IDENTIFICATION OF RECOMBINANT ANTIGENS OF MYCOBACTERIUM LEPRAE WHICH REACT WITH ANTIBODIES FROM LEPROMATOUS AND BORDERLINE LEPROSY PATIENTS

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ABSTRACT

Recombinant antigens of Mycobacterium leprae were identified by screening a lambda gt-11 M. leprae library with absorbed lepromatous (LL), borderline tuberculoid (BT) leprosy and a healthy contact sera in separate experiments. Six, three, and one clones were respectively isolated by successive screenings of the plaques by immunodetection of antigens on nitrocellulose filters. Of these, a total of six lysogens were generated. Four were made from the clones isolated using LL serum and two were made from clones isolated using BT serum. Analysis of the apparent molecular mass of the recombinant proteins by either SDS-PAGE or Western blotting, showed the fusion proteins to be in the range of 116 kD to 144 kD. The reactivity of recombinant proteins with patients sera throughout the spectrum of the disease was found to be heterogenous regardless of the molecular sizes of the proteins. The characterization of the clones with regard to DNA insert mass was made by amplification with Taq polymerase chain reaction. The analysis on 1.5% agarose gel electrophoresis showed the range to be from 0.3 Kb to 2.0 Kb. The proliferation of T-cell as a result of stimulation by the recombinant proteins (antigens) looks promising and further study for confirmation is in progress.

INTRODUCTION

Leprosy has been a word of many meanings. In every culture the disease has caused such revulsion that many disfiguring skin conditions may at times have been called leprosy, and this stigma caused by leprosy has been a major obstruction to its treatment and control. The literature on the history of the disease known as leprosy is old and extensive, as well as confused, especially in its biblical and mediaeval aspects (Skinsnes, 1873; Browne, 1975).

In 1847, Danielssen and Boeck (reviewed by Browne, 1985) described well the skin manifestations of advanced low resistant leprosy patients and recognized the importance of the consequences of peripheral nerve damage and they thought that the disease was hereditary.

Armauer Hansen (1841-1912) carefully examined under the microscope materials obtained from skin lesions of patients diagnosed as suffering from leprosy in his Bergen laboratory. He applied strict logic to the various arguments and found that heredity could not account for the observations he had made. Hansen probably saw leprosy bacilli in 1872 and mentioned recognition of these organisms in his 1873 annual report. The following year (1874) he published his tentative conclusion that the rod-shaped bodies consistently observed in his material were the cause of leprosy (Harboe, 1973). However, the primitive staining methods at his disposal were not adequate and characterization of the correct morphology of the organisms had to await the discovery of the alinine dyes.

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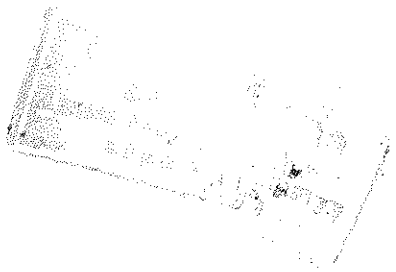
CHAPTER I

INTRODUCTION



In acid-fast staining, Mycobacterium leprae, can be recognized as a rod-shaped morphology measuring 1-8µm by 0.3µm with parallel sides and rounded ends, often showing an unusually beaded appearance (Ridley, 1988).

The ultrastructure of M. leprae, and of mycobacteria in general, has been studied in relation to their biochemistry (Draper, 1982); cell division (Fukunishi, 1985); intracytoplasmic inclusions (Hirata, 1985); cell wall and environment (Silva and Macedo, 1983).

Taxonomically M. leprae belongs to the Family Mycobacteriacae in the Order Actinomycetales (Rees, 1985). It is an obligate intracellular parasite, predominantly residing in macrophages where the organisms commonly occur in clumps or 'globi' which may become very large containing hundreds of bacteria.

The exact mechanism of transmission of the disease (leprosy) caused by M. leprae is not known although skin to skin contact has long been suspected (Job, 1981), inspite of the fact that in most instances bacilli are not in large numbers on the surface of the disease skin. Insects have been suspected as vectors by some (Dungal, 1960 and 1961), but a careful study of this possibility in India tended to exclude such transmission as a major factor (Kirchheimer, 1973). Soil has been mentioned as a possible source (Ramu, 1981), as have been various foodstuffs. But the present consensus is that the most likely mode of transmission involves inoculation through the upper respiratory tract in the form of air-borne droplets (Rees and McDougall, 1977; Chehl et al., 1985). Ultimately it may be found that the disease is spread by

more than one manner. For example, in addition to the above suggested mechanisms of transmission, armadillo (Dasypus novemcinctus to human transmission is becoming increasingly suspected in Louisiana and Texas (Job et al., 1989).

Although the distribution of leprosy is worldwide, it is more prevalent in India, Southeast Asia, and Sub-Saharan Africa (Trautman, 1989). Ethiopia is also among the countries in which leprosy is an important disease with high prevalence in the highland regions (Fig. 1). The national leprosy control programme annual report of 1984 (Table 1), shows that the number of registered cases obtained from different regions of the country was 70,809 (Ohman, 1986). However, reports of recent years show the trend of slight decreasing of case rates (Berhe et al., 1990).

Globally more than 1.6 billion people live in countries where the estimated prevalence of leprosy is greater than one case per 1,000 population (WHO, 1988)(Figure 2).

Although it can affect all ages, leprosy is rarely reported in infants. Incidence rates generally rise to a peak between 10 to 20 years of age, and then fall (WHO, 1985). This has been observed in populations of Burma, South India, Norway and the Philippines.

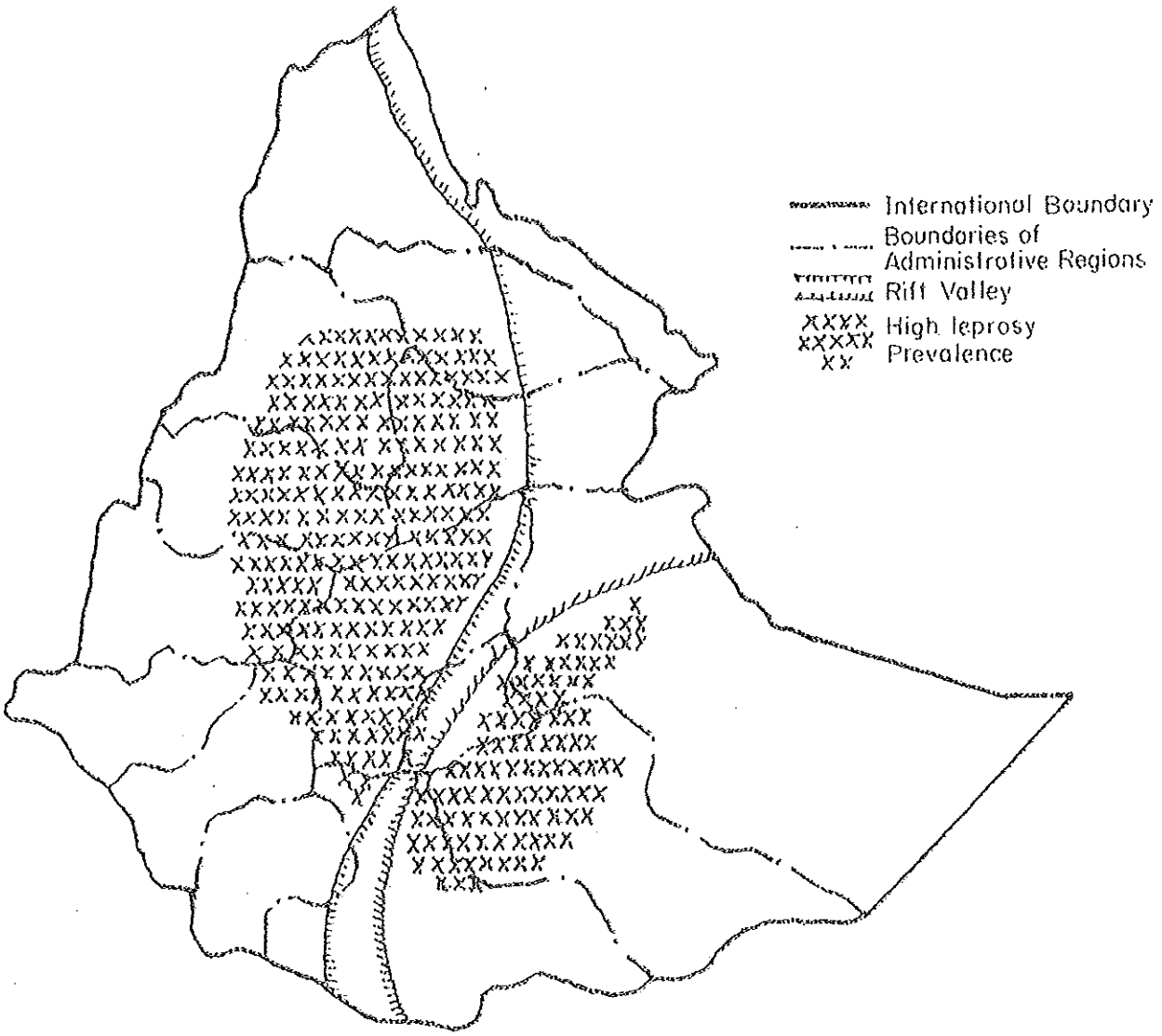


Figure 1: Ethiopia - showing areas of high leprosy prevalence corresponding to the highland regions of the country. Source Berhe et. al. (1990).

Table 1

Leprosy prevalence rate per thousand population in Ethiopia by administrative region

Administrative region	Population	No. of Patients reported	Prevalence rate per thousand	New Case reported
Arssi	1,662,233	1,770	1.1	306
Bale	1,006,491	2,506	2.5	135
Gondar	2,905,362	5,893	2.0	420
Eritrea	1,248,034	784	0.3	-
Gamo Gofa	1,248,034	20	0.02	-
Gojjam	3,244,882	14,219	4.4	1,225
Harrarghe	4,151,706	6,342	1.5	471
Illubabor	963,327	-	-	-
Keffa	2,450,369	16	0.01	-
Shoa rural	6,677,990	2,066	1.9	1,112
Addis Ababa	1,412,575	5,958	1.5	88
Sidamo	3,790,579	500	1.6	474
Tigrai	2,409,700	3,682	0.2	49
Wollega	2,369,677	14,514	1.6	285
Wollo	3,369,677	-	4.0	945
Assab Admin.	89,300	-	-	-
Country	40,606,843	70,809	1.7	5,306

Source: An introduction to Leprosy (with special reference to Leprosy in Ethiopia and to Leprosy research) (Ohman, 1986).

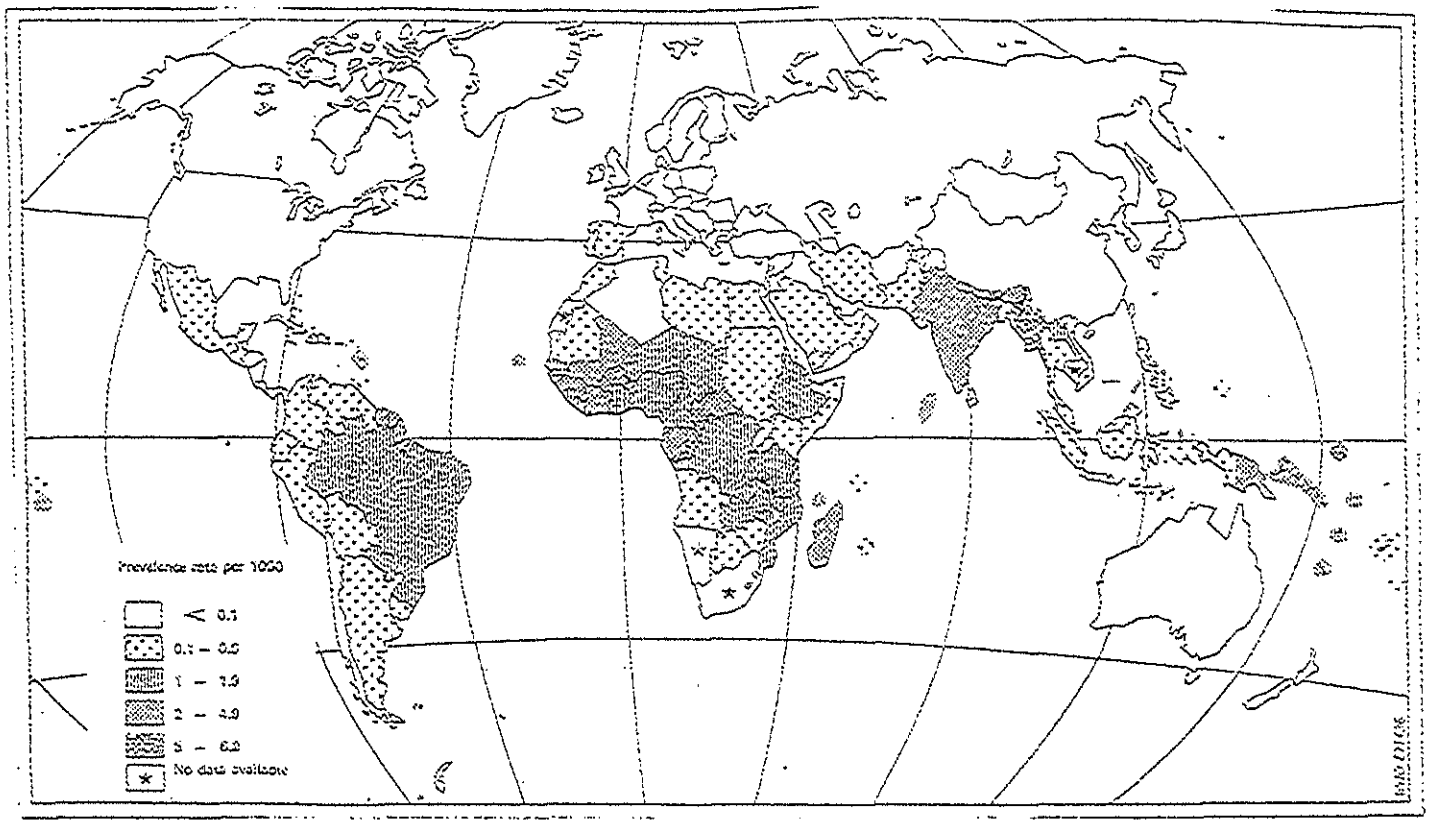


Figure 2. Prevalence of registered leprosy cases in the world, 1987.
Source: WHO Expert Committee on Leprosy, Sixth report (1988).

Both the incidence and the prevalence of leprosy appear to be higher in males than in females in most regions of the world, with the exception of certain populations in Africa where higher rates have been reported among females. This sex difference is greater in adults than in children and is more so for lepromatous than tuberculoid leprosy (WHO, 1985).

For several years man was considered to be the only reservoir of M. leprae. There is now evidence that armadillos in southern and central parts of the United States of America are naturally infected with an organism that is indistinguishable from M. leprae (Walsh et al., 1975). Natural M. leprae-like infections have also been reported in two primates, mangabey monkeys, Cercocebus atys (Meyers et al., 1985), and chimpanzees (Donham and Leininger, 1977).

Despite these reports of possible reservoirs of M. leprae other than man, the epidemiological evidence suggests that human infections are the most important, if not the only, source of infection for man.

The pathology of leprosy shows that most people who are infected with M. leprae develop a subclinical infection i.e., most of them recover naturally without ever having symptoms or signs of disease, while few people develop the disease, leprosy. The clinical pattern of leprosy depends upon the response of the host to the organism.

The earliest clinically detectable lesions are usually in the

skin and from the onset small cutaneous nerve fibres are involved. The extensive peripheral nerve damage was reviewed by Job (1989) at great depth.

In addition to skin and peripheral nerves, M. leprae invades eyes, nose, larynx, mouth, hands, soft palate, testes, adrenal glands, kidneys and the organs of the reticuloendothelial system namely, lymph nodes, liver, spleen, and bone marrow (Bryceson and Pfaltzgraff, 1990).

Clinical symptoms and signs, in leprosy, are extremely varied with respect to both their nature and extent. Many attempts have been made to produce a systematic classification of the disease. The most useful and detailed system is the Ridley-Jopling classification (Ridley-Jopling, 1966). However, before clinical and histopathologic manifestations of well-defined nature develop, patients may display one or more ill-defined hypopigmented macules. These patients are classified as having indeterminate leprosy. At this early stage, the only convincing clinically demonstrable abnormality that points to leprosy may be some sensory loss.

Ridley-Jopling (1966) initially classified patients with leprosy into five categories using histopathological criteria, and more recently, Ridley (1974) expanded the classification into a six-group system, namely, polar tuberculoid (TT), Borderline tuberculoid (BT), Borderline borderline (BB), Borderline lepromatous (BL), subpolar lepromatous (LLs) and polar lepromatous (LLp). This type of classification is widely used for research purpose.

Immune responses following infection with M. leprae are complex, being partly cellular and partly humoral in nature (Harboe, 1985). Cellular immune response involves the generation of various subsets of T lymphocytes with various functions. Some induce resistance, whereas a too intensive recruitment of T suppressor cells may inhibit the development of immunity and may thus be an important element in the development of multibacillary forms of the disease (Modline et al., 1986).

Different spectra of the disease manifest different immunological features. Patients with BT and TT leprosy express strong levels of cell-mediated immunity and have the capability of restricting the growth of M. leprae (Myrvang et al., 1973b; Bloom and Godal, 1983; Ottenhoff et al., 1989). Immunologically borderline borderline (BB) patients are characterized by varying degrees of cellular immunity towards M. leprae but most of all by marked tendency to develop delayed type hypersensitivity (DTH) reactions against M. leprae or antigens released from M. leprae containing cells (Bullok, 1978). Borderline lepromatous (BL) leprosy patients express high humoral immune response and low cellular immune response (Bjune et al., 1976). Contrary to the tuberculoid form of the disease, patients with lepromatous (LL) leprosy express a strong humoral immune response and specific unresponsiveness in lymphocyte transformation and leukocyte migration inhibition tests (Gills and Godal, 1986; Ottenhoff et al., 1989; Converse et al., 1988; Haregewoin et al., 1983; Haregewoin et al.,1984). Evidences from in vitro and in vivo

studies have shown that unresponsiveness in lepromatous leprosy often results from a deficiency of interleukin-2 (IL-2) production (Haregewoin et al., 1983; Haregewoin et al., 1984). These evidences have indicated that the immunological deficiency in lepromatous leprosy is due to a defect in the T cell population.

Although several efforts have been made to study the nature of M. leprae and the disease it causes, it is still one of the few bacterial pathogens that have not been cultured in vitro (Watson and Booth, 1987). As a result, several investigations including techniques for early diagnosis as well as identification and characterization of antigenic determinants of this organism which are necessary to elicit a protective immune response in the infected host are severely hampered. However, a number of investigators have identified antigenic determinants from extract of M. leprae cells from human biopsies (Abe, 1970) or bacilli grown in experimentally infected armadillos and nude mouse (Britton et al., 1988, Closs et al., 1970; Ehrenberg and Gebre, 1987). Even after the development of experimentally infected armadillos as a means to propagate larger numbers of M. leprae cells (Kirchheimer et al., 1972; Store, 1971) the number of bacilli available for research purposes have been limited. Hence, most studies have been restricted to characterization of antigens by immunoelectrophoresis (Britton et al., 1988, Kronvall et al., 1976; Vega-Lopez et al., 1988) and SDS-polyacrylamide-gel-electrophoresis (Chakrabarty et al., 1982; Klaster et al., 1984). Immunodiffusion (Abe, 1970); and monoclonal antibodies (Sinha et al., 1983; Ivanyi et al., 1983)

have also been used to identify antigenic determinants of M. leprae.

The application of molecular biology to the study of M.leprae has recently been developed with preliminary studies on the nucleic acids of M. leprae, cloning and expression of a number of M. leprae genes.

One of the most powerful tools available to molecular biologists is the ability to take DNA from one type of cell and transfer it to another cell in which it can be replicated in limitless amounts as part of host cell's own replication machinery. This strategy has been used to construct the genomic libraries of M. leprae (Clark-Curtiss et al., 1985). Ideally, libraries would be constructed such that the host cells, e.g. Escherichia coli, contain segments of M. leprae DNA, and that the entire M. leprae genome is represented. Single E. coli cell which contains M. leprae gene of insert can be grown in large amounts, and the nucleic acid of that specific gene or the corresponding gene product can be isolated and analysed.

Preliminary experiments had indicated that M. leprae promoters (sequences of DNA which are responsible for controlling the expression of genes) do not function very efficiently in E. coli (Clark-Curtiss et al., 1985; Lamb and Colston, 1986). Thus, while the genes can be cloned they are not expressed (they do not produce the corresponding protein product). One way of overcoming this problem was to place the M. leprae gene under the control of the foreign promoter (Streptococcal promoter) which is known to work

efficiently in E. coli (Jacobs et al., 1986).

Young et al. (1985) used a somewhat different approach. They took small fragments of M. leprae DNA and inserted them into lac Z gene encoding for ß-galactosidase of Lambda gt-11 (λ gt-11), a bacteriophage vector that is capable of driving the expression of foreign insert DNA with E. coli transcription and translation signals (Figure 3). The ligated recombinant DNA is packaged into phage heads and this material is used to infect E. coli cells. The M. leprae DNA library constructed in this manner contains 2.5×10^6 individual recombinant phage (Young et al., 1985).

Thus, the development of recombinant DNA technology afforded a way to identify M. leprae genes by analysis of the gene products expressed in E. coli. The isolation and identification of immunologically relevant antigens have received greater attention because of their immunodiagnostic and immunoprophylactic applications. Using the Lambda gt-11 approach described above, Young et al. (1985) were able to detect the expression of five M. leprae proteins (65 kD, 36 kD, 28 kD, 18 kD and 12 kD), which were identified using monoclonal antibodies (in most cases, the M. leprae proteins are expressed as fusion with ß -galactosidase).

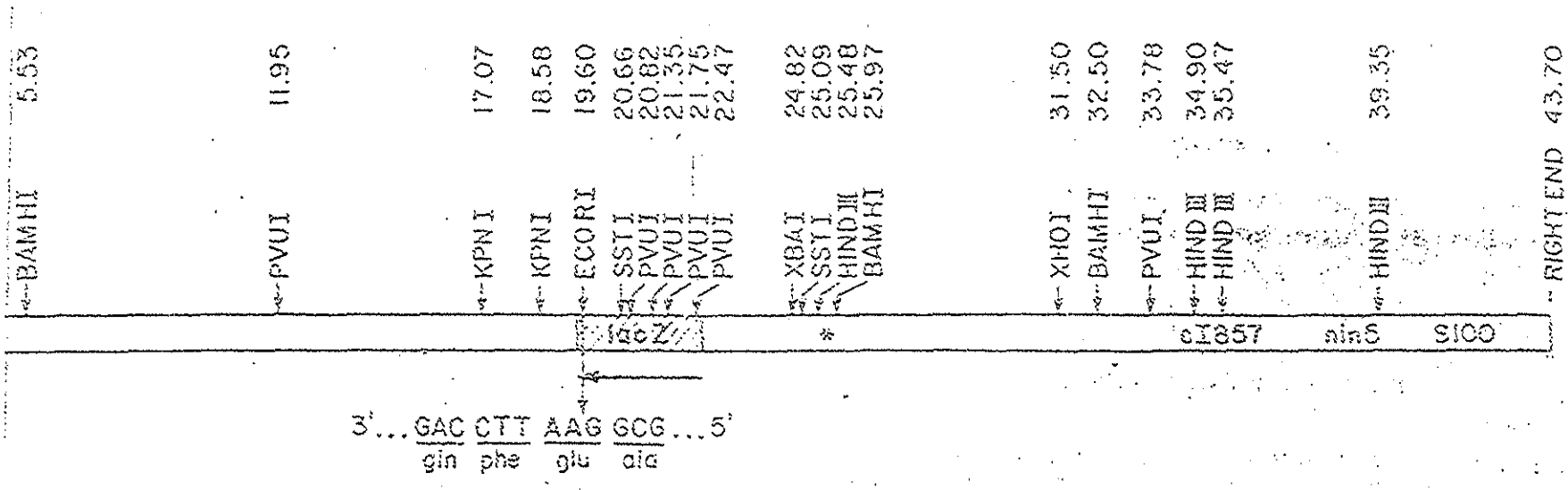


Figure 3: Features of λ gt-11. Restriction endonuclease cleavage sites are designated in Kb from the left & attachment site. The transcriptional orientation of Lac Z ( ) is given by the horizontal a The sequence of the unique E. coRI site (boldface letters), the nucleotides that immediately sur it, and the amino acids are shown below the phage map. Source: Young and Davis, 1983.

The cloning and expression of M. leprae DNA in E. coli has enabled the production of larger amounts of antigens of the uncultivable leprosy bacillus than had previously been conceivable (Young et al., 1985). The availability of antigens produced by recombinant DNA technology may make it possible to address some problems in leprosy that could not be approached by other means. In paucibacillary cases, it is often difficult to demonstrate the bacilli or their products and this posses diagnostic problems in the absence of classical clinical pathological features as in early leprosy. Thus, DNA technology can provide immense help in solving such problems as well as enabling to develop simple and specific seroepidemiological assays using recombinant protein antigens, in order to screen populations for individuals producing antibodies to M. leprae specific antigenic determinants. This would make early diagnosis of leprosy feasible, permitting early treatment to reduce its transmission and prevent the development of nerve damage and deformity.

Considerable and significant progress has been achieved in analysing the M. leprae 65 kD antigen which has been shown to be a highly conserved protein belonging to a family of heat-shock stress proteins and is present in several other organisms (Shinick et al., 1988; Vodkin and Williams, 1988). This protein has been found to be highly immunodominant for both T and B-cell responses and has been shown to have species and genus specific epitopes (Thole et al., 1987; Lamb et al., 1988). Another protein designated as 18 kD antigen has also been well characterized and T-

cell clones responsive to this antigen have been identified (Booth et al., 1988; Mustafa et al, 1986).

Clark-Curtiss and Docherty (1989) have screened DNA insert fragments selected from genomic libraries, as probes for M. leprae. One of these probes, PYA 1065, has been tried on biopsy material and can pick up about 4×10^3 M. leprae cells in a blot. Clark-Curtiss and Docherty (1989) have also used restriction fragment length polymorphism of selected genes/gene fragments of M. leprae to analyse isolates from different sources, and reported that the technique is promising to distinguish M. leprae from other mycobacteria.

Ribosomal RNA (rRNA) and rRNA genes of M. leprae along with other mycobacteria are being investigated (Estrada-Iris et al., 1986; Smida et al., 1988) and conserved as well as non-conserved sequences in the rRNA of M. leprae have been reported. Stepwise procedure for isolation of different types of RNA (including rRNA) and DNA has also been described (Katoch and Cox, 1986). This is a promising area for developing probes based on rRNA genes and rRNA.

There is also good hope for new methods which enable us to detect the presence of M. leprae in tissue with much greater degree of sensitivity than is currently possible. For example, a technique called the 'polymerase chain reaction' (PCR) involves the amplification of a single stretch of nucleic acid sequence to several thousand copies (Saiki et al., 1988), which can then be readily detected. Thus, in theory, it could be possible to detect the presence of a single bacillus as described by some

investigations (Hartskeerl et al., 1989; Williams et al, 1990).

Although significant progress has been achieved, still a lot more needs to be done, to reach the target of identification and production of immunologically relevant recombinant proteins which are useful for diagnostic purposes. Therefore, one of the options to reach this target is screening lambda gt-11 M. leprae with leprosy patients' sera an aspect on which not much has been done so far. Therefore, screening the library with sera from leprosy patients may permit the identification and production of large amounts of recombinant proteins which can be used from diagnostic purpose. Hence, the objectives of this study are,

a) To screen lambda gt-11 M. leprae expression library with leprosy patients'sera and identify clones expressing antigens recognized by antibodies.

b) To make lysogens of clones and produce larger amounts of recombinant antigens for analysis.

c) To characterize the lambda clones with regard to DNA insert and recombinant antigen.

MATERIALS AND METHODS

Recombinant DNA Library and Bacterial Strains:

Lambda gt-11 *M. leprae* genomic library that was made available to Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia, through a generous gift of R.A. Young (White Head Institute for Biomedical Research, Cambridge, MA) was used. Bacterial strains *Escherichia coli* Y1090 (pMC9)/*lac* U169, *pro* A+, *lon*, *ara* D139, *StrA*, *supF*, [trp C 22:: Tn10]) and *E. coli* Y1089 (pMC9)/*lac* U169, *ProA+*, *lon*, *ara* D139, *strA*, *hfl* A150, [chr:: Tn10]) originated from strains of Young and Davis (1983) and were obtained from Promega (Falkenberg, Sweden). *E.coli* Y1090 was used for propagation of lambda gt 11 while *E.coli* Y1089 was for lysogen.

Preparation of Laura Broth (LB)-agar medium

LB-medium (1% tryptone, 1% NaCl, 0.5% yeast extract, 0.15% agar [pH 7.5]) was dissolved in distilled water and autoclaved. For plating, 1.5% bacto-agar was added to LB medium before autoclaving. After autoclaving, the temperature of the medium was lowered to 55^{0}C in a water bath and 100mg/ml ampicillin to a final concentration of 0.04mg/ml in medium was added.The medium was then poured into 92mm petri dishes and allowed to solidify under sterile condition in the hood. The petri dishes were covered with plastfolie (GLAD, Dusseldorf, F.R. Germany) and stored at 4^{0}C.

Preparation of E. coli Y1090 and Y1089 for infection

The strains of *E. coli* were grown at 37^{0}C by streaking on LB-agar plates using standard techniques (Maniatis *et al.*, 1982) for single colony isolations. A single colony was transferred to 50ml

CHAPTER 2

MATERIALS AND METHODS

blue cap vial containing 10ml LB (pH 7.5) supplemented with 0.2% maltose and 10mM $MgSO_4$ and grown overnight at 37°C with shaking in an orbital incubator (Gallenkamp,U.K) (Young et al., 1985). The cells were spun down for 20 minutes at 2000 revolutions per minute (rpm) in TJ-6 rotor (Beckman, Switzerland). The cells were resuspended in 4ml 10mM $MgSO_4$ after the supernatant was discarded.

Patients' Sera:

Sera from patients were kindly provided by ALERT/AHRI Serum Bank, Addis Ababa, Ethiopia. The patients were classified by using the standard clinical and histopathologic criteria. The different sera, that is, from 11 lepromatous (LL), 11 borderline lepromatous (BL), 1 borderline borderline (BB), 13 borderline tuberculoid (BT), 1 polar tuberculoid (TT) leprosy patients and 3 healthy contacts (HC) were prepared and designated LL, BL, BB, BT, TT and HC, respectively.

Sera from one lepromatous, one borderline tuberculoid leprosy patients and one healthy contact were separately prepared in the following manner to remove anti-E.coli antibodies prior to screening lambda gt-11 M. leprae library. That is, E. coli Y1090 was infected with lambda gt-11 which was diluted approximately to 10^4 plaque forming units (pfu) per plate in autoclaved sample medium (SM) which was made up of 5.8g NaCl, 2g $MgSO_4.7H_2O$, 50ml 1M Tris/HCl(pH 7.5) and 5ml of 2% gelatin in a liter of distilled water. Lambda gt-11 was adsorbed to the bacterial cells by incubation at 37°C for 15 minutes. The infected cells were mixed with 0.8% agarose (Pharmacia; Uppsala, Sweden) dissolved in LB

solution (top agarose) and poured on LB-agar plates. The plates were incubated at 42°C for 10 hours until confluent lysis was formed. 82.5 mm diameter disks of nitrocellulose (NC) filters (Bio-Rad laboratories, Richmond, CA), were laid on the top of plates and incubated at 37°C for 1 hour on one side and 1/2 hour on the reverse side. The filters were then removed from the plates and blocked overnight with 5% OA/TBS/N_3 (5% ovalbumin, Tris buffer saline [50mM Tris/HCl pH 8.0, 150mM NaCl], 0.01% NaN_3). For this purpose 20 NC-filters each for borderline tuberculoid and, a healthy contact serum, 10 NC- filters for lepromatous serum were used. 100µl of each serum was diluted in 4ml 5% OA/TBS/N_3 and each filter was soaked with respective serum for 2-3 hours at room temperature with gentle shaking. The sera were then collected and stored at 4°C to be used when required for screening.

The remaining sera were absorbed with ß -galactosidase (Sigma Chemical Co., U.S.A.) and *E. coli* Y1089 by incubating overnight at 4°C with gentle shaking. The sera were then diluted in 5% OA/TBS/N_3 and used for screening the recombinant proteins.

Screening lambda gt-11 *M. leprae* Library with sera:

A single colony of *E. coli* Y1090 was grown for infection as described previously. The screening of the library was made partly using the methods described by Young *et al*. (1985). The lambda gt-11 *M. leprae* was diluted approximately to 10^4 plaque forming units (pfu) per plate which were a total of 5 x 10^4 pfu for lepromatous serum and 6 x 10^4 pfu for each borderline tuberculoid and healthy contact serum in SM. The cells and phages were mixed and incubated

at 37°C for 15 minutes to facilitate adsorption of the phage to cells. The infected cells were mixed with 3.5ml of 0.8% agarose and poured on LB-agar plates which were prepared as described previously. The plates were incubated at 42°C for 3.5 hours. When tiny plaques appeared, NC-filters that had been soaked in 10mM IPTG (Isopropyl-ß-D-thiogalactopyranoside)(Sigma Chemical CO, U.S.A) and subjected to 37°C for 15 minutes to dry were laid on the individual plates. The plates were incubated with the filters at 37°C for 4 hours. After the filters together with agar were marked using a needle, the filters were removed from the top of the agar and blocked overnight with 5% OA/TBS/N_3 with gentle shaking at room temperature, while the plates were stored at 4°C.

The next day, the filters were incubated with either lepromatous or borderline tuberculoid or healthy contact serum from which anti-E. coli antibodies were removed (i.e., after dilution(1:200) in 5% OA/TBS/N_3). The incubation took place at room temperature with gentle shaking for 4 hours. The filters were then washed 4 times in TBS/N_3 (50mM Tris/HCl pH 8.0], 150mM NaCl, 0.01% NaN_3) and incubated for one hour with Goat gamma chain specific affinity-purified antibodies to Human Immunoglobulin G (IgG), conjugated to alkaline phosphatase (Calbiochem, LaJolla, CA) diluted to 1:4000 in 5% OA/TBS/N_3; and then washed 5 times in TBS/N_3. Positive reactions were detected on the filters after addition of alkaline phosphatase substrate (i.e.,33µl NBT(nitroblue tetrazolium) and 16.5µl BCIP (4-bromo-5-chloro-3-indolyl phosphate) (Promega, U.S.A.) which were mixed in 5ml alkaline phosphatase

buffer (100mM Tris/HCl, 100mM NaCl, 5mM $MgCl_2$)(pH 9.5). The corresponding plaques giving positive reactions on NC-filters were picked from the original plates with end cut tips of pasteur pipette and suspended in 500µl SM supplemented with 30µl Chloroform. The clones were purified by successive screening using lower titers of phage. Purified clones were plated on the media containing the chromogenic indicator, X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside [Sigma Chemical Co, U.S.A.]) and IPTG to confirm the fusion of foreign DNA segment with ß-galactosidase. Then, the clones were amplified by following the standard methods (Maniatis et al., 1982). Briefly, E. coli Y1090 was infected, plated on LB-agar plates and incubated at 42°C for 8-10 hours. The lawns were overlaid with 5ml SM and incubated at room temperature for an additional two hours with gentle shaking. The lambda phage solution was removed from each plate of respective clone into sterile glass vials and stored at 4°C after 0.3 ml chloroform was added. The titers of amplified clones were determined experimentally by infecting E. coli Y1090.

PREPARATION AND ANALYSIS OF PROTEINS

A. Generation of Lambda gt-11 Recombinant lysogen in E.coli Y1089

E. coli Y1089 was prepared for infection as described previously (Maniatis et al., 1982); and lysogens were essentially generated by standard methods (Huynh et al, 1985). Briefly, 200µl of bacterial suspension was diluted to 2ml in LB-solution containing 10mM $MgSO_4$. Lambda gt-11 recombinant phage was also

diluted in LB-solution and 50µl of diluted bacterial suspension was infected with lambda gt-11 recombinant phage at a multiplicity of five to ten by incubation at room temperature for 15 minutes to facilitate the adsorption of phage to bacterial cells. The culture was plated out at a density of approximately 200 colonies per plate and incubated overnight at 30°C. The colonies were then replica-plated on two LB-agar plates from the original plates using Repliplates (FMC, Bioproducts, California, U.S.A.). The first plate was incubated at 42°C and the second at 30°C. Colonies that grew at 30°C but not at 42°C were suspected to be lysogens. The colonies were picked with sterile tooth pick and streaked on other LB-agar plates. They were allowed to grow at 30°C. Finally, to be sure whether the picked colonies were real lysogens, a single colony was picked into 10 ml LB-medium (solution) and allowed to grow overnight at 30°C in a shaker. The bacterial culture was induced at 42°C for 2 hours. By adding 0.2ml chloroform it was spun at 2000 rpm for 20 minutes in a TJ-6 rotor (Beckman, Switzerland). Then, 50µl of the supernatant was used to infect 50 µl of E. coli Y1090 (incubated at 42°C) to see generation of plaques confirming that the colony growing at 30°C, but not at 42°C, is a lysogen.

B. Preparation of fusion protein from lysogen:

The techniques used for the preparation of fusion proteins were adapted from Huynh et al. (1985). A single colony of the E.coli Y1089 lysogen was put into 100ml LB-medium (pH7.5) and incubated at 30°C with good aeration. When the culture grew to an optical

density of 0.5 at 600 nm, the temperature was increased at 42°C - 45°C as rapidly as possible. The culture was incubated at the elevated temperature for 20 minutes with good aeration. IPTG was then added to a concentration of 10mM and the culture was incubated at 37°C - 38°C for approximately 1 hour. Then the cells were harvested as rapidly as possible by spinning at 5000 rpm for 10 minutes at room temperature. The cells were rapidly resuspended in 1/25 of the original culture volume in TEP buffer (100mM Tris/HCl (pH 7.4], 10mM EDTA(ethylenediaminetetra acetate)(pH8.0), 1mM Phenylmethylsulfonylfluoride [PMSF]). The suspended cells were frozen at -70°C and stored at the same temperature. The bacteria were then thawed and sonicated for 5 - 10 seconds at 100 watt to lyse the cells and reduce the viscosity. The sonicated extract was spun at 10,000rpm for 10 minutes and 3 volumes of saturated $(NH_4)_2SO_4$ solution was added to each volume of extract, mixed and chilled at 4°C for 60 minutes. The solution was again spun down at 10,000 rpm for 20 minutes and the pellet was resuspended in TEP after pouring off the supernatant.

C. Purification of fusion proteins:

Samples of fusion proteins were diluted in TBSN (50mM Tris/HCl [pH 7.3], 150 mM NaCl, 0.1% nonidet P-40 [Sigma Chemical, Co. U.S.A.). They were loaded onto the anti-ß -galactosidase, protosorb lac Z immunoaffinity columns (Promega, U.S.A.) after the columns were equilibrated in cold TBSN, and allowed to flow through the columns. The columns were washed several times with TBSN and the proteins were eluted by 0.1M $NaHCO_3$ /Na_2CO_3 (pH 10.8)

followed by TBS. The recombinant proteins were collected in 1ml eppendorff tubes and 200µl of 1M Tris/HCl (pH 7.4) was added to lower the pH.

The concentration of recombinant proteins was determined using bicinchonic acid (BCA) (Pierce, U.S.A.) protein assay reagents with BSA (bovine serum albumin) as standard. The optical density was determined at 570nm using Titertek Multiskan (Flow, U.K.) ELISA (enzyme linked immunosorbent assay) reader.

D. Analysis of fusion proteins by SDS-PAGE and Western blotting:

8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described earlier by Laemmli (1970). The separating gel was prepared by mixing 13.3ml 30% Acrylamide/ 0.8% Bisacrylamide, 6.25 ml of 3M Tris (pH 8.8), 0.5ml of 10% sodium dodecyl sulfate (Bio-Rad Richmond, CA), 0.25ml of 10% ammonium persulphate(APS) and 0.025 ml of N,N,N'N'tetramethylethylenediamine (TEMED)(Sigma Chemical Co., U.S.A.) in 30.75 ml distilled water. 4% stacking gel was also made by mixing 3.75ml 30% acrylamide, 3.75ml of 1M Tris pH6.8, 0.3ml of 10% SDS, 0.2ml of 10%APS and 0.02ml TEMED in 22.5ml distilled water. The samples of recombinant proteins were mixed with sample buffer(1x= 2ml 10% SDS, 1ml glycerol,0.1ml 1% Bromophenol blue,0.63ml 1M Tris(pH 6.8) in 6.27ml distilled water) and 5% ß-mercaptoethanol. ß-galactosidase (Sigma) and high molecular weight markers (α-macroglobuline, ß-galactosidase, Fructose-6-phosphate kinase, pyruvate kinase) were also prepared and loaded to the gel

with the samples and run in 1x electrode buffer (10 x 30.3g Tris base, 144.2g glycine and 10g SDS in a liter of distilled water) using (Bio-Rad, U.K.) power supply at 150 volt. The gels were stained with coomassie brilliant blue (Merk, F.R. Germany) or the proteins were blotted to nitrocellulose membrane type BA.83, 0.2µm pore size, (Schleicher and Schuell,F.R.Germany) at 0.2 Amp for 2 hours in 1x blotting buffer (57.6g glycine, and 12.2g Tris base dissolved in distilled H_2O and supplemented with methanol) (Towbin et al., 1979). The filter was blocked overnight with 5%OA/TBS/N_3 by gentle shaking; incubated with 2.3mg/ml anti-ß-galactosidase (Mouse MAb, Promega, U.S.A.) diluted to 1:500 in 1% OA/TBS/N_3 and washed 4 times with TBS. The NC blot was probed with 1mg/ml anti-mouse IgG conjugated to alkaline phosphatase (Promega, U.S.A.) diluted to 1:5000 in 1% OA/TBS/N_3 followed by washing 5 times with TBS. The bands appeared after adding alkaline phosphatase substrate (NBT and BCIP mixed in alkaline phosphatase buffer [pH 9.5]).

Screening Recombinant Antigens with different patient sera:

0.5µg of recombinant antigens were dropped in array of "squares" on NC-filter disks and blocked overnight with 5% OA/TBS/N_3 with slight shaking. The filters were incubated for 4 hours with sera from lepromatous, borderline lepromatous, borderline borderline, borderline tuberculoid, tuberculoid leprosy patients and healthy contacts diluted 1:100 (which were adsorbed to ß - galactosidase and E. coli Y1089 by incubating them overnight at 4°C with gentle shaking). The filters were washed 4 times with TBS

and incubated for one hour with Goat-anti-Human IgG alkaline phosphatase conjugated. After washing 5 times with the same buffer the alkaline phosphatase substrate (NBT and BCIP) was added to visualize the development of signals.

Analysis of insert size of recombinant DNA:

Lambda gt-11 M. leprae recombinant clones and lambda gt-11 vector (phage) were independently plated on X-gal containing medium at high dilution following standard techniques (Maniatis et al., 1982). Amplification of DNA with Thermus aquaticus polymerase chain reaction (Taq PCR) was basically performed as described by Saiki et al. (1988). Well isolated plaques were selected at random and picked with the end cut of pasteur pipette into 100 µl reaction mixtures (50mM KCl, 10mM Tris(pH8.4), 2.5mM $MgCl_2$, each primer (24 base Lambda gt-11 forward and reverse) at 1mM, each dNTP (dATP, dCTP, dTTP, dGTP) (Promega Kit, U.S.A.) at 1mM, gelatin at 200 µg/ml) and boiled for 5 minutes. Four units of 4500 units/ml Taq polymerase (Promega Kit, U.S.A) was added and the samples were overlaid with 50µl heavy mineral oil (Sigma Chemical Co., U.S.A.) then subjected to 30 cycles of amplification (i.e., 95°C, 30 seconds: for denaturation; 55°C, 30 seconds for annealing and 63° C, 2 minutes for synthesis of new strand).

The amplified products of PCR were analyzed with agarose gel electrophoresis by adding 10µl of each reaction products and molecular standard (500µg/ml lambda DNA-Hind-III digest; Pharmacia, U.S.A.) to individual well of 1.5% agarose after mixing with 2µl of DNA gel loading buffer (0.25% bromophenol blue, 0.25%

xylene-cyanol, 40% W/V sucrose H_2O). The whole preparation was electrophoresed at 150 volt for 30 minutes in 1x TBE (0.089M Tris base, 0.089M boric acid, 0.002M EDTA) using (Bio-Rad, U.K.) power supply. The gel was stained with a fluorescent dye, ethidium bromide, and examined by ultraviolet transillumination.

CHAPTER 3

RESULTS

RESULTS

Screening Lambda gt-11 M. leprae library with sera:

In the primary screening of Lambda-gt-11 M. leprae library 11, 6 and 2 plaques were identified as positive clones with lepromatous, borderline tuberculoid leprosy patients and the healthy contact sera, respectively. Figure 4 depicts a typical NC filter screened with the absorbed lepromatous patient serum. In this test, it is appropriate to note that the probability of picking non-recombinant clones which could be recognized by plating on the medium containing X-gal exists. One such clone which produced only blue plaques was isolated. Furthermore, during successive screenings several of the initial clones may also be lost. As a result of such technical imperfections, out of 29 initial clones, only 10 individualized clones, that is, 6 clones of lepromatous, 3 clones of borderline tuberculoid patients and 1 clone of a healthy contact sera remained after successive screenings. These clones are denoted as LL_2, LL_3, LL_4, LL_7, LL_{10}, LL_{11}, BT_{13}, BT_{15}, BT_{17} and HC_{12} according to the serum used for screening the library.

The signal strength developed by different clones and the rate at which the signals appeared on NC-filters varied among clones identified with the help of different sera. However, this variation did not specifically exclude those clones isolated by the same serum. Clones isolated with lepromatous serum showed stronger signals and faster signal appearance, while those isolated with

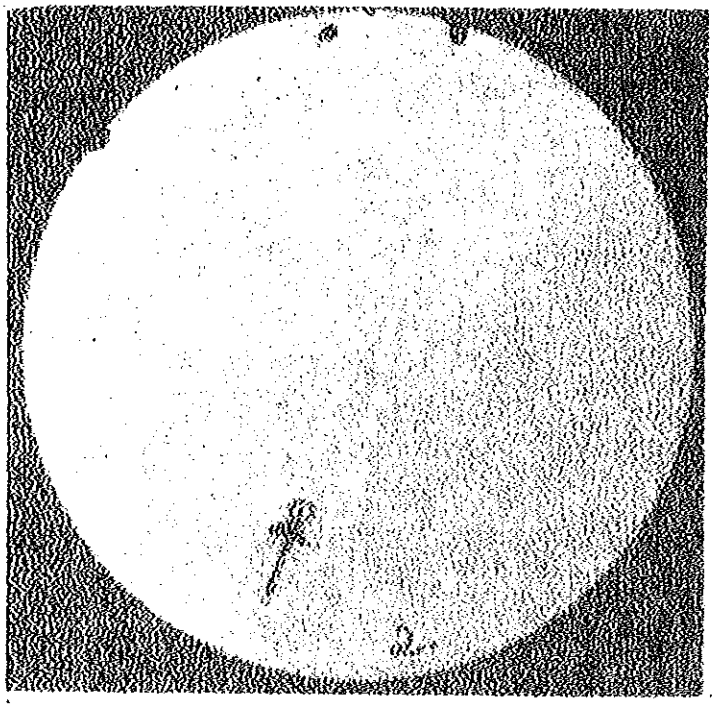


Figure 4: A representative nitrocellulose filter demonstrating the low background and good signal on screening the Lambda gt-11 *M. leprae* library with LL serum. Arrow indicates the clone LL_2 detected in that screening.

healthy contact serum showed the weakest signals and slower appearance.

In probing these clones with normal human serum, LL_{11} , BT_{13} , BT_{15} and BT_{17} showed a slight reactivity, while the rest (i.e., LL_2 , LL_3 , LL_4 , $LL_{\ldots}$, LL_{10} and HC_{18}) showed no reactivity. It was also found that none of these clones did react with goat anti-human-IgG conjugated to alkaline phosphatase.

Analysis of recombinant proteins by Coomassie staining and Western blotting:

Six lysogens (four from clones isolated by lepromatous and two from clones isolated by borderline tuberculoid patients sera) were generated. The frequency with which lysogens were produced was very low, although previous reports had shown that the generation of lysogen can be at a frequency between 10% and 70% (Huynh et al., 1985). As a result, the amount of phage used to infect E. coli Y1089 was increased from 5 times to 10 times to improve the frequency of the production of lysogen per clone. Even then, it was not possible to generate lysogens from four clones, although, it was tried several times.

The fusion proteins produced from the lysogens were analysed by SDS-PAGE followed by Coomassie blue staining (Figures 5 and 6). The figures clearly show the presence of high molecular weight fusion proteins of $(NH_4)_2SO_4$ precipitates and protosorb lac Z, immunoaffinity adsorbent purified respectively. The molecular sizes of $(NH_4)_2SO_4$ precipitates and purified proteins varied from

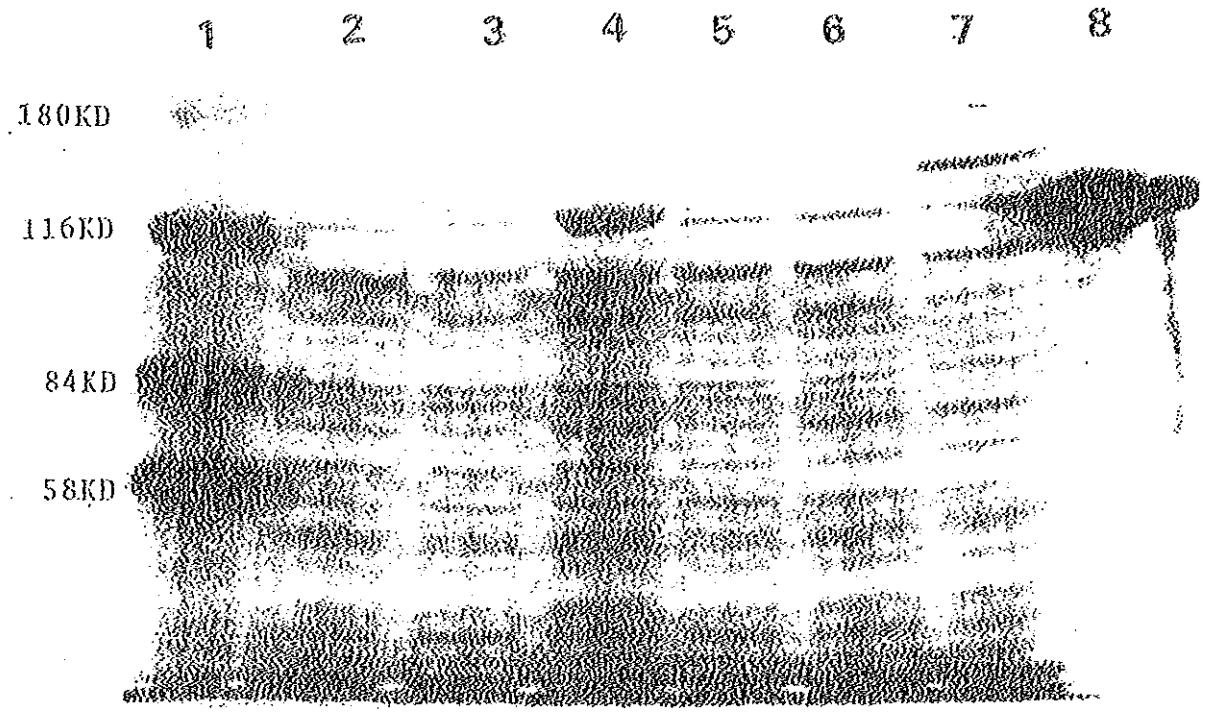


Figure 5: SDS-PAGE analysis of fusion proteins (i.e., ammonium-sulfate precipitates) followed by Coomassie brilliant blue stain. Lane 1, molecular Wt markers (α-macroglobulin(180KD), ß -galactosidase(116KD), Fructose-6-phosphate kinase(84KD) and pyruvate kinase(58KD)); Lane 2 to 7 clones, BT_{15} , BT_{13} , LL_{10} , LL_7 , LL_3 and LL_7 respectively; and Lane 8, ß -galactosidase.

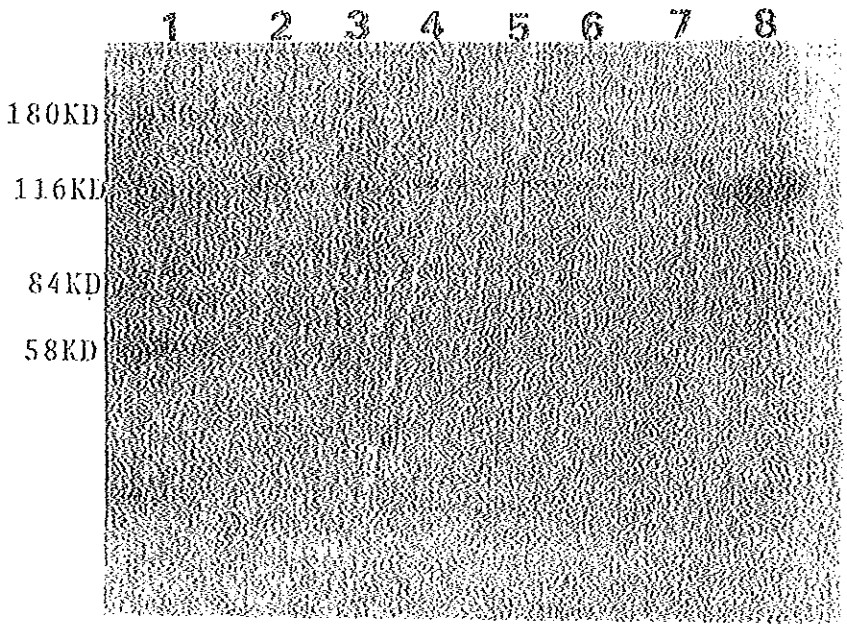


Figure 6: Lac Z immunoaffinity adsorbent purified recombinant proteins resolved on SDS-PAGE followed by Coomassie brilliant blue stain. Lane 1, molecular Wt markers; Lane 2 to 7 clones, BT_{15} , BT_{13} , LL_{16} , LL_{7} , LL_{3} and LL_{2} , respectively; and Lane 8, ß - galactosidase.

116 kD to 144 kD(kilodalton). It was evident that different lysogens generated from different clones produced varying amounts of proteins, although it was difficult to recognise their differences as the difference between the migrations of their bands could not be visualized by the resolution of the bands. Another distinct point was the formation of several bands with $(NH_4)_2SO_4$ precipitate fusion proteins (Fig. 5) as compared to immunoaffinity purified proteins (Fig. 6).

Western blot analysis confirmed the results of the molecular sizes of the fusion proteins determined by the SDS-PAGE analysis (Figure 7).

Analysis of Recombinant proteins with patients' sera:

The reactivity of recombinant antigens with different leprosy patients' sera was heterogenous (Table 2). This reactivity did not depend on the molecular weights of the recombinant proteins. The recombinant antigens generated from clones isolated by lepromatous serum strongly reacted with different leprosy patients' sera throughout the spectrum of the disease while those recombinant antigens generated from clones isolated by borderline tuberculoid serum weakly reacted with these sera. Differences in the reactivity was judged from the intensity of the signals on the NC-filters. In this experiment the recombinant antigens did not react with normal human sera (Table 2 and Figure 8).

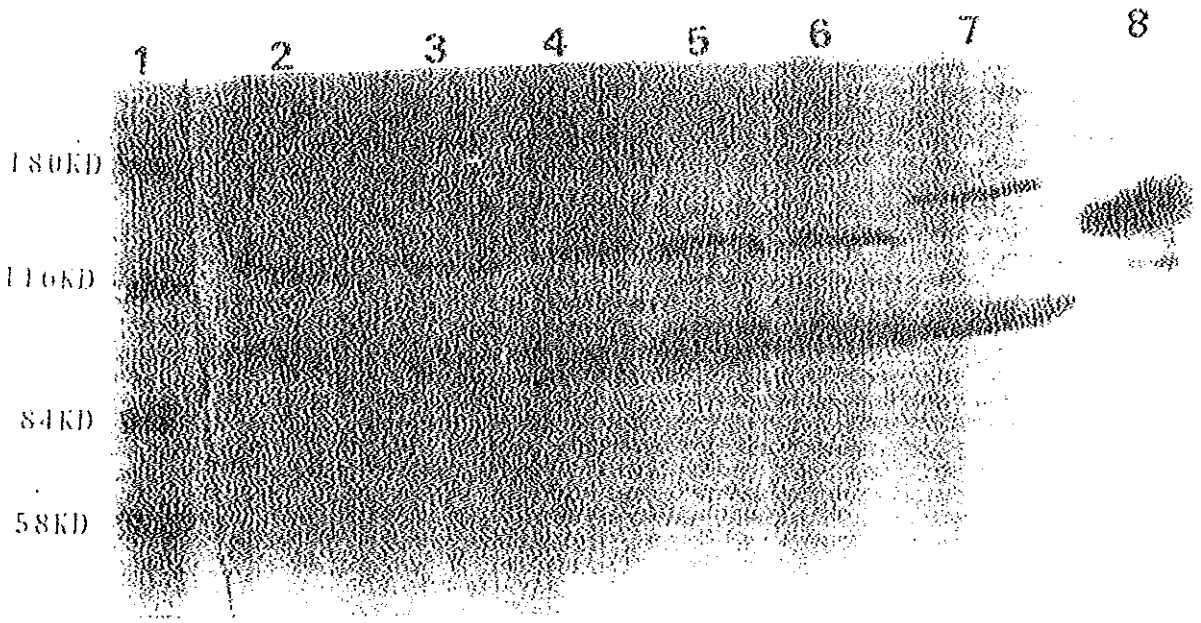


Figure 7: Western blot analysis of fusion proteins (i.e., ammonium sulfate precipitates). Crude lysate fractionated on 8% polyacrylamide gel, transferred to nitrocellulose membrane and probed with anti-β-galactosidase (mouse MAb). Lane 1 , Molecular weight markers;Lane 2 to 7 contain lysates of clones BT_{15},BT_{13},LL_{10},LL_{7},LL_{3}, and LL_{2} , respectively; and lane 8, β-galactosidase.

TABLE 2

Reactivity of recombinant antigens with sera

		RECOMBINANT ANTIGENS					
SERUM	Serum No.	LL_2	LL_3	LL_7	LL_{10}	BT_{13}	BT_{15}
LL	627/90	+++	++	+++	+++	+/-	+
	355/89	+++	-	+/-	+/-	+/-	-
	374/89	+++	+/-	+	++	+/-	+/-
	375/89	+++	+	++	++	+/-	+
	460/90	++	++	++	++	+	+
	701/90	+++	+	++	++	+	+
	403/90	++	+	+	+++	+/-	-
	391/90	++	++	++	+++	+/-	+/-
	373/90	++	+/-	+/-	+/-	+/-	+/-
	337/90	++	++	++	++	+	+
	739/90	+++	+++	++	+++	+	+
BL	326/89	+/-	+	+	++	+	+
	324/89	+	+/-	++	++	+/-	+
	342/89	+++	++	++	+	+	+
	410/89	++	+/-	+	+/-	+/-	+/-
	564/90	++	+	++	+/-	-	+
	498/90	+++	++	+	+	+	+
	631/90	+++	+	-	-	+	-
	574/90	+	+	+	++	+	+
	560/90	++	+	+	++	+	+
	410/90	++	+/-	+/-	+/-	+/-	+
	437/90	+	+	+	+/-	+/-	+
BB	867/90	++	+	++	++	+/-	+/-
BT	709/90	+	+	+++	+++	+/-	+
	771/90	++	++	++	+++	+	+
	764/90	+/-	+/-	+	+/-	+	+
	608/90	+	+	+	++	+	+
	360/90	++	+	++	++	+	+
	360/90	+	+/-	+	++	+	+
	675/90	++	++	++	++	+/-	+
	604/90	+	+/-	++	++	+	+
	517/90	+	-	-	+	-	+
	542/90	+	+	+	+	+	+
	398/90	+/-	+/-	+/-	+/-	-	-
	533/90	+	+	+	+	+	+
	1993/89	++	++	++	+	++	++

Table 2 cont.

SERUM	SERUM NO.	LL_2	LL_3	LL_7	LL_{10}	BT_{13}	BT_{15}
TT	963/88	+++	+++	+++	+++	+	++
HC	842/89	+/-	-	+/-	+/-	-	+/-
	894/89	+	-	+	+	+/-	+/-
	1189/89	+	-	+	+	+/-	+
NHS	383	-	-	-	-	-	-
	476	-	-	-	-	-	-
	54	-	-	-	-	-	-
	596	-	-	-	-	-	-
	614	-	-	-	-	-	-
	382	-	-	-	-	-	-

Key: The reactivity of individual serum with recombinant proteins (Antigens) is indicated as (+++) strong reaction, (++) intermediate reaction,(+) very weak reaction,(+/-) indeterminate,(-) negative reaction. LL,lepromatous leprosy serum; BL, Borderline lepromatous leprosy serum; BB, Borderline borderline leprosy serum;BT, Borderline tuberculoid leprosy serum; TT, Tuberculoid leprosy serum; HC, healthy contact serum; NHS, normal human serum.

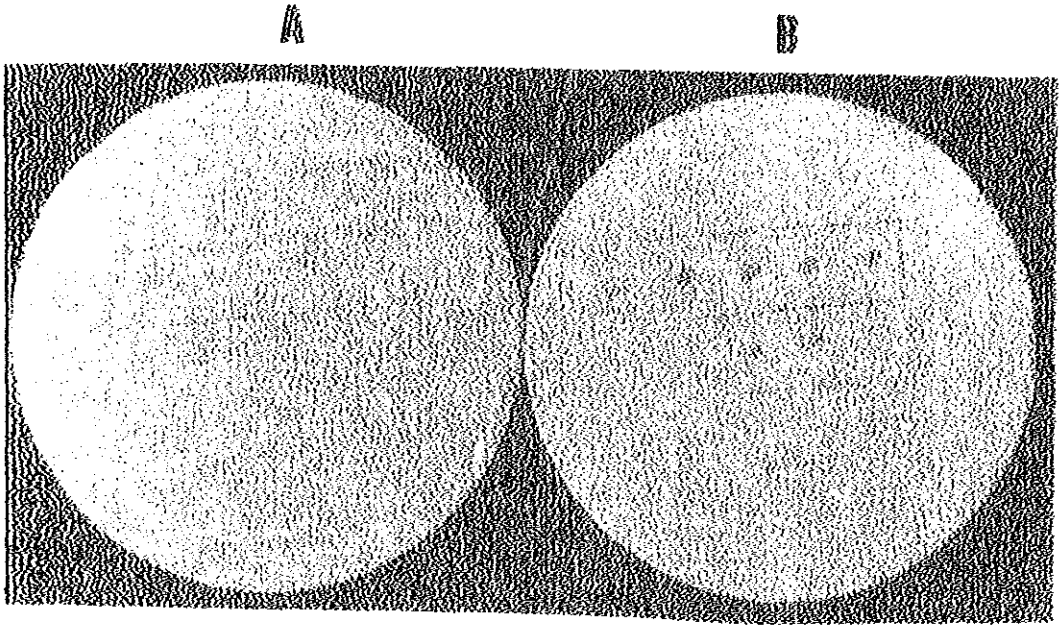


Figure 8: Representative nitrocellulose filters showing the reactivity of normal human serum (A) and lepromatous leprosy patient serum (B) with recombinant antigens. The first row represents recombinant antigens designated LL_2, LL_3, LL_9, LL_{16} and the second row represents BT_{13} and BT_{15} (i.e. from left to the right). The "square" in the third row represents B-galactosidase used as a negative control.

DNA analysis

DNA of the clones were amplified by Taq polymerase chain reaction. The different DNA insert sizes were estimated from the lambda gt-11 Hind-III digest molecular standard (Figure 9A and B) and varied from 0.3 kb to 2.0 kb (kilobase pairs), approximately. These values also included the DNA insert sizes of clones from which the generation of lysogens was difficult. Variation of the sizes of insert DNA among different clones appeared to indicate that different clones contained different insert sizes. Although some clones seem to have equal DNA insert size (Figure 9B lane 2 and 3). On the otherhand, in some, the amplification was not apparently evidenced on agarose gel electrophoresis (Fig. 9 B, lane 4).

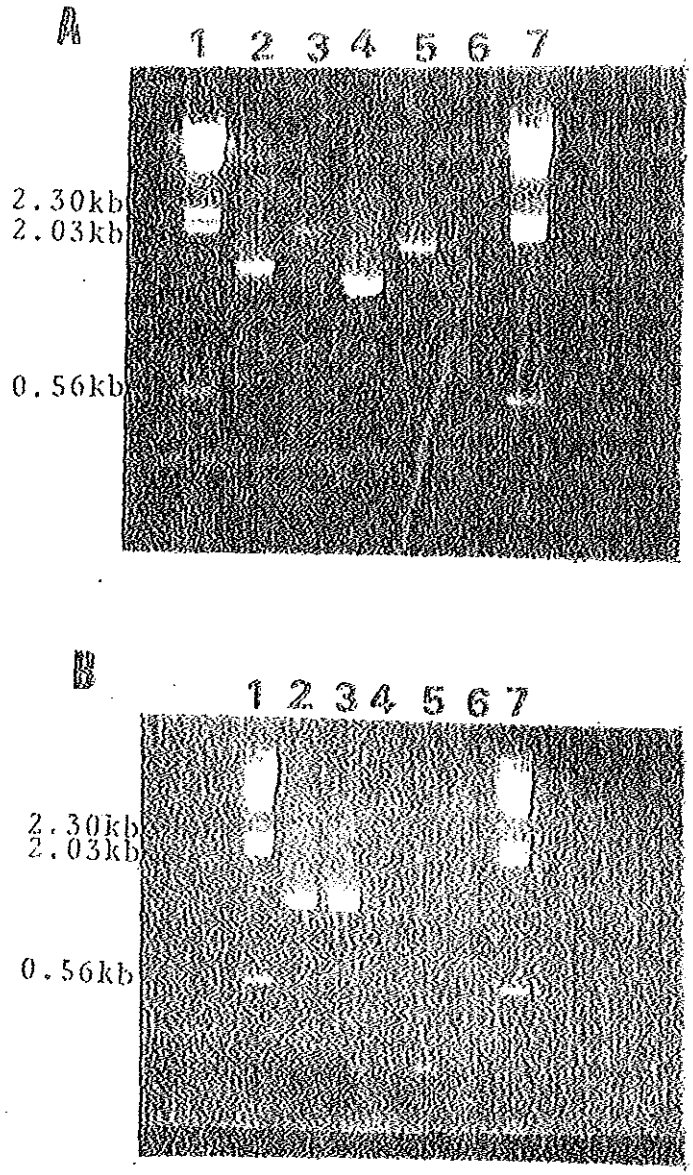
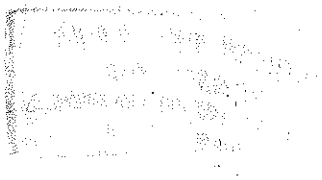


Figure 9: Insert sizes of base pairs (bp) of DNA which were amplified from the respective clones by Taq DNA polymerase chain reaction and resolved on agarose gel. A.Lane 1 and 7 molecular standard (lambda DNA Hind-III digest); lane$_2$, LL$_2$ (1.5 kb); Lane$_3$, LL$_3$ (2.0kb); Lane$_4$, BC$_{18}$ (1.4 kb); Lane$_5$, LL$_{10}$ (1.8 kb); and lane $_6$, λ gt-11. B. lane 1 and 7 molecular standard (lambda DNA Hind-III digest); Lane$_2$, LL$_4$ (1.2 kb); Lane$_3$, LL$_7$, (1.2kb); lane$_4$, LL$_{11}$ ( ); Lane$_5$, BT$_{17}$ (0.29 kb; and Lane$_6$, λ-gt-11.

CHAPTER 4

DISCUSSION



DISCUSSION

Recombinant DNA expression technology offers a strategy to thoroughly and systematically examine the antigens encoded in linear segments of a pathogen genome when antibodies are available for use as probes. The use of an efficient screening method permits a survey of antigens expressed by recombinant phages. This way individual recombinant clones that produce the antigen of interest can be isolated and subjected to further study.

In this study, recombinant clones of M. leprae were identified and characterized by screening M. leprae recombinant DNA expression library with sera of lepromatous, borderline tuberculoid leprosy patients and healthy contact whereas previously the identification and characterization of recombinant clones was predominantly based on those gene products that react with murine monoclonal antibodies (Young et al., 1985: Engers et al., 1985; Booth et al., 1988).

In screening the lambda gt 11 M.leprae library with sera from leprosy patients and healthy contact, this study has shown that it is extremely important to remove anti-E. coli antibodies from sera used for screening the library so as to minimize the background which masks the positive signals and to avoid non-specific bindings of antibodies. The necessity of such absorption of sera was also strongly emphasized by Sathish et al. (1990).

The loss of many of the clones in successive screenings encountered in this study was probably due either to the fact that many of the positive clones were comparatively slow growers, leading to an outgrowth of the recombinants or the result of

picking the wrong plaques which were found nearby the clones whose signals were expressed on the NC-filters. Similar observation was previously reported by Bramness (1989), while screening lambda gt-11 M. leprae library with rabbit antibodies.

Plating the phages (clones) on a medium containing 5-bromo-4-chloro-3-indolyl ß-D-galactoside (chromogenic indicator) seems to be necessary as an indicator to ascertain whether the picked clones contain insert or not. This method enables to distinguish the miss picks from the true positive clones, since the clones containing the insert DNA fused with ß-galactosidase fail to produce blue plaque on medium containing chromogenic indicator (Young and Davis, 1983).

As compared to the screens by lepromatous serum, only fewer clones could be detected from screens of lambda gt-11 M. leprae expressing library by borderline tuberculoid sera in this study and in that of previous works (Sathish et al., 1990). Although Hartskeerl et al. (1990) detected quite a number of clones with pooled sera from household contacts of leprosy patients by screening plasmid M. leprae libraries, only one clone could be detected in this study by screening lambda gt-11 M.leprae library with serum from a healthy contact. This discrepancy could be due to the presence of lower amounts of antibodies in borderline tuberculoid and healthy contact sera produced against M. leprae. The observed differences among signals produced by different clones could also be due to differences in the concentration of specific antibodies.

BT_{12} and BT_{13}) was also very low. This might be due to the failure of the recombinant phages to infect or to be incorporated into the chromosome of E. coli Y1089. However, following generation of lysogens, large quantities of recombinant proteins can be produced for further analysis. However, in view of a recent report that showed the possibility of isolating antigens expressed as fusion proteins and non-fusion proteins, without making lysogen (Sathish et al., 1990), the difficult task of lysogen generation may not be necessary in the future.

Analysis of the molecular sizes of proteins (produced from lysogens) by SDS-PAGE followed either by coomassie staining or western blotting revealed the fusion of foreign protein with ß - galactosidase. In the present study the formation of several bands by $(NH_4)_2SO_4$ precipitated fusion proteins as compared to the bands produced by immunoaffinity purified proteins most likely depicts the presence of E. coli proteins mixed with recombinant proteins in the crude lysate.

The recombinant proteins screened with different leprosy patients' sera were recognized by sera from different patients with great variability and levels of reactivity. The heterogenous reactivity encountered in this study has strong correlation with previous studies of western blot analysis of M. leprae antigens purified from an experimentally infected armadillo (Ehrenberg and Gebre, 1987) and the fusion proteins obtained from positively reacting Lambda gt-11 M. leprae clones (Sathish et al., 1990). The strong reactivity of recombinant proteins generated from clones

isolated by lepromatous leprosy serum with serum from tuberculoid leprosy patient was also observed in the reports of Sathish et al. (1990). Differences in reactivity could be due either to the fact that different patient sera may contain different amounts of antibodies which are more specific to some recombinant antigens of interest or the presence of secondary or tertiary structures of the recombinant antigens (Huynh et al., 1985 and Sathish et al., 1990).

In this study the identification of M. leprae antigenic determinants that may play a role at the early stage of infection was described. Since these contacts have lived in close association with leprosy patients for long periods they have a relatively higher risk of getting infected with M. leprae. Therefore, it could be assumed that the antibodies present in the observed sera are the products of a humoral response to M. leprae. However, the possibility cannot be ruled out about a portion of the antibodies being due to cross-reactive determinants with specific responses to other mycobacteria. The fact that the contacts used in this study have not yet developed leprosy, and yet possess antibodies may suggest that they have some level of immunity to the disease or are in an early stage of disease.

The DNA insert size analysis made by amplifying DNA from clones using Taq polymerase ranged from 0.3 kb to 2 kb. The insert sizes of the amplified DNAs are sufficient to include the entire coding segments. However, in some clones, DNA insert segment (fragments) were not detected after agarose gel electrophoresis, which might be due to either the absence or loss of insert

fragments in the process of handling the clones. Similar results were also reported in other studies (Sathish et al., 1990).

The analysis of the response of tuberculoid leprosy patients' peripheral blood T cells to the recombinant antigens is in progress. Preliminary results indicate that several of these antigens do elicit significant T cell proliferative responses (data not shown).

CONCLUSIONS

Screening Lambda gt-11 *M. leprae* library with sera from leprosy patients is useful to identify clones expressing immunologically reactive proteins. The generation of recombinant proteins from the isolated clones is also valuable for testing the reactivity of several leprosy patients' sera. Though, the potential diagnostic value of these recombinant antigens is promising, its confirmation needs further analysis including their specificity to react with leprosy patients' sera.

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CHAPTER 5

CONCLUSION

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