific SP receptors is higher than in $CD8^+$ T cells [26]. On the other hand, the finding of a positive correlation between the percentage of $CD4^+$ T lymphocytes and the magnitude of the SP effect in HIV patients could be consistent with this possibility, although studies with purified T cell subsets are required to investigate this hypothesis.

The concentrations of SP at which we observed the more significant inhibitory effects are slightly higher than the physiologic concentrations found in the blood of healthy subjects [27]. However, Azzari *et al.* have recently shown that the plasma levels of SP are increased in HIV-infected children [28], and thus the concentrations of SP with inhibitory properties may occur in cases of HIV infection. On the other hand, the degradation of SP depends on the activity of several enzymatic systems such as dipeptidyl peptidase IV, recently identified as the CD26 molecule present in T lymphocyte membranes [29,30]. In this respect it is interesting to note that a reduction in the number of $CD26^+$ T lymphocytes has been reported in HIV-infected patients [31,32].

In conclusion, the fact that a positive immunoregulatory neuropeptide such as SP exhibits inhibitory effects on a T cell response in ARC/AIDS patients justifies further attention to this phenomenon that could contribute to the immunosuppression observed in advanced stages of HIV infection.

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