A Genomic Study on the Cultivable and Nerve Invading *Mycobacterium HI-75* after the Recovery 3 Months of the Inoculation to Nude Mice.

Tetsuo Sakai^{1)*}, Hamit Sidik²⁾, Eiichi Matsuo²⁾

Ruriko Hayakawa¹⁾ and Akira Wakizaka¹⁾

1)Department of Biochemistry and Molecular Biology, and

2)Department of Pathology Kyorin University School of Medicine, Tokyo, Japan

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The sequence of the polymerase chain reaction (PCR) product of 16S ribosomal RNA (16S rRNA) of the leproma-derived and cultivable *Mycobacterium HI-75* (*M. HI-75*) which was obtained from the infected regions of inoculated mice, was examined and compared with that of the cultured bacteria by the direct sequencing techniques. The sequence was completely consistent with the cultured bacilli in the comparable 837 bases of 16S rRNA. The mycobacterium examined in this study was originally isolated as *M. leprae* (ML) by Skinsnes, et al. in 1975 from leproma of a lepromatous type Hansen's disease patient and therefore named as *Mycobacterium leprae HI-75* by them, and was maintained from 1984 using either Ogawa's or Sauton's media in the beginning and Ogawa's medium enriched with glucuronic acid and N-acetyl-D- glucosamine recently. Sasaki and Hamit reported the nerve invasion and the growth of the inoculated bacilli either to the nude mice or the I¹³¹ treated immunocompromised Swiss mice. We previously reported that cultured *HI-75* was most similar to *M. scrofulaceum* by the direct sequencing of the gene of 16S rRNA. The 16S rRNA obtained from the mouse tissue in the present study indicated that *M. HI-75* would be a variant of *M. scrofulaceum* possessing an ability to invade into peripheral nerve. The results suggest that the *HI-75* strain claims a nature as a pathogen to develop a leproma-like lesion.

Our previous analytical results of the sequences of 16S ribosomal RNA (16S rRNA) of *Mycobacterium HI-75* (*M. HI-75*), which was obtained from a Hansen's disease patient and was

cultured in the laboratory¹⁾, showed that the strain was most similar to *M. scrofulaceum*²⁾. In experimental models *M. HI-75* invaded into peripheral nerve and caused neuropathy^{3) 4)}. The aim of the present study is to analyze the genomic sequences of the bacillus which has been inoculated to mice and recovered from legional tissue of the mice in comparison with the sequences obtained from the cultured ones.

*Corresponding author :

Department of Biochemistry and Molecular Biology,
Kyorin University School of Medicine, 6-20-2
Shinkawa, Mitaka, Tokyo 181, Japan
Fax:+81-422-407281

MATERIALS AND METHODS

Bacterial strain and tissue preparation. The leproma-derived and cultivable *M. HI-75* have been maintained by cultivation utilizing Ogawa's medium enriched with glucuronic acid and N-acetyl-D-glucosamine. The bacillus was inoculated through the subcutaneous injection in the cheeks of mice (8wks, BALB/cA euthymic (+/+), female) and maintained for 3 months. Mouse cheek skin was recovered from infected legions and examined immediately.

Analytical procedures. 10 to 20 mg of the mouse tissue was homogenized in 100 μ l of buffer (10mM Tris-HCl, pH7.6, 10mM EDTA, pH8.0). After incubation of the homogenate with 100 μ l of lysozyme (10mg/ml) at 37°C for 1 hour, the mixture was vigorously mixed with glass beads for one minute to disintegrate the materials. The DNA was extracted with SepaGene™ (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's procedure. Then the DNA was suspended in 100 μ l of TE buffer (10mM Tris-HCl, pH7.6, 1mM EDTA, pH8.0). Polymerase chain reaction mixture contained 5 μ l of 25 mM MgCl₂, 10 μ l of 10X PCR buffer, 8 μ l of 2.5mM each dNTPs, 61.5 μ l of autoclaved ultra-pure water, 0.5 μ l of Taq DNA polymerase (2.5 unit), 5 μ l of each sense and antisense primers (20 p mole), and 5 μ l of DNA solution mentioned above for the template. The reaction was primed by a template melting step at 94°C for 5 min, followed by 35 cycles of the serial temperature changes consisting of 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min using the apparatus (Perkin Elmer, Norwalk, USA). Thus obtained PCR product was sequenced by the use of an AutoLaod™ Solid-PhaseSequencing kit (Pharmacia Biotec, Tokyo, Japan), biotinylated primers and fluorescein (FITC) conjugated primers²⁾ on a Pharmacia model A.L.F. DNA Sequencer (Pharmacia Biotec, Uppsala, Sweden). The both sequence of sense and antisense strand was analyzed and compared

with each others to confirm the accuracy. The sequences of 16 S rRNA from 8th to 341st, 518th to 928th and 1170th to 1540 bases were examined using the gene data base issued from GenBank, published data by Rogall et al. and the results of the previous report in this journal^{2) 5) 6) 7)}

RESULTS

Histopathology of the lesion of inoculated mice showed the accumulation of macrophages encompassing peripheral nerves, marked infiltration of the mycobacteria and the invasion of the bacilli in the small branches of the peripheral nerves 3 months after inoculation (Figure.1).

The sequences from 8-341st, 518-928th and 1170-1540th base regions of 16S rRNA referred to those of *E. coli* was analyzed. The sequences of *M. HI-75* obtained from legional tissue of the inoculated mice were completely consistent with those of the cultured *M. HI-75* in detectable 837 bases as previously described. These regions of 16 S rRNA contain well conserved, bacteria specific and variable species-specific regions.

DISCUSSION

The applicability of 16S rRNA sequences for bacterial classification is well accepted^{8) 9)}. 16S rRNA gene consists of genomically highly conserved primary sequences and species-specific nine variable regions. We examined the V1, V2, V4, V5, V8 and V9 regions of 16S rRNA. The sequences were completely homologous to those of 16S rRNA of *M. HI-75*, which is 5 out of 837 bases (0.6%) deviated from those of *M. scrofulaceum*. *M. leprae* differed from *M. HI-75* in 32 bases (3.8%) in the 16S rRNA sequence. In previous report we concluded that the leproma-derived and cultivable *M. HI-75* was a variant of

Fig.1. Acid-fast stains of mouse cheek skin tissue 3 months after inoculation of *M. HI-75* (X400). An arrow indicates the bacilli in a small branch of the peripheral nerve leaving the larger one intact.

*M. scrofulaceum*²⁾. Present study also suggested that the same strain of *M. HI-75* could invade into the living mouse tissue and stayed there developing lesional foci without any change in the genomic sequence of the examined ribosomal RNA gene. This would be one of the proof that *M. scrofulaceum* possibly possesses a pathogenic activity in peripheral nerve causing like that of *M. leprae*.

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感染マウスより分離した抗酸菌 HI-75 のゲノム解析

阪井哲男¹⁾ * ハミット・スディック²⁾
松尾英一²⁾ 早川るり子¹⁾ 脇坂 晟¹⁾

1) 杏林大学医学部第二生化学教室

2) 同 第一病理学教室

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キーワード：抗酸菌 HI-75：DNA シーケンス：16S リボゾーム RNA：
ハンセン病：Mycobacterium scrofulaceum

Skinsnes らによって、らい腫より分離培養された抗酸菌 HI-75 は、16S リボゾーム RNA のダイレクトシーケンスの結果、*Mycobacterium scrofulaceum* に最も近似していたことを報告した。今回、さらに抗酸菌 HI-75 をヌードマウスに接種し、感染巣の皮膚結節より同菌を採取し、同様の方法でゲノム解析を行った。その結果、両者を比較し得た 16S リボゾーム RNA の 837 塩基において両者は完全に一致していた。抗酸菌 HI-75 は *M. scrofulaceum* のヴァリエントとすれば、*M. scrofulaceum* も末梢神経に浸潤し、神経病変を惹起させる可能性が示唆された。

*Corresponding author :

〒181 東京都三鷹市新川 6-20-2

杏林大学医学部

Tel: 0422-47-5511 Fax: 0422-40-7281