Cyclosporine A treatment of leprosy patients with chronic neuritis is associated with pain control and reduction in antibodies against nerve growth factor

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Summary

Objectives Chronic neuritis (CN) is still a major problem in leprosy and is difficult to manage in patients who do not respond well to prednisone. In this study we (i) evaluate the efficacy of cyclosporine A (CyA) in controlling CN patients, and (ii) analyse the presence of anti-NGF antibodies in the sera of leprosy patients, and their behaviour during CyA treatment.

Design This was an open, prospective, non-comparative study. Sixty-seven leprosy patients in three different institutions in Pará, Brazil were studied from January, 2001 to January, 2004. Of these, 47 had no CN and 20 were leprosy patients suffering from CN and taking at least 40 mg/day prednisone to control nerve impairment and pain. Patients received 12 months reducing course CyA starting at 5 mg/kg per day. The outcome measure was sensory impairment, assessed using Semmes–Weinstein monofilament examination (SWME), muscular force and spontaneous or palpation-related pain.

Results Antibodies against NGF were detected in the sera of leprosy patients, which may explain the depletion of NGF in leprosy contributing to neuritis, inflammation and loss of cutaneous nociception. The levels of these antibodies in CN patients were slightly lower than in patients with no CN. However, anti-NGF titres in CN patients
treated with CyA were lowered to levels similar to those in the normal subjects. There was also improvement in sensory impairment, muscular force and pain.

Conclusions These data suggest that anti-NGF antibodies are present in the sera of leprosy patients and may influence the outcome of neuritis, and that CyA might be a useful drug in controlling nerve impairment and pain in leprosy patients.

Introduction

Nerve growth factor (NGF) is a trophic factor which regulates the survival of specific populations of neurons during critical periods of their development, and is essential for the outgrowth of neurites from sensory and sympathetic neurons. Other work has demonstrated that NGF is essential for maintaining the survival of the unmyelinated or myelinated nociceptive sensory neurons; it influences behaviour of immunocompetent cells, participating in the regulation of immune responses, and is also normally produced in the skin and taken up by nerve fibres via receptors on nerve terminals. NGF acts through binding to NGF receptors on the cell surface, which can be suppressed by antibodies against NGF. The presence of antibodies against NGF in the sera of patients with autoimmune diseases such as systemic lupus erythematosus, autoimmune thyroiditis and rheumatoid arthritis has already been demonstrated.

Leprosy is an important endemic disease in Brazil, and one of the most common neurodegenerative disorders of peripheral nerves. It is caused by infection with Mycobacterium leprae, a neurotropic and obligate intracellular parasite, residing primarily in myelinating Schwann cells and in macrophages. Clinical presentation is related to M. leprae tropism for the skin and peripheral nerves, which can result in chronic demyelination/remyelination, often leading to fibrosis and permanent loss of neuronal functions such as thermal sensation and touch. The disease can be determined by the host cellular immune response and is classified on the basis of cutaneous, motor and sensory alterations.

Leprosy patients may develop immunological responses against mycobacterial antigens, known as leprosy reactions, which are classified as type I, or reversal reaction (RR), and treated with prednisone. Some patients with leprosy reactions treated with prednisone do not improve clinically and need higher doses to control the reactions and the pain resulting from neuritis. Different studies in Hyderabad (India), Bangladesh, Ethiopia and Indonesia, among others, have shown that 4- to 6-month prednisolone or prednisone courses resulted in no improvement in nerve function impairment (NFI) in 12–50% of patients. Although the use of immunosuppressive agents, such as cyclosporin A (CyA), in autoimmune neuromuscular disorders has been demonstrated to be effective, CyA efficacy in controlling leprosy reactions has been controversial.

Concerning NGF, it has been shown that patients with leprosy have low levels of NGF in the skin, which may explain the early loss of pain and temperature sensation and the reduced skin flare responses, because NGF is necessary for the expression of neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) in sensory fibres.

The aim of the present study was to look for antibodies to NGF in sera of the leprosy patients and to evaluate the efficacy of CyA in the control of chronic neuritis (CN). To achieve this, we (i) analysed sera from 47 patients with different clinical forms of leprosy with no CN, for detection and quantification of anti-NGF antibodies, (ii) quantified anti-NGF antibodies in 20 patients with CN taking at least 40 mg/day prednisone, which was tapered off...
in the first 3 months to 12 months reducing course CyA starting at 5 mg/kg per day to control CN, and (iii) in 12 out of these 20 patients evaluated clinical improvement of nerve impairment and pain, and looked for correlation with serum anti-NGF levels.

Materials and methods

PATIENTS AND TREATMENT REGIMEN WITH CYA

The first twenty patients with CN who attended the Dr Marcello Candia Reference Unit in Sanitary Dermatology of the State of Pará (UREMC), in Marituba, Pará, Brazil, from January, 2001 to January, 2004 and could come for consultation at least once a month were recruited for the study. After starting CyA, eight patients did not come for consultation regularly, at least once a month, and therefore were withdrawn from the study. Dr Marcello Candia is the specialist leprosy outpatient clinic state reference unit. It is located in Marituba, in Belém metropolitan area, in the north region of Pará.

The definition of CN was: (i) enlarged peripheral nerves, with muscular force impairment or sensory loss and; (ii) self-reporting pain or in response to peripheral nerve palpation, with constant or intermittent use of prednisone in a minimal dose of 40 mg/day in the last 12 months. Patients who took three courses of the 3 months long prednisone regimen, starting at least 60 mg/day in the last 12 months, were defined as intermittent users. These patients were maintained on 40 mg/day prednisone, while introducing 5 mg/kg per day of CyA (Sigmasporin®; Sigmapharma, São Paulo, Brazil). After 30 days, the prednisone dose was reduced by 10 mg for every 15 days of treatment. After 60 days, CyA was reduced around 15% each month of treatment until reaching a minimal dose of 50 mg/day, which was maintained for 2 or 3 more months before stopping.

Clinical evaluation of the patients with chronic neuritis was done by NFI measurement, according to the Brazilian Ministry of Health guidelines, using medical and physiotherapeutic examination of pain, sensory impairment and muscular force.21,22 Pain was assessed by asking patients whether there was worsening or improvement of spontaneous or peripheral nerve palpation-related pain. Sensory impairment and muscular strength were evaluated according to the NFI evaluation, as described below.

Leprosy patients were diagnosed and classified according to the Ridley–Jopling classification criteria. Pregnant women, patients with other diseases or using other medication and children below 14 years old were ineligible for the study.

The leprosy sera used in the present study were provided by the UEPA/UFPA/MC Dermatology-Immunology Laboratory. The sera were kept frozen until use after being collected from patients at the following institutions: (i) Dr Marcello Candia Reference Unit in Sanitary Dermatology of the state of Pará, in Marituba, Pará, (ii) Tropical Medicine Nucleus at UFPA (NMT) and (iii) Basic Health Units of Marco and Guamá (UBSM and UBSG) districts in Belém, Pará. Control sera were obtained from nine healthy volunteers. The project was approved by the NMT Ethical Council (protocol number 008/00).

NEUROLOGICAL EVALUATION

All patients were evaluated neurologically. Sensory testing was performed using Semmes–Weinstein monofilament examination (SWME) with six colour graded monofilament representation on a map, and function assessment of muscular force was monitored by
voluntary motor testing (VMT) as previously described. The nerves examined were radial, ulnar and median in hands and fibular, sural and posterior tibial in feet.

Sensory examination and muscular force evaluation were graded according to clinical severity score (CSS) used to assess type 1 leprosy reactions as published by Marlowe et al., 2004.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

For coating, purified β-NGF (Sigma) was diluted to 50 ng/ml in phosphate-buffered saline (PBS), pH 7.4, 2 mmol/l EDTA and 5% fetal bovine serum (FBS), 50 μl was added to each well of a 96-well microtitration plate (Corning), and incubation was carried out for 2 h at 37°C. Sera were diluted in PBS (1:10) and 100 μl were added to each well and incubated for 2 h at 37°C. Anti-human IgG antibodies conjugated to peroxidase (Sigma) were diluted in PBS (1:1000), and 100 μl was added into each well. Incubation plates were washed 3 times with PBS containing 0.05% Tween-20, and after incubation for 2 h at 37°C, 100 μl of the enzyme substrate solution (Sigma), were added to each well. The reaction was halted by adding 50 μl of 3 mol/l HCl in each well after 30 min of incubation. Absorbance at 495 nm was read using a Multiscan photometer (Bio-Rad). Values obtained with incubation buffer coated plates and then with peroxidase-conjugated anti-human IgG antibodies were adjusted for zero absorbance.

BIOASSAY FOR PHEOCHROMOCYTOMA CELLS

PC12 cells were grown in RPMI 1640, supplemented with 5% FBS, 10% horse serum (HS), 25 μg/ml streptomycin and 25 IU/ml penicillin. The cultures were maintained in an incubator at 37°C at a humidity atmosphere of 5% CO₂. In the experiments, an identical number of cells (1–1.5 × 10⁵/plate) were plated in 35 mm diameter petri dishes (Corning), coated with poly-L-ornitine (0.1 mg/ml). Differentiation of the cultures was induced by treatment with 100 ng/ml NGF and inhibited with patients’ sera (1:25 and 1:50), in a medium supplemented with 1% HS, added every 48 h for a period of 9–11 days.

STATISTICAL ANALYSIS

CSS of patients were measured and are demonstrated at three different times: before CyA, after 3 months CyA therapy, and at the end of treatment, varying from 9 to 12 months. Student t-test was used to analyse statistical significance between after and before CyA treatment CSS. Differences between anti-NGF levels in sera of leprosy patients compared with control, and anti-NGF levels in sera of leprosy patients with CN using or not using CyA compared with control were analysed by Fisher test and Bonferroni test, following significant ANOVA. P < 0.05 was considered statistically significant.

Results

CLINICAL DATA

The following groups of patients were studied: (i) leprosy patients without reactions or CN (I = 8, TT = 12, BT = 3, BB = 7, BL = 5 and LL = 12); (ii) leprosy patients with CN...
taking at least 40 mg/day prednisone continuously or intermittently to control nerve impairment and pain, who were assigned to 12 months reducing course CyA starting at 5 mg/kg per day (BT = 2, BB = 5, BL = 4 and LL = 9). Twelve out of these 20 patients using CyA were clinically evaluated every 15 days for 12 months while using CyA, and monthly for another 12 months after withdrawal of medication. In the group of 12 patients who completed the treatment and the follow-up period, the mean age was 27.4 years (maximum: 43; minimum: 15) and the male/female ratio was 1:1.

After 30 days of CyA treatment start, all the 12 patients indicated significant pain relief. Four of these patients (33·3%) reported pain decrease, while the other eight (66·6%) reported absence of pain after this period (Table 1). From the end of the first month, the prednisone dose was decreased by 10 mg every 15 days. After 2 months of the combined treatment, only one patient had pain on palpation of the ulnar nerve. After 12 months, only one patient continued using 50 mg/day CyA because of pain. Stopping CyA led to pain increase in the right ulnar nerve after 15 days, and the patient was referred for surgery.

After the end of the treatment, nearly all patients had some muscular force improvement. Similar results were obtained by SWME analysis, which demonstrated recovery of the protecting sensation that had been reduced or lost, or improving of hypoesthetic points (Table 2). No patients worsened after CyA treatment.

DETECTION OF ANTIBODIES WITH ELISA

All patients without CyA treatment had antibodies to NGF 2–5 times higher than those in the control group (Figure 1). Patients with CN did not have significant differences when

<p>| Table 1. Patients treated with CyA: distribution of time, in months, from diagnosis to referral, time of neuritis and time to becoming pain free on CyA |
|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>n</th>
<th>Diagnosis</th>
<th>UREMC reference because of leprosy neuritis not controlled with prednisonea</th>
<th>Neuritisb</th>
<th>Pain freec</th>
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<tbody>
<tr>
<td>1</td>
<td>10/00 BB</td>
<td>07</td>
<td>15</td>
<td>01</td>
</tr>
<tr>
<td>2</td>
<td>09/00 LL</td>
<td>11</td>
<td>15</td>
<td>02</td>
</tr>
<tr>
<td>3</td>
<td>12/01 BT</td>
<td>01</td>
<td>06</td>
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<td>02/02 BL</td>
<td>13</td>
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</table>

a Period between leprosy diagnosis in Basic Health Units until patient referral to UREMC: 1–48 months (mean: 16.33 months; SD: 12.14).

b Continuous or intermittent peripheral neural pain with mean period of 22.75 months (SD: 10.81), being 48 months maximal and 6 months minimal period.

c Eleven out of 12 patients had no pain complaints after 2 months of CsA treatment, and none had to use prednisone or other anti-inflammatory drugs again during the next 12 months after withdrawal of CsA.
compared to those without CN (data not shown). Treatment of CN with CyA showed an excellent response, reducing the levels of antibodies to almost the same levels of the control group (Figure 2).

BIOLOGICAL ACTIVITY OF THE ANTIBODIES TO NGF

To evaluate the biological activity of the antibodies to NGF, we examined their capacity to inhibit the differentiation of phaeochromocytoma cells (PC12 line) induced by NGF (Figure 3). Differentiation of PC12 cells in the presence of 1:50 and 1:25 diluted serum from one lepromatous patient, without treatment and without reaction, which presented high levels

Table 2. Clinical severity score evaluation in patients put on CyA

<table>
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<th>Sensory examination</th>
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<tr>
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<tr>
<td>2</td>
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<td>Mean</td>
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<td>SE</td>
<td>1·49</td>
<td>1·35</td>
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</table>

Figure 1. Antibodies against NGF were detected in sera of leprosy patients. Anti-NGF antibodies were measured by ELISA in sera of leprosy patients without reactions or chronic neuritis, divided according to clinical forms (I = 8, TT = 12, BT = 3, BB = 7, BL = 5 and LL = 12) and compared to normal subjects (control = 9). Results are expressed in units of absorbance (495 nm) after removal of the background. *P < 0·05 compared with control, using the Fisher-test and Bonferroni-test following significant ANOVA.
of anti-NGF (Ab. = 0.871), was completely inhibited (Figure 3C and D, respectively). These results confirmed the presence of anti-NGF detected by the ELISA method.

Discussion

This study demonstrates the presence of biologically active antibodies to NGF in the sera of leprosy patients. NGF stimulates production of CGRP in human B lymphocytes and in

![Figure 2](image_url)

Figure 2. Antibodies against NGF disappear during CsA treatment. Anti-NGF antibodies were measured by ELISA in sera of normal subjects (control = 9) and compared to leprosy patients with chronic neuritis, before and after three months of CyA therapy (BT = 2, BB = 4, BL = 2 and LL = 4). Results are expressed in units of absorbance (495 nm), after removal of the background. *P < 0.05 compared with control, using the Fisher test and Bonferroni test following significant ANOVA.

![Figure 3](image_url)

Figure 3. Antibodies against NGF inhibited the differentiation of Pheochromocytoma cells (PC12 line) induced by NGF. Inhibition of the biological activity of NGF in PC12 cells, cultivated in RPMI 1690 medium, with sera of leprosy patients: (A) with 10% HS and 5% FBS; (B) with 1% HS and 100 ng/ml of NGF; and (C and D) with 1% HS, 100 ng/ml NGF and sera (1:50 and 1:25, respectively) from leprosy patients. Scale bars = 50 μm.
sensory neurons, and may influence inflammatory responses through the modulation of sensory neuropeptide synthesis. High levels of anti-NGF in the sera of leprosy patients can be a possible nerve injury mechanism, inhibiting the production of the CGRP anti-inflammatory molecule, sustaining the inflammatory pathway that results in nerve damage. Previous findings have demonstrated low levels of CGRP in the skin of leprosy patients compared with controls.

To confirm the biological activity of the anti-NGF in vitro, we blocked the NGF-induced differentiation of PC12 cells with the sera of one patient added to the PC12 cell culture. Different papers have demonstrated the inhibition of differentiation in PC12 cells or in B lymphocytes, neutralizing endogenous NGF, using anti-NGF antibodies, which is consistent with our findings.

Autoantibodies can be detected in the sera of lepromatous patients, usually decreasing during reactions, which can be correlated with an increase of immune complexes. In our study, all leprosy patients had anti-NGF antibodies, and there was a decrease in the levels of these antibodies during reactions (data not shown). Although not statistically significant, the difference found between CN and non-CN patients could be explained by the use of available anti-NGF in the formation of immune complexes during reaction.

Another remarkable point was the complete inhibition in anti-NGF production by the use of CyA, which is justifiable through immunomodulatory activity, for inhibiting T cell activation by blocking the transcription of cytokine genes, including IL-2 and IL-4, suggesting a protecting activity against neural damage by CyA in leprosy.

We had previously demonstrated that CyA could be useful in patients with type I reaction. Although the initial CyA dose of 5 mg/kg per day was not effective in avoiding the worsening condition of muscular force and sensitive function, we were able to control neuropathic pain in those patients. After long-term (1 year) follow-up of chronic neuritis patients using CyA, it was possible for us to observe the enhancement of sensory and motor function in all patients, and also the complete absence of pain in 11 of 12 patients submitted to treatment with CyA, suggesting neural symptom improvement due to low levels of anti-NGF antibodies. This correlates with the complete inhibition of anti-NGF observed in the study, and might be a mechanism of CyA action in the relief of chronic neuritis and pain.

In conclusion, we demonstrate for the first time that leprosy patients have high levels of anti-NGF that can be inhibited in vitro and in vivo by the use of CyA, which could be important for future therapeutics studies for controlling leprosy chronic neuritis.

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