Decreased concentrations of CGRP in *Leishmania major* murine cutaneous leishmaniasis

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Abstract

The expression of the sensory neuropeptide calcitonin gene-related peptide (CGRP) in the skin, secondary lymphoid organs and dorsal root ganglia (L_4–L_6) in Leishmania major-induced inflammation was evaluated by radioimmunoassay. The investigation was conducted on two mouse strains, the susceptible BALB/c and the resistant C57BL/6. The CGRP concentration in the inflamed skin of both mouse strains was decreased as early as 1 week postinfection, compared to controls. A further reduction was observed in both mouse strains throughout the 9-week study period, but was more evident in the susceptible strain. The CGRP concentration was increased in the ipsilateral dorsal root ganglia (L_4–L_6) of mice of the resistant strain 1 week postinfection, while no change was observed in the susceptible strain. In the remaining part of the study period there was a reduction in CGRP in the ipsilateral dorsal root ganglia of both mouse strains. In the spleen, a reduction was noted in the infected BALB/c at all measurement times (significant at 6 and 9 weeks), while no change was observed in C57BL/6 strain. These findings may indicate a regulatory function of CGRP in the pathophysiology of murine cutaneous leishmaniasis and hence in the disease outcome. The reduction in CGRP might also explain the defective nociception observed in patients with cutaneous leishmaniasis. © 1998 Elsevier Science Ireland Ltd.

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Leishmania (L.) major causes Old World cutaneous leishmaniasis in man. Murine infection with L. major, which is genetically determined, leads to self-limiting cutaneous or progressive visceral infection. The mouse strains C57BL/6 and BALB/c are susceptible to infection with L. major. C57BL/6 resolves infection with establishment of long-lasting immunity, whereas BALB/c mice fail to control local replication of the parasite and suffer from fatal progressive disease [12].

Cutaneous leishmaniasis is characterised by chronicity and granuloma formation [13]. The clinical picture varies from painless dry papules to purulent ulcers. In addition, peripheral nerve involvement in both murine and human cutaneous leishmaniasis, and clinical anaesthesia as well as hyperaesthesia, have been reported [11,16].

Several neuropeptides, e.g. substance P (SP), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY), have been detected in normal and inflamed skin by immunohistochemical and radioimmunological techniques [17]. CGRP, a product of an alternative processing of mRNA from the calcitonin gene, has been localised to the peripheral nervous system [14]. It is identified in small unmyelinated sensory C-fibres, which are associated with smooth muscle of blood vessels, and as free nerve endings in the epidermis and dermis [7]. Antidromic stimulation of C-fibres leads to the release of CGRP [6]. Previous reports have shown an anti-inflammatory role for CGRP in various inflammatory conditions [2–4]. Furthermore, increased synthesis and transport of CGRP has been observed in sensory nerves of inflamed tissue, and shown to be regulated by nerve growth factor (NGF) [5]. On the other hand, a decreased number of nerve fibres with immunoreactivity for CGRP has been reported in human and murine leprosy [9,10].

The immunopathology of leishmaniasis has been extensively studied, but the question of possible involvement of

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the nervous system in cutaneous leishmaniasis, and of CGRP in particular, has not yet been addressed. The aim of the present study was to investigate the expression of CGRP, as a representative of sensory neuropeptides, in a murine model of cutaneous leishmaniasis using the two different mouse strains and to correlate the concentrations in the skin, draining lymph nodes, spleen and dorsal root ganglia with the course of the disease.

Female BALB/c and C57BL/6 mice, aged 10–12 weeks, were used. The animals were housed at the Department of Neuroscience, seven per cage, at 21°C in a 12:12 h light-dark cycle and with water and food ad libidum.

Leishmania major parasites (JISHI 18 strain) were obtained from Dr. D. Evans, London School of Hygiene and Tropical Medicine, University of London. The parasites were kept virulent by monthly passage in BALB/c mice. The maintenance, cultivation and isolation of the promasti-gote stage of the L. major parasite have been described in detail [8]. For animal infection, groups (nine mice/group) of both strains of mice were injected subcutaneously with 1×10^7 stationary phase promastigotes in 0.05 ml of sterile phosphate buffered saline (PBS) in the right hind foot pad. Age-matched control animals received 0.05 ml PBS.

The skin, the draining popliteal lymph nodes, spleen and dorsal root ganglia (L_4–L_6) were excised 1, 3, 6 and 9 weeks postinfection and immediately frozen on dry ice and kept at −70°C prior to analysis.

Frozen samples were weighed and boiled for 10 min in 10 volumes of extraction buffer (2 M acetic acid, pH 3.4). After homogenisation in a polytron for 15 s, the samples were sonicated for 30 s, followed by centrifugation at $3000 \times g$ for 15 min. The supernatants were lyophilised and then diluted in 1 ml phosphate buffer pH 7.2 and kept at −20°C until analysed.

CGRP-like immunoreactivity (LI) was analysed using antiserum CGRPR8 raised in a rabbit against conjugated rat CGRP. HPLC-purified [^{125}I]-histidyl rat CGRP was used as radioligand and rat CGRP α as standard. The detection limit of the assay for rat CGRP is 9 pmol/l and the cross-reactivity of the assay to SP, neurokinin A, neurokinin B, neuropeptide K, gastrin, neurotensin, bombesin, NPY and calcitonin was less than 0.01%. The cross-reactivities to human CGRP α and β were 93 and 24%, respectively,

and to rat CGRP α and β, 100 and 120%, respectively. The intra- and interassay coefficients of variation were 8 and 14%, respectively.

Reverse-phase HPLC was performed on tissue extracts, using a Waters Delta pak C18 300 Å, with a 3.9 mm × 15 cm column eluted with a 40 min linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. Two P3500 pumps (Pharmacia & Upjohn, Uppsala, Sweden) were controlled by a GP250 gradient programmer (Pharmacia & Upjohn). A gradient of 20–50% acetonitrile was used for CGRP. Samples were passed through Millipore GS filters (0.45 μm) before chromatography, to remove particulate matter. Samples of 200 μl were injected into the columns. Fractions of 0.5 ml were collected at an elution rate of 1.0 ml/min. Each fraction was lyophilised and redissolved in 100 μl distilled water before analysis. The fractions were assayed for immunoreactivity in the tubes used for their collection.

Data represent mean ± SEM. Differences in CGRP concentrations between the groups were analysed with the Mann–Whitney U-test, and a P-value of <0.05 was considered statistically significant.

The CGRP concentration was about 1·5 times higher in the skin of C57BL/6 control animals than in the BALB/c strain controls (Tables 1 and 2).

The CGRP concentrations in the skin of the infected animals of both strains were significantly reduced throughout the study period as compared with the control animals. At 3, 6 and 9 weeks postinfection, the CGRP concentrations were about 4, 8 and 9, times lower, respectively, in the skin of the infected BALB/c mice than in the infected C57BL/6 skin (Tables 1 and 2).

In the dorsal root ganglia of the control mice of both strains, the CGRP concentrations were higher at 3, 6 and 9 weeks than at 1 week, with no difference between the strains. Regarding the dorsal root ganglia of the infected BALB/c strain, a statistically significant decrease in CGRP concentration was observed at 3, 6 and 9 weeks compared with the controls, while no difference was observed at 1 week postinfection. In the C57BL/6 strain an increase was noted at 1 week, followed by a reduction throughout the rest of the study period, with a statistically significant decrease only at 6 weeks postinfection (Tables 1 and 2).

Table 1

Tissue concentrations of CGRP-LI in L. major infected and control BALB/c mice

Group	Skin		Spleen		Dorsal root ganglia	
	Infected	Control	Infected	Control	Infected	Control
I	7.2 ± 0.4*	10.2 ± 0.8	1.0 ± 0.0	1.1 ± 0.1	36.4 ± 7.8	36.6 ± 7.0
II	1.2 ± 0.1*	9.7 ± 0.9	0.5 ± 0.1	0.8 ± 0.2	52.4 ± 6.1	178.2 ± 17.1
III	1.1 ± 0.1**	14.0 ± 1.0	0.3 ± 0.0*	0.8 ± 0.2	52.4 ± 6.1**	240.6 ± 22.9
IV	1.3 ± 0.2**	15.6 ± 0.9	0.3 ± 0.0***	1.0 ± 0.0	115.6 ± 6.6**	186.6 ± 38.7

The concentrations were obtained by RIA measurements in tissue extracts from individual animals; data are the mean ± SEM (pmol/g, $n = 7$). Differences between infected and control groups (right side vs. right side) are expressed by asterisks according to the level of significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2

Tissue concentrations of CGRP-LI in L. major infected and control C57BL/6 mice

Group	Skin		Spleen		Dorsal root ganglia	
	Infected	Control	Infected	Control	Infected	Control
I	9.2 ± 0.9**	18.0 ± 1.1	1.0 ± 0.0	1.1 ± 0.1	64.3 ± 13.6*	39.2 ± 5.9
II	4.2 ± 2.1**	17.9 ± 1.0	1.4 ± 0.3	1.0 ± 0.0	114.0 ± 7.1	140.3 ± 17.5
III	9.0 ± 0.7**	23.2 ± 2.1	0.6 ± 0.1	0.7 ± 0.1	59.4 ± 7.9**	225.9 ± 25.1
IV	11.5 ± 0.6**	15.9 ± 1.5	0.8 ± 0.1	0.9 ± 0.1	105.5 ± 5.0	129.3 ± 21.5

The concentrations were obtained by RIA measurements in tissue extracts from individual animals; data are the mean ± SEM (pmol/g, $n = 7$). Differences between infected and control groups (right side versus right side) are expressed by asterisks according to the level of significance. *$P < 0.05$, **$P < 0.01$.

The CGRP concentrations in the lymph nodes were below the detection limit. In the spleen of the infected BALB/c, although the concentrations were also low, there was a reduction in CGRP throughout the study period, which was statistically significant at 6 and 9 weeks postinfection, while no difference was observed in the infected C57BL/6 as compared with their controls (Tables 1 and 2).

Reverse-phase HPLC analysis of tissue extracts of infected mouse skin, draining lymph nodes, spleen and dorsal root ganglia revealed distinct immunoreactive components reflecting the presence of CGRP. A double peak was observed for CGRP, with the major component eluting in the position of rat CGRP α, and the minor component somewhat earlier, in a position where rat CGRP β was eluted during calibration (Fig. 1).

The present data show that CGRP in the skin of both mouse strains is depleted as early as 1 week after infection with L. major. The reason for the depletion of CGRP in the inflamed skin is not clear, but it might be due to defective storage of the peptide at the periphery as a result of peripheral nerve involvement, as previously mentioned [11,16]. A further explanation might be exhaustion of this peptide by the rapidly multiplying parasites for establishment of infection. In this context, we observed a proliferative effect of CGRP on L. major promastigotes in vitro in a preliminary study. Furthermore, the reduction in the skin CGRP concentrations was more marked and progressive with time in the susceptible mouse strain, BALB/c. This difference in CGRP concentrations between the strains might contribute, besides

other factors, to the susceptibility or resistance to L. major infection. Through induction of Th$_1$ or Th$_2$ cytokines, which have been shown to play a major role in determining the disease outcome in this model, CGRP has been shown to enhance the production of IL-6, induced by IL-1 β and TNF-α, by fibroblasts [15]. In addition, the susceptible mouse control skin had a lower CGRP concentration than the resistant mouse control skin. This might be explained by the genetic background of the two strains.

The dorsal root ganglia of the resistant strain showed an increase in CGRP at 1 week postinfection. This early high CGRP concentration might be critical for the disease outcome, since the dorsal root ganglia of the susceptible strain showed no change in CGRP, possibly as a result of an inhibitory signal delivered by the parasites to the neurones.

NGF is known to play a dynamic role in the control of the neurotransmitter peptide levels and synthesis in mature sensory neurones [18]. In a previous study [1] we observed expression of high NGF concentrations in the skin of the resistant mouse at 1 week following infection with L. major, but no change in the susceptible strain. This difference in NGF expression in the skin of these mouse strains and the probable variation in the amount of NGF retrogradely transported might further explain the difference in CGRP concentration in the dorsal root ganglia at 1 week following L. major infection.

The control mice of both strains had lower concentrations of CGRP in the dorsal root ganglia at 1 week than at later time-points in the study period. The reason for this is not
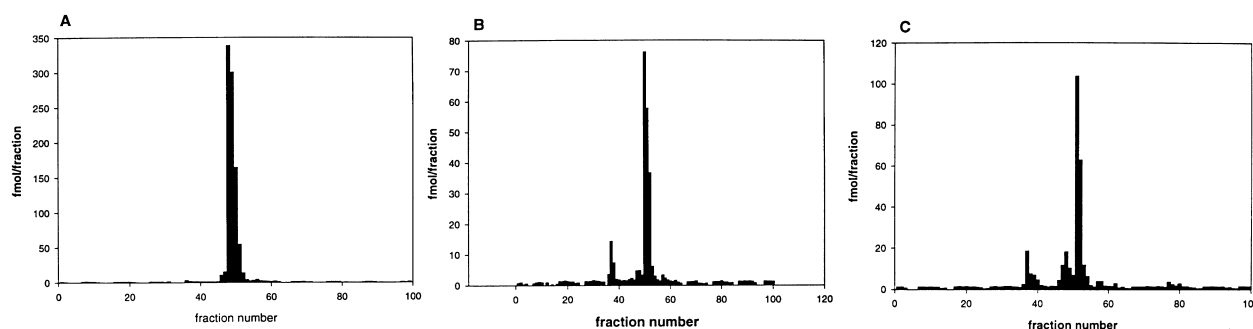

Fig. 1. Reverse-phase HPLC of CGRP-LI in standard (A) and in extracts from L. major infected skin (B) and the infected side dorsal root ganglia (C). The elution volume of rat CGRP is evident between fraction 40 and 60.

clear. It might be due to the fact that the animals had received saline 1 week before sacrifice and the resulting trauma might have activated endopeptidase enzymes, which are responsible for the peptide degradation.

The decrease in CGRP concentrations in the ipsilateral dorsal root ganglia of both mouse strains throughout the rest of the investigated period could be due to decreased synthesis and/or increased release of the peptide to the periphery in an attempt to dampen the progressing disease process. Furthermore, the reduction in the dorsal root ganglia CGRP at 1, 3 and 6 weeks postinfection was greater in BALB/c mice than in those of the C57BL/6 strain. This could also be a factor, additional to the genetic background of these animals, with a clear effect on the disease outcome, influencing the differential responses of these mice to the L. major infection.

Further support for an anti-inflammatory effect of CGRP in this murine model of cutaneous leishmaniasis is provided by the difference in CGRP concentrations in the spleen between the infected mice of the two strains. The susceptible mouse strain showed a statistically significant decline in CGRP concentrations with progression of the disease, while in the resistant strain there was no change. In our study on NGF concentrations in this model [1], a significant increase in NGF concentration was found in the spleen of the C57BL/6 strain at 1, 3 and 9 weeks postinfection, whereas the BALB/c strain showed a decrease throughout the study period. Thus, the difference in NGF concentration in the spleen might explain this observed variation in the CGRP levels, and hence the difference in the strain response to L. major challenge.

In conclusion, CGRP seems to be involved in the pathophysiology of cutaneous leishmaniasis. The differences in CGRP expression between the resistant strain C57BL/6 and the susceptible strain BALB/c, especially during the first week, might have an implication in the disease outcome. The reduction in the CGRP concentration in the skin of infected mice might also serve, among other neurotransmitters, as a basis in attempts to explain the disturbed nociception observed in patients with cutaneous leishmaniasis.

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