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A potential immunosuppressive role for vasoactive intestinal peptide (VIP) in leishmaniasis: Evidence from the use of *a selective VIP antagonist*

Ishaya H. Nock and Jayashri D. Sharma
School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110 067, India

The immunological relevance of vasoactive intestinal peptide (VIP) in the pathology of leishmaniasis has been tested. *Leishmania*-primed mouse-derived splenic cells were examined and found to exhibit suppressed and up-regulated mitogenic responses in the absence and the presence of VIP respectively. Furthermore, plasma derived from *Leishmania*-primed hamsters induced a suppressive effect on the mitogenic response of normal hamster splenic cells; this was significantly blocked by a selective VIP antagonist. The results showed the functional presence of VIP at the level of immunological disorder within a *Leishmania*-primed milieu.

THE encapsulation of the *Leishmania* parasites within the macrophage phagolysosomal vacuole of a susceptible mammalian host, harbingers a spectrum of immunological abnormalities[1-4] which favour the evolution of a defective cell-mediated immune response towards increased parasitism and disease. Parasite innate ability to effect these changes is largely ascribed to its surface antigenic molecules (membrane bond and excreted forms) such as the lipophosphoglycan (LPG), glycoprotein 63 (gp63) and acid phosphatase (AP) (see refs. 5–7). In addition, a host of serum-associated factors such as the prostaglandins, immunoglobulins, triglycerides and protein molecules[8,9], generally released in increasing quantity during infection, have also been described as important contributory molecules in the dysregulation of host immune cell functions in leishmaniasis. Indeed, speculation is that immunosuppressive factors in serum/plasma sustain the process of immunosuppression[10,11]. The functional significance accorded these factors in the overall process of immunosuppression, prompted consideration for the role of a potent immunosuppressive endogenous peptide molecule, the vasoactive intestinal peptide[12] (VIP), in the process.

The VIP is thought to influence the pathophysiology of certain tumour-related diseases[13,14] and parasitic infections[15,16]. In recent times, it has gained prominence as a molecule involved in the exacerbation of disease processes through an immunological axis[14,17], given its ability to inhibit several immunological functions such as monocyte oxidative activity[18], lymphocyte mitogenic response[19,20] and the release of lymphokines[21], and natural killer (NK) cell activity[22]. Indeed, VIP is viewed as potent in the dysregulation of host immune cell response in the human immunodeficiency virus (HIV) infection[17,23] on the basis of the presence of VIP-like substances in serum of HIV-positive patients, coupled with the resemblance of VIP and HIV-induced immunological features. Similarly, infections by *Leishmania* parasites are also associated with immunological features that parallel those induced by VIP[1-4,18-22]. In this respect, VIP may be relevant in the overall processes that tilt host immune response towards anergy in leishmaniasis.

This paper reports that splenic cells derived from immunosuppressed *Leishmania*-primed BALB/c mice exhibit certain degree of sensitivity to VIP *in vitro* and that VIP-like substances are functionally detectable in plasma of immunosuppressed *Leishmania*-primed hamsters. The results suggest that VIP is functionally present at the level of immunological disorder in leishmaniasis.

VIP (complete 28 amino acid porcine sequence), VIP_{10-28} (carboxyl partial sequence of VIP—as VIP antagonist at the receptor level[24,25]) and Aprotinin were obtained from Sigma. Radioactive labelled thymidine (^{2}H-TdR, specific activity 6.5 curie/mM) was obtained from the Bhabha Atomic Research Centre, Bombay, India. Strains of *Leishmania donovani* and *L. tropica* were obtained from the Department of Immunopathology, Post Graduate Institute of Medical Research, Chandigarh.

Experiment one: *Leishmania*-primed mouse-derived splenic cell response to mitogen in the absence or presence of VIP.

Seven-day-old cultures of *L. donovani* and *L. tropica* promastigotes were washed and suspended on 0.15 M PBS, pH 7.2, and thereafter killed by repeated freezing and thawing (5 times) for particulate antigens[26]. Sets of 2 BALB/c mice (10 g) were challenged, at various times, with 0.1 ml of promastigote suspension, equivalent to 10^8 *L. donovani* and 10^7 *L. tropica* particulate antigens, with complete Freund's adjuvant (CFA) by subcutaneous inoculation at a shaved area at the base of the tail. Seven days post-initial-challenge, mice were rechallenged with 0.1 ml of 10^6 particulate antigen suspension admixed with incomplete Freund's adjuvant (IFA). Control mice (3) were inoculated with CFA and IFA

only. Two, 4 and 6 weeks post-initial-challenge, mice were killed by cervical dislocation on the same day for splenic cells[27]. Two million (2×10^6) cells were tested for their ability to respond to Con A (2.5 μg/ml; pre-determined optimal mitogenic dose) in the absence or presence of 10^{-7} M VIP. In some VIP-treated cultures, 10^{-8} M VIP antagonist (pre-determined VIP antagonising dose) was added to block any VIP action. Splenic cells mitogenic responses were measured by the amount of ^{3}H-TdR (1 μcu/ml) incorporated by the cells for 24 h in a 72 h incubation period at 37° C 5% CO_2 and 95% air[28].

Experiment two: Functional detection of VIP in *Leishmania*-primed hamster-derived plasma.

Sets of two Golden hamsters (100 g) were challenged, at various times, with suspensions of *L. donovani* and *L. tropica* (as in experiment one) by subcutaneous inoculation at the footpad. Two, 4 and 6 weeks post-initial-challenge, animals were immobilized by chloroform anesthesia. Blood was collected by cardiac puncture with sterile syringe into sterile glass tubes containing 50 U/ml heparin and 500 KIU/ml aprotinin. (Aprotinin, a antiprotease, was added to inhibit the proteolytic degradation of VIP that may be present in plasma[29].) Blood was centrifuged at 2000 rpm for 10 min at 4° C to obtain plasma. Plasma samples from the same animal group were pooled and stored at −20° C until required. 2×10^6 cells from these animals were obtained and tested for their ability to respond to Con A (5 μg/ml) in culture medium (RPMI 1640) supplemented with either 5% FCS, or 5% FCS plus 5% plasma obtained from the *Leishmania*-primed hamsters. VIP antagonist at 10^{-8} M was added to the cultures to probe for the functional presence of VIP in plasma. (Experiments 1 and 2 were each done three times.)

To test whether priming of mice with *Leishmania* antigens induced the release of endogenous VIP above normal physiological level, the sensitivity to VIP by *Leishmania*-primed mouse-derived splenic cells were tested *in vitro* for their ability to respond to mitogenic stimulation in the absence or presence of VIP. The results obtained (Table 1) showed that *Leishmania*-primed splenic cells with suppressed mitogenic response of 30% (as was the case with *L. donovani* and 6 week *L. tropica*-primed cells) exhibited an up-regulated response on stimulation by Con A in the presence of VIP (but not Secretin, a VIP structural analogue; data not shown). VIP antagonist blocked, by a significant degree ($P < 0.001$), VIP-induced up-regulated response.

Since 1% to 5% serum or plasma derived from either *Leishmania*-infected patients or experimental animals is known to markedly inhibit lymphocyte mitogenic response[9–11], it was examined whether this inhibitory effect was partly induced by VIP that may be present in

Table 1. Response of *Leishmania*-primed BALB/c splenic cells to mitogen in the absence of VIP and VIP_{1-28}

Cell treatment	CFA (6 wk)	*L. donovani* antigens at week: 2	4	6	CFA (6 wk)	*L. tropica* antigens at week: 2	4	6
	Tritiated thymidine uptake by splenic cell primed to:							
Con A (2.5 μg/ml)	25201 ± 4301 (0 ± 17)	17713 ± 2920[c] (−30 ± 5)*	14389 ± 3250[c] (−43 ± 10)*	10381 ± 1714[c] (−58 ± 10)*	25201 ± 4301 (0 ± 17)	20479 ± 3473[a] (−12 ± 2)*	17109 ± 2430[c] (−27 ± 4)*	13017 ± 1722[c] (−44 ± 9)*
+ VIP 10^{-7} M)	15905 ± 3812[c] (−37 ± 9)**	19979 ± 3099[a] (+13 ± 2)**	22073 ± 4017[c] (+53 ± 10)**	18457 ± 2665[c] (+78 ± 11)**	15905 ± 3812[c] (−37 ± 9)**	11330 ± 2411[c] (−45 ± 10)**	12452 ± 1639[c] (−27 ± 6)**	20757 ± 3547[c] (+59 ± 10)**
+ VIP (10^{-7} M) + VIP_{10-28} (10^{-8} M)	20878 ± 3397[b,e] (−17 ± 3)**	18303 ± 1704[d] (+3 ± 3)**	18077 ± 1877[c,e] (+26 ± 3)**	15028 ± 2184[c,e] (+44 ± 15)**	20878 ± 3397[b,e] (−17 ± 3)**	16742 ± 2326[c,e] (−18 ± 3)**	15204 ± 2711[a,e] (−11 ± 2)**	16657 ± 3617[c,e] (+28 ± 6)**

Figures (open) are means ± SD of total counts (cpm) of triplicate cultures from three mice (3 independent determinations, n = 18)
* % change (means ± SD) in ^{3}H-TdR uptake against CFA-primed cells treated with Con A only.
** % change (means ± SD) in ^{3}H-TdR uptake against corresponding Con A treated cultures
[a] $P < 0.05$, [b] $P < 0.002$, [c] $P < 0.0001$ against CFA-primed cells treated with Con A only.
[d] $P < 0.01$, [e] $P < 0.001$ against corresponding VIP-treated cultures (Stundent's t test).

Table 2. Effect of *Leishmania*-primed hamster-derived plasma on normal splenic cell response to Con A and VIP

Cell treatment	^{3}H-TdR uptake by normal splenic cells cultured in medium supplemented with: 5% FCS and 5% plasma-derived from hamster-primed to:					
			L. donovani antigens for:		*L. tropica* antigens for:	
	5% FCS	CFA (6 wk)	4 wk	6 wk	4 wk	6 wk
Con A (5 μg/ml)	17004 $\pm$ 2747 (0 $\pm$ 16)	13633 $\pm$ 2656[b] (−20 $\pm$ 4)	7972 $\pm$ 792[c] (−52 $\pm$ 5) −35 $\pm$ 5*	4431 $\pm$ 563[c] (−74 $\pm$ 9) −55 $\pm$ 12*	10741 $\pm$ 1218[c] (−37 $\pm$ 4) −20 $\pm$ 2*	6412 $\pm$ 946[c] (−62 $\pm$ 9) −35 $\pm$ 6*
+ VIP_{10-28} (10^{-8} M)	15497 $\pm$ 2894 (−9 $\pm$ 1)	14001 $\pm$ 3110[a] (−18 $\pm$ 4)	10956 $\pm$ 1683[c,e] (−36 $\pm$ 6)	7737 $\pm$ 1119[c,e] (−54 $\pm$ 8)	11632 $\pm$ 2424[c] (−32 $\pm$ 7)	8757 $\pm$ 528[c,d] (−49 $\pm$ 9)

Figures (open) are means $\pm$SD of total counts (cpm) of triplicate cultures of splenic cells from normal hamster (3 independent determinations, $n = 9$).
Figures (parenthesis) are % change (means $\pm$ SD) in ^{3}H-TdR uptake against cultures with 5% FCS and Con A only.
* % change (means $\pm$ SD) in ^{3}H-TdR uptake for splenic cells of hamsters from which plasma was obtained.
[a] $P < 0.03$; [b] $P < 0.02$; [c] $P < 0.001$ against control (5% FCS + Con A treated cultures).
[d] $P < 0.01$; [e] $P < 0.001$ against corresponding cultures with VIP_{10-28} (Student's t test).

plasma. VIP functional presence in the inhibitory action induced by plasma was probed by the addition of VIP antagonist to cultures supplemented with plasma derived from *Leishmania*-primed hamsters with suppressed mitogenic splenic cell response. The results (Table 2) showed that the addition of the antagonist in such cultures significantly reduced plasma-induced suppressive effect on mitogenic response by a varying degree. The effect induced by VIP antagonist was quite in evidence in cultures containing plasma whose inhibitory potency on normal splenic cell response was $\geq 52\%$, i.e. plasma from 4 and 6 weeks *L. donovani* and 6 weeks *L. tropica*-primed hamsters. For instance, plasma from 6 weeks *L. donovani* and *L. tropica*-primed hamsters inhibited normal splenic cell mitogenic response by $74 \pm 9\%$ and $62 \pm 9\%$; VIP antagonist reduced this effect to $54 \pm 8\%$ and $49 \pm 9\%$; a significant difference in shift (blockage) of 20% and 13% respectively.

The enhanced response exhibited by immunosuppressed *Leishmania*-primed splenic cells to Con A in the presence of VIP (Table 1) strongly suggests that endogenous VIP, in all probability, was released by leishmanial induction. By this release, splenic cells got exposed and sensitized to VIP thus giving rise to the observed enhanced response to mitogenic stimulation in the presence of VIP *in vitro*. This viewpoint finds support from recent works which showed that NK cell cytotoxicity to k562 target cells[30] and rabbit splenic cell mitogenic response[31] were greatly enhanced through short-term pre-treatment or exposure to VIP.

Given that the enhanced response was significantly reduced by VIP_{10-28} (a well-proven VIP antagonist at the receptor level[24,25]) suggests that VIP-induced effect was relayed at the receptor level on mononuclear cells and may thus reflect changes in receptor number on the surface of the primed cells. Increase in VIP plasma concentration has been shown to correlate with increased VIP receptor number on mononuclear cells plus monocyte oxidative failure[32,33].

Additional evidence that favours VIP functional presence in the overall suppression of immunological functions in leishmaniasis is found in the latter experiment in which VIP antagonist, probed the functional presence of VIP in the suppressive action induced by plasma. This evidently lends support to the functional presence of VIP or VIP-like substances in the immunosuppressive action of plasma during leishmaniasis.

The *Leishmania*-primed organismal milieu may thus be seen as that which favours increased release of endogenous VIP towards increased immunological disorder and disease. Supporting evidence in this regard is, furthermore, drawn from other works which show that *Leishmania* infection is associated with increased production and release of prostaglandins[11,12]. The prostaglandins can induce the release of VIP[34].

In conclusion, the overall results favour the inclusion of VIP in the list of molecules that modulate host immune cell functions towards anergy in leishmaniasis.

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Characteristics of larval mark and origin of radii

K. K. Tandon and M. S. Johal
Department of Zoology, Panjab University, Chandigarh 160 014, India

The origin of primary radii in relation to the presence and absence of larval mark in Indian carps based on SEM studies has been described.

THE occurrence of juvenile ring or larval mark is known in most freshwater fishes[1–3] in addition to annuli and false mark on the scales, but its characteristics have not been defined except that it occurs not far from focus inside the first annulus and may be confused with the latter[1]. The scales of *Labeo rohita* (Hamilton) and *Puntius sarana sarana* (Hamilton) were studied using a JEOL-JSM-255 scanning electron microscope. The central part of the scale was mounted on a metallic stub coated with a layer of carbon-gold 100 Å thick under vacuum and a secondary electron image was obtained at an accelerating voltage of 15 kV. The scales where larval ring or mark occurs, the circuli are crowded in the anterior part of the scale and diverge posteriorly. When larval growth stops, a perfect ring is formed beyond which the fish shows rapid growth as evidenced by wide spaces between circuli, the primary radii ori-

Table 1. Back-calculated lengths of *Labeo rohita* and *Puntius sarana sarana* from different water bodies

Locality	Back-calculated lengths (cm) l_1	l_2	l_3	l_4	l_5	l_6	l_7	l_8
Labeo rohita								
Harike[9] Wetland	31.76	49.56	64.08	70.66	74.58	79.91	83.40	87.45
h (cm)	31.76	17.80	14.52	6.58	3.92	5.33*	3.49	4.05*
River Ghagger[9] at Rang Mahal, Rajasthan	28.00	43.13	54.96	64.90	75.46	79 80	83.01	85.38
h (cm)	28.00	15.13	11.83	9.94	10.56*	4.34	3.21	2.37
Puntius sarana sarana								
River Ghagger[10] at Rang Mahal, Rajasthan	16.41	26.22	32.11	36.08				
h (cm)	16.41	9.81	5.89	3.97				
Sukhna Lake[10], Chandigarh	13.21	20.99	26.39					
h (cm)	13.21	7.78	5.40					

$l_1, l_2, \ldots, l_n$ are the average back-calculated lengths in the respective years of life using Fraser's[4] equation.
h is annual increment.
*Phenomenon of growth compensation.