を経て成立したと考えられている。約90万年前、現在のインドネシアを基点とする民族が中国南部に進出し、その後更に北上した。その一部は台湾、沖縄を経由して一万年前より日本全土に分布し縄文人を形成した。その後2,300年から1,700年前にかけて極東地域北部において寒冷地適応した民族の一部が朝鮮半島を経由して日本に渡来し、弥生人となり縄文人を駆逐ないし混血し、現代の本州を中心として分布する日本人が成立した。現在の沖縄の人々およびアイヌの人々は縄文人の形質を受け継ぐヒトであるとされる。本州ではほとんどの株が4型であるのに対し、沖縄の分離株は全て3型であり、かつまた韓国由来の株は全て4型であったことは日本に分布するらい菌は現代の日本人の成立した過程と相関して2通りの方法により大陸よりもたらされたと推察される。沖縄に分布する3型らい菌は台湾を経て進入し、一方本土に分布する4型らい菌は弥生人となった大陸人ともに朝鮮半島を経て渡来したと考えられた。

我々と同じモンゴロイドが約12,000年前の氷河期にベーリンジアと呼ばれる海峡を渡ってアメリカ大陸に渡り、南アメリカに達しインディオと呼ばれる民族を形成しているが、ヒト白血病ウィルス（HTLV-1）はその移動ともにアメリカ大陸に伝播したことが示された[38]。それらの歴史からしてアメリカ大陸に分布するらい菌がどのような*rpoT*遺伝子型を示すのか当然興味が持たれるところであり、我々はパラグアイ、ペルー、メキシコから得たらい菌についてその型別を行った。パラグアイ（20株）とペルー（25株）から得たらい菌はすべて3型であったが、メキシコからの27株中25株は4型を示し、同じラテンアメリカの国に属しながら、まったく異なる分布を示した（未発表）。アメリカ大陸には大きく分けて三つのモンゴロイドのグループが移動したことが知られており[39]、らい菌の異なる型の分布は異なるグループによってもたらされ、形成されたことが推察される。メキシコにおける4型のらい菌の優勢は更に興味ある考えを示した。メキシコのハンセン病の由来は植民地時代にスペインからもたらされたとする説とフィリピンからもたらされたとする2説が言われているが、もしフィリピンに由来するものであるのなら、フィリピンのらい菌がすべて3型であったことから少なくとも3型が多数を占めるはずであるが、4型が多数を占めたことは同国のハンセン病はフィリピンに由来するものではないことを示した。

近年の分子生物学の成果を取り入れることにより、PCRによるらい菌の検出、遺伝子型別が可能となり、これまで解析し得なかったいくつかの課題について明確な結果が示されつつあるが、ハンセン病の分子疫学はいまだ緒についたばかりであり、さらに深く検討されることが求められる。遺伝子型別にしても、目下利用可能な手法のほかに更に分離株を細分可能にする方法が望まれる。たとえばTTC遺伝子型別によって同一型に分類されるらい菌を更に細分可能であれば、上述の10コピーのTTC繰り返しを示した親子の感染例が真に同一のらい菌であるかどうかの判定が可能となる。今知られている遺伝子多型の他にいくつかの多型性を示す部位の存在の可能性が示唆されており、今後それらを用いることにより一層詳しいハンセン病の疫学解析がなされ、根本的ハンセン病対策の構築に向けた成果が得られることが期待される。

文　献

1）Cole ST, Eiglmeier K., Parkhill J, James KD, Thompson NR, Wheeler PR, Honore N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Conner R, Davis RM, Devlin K, Duthoy S, Feltwell T, Praser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simson S, Simmonds M, Skelton J, Squares R, Sequares R, Stevens K, Taylor K, Whitehead S, Woodward JR and Barrel BG. Massive gene decay in the leprosy bacillus. Nature 409:1007-1011, 2001.

2）Engers HD, Abe M, Bloom BR, Briton W, Buchanan TM, Khanolkar SK, Young DB, Closs O, Gillis T, Horboe M, Ivanyi J, Kolk AHJ and Shepard CC. Results of a World

Health Organization-sponsored workshop on monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. 48:603-604, 1985.

3) Mehra V, Sweetser D and Young RA: Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. 83:7013-7017, 1986.

4) De Wit MYL and Klatser PR. Purification and characterization of a 36 kDa antigen of *Mycobacterium leprae*. J Gen. Microbiol. 134: 1541-1548, 1988.

5) Booth RJ, Harris DP, Love JM and Watson JD. Antigenic proteins of *Mycobacterium leprae* : complete sequence of the gene for the 18kDa protein. J Immunol. 140:597-601, 1988.

6) Woods SA and Cole ST. A family dispersed repeats in *Mycobacterium leprae*. Mol. Microbiol. 4:1745-1751, 1990.

7) Woods SA and Cole ST. A rapid method for the detection of potentially viable *Mycobacterium leprae* in human biopsies: a novel application of PCR. FEMS Microbiol. Lett. 65: 305-310, 1989.

8) Hartskeerl RA, de Wit MYL and Kaltser P. Polymerase chain reaction fro the detection of *Mycobacterium leprae*. J Gen. Microbiol. 135:2357-2364, 1989.

9) Williams DL, Gillis TP, Booth RJ, Looker D and Watson JD. The use of a specific DNA probe and polymerase chain reaction for detection of *Mycobacterium leprae*. J Infect. Dis. 162:193-200, 1990.

10) Plikaytis BB, Gelber RH and Shinnick TM. Rapid and sensitive detection of *Mycobacterium leprae* using a nested-primer gene amplification assay. J Clin. Microbiol. 28:1913-1917, 1990.

11) Abe M, Ozawa T, Minagawa F and Yoshino Y. Immunoepidemiological studies on subclinical infection in leprosy II. Geographical distribution of seropositive responders with special reference to their possible source of infection. Jap. J Lepr. 59:162-168, 1990.

12) Cho SN, Kim SH, Cellona RV, Chan GP, Fajardo TT, Walsh GP and Kim JD. Prevalence of IgM antibodies to phenolic glycolipid I among household contacts and controls in Korea and the Philippines. Lepr. Rev. 63:12-20, 1992.

13) van Beers SM, Izumi S, Madjid B, Maeda Y, Day R and Klatser PR. An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int. J Lepr. 62:1-9, 1994.

14) Izumi S, Budiawan T, Saeki K, Matsuoka M and Kawatsu K. An epidemiological study on *Mycobacterium leprae* infection and prevalence of leprosy in endemic villages by molecular biological technique. Ind. J Lepr. 71: 37-43, 1999.

15) Barton RPE. A clinical study of the nose in lepromatous leprosy. 45:135-144, 1974.

16) Davey TF. The nose in leprosy: steps to a better understanding. Lepr. Rev. 454:97-103, 1974.

17) Pattyn SR, Ursi D, Leven M, Grillone S and Reas V. Detection of *Mycobacterium leprae* by the polymerase chain reaction in nasal swabs of leprosy patients and their contacts. Int. J Lepr. 61:389-393, 1993.

18) de Wit MYL, Douglas JT, Mcfaden J and Klatser P. Polymerase chain reaction for detection of *Mycobacterium leprae* in nasal swab specimens. J Clin. Microbiol. 31:502-506, 1993.

19) Klatser P, van Beers S, Madjid B, Day R and de Wit MYL. Detection of *Mycobacterium leprae* nasal carriers in populations for which leprosy is endemic. J Clin. Microbiol. 31:2947-2951, 1993.

20) 佐伯圭介、Budiawan T、松岡正典、和泉眞蔵。生活環境中に存在するらい菌の疫学的意義。日本皮膚科学会雑誌 110：153-160, 2000.

21) Kazda J, Irgens LM, Muller K. Isolation of non-cultivable acid-fast Bacilli in sphagnum and moss vegetation by foot pad technique

on mice. Int J Lepr. 48:1-6, 1980.

22) Grange JM. Environmental mycobacteria and human disease. Lepr. Rev. 62:353-361, 1991.

23) Maniar AC and Vanbuckethout LR. *Mycobacterium kansasii* from an environmental source. Can. J Public Health 67:59-60, 1976.

24) Ross BC, Johnson PDR, Oppedisano F, Marino L, Sievers A, Stinear T, Hayman JA, Veitch MGK and Robins-Brown RM. Detection of *Mycobacterium ulcerans* in environmental samples during an outbreak of ulcerative disease. J Appl. Environ. Microbiol. 63:4153-4138, 1997.

25) Matsuoka M, Izumi S, BudiawanT. Nakata N. Saeki K. *Mycobacterium leprae* DNA in daily using water as a possible source of leprosy infection. Ind. J Lepr. 71:61-67, 1999.

26) Fine PEM. Leprosy: the epidemiology of a slow bacterium. Epidemiol. Rev. 4:161-188, 1982.

27) Clark-Curtis JE and Walsh GP. Conservation of genomic sequences among isolates of *Mycobacterium leprae*. J Bacteriol. 171:4844-4851, 1989.

28) Williams DL, Gillis TP and Portales F. Geographically distinct isolates of *Mycobacterium leprae* exhibit no genotypic diversity by restriction fragment length polymorphism analysis. Mol. Microbiol. 4:1653-1659, 1990.

29) Shin YC, Lee H, Walsh GP, Kim JD and Cho SN. Variable numbers of TTC repeats in *Mycobacterium leprae* DNA from leprosy patients and use in strain differentiation. J Clin. Microbiol. 38:4543-4538, 2000.

30) Matsuoka M, Maeda S, Kai M, Nakata N, Chae GT, Gillis TP, Kobayashi K, Izumi S and Kashiwabara Y. *Mycobacterium leprae* typing by genomic diversity and global distribution of genotypes. Int. J Lepr. 68:121-128, 2000.

31) Matsuoka M, Zhang L, Budiawan T, Saeki K and Izumi S. Analysis of leprosy transmission based on the genotyping of *Mycobacterium leprae* by TTC repeats polymorphism. J. Clin. Microbiol, in press.

32) Noordeen SK, Infectivity of leprosy. In: Chatterjee BR (ed) A window on leprosy. Chandi Memorial leprosy foundation New Delhi, pp59-63, 1978.

33) Fine PEM, Sterne JAC, Ponnighous JM, Bliss L, Saul L, Chihana A, Munthali M and Warndorf DK. Household and dwelling contact as a risk factors for leprosy in Northern Malawi. Am. J Epidemiol. 146:91-1028, 1997.

34) Schroder NW, Hermann C, Hamann L, Gobel UB, Hartung T and Schumann RR. High frequency of polymorphism Arg753Gln of the Toll-like -receptor gene detected by a novel allele-specific PCR. J Mol. Med. 81:368-372, 2003.

35) Miura T, Fukagawa T, Igarashi T, Yamashita M, Ido E, Funahashi S, Ishida T, Washio K, Ueda S, Hashimoto K, Yoshida M, Osame M, Singhal BS, Zaninivic V, Cartier L, Sonoda S, Tajima K, Ina Y, Gojyobori T and Hayami M. Philogenic subtype of human T-lymphotropic virus type I and their relations to the anthropological background. Proc. Natl. Acad. Sci. 91:1124-1127, 1994.

36) Falsh D, Wirth T, Linz B, Prichard JK, Stephens M, Kidd M, Blaster MJ, graham DY, Vacher S, Perez-Perez GI, Yamaoka Y, Megraud F, Otto K, Reichard U, Katzowitsch E, Wang X, Achtman M and Suerbaum S. Traces of human migrations in populations. Science 299:1528-1529, 2003.

37) 埴原和郎　日本人の骨とルーツ。角川書店, 1997.

38) Sonoda S, Li HC, Crayier L, Nunez L and Tajima K. Ancient HTLV type 1 provirus DNA of Andean mummy. AIDS Res. Human Retroviruses 16:1753-1756, 2000.

39) Greenberg JH, Turner II CG and Zegura SL. The settlement of the Americas: A comparison of the linguistic, dental and genetic evidence. Curr. Anthropol. 27: 477-497, 1986.

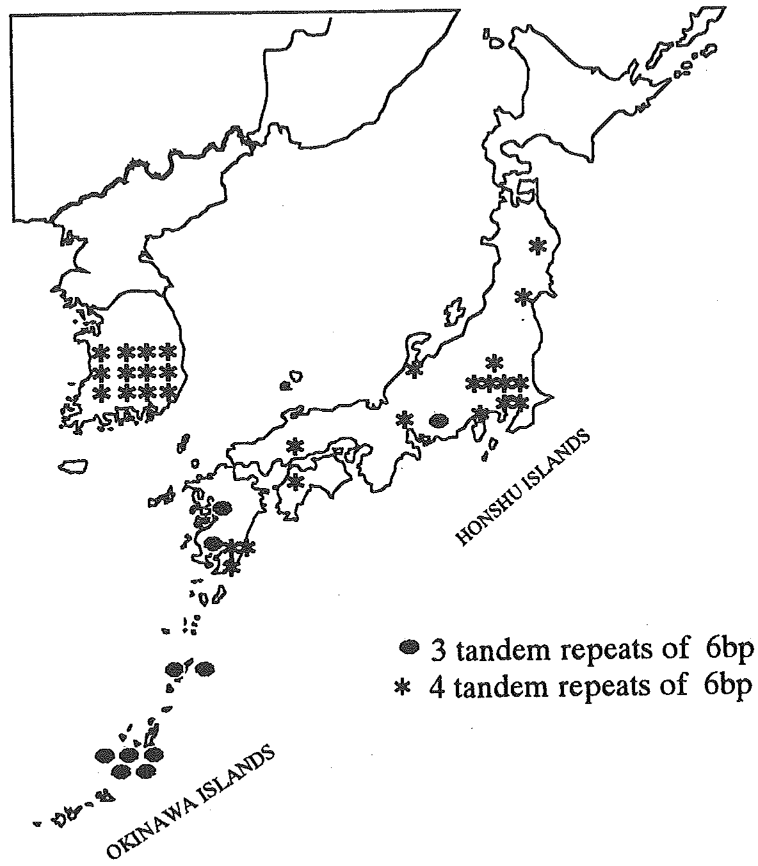

図１．日本と韓国における異なる *rpoT* 遺伝子型の分布

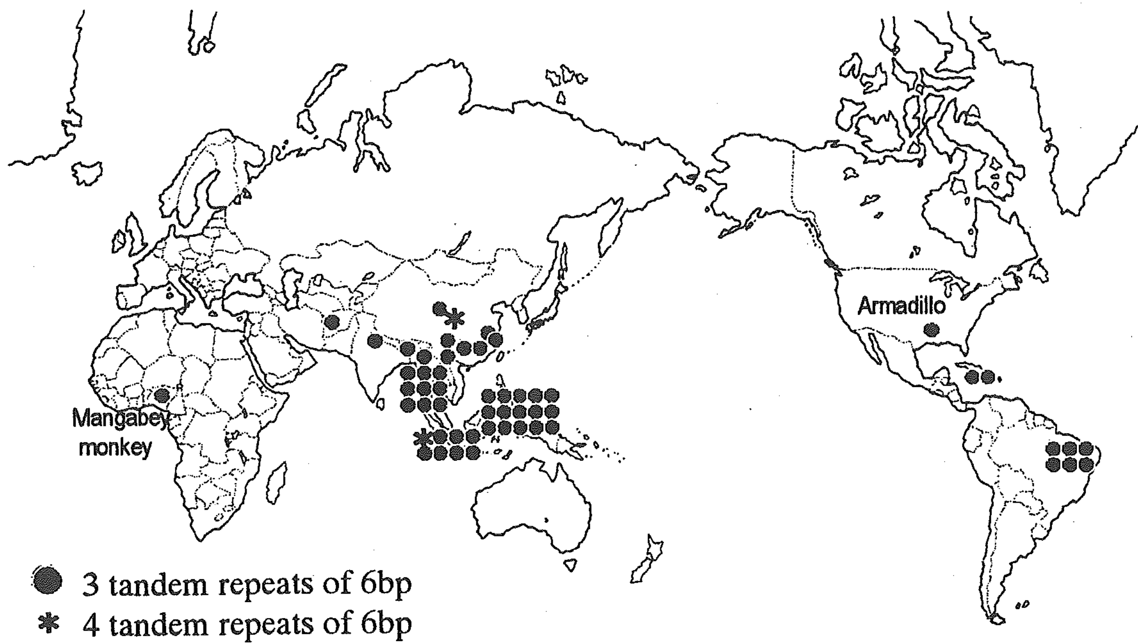

図２．世界における異なる *rpoT* 遺伝子型の分布

Molecular epidemiology of the leprosy

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Key words : Leprosy, *Mycobacterium leprae*, Genotyping, Transmission

Application of molecular biological techniques to the epidemiological study of leprosy is described. Studies of detecting *Mycobacterium leprae* DNA in samples of the nasal mucus are discussed in terms of the epidemiology and the significance of high prevalence. Epidemiological studies on the transmission of leprosy and correlation between geographic distribution of different *M. leprae rpoT* genotypes and prehistoric spread of the leprosy by genotyping based on the genomic polymorphism are introduced.

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教育講演23

ハンセン病の新患が来たら

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ハンセン病の疑いがある患者が受診したとき，どう診断を進め，治療していくかを，診察の流れに沿って説明した．これらは，ハンセン病の再発例や回復者（治癒した患者）が一般病院の皮膚科へ受診したときにも役立つと考える．

1. 皮疹の印象

ハンセン病の皮疹は多彩で，これがあればハンセン病を疑うといった単一の皮疹はない．痒みのない紅斑・低色素斑・丘疹・環状疹，肉芽腫様の小結節・結節は，一般皮膚疾患の皮疹とどこか違うという印象を与える．有痛性の紅斑は結節性紅斑と違って全身に多発し，発熱・関節痛・眼の充血を伴い，薬疹や血管炎，結節性紅斑にしては？　となる．糖尿病でもないのに熱傷や潰瘍に痛みがない，虫が這うような違和感やしびれ感，皮疹部の脱毛など，一般の皮膚疾患にはない症状がみられることがある．

皮疹部に知覚障害があるとハンセン病の疑いが強くなるが，ハンセン病の皮疹は必ずしも知覚の異常を伴わない（とくに多菌型）ことに留意しておく必要がある．

2. 問診のポイント

病歴，出身地，生活歴，家族歴，既往歴，治療歴に注意する．病歴では，主訴の症状が出る以前の初期症状を探るのが重要である．患者がハンセン病の症状と思わず，言わないこともあるので，初期に生じやすい皮疹や末梢神経障害について尋ねるようにする．

日本人の新患は沖縄出身者が多い傾向があるが，散発的にいろんな地域から出ている．外国人では，中南米の日系人や東南アジア出身者が新患の多数を占めている．日本人でもこれらの地域での生活歴がある新患が出ている．家族や周囲にハンセン病の人がいなかったか，これまでハンセン病の診断や治療を受けていないか（とくに再発や後遺症の可能性がある人，外国人）なども確かめておく．

3. 知覚の障害

末梢神経の傷害による支配領域の知覚障害と，皮疹部の知覚障害がある．触覚・温冷覚・痛覚の表在性知覚が侵され，分離麻痺もみられる．触覚を定量的に測定できるモノフィラメント，ボールペン式に0・10・50・60度を選んで調べることができる温覚計が便利である．多菌型（LL・BL）の皮疹は知覚障害がないことが多いので，皮疹に知覚があるからといってハンセン病を否定できる訳ではない．

皮疹に関わりない知覚障害があると，ハンセン病の疑いが強くなる．四肢末梢部では，皮疹の出現に先立って知覚の障害が現れることも多い．発汗低下による皮膚の乾燥，神経痛，錯知覚（知覚過敏，異常知覚など）を伴う場合がある．

4. 菌検査

皮膚の菌検査は皮疹部のほかに，菌検出の可能性が高い部位（眉間あたり，耳朶，手首伸側尺側など）でも行う．15号メス刃で長さ2ミリほど，真皮の深めまで皮膚を切開し，刃を創線と直角に立てて組織液を掻き取る．これをスライドグラスに塗りつけ，チール・ニールセン染色で染めて菌数を数えて菌指数（BI）を出す．組織液採取時に血液を混入しないこと，染色では脱色を軽くすることがコツである．組織液を取ったメス刃を70％エタノール液1ml容器に入れておくと，薬剤耐性の検査に使用できる．

菌陽性なら多菌型（MB），陰性なら少菌型（PB）になる．初めから菌陰性の型があるので，この方法で菌を検出できなくてもハンセン病を否定することはできない．

5. 組織検査

生検による組織検査ができれば，皮膚科医にとってハンセン病の診断は難しくない．できればパンチではなくメスを使って，辺縁から中心にかけて紡錘状に生検するのが望ましい．生検組織の菌検査は，チール・ニールセン染色では菌が染まりにくいのでファイト染

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色法を用いる方がよい．組織所見は，肉芽腫の性状，リンパ球の浸潤，末梢神経枝・付属器や表皮直下層の変化などを調べる．

6. 臨床検査

ハンセン病の診断を確定できる血清診断法はない．らい菌特異抗原 PGL-Ⅰへの抗体価は補助診断および病気の動きの指標として用いられている．反応性炎症では CRP が変動し，多菌型ではときに梅毒血清反応の偽陽性，免疫グロブリン上昇，自己抗体がみられる．

7. 病型を決めて治療法を選択

臨床症状と菌検査，組織所見から病型を決定し，治療薬と治療期間を設定する．病型は Ridley-Jopling 分類（LL，BL，BB，BT，TT，I）と WHO の MDT のための分類（MB，PB，SLPB）を併用する．日本のハンセン病診療ガイドラインでは，MDT に準拠しながら治療期間をより弾力的にして，臨床症状の消失，菌検査陰性化などの臨床的治癒達成まで化学療法を行うことになっている．MDT/WHO では，MB で12カ月，PB で6カ月，SLPB では1日，規定の治療を行う．

反応性病変の際には末梢神経障害，眼の炎症のコントロールを優先する．ステロイド剤，サリドマイド，NSAID，クロファジミンなどが使用される．

8. 治癒判定

MDT/WHO では一定の治療を終えたら，臨床症状があっても治癒と判定して患者登録から外すことになっている．日本では臨床症状の消失，菌検査陰性化で治癒とする．しかし患者には，顔や四肢の変形や末梢神経障害，視力障害が元のように回復してから治癒と言ってほしいとの心理があることを理解しなければならない．

9. 後遺症・合併症

さまざまの後遺症・合併症が起こるので，治療中も注意を怠らず，障害予防・障害悪化予防に努める．治癒判定後もケアが必要なことが多い．末梢神経障害による外傷，熱傷，慢性潰瘍はさらに障害度を高め，QOL を低下させるので，日常生活の指導も必要になる．

文献

1) ハンセン病治療指針：日本ハンセン病学会誌, **69**：157-177, 2000.
2) ハンセン病治癒判定基準：日本ハンセン病学会誌, **71**：235-238, 2002.
3) ニューキノロン使用指針：日本ハンセン病学会誌, **73**：65-67, 2004.
4) 医療従事者向けのハンセン病診療手引き：厚生労働科学特別研究事業，ハンセン病患者及び元患者に対する一般医療機関での医療情報提供体制に関する研究報告書, 2004.
（入手はハンセン病研究センター石井則久医師：TEL042-391-8211へ）

ニューキノロン使用指針

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キーワード：ハンセン病、ニューキノロン、オフロキサシン（OFLX）、使用指針、
多剤併用療法（MDT）

日本ハンセン病学会は、2000年に「ハンセン病治療指針」[1)]発表し、化学療法をはじめ診断と治療、後遺症の予防と治療についてのガイドラインを、2002年には、治療指針に基づいて治療を受けた患者の「ハンセン病治癒判定基準」[2)]を示した。ハンセン病の治療は、治療指針またはWHO/MDT（1997）[3)]に基づいて治療されるが、すでにMDT３薬中２薬に対しては多くの耐性報告がある。その対応策としてニューキノロン系薬であるオフロキサシン（OFLX・商品名タリビット）が多用された結果、OFLX耐性も増加してきている。厚生労働省「新興・再興感染症研究事業ハンセン病感染の実態把握及びその予防（後遺症の予防を含む）・診断・治療法に関する研究」の一環として、ニューキノロンの使用基準に関する小委員会はOFLXの耐性症例を調査しOFLX耐性の発生を防止する方法を検討した。その結果を踏まえて、小委員会はここにニューキノロンの使用指針を提示する。

１．OFLXの承認条件[4)]（2000年９月改定・第３版インタビューフォームより）

OFLXは、らい菌に対しDNA gyraseを抑制しDNA複製阻害により殺菌作用を示す。しかし*in vitro*法及びヌードマウス足蹠法では抗らい菌活性が弱いことから、厚生労働省はOFLXの保険診療の適用にあたって、効能または効果に以下の承認条件を付けた。

1）用法・用量：一日400〜600mg（４〜６錠）を２、３回に分割して経口投与する。ハンセン病については、原則として他の抗ハンセン病剤と併用すること。

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2）重要な基本的注意：承認条件；ハンセン病の治療にあたっては、本剤による治療についての科学的データの蓄積が少ないことを含め、患者に十分な説明を行い、インフォームド・コンセントを得ること。

2．OFLX耐性化の原因

OFLX耐性確認症例の調査から、耐性化の主な原因として、低用量投与と不規則服用が考えられた。低用量投与とは、たとえば100〜300mg/日/毎日、200mg/日/週2回投与などの方法である。OFLXの使用には、耐性発生防止のために次項のような点に注意をする必要がある。

3．OFLXまたはLVFXの治療効果の検討および使用基準

ハンセン病の抗菌化学療法では、他の感染症と比べ治療期間が長くなる。従って患者の理解と協力が得られるように、薬剤投与量を守ることと長期の治療期間の必要性、及び副作用についてよく説明する必要がある。

①ハンセン病の治療は「ハンセン病治療指針」に基づいて行うが、OFLXの使用が必要と判断した場合は、OFLXの単剤投与は行わず他剤との併用療法を原則とする。

②使用量については低用量投与にならないよう注意する。OFLXは最少量400mg/日の毎日投与を行うか、OFLXの一方の光学活性体であるレボフロキサシン（LVFX・商品名クラビット）を200〜300mg/日の毎日投与を行う。

③治療開始6ヶ月目に第一回の治療効果を判定する。治療開始後6ヶ月で臨床症状の改善や菌指数の低下傾向が見られないと判断した場合は、キノロン耐性を疑いキノロン耐性遺伝子検出の検査を依頼する。以降3〜6ヶ月毎に再検討を加え、使用が2年を超えないことが望ましい。菌指数は1年で1低下するのを基準として効果を判定する。

OFLX耐性が認められた場合は、DDS・RFP・B663を基本にミノサイクリン（MINO・商品名ミノマイシン）またはクラリスロマイシン（CAM・商品名クラリス）など作用機序の相違する薬剤に変更が望ましい。OFLX耐性が確認できなかった場合は、抗らい菌活性の強いスパルフロキサシン[5]（SPFX・商品名スパラ・200mg/日/毎日)またはガチフロキサシン[6]（GFLX・商品名ガチフロ、400mg/日/毎日)に変更が望ましい（この2薬剤については「使用上の注意」を参照すること）。

④ニューキノロン系薬であっても抗らい菌活性が弱いか、ほとんど活性がない薬剤もあるので注意を要する。現在まで抗らい菌活性が確認されているニューキノロン系薬はOFLX、LVFX、SPFX、GFLXである。また抗らい菌活性を持つ薬剤は限られているので新たな薬剤の導入にあたっては慎重を期し、適正使用に留意すること。

文　献

1）後藤正道・石田　裕・儀同政一・長尾榮治・並里まさ子・石井則久・尾崎元昭：ハンセン病治療指針、日本ハンセン病学会雑誌、69(157-177)2000.

2）並里まさ子・後藤正道・儀同政一・細川篤・杉田泰之・石井則久・長尾榮治・尾崎元昭：ハンセン病治癒判定基準、71(235-238)2002.

3）WHO expert committee on leprosy: seventh report, Technical report series 874, WHO Geneva 1998.

4）医薬品インタビューフォーム　タリビット錠　オフロキサシン錠　2000年9月改定（第3版）

5）Gidoh,M and Tsutsumi, S.: Activity of Sparfloxacin against *Mycobacterium leprae* inoculated into footpads of nude mice. 63:108-116(1992).

6）儀同政一: ハンセン病薬物療法の改善に関する基礎的研究、特にAM-1155, DU-6859a 及びCAMの抗らい菌活性、67(47)1998.

Guideline for the Treatment of Leprosy by New Quinolones

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Ofloxacin(OFLX) is often applied today as a substitution drug of MDT for drug resistance to dapsone, rifampicin or clofazimine. However, OFLX resistance is also becoming a great concern. Low and/or irregular administration are considered to be the major causes of OFLX resistance. OFLX should be used as a combined therapy, and minimal daily dose of 400mg of OFLX or 200~300mg of levofloxacin is required. Quinolone resistance should be considered when no improvement of clinical and/or bacterial index is observed after the treatment for 6 months. In such cases, resistance gene detection is necessary.

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Studies of Lipoproteins of *Mycobacterium leprae*

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Key words: lipoproteins, mycobacteria, host defense, cytokine

The deciphering of the genomic sequence of *Mycobacterium leprae* has made possible to predict the possible lipoproteins. The consensus sequence at the N-terminal region of the protein, including the cysteine residue to which the lipid moiety gets attached, provides a clue to the search. As such, more than 20 putative lipoproteins have been identified from *Mycobacterium leprae* genomic sequence. Lipoprotein LpK (*Accession no. ML0603*) which encodes for 371 amino acid precursor protein, was identified. Expression of the protein, in *Escherichia coli* revealed a 33 kD protein, and metabolic labeling experiments proved that the protein was lipidated. The purified lipoprotein was found to induce production of IL-12 in human peripheral blood monocytes which may imply that *M. leprae* LpK is involved in protective immunity against leprosy. Pursuit of such lipoproteins may reveal insights into the pathogenesis of the disease.

Introduction

According to World Heath Organization (WHO) epidemiological survey report, the number of leprosy patients in the world was around 534000 at the beginning of 2003, as reported by 110 countries. About 620000 new cases were detected during 2002 (http://www.who.int/lep/). Inspite of the intensive leprosy control measures taken, there is no evidence as yet of a reduction in the number of new cases [1)]. The situation implies that there is a need to develop new vaccines and immunotherapeutic tools to control the disease. Moreover there is increased concern about the disease due to the complications due to severe reactions, peripheral nerve injury due to the tropism of the bacilli to invade Schwann cells [2-4)] and emergence of drug resistant bacilli [5)].

Bacterial lipoproteins containing N-acyl diglyceride-cysteine residues, flanked by characteristic amino acids motif that are required for post-translational processing via the signal peptidase II [6, 7)], have been extensively studied in Gram-positive and Gram-negative bacteria. Membrane located 17 kD lipoprotein of *Francisella tularensis* reported by Sjosted *et al.* was found to be T cell stimulatory [8, 9)]. Lipoproteins released by pathogenic *E. coli*, *Salmonella typhimurium* and *Yersinia enterocolitica*, were found to induce proinflammatory cytokine in macrophages and ameliorate pathologic changes associated with gram negative bacterial infection in mice[10)].

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Borrelia burgdorferi and *Treponema pallidum*, the etiological agents of lyme disease and syphilis, respectively, are known to possess abundant lipoproteins [11], which act as major antagonists with the ability to influence both innate and adaptive immune responses during infection [12]. The only two well studied mycobacterial lipoproteins, are the 19 kD and 38 kD lipoproteins of *Mycobacterium tuberculosis* [13-15]. These lipoproteins are therefore presumed to be involved in the host responses, inducing interleukin-12 (IL-12) from the host cells. Since IL-12 has T cell stimulatory properties, which in turn elicits production of interferon- γ (IFN- γ), and facilitates development of Th1 cells [16-18], these lipoproteins may be involved in the induction of cellular responses to mycobacteria and thereby contributing to the development of protective immunity[19, 20]. Identification of lipoproteins in *M. leprae* seems inevitable especially in terms of host defense and for the development of new vaccines against leprosy.

Analysis of a *M. leprae* lipoprotein

To date, relatively few lipoproteins of mycobacteria have been described. The database of the *M. tuberculosis* genome (http://www.sanger.ac.uk/Projects/M_tuberculosis/) revealed that there are about a hundred putative lipoprotein coding genes, but only about 40 genes have been identified in *M. leprae* genome [21] and almost half of the genes identified are pseudogenes. Table 1 shows the list of the putative lipoproteins. One of the predictable lipoprotein was found to be partially homologous to the precursor of the glutamine binding protein, the other one was a possible transport lipoprotein and the third one was a putatitve secreted protease. But all other lipoproteins had no homology to any other protein of known function. One of the more interesting candidates is the gene annotated as *lpk* (Accession No. ML0603) [22]. The N-terminal residues of LpK showed typical features of a signal peptide with a consensus sequence (MISALMVAVAC) for the lipid modification. A sequence homologue of *lpk* was identified in the *M. tuberculosis* genome database using the BLASTN search tool. *M. tuberculosis* Rv 2413c (EMBL:AL123456, 316 amino acids) has 83.5% identity in the 316 amino acid overlap. However, the homologue has no consensus sequence for lipid modification. The fact that the lipid consensus sequence was missing is quite surprising since many of the *M. leprae* genes when compared to those of *M. tuberculosis* genes are pseudogenes as analyzed from the gene databases [21]. This fact may indicate that this lipoprotein may be specific to *M. leprae* and have a significant role in bacteria, specifically related to the unique features of the organism such as proclivity for Schwann cell invasion or development of reactions. Since it is not feasible to obtain adequate amount of protein from *M. leprae* for analyses, the gene was cloned and the protein expressed and purified in *E. coli* (Fig.1). The basic lipoprotein nature of LpK was verified experimentally. Metabolic labelling of the bacterial protein with radioactive glycerol provided presumptive evidence of a covalent linkage of lipid to LpK.

Murine experiments with infectious pathogens, indicate that IL-12 plays an important role in initiation and regulation of the T cell responses such as Th1 [23, 24]. *In vitro* experiments with *M. tuberculosis* suggested that IL-12 is induced rapidly after infection [16, 25, 26], and in *in vivo* IL-12 was crucial for the development of protective immunity against tuberculosis [27]. When we examined whether IL-12 was inducible by LpK in human monocytes, LpK induced IL-12 at a significantly high level, a level that could be maintained even in the presence of polymyxin B (Fig. 2). Another *M. leprae* putative lipoprotein (gene product of Accession No. ML1699) was expressed in inclusion bodies of *E. coli*. The purified protein, of molecular weight 39 kD, did not induce any significant amount of IL-12 in human monocytes. The reason for non-inducing capability of the purified 39 kD protein, may be the lack of lipidified region, although the exact reason remains unclear.

Discussion

M. tuberculosis 19 kD lipoprotein is both cell wall associated and secreted lipoprotein which stimulate proliferation of human T cells and promotes neutrophil priming and activation [14, 28]. It is also known to induce apoptosis in macrophages through TLR2 ligation [29]. Recently, the synthetic lipopeptide consisting of the N-terminal portion of *M. leprae* 19 kD lipoprotein is shown to induce apoptosis in human Schwann cells, also through TLR2 [30]. At present, TLR2 seems to be the only receptor known to be involved in signaling of bacterial lipoproteins and lipopeptides [31]. In likewise manner, TLR2 seems to be the receptor on antigen presenting cell, which is involved in *M. leprae* LpK lipoprotein signaling. But blocking of TLR2 with its antagonistic antibody does not completely inhibit the T cell activating ability of lipoprotein. Therefore other receptors as yet unknown, may be required for the signaling. Also, TLR2 seems to associate with TLR1 and recognise the native 19 kD *M. tuberculosis* lipoprotein and synthetic triacylated but not the diacylated lipopeptide [32, 33]. Such type of inter-related receptors may also be worth investigating.

Display of outer surface protein A (OspA) antigen as membrane associated lipoprotein by *M. bovis* bacillus Calmette-Guerin seem to be necessary for protection against *Borrelia burgdorferi* infection (Lyme disease) [34]. But there are a few reports which considers the involvement of lipoprotein deleterious to protection against disease [35]. Therefore it would be necessary to see whether the display of *M. leprae* lipoproteins could enhance host defense-associated immunity as well as serve in protection against the disease in *in vivo*.

IL-12 production in mycobacterial diseases is known to contribute to antimycobacterial defenses [17, 36, 37], by triggering of interferon- γ which, in turn, can reduce, for example, the bacillary load in lepromatous leprosy patients [16]. In this respect, we can anticipate that lipoproteins may have the potential to be used as an immunotherapeutic agent against leprosy. We may have to investigate the IL-12 inducing ability of other lipoproteins of *M. leprae* and the detailed mechanism by which the signal is transduced.

In conclusion, LpK, induced the production of IL-12 which may indicate a significant role in the induction of cellular responses leading to the development of protective immunity against the intracellular organism. Although the engagement of lipoproteins in the pathogenesis of leprosy is still to be evaluated, ongoing studies are conducted to evaluate its immunogenic role on leprosy.

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References

1) World Health Organisation. Leprosy-Global situation. Wkly Epidemiol Rec 77: 1-8, 2002.
2) Rambukkana A, Salzer JL, Yurchenco PD, Tuomanen EI. Neural targeting of *Mycobacterium leprae* mediated by the G domain of the laminin- α 2 chain. Cell 88: 811-821, 1997.
3) Ng V, Zanazzi G, Timpl R, Talts JF, Salzer JL, Brennan PJ, Rambukkana A. Role of the cell wall phenolic glycolipid-1 in the peripheral nerve predilection of *Mycobacterium leprae*. Cell 103: 511-524, 2000.
4) Job CK. Nerve damage in leprosy. Int J Lepr Other Mycobact Dis 57: 532-539, 1989.
5) Maeda S, Matsuoka M, Nakata N, Kai M, Maeda Y, Hashimoto K, Kimura H, Kobayashi K, Kashiwabara Y. Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrob Agents and Chemotherap 45: 3635-3639, 2001.
6) Wu HC, Tokunaga M. Biogenesis of lipoproteins in

bacteria. Curr Top Microbiol Immunol 125: 127-157, 1986.

7) Sankaran K, Wu HC. Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. J Biol Chem 269: 19701-19706, 1994.

8) Sjostedt A, Tarnvik A, Sandstrom G. The T-cell-stimulating 17-kilodalton protein of *Francisella tularensis* LVS is a lipoprotein. Infect Immun 59: 3163-3168, 1991.

9) Sjostedt A, Sandstrom G, Tarnvik A. Humoral and cell-mediated immunity in mice to a 17-kilodalton lipoprotein of *Francisella tularensis* expressed by *Salmonella typhimurium*. Infect Immun 60: 2855-2862, 1992.

10) Zhang H, Niesel DW, Peterson JW, Klimpel GR. Lipoprotein release by bacteria: Potential factor in bacterial pathogenesis. Infect Immun 66: 5196-5201, 1998.

11) Radolf JD, Arndt LL, Akins DR, Curetty LL, Levi ME, Shen Y, Davis LS, Norgard MV. *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytes/macrophages. J Immunol 154: 2866-2877, 1995.

12) Infante-Duarte C, Kamradt T. Lipopeptides of *Borrelia burgdorferi* outer surface proteins induce Th1 phenotype development in alphabeta T-cell receptor transgenic mice. Infect Immun 65: 4094-4099, 1997.

13) Oftung F, Wiker HG, Deggerdal A, Mustafa AS. A novel mycobacterial antigen relevant to cellular immunity belongs to a family of secreted lipoproteins. Scand J Immunol 46: 445-451, 1997.

14) Young DB, Garbe TR. Lipoprotein antigens of *Mycobacterium tuberculosis*. Res Microbiol 142: 55-65, 1991.

15) Mohagheghpour N, Gammon D, Kawamura LM, van Vollenhoven A, Benike CJ, Engleman EG. CTL response to *Mycobacterium tuberculosis*: identification of an immunogenic epitope in the 19-kDa lipoprotein. J Immunol 161: 2400-2406, 1998.

16) Zhang M, Gately MK, Wang E, Gong J, Wolf SF, Lu S, Modlin RL, Barnes PF. Interleukin 12 at the site of disease in tuberculosis. J Clin Invest 93: 1733-1739, 1994.

17) Sieling PA, Wang XH, Gately MK, Oliveros JL, McHugh T, Barnes PF, Wolf SF, Golkar L, Yamamura M, Yogi Y, et al. IL-12 regulates T helper type 1 cytokine responses in human infectious disease. J Immunol 153: 3639-3647, 1994.

18) Vordemeier HM, Harris DP, Roman E, Lathigra R, Moreno C, Ivanyi J. Identification of T cell stimulatory peptides from the 38-kDa protein of *Mycobacterium tuberculosis*. J Immunol 147: 1023-1029, 1991.

19) Ngamying M, Varachit P, Phaknilrat P, Levy L, Brennan PJ, Cho SN. Effects of vaccination with several mycobacterial proteins and lipoproteins on *Mycobacterium leprae* infection of the mouse. Int J Lepr Other Mycobact Dis 69: 43-45, 2001.

20) Fonseca DP, Benaissa-Trouw B, van Engelen M, Kraaijeveld CA, Snippe H, Verheul AF. Induction of cell-mediated immunity against *Mycobacterium tuberculosis* using DNA vaccines encoding cytotoxic and helper T-cell epitopes of the 38-kilodalton protein. Infect Immun 69: 4839-4845, 2001.

21) Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honore N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG. Massive gene decay in the leprosy bacillus. Nature 409: 1007-1001, 2001.

22) Maeda Y, Makino M, Crick DC, Mahapatra S, Srisungnam S, Takii T, Kashiwabara Y, Brennan PJ. Novel 33-kilodalton lipoprotein from *Mycobacterium leprae*. Infect Immun 70: 4106-4111, 2002.

23) Dockrell HM, Young SK, Britton K, Brennan PJ, Rivoire B, Waters MF, Lucas SB, Shahid F, Dojki M, Chiang TJ, Ehsan Q, McAdam KP, Hussain R.

Induction of Th1 cytokine responses by mycobacterial antigens in leprosy. Infect Immun 64: 4385-4389, 1996.

24) Manetti R, Gerosa F, Giudizi MG, Biagiotti R, Parronchi P, Piccinni MP, Sampognaro S, Maggi E, Romagnani S, Trinchieri G, et al. Cloning and expression of murine IL-12. J Exp Med 148: 3433-3440, 1992.

25) Fulton SA, Johnsen JM, Wolf SF, Sieburth DS, Boom WH. Interleukin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: role of phagocytosis. Infect Immun 64: 2523-2531, 1996.

26) Gately MK, Brunda MJ. Interleukin-12: a pivotal regulator of cell-mediated immunity. Cancer Treat Res 80: 341-366, 1995.

27) Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. J Exp Med 186: 39-45, 1997.

28) Neufert C, Pai RK, Noss EH, Berger M, Boom WH, Harding CV. *Mycobacterium tuberculosis* 19-kDa lipoprotein promotes neutrophil activation. J Immunol 167: 1542-1549, 2001.

29) Lopez M, Sly LM, Luu Y, Young D, Cooper H, Reiner NE. 19-kDa *Mycobacterium tuberculosis* protein induces macrophage apoptosis through Toll-like receptor-2. J Immunol 170: 2409-2416, 2003.

30) Oliveira RB, Ochoa MT, Sieling PA, Rea TH, Rambukkana A, Sarno EN, Modlin RL. Expression of Toll-like receptor 2 on human Schwann cells: a mechanism of nerve damage in leprosy. Infect Immun 71: 1427-1433, 2003.

31) Hertz CJ, Kiertscher SM, Godowski PJ, Bouis DA, Norgard MV, Roth MD, Modlin RL. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. J Immunol 166: 2444-2450, 2001.

32) Krutzik SR, Ochoa MT, Sieling PA, Uematsu S, Ng YW, Legaspi A, Liu PT, Cole ST, Godowski PJ, Maeda Y, Sarno EN, Norgard MV, Brennan PJ, Akira S, Rea TH, Modlin RL. Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. Nat Med 9: 525-532, 2003.

33) Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 169: 10-14, 2002.

34) Grode L, Kursar M, Fensterle J, Kaufmann SH, Hess J. Cell-mediated immunity induced by recombinant *Mycobacterium bovis* Bacille Calmette-Guerin strains against an intracellular bacterial pathogen: importance of antigen secretion or membrane-targeted antigen display as lipoprotein for vaccine efficacy. J Immunol 168: 1869-1876, 2002.

35) Hovav AH, Mullerad J, Davidovitch L, Fishman Y, Bigi F, Cataldi A, Bercovier H. The *Mycobacterium tuberculosis* recombinant 27-kilodalton lipoprotein induces a strong Th1-type immune response deleterious to protection. Infect Immun 71: 3146-3154, 2003.

36) Modlin RL, Barnes PF. IL12 and the human immune response to mycobacteria. Res Immunol 146: 526-531, 1995.

37) Cooper AM, Roberts AD, Rhoades ER, Callahan JE, Getzy DM, Orme IM. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. Immunology 84: 423-432, 1995.

TABLE 1. The putative lipoproteins of *M. leprae*[1]

No.	CDS Number (*M. leprae*)	No. of amino acid residues	Products
1	ML0136	233	Putative lipoprotein (lppX)
2	ML0246	218	Putative lipoprotein (lpqT)
3	ML0319	183	Putative lipoprotein (lpqE)
4	ML0489	556	Hypothetical lipoprotein
5	ML0557	238	Putative lipoprotein (lprG)
6	ML0603	371	Lipoprotein
7	ML0775	589	Putative lipoprotein (lpqB)
8	ML0902	239	Putative lipoprotein
9	ML1086	468	Probable transport protein
10	ML1093	285	lipoprotein
11	ML1099	202	Putative lipoprotein
12	ML1115	188	Possible lipoprotein
13	ML1116	187	Lipoprotein (lrpC)
14	ML1177	126	Possible lipoprotein
15	ML1315	194	Probable lipoprotein (lppK)
16	ML1339	525	Putative secreted protease
17	ML1427	445	Possible transport protein
18	ML1699	302	Putative lipoprotein
19	ML1966	161	Possible lipoprotein (lpqH)
20	ML2010	153	Putative lipoprotein
21	ML2446	441	Possible lipoprotein
22	ML2593	393	Putative lipoprotein (lprK)

[1]CDS from *M. leprae* Sanger database and number of amino acids in the prolipoprotein forms of the *M. leprae* lipoproteins are shown.

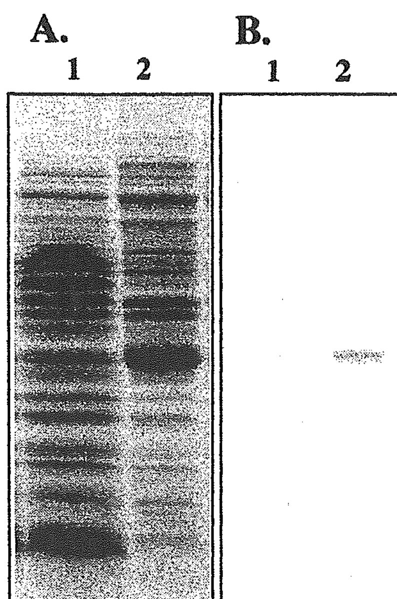

Fig. 1 : Expression and detection of *M. leprae* LpK in *E. coli*, A. Coomassie stain : 1, mock transformed and 2. *lpk* transformed *E. coli* extract. B. Western blot of the same, using monoclonal anti-His tag antibody.

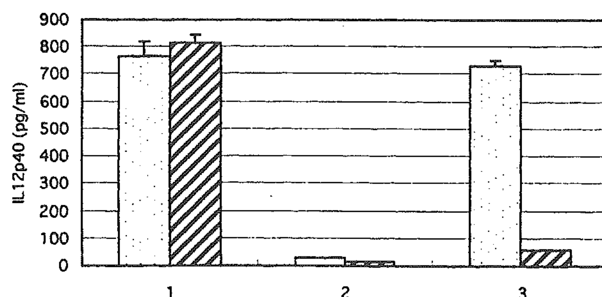

Fig. 2 : IL-12 p40 production is induced by *M. leprae* lipoprotein LpK : IL-12 p40 cytokine induction from human blood monocytes was observed using 1-LpK, 2-gene product of ML1699, 3-LPS. Hatched bar indicates the production of IL-12 p40 in the presence of polymyxin B.

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らい菌のリポ蛋白に関する研究

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ハンセン病の病原体であるらい菌の生体防御に関わる因子として、リポ蛋白に着目した。現在までに、結核菌の分子量19kDのリポ蛋白が、感染免疫反応に重要な役割をしているインターロイキン12（IL-12）を強く誘導することが報告されている。近年、らい菌のゲノムプロジェクトのデーターベースが完成され、脂質附加を受けることが予想される幾つかのリポ蛋白をコードするらい菌遺伝子を探索することができた。その結果、らい菌の33kDリポ蛋白はIL-12を強く誘導し、生体防御反応に密接に関与しているものと想定された。

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Role of the polypeptide region of a 33 kDa mycobacterial lipoprotein for efficient IL-12 production

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Abstract

Mycobacterium leprae lipoprotein, LpK, induced IL-12 production from human monocytes. To determine the components essential for cytokine production and the relative role of lipidation in the activation process, we produced lipidated and non-lipidated truncated forms of LpK. While 0.5 nM of lipidated LpK-a having N-terminal 60 amino acids of LpK produced more than 700 pg/ml IL-12 p40, the non-lipidated LpK-b having the same amino acids as that of LpK-a required more than 20 nM of the protein to produce an equivalent dose of cytokine. Truncated protein having the C-terminal 192 amino acids of LpK did not induce any cytokine production. Fifty nanomolar of the synthetic lipopeptide of LpK produced only about 200 pg/ml IL-12. Among the truncated LpK, only LpK-a and lipopeptide stimulated NF-kB-dependent reporter activity in TLR-2 transfectant. However, when monocytes were stimulated with lipopeptide in the presence of non-lipidated protein, they produced IL-12 synergistically. Therefore, both peptide regions of LpK and lipid residues are necessary for efficient IL-12 production.
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Keywords: Lipoprotein; IL-12; Mycobacteria; TLR-2

1. Introduction

Bacterial lipoproteins, containing *N*-acyldiglyceride-cysteine residues at their amino termini, have been well studied in gram-positive and gram-negative bacteria [1,2]. Acylation of the amino group of cysteine in the consensus lipid-binding sequence takes place by attachment of the diacylglycerol moiety in a thioether linkage and subsequent cleavage of the proprotein by a specific signal peptidase. One of the functional characteristics of such acylated proteins is the production of interleukin-12 (IL-12)[1] from host antigen (Ag)-presenting cells (APCs). Lipoproteins stimulated APCs and these APCs in turn activated both type 1 $CD4^+$ and $CD8^+$ T cells, to produce interferon-γ (IFN-γ), which endows bacteriocidal activities to APCs mainly macrophages. Therefore, lipoproteins play a central role as an inducer of host defense activities to control the growth of intracellular parasitic bacteria such as mycobacteria. Such lipoproteins were isolated from *Mycobacterium tuberculosis*, of which the 19- and 38-kDa proteins have been reported to be capable of activating both innate and adaptive immunity [3–5]. There are only a few reports of lipoprotein from other mycobacterial species, but recently we have identified a novel 33 kDa lipoprotein, LpK, from *Mycobacterium leprae* [6].

Mycobacterium leprae induce a chronic infectious disease, termed leprosy which has been characterized by

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[1] *Abbreviations used:* IL-12, interleukin-12; M., *Mycobacterium*; Ag, antigen; APC, antigen-presenting cell; IFN, interferon; PVDF, polyvinyl difluoride; PBMC, peripheral blood mononuclear cell; LPS, lipopolysaccharide; PG, peptidoglycan; TLR, toll-like receptor; DCs, dendritic cells.

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progressive peripheral nerve injury and skin lesions [7]. One representative spectrum of the disease is a paucibacillary form of leprosy, in which the disease lesion is localized. The localization of the lesion is a consequence of the suppression of bacterial spread and, in this process, IL-12 producing APCs seem to play a central role in activating innate and type 1 cellular immunity [8–11]. Since the newly identified lipoprotein LpK was found to be capable of inducing IL-12 production in human peripheral monocytes, it can be predicted that LpK is one of the antigens in *M. leprae* with the potential to contribute to the host defense against leprosy.

Although it may be assumed that the immuno-dominant region of the lipoprotein is the lipid region, the immuno-dominant region of LpK in terms of IL-12 production has not been studied, and it remains to be clarified whether the acylated lipopeptide region alone could represent the immuno-stimulatory domain of the lipoprotein.

In this study, we expressed various forms of truncated LpK, assessed its IL-12 producing activity and attempted to clarify the role of peptide lipidation in the context of cytokine production.

2. Materials and methods

2.1. Bacterial strains, plasmids, and lipopeptides

Escherichia coli DH5α strain (Toyobo, Tokyo, Japan) was used for all cloning and recombinant expression experiments. The plasmids used for the expression in *E. coli* were pGEM-T Easy Vector (Promega, Madison, WI), and pGFPuv (Clontech, Palo Alto, CA). Clones were selected on Luria–Bertani medium agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) or broth supplemented with ampicillin at 100 μg/ml. All other chemicals were purchased from Wako Chemicals (Richmond,VA), Sigma–Aldrich (St. Louis, MO) or Amersham–Pharmacia (Piscataway, NJ). The LpK lipopeptide containing the N-terminal 12 amino acids of LpK was synthesized by Bachem AG (Germany). The structure of the lipopeptide is as follows: Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Leu-Pro-Asp-Trp-Leu-Ser-Gly-Phe-Leu-Thr-Gly-Gly-OH. The corresponding unlipidated LpK peptide containing only the N-terminal 12 amino acids was also synthesized.

2.2. Cloning and sequencing of the truncated forms of the lpk gene

To clone the *lpk* gene, the DNA of interest was amplified by PCR by taking the genomic DNA from *M. leprae* (Thai-53 strain) as a template for PCR, and the expressed LpK lipoprotein was purified as previously described [6]. The primers used for the amplification of the gene coding protein constructs in Fig. 1 were as follows: For LpK-a, the sense primer 5′ACATGCA TGCCCTGGTGTTGGTCCTGTGG3′ (a-s) and the antisense primer 5′CGGAATTCTTAGTGATGGTGA TGGTGATGGCCTGCCCGCTGCCG3′ (a-as) were used. For LpK-b amplification, primers 5′ACATGCA TGCCCTGTTGCCTGATTGGTTGT3′ (b-s) and the antisense primer a-as were utilized. Similarly, for LpK-c, the sense primer used was a-s and antisense 5′GGAA TTCTTAGTGATGGTGATGGTGATGGCTAAGCT TAGTGATCC3′ (c-as), for LpK-d, primers used were b-s and c-as. LpK-e utilized the sense primer 5′ACAT

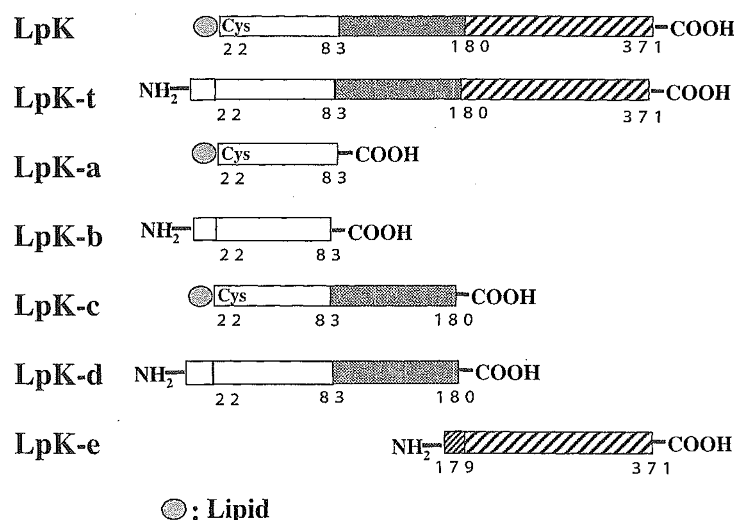

Fig. 1. Schematic representation of the constructs of LpK and truncated LpK. The lipidated constructs are LpK, LpK-a, and LpK-c. Non-lipidated constructs include LpK-t, -b, -d, and -e. The thatched region indicates the C-terminal half of the LpK protein. Numbering shows the position of the amino acid of LpK in the prolipoprotein form.

GCATGCCCTTAGCGAGCGTACTGA3′ and the previously described antisense primer for LpK amplification [6]. For LpK-t, the sense primer a-s and the same antisense primer for LpK amplification was used. All antisense primers contained the histidine tag coding sequence at the C-terminus of the protein for easy protein detection. The gene was first cloned into pGEM-T Easy Vector (Promega), and further inserted into the expression vector. All other genetic manipulations were done according to established cloning techniques [12]. All lipidated and non-lipidated *lpk* genes were expressed in *E. coli.* Restriction enzymes were purchased from New England Biolabs (Beverly, MA), Takara Shuzo (Shiga, Japan) or Toyobo (Osaka, Japan) and used according to the manufacturer's specifications. For DNA sequencing, plasmid DNA samples were purified using a Qiagen MiniPrep Kit (Qiagen, Valencia, CA). DNA sequence analysis was performed on an ABI Prism Genetic Analyser (PE Biosystems, Foster City, CA) using the dideoxy dye termination PCR method.

2.3. Detection of the expressed proteins and protein purification

Escherichia coli transformants were lysed in 6 M urea, 0.5% CHAPS, and 1 mM DTT containing 50 mM Tris–Cl and run on a 12% SDS–polyacrylamide gel [13]. The resolved proteins were transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA). The proteins were then detected using penta-His mAb (Qiagen), and color developed with 5-bromo,4-chloro, 3-indoylphosphate/nitroblue tetrazolium (BCIP/NBT). The overexpressed protein was also gel filtrated through a HiLoad 26/60 Superdex 75 prep grade column (Amersham–Pharmacia), using buffer containing 6 M urea, 50 mM Tris–Cl (pH 8.0), and 0.1% CHAPS at a flow rate of 2 ml/min. After collecting around 30 fractions, SDS–polyacrylamide gel electrophoresis was performed; the proteins were stained either by Silver Stain 'Daiichi' (Dai-ichi Pure Chemicals, Tokyo, Japan) or Coomassie blue brilliant stain. Western blotting was performed using a penta-His mAb. The fraction containing the desired protein was used for further evaluation. By SDS–PAGE of the protein and further staining with a silver stain, no apparent contamination of *E. coli*-derivatives was observed. The concentrations of LpK and its mutant proteins were determined using a Bio-Rad Protein Assay kit according to the manufacturer's instructions.

2.4. Measurement of IL-12 production by human PBMC

Human PBMCs from healthy individuals were isolated on Ficoll–Paque Plus (Amersham–Pharmacia, Upsala, Sweden) and cultured for 1 h in 10 cm dishes. The non-adherent cells were removed by washing several times with RPMI 1640 (Sigma) containing 2% FCS. By flow cytometric analyses, among the plastic adherent cells, 95–98% of the cells were CD14 positive. T cells and B cells constituted less than 1% and $CD1a^+$ dendritic cells constituted less than 0.1% of the adherent cell population. These adherent cells were then detached and cultured in triplicate in 96-well plates (10^5 cells/well) with purified lipoproteins at various concentrations. Twenty to twenty-four hours later, the culture supernatants were collected and assayed for human IL-12 p40 production using an OptEIA Set (Pharmingen, SanDiego, CA). The amount of lipopolysaccharide (LPS) in the purified lipoprotein was measured quantitatively with a Limulus Amoebocyte Lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be < 10 pg/μg protein, an amount that did not stimulate IL-12. Also, the contribution of $CD1a^+$ dendritic cells within the plastic adherent cells in the IL-12 production was examined. No significant difference in the cytokine production was observed by depleting the $CD1a^+$ cells using immunomagnetic beads (Dynabeads 450, Dynal, Oslo, Norway).

2.5. Cell transfection and luciferase assay

Human embryonic kidney cells (HEK293) were obtained from the American Type Cell Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% FCS, 50 mg/ml penicillin/streptomycin, and non-essential amino acids (Invitrogen, Carlsbad, CA), at 37 °C in a humidified incubator of 5% CO_2. The cDNA of human Toll-like receptor 2 (TLR) was PCR-amplified using a human spleen cDNA library (BD Biosciences, San Jose, CA) and inserted into pCIneo (Promega, Madison, WI). HEK293 cells (2×10^4) were transiently transfected with a mixture of plasmids: 200 ng pCIneo hTLR2, 25 ng p5× NF-kB-luc (Stratagene, La Jolla, CA), and 10 ng pRL-TK-*Renilla* luciferase plasmid (Promega) using the FuGENE 6 reagent (Roche molecular Biochemicals, Indianapolis, IN), as previously described [14]. Thirty-six hours after transfection, cells were treated with or without various amounts of LpK and its truncated forms, or peptidoglycan (PG) as positive control (for TLR2-dependent luciferase activity) for further 6 h. The cells were lysed in 70 μl of 1× passive lysis buffer (Promega) and luciferase activity in 10 μl of the cell lysate was measured using Promega Dual-Luciferase Reporter Assay System according to the protocol provided by the manufacturer. Data were expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency.

2.6. Statistical evaluation

The Student's *t* test was applied to reveal statistically significant differences.