

Evaluation of anti-phenolic glycolipid-I IgM and CD4/CD8 T cell subsets value as high risk determination indicator for individuals residing in a leprosy endemic area in Indonesia

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Abstrak

Penelitian ini menilai antibodi IgM terhadap Phenolic Glycolipid-I (PGL-I) *M. leprae* dan jumlah subset sel T (CD4 dan CD8) untuk menentukan derajat resiko menderita kusta di antara individu melalui survei penduduk di daerah endemik Kusta. Di antara 2349 dari 3074 penduduk yang diperiksa, angka seropositif pada penduduk tersebut secara umum dan kontak serumah sangat tinggi dan ditemukan secara berturut-turut sebesar 40,2% dan 50,4%. Angka rata-rata CD4 dan CD8 dari subset sel T pada penduduk secara umum ditemukan berturut-turut sebesar 849 ± 20 dan 646 ± 19 . Angka rata-rata CD4 dan CD8 dari subset sel T pada kontak serumah ditemukan berturut-turut sebesar 785 ± 13 dan 572 ± 15 . Tidak ada perbedaan bermakna dalam jumlah subset sel T (CD4 dan CD8) antara penduduk secara umum dan kontak serumah. Pada penduduk secara umum, angka rata-rata CD4 dan CD8 pada individu dengan antibodi IgM terhadap PGL-I positif berbeda secara bermakna dibandingkan dengan pada individu dengan antibodi IgM terhadap PGL-I negatif. Pada penelitian ini antibodi IgM positif tidak selalu menggambarkan manifestasi klinis kusta, namun penemuan IgM positif disertai rendahnya jumlah CD4 dari subset sel T masih mungkin menunjang teori tentang resiko tinggi untuk menjadi kusta tipe lepromatosa.

Abstract

This study reports the evaluation of the presence of IgM antibody to Phenolic Glycolipid-I (PGL-I) of *M. leprae* and T cell subsets (CD4 and CD8) count to determine the high risk to contract leprosy among individuals through a total population survey in an area where leprosy is endemic, in Indonesia. Among the 2349 individuals of the total population of 3074 people, the seropositive rates in general inhabitants and household contacts were very high (40.2% and 50.4%, respectively). The mean CD4 and CD8 T cell subset count in general inhabitants was 849 ± 20 and 646 ± 19 , respectively. The mean of CD4 and CD8 T cell subset count in household contacts was 785 ± 13 and 572 ± 15 , respectively. No statistically significant differences were found in peripheral blood CD4 and CD8 count between general inhabitants and household contacts. In general inhabitants the mean CD4 and CD8 count showed significant difference between individuals with positive IgM antibodies to PGL-I compared to individuals with negative IgM antibodies to PGL-I. Although individuals with positive IgM antibodies to PGL-I might not suffering clinical manifestation of leprosy, the finding of positive IgM and low number of CD4 count still support the theory of a high risk to contract lepromatous leprosy.

Keywords: general inhabitant, household contact, lepromatous leprosy, tuberculoid leprosy

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. The disease has a broad spectrum of clinical manifestation from the tuberculoid form, with low bacillary load, to the lepromatous form, with high bacillary load, and may lead to irreversible physical deformities and extensive damage of peripheral nerves. The spectrum of this disease activity is best classified to five groups as follows: full tuberculoid (TT), Borderline tuberculoid (BT), mid borderline (BB), borderline lepromatous (BL), and full lepromatous (LL).¹

In TT and BT leprosy the T lymphocytes of CD4+ subset are the most abundant lymphocytes which are typically present in the central region of the granulomas, while the CD8+ T lymphocytes are present as a mantle around the epitheloid structures. A characteristic serologic feature of TT and BT leprosy is low antibody response to *M. leprae*.

In LL leprosy there is poor cell-mediated immune response and high antibody response to *M. leprae*. It has been reported previously that the detection of IgM antibodies to phenolic glycolipid-I (PGL-I) using *Mycobacterium leprae* particle agglutination (MLPA) test in the community may be useful for the follow up of subclinical infection of leprosy among individuals living in an endemic area.^{2,3} We have also demons-

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trated the homogeneity of seropositivity among populations at village level in South Sulawesi, Indonesia, and suggested that seropositivity in a population meant an endemic exposure to *M. leprae*. Indeed, we also found evidence of cluster of leprosy in certain villages.^{4,5}

The subset of CD4+ T cell became central to considerations of protection and pathology in human disease. Therefore we analyze the correlation between positivity of IgM antibodies to Phenolic Glycolipid-I (PGL-I) of *M. leprae* and T cell subset (CD4 and CD8) count to determine the risk factors among individuals through a total population survey in an area where leprosy is endemic, in Indonesia.

METHODS

Field study

An isolated village in a rural area of South Sulawesi, Indonesia, with 3074 inhabitants was selected for the survey. The leprosy prevalence was expected to be 0.64/1,000 on the basis of available information from the local health service. Before the study was undertaken, the individuals of the village were informed on the purpose of the study, and written consent was obtained from all participants. Only individuals living in the village for at least three months and giving their consent were included in the study.

Clinical examination was carried out by experienced leprosy worker and diagnosis of leprosy was based on the classification of Ridley and Jopling.¹ Slit skin smears were taken from earlobes and a sample from one lesion at least, from all leprosy patients for the determination of the bacterial index (BI).

Blood collection

Blood samples were collected by venipuncture (1.0 ml). Samples were centrifuged 3000 rpm for 5 minutes to separate the serum and transferred to 500 µl vials (Sarstedt, Assist Trading Co., Ltd.) on the same day. All sera were stored at -4°C for further analysis.

The samples collected from individuals were coded, and serological tests were performed without prior knowledge of the classification of the samples.

MLPA test

The Serodia-leprae microtitre particle agglutination test kit for the detection of anti-PGL-I antibodies was purchased from Fujirebio Inc., Japan.⁶

Serum samples were diluted to 1:16 and 1:32 in 96 well U-bottom microplates. Twenty five µl of unsensitized gelatin particle and 25 µl of the particles sensitized with synthetic trisaccharide of PGL-I (NT-P-BSA) were mixed with 25 µl of 1:16 and 25 µl 1:32 diluted samples, respectively. After being incubated for 2 hours at room temperature, the plate was read for agglutination using a mirror reader. Serum samples showing agglutination at a titre of $\geq 1:32$ were considered positive. Positive sera were serially diluted to a concentration of 1:1024 for quantitative testing.

ELISA

IgM and IgG antibodies to PGL-I and LAM-B in serum was measured by indirect ELISA. An ELISA plate (Nunc-Immunoplate Maxsorp F96) was coated with 50 µl of PGL-I or LAM-B solution (0.1 µg/ml in carbonate buffer, pH 9.6), and then the free surface of the well was blocked by incubation with 1% BSA at 37°C for 60 minutes. The test serum, diluted to 1:300 with dilution buffer (PBS with 20% fetal calf serum and 0.5% Tween 20), was added to the well and incubated at 37°C for 60 minutes. After rinsing the plate with washing buffer, peroxidase-conjugated anti-human IgM or IgG antiserum (Dako immunoglobulins A/S) was added to the well and incubated at 37°C for 60 minutes. After the excess conjugate was removed using washing buffer, substrate solution (0.4 mg of o-phenylenediamine per ml and 0.4 µl of hydrogen peroxide per ml in 0.1 M citrate-phosphate buffer, pH 5.0) was added and the plate was developed in the dark for 15 to 30 minutes. The reaction was stopped with 1.25M sulfuric acid. The absorbance was then measured with a microplate photometer at 490 nm.

Counting of CD4/CD8 T cell subset

The counting of CD4/CD8 T cell subset was established in Dynabeads T4-T8 Quant (Dynal kit, Oslo, Norway). Briefly, 0.5 ml of blood was collected by venipuncture and counts of CD4/CD8 were performed at the same day. The Dynabeads T4-T8 kit uses magnetic particles coated with antibodies to the CD4 and CD8 antigen to capture and isolate T4 and T8 lymphocytes from whole blood. The isolated cells are lysed and counted directly in haemocytometer after staining with Sternheimer-Malbin solution.

First 250 µl blood in EDTA was rotated for 2 minutes at room temperature and 225 µl washing/dilution buffer was added. For monocytes depletion 25 µl Dynabeads anti CD14 was added and incubated in a tilting and rotating action for 10 minutes. The tube was

placed in the Dyal MPC-Q for 2 minutes and carefully 200 μ l of monocyte-depleted blood was transferred into each of two supplied microtubes. The tube containing the rosetted monocytes was disposed.

For the isolation of CD4/CD8 T cell subset, into one of the two microtubes containing 200 μ l monocyte depleted blood, 200 μ l washing/dilution buffer and 25 μ l Dynabead CD4 was added, and to the other 25 μ l Dynabead CD8. The tubes were capped and mixed carefully by turning and incubated for 10 minutes. The tubes were placed in the Dyal MPC-Q for 2 minutes and the supernatant was discarded. The isolated cells were washed by adding 500 μ l washing/dilution buffer and placed in the Dyal MPC-Q for a few minutes and the washing step was repeated once. The cells were resuspended in 80 μ l lysis buffer and vortexed, and leaved for 5 minutes. Again the cells were vortexed and the tubes were leaved in the MPC-Q for 1 minute to separate the Dynabeads from nuclei. Twenty μ l Sternheimer-Malbin staining solution was added and the nuclei were counted in a haemocytometer.

Data analysis

All data were recorded on special forms and analysed using a statistical software package (Epi-Info, version 6.02) in appropriate hardware. Statistical analysis was applied as indicated in the results. All *P* presented are two-tailed *P* value.

RESULTS

Sample size and incidence rate

At this survey, from 3047 people that were registered a total of 2349 inhabitants (76% of registered population) met the inclusion criteria and was clinically examined for signs of leprosy. Twenty nine new leprosy patients were detected during the survey and 2 patients had been identified years before by the local health service, giving a total of 31 leprosy patients (20 males and 11 females), ranging in age from 12 to 67 years. Based on the slit skin smear results and clinical diagnosis, 27 patients were classified as tuberculoid and four as lepromatous (bacterial indices of slit skin smears ranging from 1.0 to 4.0). There was a significant difference in leprosy patients between males and females ($P < 0.05$). The incidence rate among the population was thus 10.0/1000 population.

The male : female ratio in the examined population was 0.82 compared with 0.90 in the registered population (Figure 1).

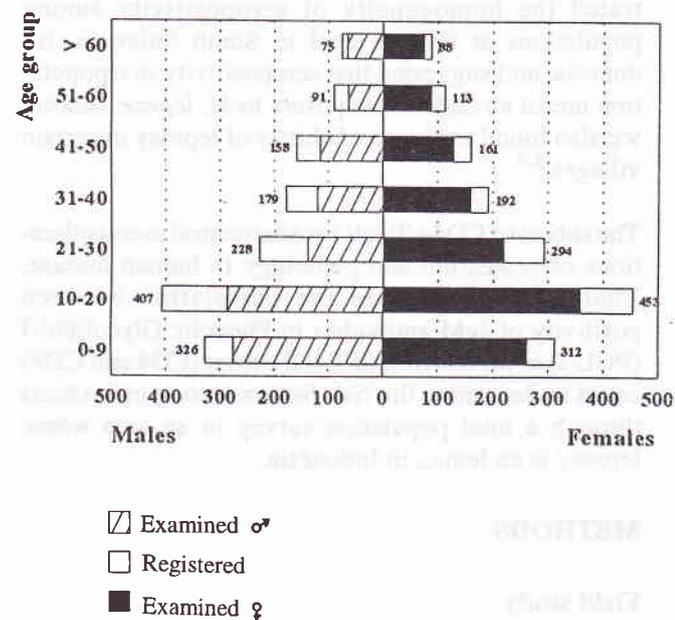


Figure 1. Distribution of registered and examined population according to age and sex in Tukamasea village

Difference in sex group among registered population and examined population is not significant (Fischer exact test, $P > 0.05$)

Seropositivity rate to *M. leprae* related antigens in general inhabitants and household contacts

As it was not feasible to test all individual samples in this survey by *Mycobacterium leprae* particle agglutination MLPA test and ELISA, we examine 1483 general inhabitants (NC group) and 73 household contacts (HC group). As shown in Table 1, using ELISA to detect anti PGL-I IgG antibody, the seropositivity rate in male and female in NC group was 7.8% and 6.4%, respectively. However, the rate of IgM positive in male and female in NC group was 31.7% and 46.5%, respectively. The rate of IgM positive in female was significantly higher than in male in the NC group (Fischer exact test, $P < 0.01$).

Using ELISA to detect anti LAM-B IgG antibody, no difference was found in seropositivity rate of LAM-B IgG between male and female in both the NC and the HC group (Fischer exact test, $P > 0.01$), while the seropositivity rate of PGL-I IgM in female was significantly higher than in male in the HC group (Fischer exact test, $P < 0.01$).

Table 1. MLPA and ELISA results using *M. leprae* antigen in general inhabitants and household contacts

Group	Antigen	IgM/IgG	Methods	Male		Female	
				Positive ‡	Negative ‡	Positive‡	Negative‡
NC*	PGL-I	IgG	ELISA	51 (7.8)	602 (92.2)	53 (6.4)	777 (93.6)
	PGL-I	IgM	ELISA	207 (31.7)	445 (68.3)	386 (46.5)	444 (53.5)
	PGL-I	IgM	MLPA	210 (32.2)	443 (67.8)	400 (48.2)	430 (51.8)
	LAM-B	IgG	ELISA	140 (21.4)	513 (78.6)	137 (16.5)	693 (83.5)
HC†	PGL-I	IgG	ELISA	5 (16.7)	25 (83.3)	3 (7.0)	40 (93.0)
	PGL-I	IgM	ELISA	10 (33.3)	20 (66.7)	29 (67.4)	14 (32.6)
	PGL-I	IgM	MLPA	11 (36.7)	19 (63.3)	30 (69.8)	13 (30.2)
	LAM-B	IgG	ELISA	5 (16.7)	25 (83.3)	6 (14.0)	37 (86.0)

* NC = General Inhabitants, HC† = Household contacts, ‡Absolute number (%), Cut off for positive to PGL-I IgG (ELISA) = 80, PGL-I IgM (ELISA) = 380, PGL-I IgM (MLPA) = 32, LAMB IgG (ELISA) = 250

Using MLPA test to detect anti PGL-I IgM antibody, the seropositivity rate in female was significantly higher than in male in both the HC and the NC group (Fischer exact test, $P < 0.01$).

In addition, no significant difference was found in seropositivity rate between HC group and NC group by ELISA or MLPA (Fischer exact test, $P > 0.01$).

Number of CD4 and CD8 T cell subset in general inhabitants and household contacts

Among the 1483 general inhabitant (NC group) and 73 household contact (HC group) samples tested by Dynabead method, the mean of CD4 and CD8 count in the NC group was 849 ± 20 and 646 ± 19 , respectively; while CD4/CD8 ratio in the NC group was 1.45 ± 0.058 . The mean of CD4 and CD8 count in the HC group was 785 ± 13 and 572 ± 15 , respectively; while CD4/CD8 ratio in the HC group was 1.44 ± 0.074 . No statistically significant difference in the mean of CD4 and CD8 count was found between the two groups (Student's t test, $P > 0.01$) (Table 2).

Table 2. CD4 and CD8 count in general inhabitants and household contacts

Group	CD4 count*	CD8 count*	CD4/CD8 ratio*
General Inhabitants	849 ± 20	646 ± 19	1.45 ± 0.058
Household contacts	785 ± 13	572 ± 15	1.44 ± 0.074

* Mean \pm SD

No statistically significant difference in CD4 and CD8 count between the two groups

Correlation between seropositivity and CD4 and CD8 count

The correlation between seropositivity and CD4 and CD8 count is shown on Table 3. There was significant difference in the mean of CD4 and CD8 count between anti PGL-I IgM seropositive and seronegative (Student's t test, $P < 0.01$).

However, there was no significant difference in the mean of CD4 and CD8 count between anti PGL-I IgG seropositive and seronegative (Student's test, $P > 0.01$).

There was no significant difference in the mean of CD4 and CD8 count between anti LAM-B IgG seropositive and seronegative (Student's t test, $P > 0.01$).

Table 3. Correlation between seropositivity and CD4 and CD8 count

Antibodies Positive/Negative	CD4 count*	CD8 count*	CD4/CD8 ratio*
Anti PGL-I IgM (+)	$949 \pm 10^*$	735 ± 16	1.28 ± 0.06
Anti PGL-I IgM (-)	635 ± 12	528 ± 9	1.21 ± 0.09
<i>P</i> value†	sign	sign	ns
Anti PGL-I IgG (+)	893 ± 26	683 ± 14	1.35 ± 0.10
Anti PGL-I IgG (-)	855 ± 14	648 ± 15	1.46 ± 0.08
<i>P</i> value	ns	ns	ns
Anti LAMB IgG (+)	821 ± 17	644 ± 23	1.35 ± 0.05
Anti LAMB IgG (-)	867 ± 28	658 ± 27	1.47 ± 0.08
<i>P</i> value	ns	ns	ns

* Mean \pm SD (of the number or ratio of T cell subset, using Dynabeads method)

† The difference between individuals with positive and negative antibodies, in the number of CD4/CD8 count and CD4/CD8 ratio was measured by student's t test, sign = significant, ns = non significant

DISCUSSION

In this study, we demonstrated the applicability of MLPA test and CD4/CD8 T cell subset as high risk indicator in subclinical infection among individuals by active survey in an area where leprosy is endemic.

The population in this survey did not differ from the total population both in sex and age group and the sample could be considered as representable. Therefore it is justified to extrapolate the serological results to the whole population of this village, giving an incidence rate of 10.0/1000 population.

In this survey, we found that the seropositivity rate to PGL-I IgM antibody by MLPA test among general inhabitants was 40.2%. In a previous survey, in the area of South Sulawesi, Indonesia, we found that the seropositivity rate by the same test among general population was 31.9%.² The results of other epidemiological studies performed in North Sulawesi also showed that patient to patient contact is the major determinant in leprosy incident, with implication for future control. The above study revealed the contact with a leprosy patient is the major determinant in leprosy incident, whereby the contact was not limited only to household relationships, but also includes neighbour and social relationships.⁷ Thus, many normal persons in the community in an area of high leprosy prevalence have increased anti PGL-I IgM, presumably as a consequence of previous subclinical infection. Our results however revealed that many subjects with positive anti PGL-I IgM did not show any clinical sign. Therefore, one can hypothesize that the majority of individual exposed to *M. leprae* can defend themselves effectively against the development of the disease. However, it remains unclear why in some individual the disease could develop progressively to lepromatous. It might be suggested that the clinical manifestation of this disease might be determined by a complex of immune mechanisms, involving the mechanism by which this bacteria evaded or triggered the cell mediated or humoral immune response.⁸ In this study, we found that the mean of CD4 and CD8 count in anti PGL-I IgM seropositive was significantly higher than in the seronegative individuals. While the immunological significance of this correlation remains obscure, this study revealed that in most of tuberculoid type of leprosy that was found by active survey they have high cellular immunity to *M. leprae*. Thus, it seems that cell mediated mechanisms of immunity rather than humoral are associated with the protection and clinical status of disease.

In this study, the seropositivity rate in general inhabitants and household contacts in females were significantly higher than in males. This is in accordance with previous reported studies,^{9,10} and our former observations.³ The higher MLPA, ELISA, anti PGL-I IgM and ELISA anti LAM-B IgM positivity rates among females did not reflect a higher case rate in our study. Higher innate IgM levels among females compared to males is the most simple explanation for this phenomenon.¹¹ However, other factors related to the endemicity and the specific life style (if any) of the population should be taken into account. In contrast, there was no significant difference in ELISA anti PGL-I IgG or ELISA anti LAM-B IgG between males and females.

Our previously described MLPA test was suitable for detecting IgM antibody in a large population in leprosy endemic areas,³ and no clustering of MLPA seropositivity was observed among the population.⁴

Several reports indicated that the predominant anti PGL-I response in most human leprosy patients was of the IgM type,¹² and that anti PGL-I IgM levels were correlated with disease classification, increasing from the tuberculoid toward the lepromatous pole of the disease spectrum.¹³

To better understand the correlation of IgM antibodies and CD4 and CD8 count with the maintenance of subinfection, we applied MLPA test and Dynabead method through a survey and we will reexamine the same individuals living in an area in which leprosy is endemic.

CONCLUSION

In general inhabitants the mean of CD4 and CD8 count showed significant difference between individuals with positive IgM antibodies to PGL-I compared to individuals with negative IgM antibodies to PGL-I. Although individuals with positive IgM antibodies to PGL-I might not suffering clinical manifestation of leprosy, the finding of positive IgM and low number of CD4 count still support the theory of a high risk to contract lepromatous leprosy.

Acknowledgments

We thank Dr. Paul R Klatser, Dr. Stella M van Beers and Dr. Shinzo Izumi for critical reading of the manuscript. We would like to express our gratitude to the Head of the South Sulawesi Office of the Ministry of Health, Republic of Indonesia and the Head of

Leprosy Unit of the Ministry of Health, Republic of Indonesia for their cooperation. The financial support of The Netherlands Leprosy Relief Association (NSL) and Pfizer Health Research Foundation of Japan is greatly appreciated.

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