PN-ABD-581
63349
8.75

RESEARCH INSTITUTE FOR HEALTH SCIENCES (RIHES)

CHIANG MAI UNIVERSITY

****

Progress Report No 2

The Sero-epidemiology of Leprosy

Program in Science and Technology Cooperation

United States Agency for International Development

Submitted By: Dr Vinai Suriyanon, Principal Investigator

Date: 31 August 1989

Rec'd in SCI OCT 4 - 1989

Project Profile

Country: Thailand

Grant Number: 936-5542-G-00-8049-00

Project Title: Sero-epidemiology of Leprosy

Project Leader: Vinai Suriyanon, MD

Organization: Research Institute for Health Sciences (RIHES)
Chiang Mai University

Co-Investigators: Kriangsak Praputpittaya, PhD
Namtip Sriruk, MSc

Project Consultants: Kenrad E Nelson, MD
James T Douglas, PhD
Patrick J Brennan, PhD
Kamthorn Thamprasert, MD
Sumalee Siriaungkul, MD
Trevor Smith, MD
J Ivanyi, MD

Authorized Officer: Chirasak Khamboonruang, MD, PhD
Director, RIHES

Total Project Budget: Bt 3,748,500 (US$149,940)

Project Duration: 1 August 1988 to 31 December 1992

Reporting Period: 1 February 1989 to 31 July 1989

Budget Allocation for This Period: Bt 470,126

Contents

01. Background/Information:

Leprosy is an important public health problem that is widespread in the developing world and is one of the leading causes of chronic disability and blindness. Although several chemotherapeutic agents are available for the treatment of leprosy, delayed diagnosis and drug resistant organisms render drug treatment of diagnosed cases as an ineffective means of control of the disease.

Recently Hunter and Brennan have isolated and characterized a cell wall component of M.leprae, namely phenolic glycolipid (PGL-1)(1). This compound contains a trisaccharide which is unique to M.leprae, and is antigenic in persons infected with this organism. Subsequently they have synthesized disaccharide and trisaccharide containing the unique carbohydrate structure found in M. leprae PGL-1 and conjugated the synthetic saccharides to protein, such as bovine serum albumin (BSA) (2,3). These synthetic neoglycoproteins, Natural Disaccharide-Octyl-Bovine Serum Albumin (ND-O-BSA) and Natural Trisaccharide-Octyl-Bovine Serum Albumin (NT-O-BSA), have been used for serologic studies in leprosy. Hunter et al have recently isolated and partially characterized another cell wall component of M.leprae, named lipoarabinomannan B (LAM-B) (4). The group specific arabinomannan of the genus Mycobacterium is apparently a membrane associated and highly immunogenic. It has been shown to be useful adjunct to PGL-1 and related saccharides in detection of leprosy (5).

In studies done in Micronesia and Sri Lanka 37 out of 39 new cases from whom sera have been collected prior to clinical onset of the disease had elevated antibodies to PGL-1 from 3 months to two years before the development of clinical signs of leprosy (6). These results are very promising. We therefore plan to do a longitudinal study of household contacts of leprosy cases in Northern Thailand and controls unexposed to household cases of leprosy to assess the efficacy of serologic screening of antibodies to M.leprae specific antigens in detecting preclinical or subclinical infections with M.leprae.

Objectives

1. To determine the prevalence of serum antibodies to M.leprae specific antigens (PGL-1, ND-O-BSA and NT-O-BSA) in household contacts of leprosy patients and controls without known household exposure to leprosy.

2. To determine the prevalence of serum antibodies to the highly immunogenic lipopolysaccharide of M.leprae, lipoarabinomannan B (LAM-B) among household contacts of leprosy cases and controls without known household exposure to leprosy.

3. To determine the comparative efficacy of filter paper strips containing dried blood in detecting antibodies to M.leprae PGL-1, ND-O-BSA, NT-O-BSA and LAM-B in leprosy patients and their household contacts.

4. To determine the predictive value of seropositivity of household contacts of leprosy cases and controls.

Materials and methods

All leprosy patients who have been first diagnosed and treated either at McKean Rehabilitation Center or at the Ministry of Public Health's Leprosy Control Center, Region 10, during the last 6 years (1982-1987) as well as new patients seen during the first 2 or 3 years of the project (1988-1990) will be recruited for this study. These leprosy patients will be examined by a physician and classified by leprosy type according to the five group scheme of Ridley and Jopling. Ten cc. of blood for serologic tests and 4 mm punch biopsy of the skin for histopathologic examination will be obtained from each of them. Slit skin smears from six sites to measure bacteriological index (BI) and morphological index (MI) will also be done. Arrangements then will be made to visit the household of each patient and a roster will be constructed of all the other household members who are living with the patient(s). Household members who at the present time are not living with the patient(s), but at sometime in the past within the period starting 5 years back from the first diagnosis of leprosy have lived in association with the index case(s) for at least 1 year, will also be enrolled in our study. Each of these household residents will be examined by the field team for evidence of leprosy. All contacts with suspicious skin lesions will be brought to McKean hospital where a complete physical examination and skin biopsy will be done by a physician. From each contact, 10 cc of venous blood will be obtained for serologic studies. In small children from whom it is difficult or not possible to obtain blood by venepuncture, fingerstick specimens will be used instead.

For the control study, villages where there are no known leprosy cases will be selected. People in these villages will serve as our normal control subjects. Examination and bleeding will be done on them in the same way as on the index cases and their household contacts.

Blood specimens from leprosy patients, their household contacts, and controls will be tested for antibodies to M.leprae PGL-1, ND-O-BSA, NT-O-BSA and LAM-B by ELISA techniques using the methods as described by Cho et.al. (7) with some modification. Each study subject will be followed up once a year. All procedures done during initial study except skin biopsy will be repeated in the follow up study.

Progress of project activities and results

1. We have finished our preliminary survey work on households of all leprosy patients living in Chiang Mai, Lamphun, Lampang, and Chiang Rai Provinces, who were first diagnosed and treated during the period from 1982 to 1987. We surveyed a total of 912 leprosy households. Among these, 213 households were excluded for various reasons: incorrect address, change of residence, poor

communication or language problems, lack of household contacts, history of treatment of index case before 1982, transportation problems, and so forth. Therefore, only 506 households were selected as eligible for inclusion in the field study. During the survey we informed patients and their household members about the nature and purpose of our research project and we asked for their willingness to participate in the study. Four hundred fifty-nine (549) households or 90.7% expressed their willingness to participate in the research study. Only 42 out of 459 households (9.3%) declined to participate.

2. Enrollment of newly diagnosed leprosy patients is continuing. Up to now 56 patients have been recruited. Blood specimens and skin biopsies have been obtained from them.

3. The study of household contacts of leprosy patients (both new and old) has begun. At the time of writing 177 households have been studied. Blood specimens have been collected from 194 index cases and 601 household contacts. The sera are being stored in the freezer and will be tested for M. leprae antibodies later.

4. To prepare for the study of normal controls, we have surveyed 2 villages where there are no known cases of leprosy. These 2 villages consist of 179 and 122 households respectively and have a total population of 1200 residents. We are making arrangements to do a complete study of these normal controls.

5. To determine the sensitivity and specificity of the ELISA method, we assayed antibodies against three M. leprae specific antigens (viz., PGL-1, ND-O-BSA, and NT-O-BSA) and one cross-reactive mycobacterial antigen (i.e., LAM-B) in sera from 80 leprosy patients (viz., 28 new lepromatous, 34 old lepromatous and 18 tuberculoid leprosy patients), 25 tuberculosis patients, and 33 normal healthy persons. In this study, we categorized lepromatous leprosy patients (LL/BL) arbitrarily into 2 groups. Those who had never been treated before the study and/or those who had been treated less than one year were called "new" lepromatous leprosy patients. On the other hand, those who had been treated more than one year were called "old" lepromatous leprosy patients. Leprosy sera were collected from patients seen at McKean Hospital and TB sera were kindly provided by Dr Sanit Makonkawkeyoon, head, Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University. Of 33 normal control sera, 20 were collected from blood bank donors of Chiang Mai University Hospital, and 13 from the Research Institute for Health Sciences (RIHES) personnel. The results of this study are shown in Tables 1 and 2, Figs. 1, 2, and 3.

By taking x + 3SD of normal controls as a cutoff point for positivity, it was found that in the studies for antibodies against the three M leprae specific antigens (Table 1) the positivity rates were highest on new lepromatous leprosy patients. In this group, the positivity rates of IgM antibodies (=82%) were higher than those of IgG and IgA antibodies. In tuberculoid leprosy, the positivity rates of IgM antibodies were not different from those of IgG and IgA antibodies. All tuberculosis patients and normal controls did not have IgM antibodies, but some of them had positive IgG and IgA antibodies against M. leprae specific antigens. This suggests that the tests for IgM antibodies against M leprae specific antigens are more sensitive and more specific than those for IgG and IgA antibodies. We can also conclude from this study that the same serologic reactivity was observed against the three M. Leprae

specific antigens. For convenience and economics we will confine our studies on the household contacts of leprosy patients to testing for IgM antibodies against only one of the M. leprae specific antigens, for example, NT-O-BSA.

The serologic reactivity against LAM-B was somewhat different (Table 2). The positivity rates of IgG antibodies were higher than those of IgM antibodies. In our future work we will therefore assay only IgG antibodies against LAM-B. In new lepromatous and tuberculoid leprosy patients, the positivity rates of IgG antibodies against LAM-B were 50% and 44% respectively. Forty-eight percent (48%) of tuberculosis sera were positive for IgG antibodies against LAM-B. None of the sera from normal healthy individuals had positive IgG antibodies against this cross-reactive antigen.

The relationship between the duration of treatment and antibody levels in lepromatous leprosy was also studied (Table 2, Figs. 1, 2, and 3). New lepromatous leprosy patients had significantly higher levels of IgM antibodies ($p < 0.005$) and IgA antibodies ($p < 0.005$) against M. leprae specific antigens and also higher levels of IgM antibodies ($p < 0.005$) and IgG antibodies ($0.05 < p < 0.10$) against the cross-reactive antigen than the old lepromatous patients who had been treated more than one year. This suggests that leprosy treatment can cause a decline in M. leprae antibody levels.

6. We have assessed the efficacy of filter paper strips containing blood obtained by fingerstick in detecting antibodies against M. leprae antigens. Blood specimens were collected in vacutainer tubes by venepuncture and on filter paper strips by fingerprick from 31 lepromatous (both new and old) and 10 tuberculoid leprosy patients, and 12 normal healthy persons. The specimens were tested for IgM antibodies against ND-O-BSA by the ELISA method. Results of this study are shown in Fig. 4 and Table 3.

This study showed that there was a very good correlation between the antibody levels in blood collected on filter paper strips and those in serum ($r = 0.979$) (Fig. 4). Moreover, the positivity rate of samples obtained from 31 lepromatous leprosy patients was higher in blood samples collected on filter paper by fingerstick, as compared to serum and plasma (the positivity rates equaled 55%, 35% and 48% respectively). It can therefore be concluded that blood collection on filter paper by fingerstick can substitute venepuncture in the study to determine antibodies against M. leprae antigens. This finding is very useful and can be applied to other leprosy field studies, particularly those with small children from whom it is difficult or impossible to obtain blood by venepuncture.

7. Some of the blood specimens from leprosy patients and their household contacts that are enrolled in our study were tested for IgM antibodies against NT-O-BSA. This preliminary study shows that 15 out of 25 sera (= 60%) from index cases and 13 out of 81 sera (= 16%) from household contacts were positive to NT-O-BSA. This finding of a 16% positivity rate in household contact sera is very interesting. However, its significance cannot be determined at this early point in the study. Study of larger numbers of household contacts and their clinical follow-up will enable us to better understand the significance of positive antibodies to M. leprae antigens in healthy persons. The seropositivity rate in leprosy sera was only 60%. This is not surprising because many of the patients who were included in this preliminary study had been on treatment over a long period and some of them

might even have completed their course of treatment. Studies by other workers (8), as well as our own study, have shown that M. leprae antibody will decline with treatment.

8. To overcome our limitation in existing manpower for the field work, we have hired a new nurse, Ms Pinthong Sukavanija. She started work in March 1989.

9. Dr J T Douglas, project consultant from the University of Hawaii at Manoa, Honolulu, Hawaii, visited us from July 31 to 2 August 1989. During his visit, Dr Douglas offered much useful advice on the laboratory and field work. His report will be included in the next progress report (No. 3).

Conclusions/Remarks

1. We have finished our leprosy household surveys in Chiang Mai, Lamphun, Lampang, and Chiang Rai Provinces. Four hundred fifty-nine (459) out of 506 household (= 90.7%) that were selected as eligible for inclusion in the field study indicated their willingness to participate.

2. Fifty-six (56) newly diagnosed leprosy patients were enrolled in the study.

3. We have done household surveys in 2 villages where there are no known cases of leprosy. People in these villages will serve as our normal control subjects.

4. To determine the sensitivity and specificity of the ELISA method, we assayed antibodies against three M. leprae specific antigens (PGL-1, ND-O-BSA and NT-O-BSA) and one cross-reactive mycobacterial antigen, LAM-B in 80 leprosy sera, 25 TB sera and 33 normal control sera. It has been found that the tests for IgM antibodies against M.leprae specific antigens are more sensitive and more specific than those for IgG and IgA antibodies. The serological reactivity against each of the three different M. leprae specific antigens is the same. In the study with LAM-B, the positivity rates of IgG antibodies are higher than those of IgM antibodies.

5. We have assessed the efficacy of filter paper containing blood obtained by fingerstick in detecting antibodies against M. leprae antigens. The study has shown a very good correlation between the antibody levels in blood collected on filter paper by fingerstick and those in sera collected by venepuncture (r = 0.979). We concluded that blood collection on filter paper by fingerprick can be substituted for venepuncture in serologic studies of leprosy.

6. We have completed studies on 177 leprosy households and have drawn blood from 194 index cases and 601 household contacts. Our preliminary study of 81 household contact sera shows that 13 of them (=16%) were positive to NT-O-BSA.

Work plan for the next period

1. Enrollment of newly diagnosed leprosy patients and the study of their household contacts will be continued.

2. Study of household contacts of old leprosy patients will be continued.

3. Blood specimens from leprosy patients and their household contacts will be tested for IgM antibodies against NT-O-BSA and for IgG antibodies against LAM-B. The prevalence of seropositivity in household contacts of leprosy patients will be determined.

4. Will start the study on control households in the two villages where there are no known cases of leprosy and where we have done a preliminary survey.

5. Follow up study on subjects that were enrolled earlier, or one year previously, will be started.

Presentations and publications resulting from this work

1. Suriyanon V, Praputpittaya K, Hirunpetcharat C, Suphawilai C, and Masarat A. Serological reactivity against Mycobacterium leprae antigens in leprosy patients. Abstracts of the 2nd National Seminar on Leprosy Control in Thailand, p 185, December 1988.

2. Praputpittaya K, Suriyanon V, Hirunpetcharat C, Rungruengthanakit K, and Masarat A. "A comparison of the reliability of blood collection by fingerstick on filterpaper as against venepuncture in a study to determine IgM antibodies against Mycobacterium leprae specific antigens". Second National Seminar on Leprosy Control, Chiang Mai, December 1988.

3. Praputpittaya K, Suriyanon V, Hirunpetcharat C, Rungrengthanakit K, and Suphavilai, C. Comparison of IgM, IgG and IgA responses to M. leprae specific antigens in leprosy. Submitted for publication.

References

1. Hunter SW, Brennan PJ. A novel phenolic glycolipid from Mycobacterium leprae possibly involved in immunogenicity and pathogenicity. *J. Baterial 147*:728-735, 1981.

2. Cho S-N, Chatterjee D, Brennan PJ. A simplified serological test for leprosy based on a 3, 6-di-o-methyl glucose containing antigen. *Am. J. Trop. Med. Hyg. 35*:167-172, 1986.

3. Chatterjee D, Douglas JT, Cho S-N, Rea TH, Gelber RH, Apinall GO, Brennan PJ. Synthesis of neoglycoproteins containing the 3, 6-di-o-methyl-B-D-lucopyranosyl epitope and their use in serodiagnosis of leprosy. *Glycoconjugate J. 2*:187-208, 1985.

4. Hunter SW, Gaylord H, Brennan PJ. Structure and antigenicity of the phosphorylated lipopoly-saccharide antigens from the leprosy and tubercle bacilli. *J. Biol. Chem. 261*:12345-12351, 1986.

5. Levis WR, Muker HC, Levis GS, Sersen E, Brennan PJ, Fried P. Mycobacterial carbohydrate antigens for serologic testing of leprosy. Presented at the U.S./Japan Leprosy Conference in Bethesda MD in July 1987.

6. Epidemiology of leprosy in relation to control: *WHO Technical Report Series No. 716*, 1985 p 23.

7. Cho S-N, Yanagihara D, Hunter SW, Gelber RH and Brennan PJ. Serological specificity of Phenolic Glycolipid I from Mycobacterium leprae and use in serodiagnosis of leprosy. *Infect. Immunity. 41(3)*:1077-1083, 1983.

8. Bach MA, Wallach D, Flageul B, Hoffenbach A and Cottenot F. Antibodies to phenolic glycolipid-1 and to whole Mycobacterium leprae in leprosy patients: Evolution during therapy. *Int J Leprosy 54(2)*: 256-267, 1986.

11 September 1989
p2prog.vinai2

TABLE 1 Levels of antibodies against M. leprae specific antigens (OD 492) in sera of leprosy patients, T.B. patients and normal controls.

Subjects	No. sera tested	IgM			IgG			IgA		
		$\bar{X}\pm S.E$	No Positive	% Positive	$\bar{X}\pm S.E$	No Positive	% Positive	$\bar{X}\pm S.E$	No Positive	% Positive
PGL-1										
LL/BL	28	0.766±0.124	23	82	0.180±0.032	14	50	0.127±0.014	14	50
TT/BT	18	0.181±0.078	5	28	0.211±0.059	9	50	0.083±0.006	3	17
T.B.	25	0.044±0.003	1	4	0.100±0.008	8	32	0.078±0.006	5	20
N.C.	33	0.047±0.003	0	0	0.050±0.004	0	0	0.065±0.002	0	0
ND-O-BSA										
LL/BL	28	0.881±0.127	23	82	0.213±0.054	15	54	0.139±0.032	18	64
TT/BT	18	0.201±0.086	4	22	0.289±0.102	6	33	0.032±0.009	5	28
T.B.	25	0.031±0.006	0	0	0.075±0.016	4	16	0.027±0.002	2	8
N.C.	33	0.051±0.006	0	0	0.041±0.002	0	0	0.022±0.001	0	0
NT-O-BSA										
LL/BL	28	0.815±0.121	23	82	0.257±0.078	20	71	0.150±0.032	19	68
TT/BT	18	0.205±0.088	5	28	0.073±0.073	8	44	0.025±0.010	2	11
T.B.	25	0.029±0.004	0	0	0.049±0.008	5	20	0.020±0.002	1	4
N.C.	33	0.050±0.005	0	0	0.029±0.002	0	0	0.012±0.002	1	3

LL/BL = Lepromatous leprosy patients, untreated and/or treated less than one year.

TT/BT = Tuberculoid leprosy patients.

TB = Tuberculosis patients.

NC = Normal controls.

RIHES Chiang Mai
7 September 1989
g:a7table.doc

10

Table 2 Levels of anti LAM - B antibodies (O D492) in sera of leprosy patients, T.B. patients and normal controls

Subjects	No. of Sera tested	IgM			IgG		
		X ± SE	No. Positive	% Positive	X ± SE	No. Positive	% Positive
LL/BL- new	28	0.256 ± 0.053	14	50	1.156 ± 0.135	14	50
- old	34	0.112 ± 0.010	6	18	0.870 ± 0.103	13	38
TT/BT	18	0.140 ± 0.047	2	11	0.896 ± 0.147	8	44
T.B.	25	0.079 ± 0.006	1	4	1.001 ± 0.136	12	48
N C	33	0.083 ± 0.004	1	3	0.298 ± 0.037	0	0

LL / BL = Lepromatous leprosy patients

TT / BT = Tuberculoid leprosy patients

TB = Tuberculosis patients

NC = Normal controls.

RIHES Chiang Mai
7 September 1989
s0vinai.sep

Table 3 Levels of IgM antibodies against ND-O-BSA (O.D 492) in blood specimens collected by venepuncture and finger stick

Samples	N.C.	LL / BL		
	X ± S.E	X ± S.E	No. Positive	% Positive
Serum (venepuncture)	0.062 ± 0.010	0.268 ± 0.074	11	35
Plasma (venepuncture)	0.039 ± 0.008	0.278 ± 0.074	15	48
Blood on filter paper (fingerstick)	0.023 ± 0.005	0.200 ± 0.057	17	55

N.C. = Normal controls (N = 12)

LL / BL = Lepromatous leprosy patients (N = 31)

RIHES Chiang Mai
7 September 1989
s0vinai.sep

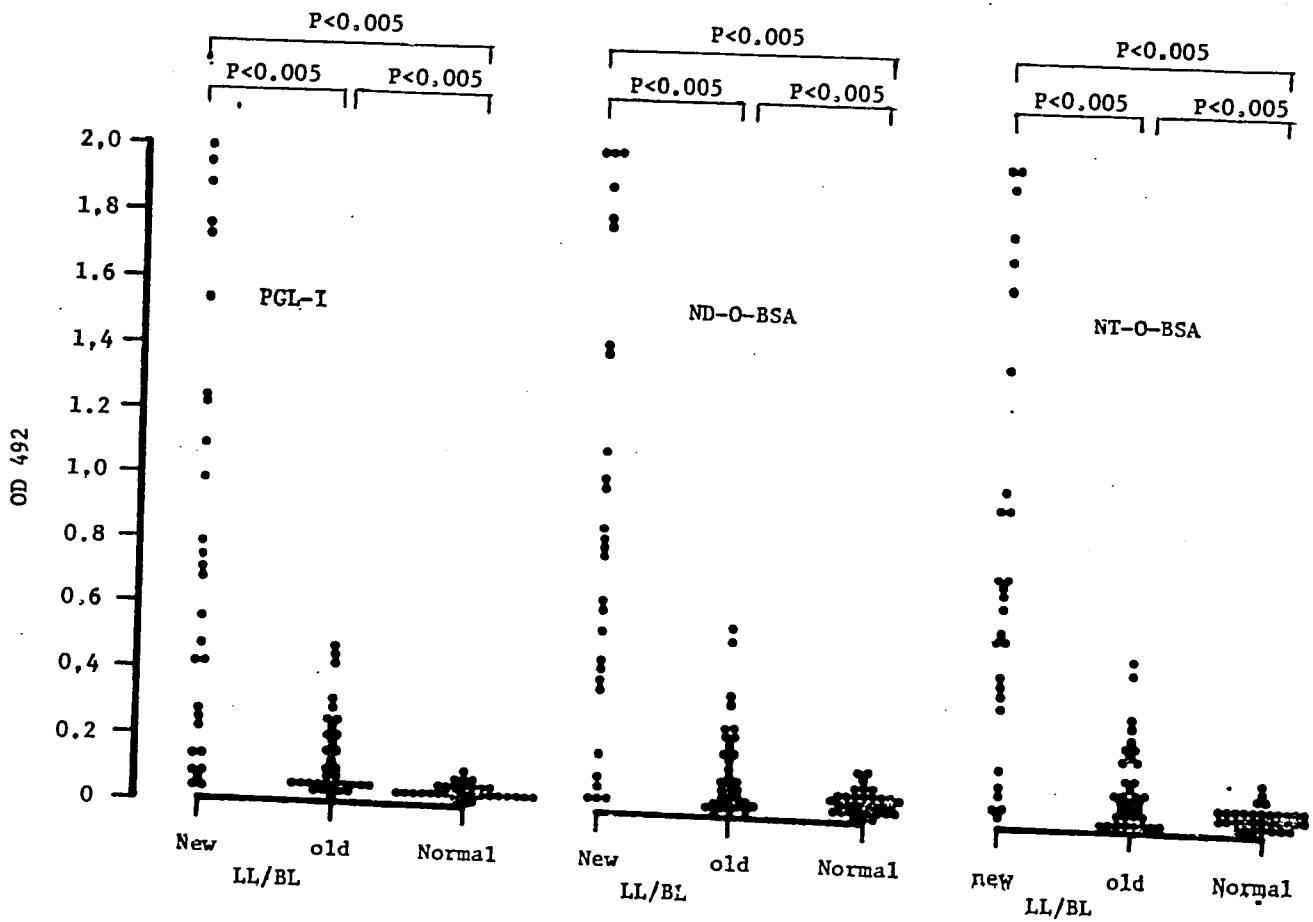


Fig. 1 IgM antibody activity against M.leprae specific antigens (PGL-I, ND-O-BSA and NT-O-BSA) in sera of lepromatous leprosy patients (LL/BL) and normal controls.

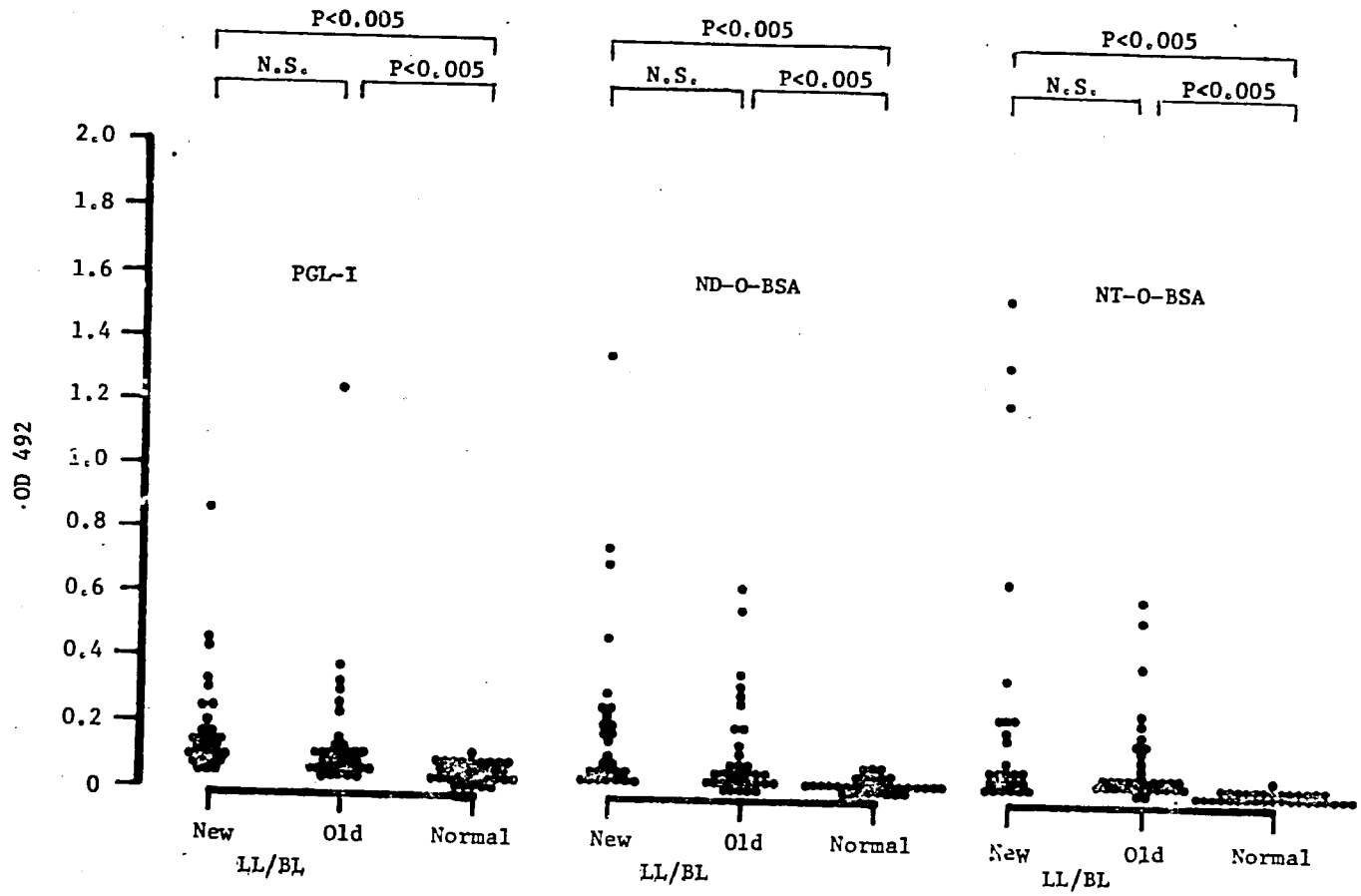
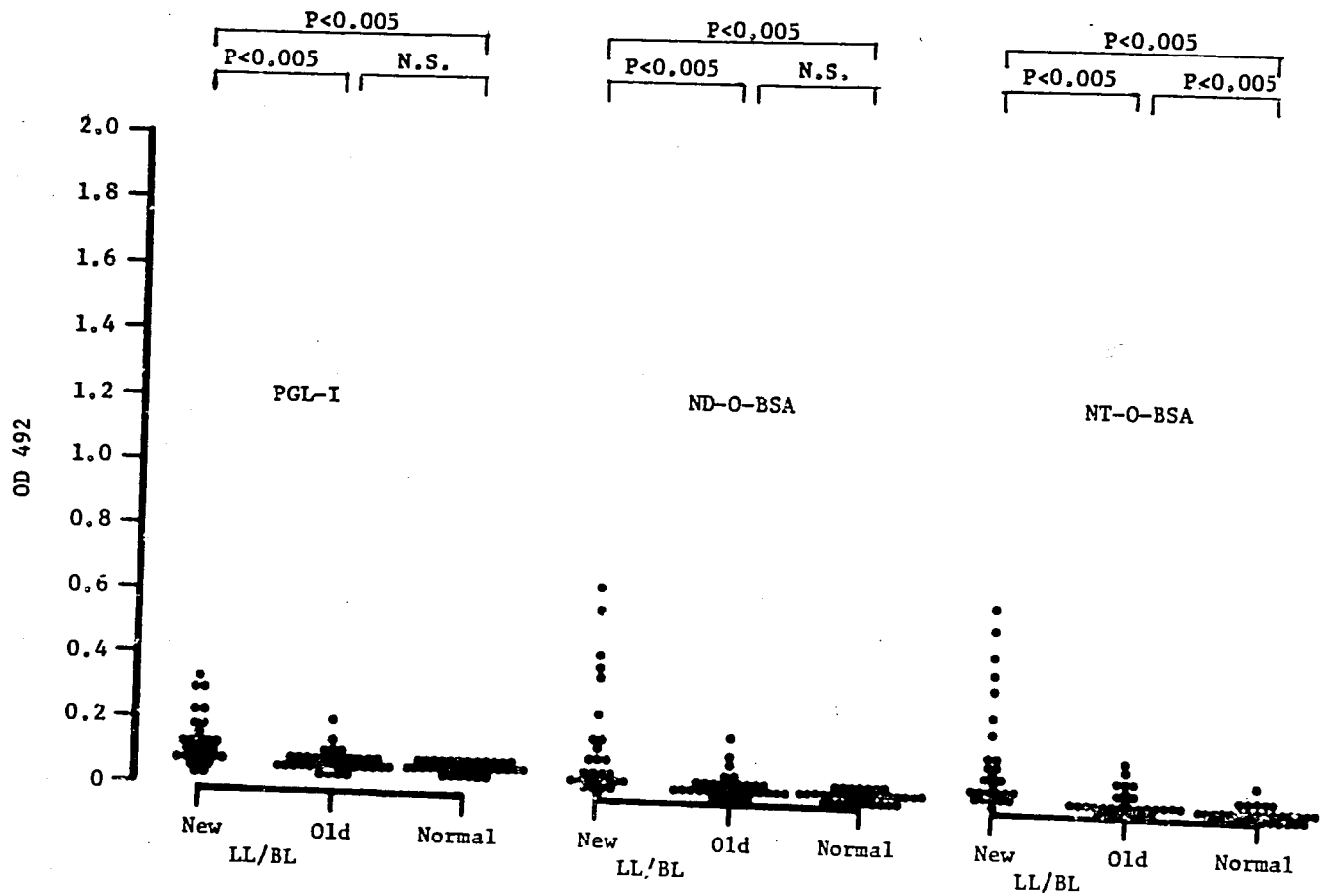
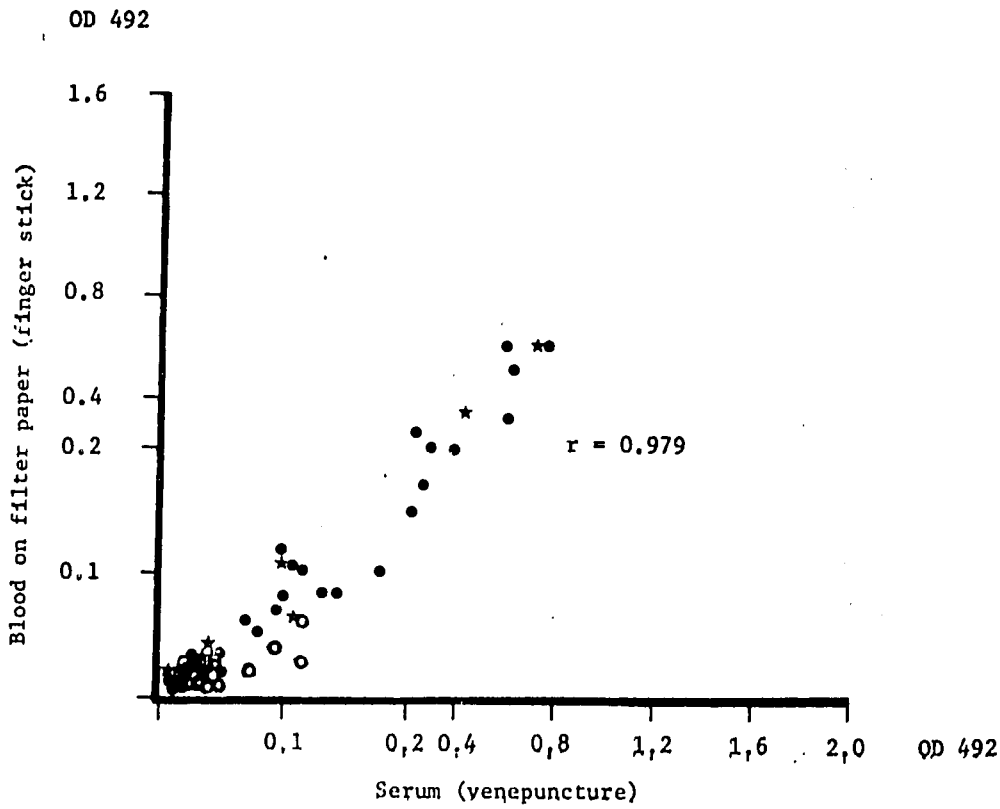


Fig. 2 IgG antibody activity against M.leprae specific antigens (PGL-I, ND-O-BSA and NT-O-BSA) in sera of lepromatous leprosy patients (LL/BL) and normal controls.



Fig, 3 IgA antibody activity against M.leprae specific antigens (PGL-I, ND-O-BSA and NT-O-BSA) in sera of lepromatous leprosy patients (LL/BL) and normal controls,



Fig, 4 Correlation between antibody levels in blood specimens collected by venepuncture and finger stick from lepromatous leprosy (●), tuberculoid leprosy (★) and normal controls (○),

8.700

RESEARCH INSTITUTE FOR HEALTH SCIENCES (RIHES)

CHIANG MAI UNIVERSITY

****

Progress Report No 1

The Sero-epidemiology of Leprosy

Program in Science and Technology Cooperation

United States Agency for International Development

Submitted By: Dr Vinai Suriyanon, Principal Investigator

Date: 28 February 1989

MAR 15 1989

17

Project Profile

Country: Thailand

Grant Number: 936-5542-G-00-8049-00

Project Title: Sero-epidemiology of Leprosy

Project Leader: Vinai Suriyanon, MD

Organization: Research Institute for Health Sciences (RIHES)
Chiang Mai University

Co-Investigators: Kriangsak Praputpittaya, PhD
Namtip Sriruk, MSc

Project Consultants: Kenrad E Nelson, MD
James T Douglas, PhD
Patrick J Brennan, PhD
Kamthorn Thamprasert, MD
Sumalee Siriaungkul, MD
Trevor Smith, MD
J Ivanyi, MD

Authorized Officer: Kosin Amatayakul, MB, ChB (Edin), FRCOG
Acting Director, RIHES

Total Project Budget: Bt 3,748,500 (US$149,940)

Project Duration: 1988-1992

Reporting Period: 1 August 1988 to 31 January 1989

Budget Allocation for This Period: Bt 877,689

18

Contents

01. Background/Information:

Leprosy is an important public health problem that is widespread in the developing world and is one of the leading causes of chronic disability and blindness. Although several chemotherapeutic agents are available for the treatment of leprosy, delayed diagnosis and drug resistant organisms render drug treatment of diagnosed cases as an ineffective means of control of the disease.

Recently Hunter and Brennan have isolated and characterized a cell wall component of M.leprae, namely phenolic glycolipid (PGL-1)(1). This compound contains a trisaccharide which is unique to M.leprae, and is antigenic in persons infected with this organism. Subsequently they have synthesized disaccharide and trisaccharide containing the unique carbohydrate structure found in M. leprae PGL-1 and conjugated the synthetic saccharides to protein, such as bovine serum albumin (BSA) (2,3). These synthetic neoglycoproteins, Natural Disaccharide-Octyl-Bovine Serum Albumin (ND-O-BSA) and Natural Trisaccharide-Octyl-Bovine Serum Albumin (NT-O-BSA), have been used for serologic studies in leprosy. Hunter et al have recently isolated and partially characterized another cell wall component of M.leprae, named lipoarabinomannan B (LAM-B) (4). The group specific arabinomannan of the genus Mycobacterium is apparently a membrane associated and highly immunogenic. It has been shown to be useful adjunct to PGL-1 and related saccharides in detection of leprosy (5).

In studies done in Micronesia and Sri Lanka 37 out of 39 new cases from whom sera have been collected prior to clinical onset of the disease had elevated antibodies to PGL-1 from 3 months to two years before the development of clinical signs of leprosy (6). These results are very promising. We therefore plan to do a longitudinal study of household contacts of leprosy cases in Northern Thailand and controls unexposed to household cases of leprosy to assess the efficacy of serologic screening of antibodies to M.leprae specific antigens in detecting preclinical or subclinical infections with M.leprae.

Objectives

1. To determine the prevalance of serum antibodies to M.leprae specific antigens (PGL-1, ND-O-BSA and NT-O-BSA) in household contacts of leprosy patients and controls without known household exposure to leprosy.

2. To determine the prevalance of serum antibodies to the highly immunogenic lipopolysaccharide of M.leprae, lipoarabinomannan B (LAM-B) among household contacts of leprosy cases and controls without known household exposure to leprosy.

19

3. To determine the comparative efficacy of filter paper strips containing dried blood in detecting antibodies to M.leprae PGL-1, ND-O-BSA, NT-O-BSA and LAM-B in leprosy patients and their household contacts.

4. To determine the predictive value of seropositivity of household contacts of leprosy cases and controls.

Materials and methods

All leprosy patients who have been first diagnosed and treated either at McKean Rehabilitation Center or at the Ministry of Public Health's Leprosy Control Center, Region 10, during the last 6 years (1982-1987) as well as new patients seen during the first 2 or 3 years of the project (1988-1990) will be recruited for this study. These leprosy patients will be examined by a physician and classified by leprosy type according to the five group scheme of Ridley and Jopling. Ten c.c. of blood for serologic tests and 4 mm punch biopsy of the skin for histopathologic examination will be obtained from each of them. Slit skin smears from six sites to measure bacteriological index (BI) and morphological index (MI) will also be done. Arrangements then will be made to visit the household of each patient and a roster will be constructed of all the other household members who are living with the patient(s). Household members who at the present time are not living with the patient(s), but at sometime in the past within the period starting 5 years back from the first diagnosis of leprosy have lived in association with the index case(s) for at least 1 year, will also be enrolled in our study. Each of these household residents will be examined by the field team for evidence of leprosy. All contacts with suspicious skin lesions will be brought to McKean hospital where a complete physical examination and skin biopsy will be done by a physician. From each contact, 10 cc of venous blood will be obtained for serologic studies. In small children from whom it is difficult or not possible to obtain blood by venepuncture, fingerstick specimens will be used instead.

For each index household a control household that does not contain or has not been exposed to any known leprosy patient, will be selected. The control household will contain a person who is the same age (plus or minus five years) and sex as the index case and should have the same socioeconomic status as the index household. The household size as well as the number of persons per room should be about the same in both groups. Examination and bleeding similar to those in index household will be done on the members of the control household.

Blood specimens from leprosy patients, their household contacts, and controls will be tested for antibodies to M.leprae PGL-1, ND-O-BSA, NT-O-BSA and LAM-B by ELISA techniques using the methods described below. Each study subject will be followed up once a year. All procedures done during initial study except skin biopsy will be repeated in the follow up study.

10

Progress of project activities and results

1. Clinical, field and laboratory personnel have been recruited. Dr Chawin Lerstsrimongkol from the Department of Family Medicine, Faculty of Medicine, Chiang Mai University, will help Dr Vinai Suriyanon in clinical work. Dr. Kamthorn Thamprasert who was named as our consultant in histopathology is presently on study leave in Australia. We have recently received a letter of award from WHO TDR Programme for Dr. Kamthorn's specialized training in histopathologic classification of leprosy at the Armed Forces Institute of Pathology (AFIP), Washington DC. and the Leprosy Atelier, University of Hawaii in U.S.A. We are now making arrangements for his training at these two institutions. This specialized training in leprosy histopathology will start soon after his training in general pathology in Australia. Dr. Kamthorn will therefore not be back to Thailand until late 1989. During his absence, Dr. Sumalee Siriaungkul, another board certified pathologist working in the Department of Pathology, Faculty of Medicine, Chiang Mai University, will serve as our consultant in histopathology.

2. Miss Namtip Sriruk our nurse epidemiologist and her colleagues who are permanent staff of the Field Research Section of Research Institute for Health Sciences have been trained in clinical diagnosis and classification of leprosy at McKean Hospital by Dr. Vinai Suriyanon and Dr. Trevor Smith. They are gaining clinical experience in leprosy and all now have the capability in clinical recognition and classification of leprosy. In early August 1988 they attended a two day workshop on leprosy that was organized by the Ministry of Public Health at Chiang Mai Provincial Office of Public Health. From 12 to 14 December 1988 they attended the Second National Seminar on Leprosy Control in Thailand at Chiang Mai Phukam Hotel in Chiang Mai.

3. To prepare for our future work in the field we reviewed the records of all leprosy patients seen at McKean Hospital as well as those reported directly to the Ministry of Public Health's Leprosy Center. Region 10 at Lampang since 1982. After the record review, a leprosy household survey was carried out. At the time of this writing we have just finished our survey in Chiang Mai. We surveyed a total of 444 leprosy households. Among these 213 households were excluded for various reasons: incorrect address, change of residence, poor communication or language problem, lack of household contacts., death of index case, history of treatment of index case before 1982, transportation problems and so forth. Therefore only 231 households were selected as eligible for inclusion in the field study. During the survey we explained to the patients and their household members the nature and purpose of our research project and we asked for their willingness to participate in the study. Two hundred households or 86.6% expressed their willingness to participate in the research study. Only 31 (13.4%) out of 231 household eligible households declined to participate. We are now doing the household survey in Lampang and plan to extend the survey to other provinces such as Lamphun and Chiang Rai which are not very far from Chiang Mai. We hope to finish the survey and be able to start the actual field study soon.

4. Since the beginning of the project, 33 newly diagnosed leprosy patients have been examined by Dr. Vinai Suriyanon. Blood specimens and skin biopsies have been obtained from them. These patients are to be enrolled in our study. We are making an arrangement to visit their households.

5. Our laboratory personnel are now well prepared to do the serologic tests in leprosy research. We have standardized and established the ELISA method for determination of antibodies to PGL-1, NT-O-BSA, ND-O-BSA and LAM-B.

ELISA methods for determination of antibodies against M. leprae antigens:

5.1. Antigens: Phenolic glycolipid I (PGL-1), natural trisaccharide-octyl-bovine serum albumin (NT-O-BSA), natural disaccharide octyl-bovine serum albumin (ND-O-BSA) and LAM-B were kindly supplied by Dr P J Brennan of Colorado State University, Fort Collins, Colorado, USA.

5.2. ELISA Conditions: The ELISA, using antigens mentioned above, was standardized and carried out according to the methodology described by Cho et al. (1983) (7) with some modifications.

5.3. Standardization of PGL-1 ELISA: Stock solution of PGL-1 kept in chloroform:methanol mixture (2:1 by volume) at 4°C, was diluted in absolute ethanol at various concentrations (0, 0.5, 1, 2, 5, and 10 microgram/ml). One hundred microliters of each dilution were added duplicately to wells of flat-bottom 96-well microtiter plates (Nunc). Plates were incubated at 60°C for 2 hours and then washed four times with phosphate buffer saline (PBS). Non-specific sites were blocked with 5% bovine serum albumin in PBS (5% BSA/PBS) at 37°C for 1 hour in a moist chamber. Plates were aspirated and 100 microliters of pooled sera from lepromatous and normal individuals (for positive and negative controls respectively), diluted in 20% normal goat serum in PBS (20% NGS/PBS) were added to wells and incubated at 37°C for 1 hour. Plates were washed with PBS and goat anti-human IgM, peroxidase-conjugated (Cappel Laboratories) at various dilutions in 20% NGS/PBS were added at 100 microliter per well. After one hour incubation at 37°C, plates were washed and 100 microliters of o-phenylene-diamine-H_2O_2 (OPD/H_2O_2) substrate in citrate phosphate buffer, pH 5.0, were added and incubated at 37°C for 30 minutes. Enzyme-substrate reaction was stopped with 2.5N H_2SO_4 and optical density (O.D.) was read at 492 nm using a Titertek Multiscan Reader (Flow Laboratories).

5.4. Standardization of NT-O-BSA and ND-O-BSA ELISA: NT-O-BSA and ND-O-BSA were variously diluted in carbonate-bicarbonate buffer, PH 9.6, and were subsequently added to wells of microtiter plates and incubated at 4°C overnight. Subsequent procedures identical to those described for PGL-1 were performed, except that in this case 1% BSA/PBS was used as a blocking agent and 10% NGS/PBS was used for diluting sera.

5.5. Standardization of LAM-B ELISA: LAM-B antigen was diluted at a concentration of 0.5 micrograms/ml in carbonate-bicabonate buffer, pH 9.6, as recommended by Dr Brennan. It was then added to wells of microtiter plates at 100 microliters/well, and incubated at 4°C overnight. Subsequent procedures were identical to those performed for NT-O-BSA and ND-O-BSA. The optimal condition for detection of IgG antibodies against LAM-B antigen was evaluated using goat anti-human IgG, peroxidase conjugated, at various dilutions.

22

5.6. To determine the optimal dilutions of M. leprae antigens, test sera as well as antihuman globulin conjugates to be used in the ELISA method, we have done the following trial experiments:

5.6.1. Detection of antibodies against M.leprae specific antigens.

Pooled sera from lepromatous leprosy patients and normal healthy persons were diluted 1:300 and 1:600 ELISA tests then were done on these diluted sera using various concentrations of M.leprae specific antigens and antihuman globulin conjugates.

Figures 1, 2 and 3 show the O.D. readings of pooled lepromatous leprosy sera and pooled normal sera in the ELISA tests for IgM antibodies against PGL-1, NT-O-BSA and ND-O-BSA respectively.

The following observations should be noted.

a. Higher O.D. readings were observed in pooled LL sera diluted at 1:300 than those diluted at 1:600 (Figures 1, 2 and 3).

b. In testing pooled LL sera for antibodies against PGL-1, NT-O-BSA or ND-O-BSA no striking differences in O.D. readings were observed when we used antihuman IgM conjugates at different dilutions of 1:500, 1:1000 and 1:2000 (Figures 1, 2 and 3).

c. In ELISA tests to detect antibodies against PGL-1. (Figure 1) we have observed nearly the same O.D. values in pooled LL sera when tested with the antigen at concentrations of 2 ug/ml, 5 ug/ml and 10 ug/ml. When the sera were tested with PGL-1 at concentration less than 2 ug/ml the O.D. values were lower.

d. In the test to detect the antibodies against NT-O-BSA in pooled LL sera (Figure 2) the O.D. values at 1:2500 and 1:5000 dilutions of the antigen were not different. The O.D. values at 1:10000 dilution of NT-O-BSA were however lower.

e. In testing pooled LL sera for antibodies against ND-O-BSA (Figure 3) at different antigen dilutions of 1:500, 1:1000 and 1:2000 almost the same O.D. values were observed. At further dilution of the antigen ND-O-BSA to 1:4000, the observed O.D. values were lower.

5.6.2. Detection of antibodies against LAM-B.

Pooled LL and pooled normal sera were diluted 1:300. ELISA tests were then done on these diluted sera using 0.5 ug/ml LAM-B (as recommended by Brennan) and various dilutions of antihuman IgG conjugate. The results were shown in Figure 4.

Conclusions/Remarks

1. Clinical, field and laboratory personnel have been recruited.

2. An initial survey of leprosy households in Chiang Mai has been finished. 200 out of 231 households (=86.6%) that were selected as eligible for inclusion in the field study expressed their willingness to participate.

3. Thirty three newly diagnosed leprosy were enrolled in our study.

4. The ELISA method for determination of antibodies to PGL-1, NT-O.BSA, ND O-BSA and LAM-B have been standardized and established. It is generally accepted that the ELISA method should be standardized such that the O.D. readings of positive control sera (or pooled LL sera) are higher than 1.00 and those of negative control sera (or pooled normal sera) are less than 0.2. To be economical and in keeping with this general idea, we choose the following as our standard dilutions of *M.leprae* antigens, test sera and antihuman globulin conjugates for the ELISA test.

	Optimal concentration or dilution
PGL-1	2 ug/ml
NT-O-BSA	1:5000
ND-O-BSA	1:2000
LAM-B	0.5 ug/ml
Test sera	1:300
Antihuman IgM conjugate (For ELISA tests to detect IgM antibodies against PGL-1, NT-O-BSA, ND-O-BSA)	1:2000
Antihuman IgG conjugate (For ELISA test to detect IgG antibodies against LAM-B)	1:30,000

24

Work plan for the next period

1. Leprosy household survey in Lampang will be continued. The survey will be extended to other provinces such as Lamphun and Chiang Rai which are not very far from Chiang Mai so that adequate numbers of subjects are recruited.

2. Complete study of household contacts of leprosy patients and controls will be started soon after we have finished the household survey.

3. Enrollment of newly diagnosed leprosy patients will be continued, and study of their household contacts will be carried out.

4. To determine the sensitivity and specificity of the ELISA method, antibodies against PGL-1, NT-O-BSA, ND,O-BSA and LAM-B will be assayed in a number of sera from leprosy patients, T.B. patients and normal healthy persons.

5. We will assess the efficacy of filter paper strips containing blood obtained by fingerstick in detecting antibodies aganist M.leprae antigens. Blood specimens collected by venepuncture and fingerstick from the same lepromatous patients will be tested for antibodies by ELISA method. The correlation between the O.D values in blood specimens obtained by the two different methods will be determined.

6. To accomplish our goal to study 500 index families and 500 control families we need at least 3 field teams, each of which should have one nurse. At the present time there are 7 field research projects going on at the Research Institute for Health Sciences (RIHES). The only unit that is responsible for all the RIHES field work is the socalled "Field Research Section" headed by Mrs. Somsri Ruckphaopunt. This section has currently only 8 nurses. It is therefore unlikely that we can recruit 3 nurses from the existing staff to work in the field for this project all the time. The problem of shortage of manpower, if not corrected, will undoubtedly slow down the work. To keep up with the plan, we will have to hire a new nurse who can work full time for this project.

References

1. Hunter SW, Brennan PJ. A novel phenolic glycolipid from Mycobacterium leprae possibly involved in immunogenicity and pathogenicity. *J. Baterial 147*:728-735, 1981.

2. Cho S-N, Chatterjee D, Brennan PJ. A simplified serological test for leprosy based on a 3, 6-di-o-methyl glucose containing antigen. *Am. J. Trop. Med. Hyg. 35*:167-172, 1986.

3. Chatterjee D, Douglas JT, Cho S-N, Rea TH, Gelber RH, Apinall GO, Brennan PJ. Synthesis of neoglycoproteins containing the 3, 6-di-o-methyl-B-D-lucopyranosyl epitope and their use in serodiagnosis of leprosy. *Glycoconjugate J. 2*:187-208, 1985.

4. Hunter SW, Gaylord H, Brennan PJ. Structure and antigenicity of the phosphorylated lipopoly-saccharide antigens from the leprosy and tubercle bacilii. *J. Biol. Chem. 261*:12345-12351, 1986.

5. Levis WR, Muker HC, Levis GS, Sersen E, Brennan PJ, Fried P. Mycobacterial carbohydrate antigens for serologic testing of leprosy. Presented at the U.S./Japan Leprosy Conference in Bethesda MD in July 1987.

6. Epidemiology of leprosy in relation to control: *WHO Technical Report Series No. 716*, 1985 p 23.

7. Cho S-N, Yanagihara D, Hunter SW, Gelber RH and Brennan PJ. Serological specificity of Phenolic Glycolipid I from Mycobacterium leprae and use in serodiagnosis of leprosy. *Infect. Immunity. 41(3)*:1077-1083, 1983.

28 February 1989
p2prog.vinai

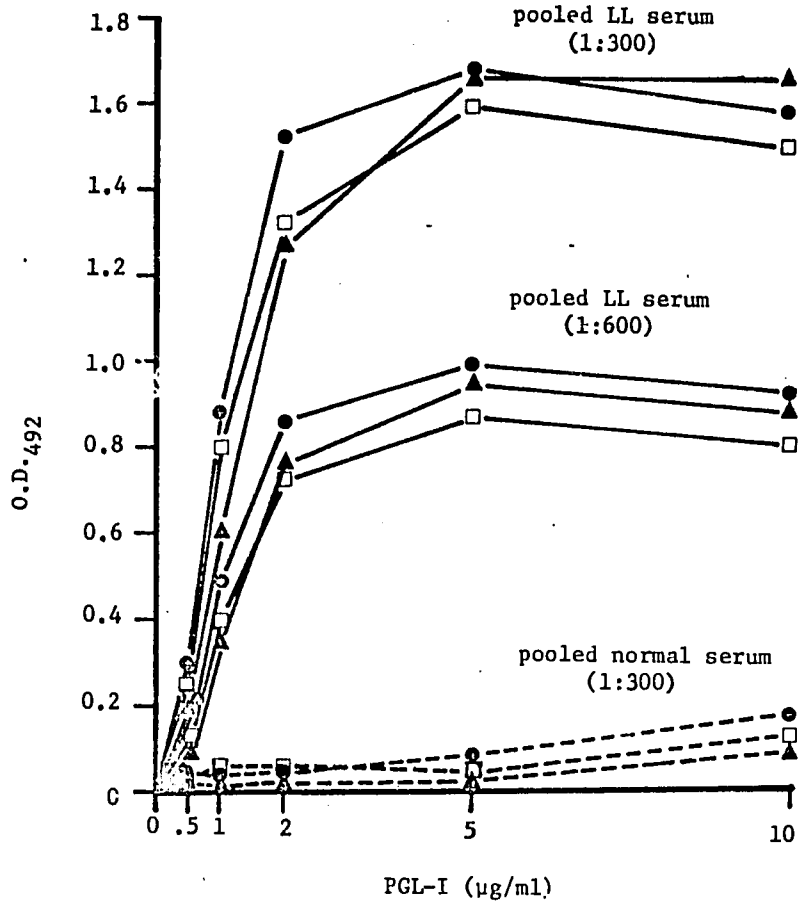


Figure 1: *Standardization of IgM antibodies against PGL-1 antigen with pooled sera of lepromatous leprosy (LL) and normal individuals in ELISA technique. Anti-human IgM, HRP-conjugated, was diluted at 1:500 (▲), 1:1000 (●), and 1:2000 (□).*

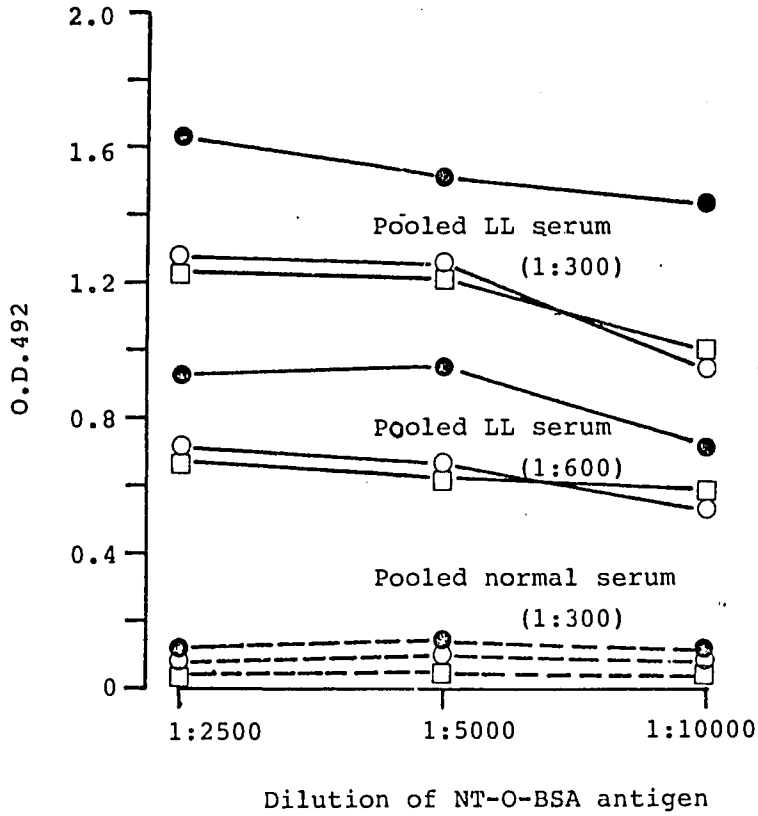


Figure 2: *Standardization of IgM antibodies against NT-O-BSA antigen with pooled sera of lepromatous leprosy (LL) and normal individuals in ELISA technique. Anti-human IgM, HRP-conjugated, was diluted at 1:500 (●), 1:1000 (○), and 1:2000 (□).*

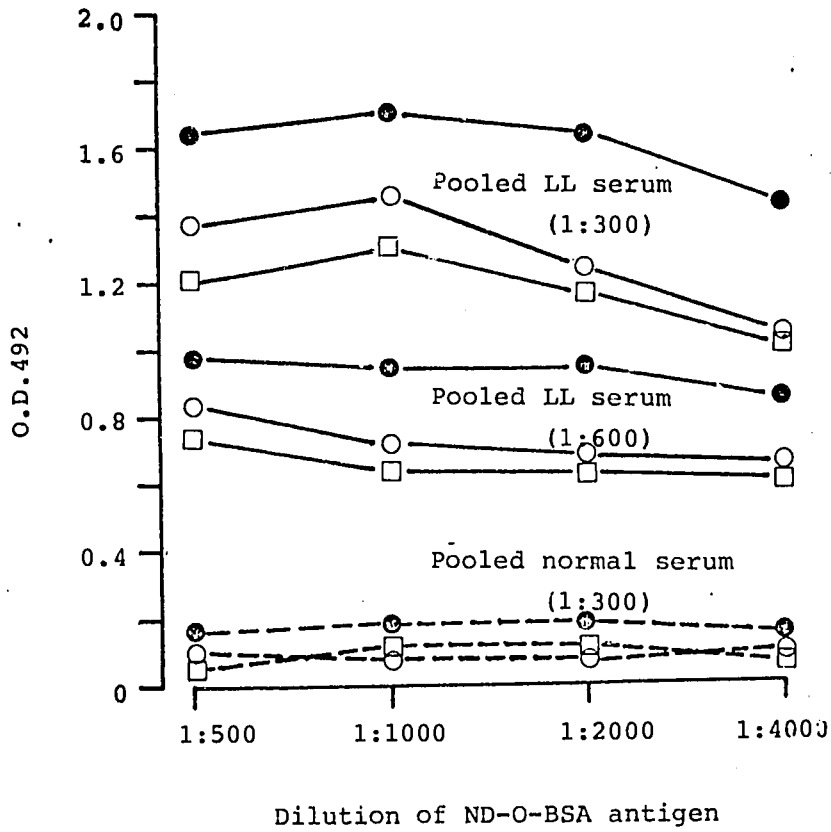


Figure 3: *Standardization of IgM antibodies against ND-O-BSA antigen with pooled sera of lepromatous leprosy (LL) and normal individuals in ELISA technique. Anti-human IgM, HRP conjugated, was diluted at 1:500 (●), 1:1000 (○), and 1:2000 (□).*

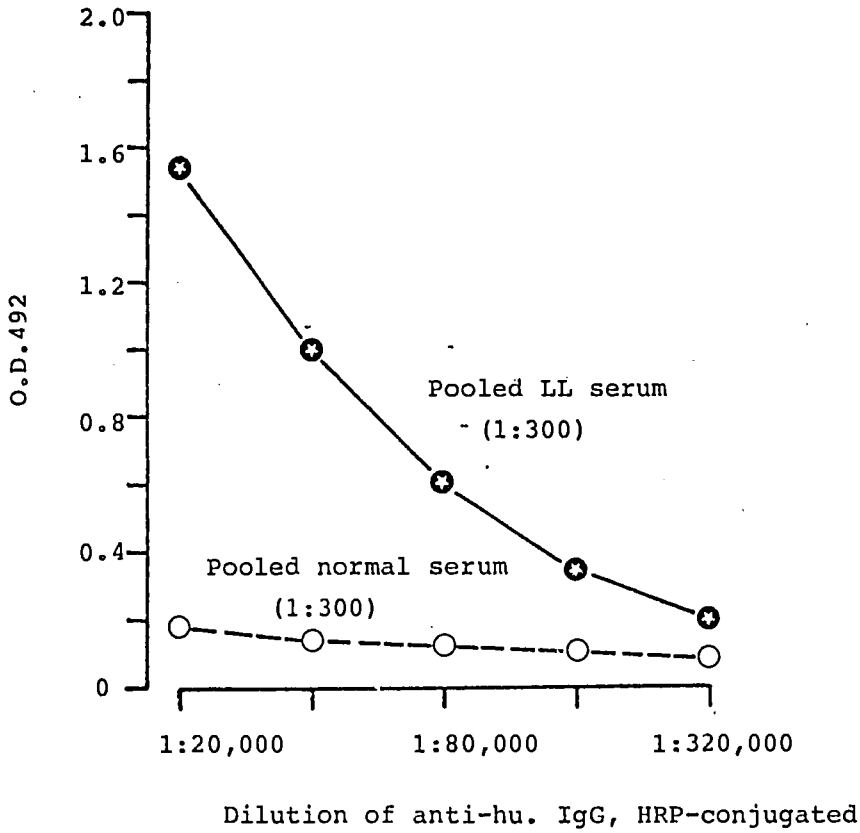


Figure 4: *Standardization of IgG antibodies against LAM-B antigen with pooled sera (1:300) of lepromatous leprosy (LL) and normal individuals in ELISA technique. The LAM-B antigen (0.5 microgram/ml) was tested against anti-human IgG HRP at various dilutions.*