

Differential Expression of Nerve Growth Factor in *Leishmania Major* Murine Cutaneous Leishmaniasis

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The cross-talk between the immune and nervous systems is becoming an interesting field of research and there is accumulating evidence supporting this notion. In the present study we investigated the levels of nerve growth factor in a murine model of cutaneous leishmaniasis, using a two-site ELISA. Two strains of inbred mice were used for this purpose, namely BALB/c and C57BL/6, genetically susceptible and resistant, respectively, to infection with *Leishmania major*. This work demonstrates a difference in expression of nerve growth factor in the skin and secondary lymphoid organ microenvironment, as well as in the serum, between these mouse strains. The high nerve growth factor levels in the microenvironment seem to be important and possibly critical for the outcome of the disease. Compared with controls, the resistant strain, C57BL/6, expressed significantly increased nerve growth factor levels in the skin, secondary lymphoid organs and serum at 1 week post-infection, whereas the susceptible strain, BALB/c, showed no change in the skin and a slight increase in the lymphoid organs and serum at this time-point. These high nerve growth factor levels in the early stage of the disease, whether produced directly by the inflammatory cells or indirectly through its induction by other cytokines or both, might indicate a contribution of this neurotrophic factor to differentiation of naive T lymphocytes into either Th₁ or Th₂ subsets that fundamentally govern the disease outcome. The expression of significantly elevated nerve growth factor levels in the skin and lymphoid organs of C57BL/6 at the late studied time points might suggest a role for nerve growth factor in the resolution of the disease process, which is usually evident from 6 weeks post-infection in this model. The high nerve growth factor levels expressed in the skin, lymph nodes and serum of BALB/c at late stages of the disease may be explained as an attempt to counteract the progression and dissemination of the disease.

This investigation adds further experimental evidence for an anti-inflammatory effect of nerve growth factor, possibly through its action as a link between the nervous and immune systems. **Key words:** mice; *Leishmania major*; ELISA; nerve growth factor.

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Leishmania (L.) major is a protozoan parasite that infects mononuclear phagocytic cells in its vertebrate host and causes Old World cutaneous leishmaniasis in man. Murine infection with *L. major*, the outcome of which is genetically determined (1), leads to both self-limiting cutaneous and progressive visceral infection. Both C57BL/6 and BALB/c mice are susceptible to infection with *L. major*. C57BL/6 mice resolve the infection with establishment of long-lasting immunity, whereas

BALB/c mice fail to control the local replication of the parasite and succumb to progressive disease (2). The immune response in cutaneous leishmaniasis is mainly cell-mediated, T lymphocytes and macrophages playing a crucial role in the outcome of the disease (3).

The immunological aspects of leishmaniasis have been extensively studied, but the possible role of the nervous system in this disease, represented by the nerve terminals innervating the skin and lymphoid organs, has been insufficiently investigated. Previous reports (4, 5) and our own (unpublished) data have pointed to peripheral nerve involvement in both human and murine skin in *L. major* cutaneous leishmaniasis. It is increasingly apparent that the nervous and immune systems influence each other's functions, and the sympathetic innervation of both primary and secondary lymphoid organs represents an important functional link between them (6, 7).

Factors known to be involved in the regulation of immune and inflammatory responses are able to influence the expression of nerve growth factor (NGF). On the other hand, NGF, which was initially identified as a trophic factor that is essential for the development and maintenance of sympathetic and sensory neurones in both the central and peripheral nervous system (8), has been shown to be produced, among other cellular sources, by peritoneal and brain macrophages (9) and to have biological effects on cells of the immune system (10–12).

This study was designed to determine the concentrations of NGF in the skin, draining lymph nodes, spleen and serum in two different strains of mice infected with *L. major*, using a highly sensitive two-site enzyme-linked immunosorbent assay (ELISA), and to correlate these levels with progression and resolution of the disease at specified time points.

MATERIALS AND METHODS

Mice, parasite and infection

Female BALB/c and C57BL/6 mice, aged 10–12 weeks, were used for this investigation. The animals were maintained in the animal facility at the Department of Pharmacology, Institute of Neuroscience, Karolinska Institute.

L. major parasites (JISHI 18 strain) were obtained from Dr. D. Evans, London School of Hygiene and Tropical Medicine, University of London. The parasite was kept virulent by monthly passage in BALB/c mice. The maintenance, cultivation and isolation of the promastigote stage of the *L. major* parasite have been described in detail (13). For animal infection, groups of 6 mice of both strains 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. Age-matched uninfected control animals of either strain received only 0.05 ml PBS. The progression or resolution of the disease was assessed weekly by measuring the thickness of the infected foot pad by a caliper (Starrett, Starrett Co., Athol, MA, USA) in comparison with the control foot pad.

The animals were sacrificed by cervical dislocation 1, 3, 6 and 9

weeks post-infection. Samples of the skin, draining popliteal lymph nodes and the spleen were excised and immediately frozen on dry ice. In addition, blood samples were drawn and centrifuged at 10,000 g for 10 min, and the sera were collected. All the samples were kept at -70°C until analysed.

Reagents

Mouse NGF- β was purified from the submandibular gland, as described previously (14). Monoclonal anti- β (2.5S, 7S) NGF, clone 27/21, which specifically reacts with the β -subunit of NGF from the mouse, both in the 2.5S and in the 7S form, anti- β (2.5S, 7S) NGF- β -gal (clone 27/21) conjugated with β -galactosidase, the substrate chlorophenol red- β -D-galactopyranoside (CPRG) and the enzyme protease inhibitor aprotinin were all purchased from Boehringer Mannheim (Mannheim, Germany).

Extraction of tissue samples

The frozen skin, lymph node and spleen samples were weighed and then homogenized with extraction buffer (100 mM Tris-HCl, 400 mM NaCl, 2% bovine serum albumin, 0.05% sodium azide, pH 7.0) (1:1) in a homogenizer (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany) for 30 s, followed by sonication in an ultrasonicator (Soniprep 150, MSE, Crawley, UK) for a further 30 s. The homogenate was centrifuged for 20 min at 4°C and 50,000 g, and the clear supernatant was then mixed 1:10 with 20 mM CaCl_2 containing 0.2% triton X-100 and kept at -20°C until analysis.

The frozen serum samples were treated immediately before analysis with 20 μg aprotinin/ml sample and incubated for 1 h at room temperature and subsequently overnight at 4°C . The samples were then centrifuged for 20 min at 4°C and 2,000 g and the supernatants were diluted 1:1 with double concentrated sample buffer (100 mM Tris-HCl, 400 mM NaCl, 20 mM CaCl_2 , 2% bovine serum albumin, 0.1% Triton X-100 and 0.1% sodium azide, pH 7.0).

ELISA for NGF- β

For determination of NGF- β levels in the tissue samples, a two-site immunoassay described earlier (19) was used and the manufacturer's instructions were followed with slight modifications. In brief, a flat bottom 96-well microplate (Coster, Cambridge, MA, USA) was coated with 100 μl of the primary antibody in coating buffer (50 mM $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, 0.1% sodium azide, pH 9.6) at a concentration of 1 $\mu\text{g}/\text{ml}$, and incubated for 2 h at 37°C . After removal of the coating solution, saturation of the non-specific binding sites was achieved by incubation with blocking solution (0.5% bovine serum albumin added to the coating buffer) for 30 min at 37°C . The plate was then washed four times with a solution of 50 mM Tris-HCl, 200 mM NaCl, 0.1% Triton X-100 and 0.05% sodium azide, pH 7.0, and then incubated with 100 μl of the standard or samples overnight at 4°C . After washing, the plate was incubated with anti-NGF- β -gal at a dilution of 1:100 in conjugate buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl_2 , 1% bovine serum albumin, 0.1% Triton X-100, 0.05% sodium azide, pH 7.0) for 4 h at 37°C . As a final step, the plate was washed and incubated with substrate at a dilution of 2:1 in substrate buffer (100 mM HEPES, 150 mM NaCl, 2 mM MgCl_2 , 1% bovine serum albumin, 0.1% sodium azide, pH 7.0) for 1 h and the developing colour was determined photometrically by an ELISA reader (Vmax, Molecular Devices, Sunny Wale, CA, USA) at 570 nm.

Statistics

Data are presented as mean $\pm$ SEM. Differences between groups were analysed statistically using the Wilcoxon rank sum test, and a p -value < 0.05 was considered as statistically significant.

RESULTS

Skin

The expression of NGF differed between the infected mice of the two strains compared with their controls. One week after

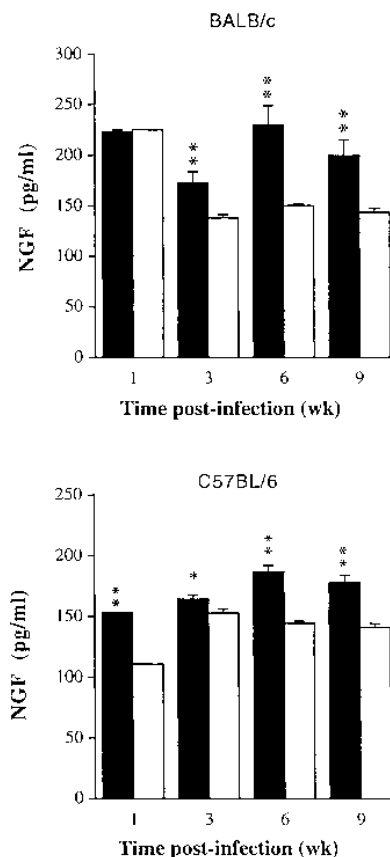


Fig. 1. Expression of NGF in the foot pad skin of the susceptible, BALB/c ($n=6$), and resistant, C57BL/6 ($n=6$), mouse strains (■) and their age- and sex-matched controls (□) ($n=6$ in each strain). * < 0.05 , ** < 0.01 compared with controls.

infection there was no change in the NGF level in the susceptible strain, BALB/c, compared with the control; the latter showed a high level at that time-point compared with the rest of the investigated period (Fig. 1). In contrast, the inflamed skin of the resistant strain, C57BL/6, displayed enhanced NGF expression compared with that of the control animals. This first week coincides with clinical establishment of the infection, observed as an increase in the foot pad thickness in the infected animal. Three weeks post-infection the infected animals of both strains showed significantly higher NGF levels than the controls, and this was more apparent in the susceptible strain. During the rest of the study period, increased NGF levels were observed in the inflamed skin of both mice strains.

Lymph node

One week after the infection an increase in the NGF level was observed in the draining popliteal lymph nodes of the infected mice of both strains, with a statistically significant difference in the resistant strain compared with controls (Fig. 2). Three weeks post-infection the NGF level was significantly higher in the infected mice than in the controls in both strains. At six weeks, the infected animals of both strains showed a rise in

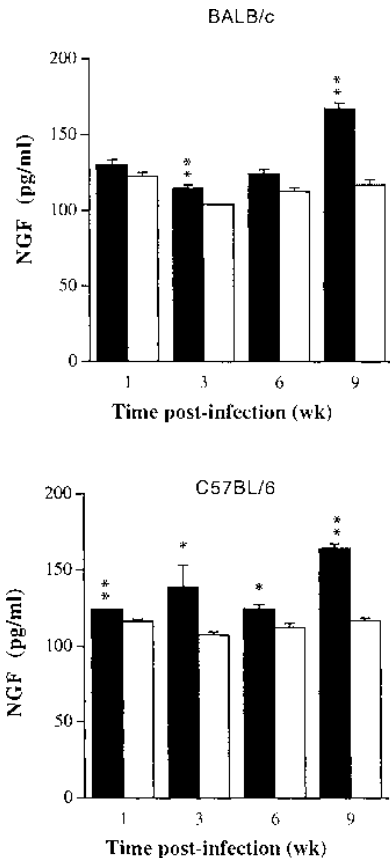


Fig. 2. Expression of NGF in the popliteal lymph nodes of the susceptible, BALB/c ($n=6$), and resistant, C57BL/6 ($n=6$), mouse strains (■) and controls (□). * <0.05 , ** <0.01 compared with controls.

NGF in the lymph nodes, which was statistically significant in the resistant C57BL/6 strain, and 9 weeks post-infection the infected mice of both strains exhibited significantly higher NGF levels than their controls.

The 9-week time-point corresponds to the beginning of the disease resolution process in the resistant strain, as observed by regression of the foot pad swelling, while it represents the time of disease dissemination in the susceptible strain. The spread of disease in the susceptible strain was evident at this period, with an increase in foot pad thickness, ulceration of skin lesions, enlargement of the draining popliteal lymph nodes and splenomegaly in comparison with the control animals.

Spleen

The infected mice of the susceptible strain, BALB/c, showed a tendency towards high NGF levels in the spleen at 1 week post-infection, followed by a decrease throughout the study period compared with the controls (Fig. 3). In the infected animals of the resistant strain, C57BL/6, on the other hand, there was a statistically significant rise in NGF at 1, 3 and 9 weeks post-infection, while at 6 weeks there was a slight decrease in comparison with the controls.

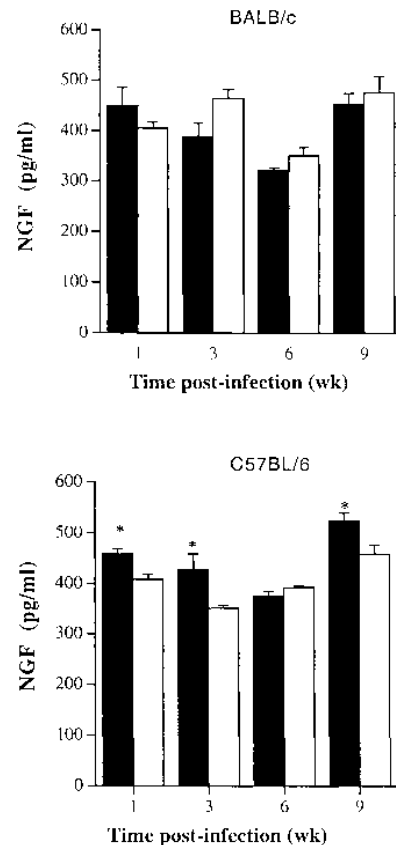


Fig. 3. Expression of NGF in the spleen in the susceptible, BALB/c ($n=6$), and resistant, C57BL/6 ($n=6$), mouse strains (■) and controls (□). * <0.05 compared with controls.

Serum

The control mice of both strains exhibited increased NGF values in the serum at 3, 6 and 9 weeks compared with the values obtained at 1 week after infection (Fig. 4).

The infected mice of the resistant strain showed elevated serum NGF levels in comparison with the control animals 1 week after infection, while those of the susceptible strain displayed no change either at 1 or 3 weeks. Thereafter, a statistically significant decline was noted in the infected mice of the resistant strain at 3 weeks post-infection in comparison with the controls. This drop in serum NGF in the resistant mice continued up to 6 weeks following infection, while at 9 weeks post-infection the NGF value almost approached the control value. The BALB/c strain, on the other hand, exhibited a slight rise in serum NGF at 6 weeks and a statistically significant increase at 9 weeks post-infection, in comparison with the controls.

DISCUSSION

The present study shows that in murine leishmaniasis the skin, lymph nodes and spleen, as well as the serum, contain variable amounts of NGF following infection with *L. major*. The NGF might be derived from immunocompetent cells such as

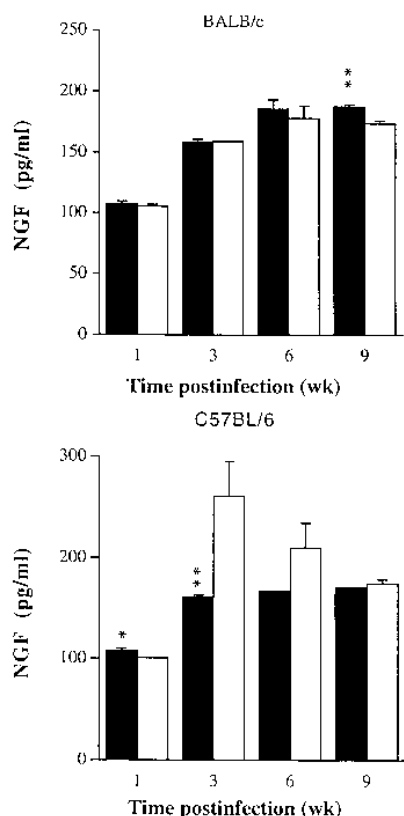


Fig. 4. Expression of NGF in the serum of the susceptible, BALB/c ($n=6$), and resistant, C57BL/6 ($n=6$), mouse strains (■) and controls (□). * <0.05 , ** <0.01 compared with controls.

monocytes/ macrophages (9), T lymphocytes (15) and mast cells (16), since all these cell types have been shown to synthesize and release this neurotrophic factor. In addition, the epidermis cannot be excluded as a source of NGF, since keratinocytes as well as skin appendages such as hair follicles from both humans and mice have been shown to express mRNA and release NGF (17, 18). Furthermore, it is documented that the inflammatory stimulus contributes to increased NGF production, and thus to its accumulation at the site of an inflammatory lesion (19). Thus, the difference in NGF levels between the mouse strains could be attributed to the difference in the degree of the inflammatory cell infiltrate and the way these cells respond to the inflammatory stimulus, especially since each mouse strain is genetically rendered either resistant or susceptible to *L. major* infection.

The present data indicate the importance and possible critical role of high levels of NGF at the time of initiation of the inflammatory response in leishmaniasis, i.e. during the first week following inoculation of the parasites. This is suggested by the finding that the resistant strain of mice, which has a capacity for spontaneous healing of skin lesions, showed significantly higher NGF levels in the skin, lymphoid organs and serum in comparison with uninfected controls of the same strain. The susceptible strain, which succumbs to the disease, displayed no significant change in the NGF levels in the organs

investigated 1 week after infection in comparison with the controls.

In the skin of the control animals of the susceptible strain the NGF value at 1 week was high in comparison with the rest of the study period and also in comparison with the corresponding controls of the resistant strain. This might be explained by a differential species response to the trauma caused by administration of the vehicle. The reason for the increase in the serum NGF levels in the control mice of both strains at 3, 6 and 9 weeks compared with 1 week is not clear, but stress conditions due to crowded housing and fighting cannot be excluded, since high serum NGF levels are usually associated with stress and anxiety (20).

The important and critical role of NGF in the first week post-infection may be explained by its possible contribution to CD4⁺ T-helper cell subset differentiation either to Th₁, which dominates the resistant strain, or Th₂, which dominates the susceptible immune responses. This is not surprising, since the post-thymic CD4⁺ T-helper subset Th₀, which differentiates upon activation to either Th₁ or Th₂, has been shown to express NGF functional receptor subunit *Trk A* upon mitogen and/or antigen and antigen-presenting cell stimulation (15). Thus the NGF action may be exerted in an autocrine manner.

The importance of macrophage cells in cutaneous leishmaniasis (21) and for the peripheral nerve injuries (22) is well documented. Whether the macrophage role in these pathological situations is mediated directly by NGF release or indirectly through synthesis of cytokines, such as IL-1 and tumour necrosis factor (TNF- α), which are known to induce NGF production (23), needs further investigation. In this context, we have previously demonstrated the possible importance of IL-1 in the initiation of a granulomatous response of human cutaneous leishmaniasis and a role for IL-6 and TNF- α in the disease resolution process (24).

Other cell types such as neutrophils, basophils and mast cells cannot be ruled out as a possible source of NGF in murine leishmaniasis, although they represent a minor percentage of the infiltrating cell population. Mast cells, for example, are well characterized as a source of NGF, as well as of the important immunoregulatory cytokines IL-4, IL-6, TNF- α and granulocyte/macrophage colony-stimulating factor (GM-CSF) (25). Furthermore, mast cells have been implicated in the augmented skin lesion size in normal and mast cell-deficient mice infected with both *L. major* (26) and *L. amazonensis* (27). On the other hand, NGF may synergise with IL-3 for the proliferation and differentiation of tissue mast cells (28). Whether mast cells are a direct source of NGF in this disease model, or whether they are an indirect inducer of neurotrophin through production of cytokines known to induce NGF synthesis and secretion, needs to be elucidated.

The infected animals of the resistant mouse strain expressed increased amounts of NGF in the skin and secondary lymphoid organs, but not in the serum, in comparison with the uninfected controls, at 6 and 9 weeks post-infection, when the disease resolution begins, indicating the possibility of an NGF contribution to the disease resolution process. In this respect, NGF has been shown to accelerate wound healing in mice (29). On the other hand, the infected mice of the susceptible strain also showed high levels of NGF in the skin and lymph nodes, as well as the serum, but not the spleen, at the specified time-points. The elevated NGF levels in the infected animals of the susceptible strain may be explained by increased release of

NGF in an attempt to restrict the disease progression and dissemination. This finding might indicate the importance of the spleen NGF levels in the disease outcome, as evident from the high NGF levels reflected by the infected mice of the resistant strain specifically at 1 and 9 weeks after infection.

Previous histopathological reports (4, 5) and our unpublished data confirm previous indications of peripheral nerve involvement in *L. major* cutaneous leishmaniasis in both humans and mice. The significantly higher NGF levels in the inflamed skin of both resistant and susceptible mice, compared with the controls, at 3, 6 and 9 weeks post-infection may be explained by the importance of NGF for the regeneration of sensory and/or autonomic nerve fibres for intensive reinnervation of the skin during wound healing, as previously reported (30).

In conclusion, this work shows the presence of the neurotrophic factor NGF in the skin and lymphoid organs of *L. major* murine cutaneous leishmaniasis and the importance of this factor in the pathogenesis of the disease, especially in its early stage, which essentially governs the outcome of the disease. NGF, which is well characterized as a neurotrophic peptide, which is essential for differentiation and survival of cellular elements in the mammalian central and peripheral sympathetic and sensory nervous systems (30), may act as a link in the bidirectional communication between the nervous and immune systems in the pathogenesis and resolution of cutaneous leishmaniasis.

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