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Selective effects of vasoactive intestinal peptide on the mitogenic response of murine T cells

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SUMMARY

Murine lymphocytes have been shown previously to possess high-affinity specific receptors for the neuropeptide vasoactive intestinal peptide (VIP). This study examines the cellular basis for modulation of concanavalin A (Con A)-induced T-cell responses by this neuropeptide. VIP was most effective as an inhibitor when added at the initiation of the mitogen response. The loss of potency when VIP was added later in the response was accompanied by a decrease in the affinity of stimulated cells for the neuropeptide. The inhibitory influence of VIP was reversible if the neuropeptide was removed from stimulated cell cultures up to 6 hr after the initiation of stimulation. In contrast, VIP-mediated inhibition was fully developed once the stimulated cells had been exposed to the neuropeptide for 24 hr. The presence of VIP led to a decreased production of interleukin-2 (IL-2) by the stimulated lymphocytes, but did not affect the expression of Con A-induced suppressor cell activity by cultured lymphocytes. Studies of the effect of selective, complement-mediated killing of cells with Thy 1, L3T4 and Lyt 2 monoclonal antibodies showed that the majority of the VIP bound by the lymphocytes was accounted for by binding to $L3T4^+$, $Lyt\ 2^-$ T cells. It was concluded that VIP exerts its influence over Con A-stimulated proliferation by selective regulation of T-cell subsets.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is a 28 amino acid neuropeptide found within neurons of both the central and peripheral nervous systems (Hokfelt *et al.*, 1978; Larrson *et al.*, 1979; Polak & Bloom, 1982) that has been implicated as an immunoregulatory peptide in both mouse and man. Specific receptors for VIP have been demonstrated on T cells of the secondary lymphoid tissues of mice (Ottaway & Greenberg, 1984) and on human peripheral blood mononuclear cells (Guerrero *et al.*, 1981; Ottaway *et al.*, 1983), human peripheral blood T cells (Danek *et al.*, 1983), and a human T cell-derived culture line (Molt 4b) (Beed *et al.*, 1983). The addition of VIP to cultures of lymphocytes from murine secondary lymphoid organs results in dose-dependent inhibition of the proliferative response of these cells to the T-cell mitogens concanavalin A (Con A) and phytohaemagglutinin (PHA) (Ottaway & Greenberg, 1984), and immunoglobulin production by Con A-stimulated murine lymphocyte cultures can be modulated by VIP (Stanisz, Befus & Bienenstock, 1986).

The aim of the studies reported here was to delineate further the cellular means through which the interaction of lymphocytes with the neuropeptide VIP alters the mitogen response of T cells. The effect of VIP on the response of mesenteric lymph node cells to Con A was chosen for detailed examination for two reasons. First, VIP receptors are well represented on these cells and appear to be similar to those occurring on T cells throughout the secondary lymphoid organs (Ottaway & Greenberg, 1984). Second, although all of the mechanisms contributing to T-cell activation are not yet understood, many aspects of the events involved in the response of lymphocytes to Con A stimulation are now well characterized.

The results suggest that VIP inhibition of the proliferative response to Con A is largely due to selective interference with the response of $L3T4^+$ helper/inducer cells. These studies support the notion that the interaction of VIP with selected subpopulations of T cells may be important in the neurophysiological regulation of immune responses *in vivo*.

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MATERIALS AND METHODS

Mice

Female BALB/c mice 6–8 weeks of age purchased from Canadian Breeding Laboratories (Montreal, Quebec) were used throughout the studies.

Cell suspensions

Single cell suspensions of lymphocytes from mesenteric lymph nodes were prepared at room temperature by gentle teasing of the tissues in RPMI-1640 medium (Gibco Canada Ltd, Burlington, Ontario) containing 5% fetal calf serum (FCS) as described elsewhere (Ottaway & Greenberg, 1984).

Mitogen-stimulated cultures

Lymphocytes were washed three times in RPMI-1640 and resuspended in complete medium consisting of RPMI-1640 medium supplemented with 10% FCS, penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St Louis, MO). Microcultures were performed in flat-bottomed plates (Falcon 3070; Falcon Plastics Co., Oxnard, CA) at a final concentration of $2{\cdot}5 \times 10^5$ cells/ml in 0·2-ml volumes. Cultures were stimulated with maximal stimulatory concentrations of concanavalin A (Con A; 2·5 μg/ml; Sigma) as described elsewhere (Ottaway & Greenberg, 1984). Cultures were incubated at 37° in a 5% CO_2 atmosphere for 3 days and labelled by adding 1 μCi [^{3}H]TdR/well (15 Ci/mmol; New England Nuclear Corp., Lachine, Quebec) for a further 18 hr of incubation. The cultured cells were harvested onto glass-fibre filters (Titertek, Skatron, Flow Lab., Rockville, MD) and the radioactivity was determined in a Packard Tricarb scintillation counter. Cultures were performed in quadruplicate and the results were expressed as the difference between the excess of c.p.m. in mitogen-stimulated versus mitogen-unstimulated cultures.

For experiments in which the reversibility of the effect of VIP, or the effect of stimulation of cells on ^{125}I-VIP binding, was studied, cells were cultured in 17 × 100 mm polypropylene tubes (Falcon 2059; Falcon Plastics) in 12-ml volumes in complete medium with or without Con A (2·5 μg/ml) and VIP for various lengths of time. The cells were then washed three times in RPMI containing 30 mM alpha-methyl-mannoside (Sigma) at 37° and resuspended in either complete medium for microcultures, or in binding medium.

Suppressor cell activity assay

The suppressive activity of Con A-treated cultured lymphocytes was assessed using modifications of described methods (Rich & Rich, 1975; Jandinski *et al.*, 1976). Lymphocytes, 3×10^6 cells/ml, were incubated in complete medium in 17 × 100 mm tubes at 37° for 48 hr without Con A (control cells), or without Con A in the presence of 10^{-7} M VIP, or with Con A in the presence or absence of 10^{-7} M VIP. Afterwards the cells were harvested and treated with mitomycin *C* (50 μg/ml; Sigma; Swain *et al.*, 1977) for 30 min at 37° and then washed three times at 37° with RPMI-1640 containing 30 mM alpha-methyl-mannoside and resuspended in complete medium. These cells were then co-cultured in quadruplicate samples at a final concentration of $2{\cdot}5 \times 10^5$ cells/ml with fresh responder lymphocytes at $2{\cdot}5 \times 10^5$ cells/ml in the presence or absence of Con A (2·5 μg/ml) in 0·2 ml volumes in complete medium. The cells were cultured, labelled, harvested and counted as described above. The percentage suppression of the responder cells was calulated as follows:

$$\% \text{ suppression} = 100 - (\% \text{ of control})$$

where the percentage of control =

$$\frac{(\text{c.p.m.} + \text{test cells} + \text{Con A} - \text{c.p.m.} + \text{test cells} - \text{Con A})}{(\text{c.p.m.} - \text{test cells} + \text{Con A} - \text{c.p.m.} - \text{test cells} - \text{Con A})} \times 100.$$

Measurement of IL-2 activity

Supernatants were collected by centrifugation and millipore filtration of lymphocyte suspensions cultured at $2{\cdot}5 \times 10^5$/ml in 3-ml volumes in complete medium with or without Con A (2·5 μg/ml) and VIP for various lengths of time. These supernatants were kept frozen at −20° until assayed for T-cell growth-stimulating activity of IL-2 using the IL-2-dependent T-cell line HT-2 (Watson, 1979). Two-fold serial dilutions in culture medium were added in 0·1 ml to microtitre wells containing 10^4 HT-2 cells in 0·1 ml. These were incubated for 18 hr and labelled by adding 1 μCi [^{3}H]TdR/well for 6 hr and then harvested and counted as above. Supernatants from the gibbon cell line MLA-144 grown to saturation were collected, filter sterilized, and used as a source of IL-2 to maintian the HT-2 line, and as a positive control for IL-2-induced proliferation of the HT-2 microcultures.

Vasoactive intestinal peptide

Synthetic VIP was purchased from Peninsula Laboratories (San Carlos, CA). Radio-iodinated VIP with a specific activity of 1 nCi/fmol was prepared as described previously (Ottaway *et al.*, 1983).

Binding studies

Binding studies were carried out using $7{\cdot}5 \times 10^{-11}$ M labelled VIP in the presence or absence of unlabelled VIP in binding medium consisting of RPMI-1640 containing 5% bovine serum albumin (Sigma) as described previously (Ottaway & Greenberg, 1984). Briefly, lymphocytes were washed three times in RPMI-1640, resuspended in the binding medium at 5×10^6 cells/ml, and then incubated with radiolabelled VIP for 60 min at room temperature in the presence or absence of various concentrations of unlabelled VIP. Cell-associated labelled peptide was separated by centrifugation at 6500 ***g*** for 1 min and then washed three times with binding medium at 4° to remove unbound label. The radioactivity of the cell sediments and supernatants was determined and specific binding was measured as the difference between cell-bound radioactivity found in the absence and presence of 10^{-7} M unlabelled VIP as a competitor (Ottaway & Greenberg, 1984). Binding assays with HT-2 cells at concentrations of 0·3 to 1×10^7 cells/ml were done similarily.

Complement-mediated cytotoxicity

Cells to be treated were washed three times in RPMI-1640 at 37° and resuspended in RPMI-1640 containing 0·3% BSA. Monoclonal anti-Thy 1.2 and anti-Lyt 2.2 antibodies were purchased from New England Nuclear Corp. Monoclonal anti-L3T4 was purchased from Becton-Dickinson Immunocytometry Systems (Mountain View, CA). These were used at final dilutions of 1/200, 1/1600, 1/100, respectively, for $1{\cdot}5 \times 10^7$ cells/3 ml. These dilutions were found to be maximally effective in producing cytotoxicity of thymus cells from the animals used here. Cytotoxicity was developed using low toxicity rabbit complement (C; Cedarlane Laboratories, Hornby, Ontario) at a final dilution of 1/10. Treated cells were then washed and resuspended in binding medium and their viability and ability to bind ^{125}I-VIP was assessed.

RESULTS

Effect of time of addition of VIP on Con A stimulation of lymphocytes

At intervals after the stimulation of lymphocytes by Con A, VIP was added to the cultures (Table 1). There was a time-dependent decrease in the efficiency with which VIP inhibited the response.

Table 1. Effect of adding VIP to Con A stimulated cultures at different times*

Time VIP added	% inhibition of response
0 hr	73·5 ± 7·8
3 hr	71·0 ± 2·4
6 hr	66·2 ± 2·5
24 hr	46·1 ± 6·4

* Results are the means ± SD of three experiments. Cells were cultured with or without Con A (2·5 μg/ml) and either VIP (10^{-7} M final) or an equivalent volume of medium was added at the times shown after the initiation of the cultures.

This apparent loss of inhibitory potency was confirmed when different concentrations of VIP were added to stimulated cultures either at the start of the reaction, or 24 hr after exposing the cells to Con A (Fig. 1).

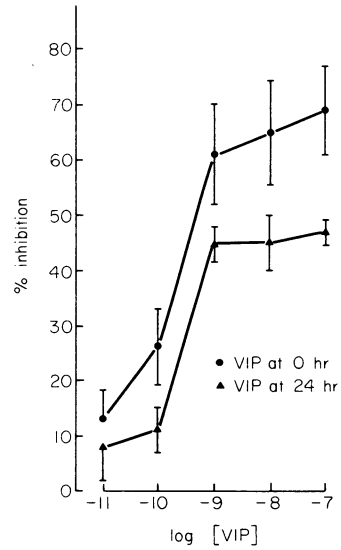

Figure 1. Potency of VIP as an inhibitor of Con A stimulation when added at different times. Mesenteric lymph node lymphocytes were cultured in the presence or absence of Con A (2·5 μg/ml) and different concentrations of VIP were added either at the start of the cultures or 24 hr after the initiation of the cultures. The results are the means ± SD of three experiments.

Alteration of VIP binding to lymphocytes during Con A stimulation

To investigate potential alterations in the interaction of VIP with cultured lymphocytes during stimulation, the stability of specific VIP binding to the cells after Con A stimulation was examined. The ability of stimulated lymphocytes to specifically bind ^{125}I-VIP was decreased by approximately 30% by 6 hr after stimulation (data not shown), and by 24 hr after the initiation of stimulation the ability of the lymphocytes to bind VIP was approximately 50% of that displayed by unstimulated cells (Fig. 2). The stimulated lymphocyte suspensions showed lower amounts of specific binding of VIP throughout a wide range of VIP concentrations (Fig. 2), and Scatchard analyses of these tracer dilution experiments indicated that, compared with fresh cells, the Con A-stimulated lymphocytes had a marked decrease in their affinity for VIP without any significant change in total binding capacity (Table 2).

The previous finding of a decline in the efficiency with which VIP can inhibit the Con A response at different times after the initiation of the reaction is consistent with this decreased affinity of the stimulated cells for the neuropeptide after stimulation.

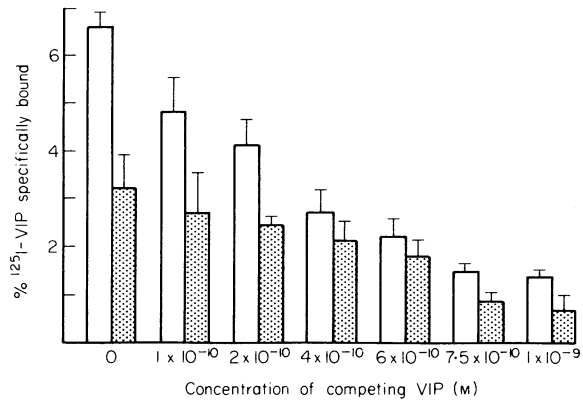

Figure 2. Binding of VIP to unstimulated and Con A-stimulated mesenteric lymph node lymphocytes. Unstimulated MLN (open areas) and MLN stimulated for 24 hr with 2·5 μg/ml Con A (shaded areas) were incubated with ^{125}I-VIP in the presence or absence of various concentrations of unlabelled VIP and the percentage of the added ^{125}I-VIP that was specifically bound to the cells was determined. The results are the means (± SD) of triplicate determinations for three experiments.

Reversibility of VIP inhibition

To examine the reversibility of the effect of VIP on the Con A-induced proliferation, lymphocytes were cultured in the presence of VIP and Con A for various lengths of time, then the VIP was removed and the cells re-exposed to stimulating concentrations of Con A (Table 3). Washing of these cells was carried out at 37°, under conditions that have previously been shown to lead to the rapid dissociation of cell-bound VIP (Ottaway & Greenberg, 1984). The results show that the inhibitory effect of VIP was totally reversible when the neuropeptide was removed from the cultures at 6 hr after starting the reactions (Table 3). However, when the cells had been exposed to both VIP and Con A for 24 hr and then washed, the cultures still demonstrated a fully inhibited response (Table 3).

VIP does not affect the expression of suppressor cell activity

Because Con A stimulation of lymphocytes induces the appearance of suppressor cells capable of inhibiting the response of lymphocytes to a variety of stimuli (Jandinski *et al.*, 1976), it was postulated that the inhibitory effect produced by VIP may reflect enhanced suppressor activity. To test this, the effect of VIP on the expression of suppressor-cell activity by cells stimulated with different concentrations of Con A was examined (Table 4). The presence of VIP did not disrupt the effect

Table 2. Effect of Con A stimulation on VIP-binding characteristics of mesenteric lymph node lymphocytes*

Cells	K_D (10^{-10} M)	Binding sites/cell
Unstimulated	1·8±0·2	2150±300
+Con A × 24 hr	4·5±0·5	2710±360

* Results are the means±SD for three tracer dilution experiments with each type of cell suspension. K_D is the dissociation constant and binding sites per cell is the receptor density determined from Scatchard plots of the data in Fig. 2.

Table 3. Effect of removing VIP from Con A cultures at different times*

Time VIP removed	% inhibition of response
6 hr	7·3±12·0
24 hr	69·3±11·4
Not removed	68·4± 8·1

* Results are the means±SD of three experiments. Cells were cultured with or without VIP (10^{-7} M) and Con A (2·5 μg/ml) for the times shown.

Table 4. Effect of VIP on the expression of suppressor cell activity*

Preincubation conditions: Con A (μg/ml)	VIP (M)	% suppression of responder cells
0	0	0†
0	10^{-7}	−2±8
2·5	0	54±9
2·5	10^{-7}	66±5
5	0	51±9
5	10^{-7}	53±6
10	0	43±9
10	10^{-7}	45±7

* Results are the means±SD for three experiments.
† By definition.

that cells incubated in the absence of Con A had on responder cells, and similarly, VIP had no effect on the Con A concentration-dependent expression of suppressing activity (Table 4).

This negative finding suggests that the inhibition exerted by VIP in the routine Con A-stimulated cultures is not dependent on increased activity of suppressor cells, and more probably results from neuropeptide influences over the induction of the proliferative response.

The effect of VIP on interleukin-2 production

Supernatants harvested after stimulation of lymphocytes with Con A in the presence of VIP were less able to support the growth of IL-2-dependent HT-2 cells than were supernatants obtained from parallel cultures stimulated with Con A alone (Fig. 3). Moreover, the effectiveness of these supernatants in supporting IL-2-dependent proliferation was decreased in a dose-dependent manner when generated in the presence of different concentrations of VIP (Fig. 3). In other experiments it was found that HT-2 cells did not have the ability to specifically bind VIP and that the direct addition of varying concentrations of VIP to HT-2 microcultures did not affect their response to supernatants from either Con A-stimulated cultures or MLA-144 cell cultures (data not shown). Thus, it was concluded that any VIP that might have been carried over with the tested supernatants was not affecting the responsiveness of the HT-2 cells to IL-2, and that the exposure of lymphocytes to VIP in the Con A-stimulated cultures had resulted in a decreased and delayed production of IL-2 by the lymphocytes (Fig. 3).

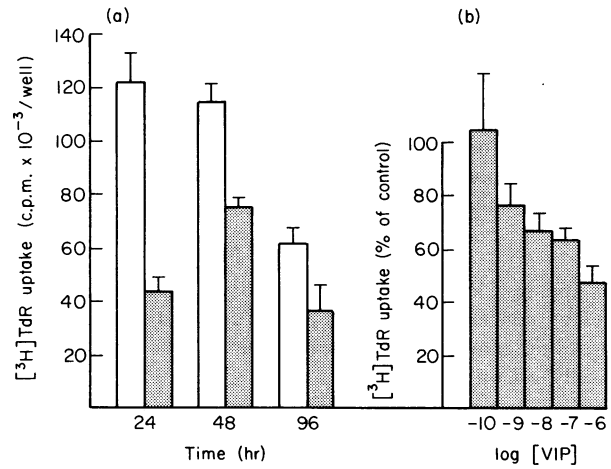

Figure 3. The effect of VIP on IL-2 activity of culture supernatants. (a) MLN cultures were stimulated with Con A in the presence (■) or absence (□) of VIP (10^7 M) and the culture supernatants were collected at the times shown. The ability of these supernatants to support the proliferation of IL-2-dependent HT-2 cells was determined. The results are the means (±SD) of triplicate determinations of three experiments for each condition. (b) MLN cultures were stimulated with Con A for 48 hr in the presence of various concentrations of VIP. The supernatants were collected and tested for their ability to support the proliferation of HT-2 cells. The results are the means (±SD) for triplicate determinations of three experiments in which the response was compared to that obtained with parallel cultures stimulated with Con A alone.

VIP binding to T-cell subpopulations

The effect of selective killing of subpopulations of cells on the specific binding of lymphocyte suspensions was examined. When cell suspensions were treated with complement alone, there was no disruption of the ability of the cells to bind ^{125}I-VIP (Fig. 4). Treatment of lymphocyte suspensions with monoclonal antisera to Thy 1.2, L3T4, Lyt 1.2, or Lyt 2.2 determinants, likewise, produced no significant alteration in the specific VIP binding expressed by the cells.

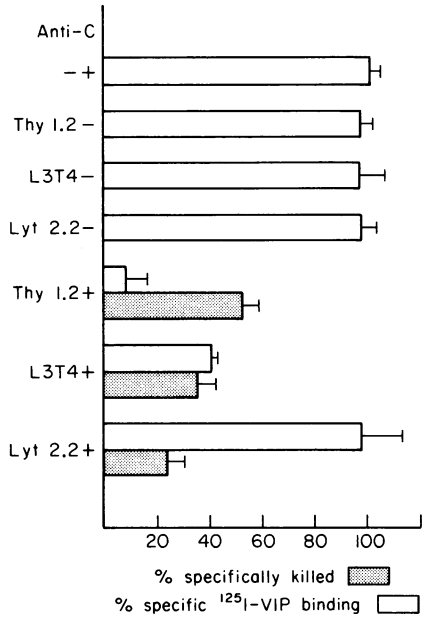

Figure 4. The effect of selective T-cell killing on specific ^{125}I-VIP binding. Mesenteric lymph node lymphocytes were treated with the monoclonal antibodies shown in the presence or absence of complement (C) and specific killing and specific binding of VIP to the treated cells examined. The results are the means ± SD of three experiments.

When cell suspensions were treated with anti-Thy 1.2 antibody plus complement, however, about 50% of the cells were killed, and more than 90% of the specific binding of VIP by the cells was prevented (Fig. 4). Treatment of the cells with anti-L3T4 in the presence of complement eliminated more than one-half of the observed specific VIP binding while killing only a minority of the cells. In contrast, treatment with anti-Lyt 2.2 antibody and complement produced no significant alteration in the specific VIP binding of the cell suspensions, although a similar proportion of the cells were killed. (Fig. 4).

These observations demonstrate two important features with respect to VIP binding. First, the effect of Thy 1.2 treatment in the presence of complement confirms our assertion from previous work that T cells rather than B cells principally account for specific VIP binding (Ottaway & Greenberg, 1984). Second, the differential effect of anti-L3T4 and anti-Lyt 2.2 killing on VIP binding suggests that the majority of the T cells responsible for VIP binding are of the L3T4$^+$, Lyt 2$^-$ phenotype.

DISCUSSION

The purpose of this study was to examine the means by which the neuropeptide VIP exerts an inhibitory effect on the proliferative response of lymphocytes stimulated with Con A. Previous work has demonstrated a close correspondence in the concentration dependence of VIP as an inhibitor of radiolabelled VIP binding and as an inhibitor of Con A-induced proliferation (Ottaway & Greenberg, 1984). Taken together with the present observation of concomitant changes in the affinity of cells for VIP (Fig. 2, Table 2) and the ability of VIP to inhibit the proliferative response (Table 1, Fig. 1) subsequent to the onset of Con A stimulation, these findings support the notion that the inhibitory influence of the neuropeptide is mediated through occupancy of the VIP receptors present on the cells.

The molecular mechanisms by which VIP binding can alter the response of lymphocytes to Con A are not fully understood, but rapid enhancement of adenylate cyclase activity has been shown in the presence of VIP, in plasma membrane preparations of normal human T cells and Molt 4b cells (O'Dorisio *et al.*, 1981). Furthermore, rapid activation of cyclic AMP-dependent protein kinase by VIP has also been demonstrated in human peripheral blood mononuclear cells (Guerrero *et al.*, 1984) and Molt 4b lymphoblasts (O'Dorisio *et al.*, 1985). It is likely that a similar pattern of enzyme activation by VIP can occur in mouse T cells to produce intracellular messengers as a consequence of surface receptor occupancy.

The observation that the effect of VIP was reversible up to 6 hr after initiation of the Con A response, but not reversible after the cells had been exposed to VIP during the first 24 hr of Con A stimulation (Table 3), suggests that further effects on other cellular processes or products also may be necessary for VIP to be able to express its inhibitory effects.

VIP had no apparent influence on the expression of suppressor-cell activity by cells stimulated with Con A (Table 4). The lack of an effect of VIP on the development of this functional capability of Con A-stimulated cells is consistent with the observations that indicate that VIP binds principally to T cells of the L3T4$^+$, Lyt 2$^-$ phenotype and not to Lyt 2$^+$ cells (Fig. 4), because, in the context of polyclonal activation by Con A, suppressor cell activity is a property of Lyt 2$^+$ cells (Jandinski *et al.*, 1976).

It is recognized that caution must be used in attributing specific cell functions to particular phenotypes (Ledbetter *et al.*, 1980; Swain, 1981), but the availability of an antibody to the L3T4 antigenic determinant (Dialynas *et al.*, 1983a, b) has permitted the assignment of most peripheral T cells to two mutually exclusive subsets: L3T4$^+$, Lyt 2$^-$ and L3T4$^-$, Lyt 2$^+$. These determinants appear to contribute to different functional capabilities. The L3T4-bearing subset is restricted to class II major histocompatibility complex molecules and appears to contain the majority of the T-helper/inducer cell population, whereas the Lyt 2-bearing subset is restricted to class I molecules and contains the majority of cytotoxic cells but few helper cells (Swain, 1981; Dialynas *et al.*, 1983a, b; Wilde *et al.*, 1983; Marrack *et al.*, 1983; Swain *et al.*, 1984).

The apparent inability of Lyt 2$^+$ cells to bind VIP and the observed failure of VIP to affect the expression of suppressor activity suggests that the inhibitory effect of the neuropeptide on Con A activation is exerted at the level of the induction of the proliferative response. Lymphokine production is an important feature of both the induction and regulation of T cells during the response to mitogens or antigens. In particular, IL-2 production is believed to play a fundamental and obligatory role in a variety of T-cell responses, including mitogen-stimulated proliferation (Gillis, 1983; Larrson, 1981; Lipkowitz *et al.*, 1984; Smith, 1984). It is likely, therefore, that the observed dose-dependent reduction in the availability of IL-2 during lymphocyte stimulation in the presence of VIP (Fig. 3) plays an important role in the inhibitory effect that the neuropeptide has on Con A-induced proliferation.

Although IL-2 can be produced by a variety of cell types under different circumstances, current evidence suggests that IL-2 production in the context of Con A stimulation of murine

cells is predominantly due to the activity of L3T4$^+$, Lyt 2$^-$ cells. Limiting dilution analyses have demonstrated that the vast majority of precursors for Con A-responsive IL-2-producing cells are in the Lyt 2$^-$ subset (Miller, 1983), and subset fractionation studies have shown that, although both L3T4$^+$ and Lyt 2$^+$ populations can produce IL-2 in response to Con A stimulation, the quantities produced by the L3T4$^+$ subset were much greater than that produced by the Lyt 2$^+$ subset (Malek, Schmidt & Shevack, 1985).

The results presented here indicate that L3T4$^+$ cells account for the majority of T cells that are capable of binding the neuropeptide (Fig. 4). Thus, the most direct working hypothesis by which to understand the inhibition of Con A-induced proliferation by VIP is that receptor occupancy leads to receptor-transduced events which alter IL-2 production by those L3T4$^+$ cells that bear receptors for the neuropeptide.

This study shows that VIP binds differentially to subpopulations of peripheral T cells, and that VIP has a selective effect on the development of different functional activities after stimulation by Con A. Since Con A stimulation may induce proliferation and differentiation processes that are similar in principle to those occurring in response to specific antigens, it is conceivable that this neuropeptide may play a regulatory role in immune responses *in vivo*. VIP-containing nerves are especially abundant in mucosal tissues of the gut, upper respiratory tract, salivary glands and the genital tract (Polak & Bloom, 1982), and the selective interaction of this neuropeptide with T cells in these tissues, therefore, may be of importance in the regulation of mucosal immune responses.

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