In Vitro Leishmania major Promastigote-Induced Macrophage Migration is Modulated by Sensory and Autonomic Neuropeptides

A. A. AHMED*, A. WAHBI†‡, K. NORDLIND§, A. KHARAZMI¶, K.-G. SUNDQVIST**, V. MUTT†† & S. LIDÉN*

**Department of Dermatology, Karolinska Hospital, Stockholm, Sweden; †Department of Clinical Immunology, Huddinge Hospital, Huddinge, Sweden; Departments of ‡Pediatric and §Dermatology, University Hospital, Uppsala, Sweden; **Department of Clinical Immunology, University of Umeå, Umeå, Sweden; ¶Department of Clinical Microbiology, National University Hospital (Rigshospitalet Afsnit 7806), Copenhagen, Denmark; and ††Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden*

(Received 19 January 1998; Accepted in revised form 19 March 1998)

Ahmed AA, Wahbi A, Nordlind K, Kharazmi A, Sundqvist K-G, Mutt V, Lidén S. *In Vitro Leishmania major* Promastigote-Induced Macrophage Migration is Modulated by Sensory and Autonomic Neuropeptides. Scand J Immunol 1998;48:79–85

Recruitment, migration and adherence of macrophages and their interaction with inoculated promastigotes are key steps in the initiation of the inflammatory process in cutaneous leishmaniasis. Parasite- and nervous system-derived factors might be involved in this process. In the present study the chemotactic activities of live, killed and sonicated *Leishmania major* promastigotes and of the promastigote culture supernatant as well as the *L. major* surface protease gp63 towards a murine macrophage cell line, Raw 264.7, were investigated, using the Boyden technique. The sensory neuropeptides SOM, CGRP and SP, and the autonomic neuropeptides VIP and NPY, were also investigated for possible modulatory effects on this chemotaxis, using the living promastigotes. Living promastigotes were the most efficient attractants for macrophages compared with other forms of the parasites. Prior incubation of the macrophages with the parasites completely abolished the chemotactic activity. This might indicate that the living promastigote chemotaxis is a receptor-mediated process. On the other hand, paraformaldehyde-killed promastigotes not only failed to induce macrophage chemotaxis but also inhibited it in comparison with the control. The surface protease gp63 tended to inhibit the macrophage chemotactic activity and the sonicate tended to stimulate it compared with controls. The culture supernatant had no effect, indicating that the chemoattractive factors putatively synthesized by the living promastigotes are not released to the surrounding medium. Somatostatin inhibited *L. major* promastigote-induced macrophage migration at a high concentration, 10^{-6} M, while substance P inhibited it at both low concentrations, 10^{-10} and 10^{-9} M, and a high one, 10^{-6} M, the last-mentioned having the greatest inhibitory effect. A stimulatory effect of calcitonin gene-related peptide was found at high concentrations, 10^{-5} and 10^{-6} M. Vasoactive intestinal peptide stimulated macrophage chemotactic activity at both a high, 10^{-5} M, and at a low, 10^{-9} M, concentration, the same concentration at which neuropeptide Y exerted its maximum inhibitory effect.

Dr A. A. Ahmed, Department of Dermatology, Karolinska Hospital, Box 120, S-171 76 Stockholm, Sweden

INTRODUCTION

Leishmaniases are caused by the intracellular protozoan parasites belonging to the genus *Leishmania*. *Leishmania major, L. tropica* and *L. aethiopica,* are the classic causative agents of cutaneous leishmaniasis in the Old World [1]. The flagellated promastigotes, which develop in the gut of the female sandfly of the genera Phlebotomus, invade and multiply within the macrophages of the skin after transformation into the non-flagellated amastigote form [2]. Thus, macrophages play a central role in the course of infection, because they serve not only as the host target cells for the parasites but also as antigen-presenting cells for T lymphocytes, which govern the specific cellular immune response, and thereafter, following adequate activation, they act as effector cells for intracellular parasite killing [3].

Chemoattraction of the host target cells towards the parasite not only ensures the parasite entry into the right cell type, but also minimises extracellular existence of the parasites and hence also extracellular lysis [4–7] or uptake and killing by neutrophils [8]. However, the signals initiating and regulating the recruitment of various cell types and their migration towards the parasite inoculation site are only poorly understood. Contributions of parasite-derived factor(s) in the chemotaxis process through soluble mediator(s) and/or receptor/ligand interactions for migration, as suggested previously [9], need to be considered at least for the target cells, macrophages.

The intimate link of the neuroendocrine and immune systems in a bidirectional communication is now accepted. The idea of a modulating effect of the nervous system on immune and inflammatory responses has been supported by the identification of neuropeptide receptors on immunocompetent cells [10] and the finding that neuropeptides are able to regulate leucocyte functions [11]. For example, neuropeptides have been shown to modulate lymphocyte and macrophage functions, such as adherence and migration [12], their chemoattractant function [13], phagocytosis [14], production of oxygen and nitrogen radicals [15], and production of cytokines [16] and immunoglobulins [17, 18]. Moreover, neuropeptides have been shown to be synthesised by immune cells such as mast cells [19], eosinophils [20], T cells [21] and macrophages [22], and an autocrine action has been suggested. Thus, it has been postulated that neuropeptides play an important regulatory role in inflammatory conditions [23].

Recruitment, adherence and migration of macrophages to the site of promastigotes' inoculation in the skin represent the initial and critical steps in the inflammatory process in cutaneous leishmaniasis. Whether inoculated promastigotes have any role *in vivo* in this migration was the basis for the present investigation. The study was designed to investigate the chemoattractant capacities of living, compared with sonicated and killed *L. major* promastigotes and of the parasite culture supernatant and the parasite surface protease gp63 for a murine macrophage cell line, Raw 264.7, *in vitro*. In addition, it was evaluated whether the sensory neuropeptides, substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin (SOM), and the autonomic neuropeptides, vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY), which have been localized in normal and inflamed skin of humans by immunohistochemical and radioimmunoassay techniques (see, e.g. [24]), have modulatory effects on the suggested promastigote-induced migration of macrophages.

MATERIALS AND METHODS

Cells and parasites. A murine monocyte/macrophage cell line, Raw 264.7, was obtained from the American Tissue Type Culture Collection (ATCC) (Maryland, USA). This cell line is an Abelson leukaemia virus-transformed macrophage cell of BALB/c origin. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For various assays, the cells were detached by vigorous pipetting and resuspended at the desired concentration of 2×10^6/ml in DMEM + 5% FBS.

L. major parasites (JIHI 18 strain) were obtained from Dr D. Evans, London School of Hygiene and Tropical Medicine, University of London, UK. The parasites were kept virulent by monthly passage in BALB/c mice. The maintenance, cultivation and isolation of the promastigote stage of the *L. major* parasite has been described in detail by Louis *et al*. [25].

Preparation of neuropeptides. SOM_{1-14} (batch no. A11227) was purchased from Novabiochem (Läufelingen, Switzerland). Human CGRP II, SP and NPY (lot nos. 032880, 035262 and 035598, respectively) were obtained from Peninsula (Merseyside, England). Native VIP was purified from porcine upper intestine by sequential chromatography and countercurrent distribution and was found to be homogeneous by high performance liquid chromatography and capillary electrophoresis, essentially as described [26]. The neuropeptides were dissolved in sterile distilled water, aliquoted at 10^{-4} or 10^{-5} molar concentrations and stored at −70 °C until used. They were then diluted with sterile phosphate buffered saline (PBS) to a working concentration immediately before the migration assay.

Preparation of the sonicate. *L. major* promastigote-derived sonicate was prepared as described by Sørensen *et al*. [9]. Briefly, stationary-phase promastigotes were sonicated five times for 45 s at 25 000 Khz on ice, using an ultrasound disintegrator (Rapidis 350, 19-mm probe with a 9.5 mm tip). The lysate was then centrifuged at 48 000 ***g*** for 1 h at 4 °C. The supernatant was sterile filtered using a 0.22 μm Millipore filter. The colloidal/antigen concentration was 70 μg/ml as measured by refractometry, using human immunoglobulin as a standard. The sterility was checked at every point in the preparation procedure by plating the sonicate on 5% blood agar for bacterial growth and by gram staining. The sonicate was then stored at −20 °C before use.

Purification of gp63. *L. major* gp63 was prepared and purified under aseptic conditions as described previously [27–29]. In brief, gp63 was cleaved from parasite membranes using phospholipase C, type III (Sigma, St. Louis, MO, USA) and purified by affinity chromatography on a concanavalin-A-conjugated Sepharose 5B column and FPLC ion-exchange chromatography on a Mono Q column (Pharmacia & Upjohn, Uppsala, Sweden). The purity was checked by SDS-PAGE and silver staining of the gel. The enzymatic activity was evaluated in an azocasein assay [30]. The protein concentration was determined by use of a commercially available protein quantification kit (Bio-Rad, Munich, Germany).

Chemoattractant assay. The chemoattractant activity of living *L. major* promastigotes was assessed according to the original technique described by Boyden [31], which consists basically of the use of a chamber with two compartments separated by a polycarbonate Millipore filter with a pore diameter of 8 μm. The lower surface of the filter was coated with fibronectin (which in itself has a chemotactic effect on monocytes [32]), which was found to give the best results as compared with the other tested matrices such as collagen or laminin. Fifty microlitres of resting macrophages or of macrophages activated by incubation with promastigotes for 2 h at 37 °C and 5% CO_2 were deposited in the upper compartment of the Boyden chamber and 30 μl of either living (2×10^7/ml) promastigote suspension, the concentration that was used for both *in vivo* and *in vitro* challenge in previous studies, sonicated (70 μg/ml), killed promastigotes (2×10^7/ml), gp 63 (0.2 mg/ml), or *L. major* supernatant, in DMEM + 5% FBS, was placed in the lower compartment. The chambers were then incubated for 4 h at 37 °C. Following the incubation period the filters were washed, fixed in methanol for 10 min and stained, and a homogenous cell population on the lower surface of the filter were counted in at least two randomly selected areas of 9 × 12 cm.

The modulatory effects of the neuropeptides at various concentrations, namely 10^{-5}–10^{-11} M for SOM and 10^{-5}–10^{-10} M for CGRP, SP, VIP and NPY, were evaluated on the living *L. major* promastigote-induced macrophage migration. The various neuropeptides were dissolved in DMEM + 5% FBS containing 2×10^{7}/ml of *L. major* parasites. As a control DMEM + 5% FBS was used throughout the experiments.

Statistical analysis. In order to evaluate hypotheses of an overall concentration-dependent effect of the actual peptide on *L. major*-induced macrophage migration, analysis of variance for repeated measurements was used. If such an effect was found, a test between each concentration and the control was performed. In order to control the significance level in the pairwise comparisons the method proposed by Dunnett [33] was used. All analyses were carried out by use of the SAS system, and a $P \leq 0.05$ was the adopted level of significance.

RESULTS

Macrophage chemotactic activity

In an attempt to determine whether *L. major*-activated macrophages have a better potential for migration than the resting ones, macrophages were incubated with stationary phase promastigotes for 2 h at 37 °C before the migration assay. This prior incubation of macrophages completely abolished their capacity for chemotaxis towards the living promastigotes.

Living *L. major* promastigotes had a pronounced chemoattractive effect on the resting macrophages, while the paraformaldehyde-killed parasites significantly inhibited macrophage chemotaxis compared with the controls (Fig. 1). The sonicate derived from *L. major* promastigotes showed a chemoattractant tendency, but this was not statistically significant in comparison with the control. The cell surface glycoprotein gp 63 showed an inhibitory tendency, while the promastigote culture supernatant had no effect.

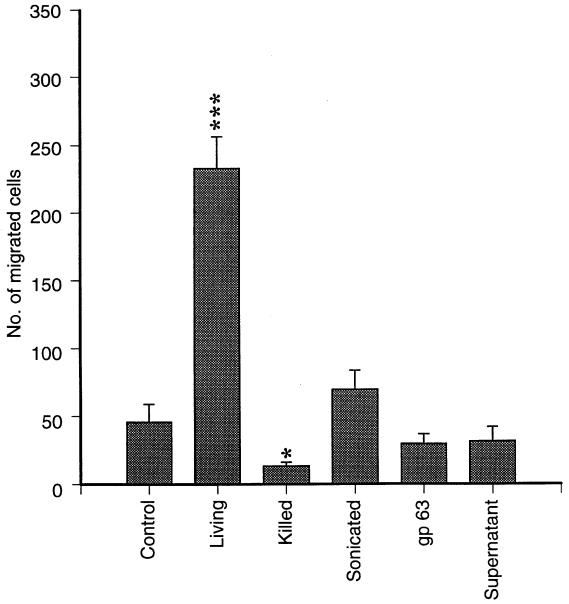

Fig. 1. Chemotactic activities of living *L. major* promastigotes (Living), paraformaldehyde-killed parasites (Killed), *L. major* promastigote-derived sonicate (Sonicated), surface protease gp63 and *L. major* promastigote culture supernatant for a murine macrophage cell line in comparison with the control (DMEM + 5% FBS). Data represent mean ± SEM of three separate experiments run in triplicate. * $P<0.05$, *** $P<0.001$.

Effects of neuropeptides

SOM inhibited the macrophage migration induced by the living *L. major* promastigotes at a high concentration, 10^{-6} M (Fig. 2A). CGRP, on the other hand, showed a tendency towards stimulation at all concentrations tested, but the effect was only statistically significant at the highest concentrations, 10^{-6} and 10^{-5} M (Fig. 2B). In contrast, SP was inhibitory at both low concentrations, 10^{-10}–10^{-9} M, and at a high concentration, 10^{-6} M (Fig. 2C). The autonomic neuropeptides VIP and NPY showed opposite effects. VIP stimulated the macrophage migration at 10^{-9} and 10^{-5} M (Fig. 2D), while NPY inhibited it at low concentrations, 10^{-10} and 10^{-9} M, the maximal inhibitory effects occurring at 10^{-9} M (Fig. 2E).

DISCUSSION

The present data provide evidence that viability of the *L. major* promastigotes is essential for effective attraction of macrophages. The mechanism(s) by which the living *L. major* promastigotes induce their chemoattractant effect on macrophages is not clear. Upon entering the vertebrate host, promastigotes may activate complement via the alternative [34] or classical [35] pathway. This complement activation, among an array of other factors, creates a C5a gradient along which macrophages are chemotactically attracted [36]. Furthermore, differential expressions of chemokines such as macrophage chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and -1β, RANTES (Regulated on Activation, Normal T cell Expressed and presumably Secreted), I-309, interleukin (IL)-8, and adhesion molecules, have been reported in American cutaneous leishmaniasis [37, 38]. The chemotaxis could also occur through continuous synthesis by living promastigotes of a cell membrane-associated factor(s), because promastigote culture supernatant failed to modulate the macrophage migration, indicating that the factor(s) responsible for chemotaxis is not released into the surrounding medium. The latter suggestion sounds realistic, as preincubation of macrophages with promastigotes for 2 h completely abolished the chemotactic capacity of these cells towards the living parasites. This indicates that the chemoattractant activity of the living promastigote is a receptor-mediated process, which possibly becomes saturated during the parasite-macrophage preincubation period, resulting in abolishment of the macrophage chemotactic capacity towards parasites. Furthermore, the promastigote-derived sonicate had a stimulatory effect, although not significant, which is consistent with a previous observation of a chemoattractant effect

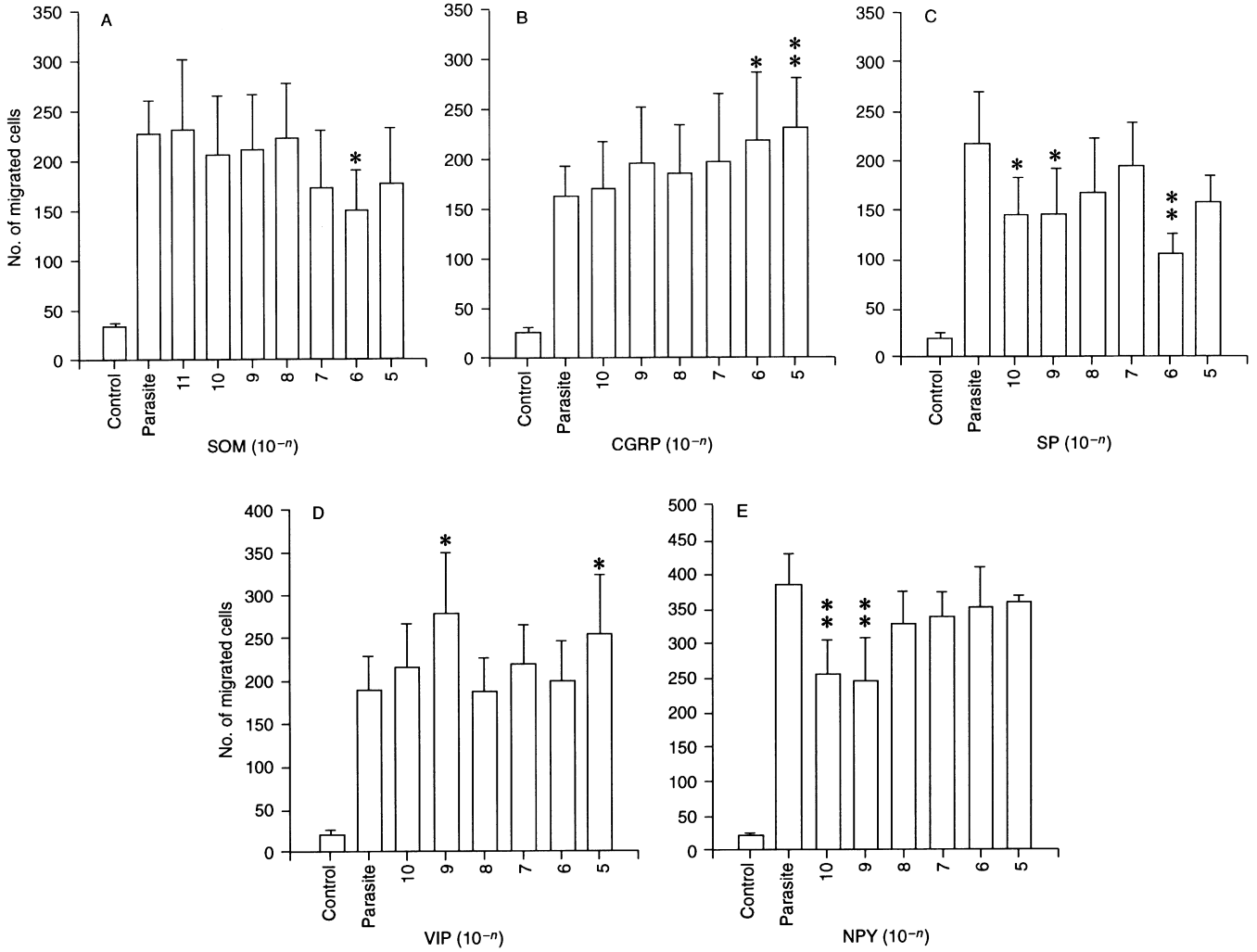

Fig. 2. The effects of SOM (A), CGRP (B), SP (C), VIP (D) and NPY (E) on macrophage migration induced by living *L. major* promastigotes in comparison with the control (DMEM + 5% FBS). Data represent mean ± SEM of five separate experiments run in triplicate. * $P < 0.05$, ** $P < 0.01$.

of *L. mexicana amazonensis*-derived sonicate on human monocytes and neutrophils [9].

On the other hand, paraformaldehyde-killed promastigotes significantly inhibited the macrophage chemotactic activity, indicating that the living promastigote chemotactic factor(s) may be proteinacious in nature. The inhibitory effect of paraformaldehyde-killed promastigotes on macrophage attraction might be due to the failure of the dead promastigotes to synthesise chemoattractive factors on their surfaces, which normally might occur in response to a macrophage signal. Another explanation could be that paraformaldehyde destroys or masks the chemoattractive receptors on the parasite cell surface, with subsequent exposure of other inhibitory surface factors such as gp63 protease, which would block macrophage chemotactic activity. In this context, heating *L. mexicana amazonensis* derived-sonicate at 65 °C for 30 min resulted in loss of chemoattraction for monocytes, while such treatment increased that for neutrophils, suggesting that different factors are responsible for the chemotactic activities of monocytes and neutrophils [9]. Moreover, the most abundant cell surface glycoprotein, gp63 on *L. major* as well as on the other *Leishmania spp.*, showed a tendency to inhibit the macrophage attraction, which concurs with a previous report that heating of the protease gp63 at 70 °C for 15 min completely abolished the inhibitory effect on monocyte chemotaxis [28]. In addition to the inhibitory effect of gp63 on chemotaxis, it has also been shown to cleave CD_4 receptors on the surface of T cells, which might explain its inhibitory effect in this migration assay [39]. Fractionation studies are needed for characterization of the chemoattractant components of promastigotes to elucidate the mechanism for chemotaxis during this critical step of infection, which could be a successful target for future disease prevention and therapy.

Some neuropeptides have shown an ability to induce inflammatory cell influx and expression of cell adhesion molecules in normal human skin after intradermal injection of immunogens [40]. In the present study, SOM inhibited the chemotactic

migration of macrophages towards living *L. major* promastigotes, at a high concentration, 10^{-6} M, a concentration that might be available in the nerve terminals at the periphery. This corresponds with the reported direct inhibitory effect of SOM on the chemotactic response of neutrophils to IL-8 [41].

In contrast, CGRP at high concentrations, 10^{-6} and 10^{-5} M, stimulated the parasite-induced macrophage migration. This stimulatory effect conforms to a previous observation that this neuropeptide enhanced the adhesion of human leucocytes to vascular endothelial cells, possibly through a direct effect on the microvessels [42]. In addition, CGRP has been shown to induce human endothelial cell proliferation [43]. Furthermore, in both its forms, α and β, CGRP has been found to be chemotactic to human T cells in a receptor-mediated fashion, because preincubation of the cells with CGRP down-regulated the chemotactic activity towards this neuropeptide [13].

SP inhibited macrophage migration towards promastigotes at 10^{-6}, 10^{-9} and 10^{-10} M, a finding that contradicts previous reports on a chemotactic effect of SP on human T cells [44] and monocytes [45], in a receptor-mediated manner. Moreover, it enhanced granulocyte infiltration into mouse skin both through degranulation of mast cells and by a direct action on endothelial cells [46]. In addition, SP has been shown to induce expression of endothelial leucocyte adhesion molecule (ELAM)-1 [47] and P- and E-selectin on human skin microvascular endothelium [40]. Furthermore, SP receptors have been identified on human skin keratinocytes, and incubation of these cells with SP induced expression of intercellular adhesion molecule (ICAM)-1 and production of cytokines, leading to keratinocyte activation [48]. As the parasite–macrophage interactions and the subsequent entry of the parasites into their target cells constitute the initial steps in the establishment of the disease, the inhibitory effect of SP on macrophage migration towards *L. major* parasites might result in abrogation of this initial step in the disease process and hence give protection to the host.

The autonomic neuropeptides VIP and NPY exhibited mutually opposite effects. VIP stimulated *L. major*-induced macrophage migration at a low, 10^{-9} M, and at a high, 10^{-5} M, concentration. This is in line with a previous finding on VIP enhancement of adherence and chemotaxis for rat peritoneal macrophages but not for lymphocytes [12], but not with another report on an inhibitory effect of VIP on rat alveolar macrophage chemotaxis towards an endotoxin-activated rat serum [49]. Furthermore, VIP has been shown to exert dual effects on leucocyte migration, an inhibitory effect at 10^{-9}–10^{-7} M and a stimulatory effect at 10^{-14}–10^{-12} M, and the intact peptide was essential for these effects [50].

NPY inhibited macrophage migration in a concentration-dependent manner, with a maximal inhibitory effect at 10^{-10} and 10^{-9} M. The latter was the same concentration at which VIP exerted a significant stimulatory effect. It has been suggested that VIP and NPY may be released simultaneously from sympathetic nerve terminals to act either as agonists or antagonists for each other in the microenvironment [51]. The inhibitory effect of NPY is in contrast with a previous report on an ability of NPY to enhance the adhesion of human peripheral mononuclear cells and cells of the monocyte cell line U 937 to cultured human umbilical vein endothelial cells (HUVECs) in a dose- and time-dependent manner. The enhancing effect of NPY on the adhesiveness of these cells to HUVECs was not due to induction of adhesion molecules but rather to a direct action on the endothelial cells [52].

The mechanisms underlying the modulating effects of the investigated neuropeptides at variable concentrations, and the lack of a dose-dependent effect, on *L. major*-induced macrophage migration are not known. In view of that, the neuropeptides might exert their effects both through receptor- and nonreceptor-mediated mechanisms [53], the possibility of a receptor-mediated mechanism of action seems likely at low, 10^{-10}–10^{-9} M, concentrations, as most of the neuropeptide receptor Kd values are around 10^{-9} M. On the other hand, nonreceptor mechanisms such as a direct action on cell membrane and calcium channels via its charge, is more likely with high, 10^{-6} and 10^{-5} M, concentrations.

The discrepancy between the present and previous findings on the effects of neuropeptides on various cell populations could be attributed to the different experimental procedures and the origin of cell population used. The previous studies used normal T cells, granulocytes or keratinocytes from human or mouse skin, while the present study was carried out with macrophages challenged with *L. major* parasites.

In conclusion, our findings provide evidence for an ability of *L. major* promastigotes to attract macrophages. The nervous system, represented by the sensory and autonomic nerve fibres and their contents of neuropeptides at the periphery, may modulate these initial steps in the host–parasite interactions and, possibly, the subsequent development of disease.

ACKNOWLEDGEMENTS

This study was supported by grants from the Welander/Finsen Foundation, the Swedish Society for Medical Research, the Danish Biotechnology Programme, Centre for Cell Communication, and funding from the Karolinska Institute.

REFERENCES

1 Liew FY, O'Donnell CA. Immunology of leishmaniasis. Adv Parasitol 1993;32:161–236.
2 Reiner SL, Locksley RM. The regulation of immunity to *Leishmania major*. Ann Rev Immunol 1995;13:151–77.
3 Russell DG, Talamas-Rohana P. Leishmania and the macrophage: a marriage of inconvenience. Immunol Today 1989;10:328–32.
4 Alexander J, Russell DG. The interaction of Leishmania species with macrophages. Adv Parasitol 1992;31:175–239.
5 Celada A, Nathan C. Macrophage activation revisited. Immunol Today 1994;15:100–2.
6 Chang K-P. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int Rev Cytol (Suppl) 1983;14:267–308.

7 Reiner NE. Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. Immunol Today 1994;15:374–81.
8 Chang K-P. Leishmanicidal mechanisms of human polymorphonuclear phagocytes. Am J Trop Med Hyg 1981;30:322–33.
9 Sørensen AL, Kharazmi A, Nielsen H. *Leishmania* interaction with human monocytes and neutrophils: Modulation of the chemotactic response. APMIS 1989;97:754–60.
10 Carr DJJ. Neuroendocrine peptide receptors on cells of the immune system. In: Blalock JE, ed. Neuroimmunoendocrinology. Basel:Karger, 1992; 84–105.
11 Goetzl EJ, Adelman DC, Sreedharan SP. Neuroimmunology. Adv Immunol 1990;48:161–90.
12 Fuente MDL, Delgado M, Rio MD *et al.* Vasoactive intestinal peptide modulation of adherence and mobility in rat peritoneal lymphocytes and macrophages. Peptides 1994;15:1157–63.
13 Foster CA, Mandak B, Kromer E, Rot A. Calcitonin gene-related peptide is chemotactic for human T lymphocytes. Ann NY Acad Sci 1992;657:397–404.
14 Fuente MDL, Delgado M, Rio MD, Martínez C, Hernanz A, Gomariz RP. Stimulation by vasoactive intestinal peptide (VIP) of phagocytic function in rat macrophages. Protein kinase C involvement. Regul Pept 1993;48:345–53.
15 Wiik P. Vasoactive intestinal peptide inhibits the respiratory burst in human monocytes by a cyclic AMP-mediated mechanism. Regul Pept 1989;25:187–97.
16 Lotz M, Vaughan JH, Carson DA. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. Science 1988;241:1218–21.
17 Blum AM, Metwali A, Mathew R, Elliot D, Weinstock JV. Substance P and somatostatin can modulate the amount of IgG2a secreted in response to Schistosome egg antigens in murine *Schistosomiasis mansoni*. J Immunol 1993;151:6994–7004.
18 Kimata H, Yoshida A, Ishioka C, Mikawa H. Differential effect of vasoactive intestinal peptide, somatostatin, and substance P on human IgE and IgG subclass production. Cell Immunol 1992;144:429–42.
19 Blalock JE. Production of peptide hormones and neurotransmitters by the immune system. In: Blalock JE, ed. Neuroimmunoendocrinology. Basel:Karger, 1992; 1–24.
20 Weinstock JV. Production of neuropeptides by inflammatory cells within the granulomas of murine *Schistosomiasis mansoni*. Eur J Clin Invest 1991;21:145–53.
21 Leceta J, Martínez C, Delgado M, Garrido E, Gomariz RP. Expression of vasoactive intestinal peptide in lymphocytes: A possible endogenous role in the regulation of immune system. Adv Neuroimmunol 1996;29:29–36.
22 Weinstock JV, Blum AM, Malloy T. Macrophages within the granulomas of murine *Schistosoma mansoni* are a source of a somatostatin 1–14 like molecule. Cell Immunol 1990;131:381–90.
23 Johnson HM, Downs MO, Pontzer CH. Neuroendocrine peptide hormone regulation of immunity. In: Blalock JE, ed. Neuroimmunoendocrinology. Basel:Karger, 1992; 49–83.
24 Wallengren J, Ekman R, Sundler F. Occurrence and distribution of neuropeptides in human skin. Acta Derm Venereol (Stockh) 1987;67:185–92.
25 Louis JA, Moedder E, Behin HD. Regulation of protozoan parasite antigens by murine T lymphocytes. 1. Induction of specific T lymphocyte dependent proliferative response to *Leishmania tropica*. Eur J Immunol 1979;9:841–7.
26 Said SI, Mutt V. Isolation from porcine-intestinal wall of a vasoactive octacosapeptide related to secretin and to glucagon. Eur J Biochem 1972;28:199–204.
27 Bouvier J, Etges RJ, Bordier C. Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes. J Biol Chem 1985;260:15504–9.
28 Russell DG, Wilhelm H. The involvement of the major surface glycoprotein (Gp63) of *Leishmania* promastigotes in attachment to macrophages. J Immunol 1986;136:2613–20.
29 Sørensen AL, Hey AS, Kharazmi A. *Leishmania major* surface protease Gp63 interferes with the function of human monocytes and neutrophils *in vitro*. APMIS 1994;102:265–71.
30 Prestidge L, Gage V, Spizizen J. Protease activities during the course of sporulation in *Bacillus subtilis*. J Bacteriol 1971;107:815–23.
31 Boyden SV. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med 1962;115:453–66.
32 Hauzenberger D, Klominek J, Sundqvist K-G. Functional specialization of fibronectin-binding β_1-integrins in T lymphocyte migration. J Immunol 1994;153:960–71.
33 Montgomery DC. Design and analysis of experiments. New York: John Wiley & Sons, 1991.
34 Mosser DM, Edelsen PJ. Activation of the alternative complement pathway by *Leishmania* promastigotes: Parasite lysis and attachment to macrophages. J Immunol 1984;132:1501–5.
35 Mosser DM, Burke SK, Coutavas EE, Wedgewood JF, Edelsen PJ. *Leishmania* species: Mechanisms of complement activation by five strains of promastigotes. Exp Parasitol 1986;62:394–404.
36 Snyderman R, Pike MC, McCarley D, Lang L. Quantification of mouse macrophage chemotaxis *in vitro*: Role of C5 for the production of chemotactic activity. Infect Immun 1975;11:488–92.
37 Ritter U, Moll H, Laskay T *et al.* Differential expression of chemokines in patients with localized and diffuse cutaneous leishmaniasis. J Infect Dis 1996;173:699–709.
38 Tapia FJ, Caceres-Dittmar G, Sánchez MA, Fernández CT, Rondón AJ, Convit J. Adhesion molecules in lesions of American cutaneous leishmaniasis. Exp Dermatol 1994;3:17–22.
39 Hey AS, Theander TG, Hazrati SM, Hviid L, Kemp M, Kharazmi A. Cleavage of CD-4 molecules on human T cells by *Leishmania major* and *Leishmania donovani* surface glycoprotein gp63. J Immunol 1994;152:4542–8.
40 Smith CH, Barker JNWN, Morris RW, MacDonald DM, Lee TH. Neuropeptides induce rapid expression of endothelial cell adhesion molecules and elicit granulocytic infiltration in human skin. J Immunol 1993;151:3274–82.
41 Peluso G, Petillo O, Melone MA, Mazzarella G, Ranieri M, Tajana GF. Modulation of cytokine production in activated human monocytes by somatostatin. Neuropeptides 1996;30:443–9.
42 Sung C-P, Arleth AJ, Aiyar N, Bhatnagar PK, Lysko PG, Feuerstein G. CGRP stimulates the adhesion of leukocytes to vascular endothelial cells. Peptides 1992;13:429–34.
43 Haegerstrand A, Dalsgaard C-J, Jonzon B, Larsson O, Nilsson J. Calcitonin gene-related peptide stimulates proliferation of human endothelial cells. Proc Natl Acad Sci USA 1990;87:3299–303.
44 Foster CA, Mandak B, Kromer E, Rot A. The neuropeptide substance P is chemotactic for human T cells. J Invest Dermatol (abstr) 1991;29:537.
45 Ruff MR, Wahl SM, Pert CB. Substance P receptor-mediated chemotaxis of human monocytes. Peptides 1985;6:107–11.
46 Iwamoto I, Tomoe S, Tomioka H, Yoshida S. Substance P-induced

granulocyte infiltration in mouse skin: the mast cell-dependent granulocyte infiltration by the N-terminal peptide is enhanced by the activation of vascular endothelial cells by the C-terminal peptide. Clin Exp Immunol 1992;87:203–7.

47 Matis WL, Lavker RM, Murphy GF. Substance P induces the expression of an endothelial-leukocyte adhesion molecule by microvascular endothelium. J Invest Dermatol 1990;94:492–5.

48 Viac J, Guénche A, Doutremepuich JD, Reichert U, Claudy A, Schmitt D. Substance P and keratinocyte activation markers: an *in vitro* approach. Arch Dermatol Res 1996;88:85–90.

49 Litwin DK, Wilson AK, Said SI. Vasoactive intestinal polypeptide (VIP) inhibits rat alveolar macrophage phagocytosis and chemotaxis *in vitro*. Regul Pept 1992;40:63–74.

50 Bondesson L, Nordlind K, Lidén S, Gafvelin G, Theodorsson E, Mutt V. Dual effects of vasoactive intestinal polypeptide (VIP) on leucocyte migration. Acta Physiol Scand 1991;141:477–81.

51 Lasanen I, Tammela TLJ, Liesi P, Waris T, Polak JM. The effect of acute distension on vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and substance P (SP) immunoreactive nerves in the female rat urinary bladder. Urolog Res 1992;20:259–63.

52 Sung C-P, Arleth AJ, Feuerstein GZ. Neuropeptide Y upregulates the adhesiveness of human endothelial cells for leukocytes. Circul Res 1991;68:314–8.

53 Agro A, Stanisz AM. Neuroimmunomodulation: classical and nonclassical cellular activation. Adv Neuroimmunol 1995;5:311–9.