研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
石井則久 鈴木幸一	ハンセン病	川田　暁編	よくわかる病態生理9皮膚疾患	日本医事新報社	東京	2008	187-190

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Y. Fukutomi, Y. Maeda, M. Matsuoka, M. Makino.	Temperature dependency for survival of *Mycobacterium leprae* in macrophages.	Jpn. J. leprosy	78	7-16	2009
Matsuoka M.	Recent advances in the molecular epidemiology of leprosy.	Jpn. J. leprosy	78	67-73	2009
Y. Miyamoto, T. Mukai, Y. Maeda, M. Kai, T. Naka, I. Yano, M. Makino.	*Mycobacterium avium* complex *gtfTB* gene encodes glucosyltransferase required for the biosynthesis of serovar 8-specific glycopeptidolipid.	J. Bacteriol.	in press		2009
M. Makino, Y. Maeda, M. Kai, T. Tamura, T. Mukai.	GM-CSF mediated T cell activation by macrophages infected with recombinant BCG that secretes major membrane protein-II of *Mycobacterium leprae*.	FEMS Immunol. Med. Microbiol.	in press		2009
Terao K.	Dynamic changes in early development of immune system in macaque monkeys –The significance from standpoint of the preclinical toxicity test using nonhuman primates.	J. Toxico. Sci.	in press		2009
Matsuoka M. Khin S. A. Kyaw K. Tan E. V. Balagon M. V. Saunderson P. Gelber R. Makino M. Nakajima C. Suzuki Y.	A novel method for simple detection of mutations conferring drug resistance in *Mycobacterium leprae*, based on a DNA microarray, and its applicability in developing countries.	J. Med. Microbiol.	57	1213-1219	2008

M. Kai, N. P. N. Ha, H. T. T. Huong, N. H. An, Y. Fukutomi, Y. Maeda, Y.Miyamoto, T. Mukai, T. Fujiwara, N. T. Tan, M. Makino.	Serological diagnosis of leprosy in patients in Vietnamese by enzyme-linked immunosorbent assay with *Mycobacterium leprae*-derived major membrane protein-II.	Clin. Vaccine Immunol.	15	1755-1759	2008
T. Mukai, Y. Maeda, T. Tamura, Y. Miyamoto, M. Makino.	$CD4^+$ T cell activation by antigen-presenting cells infected with urease-deficient recombinant *Mycobacterium bovis* bacillus Calmette-Guérin.	FEMS Immunol. Med. Microbiol.	53	96-106	2008
N. Fujiwara, N. Nakata, T. Naka, I. Yano, M. Doe, D. Chatterjee, M. McNeil, P. J. Brennan, K. Kobayashi, M. Makino, S. Matsumoto, H. Ogura, S. Maeda.	Structural analysis and biosynthesis gene cluster of an antigenic glycopeptidolipid from *Mycobacterium intracellulare*.	J. Bacteriol.	190	3613-3621	2008
Bang PD, Suzuki K., Ishii N., Khang TH.	Leprosy situation in Vietnam-reduced burden of stigma.	Jpn. J. Leprosy	77	29-36	2008
Tanigawa K., Suzuki K., Nakamura K., Akama T., Kawashima A., Wu H., Hayashi M., Takahashi S., Ikuyama S., Ito T., Ishii N..	Expression of adipose differentiation-related protein(ADRP) and perilipin in macrophages infected with *Mycobacterium leprae*.	FEMS Microbiol. Lett.	289	72-79	2008
儀同政一	ニューキノロン系抗菌薬の構造式と抗らい菌活性の相関	日本ハンセン病学会雑誌	78	17-23	2009
鈴木幸一、 永岡　譲、 森　修一、 石井則久	2007年における世界のハンセン病の現況について	日本ハンセン病学会雑誌	77	15-23	2008

谷川和也、 鈴木幸一、 川島　晃、 三島眞代、 Huhehasi Wu、 赤間　剛、 武下文彦、 石井則久	らい菌感染マクロファージにおける細胞内寄生と排除に関わる分子機構	日本ハンセン病学会雑誌	77	57-61	2008
石井則久、 関根万里、 渡辺朋美、 朝比奈昭彦	輸入皮膚感染症	臨床皮膚科	62 (増刊号)	22-26	2008
石井則久	ハンセン病の最近の話題	皮膚の科学	7	416-420	2008

Ⅳ. 研究成果の刊行物・別刷

ハンセン病

（石井則久・鈴木幸一）

◉ ハンセン病はらい菌による，皮膚と末梢神経を主な病変の場とする慢性感染症である。

◉ 有効な治療薬の無かった時代には顔面・手足などの変形が起こり，法律なども制定され，偏見・差別の対象になった。

らい菌と免疫

ハンセン病（Hansen's disease, leprosy）の原因菌であるらい菌（*Mycobacterium leprae*）は，1873年に菌が発見されて以来現在に至るまで人工培養に成功していないが，全遺伝子塩基配列は2001年に解読された。らい菌の増殖は遅く（約12日で二分裂する），至適発育温度は31℃前後で，主にマクロファージ内寄生菌である。また，らい菌と末梢神経のシュワン細胞との親和性が高いため，末梢神経障害が起こる。

らい菌を排除する免疫能は比較的強いと考えられ，らい菌は容易に排除されるが，免疫能が完全でない小児期に大量・頻回にらい菌を吸入すると，数年から10年以上の潜伏期間の後に発症する。発症に影響を与える因子としては，個々人のらい菌に対する特異的な細胞性免疫能のほか，公衆衛生の程度，経済状態，栄養状態などの環境・社会的因子が論じられている。また，感受性遺伝子の存在も議論されている。

生体のらい菌に対する免疫能の程度によって，らい菌がほとんどいない病型（少菌型，TT型など）と，らい菌が大量に存在する病型（多菌型，LL型など）に分類される（図10-22，表10-2）。

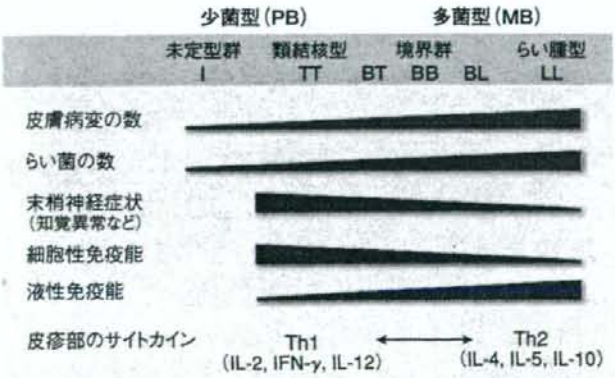

図10-22　ハンセン病の病型と宿主の免疫反応

表10-2　ハンセン病の病型分類

菌数による分類 (WHO分類)	少菌型 (paucibacillary ; PB)	多菌型 (multibacillary ; MB)
免疫学的分類 (Ridley-Jopling分類)	(I)　TT	B BT　BB　BL　　LL
細胞性免疫能	良好	低下/なし
局所の免疫	Th1, IL-2, IFN-γ, IL-12	Th2, CD8 T細胞, IL-4, IL-5, IL-10
皮膚スメア検査	陰性	陽性
らい菌	少数/発見しがたい	多数
皮疹の数	少数	多数
皮疹の分布	左右非対称性	左右対称性
皮疹の性状	紅斑(環状斑), 境界明瞭	紅斑(環状斑), 丘疹, 結節
皮疹の表面	乾燥性, 無毛	光沢, 平滑
皮疹部の知覚異常	高度(触覚, 痛覚, 温度覚)	軽度/正常
病理所見	類上皮細胞性肉芽腫 巨細胞, 神経への細胞浸潤	組織球性肉芽腫 組織球の泡沫状変化
病理でのらい菌	陰性	陽性
主たる診断根拠	皮疹部の知覚異常	皮膚スメア検査などでのらい菌の証明
感染性	なし	感染源になる

ハンセン病の疫学

日本人の新規患者は毎年数人程度で，高齢者がほとんどである。一方，在日外国人の新規患者は毎年8人程度であるが，現在世界的にはWHOのハンセン病制圧運動が展開され，新規患者は毎年27万人を下回り，後遺症対策や人権問題，スティグマ対策が課題となってきている。

社会・医学とハンセン病

ハンセン病に対する有効な治療薬がなかった時代には，病状が進み，顔面，手足に皮疹および末梢神経麻痺(痛覚障害，度重なる外傷・火傷，変形，運動障害)などを形成した。そのため外見上の問題と手足の不自由による就労の困難，住民から疎外，さらに宗教からも差別され，世界的に偏見・差別の対象となった。日本においては明治時代になって救済から隔離に進み，「癩予防ニ関スル件」，「癩予防法」，さらにハンセン病に有効な治療薬が開発されていた1953年には「らい予防法」が制定された。医学的進歩とかけ離れ，人権を無視したこの法律は1996年まで存続した。医療関係者はその間患者の人権にはほとんど関心を示さず，医学の進歩の追求を優先してきた。今後，病気に関連する法律や社会的状況などにも常に眼を開いてい

ることが必要である。

ハンセン病の同義語として「らい」,「癩」などが用いられてきたが，現在は偏見・差別を助長するものとして使わない。

診　断

出生地（国），小児期の生活歴，家族歴などの問診，自覚症のない皮疹や知覚異常による外傷や火傷，さらに神経肥厚などからハンセン病を鑑別にいれる。診療や検査，入院などに際しては通常の感染予防の対応で十分である。

ハンセン病に特徴的な皮疹はないが，皮疹にほぼ一致して知覚（触覚，痛覚，温度覚）の鈍麻や麻痺を認めることが診断上有用である。また，末梢神経の肥厚や運動障害にも注意が必要である。らい菌に対し免疫能が高いTT型（図10-23），全く反応しないLL型（図10-24），それらの中間のB群（BT型，BB型，BL型）に病型分類される（Ridley-Jopling分類）。TT型などは検査でらい菌を検出しにくいので少菌型（paucibacillary；PB），LL型

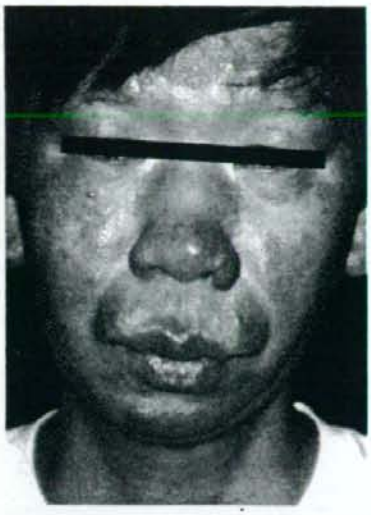

図10-24　多菌型ハンセン病（MB, LL型）
らい菌がマクロファージ内で増殖し，組織球性肉芽腫を形成するため，皮膚は光沢を帯びた紅斑や結節，神経は軽度の障害を認める。

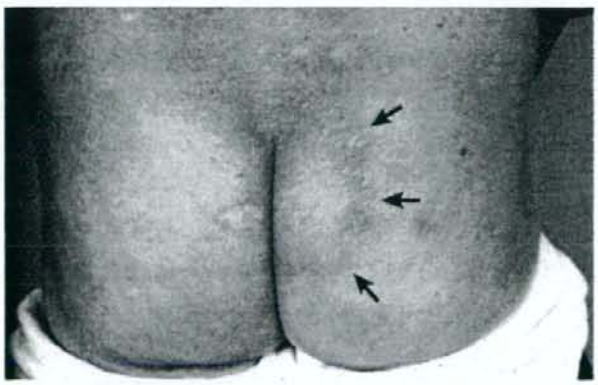

図10-23　少菌型ハンセン病（PB, TT型）
らい菌に対して免疫機能が働いている。らい菌浸潤を局所で防御して炎症を起こしているので，皮膚および末梢神経に症状（皮疹，知覚異常）が出現する。

などはらい菌を検出できるので**多菌型**(multibacillary；MB)とも分類される。このPBとMBの分類は治療法の選択にも応用され，**WHO分類**として知られている。

TT型では1ないし少数個の皮疹で，乾燥性で，辺縁やや隆起した境界明瞭な紅斑やときに環状斑として認められることが多い。皮疹部ではらい菌を排除するために類上皮細胞肉芽腫性炎症を起こし，知覚障害，発汗障害などを認める。LL型では皮疹は左右対称性で，多数の紅斑，黄褐色～淡紅色の丘疹ないし結節，板状の結節や硬結などが全身に出現する。**組織球性肉芽腫**の病像で，らい菌を排除しようとする炎症症状に乏しく，神経の障害は徐々に生じる。B群はTT型とLL型の中間の病像を示す。

治療中，あるいは治療前後にらい菌の菌体成分に対する免疫反応が活性化し，急速な末梢神経の障害(疼痛，運動障害など)や皮疹の再燃や新生，発熱などが起こることがあるが，これをらい反応と呼んでいる。

らい菌検出は，①**皮膚スメア検査**，②病理組織特殊染色(Fite染色)，③PCR検査で行う。

皮疹(自覚症なし)，神経(知覚障害，肥厚，運動障害)，らい菌検出，病理組織検査の4項目を総合して診断する。

治　療

治療の基本は，神経症状(神経炎，らい反応，後遺症など)を起こさず，らい菌を生体から排除することである。WHOの推奨する多剤併用療法を基本に行う。リファンピシン，ジアフェニルスルホン(DDS)，クロファジミンの3種類の抗菌薬を内服する。耐性菌の出現防止のため確実に内服することを指導する。らい反応や神経炎に対しては迅速にステロイド内服を行う。

Jpn.J.Leprosy 78. 7-16(2009)

Temperature dependency for survival of *Mycobacterium leprae* in macrophages

Yasuo FUKUTOMI *, Yumi MAEDA, Masanori MATSUOKA, and Masahiko MAKINO

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

[Received: 4 Aug. 2008 / Accepted: 15 Oct. 2008]

Key words : human, macrophage, mouse, *Mycobacterium leprae*, survival

Hansen's disease is caused by an infection with an intracellular pathogen, *Mycobacterium leprae*, which mainly inhabits macrophages and Schwann cells. However, little is known about the survival or growth mechanisms of the bacilli in mouse and human macrophages. In the present study, by using radiorespirometry analysis for the evaluation of the viability of *M.leprae*, we observed that *in vitro* incubation of *M.leprae*-infected macrophages at 35°C was more growth permissive than at 37°C, and supplementation with the immunosuppressive cytokine IL-10 supported the survival of the bacilli in the macrophages for 3 weeks, whereas viability of the bacilli was gradually lost if cultured without IL-10. In human macrophages, *M.leprae* retained its viability when cultured at 35°C for at least 4 weeks without IL-10. However, the viability of *M.leprae* was almost lost within 2 weeks if cultured at 37°C. These data suggest that temperature is a crucial factor for the survival of *M.leprae* in host cells.

Introduction

Hansen's disease is caused by an infection with *Mycobacterium leprae*. *M.leprae* is an intracellular pathogen, mainly residing in macrophages and Schwann cells. In patients, *M.leprae* is predominantly observed in the skin, nasal mucosa and peripheral nerves, particularly the more superficial ones. This clinical observation suggests that the optimal temperature of *M.leprae* for survival in human cells is less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse footpad where the temperature is lower than the core temperature, and the optimal temperature for the growth of *M.leprae* is reported to be in the range of several degrees above and below 30°C [2].

From another aspect, the growth of *M.leprae* seems to be largely affected by the host immune

*Corresponding author :
Department of Microbiology, Leprosy Research Center,
National Institute of Infectious Diseases
4-2-1, Aoba-cho, Higashimurayama-shi, Tokyo 189-0002, Japan
TEL : 81-42-391-8211 FAX: 81-42-394-9092
E-mail : fukutomi@nih.go.jp

response. Hansen's disease is characterized by a broad spectrum of the host immune response, such as lepromatous type (towards the increased load of bacteria) and tuberculoid type (towards the decreased bacterial load). In lepromatous type leprosy, Th-2 cytokines (IL-4, IL-5 and IL-10) are predominantly expressed in local lesions. In contrast, in tuberculoid type, Th-1 cytokines (IFN-γ, IL-2) are predominantly expressed [3]. Among cytokines, IFN-γ has been demonstrated to play a central role in activating macrophages to kill intracellular pathogens including *M.leprae*, whereas IL-10 is reported to inhibit the microbicidal activity of macrophages, resulting in the survival of the intracellular pathogen [4]. However, little is known about the survival and growth mechanisms of *M.leprae* in human macrophages since the viability of these uncultivable bacilli cannot be easily measured by in vitro study.

Previously we reported that metabolically active *M.leprae* were maintained in monolayer cultures of mouse peritoneal macrophages and supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system temperature is extremely important and 31-33°C incubation temperature is more growth permissive than 37°C [5]. In the present study, we observed that incubation of mouse macrophages at 35°C was also more permissive than at 37°C and supplemental IL-10, but not TGF-β, supported the metabolic activity of *M.leprae* in the macrophages for several weeks. Moreover, *M.leprae* from infected human macrophages cultured in vitro sustained metabolic activity for at least 4 weeks if cultured at 35°C but not at 37°C. Collectively, these data demonstrate that temperature is one of the crucial factors for *M.leprae* survival in human host cells.

Materials and Methods

M.leprae inoculum: The Thai-53 strain of *M.leprae* [6] was maintained in continuous passage in athymic *nu/nu* mice (Clea Co, Tokyo, Japan) by inoculation of bacilli into both hind foot pads. Experiments with mice were performed in compliance with the guidelines of the Experimental Animal Committee of the National Institute of Infectious Diseases. At approximately one year post inoculation, the foot pads were processed to recover *M.leprae* by Nakamura's method with a slight modification [7]. Briefly, tissue was minced and homogenized with Hanks' balanced salt solution (HBSS) containing 0.05% Tween 80. The homogenate was centrifuged at 150×g for 10 min and supernatant of the sample homogenate was treated with 0.05% trypsin at 37°C for 60min. The suspension was centrifuged at 4,000×g for 20min and sediment was re-suspended in HBSS followed by treatment with 1% sodium hydroxide at 37°C for 15min. The treated material was washed and re-suspended in HBSS at the desired bacillary concentration. Bacillary number in each foot pad was enumerated individually according to standard techniques [8].

Cytokines: Murine recombinant IL-10 was obtained from Genzyme Corp. TGF-β was obtained from Kurashiki Bouseki (Kurashiki, Japan). Both cytokines were stored at -80°C until use.

Mouse macrophage culture: Mouse peritoneal resident cells (approximately 50% macrophages) were harvested from retired ICR mice and suspended as previously described [9] at a concentration of 2×10^6/ml in RPMI 1640 (Gibco BRL, Invitrogen Corp., Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS, HyClone Laboratories, Logan UT), 25 mM N-2-

hydroxyethylpiperazine -N'- 2-ethanesulfonic acid (HEPES), 2 mM glutamine and 100μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). One half ml was seeded into 24 well tissue culture plates (Corning) containing 13 mm LUX coverslips (Nunc Thermanox coverslips, NalgeNunc, Thermo Scientific, Rochester, NY). After 20 hr adherence of the cells, macrophage monolayers were obtained after washing non-adherant cells from the coverslip with Hanks Balanced Salt Solution (HBSS, Sigma) leaving approximately 1×10^6 macrophages adhered per coverslip.

Human macrophage culture: Human peripheral blood was obtained under informed consent from healthy individuals. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, HP7 9NA, UK) gradient centrifugation[10]. The cells were suspended in AIM-V medium (Gibco BRL, Invitrogen Corp., Carlsbad, CA) and 1×10^6 PBMC were cultured in a well of a 24-well tissue culture plate (Falcon, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes) containing 13 mm LUX coverslips at 37°C in a 5%-CO_2 incubator for adherence of monocytes. After 1 hr incubation, the coverslips were washed with HBSS to remove non-adherent cells. The monocytes on the coverslips were cultured in a new 24-well plate containing RPMI1640 medium (Sigma) supplemented with 20% FBS (Whittaker Co., Walkersville, MD), 25mM HEPES, 2mM L-glutamine and 100μg/ml ampicillin in the presence of 10 ng/ml of human M-CSF (R&D Systems, Minneapolis, MN) or 40 ng/ml of GM-CSF (R&D Systems). After 7 days, the M-CSF-conditioned macrophages (M-macrophages) and the GM-CSF-conditioned macrophages (GM-macrophages) were used for infection with *M.leprae*.

Infection of macrophages with *M.leprae*: Purified mouse macrophage monolayers were infected with fresh *M.leprae* suspended in 0.5 ml medium per well. After 4 hr incubation for mouse macrophages and 20 hr incubation for human macrophages, non-phagocytosed bacilli were removed by washing and the cultures were incubated in 1.0 ml media supplemented with the appropriate cytokine in 5% CO_2 at the appropriate experimental temperatures[9]. Media were changed and cytokines were replenished at 5 days interval.

Radiorespirometry: The macrophages were lysed with 0.1 N NaOH to release the phagocytosed *M.leprae*, and the viability of the bacilli was determined by evaluating the oxidation of ^{14}C-palmitic acid to $^{14}CO_2$ by radiorespirometry as described previously [11]. Total isotope release was usually analyzed after one week of incubation at 31°C [9].

Staining of *M.leprae*-infected macrophages: Coverslips of *M.leprae*-infected adherent macrophages were prefixed with absolute methanol and acid-fast stained. The specimens were observed under Nikon Optiphot light microscopy.

Results

Viability of *M.leprae* in mouse macrophages cultured *in vitro*: Mouse peritoneal resident macrophages (1×10^6 cells per well) were incubated with freshly harvested *M.leprae* (multiplicity of infection (MOI), 5:1 or 10:1) for 4 hr to allow phagocytosis. Non-phagocytosed bacilli were washed off and the culture of the macrophages continued for up to 14 days. Viability (metabolic activity) of *M.leprae* in macrophages was assessed by radiorespirometry. As shown in Fig. 1, the viability of the bacilli was gradually decreased in macrophages cultured at 35°C. In contrast, the viability was significantly lost, if the macrophages were cultured at 37°C. Next, the mouse peritoneal

resident macrophages were incubated with 3 doses of *M.leprae* (MOI, 1:1, 4:1 and 10:1) for 4 hr to allow phagocytosis, and the culture continued for longer periods up to 21 days. Viability of *M.leprae* in macrophages was assessed at 7 day intervals. As shown in Fig. 2, in each dose of *M.leprae* infection, decrease in viability was significant after 21 days.

Effects of cytokines on viability of *M.leprae* in mouse macrophages: Supplementation of IL-10 to the infected macrophage culture was

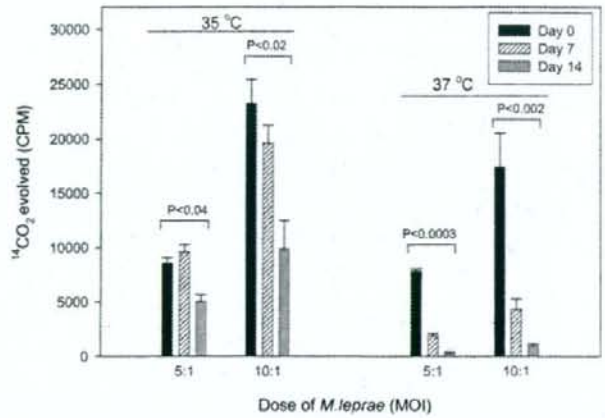

Fig.1. Viability of *M.leprae* in mouse macrophages cultured *in vitro*. Mouse peritoneal resident macrophages were incubated with 5×10^6 or 1×10^7 per well of *M.leprae* (MOI, 5:1 or 10:1), for 4 hr at 37°C to allow phagocytosis. Non-phagocytosed bacilli were washed off and the culture of the macrophages continued up to 14 days at 35°C or 37°C. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

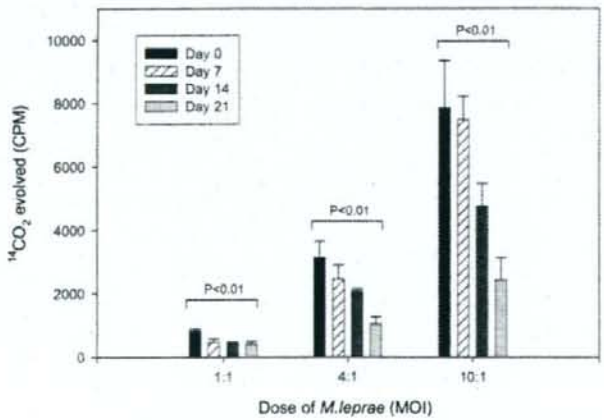

Fig.2. Viability of *M.leprae* in mouse macrophages cultured *in vitro*. Mouse peritoneal resident macrophages were incubated with 3 doses, 1×10^6, 4×10^6 and 1×10^7 per well of *M.leprae* (MOI, 1:1, 4:1 and 10:1) at 35°C for 4 hr to allow phagocytosis, and the culture continued at 35°C for longer periods up to 21 days. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

clearly associated with sustained viability of intracellular *M.leprae* cultured at 35°C (Fig.3). In the presence of 3 U/ml of IL-10, *M.leprae* maintained their viability, whereas viability was steadily lost without IL-10. We also examined the effect of TGF-β, another suppressive cytokine for macrophage activation, on the viablility of the bacilli. To the contrary, supplementation of TGF-β

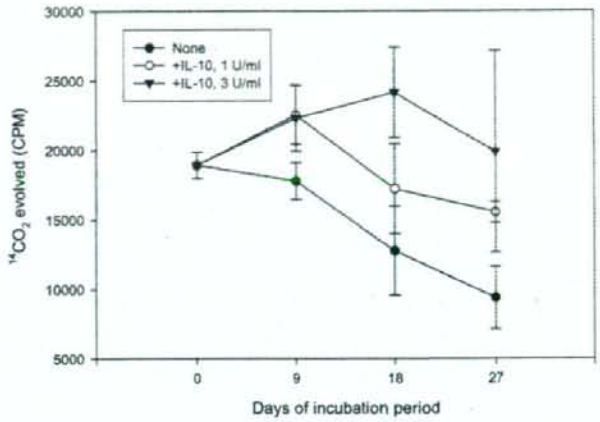

Fig.3. Effect of IL-10 on *M.leprae* survival in mouse macrophages. Mouse peritoneal resident macrophages were incubated with 1×10^7 per well of *M.leprae* (MOI, 10:1) at 35°C for 4 hr to allow phagocytosis, and the culture continued at 35°C for 9, 18 and 27 days. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

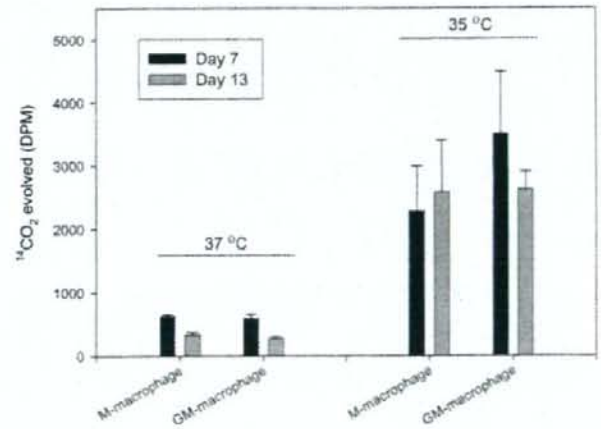

Fig.4. Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated at the same temperatures for indicated periods. By observation of the acid fast-stained cells under light microscopy, no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured between at 35°C and at 37°C. So the viability at day 0 is considered equal. After 7 days and 13 days incubation period, the cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry (dpm: disintegrations per minute).

significantly decreased the viability of *M.leprae*, when incubated for longer than 28 days post infection (Table 1).

Viability of *M.leprae* in human macrophages cultured *in vitro*: Human macrophages were obtained by culturing monocytes in the presence of either M-CSF or GM-CSF for 7 days. These macrophages (1×10^5 cells per well) were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated again at the same temperatures. By observation of the acid fast-stained cells under light microscopy,

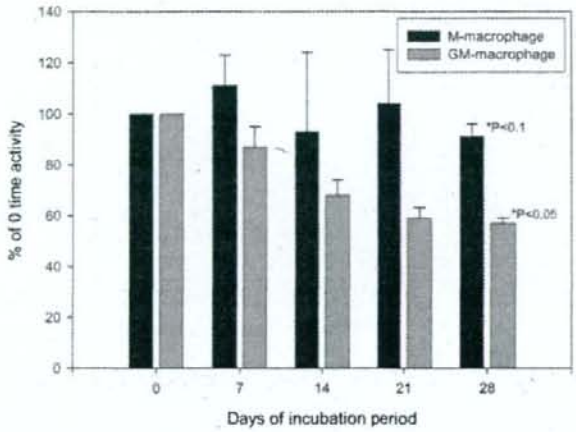

Fig.5. Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were infected with *M.leprae* (MOI, 50:1) for 20 hr at 35°C and incubated again at 35°C for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry. The results at day 7, 14, 21 and 28 are expressed as percentages of *M.leprae* metabolic activity at time 0. Radiorespirometry data obtained from *M.leprae* in M-macrophages at time 0 was 5,932±399 and those in GM-macrophages was 3,084±78. *P values calculated in comparison to day 0 viability.

Table 1. Effect of TGF-β on survival of *M.leprae* in mouse macrophages cultured at 35°C[a]

Experiment 1

Days of incubation period	At time 0	7	14	28
Medium only	5,222±936[b]	2,774±295	3,086±425	2,828±1,815
+TGF-β		2,919±535	3,119±1,339	1,973±126

Experiment 2

Days of incubation period	At time 0	14	28	49
Medium only	26,791±1,428	19,103±621	7,420±2,986	5,713±1,144
+TGF-β		14,306±2,240	3,728±410	1,594±317

[a]Mouse peritoneal resident macrophages were incubated with *M.leprae* (MOI, 1:10) for 4 hr to allow phagocytosis, and the culture continued for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.
[b]Radiorespirometry data, cpm.
Dose of TGF-β, 500pg/ml.
N.D., not determined.

no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured at 35°C and at 37°C (data not shown). Viability of *M.leprae* was assessed after 7 and 13 days. The results clearly showed that the viability of *M.leprae* incubated at 35°C was maintained, whereas the viability was lost if cultured at 37°C (Fig. 4). Next, *M.leprae*-infected human M- and GM-macrophages were cultured for prolonged periods at 35°C. Viability was sustained well for 4 weeks in human macrophages, especially in M-macrophages (Fig. 5).

Discussion

In vivo M.leprae is able to enter and survive in a wide variety of tissues and cell types[12]. The preferred host cell for the leprosy bacillus appears to be the macrophages and a number of unsuccessful attempts have been made to grow *M.leprae* in macrophages *in vitro*. For example, Sharp and Banerjee[13] employed macrophages from conventional mice and rats, *nu/nu* mice or *nu/nu* rats and armadillos. The *M.leprae* inocula were derived from 3 sources (human leproma, *nu/nu* mouse footpad and frozen infected armadillo tissue). Incubation temperature was varied from 31°C to 35°C and *M.leprae*-infected cells were maintained for up to 200 days. Fieldsteel and McIntosh [14] employed a range of rat, mouse and human tissue. The conclusion of these reports is that no significant multiplication of *M.leprae* occurred in any of the cells or tissues.

Previously, we reported that metabolically active *M.leprae* could be maintained in monolayer cultures of mouse peritoneal macrophages and that supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system, temperature is an extremely important factor for growth and 31-33°C incubation temperature is more permissive than 37°C [5]. In the present study, we further observed that incubation of mouse macrophages infected with *M.leprae* at 35°C was also more growth permissive than at 37°C. We chose 35°C as the incubation temperature, and not 31°C, because the maintenance of the integrity of the macrophage monolayer was better at 35°C than at 31-33°C. Moreover, the monolayer of *M.leprae*-infected human macrophages at 31-33°C could not be maintained for longer than one week. We observed that maintenance of the monolayer was good at 35°C, and *M.leprae* at 35°C was also more growth permissive than those at 37°C in human macrophages (Fig. 4 and 5). Our starting inoculum of *M.leprae* was freshly obtained for each experiment from infected *nu/nu* mice. We also were able to rapidly quantify the metabolic activity of *M.leprae* using the radiorespirometry technique adapted by Franzblau [11]. This assay is accurate and highly sensitive with the results available in a short duration of 1 wk (compared to 6-12 months when titrated in mouse footpads). Radiorespirometry data correlates well with other *in vitro* systems [11] but, more importantly, the data correlated well with "viability" as observed in the mouse footpad system [12].

Various clinical evidence suggests that *M.leprae* prefer a growth temperature of less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse foot pad where the temperature is lower than the body temperature [2]. In addition, *Dasypus novemcinctus*, the nine-banded armadillo has a core temperature of ~33°C, which renders it permissive as a host for the leprosy bacillus [13]. Mononuclear phagocytes in virtually every organ of the natural or experimentally infected armadillo become heavily parasitized with propagating *M. leprae* [14]. Whether intracellular or extracellular, *M.leprae* clearly prefers temperatures cooler than

normal human body temperature [12], and 37°C appeared to be highly detrimental to *M.leprae* viability. The *in vitro* results obtained in the present study confirmed the preference of lower temperature (35°C) by *M.leprae* residing in human macrophages.

In this study, supplemental IL-10, but not TGF-β supported the metabolic activity of *M.leprae* in mouse macrophages for several weeks, similar to the results obtained previously [5]. In choosing TGF-β and IL-10 as the cytokines that might bolster the intracellular survival of *M.leprae*, we were attempting to down-regulate any innate ability of the normal macrophages to cope with the organism. TGF-β is produced by activated macrophages and other inflammatory cells and has a broad array of modulatory functions on the immune response. TGF-β has been shown to interfere with macrophage antimicrobial mechanisms including the generation of reactive oxygen intermediates [15] and reactive nitrogen intermediates [16], and has been shown to enhance the intracellular growth of *M.tuberculosis* in human monocytes [17]. However, in the present studies with mouse macrophages, exogenous TGF-β had no detectable effect on sustaining intracellular *M.leprae* viability, and in fact decreased the viability (Table 1). In contrast, supplementing media with IL-10 clearly affected the long term viability of *M.leprae* in mouse macrophages (Fig. 3). IL-10 is produced by T cells, B cells and macrophages [18, 19]. IL-10 has been shown to be a potent down-regulator of cell-mediated immunity to intracellular pathogens [20]. *In vivo*, endogenous IL-10 dampened the cell-mediated immune response to avirulent mycobacterial infection [4] and appeared to lead to loss of control of *M.tuberculosis* infection with widespread dissemination [21]. IL-10 functions in part at the level of the macrophage by attenuating iNOS mRNA expression, iNOS activity and, by inference, NO production [22]. In human macrophages, however, the viability of *M.leprae* was maintained for 4 weeks in the absence of IL-10 (Fig. 5), suggesting that human cells seem to be better hosts than mouse cells for *M.leprae* survival. Viability of *M.leprae* in M-macrophages seems to be maintained for a longer period (up to one month) than that in GM-macrophages (Fig. 5). One of the reasons for this may be due to the production of IL-10 by M-macrophages [23], although the mechanism by which IL-10 contributes to the maintenance and growth of *M.leprae* is unclear.

In conclusion, the present study showed that the metabolism, and presumably the viability, of *M.leprae* could be sustained under culture conditions at 35°C, which is also a moderate temperature necessary to maintain the integrity of macrophages.

Acknowledgments

The study was supported partly by a Health Science Research Grant of Emerging and Re-emerging Infectious Diseases, from the Ministry of Health, Labour and Welfare of Japan. We are also grateful to the Japanese Red Cross Society for kindly providing PBMCs from healthy donors.

References

1) Brand PW: Temperature variation and leprosy deformity. Int J Lepr 27:1-7, 1959.
2) Shepard CC: Temperature Optimum of *Mycobacterium leprae* in Mice. J Bacteriol 90:1271-1275, 1965.
3) Yamamura M, Uyemura KD, Aeans RJ, Weinberg K, Rea TH, Bloom.BR, Modlin RL: Defining protective response to pathgens: cytokine profiles in leprosy lesions. Science 254:277-282, 1991.

4) Sharma S, Bose M: Role of cytokines in immune response to pulmonary tuberculosis. Asian Pac J Allergy Immunol 19: 213-219, 2001.

5) Fukutomi Y, Matsuoka M, Minagawa F, Toratani S, McCormick G, Krahenbuhl J: Subversion of macrophage anti-microbial function bolsters intracellular survival of *M.leprae*. Int J Lepr 72:16-26, 2004.

6) Kohsaka K, Mori T, Ito T: Lepromatoid lesion developed in nude mouse inoculated with *Mycobacterium leprae*. Lepro 45:177-187, 1976.

7) Nakamura M: Elimination of contaminants in a homogenate of nude-mouse footpad experimentally infected with *Mycobacterium leprae*. Jpn J Lepr 64:47-50, 1994.

8) Shepard CC, McRae DH: A method for counting acid-fast bacteria. Int J Lepr 36:78-82, 1968.

9) Adams LB, Franzblau S, Taintor R, Hibbs J Jr, Krahenbuhl JL: L- arginine-dependent macrophage effector functions inhibit metabolic activity of *Mycobacterium leprae*. J Immunol 147: 1642- 1646, 1991.

10) Maeda Y, Mukai T, Spencer J, Makino M: Identification of an immunomodulating agent from *Mycobacterium leprae*. Infect Immun 73:2744-2750, 2005.

11) Franzblau SG: Oxidation of palmitic acid by *Mycobacterium leprae* in an axenic medium. J Clin Microbiol 2618-2624, 1988.

12) Truman RW, Krahenbuhl JL: Viable *Mycobacterium leprae* as a research reagent. Int J Lepr 69: 1-12, 2001.

13) Kirchheimer W F, Storrs EH: Attempts to establish the armadillo (*Dasypus novemcinctus*) as a model for the study of leprosy. Int J Lepr 39:693-702, 1971.

14) Fieldsteel AH, McIntosh AH: Attempts to cultivate and determine the maximum period of viability of *M.leprae* in tissue culture. Int J Lepr 40: 271-277, 1972.

15) Tsunawaki S, Sporn M, Ding A, Nathan C: Deactivation of macrophage by transforming growth factor-beta. Nature 334:260-262, 1988.

16) Ding A, Nathan CF, Graycar J, Derynck R, Stuehr DJ, Srimal S: Macrophage deactivating factor and transforming growth factors-beta 1, - beta 2 and -beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. J Immunol 145:940-944, 1990.

17) Hirsch CS, Yoneda T, Averill L, Ellner JJ, Toosi Z: Enhancement of tintracellular growth of *Mycobacterium tuberculosis* in human monocytes by transforming growth factor-1. J Inf Dis 170:1229-1237, 1994.

18) Fiorentino DF, Zlotnik A, Viera P, Mosmann TR, Howard M, Moore KW, O'Garra A: IL-10 acts on the antigen presenting cell to inhibit cytokine production by Th1 cells. J Immunol 146:3444-3451, 1991.

19) O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M: Ly-1 B (B-1) cells are the main source of B cell - derived IL-10. Eur J Immunol 22:711-717, 1992.

20) Redpath S, Ghazal P, Gascoigne NR: Hijacking and exploitation of IL-10 by intracellular pathogens. Trends Microbiol 9:86-92, 2001.

21) Dugas N, Palacios-Calender M, Dugas B, Riveros-Moreno V, Delfraissy J, Kolb J, Moncada S: Regulation by endogenous IL-10 of the expression of nitric oxide synthase induced by ligation of CD23 in human macrophage. Cytokine 10:680-689, 1998.

22) Huang C, Stevens B, Nielsen E, Slovin P, Fang X, Nelson D, Skimming J: Interleukin-10 inhibition of nitric oxide biosynthesis involves suppression of CAT-2 transcription. Nitric Oxide 6:79-84, 2002.

23) Makino M, Maeda Y, Fukutomi Y, Mukai T: Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages. Microbes Infect 91:70-77, 2007.

マクロファージ内におけるらい菌生存の温度依存性

福富康夫*[1)]、前田百美[1)]、松岡正典[2)]、牧野正彦[1)]
国立感染症研究所ハンセン病研究センター
1）病原微生物部
2）生体防御部

〔受付：2008年8月4日、掲載決定：2008年10月15日〕

キーワード：生存、ヒト、マウス、マクロファージ、らい菌

ハンセン病は細胞内寄生菌であるらい菌によって引き起こされる感染症である。らい菌は主にマクロファージとシュワン細胞に感染する。しかしながら、マウスやヒトマクロファージ内における生存・発育機構について詳細は明らかになっていない。本研究では放射性同位元素を用いた方法によりらい菌の生存率を評価した。そして、らい菌感染マクロファージを35度で培養する方が37度で培養するよりもらい菌の生存率を高い状態に保つことができることが判明した。また、免疫抑制性サイトカインであるIL-10を添加することにより3週間程度生存が維持されることが分かった。一方、IL-10未添加の場合、生存率は徐々に低下した。ヒトマクロファージの場合は、IL-10未添加の場合でも少なくとも4週間生存は維持された。しかしながら、37度で培養すると2週間以内に生存率は著明に低下した。これらの結果から、らい菌の細胞内における生存には温度が決定的な要因のひとつであることが判明した。

* Corresponding author:
国立感染症研究所ハンセン病研究センター病原微生物部第二室
〒189-0002　東村山市青葉町4-2-1
TEL：042-391-8211　FAX：042-394-9092
E-mail：fukutomi@nih.go.jp

Jpn.J.Leprosy 78, 67-73(2009)

Recent advances in the molecular epidemiology of leprosy

Masanori MATSUOKA *
Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

[Received / Accepted: 24 Sept. 2008]

Key words : leprosy, molecular epidemiology, *Mycobacterium leprae*, SNPs, VNTRs

Recent advances in the molecular epidemiology of leprosy through genotyping of variable number tandem repeats (VNTRs) and single nucleotide polymorphisms (SNPs) are described. VNTRs with a broad range of diversity are useful genotyping tools for analyzing transmission in community areas, and SNPs and VNTRs with a small degree of variation are favorable for investigating the global transmission of leprosy. We expect that the transmission of leprosy can be fully analyzed by the application of these new methodologies.

Introduction

The number of newly detected cases of leprosy has been gradually decreasing around the world in recent years [1]. Even so, some details of disease transmission remain to be elucidated. One of the important subjects still under dispute, and essential for establishing a better strategy for preventing new cases, is the transmission mode of leprosy including the infectious source. Phenotype, serotype, phage type and genotype are robust metrics for analyzing and tracing transmission in many infectious diseases [2, 3]. These useful tools were not available for understanding the transmission of leprosy until two kinds of variable number tandem repeat (VNTR) were identified [4, 5], followed by the discovery of single nucleotide polymorphisms (SNPs) in the *Mycobacterium leprae* genome [6]. Epidemiological studies of leprosy using these two assays have been gradually accumulating since 2004, and findings generated by these breakthroughs in molecular epidemiology are now taking the place of classical epidemiological methods. An international study group for investigating the transmission of leprosy through the application of advances in molecular biology, including genotyping, has been organized and collaborative studies have begun [7]. VNTRs with broad allelic diversity are useful for analyzing transmission patterns in the community [8-11]. SNPs and VNTRs with a narrow range of diversity are practical for characterizing the global geographical distribution of different genotypes of *M. leprae*,

*Corresponding author :
Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases
4-2-1, Aobacho, Higashimurayama-Shi, Tokyo 189-0002, Japan.
TEL : +81-42-391-8211 FAX : +81-42-394-9092
E-mail : matsuoka@nih.go.jp

and are beneficial in tracing the dissemination of leprosy in prehistoric times and following current global leprosy migration patterns [12-16]. This article describes recent advances in the molecular epidemiology of leprosy.

Application of VNTRs to Analyze the Transmission of Leprosy

It has long been believed that leprosy is transmitted by heavy contact with leprosy patients; however, there are some findings which contradict this premise. For instance, a study on the geographical distribution of responders to *M. leprae* antibodies showed a distribution of positive responders in houses remote from those with known patients [17]. The limited effect of chemoprophylaxis [18] means that drugs prevent the progression of an active infection to overt disease [19] but do not interrupt transmission. Attempts to curtail transmission and reduce new infections by chemoprophylaxis in the Federated States of Micronesia (FMS) failed. The number of new cases detected in FMS has increased again after 1999 and over the last several years has risen to nearly the same level as before execution of the chemoprophylaxis program [1]. Many new cases without known contact with leprosy patients [20, 21] have also been reported. These findings suggest gaps in the traditional concept of leprosy transmission and imply the existence of an infectious source of *M. leprae* other than leprosy patients.

PCR studies have shown that in areas with a high prevalence of leprosy, many individuals harbor *M. leprae* in their nasal mucus [22, 23]. These findings indicate that people in such areas are frequently exposed to *M. leprae* infection. The hypothesis that the bacterial genotypes from household members or other contacts of patients should be identical if the bacilli are transmitted by contact with the patient, was tested by a comparison of genotypes from the nasal mucus of patients and family members, and also from new patients and the supposed index case of a multifamily case [8]. Genotyping based on the copy number of a TTC repeat [5, 8] and another genotyping method with higher discriminatory power were applied [10]. Results revealed that the genotype of *M. leprae* in the nasal mucus differed between the patient and contacts in a given household. The existence of *M. leprae* strains with allelic diversity in a multifamily case in China was also shown [11]. On the other hand, a study conducted in India revealed no divergence among the three VNTRs from two patients in a multifamily case [9]. The lack of divergence among the patients of this case could be explained by the low discriminatory power of genotyping using only three polymorphic VNTRs. In another study, isolates determined as having identical genotypes using a few VNTRs were separated into different genotypes by a method with high resolution power using a combination of other appropriate VNTRs [10]. Thus, we theorize that the Indian clinical isolates with identical genotypes might be classified into different genotypes with the improved methodology. Although VNTRs seem to fluctuate in biopsy samples from the same patient [24, 25], co-infection of *M. leprae* strains with different VNTRs may occur. While the variation of VNTRs in a given patient is often by only one or two copies, the TTC copy number of the isolate from a supposed index case was 18 and the TTC copy number of the bacilli from his son was 10 in one multifamily case report [8]. It is clear that the genotypes of the bacilli from father and son were distinct and hence did not come from the same infectious source [8]. This suggests the presence of an alternate source of infection.

Water sources used daily by people in an endemic area are thought to be a potential source