厚生労働科学研究費補助金

（新型インフルエンザ等新興・再興感染症研究事業）

ハンセン病診療のネットワーク構築

平成２３年度　分担研究報告書

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研究要旨　日本におけるハンセン病診療が、スムーズに行われるようにネットワーク構築を目指した。24 名の皮膚科医にハンセン病の講習会・実習(皮膚スメア検査、末梢神経検査、病理組織検査など)を行い、ハンセン病患者・回復者の診療体制を構築した。さらに、ハンセン病 2 型らい反応(ENL)に対するサリドマイド診療ガイドラインを作成し、サリドマイドの適正使用法を構築した。

A. 研究目的

日本におけるハンセン病診療がスムーズに行われるようにネットワークの構築を目指す。

B. 研究方法

ハンセン病診療に欠けている要素を抽出し、それらを補う資料や情報を提供し、講習会などを開催する。また、ハンセン病の新規患者については、実際に診療方法、鑑別方法、検査方法を指導し、主治医がハンセン病を理解し、自ら診療可能になるようにした。患者情報については入手しないため、倫理上の問題はない。

C. 研究結果

ハンセン病患者の減少のため、皮膚科医が診療する機会が殆どない。そのため 30 名に対してハンセン病講習会を実施した(参加者:皮膚科医 24 名、内科医 2 名、薬剤師 1 名、保健師 1 名、回

復者 2 名)。ハンセン病の知識、回復者の心情、皮膚スメア検査実習、末梢神経検査、病理組織検査などを実施し、知識・技術の伝達を行った。ハンセン病回復者から、医療面や生活面などの体験や要望をお聞きした。ハンセン病診療の座右の書として作成した「ハンセン病アトラス 診断のための指針」も配布し、当事者の他、医局員や若い皮膚科医の教育に活用することとした。

ハンセン病回復者は、過去の偏見・差別の歴史から、なかなか一般医療機関に受診する勇気がない。一般医療機関受診のチャンスを広げるため、研究分担者が厚労省疾病対策課と共同で作成した「ハンセン病療養所退所者等ハンドブック」をひき続き回復者等に配付することとした。ハンドブックにはハンセン病の再発と皮膚病に気軽に対応できる皮膚科医の一覧表を掲載した。

2011 年には 5 名の新規ハンセン病患者がいた。5名の新規患者については主治医に対して、実際の検査の実技指導、治療の指導を行い、ハンセン病を確実に診療できる体制を確立した。

顧みられない熱帯病(neglected tropical diseases)としてハンセン病と共に挙げられているブルーリ潰瘍についても検討・研究を行った。すなわち、両者は末梢神経症状を呈すること、潰瘍をおこすこと、熱帯地域に多いことなどから極めて類似した所見を示すので鑑別が困難である。日本においてもブルーリ潰瘍が現在まで 32 例報告されており、ハンセン病との鑑別に困難をきたし、両疾患の異同が問題になっている。両疾患の臨床的、菌学的な検討を行ない、臨床現場で鑑別がスムーズにできるように症例検討を行った。さらに感染源やベクターの調査のため福島県の現地での環境調査も実施した。

D. 考察

ハンセン病患者が減少し、診療する機会が減少し、教育を受けていない、一度も診療機会がない皮膚科医が大多数を占めるようになっている。またハンセン病とブルーリ潰瘍の鑑別ができない場合もおきている。また、ハンセン病の偏見・差別の歴史や、ハンセン病回復者の心情なども理解できていない。それらを解決するために、講習会を開催し、意識向上に努めた。皮膚科医は知識吸収の意欲はあり、講習会には 30 名の皮膚科医が参集した。講習会を実りあるものにするためにハンセン病回復者の方、2 名にも参加いただき、彼らの現状などについて講演いただいた。今後も講習会を通じて学習意欲を持続させるために、年に一回程度の継続した教育機会を設けることが必要である。

ハンセン病回復者を一般医療機関に受診させる(インテグレーション)事は難しいが、一歩でもそれに近づける努力は必

要である。そのため、気軽に相談できる皮膚科医名簿を公開し、ハンセン病療養所退所者等ハンドブックを配布した。これらの皮膚科医を起点として他の診療科などに受診できることを期待したい。また、ハンセン病回復者などから生の声を聞いて、患者と医師とのあるべき関係を構築することも大事である。

ハンセン病の新規患者は減少しているが、外国人患者については鑑別にハンセン病が入っているので、診断に迷うことは多くないようである。一方、日本人患者については、ハンセン病とブルーリ潰瘍や他の皮膚病との鑑別は難しく、診断が遅れる場合がある。数年に1名程度は日本人新規患者も登録されることがあり、必ず鑑別に「ハンセン病」を入れることが必要である。2011 年は 5 名の新規患者が登録されたが、フィリピン人と、ブラジル人とインドネシア人が各 1 名、沖縄県出身日本人が 2 名であった。

E. 結論

ハンセン病診療を皮膚科医が主体的に実施するためのネットワーク作りは、まだ始まったばかりであるが、皮膚科医の教育、ハンセン病回復者の一般医療機関への受診の動きを、引き続き行うことが重要である。

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H. 知的財産権の出願・登録状況

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

Chapter 3

Microbiology and Experimental Leprosy

Masanori Matsuoka

3.1 INTRODUCTION

Hansen's disease was revealed to be an infectious disease by *Mycobacterium leprae* in 1873 by Gerhard Henrik Armauer Hansen of Norway. He reported in a Norwegian medical journal that "Spedalskhedens Åarsager" or rod-shaped bodies, were present in unstained samples prepared from leproma (Pallmary, 1955). Today, the infectious disease caused by *M. leprae* is termed Hansen's disease on account of the discoverer. Microbiological research on *M. leprae* has been hampered considerably by the unfeasibility of cultivation *in vitro*. Nevertheless, based on achievements such as the development of animal models (Shepard, 1960; Kirchheimer, 1971; Kohsaka, 1976) and whole genome sequencing (Cole *et al*, 2001), new findings have been accumulated fast, especially in terms of genetic diagnoses of drug resistance, in genotyping and its application to epidemiological analyses, and microbiological characteristics —once presumed impossible.

3.2 CLASSIFICATION

According to "Bergey's Manual of Systematic bacteriology," *M. leprae* is described in Section 6 as a species of the genus *Mycobacterium* in the Mycobacteria family Mycobacteriacae. *M. leprae* is closely related to *Mycobacterium tuberculosis*.

3.3 LOCALIZATION

M. leprae is found numerously in skin leporma, nasal mucosa, visceral organs, peripheral nerve trunks, and bone marrow in lepromatous leprosy case. Sometimes more than 10^9 of the bacteria exist per gram of a nodule. The bacteria are also observed in skin lesion in the borderline type of leprosy, although they are detected only slightly in the tuberculoid type (Riley & Jopling, 1966). The amount of bacilli in the lesion is called the bacterial index (B.I.) which is used as a criterion for the classification of disease types and for monitoring the therapeutic effects. Bacilli also exists other than patients. Animals of naturally acquired case identified in nine-banded armadillos (Walsh *et al*, 1975), mangabey monkeys (Meyers *et al*, 1985), and chimpanzees (Donham & Leininger, 1977) also harbor the bacilli. Taking these finding into consideration, leprosy is regarded as a zoonosis. Detecting *M. leprae* specific DNA by PCR on nasal mucosa of most residents in areas, where Hansen's disease is prevalent, has revealed contentious contamination by bacteria and suggesting the existence of *M. leprae* in the natural environment (van Beers *et al*, 1994; Matsuoka *et al*, 1999).

A new *Mycobacterium* species, which could not be able to grow artificial media, was discovered from the patients with Lucio phenomenon. As to 20 genes and some pseudo genes showed an overall 90.9% match with *M. leprae*. The isolate is termed *M. lepromatosis* (Han *et al*, 2008, Han *et al*, 2009). Pathogenicity of this bacteria for diffuse lepromatous leprosy should be studied further.

3.4 MICROBIOLOGICAL CHARACTERISTICS

3.4.1 Morphology and structure

M. leprae is rod-shaped bacterium with round ends. of 1-8 μm length and 0.3 μm in diameter (Fig. 3.1). It produces no spores, nor does it have flagella. The bacterial cells' outermost layer has a pseudocapsule structure mainly consisted of lipids phthiocerol dimycocerosate (PDMI) and phenolic glycolipid-I (PGL-I). The bacillus is gram-positive

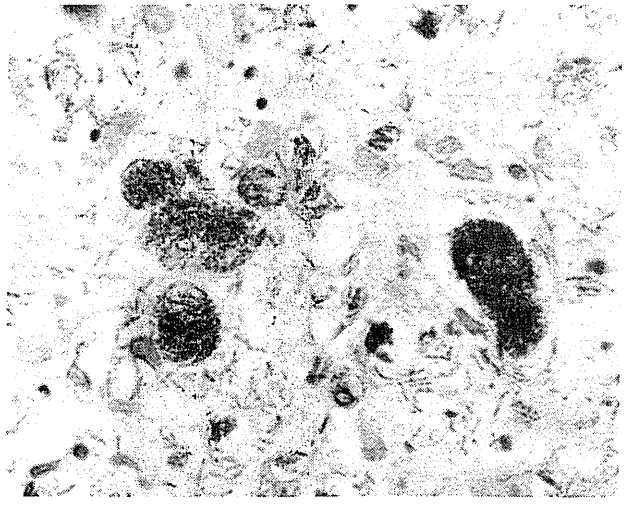

Figure 3.1 *M. leprae* in the leproma of LL patient. (Photo by Matsuoka M)

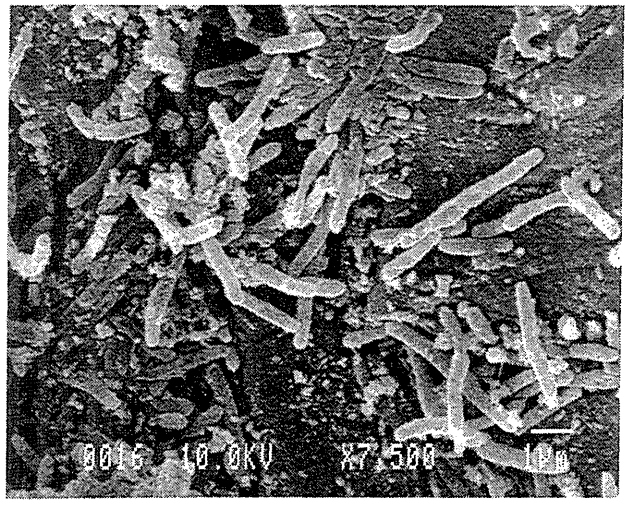

Figure 3.2 Scanning electron microscopic feature of *M. leprae*. (Photo by Amakao K)

and is stained uniformly red by the Ziehl-Neelsen method. Such bacteria are called solid bacteria; some, designated as non-solid, are granular, segmented, short rods, or are extremely elongated. Non-solid bacteria are regarded as those that have lost viability. Ratio of non-solid bacteria is presumed to be increased when the bacilli are inactivated by therapeutic effects. The ratio of the two forms of the bacteria, solid vs non-solid, is designated as the morphological index (M.I.). When treatment effects are achieved, B.I. decreases by about one per year, whereas M.I. shows more rapid change; for that reason, M.I. is sometimes used as an indicator for monitoring therapeutic effects.

M. leprae proliferates in histiocyte. It is arranged in nodes to resemble palisades or cigars; further proliferation results in the formation of globular bacterial masses once called "globi" (Fig.3.2). Neuro-tropism of the bacilli is one of the characteristics and this character is account for neuropathy in leprosy.

Electron microscopic observation shows an electron-transparent layer around the bacterial surface (Takeda *et al*, 2003) (Fig.3.3 and 3.4). This part is presumed to be related to the capsule like structure mainly consisted of PDMI and PGL-I (See Figures in chapter 4). The carbohydrate end of PGL-I is specific structure to *M. leprae*, it is used in serodiagnosis as an antigen of antibody reaction specific to *M. leprae* (Izumi *et al*, 1990). It also has been shown that PGL-I might be involved in the neurotropism of *M. leprae* by first binding to laminin 2 of the Schwann cell and then to the nerve via its receptor dystroglycan (Ng *et al*, 2000).

On the bacteria surface, "paired fibrils" and a "band structure" are observed similarly to other acid-fast bacilli. Many "paired fibrils" are present in the longitudinal direction of the cell. One to several "band structures" circularly surround the cell. A peptidoglycan layer exists on the outer periphery of the cytoplasmic membrane. Its outer layer consists of arabinogalactan and mycolic acid, constituting the 22-nm-thick cell wall. Lipoarabinomannan (LAM) sticks out of the cell membrane with phosphatidylinositol mannoside (PIM) as an anchor. The cell membrane comprises a phospholipid bilayer and various proteins, similarly to other acid-fast bacilli. It has two major membrane-bound proteins: 35-kDa (MMP-I) and 22 kDa (MMP-II).

M. leprae possesses a dopa (3,4-dehydroxyphenylalanine) oxidase that is not found in other acid-fast bacilli, which is useful for identification of the bacteria. Furthermore, *M. leprae* has superoxide dismutase activity, although its *katG* gene is shown to be a pseudogene (Nakata *et al*, 1997). Consequently, it has no catalase activity and the bacilli are resistant to INH.

3.4.2 Genetics

The *M. leprae* genome size is 3.3 Mb; it is smaller than the 4.4 Mb of *M. tuberculosis* (Cole *et al*, 2001). The *M. leprae* genome includes 1614 genes and 1133 pseudogenes, although the *M. tuberculosis* genome contains only 6 pseudogenes. The existence of numerous pseudo genes is supposed as the reason that *M. leprae* has not been grown in any artificial media. Polymerase Chain Reaction (PCR) for *M. leprae* was exploited based on the sequencing data (Woods & Cole 1989; Plikaytis *et al*, 1994) and PCR is applied widely for leprosy study such

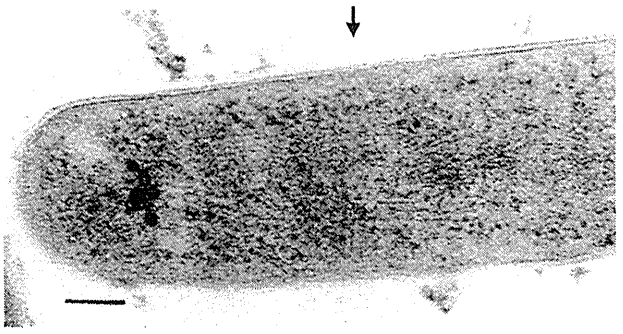

Figure. 3.3 *M. leprae* observed by transparent electron microscope. Arrow indicates electron-transparent layer. Scale bar is 100nm. (Reprint of Takeda A *et al*, 2003)

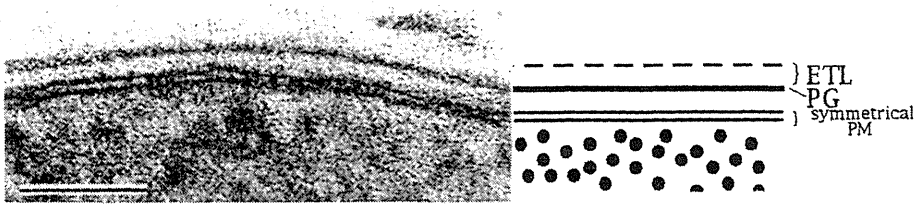

Figure 3.4 Structure of cell wall. Scale bar is 50nm. ETL; Electron-transparent layer, PG; Peptidoglycan, PM; Plasma membrane. (Reprint of Takeda A *et al*, 2003)

as molecular epidemiology (Cole *et al*, 2001, Matsuoka, 2009), drug resistance, (Williams & Gillis, 2004; Matsuoka *et al*, 2007), and specific detection of DNA from clinical samples and ancient skeletal (van Beers *et al*, 1994; Hass *et al*, 2000).

3.4.3 Growth

Although many reports have described *in vitro* culture of *M. leprae*, its cultivation has never been confirmed. Currently, *M. leprae* for laboratory use are obtained from the bacilli inoculated to the susceptible animals and grown (Kirchheimer *et al*, 1971; Kohsaka *et al*, 1976). The bacteria grown in large quantities in armadillos are utilized for purification and analyses of cell components of the bacilli. *M. leprae* grown in nude mice are useful for the experiments as for genetic analysis which could be done by small amount of bacilli.

The generation time of *M. leprae* in the mouse footpad in the logarithmic growth phase is 12-13 days (Shepard & MacRae, 1965) or 11.1±1.92 days (Levy, 1976).

Preferability of lower temperatures for the growth of *M. leprae* is known based on the findings that the favorite sites for lesions in humans are parts of lower temperature such as skin and nasal mucosa, and infection experiments in animals: in the *M. leprae* growth study in the mouse footpad done by Shepard, the growth of the bacteria was most fast when the room temperature was 20°C and the footpad temperature was 30°C. At room temperature of 10 or 30°C (footpad temperature of 25 or 36°C, respectively) bacterial growth was delayed. The relation between the site of *M. leprae* growth and the body temperature in nude mice also showed that sites at lower temperatures such as the footpad, the tail, the eyelid, and the auricle are suitable for the growth of *M. leprae* (Kohsaka *et al*, 1978). In addition, ATP contents and PGL-synthesis are lower at 37°C than at 33°C; the ability for oxidative degradation of palmitic acid, as measured using the Buddemeyer method, is highest at 31-32°C (Fukutomi *et al*, 2004).

3.4.4 Stability of viability

Viability of *M. leprae* out of the body is examined using the mouse footpad method. Results showed survival for 9 days in a dried condition, at 24-33°C; 5 months in the dark at 28-44% humidity; 60 days in saline left at room temperature or in the Hanks' solution in a refrigerator; and 7 days with exposure to sunlight for 3 hours per day (Desikan *et al*, 1995). Additionally, the bacteria survived for 7 days in nasal discharge that had been discharged from a patient and dried.

The bacteria retained infectivity to mice after storage for 12 months in broth (Bacto TB Broth; Difco Laboratories) containing 7.5% dimethyl sulfoxide in liquid nitrogen (−196°C). There was no decrease in viability for 2 weeks in Hanks' solution containing 0.1% bovine serum albumin (0°C). The decreased viability was reduced further through addition of 10% glycerin. In each case, slow freezing and rapid thawing were necessary for better maintenance of viability. *M. leprae* that had been suspended in 10% skim milk and lyophilized were able to proliferate in nude mice even after 4 years.

Nakamura's method for decontamination and

purification using 0.05% trypsin and 1% NaOH is highly effective while with viability loss of *M. leprae* is low (Nakamura, 1994). The bacterial suspension prepared by Nakmura's method and suspended in Hanks' Balance Salt Solution containing 10% glycerin followed by keeping at −84°C retained proliferative capacity in the nude mouse footpad for more than 10 years.

M. leprae is inactivated partially after one hour and completely after two hours by chlorhexidine treatment. Infectivity of the bacilli to mice was lost with single dose of 600, 900 and 1,200 mg rifampicin (Levy *et al*, 1976).

Viability or proportion of viable *M. leprae* is determined by proliferation in the mouse footpad, oxidizing palmitic acid with the release CO_2 measured using the Buddemeyer method (Franzblau, 1988), chemoluminescence measurement of the ATP amount by luciferin/luciferase reaction, and the FDA/EB fluorescent staining method. Application of RT-PCR is also used in some cases (Phenitsuksri *et al*, 2006; Martinez *et al*, 2009). Reverse transcription PCR followed by Real time PCR targeting 16s rRNA showed decline in viability during the course of multidrug therapy (Martinez *et al*, 2009).

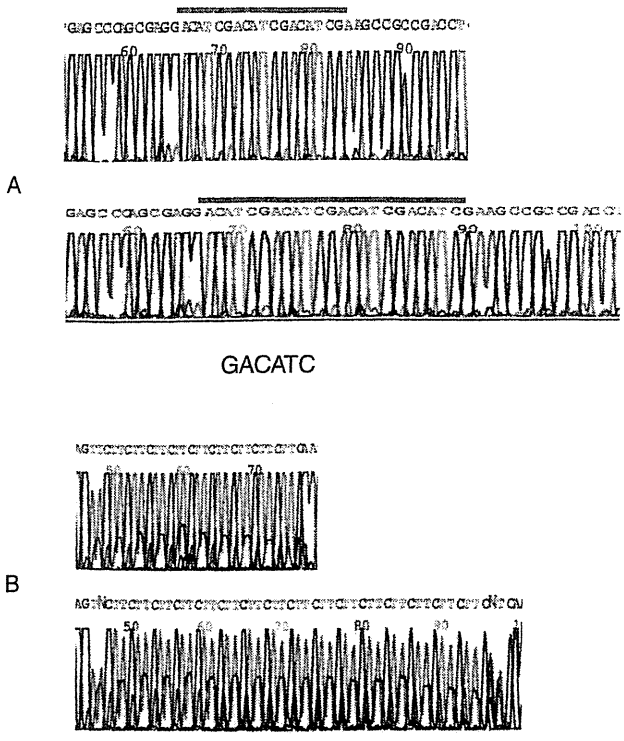

Figure 3.5 Polymorphism of variable number tandem repeats. A; Tandem repeat of 6 bp minisatellite in the *rpoT* gene, B; Tandem repeat of TTC microsatellite. (Figure by Matsuoka M)

3.5 GENETICS AND APPLICATION FOR MOLECULAR EPIDEMIOLOGY

3.5.1 Genome and application

The *M. leprae* genome size is 3.3 Mbp; it is smaller than the 4.4 Mbp of *M. tuberculosis* (Cole *et al*, 2001). The *M. leprae* genome includes 1614 genes and 1133 pseudogenes, although the *M. tuberculosis* genome contains only 6 pseudogenes. The existence of numerous pseudogenes is supposed as the reason that *M. leprae* has not been grown in any artificial media. PCR for *M. leprae* was exploited based on the sequencing data (Woods & Cole 1989; Plikaytis *et al*, 1994) and PCR is applied widely for leprosy study such as molecular epidemiology (Cole *et al*, 2001, Matsuoka 2009), drug resistance (Williams & Gillis 2004; Matsuoka *et al*, 2007), and specific detection of DNA from clinical samples and ancient skeletal (van Beers *et al*, 1994; Hass *et al*, 2000). Comparative genome analysis identified *M. leprae* specific peptides or proteins and some of them specifically react with T cell derived from leprosy patients. Those peptides are potentially eligible for developing rapid diagnostic test for the early detection of *M. leprae* infection (Spencer *et al*, 2005; Geluk *et al*, 2009).

3.5.2 Genomic polymorphism

Typing of isolates is the essential tool for epidemiological analysis as to trace infection route of infectious diseases. However, regarding the phenotypic diversity among *M. leprae* strains, only the difference in the mouse footpad growth manner is known. According to Shepard, growth in the mouse footpad differs by strains of *M. leprae*, i.e., some strains, termed fast strain, reach the higher plateau level sooner, on the contrary, some strains that reach the lower plateau level later with longer generation time. This characteristic is apparently not related to the disease type of the patient from whom the bacteria were derived, and did not change after passage in mice (Shepard & MacRea, 1971).

Genetic polymorphism of *M. leprae* was considered to be extremely limited. Isolates originated in various sources showed no polymorphism by restriction fragment length polymorphism (RFLP), which has been applied for epidemiological analysis of tuberculosis. In fact, RFLP shows no distinguishable polymorphism among *M. leprae* strains from various areas in the world, or from humans, armadillos, and mangabey monkeys (Williams,

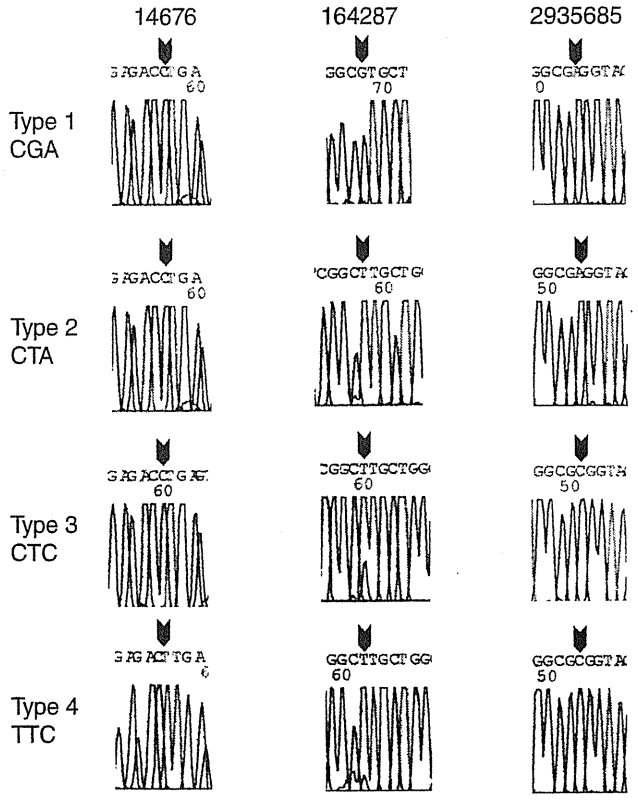

Figure 3.6 Single nucleotide polymorphism. SNP type 1 reveals GCA, SNP type 2 reveals CTA , SNP type 3 reveals CT (Picture by Matsuoka M)C, and SNP type 4 reveals TTC at the position 1467, 1642875, and 2935683 respectively. (Figure by Matsuoka M)

1990). Furthermore, no differences between isolated strains were found in the spacer regions between 16S and 23S ribosomal RNA genes (de Wit *et al*, 1994).

3.5.3 Variable number tandem repeats

Genetic identification first became possible by the discovery that strains of *M. leprae* having either 3 or 4 tandem repeats of 6 nucleotides in the *rpoT* gene (Fig.3.5). Each of the two genotypes shows a characteristic geographical distribution: strains with four repeats is predominance in eastern Asia including Honshu area of Japan; whereas almost all strains had three repeats in the other areas of the world including Okinawa (Matsuoka *et al*, 2000). Genotype of *M. leprae* in Latin America showed that most strains in Mexico had four repeats, indicating that the origin of the disease in Mexico was different from other Latin American countries examined (Matsuoka *et al*, 2005). The global geographical distribution of the respective *rpoT* genotypes in *M. leprae* is presumed to be formed by the prehistoric migration of humankind (Matsuoka *et al*, 2005).

Variable copy number of tandem repeat of three nucleotides TTC was detected in non-coding region of the *M. leprae* (Shin *et al*, 2000) (Fig. 3.5). Isolates from the Philippines showed variable repeats ranging from 10 to 37. Using this variable number of tandem repeats (VNTRS), genotype of *M. leprae* from nasal mucosa of residents and patients at the high prevalent area in Indonesia were compared. Results showed family cases in which different *M. leprae* genotypes were detected from patients and their families who resided together. In addition, patients with different TTC genotype strain were found in multifamily cases (Matsuoka *et al*, 2004). This result disagreed with the conventional conception that a heavy house hold contact with multibacillary patient is the mode of infection and suggests existence of infectious source other than patient. This supposition agreed well with idea derived from seroepidemiology (Abe *et al*, 1990). Existence of many microsatellites of up to five nucleotides or mini-satellites of more nucleotides, which is useful for genotyping was shown by in silico analysis (Groathouse *et al*, 2004). Their application is useful in the elucidation of the infection mode of Hansen's disease based on more detailed genotyping and existence of infectious source other than patient is deduced (Zhang *et al*, 2004; Matsuoka, 2009).

3.5.4 Single nucleotide polymorphisms

M. leprae isolates were classified into 4 types singlenucleotide polymorphisms (SNPs) at position 14676, 1642875, and 2935685 in genomic DNA (Fig.3.6) (Monot *et al*, 2005). The frequency of single nucleotide polymorphisms (SNPs) in the *M. leprae* genome has been shown to be one per 28 kb, which is extremely low compared to other bacteria, suggesting that *M. leprae* distributed worldwide are derived from limited clones (Monot *et al*, 2005). Based on geographic distribution of each SNPS type, type 1 to type 4, in globally different area, it is presumed that the disease originated in east Africa or the Indian subcontinent and spread worldwide with the movement of humankind. Genotyping by SNPs and polymorphism is suitable for the analysis of global transfer of leprosy (Matsuoka *et al*, 2006; Monot *et al*, 2009).

3.6 DRUG RESISTANCE

3.6.1 Drugs used in multidrug therapy and resistance

Promin was introduced into the treatment of

Hansen's disease in the 1940s. In 1953, a clinical case had already been reported in which resistance to dapsone was suspected. The resistance was subsequently proved in 1964 using the mouse footpad method. Currently, many cases of resistance have been reported for dapson, and rifampicin which are key components for the WHO multidrug therapy (MDT) (Maeda *et al*, 2001). Resistance to quinolone has also been reported in a few cases. Not only single drug resistance, multidrug resistance has been demonstrated (Cambau *et al*, 1997; Maeda *et al*, 2001; Matsuoka *et al*, 2003). Although three reports describe resistance to clofazimine; none has been confirmed by a replication study. No reports in the relevant literature have described resistance to minocycline.

3.6.2 Mouse footpad method

Drug susceptibility of *M. leprae* has been tested traditionally using the mouse footpad method since 1960s. Five thousand *M. leprae* were inoculated in the footpads of mice. The mice were given feed containing 0.01%, 0.001%, or 0.0001% dapsone; 0.01% rifampicin; 0.001% clofazimine; 0.15% ofloxacin; 0.02% sparfloxacin; or 0.08% minocycline. After 25-30 weeks, susceptibility was determined by the *M. leprae* growth in the footpads (Ji, 1987; Matsuoka *et al*, 2003).

3.6.3 Molecular biological method for detecting drug resistance

Drug resistance to dapsone, rifampicin, and quinolone was recently revealed to be occurred by amino acid substitution at each drug's target site (Williams & Gillis, 2004; Matsuoka *et al*, 2007). The genomic region that codes amino acids conferring resistance is designated as the drug resistance determining region (DRDR).

Dapsone exerts a bacteriostatic effect by competitive binding as the analog of p-aminobenzoic acid (PABA) to dihydropteroate synthase (DHPS) and thus inhibit the synthesis of folic acid. Single-nucleotide mutations in the triplets of the threonine at position 53 and of the proline at position 55 of the *folP1* gene coding DHPS result in amino acid substitutions, causing dapsone to be unable to bind to DHPS, leading to the acquisition of dapsune resistance (Fig.3.7 A) (Kai *et al*, 1999; Williams & Gillis, 2004).

Rifampicin inhibits mRNA transcription by binding to the beta subunit of RNA polymerase. A single-nucleotide mutation in DRDR of the *rpoB* gene coding the beta subunit causes resistance to

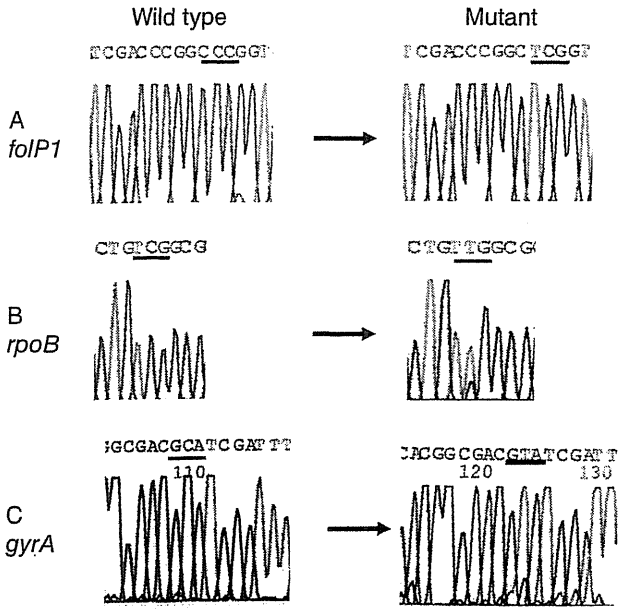

Figure 3.7 Mutation in the *folP1*, *rpoB*, and *gyrA* gene of *M. leprae* which is resistant to dapsone, rifampicin and quinolone. A; *folP1*: 55 (CCC: Pro → CTC: Leu), B; *rpoB*: 425 (Ser: TCG → TTG: Leu), C; gyrA: 91 (Ala: GCA → Val: GTA). (Figure by Matsuoka M)

rifampicin. About 70% of the rifampicin resistance of *M. leprae* is attributable to mutation at position 425 from TCG (Serine) to TTG (Leucine) (Fig. 3.7 B). Furthermore, amino acid substitutions at positions 407, 410, 420, 425 and 427 are involved in the acquisition of rifampicin resistance (Honoré & Cole, 1993; Williams & Gillis, 2004).

New quinolones inhibit DNA replication by binding to the A subunit of DNA gyrase. Amino acid, valine, substitutions at positions 91 in *gyrA* coding the A subunit have been reported for resistant strains (Cambau *et al*, 2001; Williams & Gillis, 2004) (Fig. 3.7 C). Mutations at 89, 92, and 95 are supposed to also confer quinolone resistance according to the analogy to quinolone resistance in *M. tuberculosis*.

Correlation between drug resistance and gene mutation in *M. leprae* has been examined in limited isolates because of the tedious procedure for the mouse footpad method, i.e., it has been examined in less than 180 strains including both susceptible and resistant strains. More data are necessary in the future to enhance the accuracy of the determination of resistance by the search for gene mutations, and to develop simple test methods.

3.6.4 Level of dug resistance

Relapse after the completion of MDT or after symptoms subsided has been observed. Resistant bacteria were detected at high percentages in re-

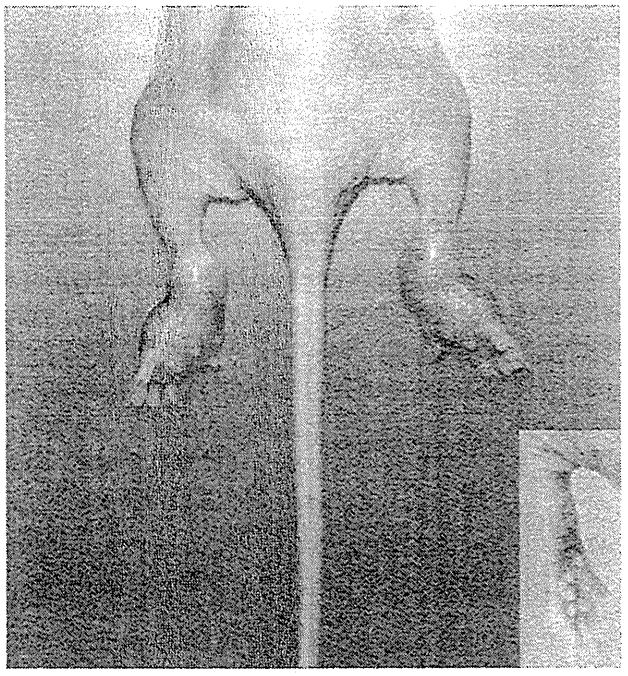

Figure 3.8 Nude mouse footpad swollen. The nude mouse was inoculated 10^7 bacilli into the footpad 11 months ago. Photo at right side is normal nude mouse footpad. (Photo by Matsuoka M)

rapsed or intractable cases in Japan (Maeda *et al*, 2001). On the other hand, follow-up for 10 years after completion of MDT in Cebu Island, Philippines showed recurrence in 10.28/100 patients/year, of which 1 of 15 cases was dapson resistant, whereas the other strains were susceptible to dapsone and rifampicin (Cellona *et al*, 2003). Comparative examination by genotypes of strains is necessary to determine whether these cases resulted from reinfection or relapse. The strain is designated as persistent, a persister, if these are caused by a susceptible strain remaining after therapy (Matsuoka *et al*, 2007). The mechanism of relapse by a persister is an important concern from the perspective of measuring Hansen's disease mainly based on chemotherapy in the future. In this context, global leprosy control of WHO launched out sentinel surveillance on 2008 to monitor the level of drug.

3.7 ANIMAL MODELS

Animal models of Hansen's disease are intended for use as pathological models as well as a means to propagate bacteria for use in experiments. Various means of inoculation to many animal species have been attempted since just after the discovery of *M. leprae* (Johnstone, 1987). Nevertheless, no reproducible experimental transmission was reported until the successful study of limited growth in mice footpads (Shepard, 1960).

3.7.1 Mice

Mice inoculated with 10^3-10^4 *M. leprae* into a footpad show limited growth of 10^5-10^6 at after about 6-8 months, although no changes recognized by naked eye exists. Therefore, bacillary growth is determined by counting the bacilli in smears of footpad homogenates (Sheparad & McRea, 1968). Real-time PCR might be applicable to quantification of the bacilli instead of counting bacilli in smear (Truman *et al*, 2008). Histopathological futures correspond to the BL type of leprosy in humans. This test system is widely applied to the isolation of *M. leprae*, screening of anti leprosy drugs, examination of drug resistance of *M. leprae*, and the like.

3.7.2 Immunocompromised mice

Based on the supposition that the limited growth of the bacteria in footpads of normal mice results from the development of immunity, susceptibility was examined in mice that were immunocompromised by thymectomy and X ray-irradiation (Rees, 1966). At 12 months after inoculation, the bacillary number reached 10^8-10^9 associated with marked swelling in the footpad. Bacteria were also detected in various parts of the body including auricles and the nose, confirming disseminated systemic infection. This model was used in experiments of infection via nasal mucosa, proving that *M. leprae* infects hosts via nasal mucosa. However, it became comparatively disused after reports of higher susceptibility of nude mice.

3.7.3 Nude mice

When the thymus' function as a tissue for lymphocyte maturation site was clarified, and when the importance of cellular immunity in the prevention of *M. leprae* infection became known, the infection experiment of *M. leprae* to athymic nude mice was conducted and succeeded in causing systemic disseminated lepromatous lesions (Fig.3.8) (Kohsaka *et al*, 1976; Chehl *et al*, 1985). Bacillary number in the footpad inoculated with 10^7 bacilli reach to 10^{11} bacilli per footpad after 11 to 12 months. In nude mice, bacterial proliferation in lower temperature parts such as the eyelid, nose, tail, testis, and auricle is extremely high (Kohsaka *et al*, 1978). This model is used for screening of therapeutics for Hansen's disease, or for the isolation of the bacteria. In addition, armadillos cannot be bred in captivity. For that reason, wild armadillos are captured for laboratory

use. They might have natural infection or might be contaminated by other acid-fast bacteria, but *M. leprae* grown in nude mice are useful as a good source of bacteria. Although total bacillary number in the footpad amounts to about 10^{12}/footpad and shows large swelling of footpad at 12 months post infection,these badilli are not suitable for experiments, since bacilli with highest viability could be obtained from moderately enlarged nude mouse footpad (Truman & Krahenbuhl, 2001). The Leprosy Research Center of the National Institute of Infectious Diseases of Japan provides the bacilli to meet the demands of researchers.

3.7.4 Armadillos

Nine-banded armadillos (Fig.3.9), which inhabit the southern US to Latin American countries, normally have body temperature as low as 30-35°C. For that reason, their susceptibility to *M. leprae* was examined, revealing extremely high susceptibility (Kirchheimer, 1971). Intravenous inoculation of a large quantity (10^9 or more) of *M. leprae* develops the disease in 90% or more of the animals. Lesions correspond to the LL type of leprosy in humans. In armadillos, unlike humans, numerous *M. leprae* are detected in almost all organs, and 10^{10} or more *M. leprae* per gram exist in the liver and spleen. The obtained bacteria are used for various experiments requiring large amounts of bacterial components.

Cases of natural infection have been identified in armadillos (Walsh *et al*, 1975). It has been reported that the infection rate in Louisiana is 10% on average (about 30% maximum). No cases of naturally acquired case of infection have been reported previously for the armadillos inhabiting in eastern states as Florida, but spreading to eastward is indicated recently (Loughry *et al*, 2009). Together with naturally acquired infection in monkeys, Hansen's disease is considered to be a zoonosis (Walsh, 1981), and infection of humans, possibly from armadillos, has also been reported (Douglas *et al*, 1987).

3.7.5 Primates

Transmission of *M. leprae* to monkeys has long been conducted. Although results in chimpanzees and in white-handed gibbons (*Hylobates ler*)appear promising, these did not attract much attention. Later, naturally acquired infection was found in mangabey monkeys (Meyers *et al*, 1985), chimpanzees (Donham & Leininger, 1977), and cynomolgus monkeys (Valverde *et al*, 1998) drawing attention to the susceptibility of primates to *M. leprae*. Additionally, rhesus monkeys and African green monkeys have been shown to be susceptible to *M. leprae* by experimental infection (Wolf *et al*, 1985). In cases of infected primates, various lesions resembling those in human disease types are observed, including neuritis and erythema nodosum leprosum (ENL) (Gormos *et al*, 1995). These are considered to be models that are especially suitable for neuropathy studies.

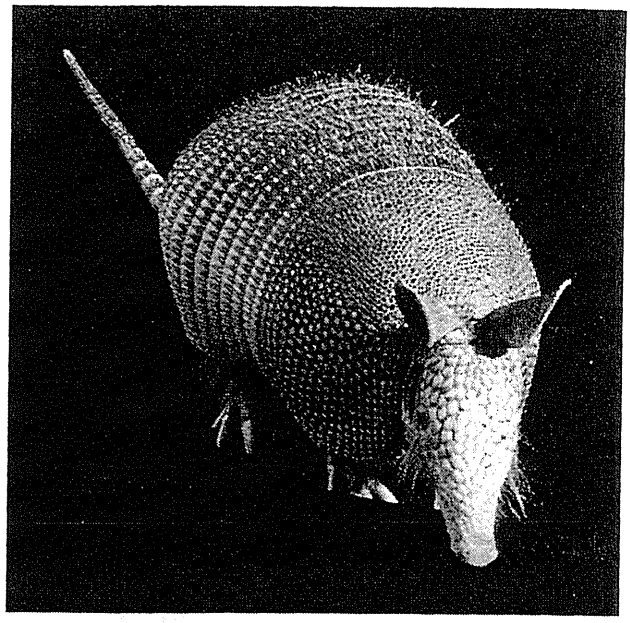

Figure 3.9 Nine banded armadillo (*dasypus novemcinctus*). (Photo by Truman R)

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3.8 INFECTION

3.8.1 Source of infection

The source of infection is presumed to be untreated patients because an untreated patient with the LL type sheds 2×10^8 cells per day from nasal mucosa. Results of many epidemiological studies show that the incidence rate is high in people in contact with LL patients (Noordeen, 1978). On the other hand, many findings as follows indicate sources of infection other than untreated patients: in areas with a high prevalence rate of the disease, *M. leprae* exists in nasal mucosa of many residents, who are positive for the specific anti-*M. leprae* antibody (Saeki *et al*, 2000; van Beers *et al*, 1994); over 16% of residents were antibody positive in an area where had been no new patients for several years (Abe *et al*, 1990); in some cases, the genotypes do not match between a patient who is presumed to be the source of infection and a cohabit-

ing family patient (Matsuoka *et al*, 2004). These facts suggest the possibility of direct infection by sheding from multibacillary patients or inapparent infection and of indirect infection by *M. leprae* that survive in environment after dischange (Desikan & Sreeratsa, 1995).

3.8.2 Infection route

Invasion of *M. leprae* into the body is presumed to be usually via nasal mucosa based on observations: lesions are already apparent in the nasal cavity before dermal lepromatous lesions are observed (Barton, 1974), and the *M. leprae* genes are sometimes detected in nasal mucosa of residents other than patients, including both contact and non-contact (van Beers *et al*, 1994; Saeki *et al*, 2000). In addition, infection established via nasal mucosa in immunocompromised mice was shown (Rees *et al*, 1966). These findings strongly support infection establishment via nasal mucosa.

3.8.3 Pathogenicity

Abe *et al* examined the antibody titer in residents of Okinawa prefecture using fluorescent leprosy antibody absorption (FLA-ABS). Results showed that 22% of residents were positive (Abe *et al*, 1990). Seroepidemiological results obtained in Indonesia also showed that many residents were infected (van Beers *et al*, 1994; Saeki *et al*, 2000). These observations indicate that *M. leprae* posseses adeguate infectivity. On the other hand, based on the discrepancy between the high antibody positive rate and the prevalence rate, it is considered that the virulence of *M. leprae* is not high and that some infected individuals develop the disease because of some immunodeficiency to the bacteria. Hansen's disease is well known for its family accumulation, in which genetic factors of characteristics to define immunological competence might play certain roles.

3.9 MICROBIOLOGICAL DIAGNOSIS

In areas where it is prevalent, Hansen's disease is diagnosed by observations of skin or neural lesions, and classified as either multibacillary or paucilbacillary type for treatment. Microbiological tests in the area of high prevalence are limited to the B.I. test by microscopy.

For definitive diagnosis, the blood antibody value against PGL-I is measured using ELISA or lateral flow test or leprosy-specific gelatin agglutination to determine the existence of infection. The lepromin reaction is used for determination of the disease type: positive is the tuberculoid type and negative is the lepromatous type.

Microbiological identification methods include: 1) amplification of specific DNA regions using PCR (Woods & Cole, 1989; Plikaytis *et al*, 1990; Kurabachew *et al*, 1998) ; 2) acid-fastness determination by Ziehl-Neelsen staining and disappearance of acid-fastness by pyridine treatment; 3) immunostaining using various monoclonal antibodies that recognize epitopes specific to *M. leprae*; 4) limited growth in the mouse footpad; and 5) non-proliferation in all artificial media including media for *M. tuberculosis*.

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