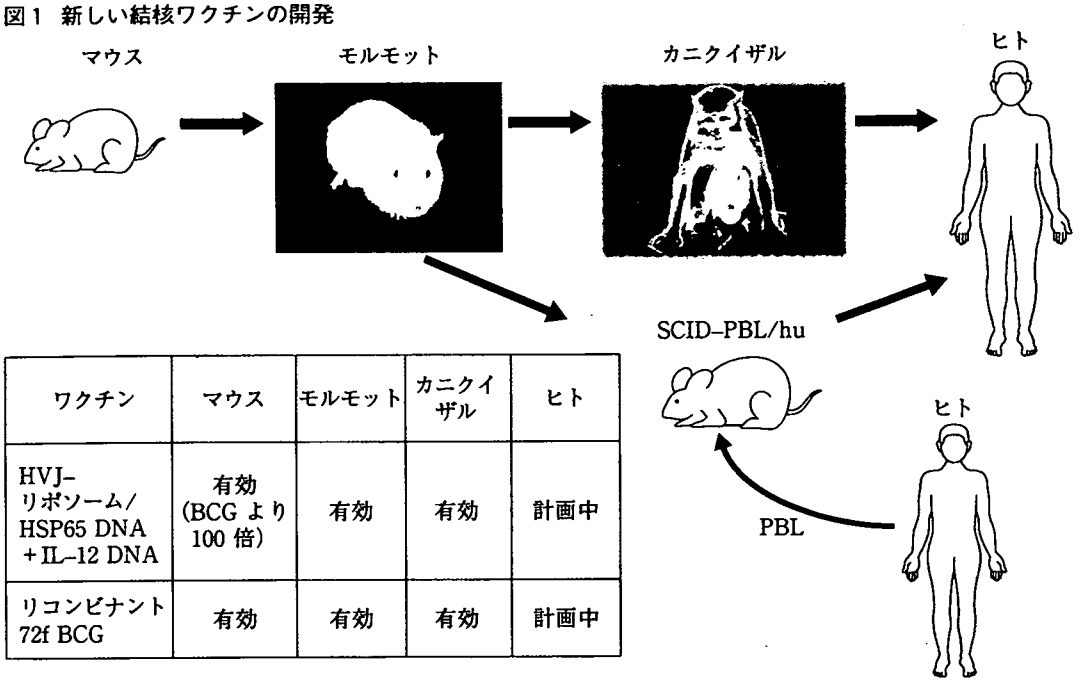
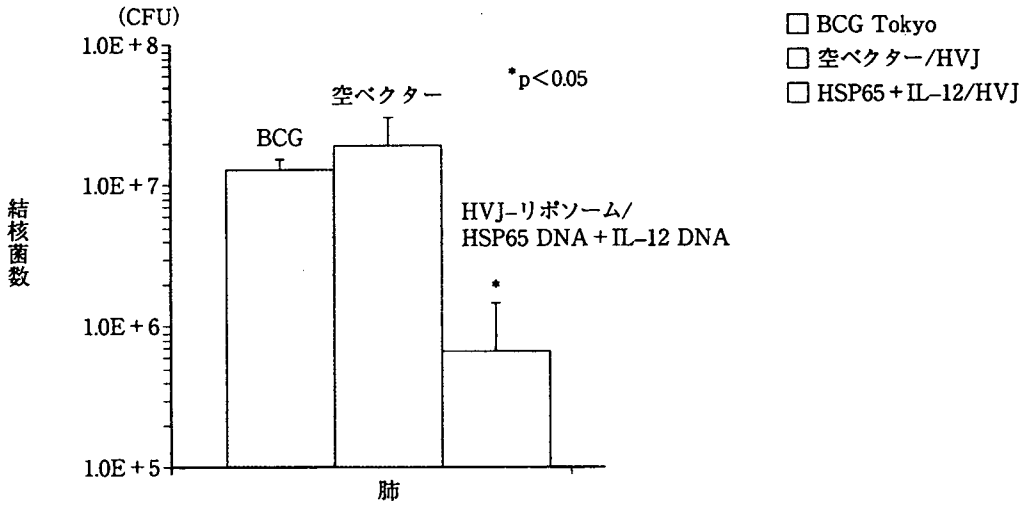
図1　新しい結核ワクチンの開発



ワクチン	マウス	モルモット	カニクイザル	ヒト
HVJ-リポソーム/HSP65 DNA+IL-12 DNA	有効(BCG より100 倍)	有効	有効	計画中
リコンビナント72f BCG	有効	有効	有効	計画中

図2　HVJ-リポソーム/HSP65 DNA＋IL-12 DNA ワクチンによる結核予防ワクチン効果
(結核感染5週後)

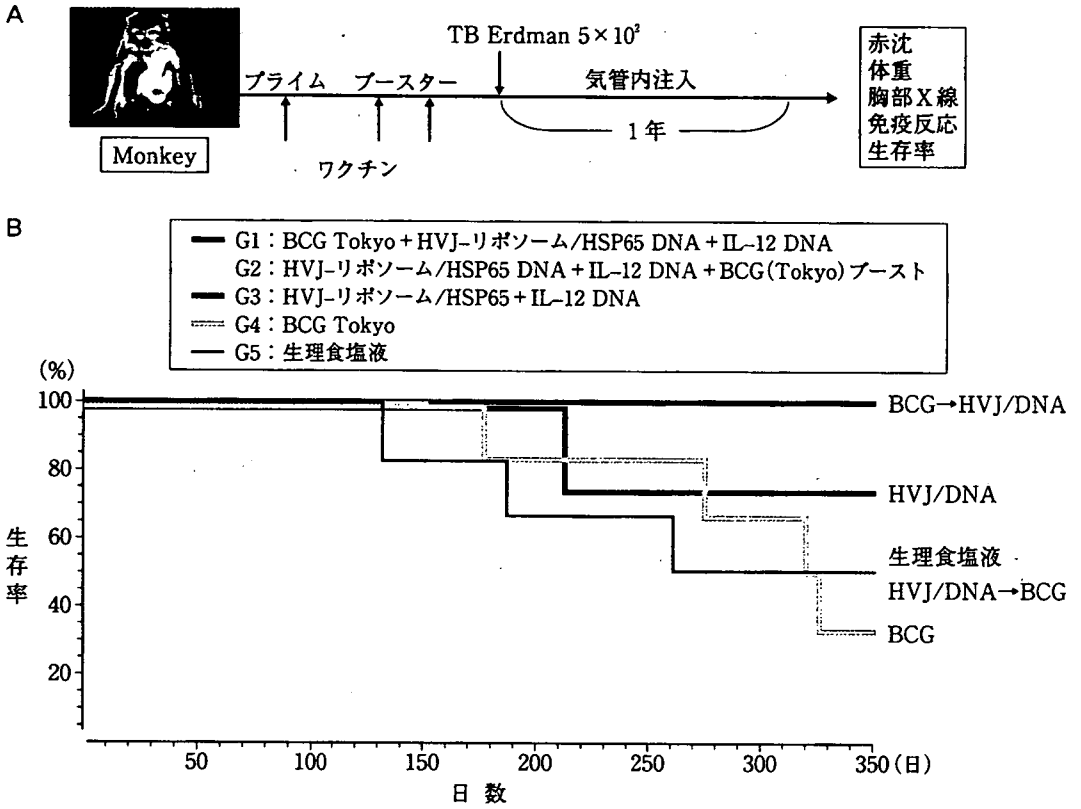


クチン効果を示した[1]．

2）リコンビナント BCG（rBCG）ワクチン

サブユニットワクチンの Mtb72f 融合タンパク質の DNA を導入した 72f rBCG の作製に成功した[4]．この 72f rBCG は，マウス，モルモットの系のみならず，カニクイザルを用いた系でも結核予防ワクチン効果を示した[4]．

図3 ヒトの結核感染に最も近いカニクイザルを用いたHVJ-リポソーム/HSP65 DNA＋IL-12 DNAワクチンの結核予防効果



4．新しいヒト生体内抗結核免疫解析モデル SCID-PBL/hu

我々が世界に先駆けて開発したSCID-PBL/huの系でも，ヒトCTL分化を介して結核予防ワクチン効果を示した[10][11]．

結核ワクチンの展望

1．新しい結核ワクチンの臨床応用

カニクイザル（cynomolgus monkey，最もヒトの肺結核に近いモデル[4][8][12][13]）を用い，BCGよりもはるかに強力な予防ワクチン効果（生存率，赤沈，体重，肺の組織）を示すワクチン2種を開発した[4]．我々はカニクイザルに対し，結核感染後1年でコントロール群（生理食塩液投与群）では4匹中4匹死亡（0％生存）したが，HSP65 DNA＋IL-12 DNAワクチン投与群では4匹中2匹生存（50％生存）を認め，ワクチン効果をサルのレベルで認めた[4]．すなわち，HVJ-リポソーム/HSP65 DNA＋IL-12 DNA予防ワクチン投与による結核感染カニクイザル生存率改善効果を得た．また，赤沈改善効果を有意差をもって示した．さらにこのワクチンを投与したカニクイザルでは，コントロール群に比較し有意差（$p<0.01$）をもってHSP65抗原に対して増殖増強反応を示した．72f融合タンパク質サブユニットワクチン，ワクシニアウイルスに85A DNAを導入したワクチンやr85B BCG（Horowitzら）は，第I相臨床試験となっている[14][15]．最も切れ味のするどい臨床応用ワクチン候補の筆頭として，HSP65 DNA＋IL-12 DNAワクチンが挙げられる[4][5][13]．

2．プライミングーブースター法
（乳幼児 BCG－成人 HVJ/HSP65 DNA＋IL-12 DNA ワクチン）

さらに BCG ワクチンをプライミングし，新しいワクチンをブースターする方法を用いた．このプライミングーブースター法で 100% の生存を示した[13]（図3）．一方，BCG ワクチン単独投与群は 33% の生存率であった[13]．このように，ヒトの結核感染に最も近いカニクイザルを用いた実験系で，強力な新しい結核ワクチンを我々は世界に先駆けて開発した．すなわち，本邦では乳幼児に BCG 接種が義務づけられていることより，プライミングワクチンとして BCG ワクチンを用い，成人ワクチン（中学生，成人，老人）として我々が開発した切れ味のするどいこの DNA ワクチンをブースターワクチンとして用いることにより，強力な新しい結核ワクチンの臨床応用が可能となる案を計画中である．

おわりに

HSP65 DNA＋IL-12 DNA/HVJ－エンベロープワクチンが明らかに優れていることより，このワクチンが結核の発症予防や治療に役立つ日が来るであろう．

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New Development of Novel Vaccines against Tuberculosis

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解説[I]

新しい結核ワクチン

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結核は世界の人口の1/3約20億人が感染し，900万人が毎年結核を発症し，200万人が死亡している世界の最大の感染症である．

BCGワクチンは，成人に対する結核予防ワクチンとしては有効でない．したがって，新しい結核ワクチン開発を行った．HSP65DNA＋IL-12DNAワクチンは，BCGよりも1万倍強力な結核予防ワクチン効果をマウスで示した．さらに，ヒトの結核感染に最も近いモデルのサルにも有効であり，臨床応用を計画中である．一方多剤耐性結核(特に超薬剤耐性結核XDR-TB等)が大問題となっている．

この結核ワクチンは，結核治療効果も示し，多剤耐性結核にも有効性が示唆された．他の結核ワクチン開発(r72fBCG等)についても述べる．

はじめに

いまだに世界の1/3の20億人が結核菌に感染しており，その中から毎年880万人の結核患者が発症し，200万人が毎年結核で死亡している，最大の感染症の一つである(WHOレポート2002年)[1~4]．本邦でも1998年から結核罹患率の増加・横ばいが認められ，1999年"結核緊急事態宣言"が厚生省より出された．結核症に対する宿主の抵抗性細胞性免疫といって過言ではない．特に獲得免疫(キラーT細胞とTh1ヘルパーT細胞)が重要であり，最近では自然免疫の結核への関与が再び重要視されている．1998年，米国CDCは結核に対し，政府・学術機関・企業が一体となって新世代の結核ワクチン開発の必要性を強く主張する発表をした．また，ACETは国民の健康に対する大敵である結核撲滅のためには，BCGに代わる有効なワクチンが必要であることを示した．しかしながら，BCGに代わる結核ワクチンは欧米でも臨床応用には至っていない．われわれはBCGよりもはるかに強力なDNAワクチンやリコンビナントBCGワクチンの開発に成功した(**表1**，**図1**)[5~8]．新しい抗結核ワクチン開発と結核感染免疫におけるキラーTの機能解明についても述べる[9, 10]．

Ⅰ．新しい結核ワクチン開発

1．現行のBCGワクチンの有用性

現行のBCGワクチンのWHO評価：BCGワクチンの評価がWHOによりなされた．すなわち大人(成人)の結核に対しては，BCGワクチンは予防効果がないという結論がWHOによって報告された．10万人を超す南インド農民を対象として実施された大規模なcontrolled trial(Chingleput study)では，全く有効性が否定される結果となった(上記WHOの報告)[10]．(ただし，小児における結核性髄膜炎や粟粒結核など播種性のものには十分な予防効果がある．)したがって，成人の結核に対し有効な新しい結核ワクチンの開発が必須である．事実1998年米国政府・ACET，CDCは政府，研究所，大学・企業の三者が一体となって，新しい結核ワクチン開発が必須であることを表明した[1, 6]．

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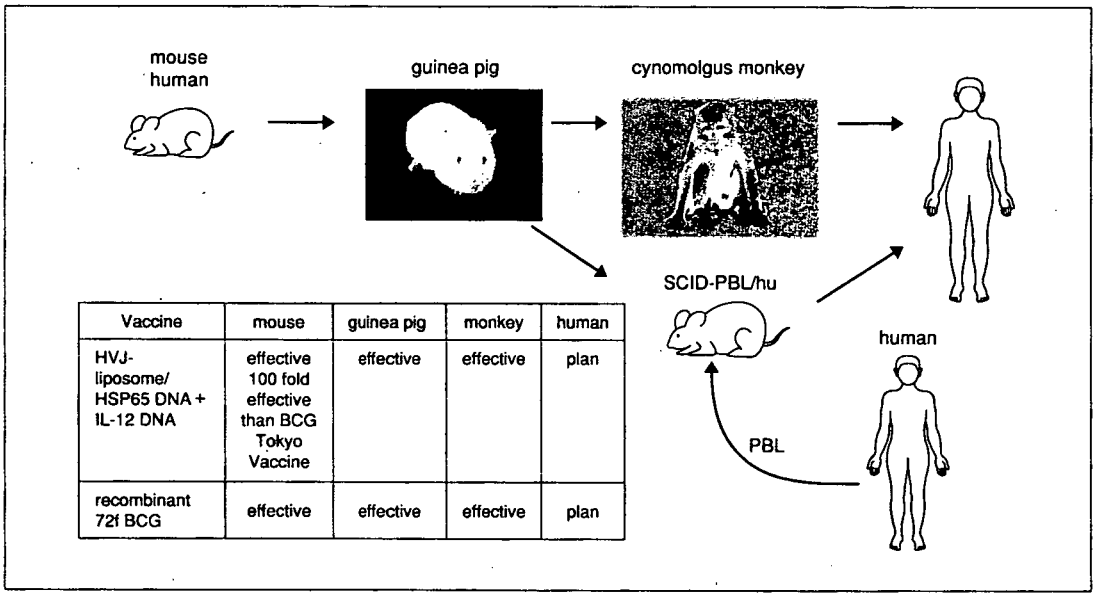
表1 The Development of Novel Vaccines for M. tuberculosis

(1) DNA vaccine HVJ-liposome/HSP65 DNA + IL-12 DNA	more effective than BCG (mouse, guinea pig, cynomolgus monkey)
(2) DNA vaccine HVJ-Envelope/HSP65DNA + IL-12 DNA	extremely stronger effect than BCG
(3) recombinant BCG vaccine ① recombinant 72f BCG ② recombinant (Ag85A + 85B + MPB51) BCG	more effective than BCG (mouse, guinea pig, cynomolgus monkey) more effective than BCG (mouse)
(4) Therapeutic vaccine IL-6 related DNA (mouse)	
(5) Priming-Booster Method BCG (priming) + Novel vaccine (booster) (cynomolgus monkey)	
(6) Novel vaccine (per os) using gene-knock out attenuated Listeria	
(7) Novel vectors AAV vector (1000 fold effective expression vector ↑), Adenovirus vector	

Selected as WHO STOP TB Partnership and WHO STOP TB Vaccines Working Group



Vaccine	mouse	guinea pig	monkey	human
HVJ-liposome/ HSP65 DNA + IL-12 DNA	effective 100 fold effective than BCG Tokyo Vaccine	effective	effective	plan
recombinant 72f BCG	effective	effective	effective	plan

図1 The Development of Novel Vaccines for M. tuberculosis using animal models

2. BCGワクチンより1万倍強力な結核予防ワクチン

BCGワクチンより1万倍強力な結核予防ワクチンを開発

われわれの国立病院機構近畿中央胸部疾患センター臨床研究センターが，画期的な結核の新しいワクチンを開発した．マウスの実験で現行のBCGワクチンを超える極めて強力な有効性(1万倍の効果)を確認した．マウスの結核感染系ではBCGワクチンをはるかに凌駕する新しい結核ワクチンは極めて少ない．われわれは，HSP65 DNA + IL-12 DNA(HVJ-エンベロープベクター)のワクチンはBCGワクチンよりも1万倍強力な結核予防ワクチンであることを世界に先駆けて明らかにした．

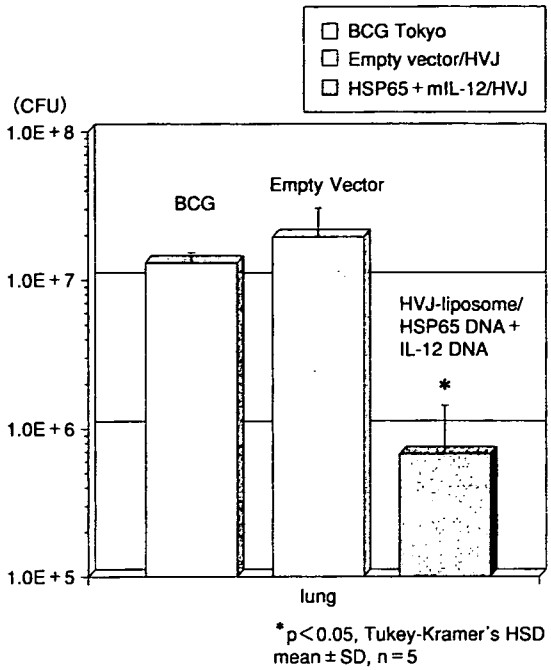


図2 Prophylactic efficacy of HVJ-liposome/HSP65 DNA+IL-12 DNA vaccine on TB-infected mice (5 weeks after TB infection)
Number of M. tuberculosis

これらの研究が国内外より極めて高く評価され，当臨床研究センターはWHO(世界保健機関)よりWHO STOP TB Partnershipに選ばれた．また，大阪大学大学院(医学系研究科)・連携大学院にも選ばれた(**表1**)．

3. 新しい結核ワクチン

結核ワクチンは①サブユニットワクチン，②DNAワクチン，③リコンビナントBCGワクチン(弱毒化結核菌を含む)，その他に大別される．

マウスではBCGワクチンをはるかに凌駕する新しい結核ワクチンは極めて少ない．われわれはHSP65 DNA+IL-12 DNA予防ワクチンにてBCGワクチンの100倍強力なワクチンの開発に成功した(**表1**，**図1**，**2**)[6, 7, 9, 11]．

1) DNAワクチン

われわれが開発した，新しい結核ワクチン，新ワクチンは，前述の如く，結核菌のHSP65というたんぱく質と免疫力を高める働きのあるインターロイキン-12を作る遺伝子(DNA)を注射するDNAワクチンと呼ばれるものである．HVJウイルスの殻を利用して，DNAを体内の細胞内に送り込んでこれらのたんぱく質を作らせ，強い免疫反応の誘導を狙った．この新しいワクチンは，結核免疫に最も重要と考えられている(結核菌に対する)CD8陽性キラーT細胞の分化を増強した．さらにIFN-γ産生T細胞の分化を増強した．

マウスの実験系：マウスに新ワクチンを接種した後，結核菌を感染させ，5週間後の結核菌の数を調べた．すると，新ワクチンを接種したマウスの菌数はBCG接種のマウスの約1千分の1で発症を抑えられる程度だった．さらに，あらかじめBCGを接種してから新ワクチンを打つと，菌数は約1万分の1まで抑えられていた．

新しい結核ワクチンの開発研究が高く評価され，WHO STOP TB VACCIENE GROUP MEETINGに選出された．

2) リコンビナントBCGワクチン

結核菌は300種以上のタンパク質を分泌するが，α抗原Ag 85Bとそのファミリー(85A，Ag85C)DNAをリコンビナントBCGに使用した[2, 5]．これらの遺伝子をPNN2シャトルベクター(大腸菌⇔好酸菌)に組み込みBCG東京菌に，遺伝子を導入した．

最近，サブユニットワクチンのMtb72f融合タンパク質[12]のDNAを導入した72fリコンビナントBCGの作製に成功した．この72f rBCGは，BA51 rBCGと同程度の極めて強力な結核菌に特異的なIFN-γ産生T細胞数の増強を誘導することをElispot Assayで明らかにした．

4. 新しいヒト生体内抗結核免疫解析モデルSCID-PDL/hu

われわれが世界に先駆けて開発したSCID-PBL/huの系で結核患者リンパ球をSCIDマウスに生着させ，結核菌タンパク質に特異的なヒトキラーT細胞誘導を示す画期的な，生体内ヒト免疫解析モデル(ヒト結核ワクチン効果解析モデル)を開発した[6, 7, 9]．

表2 Improvement of cynomolgus monkeys infected with M. tuberculosis by the vaccination with HVJ-liposome/HSP65 DNA+IL-12 DNA

Vaccine	number	survival	dead	%survival
HVJ-liposome/ HSP65DNA+ IL-12DNA	4	2	2	50%
BCG	4	2	2	50%
Control (saline)	4	0	4	0%

Ⅱ. 結核ワクチンの展望

1. 新しい結核ワクチンの臨床応用

カニクイザル(cynomolgus monkey, 最もヒトの肺結核に近いモデル. Nature Medicine 2, 430, 1996参照)を用いBCGよりもはるかに強力な予防ワクチン効果(生存率, 血沈, 体重, 肺の組織)を示すワクチン二種を開発した[8]. すなわち, 現在最も有力なものとして, HVJリポソーム/HSP65 DNA+IL-12 DNAワクチンおよび, r72f BCGワクチンがあげられる(表1). すなわち, カニクイザルに3回ワクチン投与を3週間隔で行った. 最終免疫より, 4週間後にヒト結核菌エルドマン株 5×10^2CFUを気道内注入した. 事実, われわれはカニクイザルで結核感染後1年で, コントロール群(生食投与群)では4匹中4匹死亡(0%生存)したが, HSP65 DNA+IL-12 DNAワクチン投与群は, 4匹中2匹生存(50%生存), r72f BCGワクチンで4匹中3匹生存(75%生存)を認め, これらのワクチン効果をサルのレベルで認めた[8] (表2). すなわち, HVJリポソーム/HSP65 DNA+IL-12 DNA予防ワクチン投与による結核感染カニクイザル生存率改善効果を得た(表2). また, HSP65 DNA+IL-12 DNAワクチンは血沈改善効果を有意差をもって示した. さらに, このワクチンを投与したカニクイザルでは, コントロール群に依存し有意差($p<0.05$)をもって, HSP65抗原に対し, 増殖増強反応を示した[8]. Ag85B-ESAT-6融合タンパク質についてAndersonらも報告しているが, モルモット, サルでは効果は不明である. 一方,

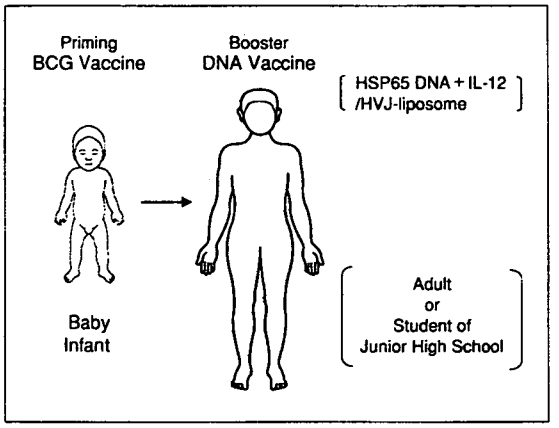


図3 Novel Prophylactic Vaccine(DNA Vaccine against TB)

HuygenのAg85A DNAワクチンはマウス・モルモットで有効であったが, サルの結核感染予防に対し有効でなかったという. 72f融合タンパクサブユニットワクチン, ワクシニアウイルスに85A DNAを導入したワクチンやr85B BCG(Horowitzら)は第Ⅰ相clinical trialとなっている[12]. A. Hillらのワクシニアウイルス-85A DNAワクチンは, アフリカでの第一相clinical trialでは, 85A DNA蛋白に対する免疫応答増殖が認められた. 最も切れ味のするどい臨床応用ワクチン候補の筆頭として, HSP65 DNA+IL-12 DNAワクチンがあげられる. リコンビナント72f BCGも有効である. さらに, われわれはHSP65 DNA+IL-12 DNAワクチンやリコンビナント72f BCGワクチンを組み合わせ, 極めて強力なワクチン開発を目指している[5~7].

2. プライミング-ブースター法(乳幼児BCG-成人HVJ/HSP65DNA+IL-12DNAワクチン)

さらにBCGワクチンと新ワクチンのプライミング-ブースター法で100%の生存を示した. このように, ヒトの結核感染に最も近いカニクイザルを用いた実験系で, 強力な新しい結核ワクチンを, われわれは世界に先駆けて開発した. すなわち, 本邦では乳幼児にBCG接種が義務づけられていることにより, プライミングワクチンとしてBCGワクチンを用い, 成人ワクチン(小学生, 中学生,

成人，老人)として，切れ味のするどいわれわれが開発したHVJ/HSP65 DNA＋IL-12 DNAワクチンをブースターワクチンとして用いることにより，強力な新しい結核ワクチンの臨床応用が可能となる案を計画中である(図3)．

おわりに

当国立病院機構近畿中央胸部疾患センターは，呼吸器疾患(結核を含む)準ナショナルセンターとなった．日本の結核患者数の60%の診断・治療を行っている，国立病院・療養所54施設を統括し，国立病院・療養所政策医療呼吸器ネットワークを用いて結核の新しい予防・治療法の確立が進展している．

サルにおいては，HSP65 DNA＋IL-12 DNA/HVJ-エンベロープワクチンが明らかにすぐれていることより，このワクチンが結核の発症予防や治療に役立つ日が来るであろう．

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学会レポート

第5回国際ワクチン学会
FIFTH WORLD CONGRESS ON VACCINES, IMMUNIZATION AND IMMUNOTHERAPY

独立行政法人国立病院機構近畿中央胸部
疾患センター・臨床研究センター・臨床研究センター長

岡田　全司

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学会
レポート

第5回
国際ワクチン学会

FIFTH WORLD CONGRESS ON VACCINES, IMMUNIZATION AND IMMUNOTHERAPY

■
独立行政法人国立病院機構近畿中央胸部疾患センター・臨床研究センター・臨床研究センター長*
岡田全司

第5回国際ワクチン学会(WC-VII；Fifth World Congress on Vaccines, immunization and immunotherapy)は2006年11月6日から9日にかけ，モントリオール大学医学部Edouard Kurstak教授をWCVII会長として，カナダ・モントリオール市フェアモント・クイーン・エリザベスホテルを会場として開催された．

プレナリーセッション4テーマ・28題，シンポジウム8テーマ・67題，ラウンドテーブル1テーマ・3題，ポスター14題と多くの演題数であった．

参加者数は約400名にのぼり，世界各国からの参加者であった．

主催国カナダ・ナショナル・チームとして，グリフィス博士などが参加，当学会委員会メンバーとして，ウェール医大・アダムス教授，米国・ブラウン大学・DeGroot教授，日本・岡田全司，米国・Mayo Clinic・G.A. ポーランド教授，米国・メルク社研究所長・シバー博士らが参加した．

本学会のテーマは「新しいワクチンの臨床開発研究，高免疫原性かつ安全なワクチン・免疫療法(遺伝子工学を活用した)」であり，まさしくこのテーマに沿った極めて興味深い発表が数多くなされた．

ウェルカム・オープニング・リマークでは有名なポーランド博士が，現在世界中を震撼させているトリ・インフルエンザワクチンについてレビューした．トリ・インフルエンザA/H5N1が200例以上，世界でヒトに感染し，50%致死率．pandemic感染の危険性を示し，さらに新しいA/H5N1の出現も報告した．米国で200万人以上が死亡し，全労働者の40%がインフルエンザに罹患することを計算した．

1．プレナリー・セッション「新規ワクチンと免疫方法」において

(1) シバー博士は非増殖性・非成果性のアデノウィルスベクター5型を用いたHIV-2ワクチンを開発し，臨床応用中である．

(2) コレラ，腸チフス，髄膜炎菌，麻疹，ウエストナイルなどに対する優れたワクチンの発表が多くのグループよりなされた．

(3) アミロイド-βペプチド(Aβ)のN末アミノ酸1-14をアルツアハマー病の治療ワクチンとして開発中．

(4) ヒト子宮頸癌の原因のヒトパピローマウイルス16型，18型，のカプシド蛋白のウイルス様粒子(VLPs)が作製され，10年間の臨床治験の結果，子宮頸部の癌化を抑制した．同様の報告がアダムス博士などにより第Ⅰ・Ⅱ相臨床試験を行い，子宮頸癌予防に95～100%の効果を得た．

2．シンポジウム〔遺伝子ワクチン，免疫修飾(活性)物質，デリバリーシステム〕において

DNAワクチンはマウスなどの小動物に強い免疫原性を示すが，non-human primateモデルにおいても免疫原性が同じか否かまだ解析されていない．したがって，ワイナー博士は，サルのモデルで種々のサイトカインIL-1～IL-27のDNAワクチンの中で，IL-12 DNAワクチンがサルの系で最も強力にキラーT細胞やヘルパーT細胞抗体産生を誘導した．また，IL-15

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DNAワクチンも優れていた．また，ベクターとしてサルの系ではアデノウィルスベクターが極めて優れていた．

・田代博士は日本のヒトインフルエンザH5N1ワクチン開発とclinical trialについて発表した．奥野博士らはモノクローナル抗体を作製し，これが他の多くの型のインフルエンザに対しても中和活性を示した．

3. [シンポジウム6：新興・再興感染症に対するワクチン]において

筆者はアエラス・グローバルTBワクチン財団のJ.C. Sadoff博士と二人でこのシンポジウムの座長を行った．さらに筆者等はこのシンポジウムで「カニクイザルを用いた新しい結核ワクチン(HVJ-エンベロープ/HSP65DNA＋IL-12DNA)の開発」の発表と「SCID-PBL/huマウスモデルを用いたSARSコロナウィルスに対するDNAワクチンとヒト型モノクローナル抗体を用いた治療法の開発」の2つの課題のシンポジストに選ばれる栄誉を得た．

カニクイザル(最もヒトの肺結核に近いモデル)を用いBCGよりもはるかに強力(マウスの系でBCGより1万倍)な予防ワクチン効果(100%の生存率)を示すワクチンを開発した．

Sadoff博士はビル・ゲイツ財団より支援の多額の研究費を用い，結核ワクチンの臨床試験第Ⅰ期を行っている．①リコンビナント72f蛋白によるサブユニットワクチン(GSK社)②リコンビナントAg85B BCGワクチン③リコンビナント　ストレプトリジンBCGワクチン④MVA85A(ワクシニアウィルスベクター)などである．

写真1　WCVII会長Karstak教授と

写真2　ウェルカム・オープニング・リマーク

写真3　コーヒーブレイクでの情報交換

・ヒト生体内免疫応答解析モデルSCID-PBL/huを用い我々のSARS(M)DNAワクチンがSARSウィルスに対する中和抗体生産を誘導する初めての発見をした．

・堀井博士はすぐれたマラリアワクチン　リコンビナントSE36蛋白ワクチン(第Ⅰ相)を開発した．

・植物に遺伝子を導入して栽培し，大量のDNAを取る方法の興味深い発表があった．

・23年前，B型肝炎まん延地区の南イタリーでB型肝炎ワクチンが開始された．開始前HB抗原キャリアーが13.4%であったのが2006年には0.9%と著明に減少した．

この国際ワクチン学会に出席し，新しいワクチン開発の進展に感銘を受けた．しかしながら，遺伝子学や免疫学等の進展により，B型肝炎やパピローマウイルスワクチンによる肝癌や子宮頚癌の制御がなされつつあるが，パンドラの箱は空になるにはほど遠い．すなわち，SARSやトリ・インフルエンザなどの新しい病気がつぎからつぎへ出てくる．したがって，ワクチン研究者は，いつでも新しい技術開発やワクチン開発に精進する必要がある感想を得た．次回の2年後，オランダ　ハーグが楽しみである．

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Original article

Detection of streptomycin resistance in *Mycobacterium tuberculosis* clinical isolates from China as determined by denaturing HPLC analysis and DNA sequencing

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Abstract

China is regarded by the World Health Organization as a major hot-spot region for *Mycobacterium tuberculosis* infection. Streptomycin has been deployed in China for over 50 years and is still widely used for tuberculosis treatment. We have developed a denaturing HPLC (DHPLC) method for detecting various gene mutations conferring drug resistance in *M. tuberculosis*. The present study focused on *rpsL* and *rrs* mutation analysis. Two hundred and fifteen *M. tuberculosis* clinical isolates (115 proved to be streptomycin-resistant and 100 susceptible by a routine proportional method) from China were tested to determine the streptomycin minimal inhibitory concentration (MIC), and subjected to DHPLC and concurrent DNA sequencing to determine *rpsL* and *rrs* mutations. The results showed that 85.2% (98/115) of streptomycin-resistant isolates harbored *rpsL* or *rrs* mutation, while *rpsL* mutation (76.5%, 88/115) dominated. MIC of 98 mutated isolates revealed no close correlation between mutation types and levels of streptomycin resistance. No mutation was found in any of the susceptible isolates. The DHPLC results were completely consistent with those of sequencing. The DHPLC method devised in this study can be regarded as a useful and powerful tool for detection of streptomycin resistance. This is the first report to describe DHPLC analysis of mutations in the *rpsL* and *rrs* genes of *M. tuberculosis* in a large number of clinical isolates.

Keywords: *Mycobacterium tuberculosis*; Streptomycin resistance; DHPLC; *rpsL*; *rrs*; MIC

1. Introduction

Tuberculosis has continued to be the most common infectious cause of death, and still has a serious impact medically, socially and financially [1]. Multidrug-resistant tuberculosis (MDR-TB), caused by tubercle bacilli that are resistant to at least isoniazid and rifampicin, is one of the most worrisome elements of the antibiotic resistance pandemic, because TB patients for whom treatment has failed have a high risk of death [1]. The global number of incident cases of MDR-TB in 2004 was estimated to be 424,203. Three countries, China, India and the Russian Federation, accounted for 261,362 MDR-TB cases, or 62% of the estimated global burden [2]. Very recently, XDR-TB has been proposed as a result of a global survey by the WHO, although its exact incidence is not known in the world. Streptomycin was the first antibiotic shown to be active against the etiologic agent of TB, *Mycobacterium tuberculosis*, and was used in control programs for many years. However, as a result of significant levels of resistance when streptomycin was used as monotherapy, and some side effects, streptomycin usage declined greatly in industrialized countries in the 1960s [3]. Recently, the emergence of

Abbreviations: MDR, multidrug-resistant; MIC, minimal inhibitory concentration; *M. tuberculosis*, *Mycobacterium tuberculosis*; TB, tuberculosis; Denaturing HPLC, denaturing high-performance liquid chromatography.

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strains of *M. tuberculosis* displaying resistance to some or all of the major anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, pyrazinamide and fluoroquinolones) has led to renewed interest in streptomycin and its derivatives, kanamycin and amikacin. However, in some countries including China, streptomycin has continued to be used commonly for tuberculosis treatment.

Streptomycin has been shown to interact directly with the 30S subunit of the ribosome, thereby interfering with protein biosynthesis [18]. The ribosome accuracy center is a highly conserved component of the translational apparatus, comprising a 16S rRNA domain and several polypeptides including the ribosomal protein S12. RNAs for ribosomal protein S12 (encoded by the *rpsL* gene) and 16S (encoded by the *rrs* gene) are the main targets of streptomycin [4]. It is now known that streptomycin binds tightly to the phosphate backbone of 16S rRNA in four different domains – helix 1, the 530 loop, the 912 loop and the 1400 region – thereby also forming both salt bridges and hydrogen bonds, and making contact with the S12 ribosomal protein [19,20], eventually leading to misreading of the genetic code during translation [20]. Mutations in genes encoding the rRNAs for ribosomal protein S12 (*rpsL* gene) and 16S (*rrs* gene, within which the parts encoding the 530 loop, the 912 loop and the 1400 region of 16S rRNA are named *rrs*A, *rrs*B and *rrs*C, respectively, in this work) lead to streptomycin resistance. This work was carried out to explore *rpsL* and *rrs* mutation in streptomycin-resistant clinical isolates from China.

There are several methods to detect various gene mutations. DNA sequencing is a good method for detecting mutation, but cannot be used routinely by many laboratories because of its relatively high cost. PCR-single-stranded conformational polymorphism (SSCP) is often used, but has a major disadvantage in that the technique is empirical and it is difficult to optimize the experimental conditions. Temperature-mediated heteroduplex analysis by denaturing high-performance liquid chromatography (TMHA-DHPLC) is a relatively new technique that uses heteroduplex formation between wild-type and mutated DNA strands to identify mutations. DHPLC was predicted to be a potentially useful genotypic screening method for gene mutations conferring drug resistance in *M. tuberculosis* [5–7] and it is cost-effective. We have developed the DHPLC method for detecting various gene mutations in *M. tuberculosis* [8,9]. The present study was focused on *rpsL* and *rrs* mutation analysis. The DHPLC method devised in this study can be regarded as a useful tool for clinical analysis of streptomycin resistance in tuberculosis. This is the first report to describe DHPLC analysis of mutations in the *rpsL* and *rrs* genes of *M. tuberculosis* in a large number of clinical isolates.

2. Materials and methods

2.1. Clinical isolates and drug susceptibility tests

M. tuberculosis H37Rv (ATCC 25618) was used as a reference strain. Streptomycin-dependent strain 18b was from the Mycobacterial Reference Center of The Research Institute of Tuberculosis. Two hundred and fifteen clinical isolates (115 proved to be streptomycin-resistant by a routine proportional method, and 100 streptomycin-susceptible) were collected from different patients with pulmonary tuberculosis (123 males and 92 females, aged 15–75 years) over a period of 3 years (2002–2004) at Beijing Tuberculosis and Thoracic Tumor Research Institute, China. Forty-three (37.3%) of 115 streptomycin-resistant isolates were MDR. MICs of streptomycin were detected by an absolute concentration method in L–J medium at concentrations of 1, 2, 5, 10, 20, 50, 80, 100, 200, 400, and 800 μg/ml.

2.2. DNA isolation and PCR amplification

Chromosomal DNA was extracted from *M. tuberculosis* H37Rv and clinical isolates by the method described previously [8,10]. For the *rpsL* gene, a 300-bp DNA fragment was generated by PCR with the primer set SM1, SM2. For the *rrs* gene, three primer sets were used to amplify regions corresponding to the 530 loop, 912 loop and 1400 region of 16S rRNA, respectively (Table 1). TaKaRa Ex *Taq* was the polymerase used for the PCR reaction.

2.3. DHPLC analysis

DNA from the reference strain was used for individual hybridization with each test isolate. DHPLC was performed with the WAVE DNA fragment analysis system (Transgenomic Inc.). The melting temperature for each gene is shown in

Table 1
Primer sets and predicted PCR products of *rpsL* and *rrs* genes

Accession number (GenBank)	Primer set (F: forward. R: reverse)	Size (bp)	Melting temperature (°C)
rpsL X80124	F 5′-ATG CCA ACC ATC CAG CAG CT R 5′-ACC GCG GAT GAT CTT GTA GC	300	65.8
rrs BX842576			
*rrs*A (530 loop)	F 5′-GAT GAC GGC CTT CGG GTT GT R 5′-TCT AGT CTG CCC GTA TCG CC	238	63.2
*rrs*B (912 loop)	F 5′-GTA GTC CAC GCC GTA AAC GG R 5′-AGG CCA CAA GGG AAC GCC TA	240	62.3
*rrs*C (1400 region)	F 5′-TTA AAA GCC GGT CTC AGT TC R 5′-TAC GCC CCA CCA GTT GGG GC	300	63.3

Table 1. The conditions for DNA hybridization and DHPLC analysis have been described in detail elsewhere [8].

2.4. DNA sequencing

rpsL and *rrs* gene PCR products of 115 streptomycin-resistant, and 100 streptomycin-susceptible clinical isolates, and the streptomycin-dependent strain 18b, were sequenced. The sequencing primer sets were the same as those used for PCR. After purification, the PCR product (5 ng) was used as a template for *Taq*Cycle Sequencing using ABI Prism Big Dye Terminator sequencing kits (Applied Biosystems). Cycle sequencing products were subsequently analyzed on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer Applied Biosystems) [9].

3. Results

3.1. rpsL mutations in clinical isolates

The results of DNA sequencing showed that all of the 100 streptomycin-susceptible isolates had wild-type *rpsL*. Of the 115 streptomycin-resistant isolates, 88 (76.5%) were found to have *rpsL* mutations, the majority at codon 43 (Fig. 1). Seven (6.1%) isolates rpsL88 mutation (AAG → AGG, Lys → Arg) and 81 (70.4%) had an rpsL43 (AAG → AGG, Lys → Arg). Seven of the isolates with an rpsL43 mutation harbored a second mutation in *rrs* and *rpsL* genes: one isolate had rpsL39 (ACC → ACT, no amino acid change), one had rpsL71 (GGC → AGC, Gly → Ser), one had rrs462 (T → G, 645 A deletion), one had rrs513 (A → C), one had rrs1400 (A → G) and two had rrs1401 (C → T) mutations. No compound mutation of codon 88 was found.

Codon 88 was the other mutation point. Seven (6.1%) isolates carried codon 88 mutation (AAG → AGG, Lys → Arg). No compound mutation of codon 88 was found.

3.2. rrs mutations in clinical isolates

No mutation was found among 100 streptomycin-susceptible isolates as evaluated by DNA sequencing. Of the 115 streptomycin-resistant clinical isolates, a total of 11 (9.5%) isolates had *rrs*A mutation (Fig. 2), among which two had compound mutation with *rpsL* codon 43 mutation as described above, and nine possessed mono-mutation. Among the mutation types, two isolates had 513 A → C and 645 deletion, five had 513 A → C, one had 516 C → T, and one had 464 A → C.

*rrs*B: Only one (0.9%) isolate showed *rrs*B 906 A → C mutation.

*rrs*C: Three (2.6%) isolates were found to have *rrs*C mutation (one with 1400 A → G, two with 1401 C → T), all of them compound with *rpsL* codon 43 mutation, as described above.

3.3. Streptomycin resistance gene mutation and MIC analysis

Fig. 3 shows the relationship between mutation types and MIC levels of 98 streptomycin-resistant isolates in which *rpsL* or *rrs* mutation was detected. Seventy-four isolates with *rpsL*43 AAG → AGG mutation were found at different MICs ranging from 20 to 800 μg/ml. The MICs of nine isolates with *rrs*A mono-mutation ranged from 50 to 800 μg/ml. There was no significant difference in MIC among the mutation types. These results revealed no close correlation between mutation type and streptomycin resistance level.

3.4. Mutations in streptomycin-dependent strain 18b

DNA sequencing results revealed one insertion of an additional cytosine residue between positions 512 and 513 in the 530 loop and one deletion of adenosine at position 645 in the *rrs*A gene. No mutation was found in *rpsL*, *rrs*B or *rrs*C.

3.5. DHPLC analysis

The results of DHPLC analysis were completely consistent with those of DNA sequencing. With *M. tuberculosis* H37Rv as a reference strain, DHPLC analysis revealed that all the streptomycin-susceptible isolates, and those isolates that were streptomycin-resistant but with no *rpsL* or *rrs* mutation, had a normal peak pattern, which was the same as that of H37Rv. All the 88 isolates that were found to carry *rpsL* mutation showed an aberrant DHPLC pattern. Four types of mutation in the *rpsL* gene were found by DNA sequencing, and four corresponding peak patterns were shown by DHPLC analysis (Fig. 4). Seventy-six codon 43 AAG → AGG mono-mutated isolates, seven codon 88 AAG → AGG mono-mutated isolates,

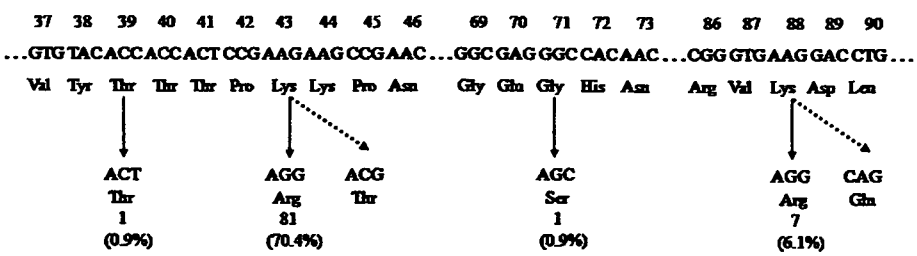

Fig. 1. Nucleotide sequence and missense mutations in the *rpsL* gene. ——▶ shows the mutations found in this work. - - - ▶ shows mutations from other reports.

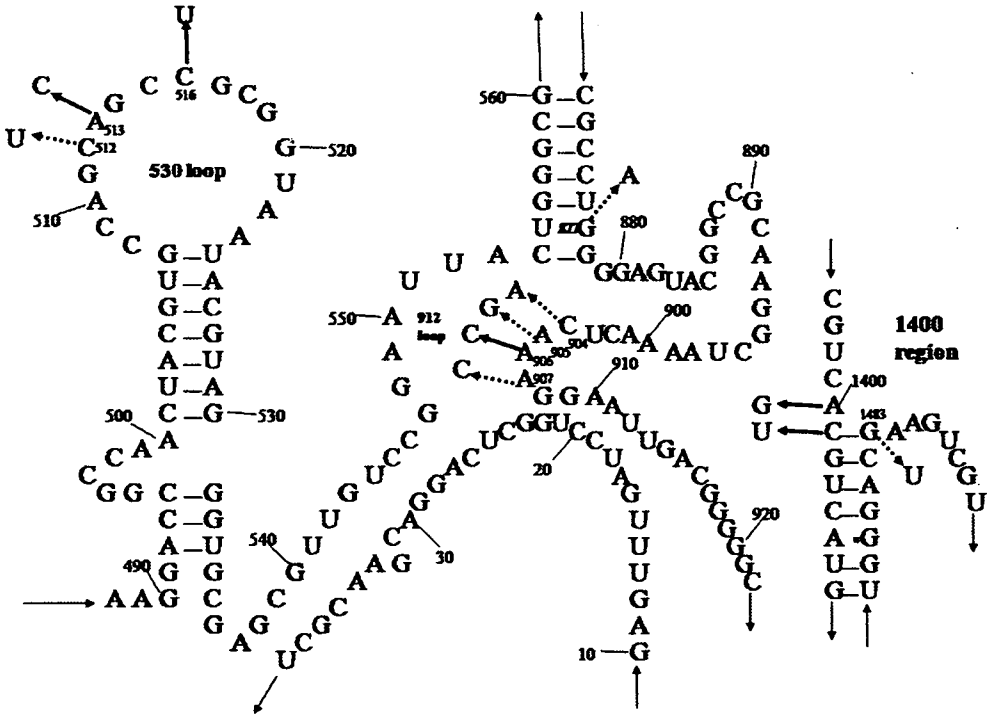

Fig. 2. Mutations located in the 530 loop, the 912 loop and the 1400 region in 16S rRNA associated with streptomycin resistance. The figure is based on a model structure of *M. tuberculosis* 16S rRNA [15]. ——▶ shows the mutations found in this work. - - - ▶ shows mutations from other reports.

one codon 43 AAG → AGG compound with codon 39 ACC → ACT mutated isolate, and one codon 43 AAG → AGG compound 71 GGC → AGG mutated isolate showed their own specific and aberrant DHPLC patterns, respectively.

Four types of *rrs*A gene mutation, one type of *rrs*B gene mutation and two types of *rrs*C gene mutation were found in this study. Their corresponding DHPLC patterns are shown in Fig. 4 and all of them differs from the susceptible reference strain, H37Rv. With regard to the streptomycin-dependent strain 18b, the results of *rpsL* and *rrs* gene DHPLC analysis are also shown in Fig. 4.

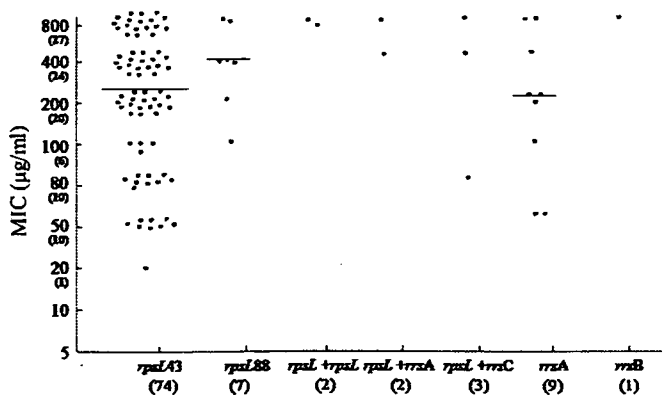

Fig. 3. Relationship between mutation types and MICs of 98 streptomycin-resistant isolates. Numbers in bracket indicate total number of clinical isolates harboring one type of mutation or one level of MIC. ——— indicates average MIC level in a group. No significant difference was found among *rpsL*43AAG ——▶ AGG, *rpsL*88AAG ——▶ AGG, *rrs*A mutation, *rrs*B mutation and three kinds of double mutations.

4. Discussion

One practical implication of the present findings is that that the DHPLC method has wide clinical application for *rpsL* and *rrs*A mutation analysis. Currently, control of MDR-TB and XDR-TB is a major issue throughout the world. It is useful to detect *rpsL* and *rrs* mutations in kanamycin- and amikacin-resistant *M. tuberculosis* strains. Kanamycin and amikacin are commonly used in second-line therapy of TB. Detection of drug-resistant phenotypes of *M. tuberculosis* using routine methods takes several weeks. The establishment of a rapid, simple and reliable method for detection of drug-resistant phenotypes of *M. tuberculosis* is one of the most urgent requirements for effective treatment of tuberculosis patients. Compared with DNA sequencing and drug susceptibility testing, DHPLC has been confirmed to be a simple, reliable and cost-effective method with high sensitivity and specificity, and has already been applied for detection of *rpoB* and *gyrA* gene mutation in order to predict rifampicin and fluoroquinolone resistance in *M. tuberculosis* [7–9]. This is the first report of the use of DHPLC for streptomycin resistance gene mutation analysis using a large series of clinical samples. The results of DHPLC are completely consistent with those of DNA sequencing: In all of the susceptible clinical isolates, no mutation was found; 84.3% of streptomycin-resistant clinical isolates revealed *rpsL* or *rrs*A mutation; one type of peak patterns corresponded to one specific mutation type. The DHPLC method devised in this study can be regarded as a useful and powerful tool for analysis of *rpsL* and *rrs* mutation in *M. tuberculosis*. It should be mentioned that the frequency of streptomycin resistance is very high in China. One obvious

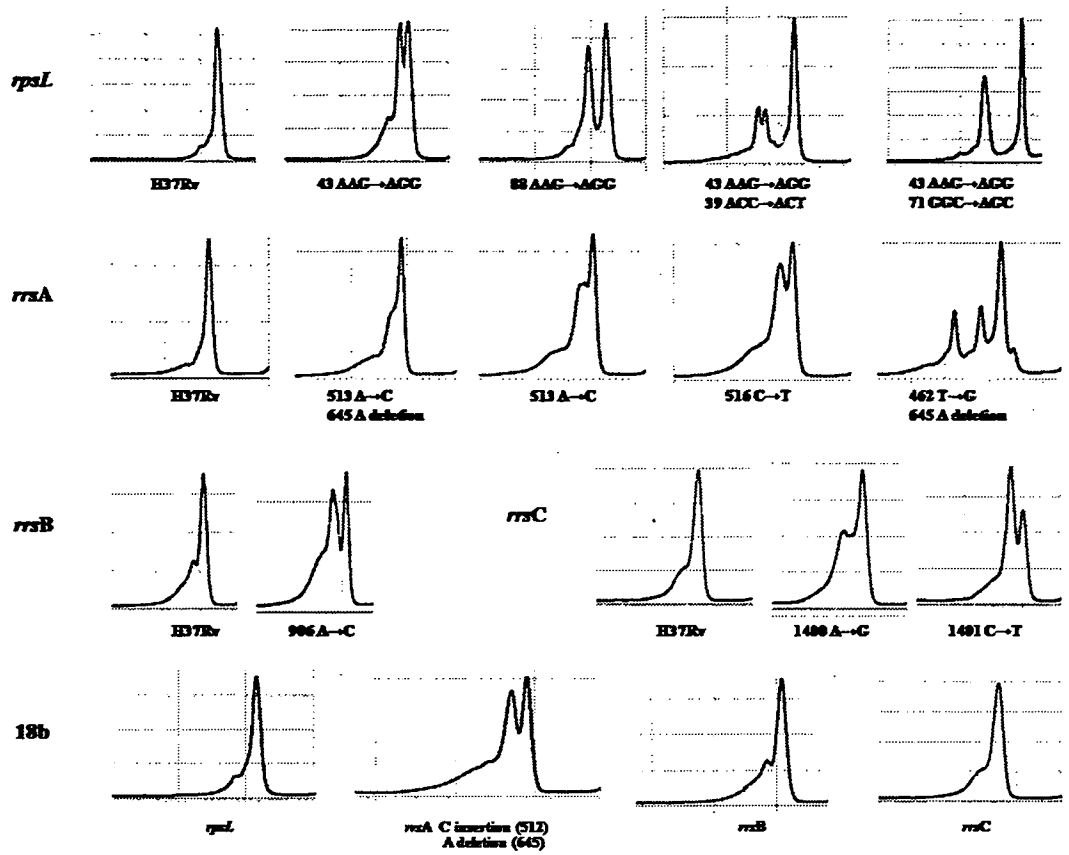

Fig. 4. DHPLC peak patterns of the *rpsL* and *rrs* genes of streptomycin-resistant clinical isolates and streptomycin-dependent strain b18.

reason is that streptomycin has been used for many years in China as an anti-TB drug. In China, Japan and other countries with a high frequency of *rpsL* and *rrs* mutation in streptomycin-resistant clinical isolates, it is anticipated that this DHPLC method will have a high predictive value.

In this study, DNA sequencing and the DHPLC method were applied to investigate the molecular mechanism of streptomycin resistance in *M. tuberculosis* clinical isolates from China. The results revealed that 85.2% of streptomycin-resistant isolates had mutation in the *rpsL* or *rrs* gene. This rate is much higher than those of 56–68% reported in America [10,11], 48% in Germany [12], 60% in France [13], 52% in Poland [14], 24% in Mexico [15], and 77.8% in Japan [16], and indicates the different geographic distribution of *rpsL* and *rrs* mutations among streptomycin-resistant *M. tuberculosis* isolates, those from China showing the highest frequency. There is a possibility that a high transmission frequency could be the cause for the high frequency of *rrs* and *rpsL* mutations in our isolates compared to those found in previous reports [10–16]. It was found in this study that *rpsL* played a more important role (76.5%, 88/115) than the *rrs* gene (*rrs*A: 9.6%, 11/115) in the mechanism of streptomycin resistance, and no *rpsL* or *rrs* mutation was found among 100 streptomycin-susceptible clinical isolates, strongly confirming and extending the findings of other investigators [11–16]. The relative proportions of *rpsL* versus *rrs* mutations in the streptomycin-resistant isolates are similar compared with other studies [11–16]. No relationship between mutation type and resistance level was found in this study, thus differing from the reports of Cooksey [5] and Bottger [17]. The reason may be related to the difference in areas from which the strains were derived, similarly to the differences in the geographic distribution of mutation types discussed above. It is also possible that there are other reasons for no relationship between MIC and mutation type. Unknown mechanisms may exist between different strain families of *M. tuberculosis*.

In this study, *rpsL* mutations were found to occur predominantly at codon 43 (91%, 81/88 isolates). Codon 88 mutation played a minor role (9%, 7/88 isolates). These results largely confirm the findings of other researchers [21]. Two new point mutations found in this study, at codon 39 (ACC → ACT) with no amino acid substitution, and at codon 71 (GGC → AGC, Gly → Ser), both compounded with codon 43 AAG → AGG, seemed to be of only minor importance. Previously reported mutations involving codon 43 AAG → ACG (Lys → Thr) and codon 88 AAG → CAG (Lys → Gln) were not found [21]. This is because they have been proved to be restrictive mutations leading to fitness cost and show attenuated virulence. Only non-restrictive mutations such as codon 43 AAG → AGG (Lys → Arg), which has unaltered virulence properties, can be widely transmitted and finally dominate in clinical isolates [22]. In this study, a total of 15 isolates

(13%) were found to have *rrs* mutation. In contrast to most bacteria that have multiple copies of the *rrs* gene, *M. tuberculosis* and other slow-growing mycobacteria have only one copy [4,21]. Mutations in the *rrs* gene, which encodes the loops of 16S rRNA, the highly conserved 530 loop, the 912 loop and the 1400 region that interact with the S12 ribosomal protein, constitute an easily selected resistance site. In *rrs*A, 513 A → C, 516 C → T, 464 A → C mutations were demonstrated in these isolates from China, while 512 C → T mutation and 491 C → T polymorphism [15,23] were not found. Since only one isolate carried *rrs*B mutation and three *rrs*C-mutated isolates were all compounded with *rpsL* codon 43 AAG → AGG mutation concurrently, it can be concluded that *rrs*B and *rrs*C mutations are not common, while the majority of *rrs* point mutations producing streptomycin resistance in *M. tuberculosis* occur in *rrs*A, which encodes the 530 loop of 16S rRNA. Two isolates had identical *rpsL* codon 43 mutation plus *rrs*C codon mutation. There is a possibility of transmission for the two isolates each with two identical mutations, although no data are available. It is reported that *rrs*C 1400 A → G mutation is the main mechanism involved in resistance to other aminoglycosides (amikacin and kanamycin) in *M. tuberculosis* [24]. It is interesting that the double mutations result in a higher MIC, although the numbers are small. Further study will be required to clarify the mechanism to undergo double mutations. In this study, 14.8% (17/115) of streptomycin-resistant isolates revealed no mutation in the *rpsL* or *rrs* gene. This observation implies that there is at least one additional mechanism conferring streptomycin resistance, and that future molecular genetic studies should be aimed at identifying the gene(s) involved. Recently, Okamoto *et al.* [25] reported that mutations within the *gidB* gene, which encodes a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA, played a role in the mechanism of streptomycin resistance. In fact, two of the 17 clinical isolates in this study that were streptomycin-resistant but with no *rpsL* or *rrs* mutation were found to harbor *gidB* gene mutation (DHPLC results not shown), and the results were also confirmed by DNA sequencing and therefore further study is needed. Apart from this, a growth inhibition experiment [17] has revealed that membrane-active agents such as Tween 80 are capable of reducing the level of resistance significantly by approximately 10-fold, supporting a hypothesis that membrane permeability may play a role in streptomycin resistance. It seems entirely possible that a combination of different resistance mechanisms may operate in a drug-resistant clinical isolate. Therefore, for example, a permeability barrier would be expected to increase the level of resistance of isolates with an altered *rpsL* or *rrs* gene. Other factors, for example a membrane efflux pump [26,27], may have some relationship to streptomycin resistance. In order to further explore the molecular mechanism of streptomycin, we have also sequenced the streptomycin-dependent strain 18b. As is the case for *E. coli*, *M. tuberculosis* also has three streptomycin phenotypes: sensitive, resistant and dependent. Streptomycin-dependent strain 18b was isolated in Japan in 1955 by Hashimoto [28], and in 1995 Cole [29] found that it possessed a novel mutation in the 530 loop of the 16s rRNA: insertion of an additional cytosine between 512 C and 513 A. In the present study, we found a second mutation in its 16s rRNA: a 645 A deletion. How streptomycin has helped to stabilize the conformational structure of 16s rRNA and S12 ribosomal protein and eventually become a necessary component for the process of translation is not clear. The mechanism responsible for the streptomycin dependence of 18b still remains to be discovered.

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Recombinant BCG Tokyo (Ag85A) protects cynomolgus monkeys (*Macaca fascicularis*) infected with H37Rv *Mycobacterium tuberculosis*

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Summary
One tuberculosis vaccine candidate that has shown a promising degree of protective efficacy in guinea pigs is recombinant BCG Tokyo (Ag85A)(rBCG-Ag85A[Tokyo]). As a next step, cynomolgus monkeys were utilized because they are susceptible to *Mycobacterium tuberculosis* and develop a continuous course of infection that resembles that in humans both clinically and pathologically. The recombinant BCG vaccine was administered once intradermally in the back skin to three groups of cynomolgus monkeys, and its protective efficacy was compared for 4 months with that of its parental BCG Tokyo strain. Vaccination of the monkeys with the rBCG-Ag85A[Tokyo] resulted in a reduction of tubercle bacilli CFU ($p<0.01$) and lung pathology in animals challenged intratracheally with 3000 CFU H37Rv *M. tuberculosis*. Vaccination prevented an increase in the old tuberculin test after challenge with *M. tuberculosis* and reaction of *M. tuberculosis*-derived antigen. Thus, it was shown in monkeys that rBCG-Ag85A[Tokyo] induced higher protective efficacy than BCG Tokyo. This warrants further clinical evaluation.

Introduction

Tuberculosis (TB) still remains a major health threat affecting millions of people worldwide. The only TB vaccine currently available is *Mycobacterium bovis* BCG. However, the efficacy of BCG against adult pulmonary TB still remains controversial.[1–4] Thus, development of a better TB vaccine is urgently required to counteract the global threat of TB.

We have previously reported the protective efficacy of a TB DNA vaccine (Ag85A) and a recombinant strain BCG Tokyo (Ag85A) in small-animal models challenged with *M. tuberculosis* Kurono strain.[5,6] We found that recombinant BCG

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