Immunopathology of leprosy: towards the search for diagnostic and prognostic biomarkers to elucidate pathobiology and their utility in patient care

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Increased chitotriosidase activity in serum of leprosy patients: Association with bacillary leprosy

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Increased chitotriosidase activity in serum of leprosy patients:

Association with bacillary leprosy

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Key words: Chitotriosidase, leprosy, macrophages, neopterin

Abbreviations: Bacteriological index (BI), mid-borderline (BB), borderline lepromatous (BL), borderline tuberculoid (BT), cell-mediated immune response (CMI), erythema nodosum leprosum (ENL), healthy controls (HC), lepromatous leprosy (LL), lipoarabinomannan (LAM), multibacillary (MB), multi-drug therapy (MDT), non-ENL BL/LL (NE), non-reactional borderline (NRB), paucibacillary (PB), phenolic glycolipid - I (PGL-I), reversal reaction (RR), tuberculoid leprosy (TT), Mycobacterium leprae (M.leprae).
Summary

Human phagocyte-specific chitotriosidase is associated with several diseases involving macrophage activation. Since macrophage activation plays an important role in the control of Mycobacterium leprae infection, we studied the association of chitotriosidase with leprosy both in serum and in situ in lesional skin biopsies from patients. Serum samples from 77 Indonesian leprosy patients (38 non-reactional and 39 reactional leprosy patients) and 39 healthy controls (HC) from the same endemic region were investigated.

The patients were classified as multibacillary (MB, n = 69) or paucibacillary (PB, n = 9) based on the bacterial index in slit-skin smears. Thirty-six of the reactional patients had erythema nodosum leprosum (ENL), while only 3 had reversal reaction (RR). Follow-up serum samples after corticosteroid treatment were also obtained from 17 patients with ENL and one with RR. Multibacillary (MB) patients showed increased chitotriosidase activity in serum as compared to paucibacillary (PB) patients and healthy controls. Although no significant difference was observed between reactional and the corresponding non-reactional groups, ENL showed significantly higher chitotriosidase activity as compared to HC. Furthermore, corticosteroid treatment resulted in significant decline of enzyme activity in ENL sera. Chitotriosidase activity correlated with levels of neopterin, another macrophage activation marker, but not with IL-6, IFN-γ, TNF-α and IL10. Immunohistochemical staining of 6 MB (LL = 5, BL = 1) lesional skin sections from archival material showed positive staining for chitotriosidase within lipid-laden macrophages suggesting that macrophages are the source of the enzyme detected in serum. Thus, serum chitotriosidase activity is potentially useful in distinguishing MB from PB leprosy and in monitoring response to therapy in ENL.

Introduction

An important aspect of leprosy is its unique spectral pathology [1,2,3]. At one pole lepromatous leprosy (LL) presents with disseminated lesions characterized histopathologically by a predominant macrophage infiltrate with foamy changes and a high load of acid-fast Mycobacterium (M.) leprae [3]. Tuberculoid (TT) leprosy, at the other end of the spectrum, has a limited distribution, with lesions showing granulomas of epithelioid macrophages and multi-nucleated giant cells surrounded by a cuff of lymphocytes, with no detectable M. leprae [3]. During the chronic course of leprosy, sudden increases in immune activity may occur which are called reactions. These are either type I (reversal reaction, RR), due to an acute increase in the cell mediated immune response (CMI), or type II (erythema nodosum leprosum, ENL), described as an immune complex mediated disease with involvement of the CMI.

Early studies provided evidence for a defect in the CMI in LL using an in vitro lymphocyte transformation assay [4]. A strong CMI response to M. leprae was however
found in TT and contacts of leprosy patients (except those of LL patients treated less than 6 months) [5]. Since the CMI response is the primary line of defence against intracellular pathogens like mycobacteria, its defect in LL patients has important consequences for disease progression. In TT patients, the strong CMI limits the spread of \textit{M. leprae}, but sometimes results in damage to nerves as a bystander effect [3].

It is ironical that the macrophage, which is an important component of the CMI response, is also one of the preferred host cells for \textit{M. leprae} [6]. Different survival mechanisms have been described for \textit{M. leprae}, either through a defective host macrophage [7] or an active subversion of the host defences [8,9]. Indirect evidence, however, suggests that macrophage activation is not completely defective in lepromatous leprosy patients since elevated levels of neopterin, a marker of macrophage activation, are observed in sera of LL – borderline lepromatous (BL) patients [10] and leprosy reactions [10,11,12], as compared to healthy controls.

Recent studies have shown that human phagocyte-specific chitotriosidase, the first discovered mammalian chitinase, is an important component of the innate immune response against fungal pathogens [13]. Chitotriosidase is an endoglucosaminidase belonging to family 18 of glycosylhydrolases and cleaves chitin [14]. The enzyme was discovered in sera of Gaucher patients, who lack the lysosomal enzyme acid $\beta$-glucocerebrosidase and therefore are unable to degrade the glycosphingolipid glucosylceramide within the lysosomes [14,15,16]. Chitotriosidase serves as a crucial macrophage-derived biomarker to monitor disease onset, progression and therapeutic response in Gaucher disease [17]. Increased chitotriosidase activity has been found within atherosclerotic lesions, in which cholesterol-laden foam cells reside [18]. Elevated serum chitotriosidase activity has been reported in malaria [19] and sarcoidosis [20,21] as well, but not in pulmonary tuberculosis, a mycobacterial disease [21]. However, chitotriosidase activity has been reported in tuberculous pleural effusions [22], suggesting that chitotriosidase production maybe local in these patients.

Since macrophage activation plays an important role in the control of \textit{M. leprae} infection, we studied the association of chitotriosidase with leprosy both in serum and \textit{in situ} in lesional skin biopsies from patients.

\textbf{Materials and Methods}

\textit{Patients and controls}

The study included 78 leprosy patients of which 72 attended the leprosy clinic at the Hasanuddin University hospital in Makassar, Indonesia. Thirty-six normal healthy controls (HC) residing in the same area as the patients were also included in the study. The
Chitotriosidase in leprosy

study was approved by the ethical committee of the Hasanuddin University and informed consent was obtained from the patients and HC. In addition, serum and skin biopsy specimen were also available from 6 patients from archival material at the Academic Medical Center, Amsterdam, The Netherlands which were used for concomitant immunochemistry to study the localization of chitotriosidase within the immune cells and chitotriosidase activity within serum (Table 1). The median age of the patients was 29 years (range: 14-80 years) and included 51 males and 27 females. The median age of the HC was 28 years (range: 19-41 years) and included 28 males and 8 females.

Every patient was clinically assessed by detailed history, medical and dermatological examinations. Bacteriological examination of slit-skin smears was carried out to determine the bacteriological index (BI). The patients were classified according to Ridley and Jopling’s five sub-group classification [1] as 23 lepromatous (LL), 43 borderline lepromatous (BL), 3 mid-borderline (BB), 2 borderline tuberculoid (BT) and 7 tuberculoid (TT) patients. Patients with a BI > 0 were further grouped as multibacillary (MB, \( n = 69 \)) and included all the LL, BL and BB patients, whereas those with BI = 0 were grouped as paucibacillary (PB, \( n = 9 \)) and included the BT and TT patients. Thirty-nine of the patients were diagnosed with reactions of which 36 had type II /erythema nodosum leprosum (ENL; LL=17, BL=19) and 3 had type I / reversal reaction (RR; BB=2, BT=1). ENL was diagnosed by the acute appearance of nodular skin lesions, accompanied by fever with or without peripheral nerve pain and nerve dysfunction. RR reactions typically presented as an acute inflammation of pre-existing lesions and/or onset of new erythematous skin lesions. For the purpose of comparisons the BL and LL patients without ENL reactions were grouped together as NE (non-ENL BL/LL, \( n = 34 \)) since this group of patients is prone to ENL. Similarly, BL, BB and BT patients without RR were grouped as NRB (non-reactional borderline, \( n = 25 \)) since this group is prone to RR. Thus the BL patients were common to both NE and NRB groups since potentially they might develop either ENL or RR [10]. The serum profiles of these groups were compared with ENL and RR patients respectively.

Leprosy was treated with MDT according to World Health Organization guidelines [23]. The study group thus consisted of 30 untreated and 48 treated patients (Fig. 1b). Reactions were treated using prednisolone starting at 40 mg/day and gradually tapering off over a period of 12 weeks [23]. In the absence of biopsy samples in the Indonesian patients, the initial clinical assessment as well as assessment of improvement was done by the clinician-in-charge. Clinical improvement of reactions was defined as complete subsidence of all reactional symptoms. Follow-up samples at the end of corticosteroid treatment were obtained from 17 patients with ENL and 1 patient with RR.

After informed consent, blood samples were collected by venipuncture, the serum was separated, aliquoted and stored in liquid nitrogen at Makassar until transported to the
Netherlands for analysis. No concomitant biopsy specimen could be obtained from the Indonesian patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Classification</th>
<th>Reaction</th>
<th>B.I.</th>
<th>Chitotriosidase</th>
<th>Immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>LL</td>
<td>ENL</td>
<td>n.a.</td>
<td>396</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>LL</td>
<td>ENL</td>
<td>n.a.</td>
<td>115.3</td>
<td>+</td>
</tr>
<tr>
<td>BR15</td>
<td>LL</td>
<td>ENL</td>
<td>4</td>
<td>531.3</td>
<td>+</td>
</tr>
<tr>
<td>BR39</td>
<td>BL</td>
<td>-</td>
<td>4</td>
<td>136.2</td>
<td>+</td>
</tr>
<tr>
<td>BR43*</td>
<td>LL</td>
<td>-</td>
<td>5</td>
<td>251.4</td>
<td>+</td>
</tr>
<tr>
<td>BR47</td>
<td>LL</td>
<td>ENL</td>
<td>5</td>
<td>456</td>
<td>+</td>
</tr>
</tbody>
</table>

**Key**
- n.a. : not available
- * : representative immunostainings depicted in Fig. 3

Table 1. Chitotriosidase levels in 6 MB patients

**Cell culture**

Monocytes were isolated and cultured as described previously (13). Briefly, the cells were cultured for 7 days in RPMI 1640 (BioWhittaker, Viviers, Belgium) supplemented with 10% human serum (HS) (BioWhittaker) to allow for chitotriosidase induction in the presence or absence of dexamethasone at the following pre-determined optimal concentration of 2 nM (Sigma-Aldrich Chemie BV, The Netherlands). CD163 cell surface expression was determined by flow cytometry according to standard procedures on a FACS Calibur and data were analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA, USA).
**Chitotriosidase assay**

Chitotriosidase enzyme activity was determined in sera of leprosy patients, or in cell lysates, using the fluorescent substrate 4-methyl umbeliferyl (4-MU) β-D-N,N’,N’’-(MU)-triacetylchitotriose [24]. Activities were calculated as nanomoles per millilitre per hour. Due to the absence of a cDNA source of the leprosy and control patients we were not able to screen for chitotriosidase deficiency and have decided to use actual enzyme activities. To exclude chitotriosidase deficient individuals from our analysis we did not take into account enzyme activities below 5nmol/ml*h.

**Cytokine and soluble serum marker assays**

The measurement of cytokines IL-6, IL-10, IFN-γ, TNF-α, cytokine receptor (sIL-6R), sCD27 and neopterin has been described previously [10,25].

**Anti-PGL-I IgM assay**

Anti-PGL-I IgM antibodies were detected as described in Brett et al. 1986 [26] using natural tri-saccharide linked to bovine serum albumin via a phenolic ring (NT-p-BSA) as a semi-synthetic analogue of PGL-I.

**Immunohistological techniques**

Formalin-fixed, paraffin embedded tissues (6μm) were stained by the Haematoxylin- Eosin (HE) technique for a light microscopic examination of the tissue morphology. The immune infiltrate was characterized by immunohistochemical staining as described earlier [27]. Briefly, the sections were deparaffinized and preincubated with 3% H₂O₂ in 0.01% sodium azide to inhibit endogenous peroxidase activity. Antigen retrieval for CD68 staining was carried out by treating the slides with 0.25% pepsin in 0.01M HCl for 10 minutes at 37°C. The sections were blocked with normal goat serum. The antibodies used for the stainings were CD68 (Dako, Denmark), mycobacterial lipoarabinomannan (LAM, clone F30-5, from Dr. A. Kolk, KIT-Biomedical Center, The Netherlands), M.leprae specific phenolic glycolipid-I (PGL-I, clone 47-21, from Dr. A. Kolk, KIT Biomedical Center, The Netherlands) and chitotriosidase (mouse monoclonal clone CH1, IgG2b). Primary mouse monoclonal antibodies were applied to the specimen followed by incubation with biotinylated rabbit anti-mouse Ig and subsequently horse radish peroxidase (HRP) labelled streptavidin. The colour reaction was developed using 3 amino-9 ethyl carbazole (AEC) as a substrate. The sections were counterstained with haematoxylin. The percentage of the various cellular populations in the lesions were determined by light microscopy in three randomly selected fields of sequential sections.

The double stainings were carried out to study the localization of chitotriosidase in...
relation to the macrophages. The staining methods were similar to that used by Verhagen et al [27]. The antibodies used in the staining were chitotriosidase (mouse monoclonal clone CH1, IgG2b) and the HAM-56 clone (Dako, Denmark) to stain macrophages, (since staining for CD68 on paraffin sections required antigen retrieval, which might affect the staining for chitotriosidase).

Statistical Analysis
Since the data did not follow Gaussian distribution, the Mann-Whitney U test was performed to compare levels of the serum markers and chitotriosidase activity between MB and PB patients and HC. Moreover, since NE and NRB patient groups are prone to ENL and RR respectively, ENL was compared with NE while RR was compared with NRB and the differences in levels of serum markers and chitotriosidase activity were analyzed using the Mann-Whitney U test. Chitotriosidase activity in sera of patients before and after treatment of ENL were compared by the Wilcoxon Rank Test for paired samples. Correlations between different serum markers and chitotriosidase activity were analysed using the Spearman’s rank correlation coefficient. A correlation was assumed when $r > 0.4$ with $P < 0.05$.

Results

Increased chitotriosidase activity in serum of leprosy patients
Serum chitotriosidase activity was significantly elevated in leprosy patients as compared to healthy controls from the same leprosy endemic area ($P < 0.0001$; Fig 1a). The patients were classified as MB, PB, ENL, RR, NE and NRB as described in detail in materials and methods. Previously we have shown that multi drug therapy (MDT) status did not significantly change levels of serum cytokines and receptors [10]. Similarly, in the present study, statistical analysis revealed that the MDT status did not significantly affect chitotriosidase activity in the patients (Fig. 1b). Hence, phenotypic patient groups were assigned irrespective of their MDT status to have statistically significant numbers of patients within each group. Median activity of serum chitotriosidase was significantly higher in MB (median: 100.5, range 3-2362) as compared to HC (median: 16, range 2.3-16) ($P < 0.0001$), but no difference was found between PB (median: 24, range 8-83) and HC. A significant difference in chitotriosidase activity was also observed between MB and PB patients ($P = 0.011$) (Fig. 1c). Whereas serum chitotriosidase activity was elevated in ENL (median: 105, range 3-2362) ($P < 0.0001$) as compared to HC, no significant difference was noted between RR (median: 8, range 4-24) and HC. On the other hand, while RR showed significantly lower chitotriosidase activity as compared to NRB (median: 99, range 10-544) ($P = 0.031$), no difference was noted between NE (median: 99, range 6-544) and ENL (Fig. 1d). The latter observation should be interpreted with caution however, because the number of RR patients in the study is low.
Chitotriosidase activity in leprosy

Fig. 1. Chitotriosidase activity in the sera of leprosy patients and healthy controls (HC). Leprosy patients (■) showed higher chitotriosidase activity than HC (▲) (A). No significant difference in chitotriosidase activity was observed between untreated [multibacillary, MB (■); paucibacillary, PB (▼)] and multi-drug (MDT) treated [MB (▲); PB (♦)] patients. Both untreated and treated MB showed increased chitotriosidase activity as compared to HC (●) (B). MB leprosy (■) displayed higher chitotriosidase activity as compared to PB leprosy (▲) or HC (▼) (C). No significant difference in chitotriosidase activity was noted between ENL (▲) and NE (■) patients. ENL showed significantly higher chitotriosidase activity compared to HC (●). However, RR (♦) showed significantly lower chitotriosidase activity than NRB (▼) patients, although the sample size for RR patients is too small to draw firm conclusions (D).

Serum samples from 17 patients with ENL and 1 with RR were available at the onset of reaction and at the end of corticosteroid treatment. Chitotriosidase activity was observed to decline significantly following corticosteroid treatment in ENL (median: 24; range: below detection-360) (P = 0.002) (Fig. 2a) and in the single RR patient (data not shown) and was not significantly different from the activity observed in HC. Furthermore, we found that
**Immunopathology of leprosy**

*in vitro* polarization of human monocytes towards macrophages with the corticosteroid dexamethasone prevented chitotriosidase induction (Fig 2b). The dexamethasone-driven polarization of the macrophages was confirmed, for instance, by analysis of the induction of CD163 at the cell surface (Fig 2c).

Fig. 2. Effect of corticosteroid treatment on chitotriosidase activity. Chitotriosidase activity in ENL before (■) and after (▲) treatment with prednisolone in comparison with HC (▼). Chitotriosidase activity declined on prednisolone treatment of ENL to levels observed in HC (A). Chitotriosidase activity during dexamethasone induced polarization of human monocytes towards macrophages. Chitotriosidase activity was suppressed (B) concomitantly with induction of CD163 (C) at the macrophage cell surface.

**Chitotriosidase activity correlates with neopterin in leprosy serum**

The results of a cross-sectional analysis of serum focusing on cytokines and soluble cellular markers have been described elsewhere [10]. A summary of the serum cytokines and soluble cellular markers is included in Table 2. Here, we analysed whether serum chitotriosidase activity correlated with any of the soluble markers of the above mentioned cross sectional analysis. Serum chitotriosidase activity correlated well with another macrophage activation product, namely neopterin (r = 0.48, P = 0.0003) (Table 3). Chitotriosidase activity did not correlate with the cytokines IL-6 (r = 0.02), IL-10 (r = 0.19), IFN-γ (r = 0.16), TNF-α (r = -0.10), the soluble cytokine receptor sIL-6R (r = 0.21) or sCD27 (r = 0.10) (Table 2). Neither BI (r = 0.36) nor levels of anti-PGL-I IgM antibodies (r = 0.11) correlated with chitotriosidase activity in sera of the patients (Table 3).
<table>
<thead>
<tr>
<th>Classification</th>
<th>IL-6 pg/ml</th>
<th>sIL-6R ng/ml</th>
<th>IL-10 pg/ml</th>
<th>IFN-γ pg/ml</th>
<th>TNF-α pg/ml</th>
<th>sCD27 U/ml</th>
<th>Neopterin n.mol/L</th>
<th>Chitotriosidase n.mol.mL⁻¹.hr⁻¹</th>
<th>PGL-I O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>54.1</td>
<td>39.6</td>
<td>10.9</td>
<td>6.3</td>
<td>17.4</td>
<td>142.6</td>
<td>24.2</td>
<td>100.5</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>0 – 8002</td>
<td>0 – 106.8</td>
<td>0 – 410.8</td>
<td>0 – 20.9</td>
<td>0 – 613.2</td>
<td>55.3 – 655.5</td>
<td>3.7 – 116.2</td>
<td>(3 – 2362)</td>
<td>0.003 – 2.812</td>
</tr>
<tr>
<td>PB</td>
<td>20.2</td>
<td>51.5</td>
<td>0</td>
<td>9.3</td>
<td>0</td>
<td>157.5</td>
<td>9.8</td>
<td>24</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>0 – 147.2</td>
<td>15.7 – 84</td>
<td>0 – 34.6</td>
<td>0 – 13.9</td>
<td>0 – 379.5</td>
<td>121.2 – 220.8</td>
<td>5.4 – 24.9</td>
<td>(8 – 83)</td>
<td>0.005 – 0.207</td>
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<td>NE</td>
<td>113.6</td>
<td>31.39</td>
<td>23.2</td>
<td>0</td>
<td>0</td>
<td>260.2</td>
<td>22.6</td>
<td>99</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>(0 – 8002)</td>
<td>(5.4 – 88.4)</td>
<td>(0 – 623.2)</td>
<td>(0 – 127.7)</td>
<td>(0 – 613.2)</td>
<td>(108.3 – 1062)</td>
<td>(4.3 – 116.2)</td>
<td>(6 – 544)</td>
<td>(0.004 – 1.396)</td>
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<tr>
<td>ENL</td>
<td>72.7</td>
<td>44.96</td>
<td>14.7</td>
<td>2.5</td>
<td>15.8</td>
<td>210.8</td>
<td>19.1</td>
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<td>0.1</td>
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<tr>
<td></td>
<td>(0 – 9834)</td>
<td>(0 – 106.8)</td>
<td>(0 – 304.7)</td>
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<td>(0 – 338)</td>
<td>(55.3 – 906.8)</td>
<td>(3.7 – 69.33)</td>
<td>(3 – 2362)</td>
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<td>NRB</td>
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<td></td>
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<td>(5.4 – 88.44)</td>
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<td>(0 – 127.7)</td>
<td>(0 – 552)</td>
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<td>(4.3 – 116.2)</td>
<td>(10 – 544)</td>
<td>(0.004 – 1.295)</td>
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<td>RR</td>
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<td>9.3</td>
<td>22</td>
<td>216.4</td>
<td>26.4</td>
<td>8</td>
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</tr>
<tr>
<td></td>
<td>(51.3 – 3214)</td>
<td>(20.1 – 98.3)</td>
<td>(0 – 95.9)</td>
<td>(0 – 14.8)</td>
<td>(0 – 548)</td>
<td>(142.6 – 272.9)</td>
<td>(9.8 – 60)</td>
<td>(4 – 24)</td>
<td>(0.022 – 0.413)</td>
</tr>
<tr>
<td>HC</td>
<td>0</td>
<td>48.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.3</td>
<td>16</td>
<td>0</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>(0 – 728.6)</td>
<td>(5.6 – 186.5)</td>
<td>(0 – 171.3)</td>
<td>(0 – 15.4)</td>
<td>(0 – 824)</td>
<td>(728.6)</td>
<td>(3.9 – 11.9)</td>
<td>(23 – 42)</td>
<td>(0 – 0.027)</td>
</tr>
</tbody>
</table>

Table 2  Median levels of cytokines, cytokine receptor, cellular activation products, PGL-I and chitotriosidase across the disease groups
Key: The values represent the median of the different parameters measured with ranges in parentheses
Immunopathology of leprosy

### Table 3 Correlation of chitotriosidase activity with cytokines, PGL-I and BI in leprosy patients

<table>
<thead>
<tr>
<th></th>
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<th>P</th>
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<tbody>
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<td>Neopterin</td>
<td>0.48*</td>
<td>0.0003*</td>
</tr>
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<td>IL-6</td>
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<td>0.9</td>
</tr>
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<td>IL-10</td>
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<td>0.25</td>
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<td>IFN-γ</td>
<td>0.16</td>
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<tr>
<td>sIL-6R</td>
<td>0.21</td>
<td>0.14</td>
</tr>
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<td>sCD27</td>
<td>0.1</td>
<td>0.57</td>
</tr>
<tr>
<td>BI</td>
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<td>0.11</td>
</tr>
<tr>
<td>PGL-I</td>
<td>0.11</td>
<td>0.42</td>
</tr>
</tbody>
</table>

**Immunohistochemical analysis of chitotriosidase in tissues of MB and PB patients**

As chitotriosidase is expressed by human macrophages and also stored in specific granules of polymorphonuclear neutrophils [13], immunohistochemical staining was performed to identify the source of the observed increase in chitotriosidase activity in leprosy patients. Positive staining for chitotriosidase protein, which appeared to be localized within macrophages, was seen in lesional skin biopsies from 6 MB patients (Figs. 3a and 3e). However, observation of serially stained sections showed that only a sub-population of the CD68+ cells expressed chitotriosidase protein, suggesting that chitotriosidase activity was only upregulated in a subset of macrophages. This positive staining in tissue sections corresponded to the high serum levels of the enzyme seen in these patients as depicted in Table 1. Immunohistochemical double staining of corresponding serial sections with antibodies to chitotriosidase and the macrophage-specific antibody HAM56 showed co-localization, confirming the macrophage localization of the enzyme (Fig. 3b). This suggests that the macrophages, present in the skin, are the source of the chitotriosidase activity detected in serum of leprosy patients. To study the localization of *M.leprae* associated antigens in relation to chitotriosidase, serial tissue sections were also stained for mycobacterial LAM (Fig. 3c) and the *M.leprae* specific glycolipid PGL-I (Fig. 3d). Positive staining for both antigens was seen in foam cells, which were also positive for the macrophage marker CD68 in serial sections (Fig. 3e), suggesting that these antigens are also localized within macrophages.
Fig. 3. Immunohistochemical staining of lesional skin from a MB patient (BR43). Single staining for chitotriosidase (A); double staining for chitotriosidase (blue) and macrophages (HAM56) (red), −> shows double stained cells (B); single staining for LAM (C); PGL-I (D) and CD68 (E). Magnifications 20X
**Discussion**

Human phagocyte-specific chitotriosidase activity is associated with several diseases involving macrophage activation [14,28,29] and is a valuable tool for monitoring the efficacy of therapy in Gaucher’s disease [15]. The crucial role of the macrophage in leprosy prompted us to look at chitotriosidase activity in the sera of leprosy patients and healthy controls mainly from leprosy endemic areas in Indonesia. The present report is, to our knowledge, the first one to describe an association of serum chitotriosidase activity with leprosy.

Serum chitotriosidase activity was elevated in MB as compared to PB patients or healthy controls in the present study. MB leprosy is characterised by an abundant macrophage infiltrate with many macrophages showing foamy morphology depending on the chronicity of the disease [1,3]. Representative immunohistochemical staining of skin biopsies from 6 MB patients (LL/BL) with high serum chitotriosidase activity showed positivity for chitotriosidase, which was localized within the macrophages (Fig. 3a, b), suggesting that these macrophages are the active producers of chitotriosidase activity detected in MB serum although observations on single stained serial sections showed only a subset of the CD68+ macrophages to be positive for chitotriosidase. Similar observations have also been reported for macrophages in atherosclerotic plaques, where only a sub-population of the cells are positive for chitotriosidase [18]. Chitotriosidase activity was higher in ENL as compared to HC although no significant difference was observed between ENL and non-ENL MB which suggests that chitotriosidase activity is independent of the reaction state of the patient. However, surprisingly, RR patients showed a trend towards decreased chitotriosidase activity as compared to NRB patients. Although this apparently contradictory result may reflect the inherently different nature of the two reactions, where RR is associated with an increased CMI whereas ENL is thought to be an immune complex disease with some involvement of the CMI, it needs to be interpreted cautiously on account of the low number of RR patients in the study.

Recently, it has been suggested that chitotriosidase RNA expression levels, derived from Kupffer cells in the liver from individuals suffering from non-alcoholic steatohepatitis and simple steatosis, correlate amongst others with TNF-α RNA expression [30]. In the present study, serum chitotriosidase activity did not correlate at all with serum cytokines (IL-6, IL-10, IFN-γ, TNF-α), the IL-6 receptor, or the T-cell activation marker CD27, in leprosy patients (Table 3). The lack of association between chitotriosidase and other indicators of inflammation suggested that chitotriosidase and cytokine response pathways could be regulated differently. It could be speculated that chitotriosidase induction is a response to lipid accumulation within the macrophages [31] irrespective of the cytokine environment in the surrounding tissue. A positive correlation was observed, however, between chitotriosidase activity and serum levels of neopterin, another macrophage activation associated molecule (Table 3) [32]. Elevated
levels of serum neopterin were previously reported in leprosy patients in general [10,11] and in particular in reactions [11,12]. As observed for chitotriosidase [17-20], elevated levels of serum neopterin were also reported in diseases associated with macrophage activation like malaria [33,34], tuberculosis [35] and coronary artery disease [36]. Serum neopterin levels were significantly higher in sarcoidosis patients with progressive disease (Stage II) as compared to patients with no indications for corticosteroid therapy (Stage 0 or Stage I) [37]. Similarly, increased chitotriosidase activity was observed in Stage II and Stage III sarcoidosis patients in a separate study [20]. Serum chitotriosidase activity also correlated with neopterin levels in Gaucher’s patients although it was weaker as compared to the correlation with angiotensin converting enzyme (ACE), adenosine deaminase (ADA) and β-hexosaminidase respectively [38]. Interestingly, in the present study, serum chitotriosidase activity did not correlate with either anti-PGL-I IgM levels or with the BI of the patients. This suggests also that the chitotriosidase activity does not reflect the actual bacterial load, but is a measure of accumulation of the lipid antigens such as LAM and PGL-I (Figs. 3d, e) in the macrophages. This may in turn explain why chitotriosidase activity persists even after completion of MDT in the patients, since *M. leprae* antigens such as LAM and PGL-I are known to persist even after completion of MDT in the patients [27]. However, in the absence of functional data, these suggestions remain speculative.

Chitotriosidase activity declined in patients with ENL on treatment with prednisolone. This was supported by *in vitro* data showing lack of induction of chitotriosidase during dexamethasone-induced polarization of monocytes to macrophages. Our previous study with the same patient group [10] showed a decline in serum IFN-γ, TNF-α and sIL-6R but not neopterin on corticosteroid treatment of ENL. This suggests that although chitotriosidase and neopterin are both products of macrophage activation, regulation of their expression may be different as also reported elsewhere [38]. In this regard, neopterin has been proposed as a marker of an active CMI response [32] since it is induced in macrophages in response to IFN-γ from T-cells. On the other hand, chitotriosidase is elevated in response to lipid accumulation within alternatively activated macrophages as is seen in Gaucher disease [31].

We have emphasised in an earlier report that a major limitation of analysis of cytokines and activation markers is that serum measurements may not adequately reflect the tissue immune response [10,27]. However, in the present report, high serum levels of chitotriosidase corresponded to positive staining for the enzyme within foamy macrophages in the MB leprosy patients. Another limitation is a lack of disease specificity whereby cytokines and other soluble markers studied in the present report may change in other immune-mediated conditions [10]. Similarly, whereas both neopterin and chitotriosidase be elevated in diseases involving macrophage activation, chitotriosidase may also be elevated in other lipid accumulation disorders [15,17-20]. Hence, neither cytokines, neopterin nor
chitotriosidase activity measurements can be used for the diagnosis of leprosy and associations of these markers with disease conditions should be interpreted with caution. However, these molecules may be useful prognostic markers in monitoring the response to corticosteroid therapy in reactions. In this regard, the patients in the present study were medically examined for signs of other concomitant infections and skin inflammations and general health conditions and controls from the same area were used to generate baseline values.

The present study does not shed any light on the role of chitotriosidase in leprosy pathology. In this respect, van Eijk et. al. [13] previously demonstrated the anti-fungal activity of human chitotriosidase in vitro and in vivo in mouse models of systemic Candidiasis and systemic Aspergillosis. Another report observed that deficiency in chitotriosidase was also associated with infections with *Wuchereria bancrofti*, the causative agent of filariasis [39]. This led to the suggestion that human chitotriosidase is a component of the innate immunity involved in protection against chitin containing pathogens. However, *M.leprae* is not known to contain chitin in its cell wall/membrane fraction [40]. It is interesting to note that variants of chitotriosidase affecting its activity are associated with Gram-negative bacteremia in children undergoing therapy for acute myeloid leukemia, suggesting more pleiotropic effects of chitotriosidase [41]. Alternatively, increased chitotriosidase in leprosy may be an indirect phenomenon related to lipid overloading of lysosomes in macrophages. However, in the context of the present study, these suggestions remain purely speculative and need to be studied further at a functional level.

In conclusion, serum chitotriosidase activity was observed to be associated with multibacillary leprosy and may be potentially useful in monitoring response to therapy in ENL reactions.
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Immunopathology of leprosy

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