

Role of PGL-I antibody detection in the diagnosis of pure neural leprosy

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Summary Pure neural leprosy (PNL) is difficult to diagnose because skin lesions and acid-fast bacilli (AFB) in slit smears are absent. At present, the gold standard for PNL diagnosis is the histopathological examination of a peripheral nerve biopsy. Even so, detection of bacteria is difficult and histological findings may be non-specific. Furthermore, nerve biopsy is an invasive procedure that is only possible in specialized centres. Therefore, there is a need for additional diagnostic methods that may help to confirm the clinical diagnosis of PNL. In the present study, an additional laboratory test, the ELISA for anti-phenolic glycolipid I (PGL-I) IgM antibodies, was performed on 103 individuals with clinical and neurophysiological signs of peripheral neuropathy, of which 67 were diagnosed as PNL patients and 36 remained as 'not diagnosed as PNL', as well as on a control group of 34 patients with other neurological diseases. An antibody response was present in 14/67 (21%) of the patients diagnosed as PNL as compared with 3/34 (9%) of controls. Anti-PGL-I positivity was observed in 5/8 (63%) of the AFB positive cases. Patients whose diagnosis was confirmed solely by *Mycobacterium leprae* PCR on the nerve sample had 4/25 (16%) seropositivity. In addition, anti-PGL-I antibodies were detected in 9/40 (23%) of the PNL patients who were PCR negative for *M. leprae* DNA. Moreover, two patients who showed clinical and electrophysiological manifestations suggestive of PNL were diagnosed with the

help of their positive test results in the anti-PGL-I ELISA. In conclusion, detection of antibodies against PGL-I in patients with peripheral neuropathy is useful as an additional laboratory test to help PNL diagnosis.

Introduction

Primary neuritic or pure neural leprosy (PNL) is one of the clinical forms of the disease caused by *Mycobacterium leprae*. It is difficult to diagnose because the cardinal features of skin lesions and acid-fast bacilli (AFB) in skin/slit smears are absent.¹⁻³ PNL is clinically defined as the loss of peripheral nerve function, either muscle weakness or reduced sensation, associated or not with nerve enlargement, with asymmetrical involvement of usually one or several nerve trunks.⁴ The prevalence of this form of leprosy is responsible for 1–16% of all leprosy cases, depending on the population studied.⁵ As peripheral neuropathy can be caused by many other diseases, such as metabolic disorders, drug reactions, hereditary diseases and other infections,¹ diagnosis of PNL cannot be firmly established on clinical features alone. Therefore other tests must be used to confirm diagnosis.

In a previous study,⁶ nerve biopsies were tested to establish a diagnosis of PNL; if either histopathological changes or *M. leprae* DNA was detected the clinical diagnosis was confirmed. However, nerve biopsy is an invasive procedure that is only possible in specialized centres. For this reason, the diagnosis of PNL is still problematic in most cases, and the use of any other diagnostic method that can help to confirm the clinical diagnosis would be helpful.

Over the past years, a large number of studies have described the detection of IgM antibodies to phenolic glycolipid-I (PGL-I), an *M. leprae* specific antigen. Numerous studies have shown that antibody levels can be used as a marker for the bacterial load, with a widely varying but positive correlation between antibody levels and bacterial index (BI).⁷ Therefore, seropositivity could be an important tool for the confirmation of PNL, as this form of leprosy is notoriously difficult to diagnose.

The aim of the present study was to determine the contribution that PGL-I antibody detection can make towards the confirmation of infection with *M. leprae* in patients who presented clinical, neurological and electrophysiological manifestations suggestive of PNL.

Patients and methods

STUDY POPULATION

All consecutive patients suspected of PNL evaluated between October 1998 and May 2003 at the Leprosy Out-Patient Clinic, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil were included in the cohort. A total of 103 individuals who presented with clinical (nerve enlargement and/or nerve pain and/or motor deficit and/or sensory impairment) and electrophysiological alterations suggestive of PNL in the absence of any cutaneous lesions, as described by Jardim *et al.*,⁶ were enrolled into the study. Jardim *et al.*⁸ described the histological results of 19 of these PNL patients in detail.

Excluded from the study were patients with evidence of any skin lesions, infiltration or a history of skin lesion(s) as well as those with neuropathy-associated diseases, such as diabetes mellitus, alcoholism, hepatitis B or C or HTLV-I infections, rheumatological/rheumatic

diseases, in addition to toxic, drug-induced or hereditary neuropathies. In addition, patients from whom no serum sample was available were excluded.

Nerve biopsy on one of the affected nerves was performed on all of the 103 individuals and PNL was diagnosed in 67 patients according to the following criteria: (i) definite PNL: detection of acid fast bacilli (AFB), and/or epithelioid granuloma, and/or *M. leprae* DNA (by PCR) in a nerve biopsy sample ($n = 36$); (ii) probable PNL: no AFB detected, negative for *M. leprae* PCR but with nonspecific inflammatory infiltrate and/or fibrosis, which strongly suggests infectious neuropathy caused by *M. leprae* ($n = 25$); (iii) possible PNL: positive serology for anti-PGL-I antibodies and/or clinical signs sufficient to diagnose leprosy despite the absence of any other alterations in nerve biopsies (such as ulnar nerve enlargement and pain, drop foot or neuritis evidenced by magnetic resonance imaging) and a negative *M. leprae* PCR result ($n = 6$).

In addition, 34 patients with neuropathies caused by diseases other than leprosy, and recruited from the Neurology Clinic at University Hospital Pedro Ernesto, Rio de Janeiro, were included as a control group. Nerve conduction evaluation was normal in all of them. Therefore, nerve biopsies were unethical. For this group only blood samples were collected. Twenty-four patients had syndromes affecting the central nervous system, in five the peripheral nervous system, and in the other five, both the central and peripheral nervous systems were affected.

The research was carried out in strict compliance with the international norms on ethics in human research and was approved by the Ethics Committee of the Oswaldo Cruz Foundation. All individuals participating in the study provided their written informed consent.

LABORATORY STUDIES

All 103 patients were untreated at the time of diagnostic biopsy. Sensory dorsal cutaneous ulnar branch (64.5%), superficial peroneal (25%) and sural nerve at the ankle level (10.5%) were selected for biopsies based on clinical or electrophysical alteration. Polymerase chain reaction (PCR) for detection of *M. leprae* DNA was performed on the nerve biopsies as described before by Santos *et al.*,⁹ and the set of primers used was sense 5'-GCACGTAAGCCTGTCGGTGG-3' and antisense 5'-CGGCCGGATCCTCGATGCAC-3'. Slit smears were taken to determine systemic bacillary load as described previously.⁶ Histopathological examination of the nerve samples was performed and the presence of AFB was detected following Ziehl-Neelsen staining.

COLLECTION OF SERUM SAMPLES

For detection of IgM antibodies against PGL-I, blood was collected and allowed to clot for 30 min before centrifugation. Serum samples were harvested and kept frozen (-20°C) until use.

PGL-I ELISA

NT-P-BSA was used as semi-synthetic analogue of PGL-I. The ELISA was performed essentially as described by Bühner *et al.*¹⁰ with the following modifications: serum dilution was 1:500, the conjugate dilution 1:10,000, and the substrate used was 0.04%

tetramethylbenzine (TMB) and 0.04% urea peroxide in 0.1 M sodium acetate citric acid buffer (pH 4.0). The cut-off value used for positivity was $OD_{450} = 0.2$.

STATISTICAL ANALYSIS

Data were analyzed using Epi-info version 6.04b and the Excel software package.

Results

LABORATORY RESULTS FOR DEFINITION OF PNL

PNL was diagnosed in 67 out of the 103 (65%) patients who were initially suspected of having leprosy according to the criteria described in the methods section. In the other 36 (35%) individuals (who also presented with clinical and neurophysiological alterations), leprosy diagnosis could not be established by any of the parameters described and they are referred to in this study as 'not diagnosed as PNL'. The results of the diagnostic tests performed are given in Table 1.

The PNL diagnosis was confirmed by the presence of AFB in eight (12%) of the 67 patients, epithelioid granuloma in 11 (16%), non-specific inflammatory infiltration and/or

Table 1. Laboratory test results for 103 patients suspected of PNL and 34 controls

| Group | n | Test results | | | | |
|----------------------|----------------|----------------|---------|---------|---------|---------|
| | | Histopathology | | | PCR | PGL-I |
| | | AFB* | EG** | I/F*** | | |
| Definite (36) | 1 | + | - | + | - | + |
| | 1 | + | + | - | + | + |
| | 1 | + | + | - | - | + |
| | 2 | + | - | + | + | + |
| | 3 | + | - | + | + | - |
| | 1 | - | + | + | - | + |
| | 2 | - | + | + | + | - |
| | 6 | - | + | + | - | - |
| | 1 | - | - | + | + | + |
| | 1 | - | - | - | + | + |
| | 5 | - | - | + | + | - |
| | 12 | - | - | - | + | - |
| Probable (25) | 4 | - | - | + | - | + |
| | 21 | - | - | + | - | - |
| Possible (6) | 2 | - | - | - | - | + |
| | 4 [#] | - | - | - | - | - |
| Total PNL | 67 | 8(12%) | 11(16%) | 46(67%) | 27(40%) | 14(21%) |
| Not diagnosed as PNL | 36 | - | - | - | - | - |
| Controls | 34 | ND | ND | ND | ND | 3 |

+ = positive result; - = negative result; ND = not done.

*AFB = acid fast bacilli; **EG = epithelioid granuloma; ***I/F = non-specific inflammatory infiltrate and/or fibrosis.

[#]Patients diagnosed based on the highly suggestive clinical signs supported by other paraclinical examinations.

fibrosis in 46 (67%), PCR positive for *M. leprae* was in 27 (40%) and 14 (21%) were seropositive for PGL-I. The antibody response was mainly present in those individuals with detectable AFB in the nerves. Figure 1 shows the overlap of test results. Four patients were histologically, serologically and PCR for *M. leprae* positive. Of these four patients, three had detectable AFB, 1 epithelioid granuloma and two non-specific inflammatory infiltration and/or fibrosis.

DETECTION OF ANTI-PGL-I ANTIBODIES IN PATIENTS WITH PURE NEURAL LEPROSY

Overall, 14/67 PNL patients (21%) had antibodies against PGL-I as compared to 3/34 (9%) of the control group ($P = 0.13$). The sensitivity and specificity of the PGL-I ELISA test were 21% and 91%, respectively. Among the definite PNL patients [criterion (i); $n = 36$], eight (22%) had anti-PGL-I antibodies, in the probable PNL group [criterion (ii); $n = 25$] a positive response occurred in four (16%), and in the possible PNL group [criterion (iii); $n = 6$] anti-PGL-I antibodies were detected in two individuals (33%). In these latter two patients, anti-PGL-I antibodies were the only indication of their previous/present exposure to the leprosy bacillus (Table 1). The rate of response in the PGL-I ELISA did not vary significantly when PNL patients, grouped according to different criteria (definite, probable and possible), were compared ($P = 0.61$).

In the control group ($n = 34$), three individuals had anti-PGL-I antibodies. One patient presented with complications in the central nervous system due to HIV infection, one had

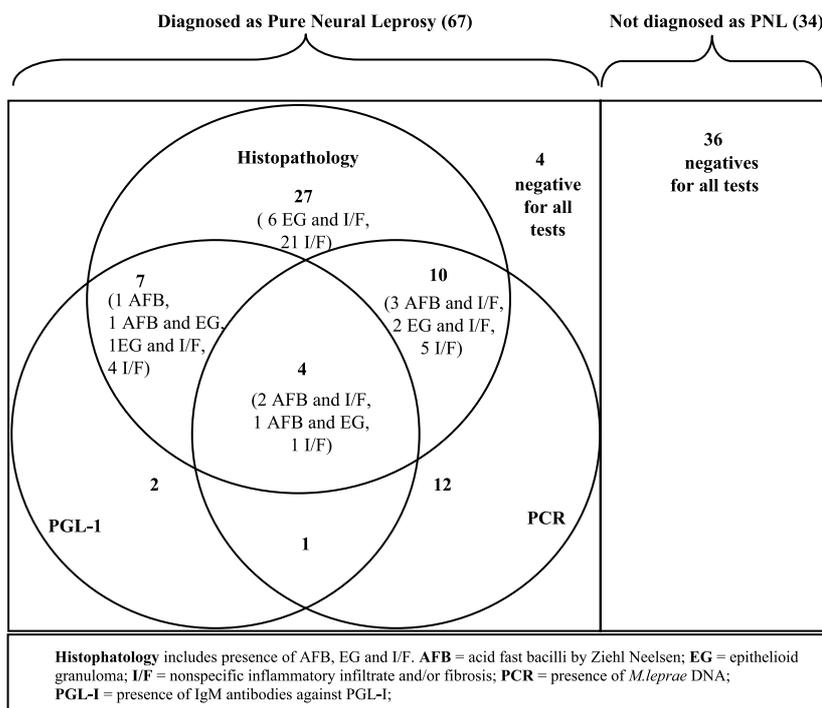


Figure 1. Overlap of laboratory test results in 103 patients suspected of PNL.

lung carcinoma and paresthesia in the feet, and the third one presented with cerebral vascular disease without any signs or symptoms of peripheral nervous system abnormality. Nerve biopsy was not performed in any of them because nerve conduction evaluation was normal in all patients.

COMPARISON OF LABORATORY TESTS WITHIN THE PNL GROUP

M. leprae infection in PNL was most frequently shown by PCR (40% of the cases), followed by anti-PGL-I antibodies (21%), and by the direct observation of the bacteria (AFB = 12%; Table 1). Sixty-three percent (5/8) of patients with confirmed leprosy infection with AFB in the nerves had antibodies against PGL-I. In contrast, seropositivity was seen in only 19% (5/27) of the PNL patients who were PCR positive for *M. leprae*. Of these five *M. leprae* PCR and PGL-I positive patients, three (60%) were also AFB positive. Thus, detection of AFB in the nerves ($n = 8$) was highly related to the positive PGL-I testing (5/8) and also to the detection of *M. leprae* DNA (6/8). On the other hand, of the 19 patients in whom PCR for *M. leprae* provided the only definite proof of PNL (criterion i), only two (11%) were seropositive.

The other histological parameters used for diagnosis showed presence of epithelioid granuloma in nerve biopsies and positive anti-PGL-I antibodies to occur in 27% (3/11) of the patients, whereas anti-PGL-I antibodies and fibrosis/infiltration were seen in 20% (9/46) of the patients. In the group of 17 definite patients with positive histopathology, six patients (35%) were also seropositive, including four who showed fibrosis/infiltration. When patients with positive AFB and/or epithelioid granuloma but with negative PCR for *M. leprae* were considered, IgM antibodies against PGL-I were found in 18% (3/17). The two patients who were both AFB and epithelioid granuloma positive were also positive in the PGL-I ELISA.

In addition, detection of antibodies as an accessory test was able to confirm PNL in a total of 23% of the PCR for *M. leprae* negative cases (9/40), 15% of the AFB negative cases (9/59), 20% of the cases with no epithelioid granuloma (11/56), and in 24% (5/21) of the fibrosis/infiltration negative cases. More interestingly, anti-PGL-I antibody levels were positive in six out of 31 (19%) patients in whom AFB, EG and PCR for *M. leprae* were negative [criteria (ii) and (iii) combined]. As for PCR for *M. leprae* testing, correlation with AFB was noted in 75% (6/8) of the cases and presence of epithelioid granuloma in 27% (3/11). In addition, detection of *M. leprae* DNA defined the diagnosis of PNL in 36% (21/59) of those who were AFB negative, in 43% (24/56) EG negative and in 67% (14/21) fibrosis/infiltration negative.

Discussion

In general, establishing the diagnosis of PNL is a challenge for clinicians, especially when patients with symptoms of nerve involvement are scarce and where laboratory facilities are not available. When clinical data alone are insufficient to establish the diagnosis confidently, these should be aided by laboratory test. The most important test is the histological examination of nerve biopsy. However, this may give non-specific results^{4,8,11} despite detailed examination. Therefore, the use of auxiliary tests, even if they show a relatively low sensitivity, can be of importance to confirm the clinical diagnosis of PNL. In the present study, in addition to histological parameters and PCR for the detection of *M. leprae* DNA in

the nerve,⁶ the detection of anti-PGL-I antibodies in patients was employed to aid the diagnosis of PNL.

The overall seropositivity found here for PNL patients (21%) is comparable to the 26% found by Roche *et al.*¹² Also, the positive response observed in our control group (9%) is within the range normally detected among controls in endemic areas.¹³

Infection by *M. leprae* in PNL was evidenced most often by PCR, followed by anti-PGL-I antibodies, and by direct observation of the bacteria (AFB). From the eight patients with AFB in their biopsy, six also showed unspecific inflammatory infiltration and only two showed epithelioid granuloma. Within the definite PNL group, a higher percentage of seropositive serum samples were detected in patients with AFB in their nerve biopsies as compared to the *M. leprae* PCR positive cases. Moreover, detection of AFB in the nerves was highly related to positive PGL-I testing (63%) as well as to the detection of *M. leprae* DNA (75%). The observation that 60% of the *M. leprae* PCR and anti-PGL-I positive cases were also AFB positive may indicate that these patients are carrying higher numbers of bacteria. However, although a significant correlation between serology results and the bacterial index has been demonstrated previously,⁷ such a parameter cannot be assessed in PNL as AFB are absent in skin smears from all of these individuals.

A higher *M. leprae* PCR positivity than seropositivity was observed in patients with negative histopathology results. Due to the high sensitivity of the PCR technique and the fact that presence of epithelioid granuloma maybe a representation of the patient's ability to mount a cellular immune response (CMI), a lower correlation between these tests and PGL-I positivity can be expected. Indeed, only two anti-PGL-I positive patients were found among those diagnosed based only on a positive PCR for *M. leprae* result ($n = 19$) and one out of seven was detected when the presence of epithelioid granuloma alone was considered. Even so, by using the PGL-I ELISA, we were able to detect a total of 23% (9/40) positive individuals among the *M. leprae* PCR negative cases.

Another interesting discrepancy encountered in PNL is the lack of correlation between the histopathological results and the Ridley–Joplin classification. Various authors have tried to include PNL patients as belonging to the TT or LL poles based on the histopathological result of nerve biopsies.^{14–16} However, as observed in this series, where two out of eight cases with epithelioid granuloma were AFB positive, Ridley–Joplin classification does not apply to the nerve biopsies.

The overall reported data indicate, in accordance with earlier observations, that in PNL patients: (i) PCR for *M. leprae* is more sensitive than AFB microscopy in the detection of *M. leprae*, thus also detecting patients with low bacillary load; (ii) serology is more often positive in patients who carry higher numbers of bacteria; and (iii) presence of epithelioid granuloma in the nerve correlates with high CMI response and is inversely related to the detection of anti-PGL-I antibodies.

Timely treatment is imperative in leprosy, especially because once nerve fibrosis occurs, damage is permanent and irreversible.¹⁷ Therefore, Jacob and Mathai¹⁸ recommended that in hyperendemic areas it would be wise to treat patients for leprosy even if nerve biopsy examination is inconclusive, provided that clinical suspicion is high and detailed investigation for other causes of peripheral neuropathy yield negative findings. As anti-PGL-I antibody testing is specific, initial suspects with confirmed peripheral neuropathy by the electrophysiological test, even in the presence of an inconclusive histopathological result, were considered to have PNL and were treated as such when their anti-PGL-I ELISA was positive. Our results show that a person with a confirmed peripheral neuropathy with negative

PCR for *M. leprae*, negative histological findings but positive PGL-I should be diagnosed as a definite case of PNL. In our study group, the remaining 36 individuals with no conclusive evidence for PNL (not diagnosed as PNL, Table 1) were kept under observation. After an average of five years follow-up, none had presented new signs or symptoms indicative of leprosy. So far, the methods used appear to be capable to differentiate leprosy from other peripheral neuropathies.

In conclusion, considering the difficulties in confirming the diagnosis of PNL in some cases, as well as the need of early diagnosis and treatment for prevention of nerve damage and the resulting complications, the use of PGL-I antibody detection as a non-invasive laboratory test is proposed to help in the diagnosis of PNL in areas where nerve biopsy is unavailable. A positive anti-PGL-I result in a patient with peripheral neuropathy in a leprosy endemic area is to be considered as PNL. However, a negative result will not exclude the possibility of this diagnosis.

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