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Utility of measuring serum levels of anti-PGL-I antibody, neopterin and C-reactive protein in monitoring leprosy patients during multi-drug treatment and reactions

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Summary

OBJECTIVE To verify the validity of measuring the levels of *Mycobacterium leprae*-specific anti-phenolic glycolipid (PGL)-I antibody, neopterin, a product of activated macrophages, and C-reactive protein (CRP), an acute phase protein, in serial serum samples from patients for monitoring the leprosy spectrum and reactions during the course of multi-drug treatment (MDT).

METHODS Twenty-five untreated leprosy patients, 15 multi-bacillary (MB) and 10 paucibacillary (PB), participated. Eight patients developed reversal reaction and five developed erythema nodosum leprosum (ENL) during follow-up. The bacterial index (BI) in slit-skin smears was determined at diagnosis and blood samples collected by venipuncture at diagnosis and after 2, 4, 6 and 12 months of MDT. PGL-I antibody and neopterin were measured by enzyme-linked immunosorbent assay, whereas the CRP levels were measured by the latex agglutination method.

RESULTS The levels of PGL-I antibodies and neopterin were higher in the sera of MB than PB patients, which correlated with the patients' BI. The serum levels of CRP did not differ significantly between the MB and PB patients. The serum levels of PGL-I and neopterin were no higher in reactional patients than non-reactional patients prone to such reactions. However, ENL patients had higher serum CRP levels than non-reactional MB patients. The serum PGL-I antibody levels declined significantly during MDT, in contrast to neopterin and CRP levels.

CONCLUSION Measuring the serum levels of PGL-I antibodies and neopterin appeared to be useful in distinguishing MB from PB patients, whereas monitoring the levels of PGL-I antibodies appeared to be useful in monitoring MB patients on MDT. Measuring serum CRP, although not useful in monitoring the patients, has limited significance in detecting ENL reactional patients.

keywords leprosy, *Mycobacterium leprae,* neopterin, C-reactive protein, multi-drug treatment

Introduction

Leprosy is a chronic infectious disease with a clinical spectrum determined by the host's cell-mediated immunity (CMI) towards *Mycobacterium leprae* (Harboe 1994; Modlin 1994). The prevalence of leprosy worldwide has declined significantly since the introduction of the World Health Organization (WHO) recommended multi-drug treatment (MDT) in 1982 (WHO 1982). However, areas of hyper-endemic infection and the high number of new cases detected in many countries (WHO 2006) remain a cause for concern.

The tissue damage occurring in leprosy is exacerbated during episodes of reactions, namely type 1 [reversal reaction (RR)] and type 2 [erythema nodosum leprosum (ENL)] reactions (Naafs 2000). The acute inflammation associated with reactions causes irreversible tissue damage and nerve destruction; thus, early detection of leprosy reactions is a key priority (Jacobson & Krahenbuhl 1999; Britton & Lockwood 2004). Permanent nerve damage can be prevented provided reactions are detected early and adequately treated (Naafs 1996).

With the current emphasis on the integration of leprosy control activities into the general health-care services (WHO 2006), we need laboratory markers to detect leprosy patients at the early stages, to aid clinical diagnosis and to monitor treatment efficacy. Several approaches have been attempted, with mixed results. The measurement of serum antibodies to PGL-I, an *M. leprae* cell wall antigen is specific (Brett *et al.* 1983) but has limited sensitivity in

Table 1 Severity score of reactional patients

Patient no.	BI	Classification	Reaction	Clinical criterion	Severity Score
1	3.0	LL	ENL	A1	2
				A2	1
				A3	1
				A4	0
2	0.25	LL	ENL	A1	2
				A2	1
				A3	1
				A4	0
3	3.2	LL	ENL	A1	2
				A2	2
				A3	2
				A4	1
4	5.0	LL	ENL	A1	2
				A2	2
				A3	2
				A4	1
5	3.3	LL	ENL	A1	2
				A2	2
				A3	2
				A4	1
6	0.16	TT	RR	A1	2
				A2	0
				A3	0
				A4	0
7	1.6	BL	RR	A1	2
				A2	1
				A3	0
				A4	1
8	0.5	BB	RR	A1	1
				A2	0
				A3	0
				A4	0
9	2.3	BB	RR	A1	2
				A2	2
				A3	0
				A4	0
10	2.0	BB	RR	A1	2
				A2	2
				A3	0
				A4	0
11	1.5	BB	RR	A1	2
				A2	2
				A3	1
				A4	1
12	0	BT	RR	A1	2
				A2	0
				A3	0
				A4	0
13	2.6	BL	RR	A1	2
				A2	2
				A3	1
				A4	1

BI, bacterial index; LL, lepromatous; ENL, erythema nodosum leprosum; TT, tuberculoid; RR, reversal reaction; BL, borderline lepromatous; BB, borderline.
A1, degree of inflammation of skin lesions: 0 = none; 1 = erythema; 2 = erythema and raised; 3 = ulceration.
A2, peripheral oedema (owing to reaction): 0 = none; 1 = minimal; 2 = visible, but not affecting function; 3 = oedema affecting function.
A3, nerve pain: 0 = none; 1 = pain on activity; 2 = pain at rest; 3 = pain disturbing sleep.
A4, nerve tenderness, worst affected nerve only: 0 = none; 1 = mild tenderness; 2 = withdrawal or wincing; 3 = not allowing palpation.

detecting paucibacillary (PB) leprosy patients (Brett *et al.* 1983; Oskam *et al.* 2003). Moreover, high antibody levels were not associated with the development of reactions in leprosy patients (Roche *et al.* 1993; Stefani *et al.* 1998). Neopterin, a monocyte-macrophage activation product, has been previously used as a marker for increased cell-mediated immune activity in leprosy and other diseases (Murr *et al.* 2002; Hoffmann *et al.* 2003). Increased levels of neopterin are associated with both ENL and RR (Hamerlinck *et al.* 1999; Faber *et al.* 2004). Similarly, increased levels of the acute phase C-reactive protein (CRP) have been associated with ENL (Foss *et al.* 1993). Other studies looked at the profiles of serum cytokines associated with the leprosy spectrum and reactions with often contradictory results (Moubasher *et al.* 1998; Faber *et al.* 2004). Thus, our aim was to verify the validity of measuring anti-PGL-I antibody, neopterin and CRP levels in monitoring the leprosy spectrum and reactions during the course of MDT in serial serum samples from patients.

Materials and methods

Patients

Twenty-five untreated patients (13 females; age range 38–76 years), attending the leprosy clinic at the Instituto Lauro de Souza Lima, Bauru, Brazil, agreed to participate in the study. Every patient was clinically assessed by taking a detailed history and thorough medical and dermatological examination. Slit-skin smears were also bacteriologically examined to determine the bacterial index (BI). Patients were classified based on the clinical findings and histopathological examination (Ridley & Jopling 1966) and included lepromatous (LL, $n = 7$), borderline lepromatous (BL, $n = 2$), borderline (BB, $n = 6$), borderline tuberculoid (BT, $n = 5$) and tuberculoid (TT, $n = 5$)

groups. Owing to the small sample size, the patients were grouped as paucibacillary (PB) (n = 10), which included TT and BT patients with 'average' BI <2+, and multi-bacillary (MB) (n = 15), including all BB, BL and LL patients with a BI of ≥2+ 'at any one site'. Patients were treated with MDT according to the WHO guidelines (WHO 1982).

Follow-up was 12 months. Serum samples were obtained from the patients before starting treatment and 2, 4, 6 and 12 months after starting MDT. During the period of follow-up, 13 patients developed a reaction (eight RR and five ENL). These patients were assessed clinically and histopathologically to confirm the presence of a reaction and to ascertain its type and severity (Table 1). ENL was characterized by an acute onset of erythematous nodules accompanied by fever. In some patients, the nodules were accompanied by mild peripheral oedema and neuritis (either spontaneous neuritic pain or pain on palpation of nerves). RR was characterized by acute onset of erythematous plaques, well defined and often oedematous. Some RR patients also presented with mild peripheral oedema with no alteration in function, neuritic pain or tenderness on palpation. Patients did not receive any extra anti-reactional therapy, such as prednisolone as the severity score of the reactions was moderate. The strategy adopted by the clinicians was to avoid anti-reactional intervention, unless the reaction became severe, and to allow natural regression of the reaction while continuing MDT.

The BL and LL patients without ENL reactions were grouped together as non-ENL BL/LL (NE, n = 4), whereas BL, BB and BT patients without RR were grouped as G16 (NRB, n = 6). This grouping was for the purpose of comparisons as ENL normally occurs in BL/LL patients while RR occurs in BL/BB/BT patients. The serum profiles of the NE and NRB groups at the onset of leprosy were compared with ENL (n = 5) and RR (n = 8) patients at the onset of the reactions, respectively.

Microscopic examination of skin smears

Slit-skin smears were taken from all leprosy patients at diagnosis from four sites, including one clinical lesion. The smears were stained using 'the Ziehl-Neelsen Carbol Fuchsin method' (Ministerio da Saude, Brasil 2000) and the mean BI was calculated according to logarithmic index proposed by Ridley and Hilson (1967).

Serological assays

The PGL-I IgM antibodies were assayed using the enzyme-linked immunosorbent assay (ELISA) kit developed by the Royal Tropical Institute Amsterdam, the Netherlands, following the protocol of Brett *et al.* (1986), Briefly, the

Table 2 Median values of PGL-I, neopterin and CRP in the sera of MB and PB leprosy patients. Values in parentheses indicate the range

	PGL-I (OD)			Neopterin (nM)			CRP (mg/dl)		
MDT (months)	MB	PB	P value	MB	PB	P value	MB	PB	P value
0	0.757 (0.026–2.643)	0.124 (0.015–0.482)	0.029*	11.06 (3.90–35.54)	6.04 (2.33–9.75)	0.004*	6.0 (0–96)	0 (0–12)	0.14
2	0.406 (0.064–2.088)	0.106 (0.012–0.394)	0.013*	12.97 (7.22–22.73)	5.99 (3.53–12.7)	0.001*	6.0 (0–96)	3.0 (0–24)	0.48
4	0.337 (0.026–1.569)	0.078 (0.018–0.405)	0.033*	10.56 (6.04–23.27)	7.19 (4.26–10.96)	0.007*	6.0 (0–48)	3.0 (0–48)	0.52
6	0.432 (0.025–2.001)	0.12 (0.024–0.437)	0.038*	9.95 (7.07–110.11)	7.13 (3.10–9.44)	0.002*	6.0 (0–48)	3.0 (0–32)	0.56
12	0.299 (0.022–1.621)	0.101 (0.019–0.376)	0.029*	12.57 (5.00–19.48)	5.79 (2.31–9.77)	0.0005*	6.0 (0–96)	0 (0–98)	0.2

PGL-I,; CRP, C-reactive protein; MB, multi-bacillary; PB, paucibacillary; MDT, multi-drug treatment.
*Significant values.

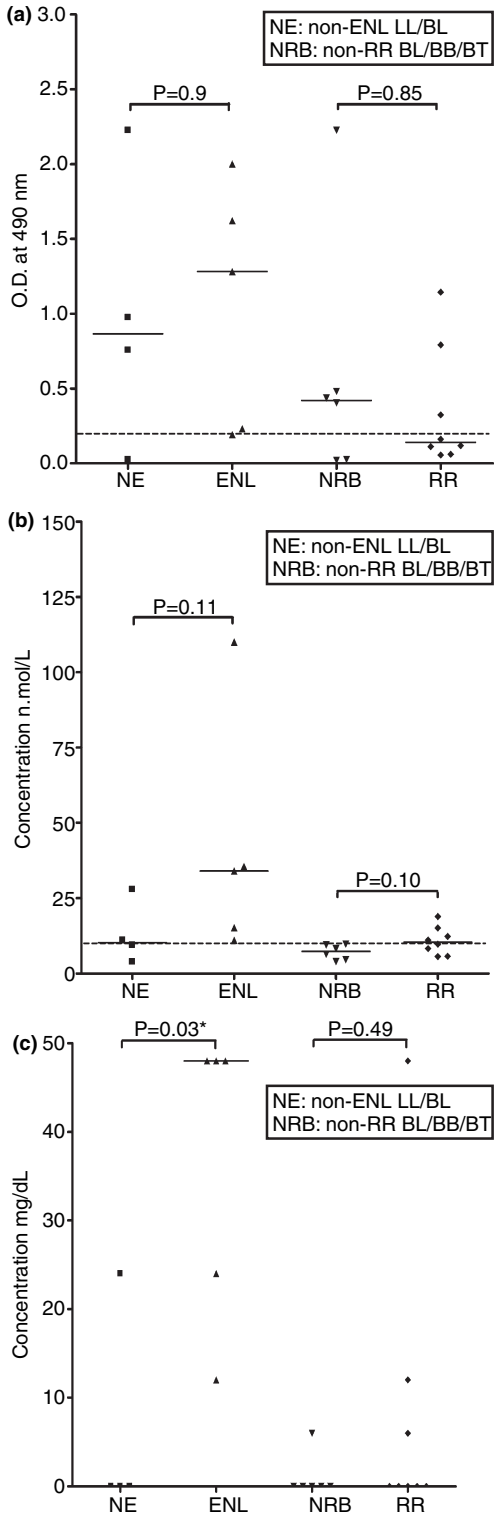

Figure 1 Comparison of PGL-I (a), neopterin (b) and C-reactive protein (c) in the sera of reactional and non-reactional patients.

wells of ELISA plates were coated with either the semi-synthetic antigen PGL-I - NTP-BSA (natural trisaccharide coupled to bovine serum albumin through a phenol group) (0.01 mg/ml carbonate buffer per 50 ?l per well, overnight at 4 °C) or with coating buffer (control). After blocking with 1% milk-PBST (phosphate buffered saline tween 20), a dilution of each sample equivalent to 1:500 serum in PBST containing 10% normal goat serum (NGS) was included (50 μl per well, 37 °C, 60 min) in four wells (a pair of each of antigen coated and buffer coated). The plates were washed with PBST and incubated with 1:10 000 dilution of peroxidase-conjugated goat anti-human IgM (Cappel/Organon Teknika, Tumhout, Belgium) in PBST-10% NGS (50 μl per well, 37 °C, 60 min). After another wash, the colour reaction was developed with 50 μl of substrate solution, *o*-phenylenediamine, was added to the wells (at room temperature for about 20 min). The reaction was stopped with 50 μl of 2.5 N H_2SO_4 and the absorbance was read at 490 nm. The mean absorbance of the control wells subtracted from that of the wells with NTP-BSA. The result was regarded positive if the optical density (OD) exceeded 0.200.

The serum neopterin levels of the patients were assessed using a commercially available ELISA kit (Neopterin ELISA, IBL, Germany) according to the manufacturer's instructions (Westermann *et al.* 2000). This ELISA is based on the competition of unlabelled neopterin from the patients' sera with enzyme-labelled neopterin for the binding sites of a neopterin-specific antibody. The upper limit of the normal range is approximately 10 nM (Hamerlinck *et al.* 1999).

The serum levels of CRP were assayed by the latex agglutination method using the rapid-latex-CRP kit (Omega Diagnostics, Scotland, UK) according to manufacturer's instructions. The sera that showed agglutination were diluted for measuring the titre. A positive result for CRP was reported if the level was at least 0.6 mg dl.

Statistical analysis

The differences in the anti-PGL-I IgM, neopterin and CRP levels were compared within the leprosy spectrum and in reactional patients. As the data did not follow Gaussian distribution, the Kruskal–Wallis test was performed to test the differences in anti-PGL-I IgM, neopterin and CRP levels across the leprosy spectrum and reactions. The NE patients are prone to ENL and NRB patients to RR. Hence ENL was compared with NE, while RR was compared with NRB. The differences in anti-PGL-I IgM, neopterin and CRP were analysed using the Mann–Whitney U test. Repeated measures of analysis of variance (ANOVA) was performed to test whether a significant change occurred in

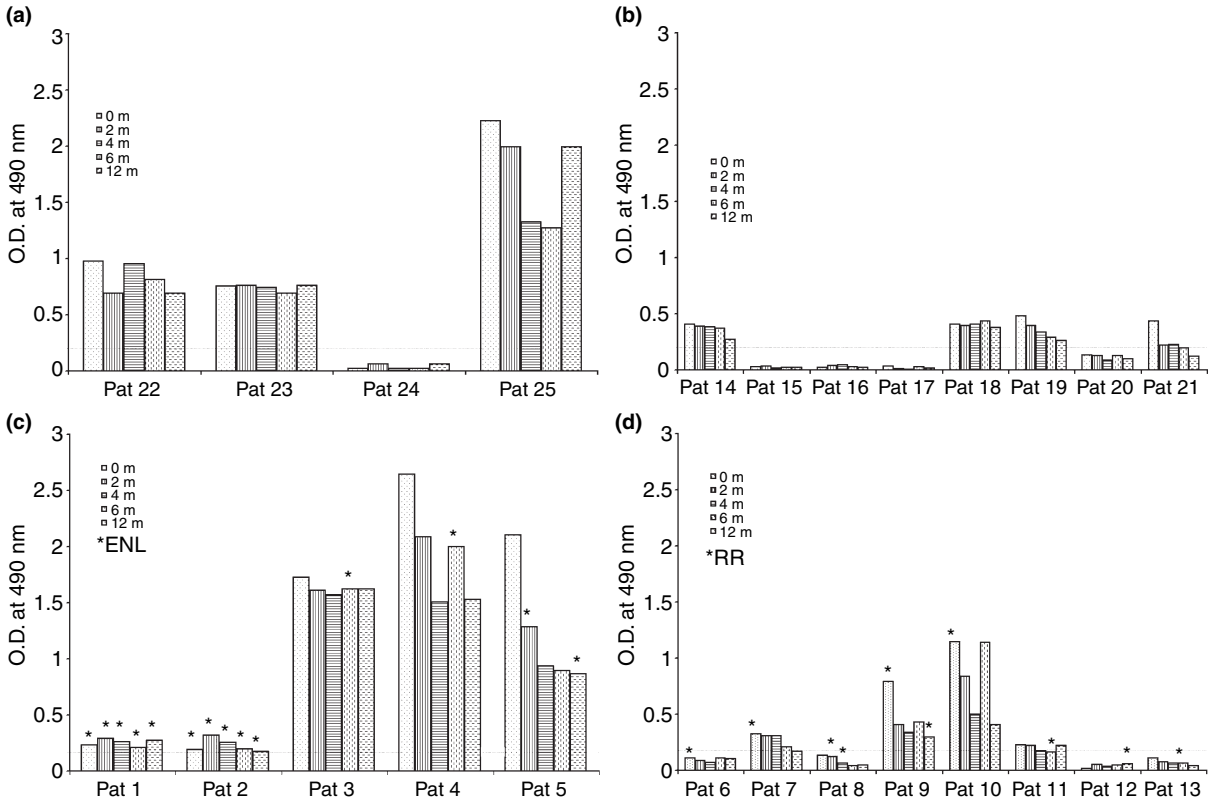

Figure 2 Serial measurements of PGL-I levels in NE (a), NRB (b), ENL (c) and RR (d) patients during MDT. NE, non-erythema nodosum leprosum; NRB, non-reactional borderline; ENL, erythema nodosum leprosum; RR, reversal reaction; MDT, multi-drug treatment.

the levels of anti-PGL-I IgM, neopterin and CRP in serial serum samples. Correlations between anti-PGL-I IgM, neopterin, CRP and BI were analysed using the Spearman's rank correlation coefficient. Differences were considered significant when $P < 0.05$.

Results

Of the 25 patients, 13 developed a reaction during the 12 months of follow-up. Eight had type 1 and five had type 2 reactions. Six of the patients who developed type 1 reaction and all patients with type 2 reaction were MB.

Serum PGL-I

The PGL-I levels were significantly higher in MB patients before and at all time points during treatment (Table 2). In untreated patients, the serum PGL-I levels and BI correlated strongly ($r = 0.72$, $P < 0.0001$). However, the serum PGL-I levels did not differ significantly between NE and ENL ($P = 0.90$) or NRB and RR patients ($P = 0.85$) (Figure 1). The PGL-I levels fell significantly ($P = 0.006$) in MB but not in PB patients during the 12-month follow-up with MDT treatment (Figure 2). In PB patients, the levels remained low throughout the period of follow-up.

Neopterin

The serum neopterin showed a similar trend to PGL-I, with levels being significantly higher in MB than in PB patients before and throughout treatment (Table 2). There was a significant correlation between the serum levels of neopterin and BI of the untreated patients ($r = 0.60$, $P = 0.001$), but not between NE and ENL ($P = 0.11$) or NRB and RR (Mann-Whitney, $P = 0.1$) (Figure 1). The neopterin levels did not fall significantly during MDT (Figure 3).

C-reactive protein

In contrast to PGL-I and neopterin, the CRP levels did not differ significantly between MB and PB patients (Table 2). Moreover, no significant correlation was seen

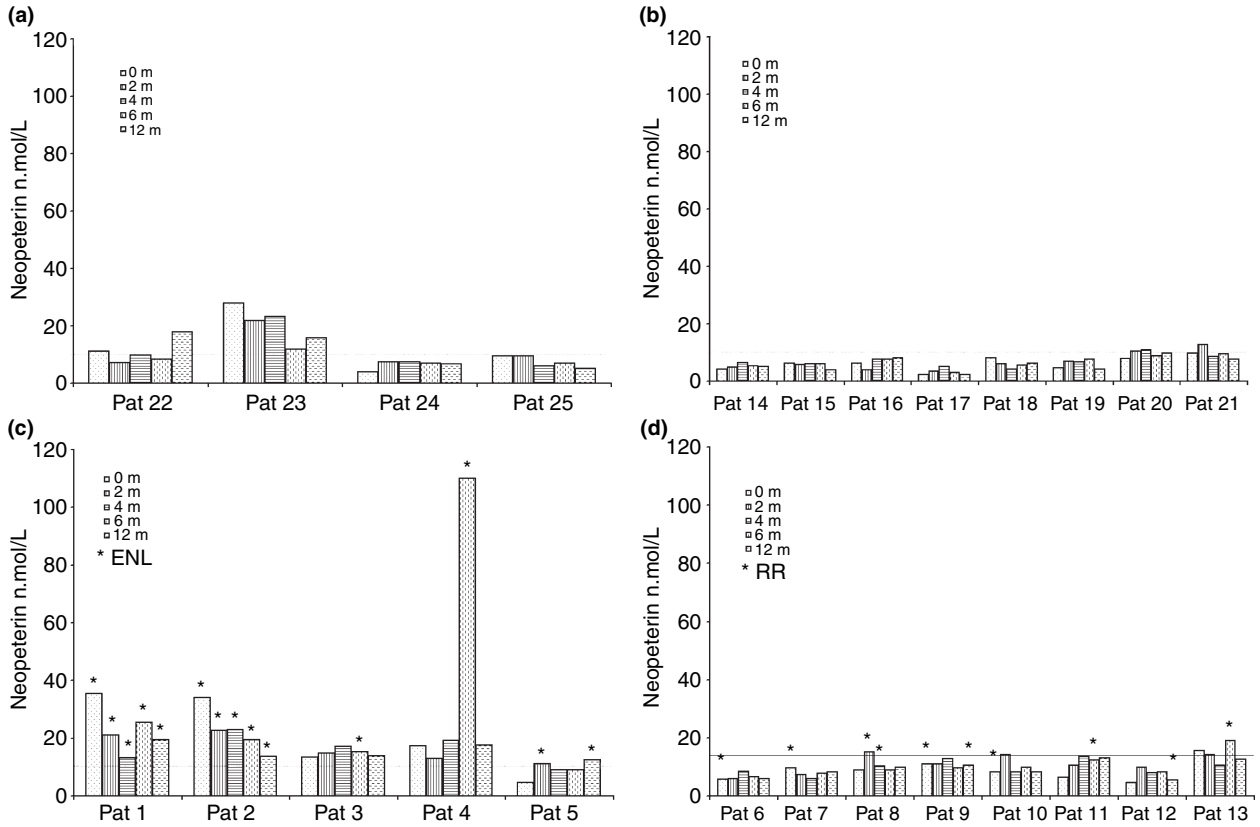

Figure 3 Serial measurements of neopterin levels in NE (a), NRB (b), ENL (c) and RR (d) patients during MDT. NE, non-erythema nodosum leprosum; NRB, non-reactional borderline; ENL, erythema nodosum leprosum; RR, reversal reaction; MDT, multi-drug treatment.

between the CRP levels and BI in the patients ($r = 0.26$). ENL patients, however, had significantly higher levels of CRP than NE patients ($P = 0.03$). No such difference was noted between NRB and RR patients ($P = 0.49$) (Figure 1). Furthermore, the CRP levels did not decline during MDT (Figure 4).

Discussion

We assessed the laboratory markers, which can be used as an adjunct to the clinical monitoring of leprosy patients on MDT and those developing reactions. The PGL-I IgM levels were significantly higher in MB patients than PB patients and showed a strong association with the bacterial load, confirming the findings of Oskam *et al.* (2003), Roche *et al.* (1993) and Stefani *et al.* (1998). The PGL-I levels showed a trend towards significant decline during the12 months of follow-up (Cho *et al.* 1991; Roche *et al.* 1993). High antibody levels were not associated with the development of reactions in the patients (Roche *et al.* 1993; Stefani *et al.* 1998). Our results suggest an important role for the measurement of anti-PGL-I antibodies in distinguishing MB from PB leprosy, as reported previously (Oskam *et al.* 2003).

Neopterin showed a trend similar to PGL-I with significantly higher levels in MB than PB patients and correlating significantly with the bacterial load. This is contradictory to a previous report where no such difference between neopterin levels in MB and PB was observed (Hamerlinck *et al.* 1999). We found no significant difference in the neopterin levels between non-reactional and reactional patients, which contrasts with previous reports where ENL (Hamerlinck *et al.* 1999) and RR (Hamerlinck *et al.* 1999; Faber *et al.* 2004) patients had significantly higher serum levels of neopterin than non-reactional leprosy patients. In contrast to Hamerlinck *et al.* (1999), we exclusively compared ENL with NE (LL and BL without reactions), while RR was compared with NRB (BL, BB and BT) as NE and NRB are prone to ENL and RR, respectively. The neopterin

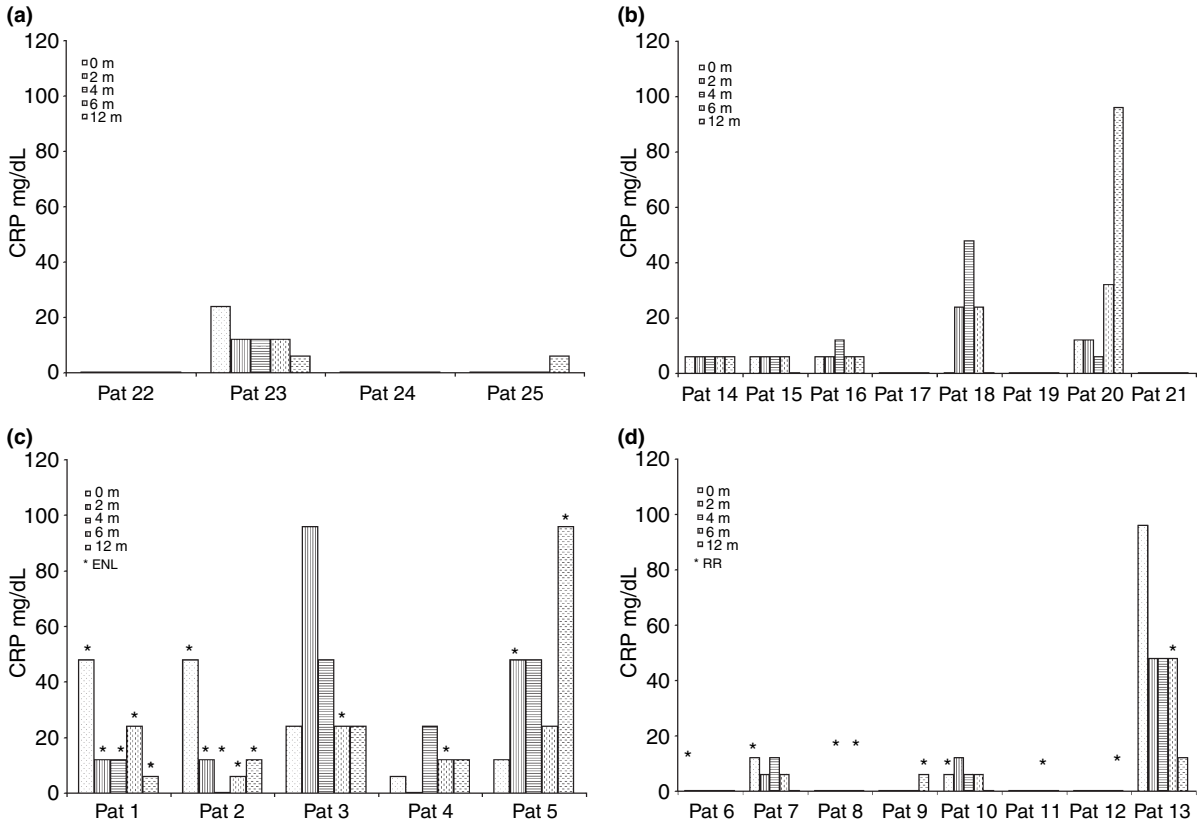

Figure 4 Serial measurements of CRP levels in NE (a), NRB (b), ENL (c) and RR (d) patients during MDT. CRP, C-reactive protein; NE, non-erythema nodosum leprosum; NRB, non-reactional borderline; ENL, erythema nodosum leprosum; RR, reversal reaction; MDT, multi-drug treatment.

levels did not decline significantly during the 12 months of follow-up of patients on MDT.

Neopterin is a monocyte/macrophage activation product and often used as a marker of CMI activity (Murr *et al.* 2002; Hoffmann *et al.* 2003). The CMI plays an important role in determining the leprosy spectrum. PB leprosy is associated with strong CMI, which declines progressively towards the lepromatous end of the spectrum (Ridley & Jopling 1966; Modlin 1994; Jacobson & Krahenbuhl 1999; Britton & Lockwood 2004). Hence, it seems paradoxical that we detected higher levels of neopterin in MB patients. These higher neopterin levels could be a result of the sheer large numbers of macrophages recruited in MB leprosy, a more generalized disease. On the other hand, PB leprosy has a more limited tissue distribution of macrophages resulting in lower overall neopterin levels in circulation despite a strong activation of the CMI.

C-reactive protein, the prototypical acute phase protein in humans, has been used to detect acute infections, assess response to treatment and evaluate the inflammatory response in chronic diseases, such as vasculitis and rheumatoid arthritis (Marnell *et al.* 2005). The serum CRP has been reported to be elevated during ENL reaction (Foss *et al.* 1993; Hussain *et al.* 1995; Memon *et al.* 1996). Similarly, we saw a significantly higher level of CRP in ENL patients than in NE patients (Memon *et al.* 1996). However, no difference in the serum CRP levels was observed either between MB and PB or RR and NRB patients nor was there any significant decline in the levels during the course of the MDT.

A major limitation of the measurement of serum neopterin and CRP in leprosy patients is the lack of disease specificity, as both products are indicative of general inflammation and likely to be elevated in all immune-mediated diseases (Hussain *et al.* 1995; Murr *et al.* 2002; Hoffmann *et al.* 2003; Marnell *et al.* 2005). Hence, the significance of serum neopterin and CRP measurement need to be interpreted with caution. Nevertheless, on the basis of previous studies (Foss *et al.* 1993; Faber *et al.* 2004; Marnell *et al.* 2005), measurement of neopterin and

CRP levels are likely to be valuable in monitoring the treatment of acute reactional episodes in serial samples, rather than as diagnostic biomarkers. Similar limitations are also involved in the measurement of other markers of cellular activation, such as cytokines, as indicators of reactions (Faber *et al.* 2004). Hence, further studies are needed to search for other bio-markers which would help in early detection of reactions in leprosy.

A limitation of our study is the small sample size, which may have resulted in some of the smaller differences between the groups to be missed owing to the lack of power of the statistical analysis. A study involving a larger number of patients is therefore desirable to unravel subtle changes in the level of the soluble products associated with the different patient groups.

In conclusion, PGL-I and neopterin serum measurements may be useful in distinguishing MB from PB patients, which can aid in choosing treatment. The CRP levels appear to have some value in the detection of ENL reactions. The results suggest a need for further research to identify other new laboratory markers for diagnosis and monitoring of leprosy and reactions.

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