Current Topics

ANTI-TUBERCULOSIS IMMUNITY BY CYTOTOXIC T CELLS・GRANULYSIN AND THE DEVELOPMENT OF NOVEL VACCINES (HSP-65 DNA+IL-12 DNA)

Masaji OKADA and Yoko KITA

Abstract CDC and ACET in U.S.A. reported that novel vaccines instead of BCG are required for the protection against infection of *Mycobacterium tuberculosis* worldwide. However, no novel vaccine for clinical use has not yet been developed in the world including U.S.A. and Europe.

We have developed a novel tuberculosis (TB) vaccine; a combination of the DNA vaccines expressing mycobacterial heat shock protein 65 (HSP65) and interleukin 12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-envelope and -liposome (HSP65+IL-12/HVJ). This vaccine provided remarkable protective efficacy in mouse compared to the BCG vaccine on the basis of C.F.U of number of TB, survival, an induction of the CD8 positive CTL activity and improvement of the histopathological tuberculosis lesions. This vaccine also provided therapeutic efficacy against multi-drug resistant TB (MDR-TB) and extremely drug resistant TB (XDR-TB) in murine models. Furthermore, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. This novel vaccine provided a higher level of the protective efficacy than BCG based upon the assessment of mortality, the ESR, body weight, chest X-ray findings and immune responses. Furthermore, the BCG priming and HSP65+IL-12/HVJ vaccine (booster) by the priming-booster method showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). Furthermore, this vaccine exerted therapeutic efficacy (100% survival) and augmentation of immune responses in the TB-infected monkeys. These data indicate that our novel DNA vaccine might be useful against *Mycobacterium tuberculosis* including XDR-TB and MDR-TB for human therapeutic clinical trials.

The review also provides recent advances of the precise studies of induction of immunity including CD8 positive cytotoxic T cells and effector molecules such as granulysin by these vaccines, against multi-drug resistant tuberculosis and extremely drug resistant tuberculosis.

Key words: Killer T cell, Granulysin, New TB vaccine

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ミニ特集「免疫と結核」

結核免疫（序論）

岡田　全司

要旨：いまだに世界の3分の1の20億人が結核菌に感染しており，その中から毎年940万人の結核患者が発症し，180万人が死亡している，最大の感染症の一つである（WHOレポート2008年）[1)～11)]。本邦でも1998年から結核罹患率が増加・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は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，Th1ヘルパーT細胞，Mφ）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。一方，DBA/1マウスやBALB/cマウスがC57BL/6マウスに比較して結核菌感受性であり，特にDBA/1マウスはBALB/cマウスよりも結核菌易感受性であることも発見した。この結核菌抵抗性は各マウスストレインのキラーT細胞誘導活性と相関することを発見した。したがって，これらの「免疫と結核」の最先端の研究を行っている研究者に執筆をお願いした。(1) 結核菌抗原認識とT細胞免疫，(2) CpGモチーフと結核免疫でTLR9によるCpG認識，(3) Lipocalin 2，SLPIと結核自然免疫，(4) キラーT細胞，granulysinによる結核免疫とワクチン（HSP 65＋IL-12 DNAワクチン等）開発，(5) 結核に対する感染防御機構，をトピックとして選んだ。

キーワーズ：結核免疫，T細胞，獲得免疫，自然免疫

Ⅰ．はじめに

結核症に対する免疫は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。

また，マクロファージ（Mφ）が結核菌増殖の場であり，Mφ-T細胞間の相互の活性化，ヘルプにより結核感染防御に重要な抵抗性を示す。

したがって，これらの「免疫と結核」の最先端の研究を行っている研究者に執筆をお願いした。

Ⅱ．結核症

（1）結核症の現状

結核症は最大の再興感染症で，HIV感染に伴う結核合併症や多剤耐性結核が大きな問題である。

2007年の本邦結核死亡率は10万人に1.7，罹患率は10万人に19.8人である。日本の結核罹患率は，欧米の約5倍も高く，アジア（中国，インド等）やアフリカ地域に多い。

感染した人の5～10%の人が発病し，発病は免れた人でも3分の1以上の人は結核菌をからだの中に抱えたまま高齢に達している。結核菌はからだの抵抗力（免疫力）によって抑え込まれ冬眠状態（dormancy）になっている。高齢，糖尿病，エイズ，副腎皮質ホルモンによる治療，慢性腎不全（人工透析），抗関節リウマチ薬の抗TNFα抗体等で免疫力が低下すると，冬眠していた結核菌が暴れ出す。

Ⅲ．獲得免疫と結核

結核感染に対する免疫力はMφ，$CD4^+$T細胞，NK細胞，γ/δT細胞，キラーT細胞（$CD8^+$Tと$CD8^-$T）および肉芽腫形成の総合的な抵抗力である（Fig. 1）。また，1998年*Nature*に結核菌H37Rvゲノム全塩基が掲載され，遺伝子レベルで結核免疫を解析しうることになった。

（1）キラーT細胞（$CD8^+$T細胞）

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（Received 21 Mar. 2010）

すなわち，結核におけるCD8+T細胞はマウスで抗結核免疫に重要である（Fig. 2）（当ミニ特集・岡田，喜多のキラーT細胞・granulysinによる結核免疫の項参照）。

MHCクラスⅠ拘束性の結核菌の38kDa蛋白，HSP65蛋白を認識するマウスCD8+キラーTや19kDa蛋白，Ag85，CFP10（Mtb11）を認識するヒトCD8+キラーTが報告されている[10]。ESAT-6抗原に対するキラーTでHLA-A2とは82～90位の9個のアミノ酸AMASTEGNVが結合してキラーT細胞がこれらを認識する。われわれは世界に先駆けて確立した，ヒト生体内結核免疫応答解析モデルSCID-PBL/huに，このESAT-6ペプチドを投与し，これに特異的でHLA-A2拘束性を示すヒトキラーTを生体内で誘導することに初めて成功した[2)12)]。

（2）ヘルパーT細胞

CD4+T細胞が結核免疫に重要であることはMHC ClassⅡ(−/−)マウスやCD4(−/−)マウス，抗CD4抗体投与マウスで明らかとなっている（Th1と結核免疫については岡田総説[1)]参照のこと）。

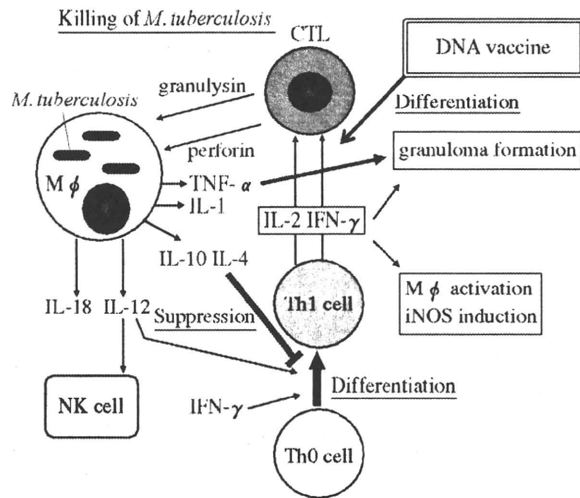

Fig. 1 Mφ and T cell immunity against tuberculosis

Ⅳ. 自然免疫と結核

（1）マクロファージ（Mφ）

結核菌の増殖場所はMφ内である。一方，Mφは異物貪食能と細胞内殺菌能および抗原提示能をもつ。したがって結核菌が優位に立つか，ヒト（生体）が優位に立つかの戦争でもある（詳細は岡田結核文献[2)3)]参照）。殺菌性ラジカルである活性酸素，各種殺菌蛋白ROIやNOなどのRNI，TACO，Nrampも結核菌の殺傷に関与する。

（2）Toll-like受容体およびPathogen Recognition Receptorとマクロファージ・樹状細胞活性化

最近発見されたToll-like receptor（TLR）ファミリーがinnate immunityの重要な役割を果たしている[13)]。

TLR（TLR1～TLR10）はそのリガンドによって大きく3つに分類される（Fig. 3A）。

このうち菌体膜由来の糖脂質を認識するTLRとしては，TLR1，TLR2，TLR4，TLR6，TLR9である。

結核菌のcell wall（LAM，mAGP，total lipid）による応答はTLR2を介する（Fig. 3B）。一方，結核生菌に対する反応にはTLR2とTLR4が必要である。病原株の*M. tuberculosis*由来のMan LAMはMφを活性化しないが，非病原性の抗酸菌は異なるglycolipid Ara LAMよりなり，これはTLR2を介してMφを活性化する。この差が発病の差となる可能性もある。結核菌体成分19kDaのlipoproteinがTLR2を介してMφを活性化する。また，抗酸菌DNAから見いだされたCpGモチーフ（パリンドローム配列）は感染防御免疫能増強することが示されていたが，CpGレセプターに対するTLR9が審良らによりクローニングされた。

TLR2の場合，細胞内領域の2つの変異（Arg753 GlnとArg677 Trp）が認められ，Arg753 Glnは敗血症にかか

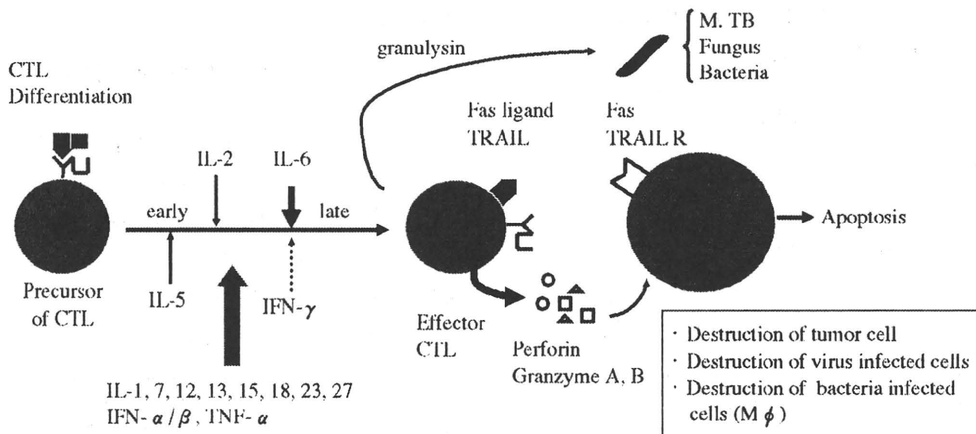

Fig. 2 Induction of CTL and granulysin

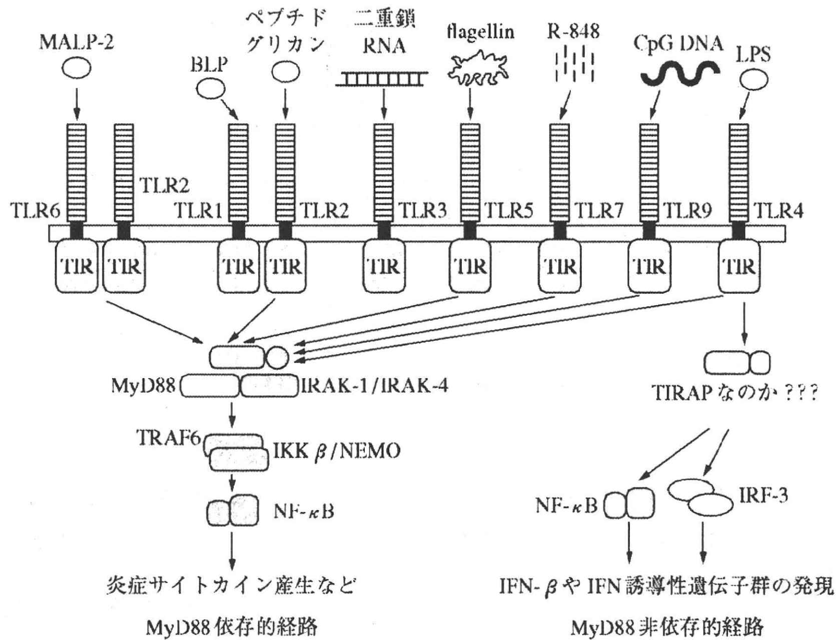

Fig. 3A TLR and pathogens

りやすく，Arg677TrpはアジアƒØ人において*M. leprae*による結節性ハンセン症と関連している。

TLRはそれぞれ病原微生物由来の構成成分を認識する。TLRシグナルを介するシグナル伝達経路にはMyD88を介するMyD88依存的経路とMyD88を介さないMyD88非依存的経路の2つが存在する。主に前者はすべてのTLRを介した炎症性サイトカインの産生を，後者は主にTLR3・TLR4を介したインターフェロン（IFN）およびIFN誘導性遺伝子群の産生を担う。

このMyD88非依存的経路を担うアダプター分子がTRIFである。TRIFがTLR3とTLR4のMyD88非依存的経路に共有されているのに対し，TRAMはMyD88非依存的（TRIF依存的）経路をTLR4シグナルに特異的にだけ与えるアダプター分子である。また，TIRAPはすべてのTLRに共有されたMyD88依存的経路を，TLR1/2/6とTLR4シグナル特異的に与える役割をもつ。われわれは竹田との共同研究でTRIF (−/−)×MyD88 (−/−)ダブルノックアウトマウスを用い，結核菌に対する易感染性を解析しつつある。

TLR以外にもPRR (pathogen recognition receptor)としてDC-SIGN，NODファミリー，マンノース受容体，スカベンジャー受容体，dectin-1があげられる。HIVや*M. tuberculosis*はDC-SIGNに結合して樹状細胞に入り込むが，その際，そのTLRによる自然免疫機構の活性化を抑制し，これらの病原体の生存を有利にする機構が働いていることが示された。NOD1，NOD2を中心とするCARDファミリーの分子は，膜貫通領域をもたず，細胞

Fig. 3B TLRと結核菌体成分

結核菌体成分	レセプター
LAM	TLR2
CWS	TLR2/4
peptidoglycan	TLR2/4
19 kDa lipoprotein	TLR2
CpG repeat	TLR9

質蛋白として存在する。NOD2は，古くより菌体由来の免疫調整物質として知られていたPGNの構成成分であるムラミルジペプチド（MDP）を認識することが示された[8]。

（3）RNAヘリケース（RIG-I, MDA5, LGP I）

TLR-3, -4, -7はそれぞれウイルス核酸成分である二本鎖RNA，一本鎖RNA，非メチル化DNAなどのウイルス構成成分を認識しI型IFN産生を誘導する。TLRはウイルスセンサーであると考えられている。一方，細胞内に存在するRNAヘリケースRIG-I (retinoic acid inducible gene I) およびMDA5 (melanoma differentiation associated gene 5) は，細胞内に侵入したウイルスを感知するシステムであり，ウイルスに特徴的な二本鎖RNAや一本鎖RNAの5′-triphosphate構造などを認識し，I型IFN産生を誘導する[14]。

（4）細胞内ウイルスセンサー〜RNAヘリケース(RIG-I, MDA5, LGP2)

RIG-Iはレチノイン酸で誘導されるDExD/boxRNAヘリケースであるが，ウイルス認識にかかわる分子として

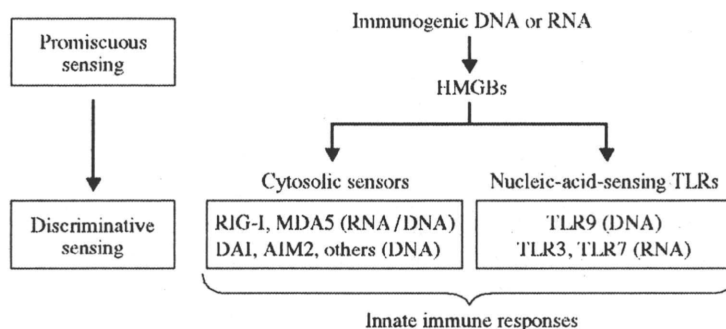

Fig. 3C Intracellular DNA sensor

発見された[14]。細胞質に存在するRIG-Iは，C末のヘリケースドメインでウイルスの複製過程でできる二本鎖RNAを認識し，N末に存在する2つのCARDドメインからIFN誘導シグナルを下流へと伝達すると考えられている。MDA5はRIG-I同様，N末に2つのCARDおよびC末にヘリケースドメインをもつ分子で，RIG-Iと相補的にウイルス認識に関与することが報告されている。今のところ人工二本鎖RNAであるpolyinosine-polycytidylic acid（ploy I：C）がMDA5のリガンドとして同定されている。LPG2は負の制御を行う。HVJ-エンベロープベクターはこのRIG-Iをより刺激する可能性が示唆されている（Fig. 3C）。

（5）細胞内DNAセンサー　DAI, AIM2, High Mobility Group Box（HMGB）

細胞内DNAセンサーとして，DAI（DNA-dependent activator of IRFs）とAIM2（absent in melanoma 2）が発見され，自然免疫と獲得免疫を誘発する。また，RLRs（RIG-I like receptor）も細胞内DNAセンサーとして作用する。

HMGBタンパク質でできるHMGB1, HMGB2およびHMGB3が核酸の万能監視役として機能することが示された。HMGBが存在しないと，核酸によるTLR3, TLR7, TLR9の活性化も低下する。HMGBは乱雑な核酸センサーであり，TLR3, 7, 9, RIG-I, MDA5, DAI, AIM2は細かい識別力のある核酸センサーである。DNAワクチンが強力な免疫活性を示すのがこの経路と関与するかどうか興味深い（Fig. 3C）[15]。

（6）樹状細胞

ウイルス感染時において，免疫担当細胞の1つである樹状細胞dendritic cell（DC）は大量のI型IFNを産生することが知られている。さらに，DCの中でもプラズマサイトイドDC（pDC）とよばれる集団が特にIFN-α産生能が高いことが明らかとなってきた。DCはこのpDCとそれ以外のコンベンショナルDC（cDC）の2つのサブセットに大きく分けることができる。cDCはGM-CSF存在下に骨髄細胞より誘導することができる。pDCはFlt-3-Lにより骨髄細胞から誘導することができ，TLR7, TLR9を高発現しているという特徴をもつ。

V．結核菌抵抗性と宿主免疫

（1）マウスの系統と結核菌抵抗性

結核菌に対する感染性抵抗性はマウスの系統により大きく異なる。結核実験によく使われるC57BL/6マウスは結核菌抵抗性である。一方BALB/cマウスは結核菌感受性であることはよく知られた事実である。その理由として，C57BL/6はI型ヘルパーT細胞（Th1）優位のマウスであり，BALB/cマウスはII型ヘルパーT（Th2）優位のマウスであることがあげられる。C57BL/6マウスのTh1機能はBALB/cマウスのTh1機能の200倍であると報告されている。

われわれは種々の系統のマウスのキラーT細胞誘導機能と結核菌感染抵抗性が相関する結果を最近得たので報告する。特にDBA/1マウスはBALB/cマウスよりも強い結核菌感受性を発見したので，その実験結果を報告する（Fig. 4, 5, 6）。

DBA/1マウス脾細胞，BALB/cマウス脾細胞，およびC57BL/6脾細胞をそれぞれPPD 5 $\mu g/ml$, 20 $\mu g/ml$, 結核死菌H37Ra 20 $\mu g/ml$, 100 $\mu g/ml$で刺激して*in vitro*培養し，5日後の結核菌抗原HSP65に対するキラーT細胞活性を^{51}Cr遊離法で測定した。

その結果，DBA/1マウスとBALB/cマウス脾細胞中のキラーT活性はC57BL/6脾細胞中のキラーT活性に比較して著明に低下していた（Fig. 5A）。さらに，メモリーキラーT細胞活性の系では，BALB/cマウス由来のメモリーキラーT誘導は認められたのに比較し，DBA/1マウス由来のメモリーキラーT細胞誘導はほとんど認められなかった（Fig. 4）。一方IFN-γ産生においては，DBA/1マウスの脾細胞のほうが，BALB/cよりもIFN-γ産生増強が強いことが示された（Fig. 6）。IL-2産生においてはBALB/cが最も低く，C57BL/6よりやや低下するものの，DBA/1は強い産生が認められた。これら

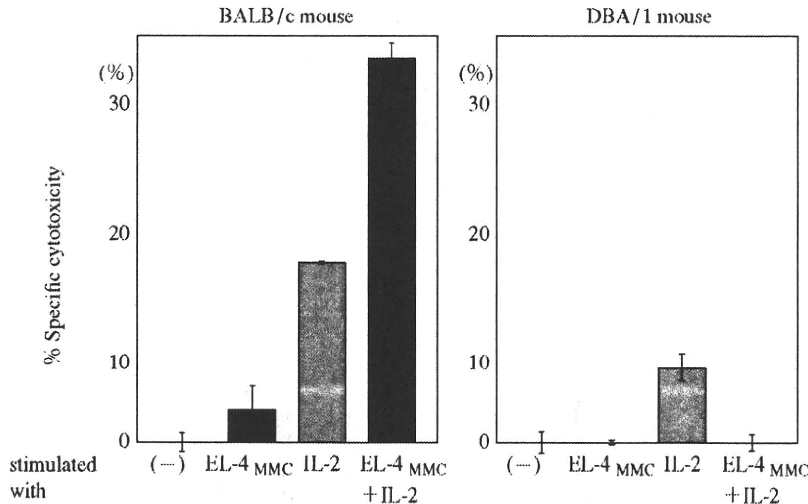

Fig. 4 Comparison of CTL induction *in vitro* against *M. tuberculosis* antigens between DBA/1 mouse and BALB/c mouse (Target EL-4)

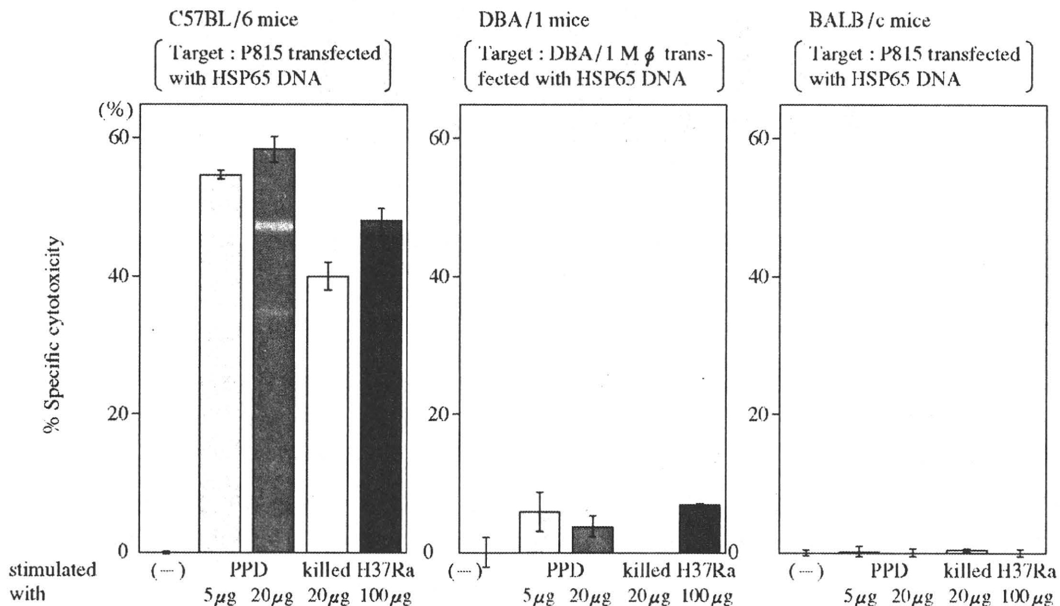

Fig. 5A Comparison of CTL induction *in vitro* against *M. tuberculosis* antigens among DBA/1, BALB/c and C57BL/6 mice

の結果より，結核菌抵抗性とキラーT細胞活性が最も相関することを明らかにした（Fig. 5B）。

Ⅵ. サイトカインと結核

（1）キラーT細胞分化とサイトカイン（キラーT細胞分化因子）

筆者らは CD8$^+$ キラーT細胞（Tc）の誘導にはヘルパーT細胞（Th 細胞）から産生されるサイトカインが必要であることをはじめて明らかにした。クラスⅡ抗原を認識しキラーT細胞分化因子を産生する Th 細胞は CD4$^+$CD8$^-$ であり，クラスⅠ抗原を認識しキラーT細胞分化因子を産生するT細胞は CD8$^+$ である。また，モノクローナル抗 IL-2 抗体を用いて，IL-2 はキラーT細胞誘導に必須な因子の一つであることを示した（Fig. 2）[16)~18)]。

さらに，IL-2 とは異なるサイトカインもT細胞分化誘導に必要であることをキラーT細胞分化因子を産生するヒトT細胞ハイブリドーマ，および IL-2 依存性ヒト Th クローンを世界に先駆けて確立し明らかにした。その解析の結果，IL-6，IFN-γ がキラーT細胞分化因子として強力なキラーT分化を誘導することを明らかにした[15)16)]。

Fig. 5B mouse strain と結核菌感受性

マウス strain	結核菌感受性免疫能			
	結核菌感受性	結核菌5×10⁵ i.v. 後生存菌数	メモリーキラーT誘導	IFN-γ 産生能
C57BL/6	＋	＞300日	＋＋＋＋	＋＋＋＋
BALB/c	＋＋	250日	＋	＋
DBA/1	＋＋＋＋	25日	−	＋＋＋

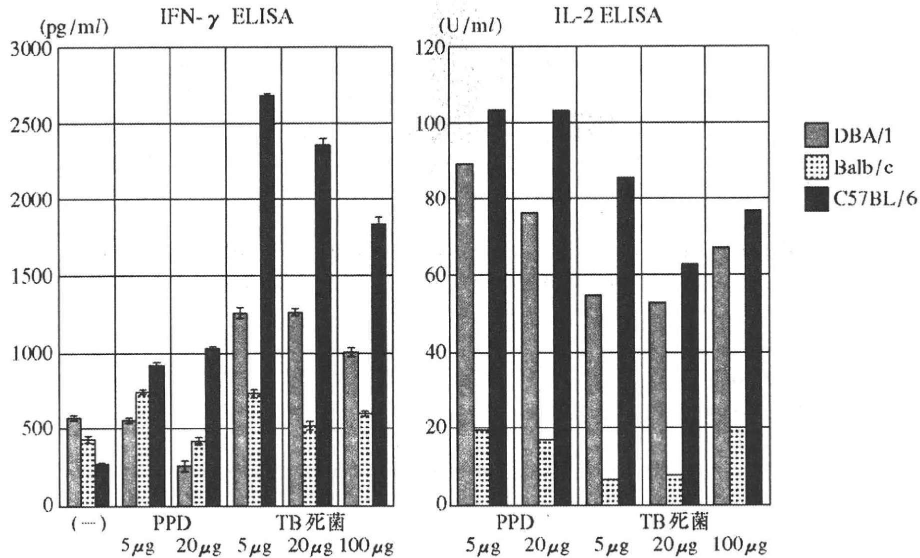

Fig. 6 IFN-γ and IL-2 productions from DBA/1, Balb/c or C57BL/6 mouse spleen cells

筆者らはIL-6がTc誘導の後期の分化段階に作用することを解明した[16]。多剤耐性結核患者PBLにおいて，これらのキラーT細胞分化因子すなわちIL-2，IFN-γ，IL-6の著明な低下を認めた[8]。また，糖尿病合併難治性結核患者ではPPD特異的キラーTの分化誘導の著しい低下を明らかにした[8]。

（2）サイトカインと結核免疫

細胞内寄生細胞（とくに結核菌）はMφに貪食されても殺菌処理されず，細胞内増殖が可能な菌である。種々の機構でMφの殺菌から逃れ，結果的に慢性持続性炎症（慢性感染症）および肉芽腫形成を誘発する。抗結核菌免疫にIFN-γ，TNF-α，IL-6，IL-12が重要であることは解析されている。

（3）IFN-γ，TNF-α，IL-6，IL-12，IL-15と結核免疫

細菌の貪食に伴ってMφが産生するサイトカインのうち，IL-12，IL-18，TNF-αやIL-1は，T細胞，NK細胞およびγδ型T細胞からのIFN-γ産生誘導に関与している。IFN-γは，Mφを活性化し貪食した菌の殺菌処理を促進するヘルパーT細胞，キラーT細胞の分化因子としても作用する。IL-12とIFN-γ産生の間にはポジティブフィードバック機構が働いてIFN-γはMφからのIL-12産生を誘導し，IL-12はT細胞からのIFN-γ産生をさらに増幅し，初期防御反応では感染局所にMφを集め，特異的防御免疫が成立する[3]。

IL-12，IL-18，IFN-γはαβ型T細胞のTh1への分化に重要なサイトカインで，IL-6やTNF-αと強調して抗原特異的なTh1を誘導する。Th1の分化誘導には樹状細胞（dendritic cells：DC）が重要で，Mφよりも高いT細胞からのIFN-γ産生誘導活性を示す。DCが末梢リンパ組織に移行して感染抵抗性T細胞の分化を誘導する。ファゴソーム内で処理された細菌由来の抗原はclass II分子に結合しCD4⁺Th1型T細胞により認識される。細胞質に存在する細菌由来抗原はプロテオソームにより分解されclass I分子と会合し，CD8⁺T細胞により認識される。CD8⁺T細胞はIFN-γを産生するとともに，殺菌能の低下したMφや菌が感染した非食細胞系細胞を破壊し，あらたに動員されてくる活性化Mφに菌を処理させ，感染防御に関与している。また，IL-15はメモリーキラーT細胞を活性化して結核免疫に寄与する。一方，IL-10は結核免疫のMφ機能を抑制する。そのほかIL-10，ファミリーサイトカインとしてIL-19，IL-22，IL-28，IL-29が報告されているが，IL-10と同様の機能

をもつのか，または，IL-28，IL-29はIFN-γとホモロジーがあり，抗ウイルス活性をもつことよりもIL-10と逆の結核免疫機能を示すか興味がある。IL-10，TGF-β，IL-4も結核に対する免疫応答を抑制する。IL-25は，IL-4，IL-5，IL-13の産生を誘導する。

結核性肉芽腫の形成にTNF-αの存在が最も重要である。

（4）IL-23，IL-27，IL-31，IL-32と結核免疫

IL-7，IL-15，IL-17，IL-23，IL-27，IL-31はキラーT分化を誘導した。一方，DNAワクチン（HSP65 DNA＋IL-12 DNAワクチン）は，特にIL-32（結核菌刺激特異的に産生。Plos Path 2006）と強いキラーT分化相乗効果を示した。

（5）ヒトサイトカイン産生異常症およびサイトカインレセプター異常と結核感染

ヒトにおいて，IFN-γ受容体遺伝子に変異がみられた先天性IFN-γレセプター欠損児に，BCGワクチン注射で重症全身性感染が認められたり，*M. avium*感染症をきたした。マウスにおいてもIFN-γ遺伝子ノックアウトマウスやIFN-γ受容体遺伝子ノックアウトマウスでは結核易感染性である[3]。

TNF-αは肉芽腫形成のみでなく慢性の長期感染結核に重要であり，抗TNF-α抗体投与マウスや，TNFレセプター（TNF-Rp55）欠失マウス，$TNF^{-}/^{-}$マウスでは結核菌感染の死亡率が著増し，肉芽腫形成も損なわれた重症の肺結核病理像を示した。さらに，IL-6遺伝子ノックアウトマウスでも結核感染の増悪をきたしたりIFN-γの産生誘導の欠損がみられ，IL-6も非特異的防御，とくにMφの活性化やキラーT細胞分化を介して特異的な結核免疫に関与している可能性もある。著者らは世界に先駆けてIL-6産生能欠損患者$IL\text{-}6^{-}/^{-}$患者を発見した。この患者は易感染性で肺炎を繰り返し発症した。興味深いことに肺炎感染時CRP陰性で発熱も認められなかった。

IL-12レセプター欠損マウスやIL-12欠損患者では結核菌感染・増殖を抑制できなかった。すなわち，IL-12も抗結核免疫に重要なサイトカインであることが示された。また，rIL-12の投与にてBALB/cマウスの結核菌抵抗性が増し，IL-12の生体内中和で感染増悪をきたす。IL-12p40とIL-12Rβ1欠損患者ではIL-12とホモロジーのあるIL-23やIL-23R欠損を伴うことが多い。IL-12欠損，IL-12R欠損患者の易結核菌感染性が$IL\text{-}23^{-}/^{-}$に起因する可能性がある。

（6）サイトカインと結核治療

①マウスの系において，著者らはアデノウイルスベクターにIL-6関連遺伝子（IL-6 DNA＋IL-6レセプターDNA＋gp130 DNA）を導入し，結核感染治療効果を世界に先駆けて明らかにした（いままで治療効果を示す治療ワクチンの報告はない）。結核菌に特異的キラーT細胞誘導活性，IL-2産生増強と相関した（IFN-γ DNAも治療ワクチン効果）。

②Condosらは多剤耐性結核の5症例に対して，IFN-γの吸入治療を試みた。5例中4例で喀痰塗抹で菌陰性化した。しかし，治療中止1カ月後より再度陽性となった。また，胸部CTでは1例で有意な改善，1例で軽度改善，3例で空洞の縮小が観察された。

③肺結核患者に，IL-12投与を行い有効例が報告されている。

④サイトカインIFN-γ，IL-2，IFN-αやG-CSFを多剤耐性結核患者に投与し，投与期間中のみ排菌数が減少した。

（7）肉芽種形成とサイトカイン

結核性肉芽種の形成にTNF-αの存在が最も重要である。近年新しい抗リウマチ薬としてモノクローナル抗TNF-α抗体がRAに有効であるが，多数の結核患者が発症することが報告されている。MCP-1やRANTESも肉芽種形成に関与する。

Ⅶ．おわりに

最近の獲得免疫と結核，自然免疫と結核，サイトカインと結核についてレビューした。Th17やregulatory T等については各論を参照されたい。

文　献

1）岡田全司：結核ワクチン.「結核」第4版，医学書院，東京，2006，50−58.

2）岡田全司：自然・獲得免疫と疾患：結核. 最新医学. 2005；60：678−696.

3）岡田全司：医学のあゆみ「サイトカイン」state of arts，医歯薬出版，東京，2004，209−213.

4）Okada M, Kita Y, Nakajima T, et al.: Novel prophylactic and therapeutic vaccine against tuberculosis. Vaccine. 2009；27：3267−3270.

5）Yoshida S, Tanaka T, Kita Y, et al.: DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation. Vaccine. 2006；24：1191−1204.

6）Okada M, Kita Y, Nakajima T, et al.: Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA＋IL-12 DNA) against tuberculosis using the cynomolgus monkey model of TB. Vaccine. 2007；25：2990−2993.

7）Kita Y, Tanaka T, Yoshida S, et al.: Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine. 2005；23：2132−2135.

8）Okada M, Kita Y：Tuberculosis vaccine development：The

development of novel (preclinical) DNA vaccine. Human Vaccine. 2010 ; 6 : 1−12.
9) Okada M, Kita Y, Nakajima T, et al.: A Novel Therapeutic and Prophylactic Vaccine (HVJ-Envelope/Hsp65 DNA+IL-12 DNA) against Tuberculosis Using the Cynomolgus Monkey Model. Procedia in Vaccinology. 2010 ; 2 : 34−39.
10) Flynn JL, Chan J : Immunology of tuberculosis. Annu Rev Immunol. 2001 ; 19 : 93−129.
11) Schluger NW, Rom WN : The host immune response to tuberculosis. Am J Respir Crit Care Med. 1998 ; 157 : 679−691.
12) Tanaka F, Abe M, Akiyoshi T, et al.: The anti-human tumor effect and generation of human cytotoxic T cells in SCID mice given human peripheral blood lymphocytes by the in vivo transfer of the Interleukin-6 gene using adenovirus vector. Cancer Res. 1997 ; 57 : 1335−1343.
13) Akira S : Toll-like receptors and innate immunity. Adv Immunol. 2001 ; 78 : 1−56.
14) 加藤博巳, 審良静男：RNAウイルス認識におけるMDA5とRIG-Iヘリケースの役割.「Annual Review免疫」. 中外医学社, 東京, 2008, 57−60.
15) Yanai H, Ban T, Wang Z, et al. : HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. Nature. 2009 ; 462 : 99−103.
16) Okada M, Yoshimura N, Kaieda T, et al. : Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. Proc Natl Acad Sci USA. 1981 ; 78 : 7718−7721.
17) Okada M, Sakaguchi N, Yoshimura N, et al. : B cell growth factors and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. J Exp Med. 1983 ; 157 : 583−590.
18) Okada M, Kitahara M, Kishimoto S, et al. : IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. J Immunol. 1988 ; 141 : 1543−1549.

Current Topics

IMMUNITY AGAINST *MYCOBACTERIUM TUBERCULOSIS* (INTRODUCTION)

Masaji OKADA

Abstract A third of world's population infected with *Mycobacterium tuberculosis*, and 2 million people die from tuberculosis every year. It is well established that protective to *M. tuberculosis* depends on both CD4^{+} and CD8^{+} T cells[1)〜8)].

In particular, acquired immunity (cytotoxic T cell, Th1 helper T cell and Mϕ) play an important role for TB infection. Recently, natural immunity also play a very attractive role for the development of TB immunity.

We found that memory CTL is most important for the protection against TB using several kinds of mice. It was demonstrated that DBA/1 mice are more sensitive to TB infection than BALB/c mice (Th2 prone mice). Induction of memory CTL in DBA/1 mice was lower than BALB/c. In contrast, IFN-γ production of DBA/1 mice was higher than BALB/c.

Therefore, famous researchers in the fields of TB immunity reviewed the recent advances of TB immunity, such as (1) T cell immunity and recognition against TB antigen, (2) TLR9 and CpG motif, (3) lipocalin2 and SLPI in natural TB immunity, (4) acquired immunity (CTL) and granulysin. The development novel vaccine (HSP65+IL-12 DNA vaccine), (5) The mechanism of protection against TB, in this mini-review series.

Key words: Immunity against M.TB, T cell, Acguired immunity, Innate immunity

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Phylogeographical particularity of the *Mycobacterium tuberculosis* Beijing family in South Korea based on international comparison with surrounding countries

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To understand the domestic population structure of *Mycobacterium tuberculosis* clinical isolates in the Republic of Korea, we genotypically analysed 80 isolates obtained from various geographical origins in the country. Of these, 64 (80.0 %) isolates were identified as Beijing family strains. It is particularly interesting that their phylogenetic classification, based on the ancient/modern separation and the presence/absence of the genomic region RD181, revealed a majority of the ancient (RD181 +) subfamily in the population. The 15 loci of variable number of tandem repeat(s) of mycobacterial interspersed repetitive units (15-MIRU-VNTR) were also analysed. Combination with the previous VNTR data reported from surrounding countries revealed that the topology of the minimum spanning tree was linked tightly not to the geographical origins of the patients but to the phylogenetic characteristics of the isolates. These results show that the phylogeographical distribution of the *M. tuberculosis* Beijing family around far-eastern Asia could be estimated using international accumulation and comparison of VNTR genotyping data.

Received 13 May 2010
Accepted 20 June 2010

INTRODUCTION

The Beijing family, a lineage of *Mycobacterium tuberculosis*, is well known for its highly endemic prevalence around East Asian countries and as a causative agent of tuberculosis (TB) (van Soolingen *et al.*, 1995). In the Republic of Korea (South Korea), TB is still a major public health concern, with 34 157 (70.3 per 100 000) registered new TB patients in 2008 and 2376 deaths attributable to TB in 2007 (Korea Centers for Disease Control & Prevention, 2008). More than 70 % of *M. tuberculosis* strains isolated from Korean pulmonary TB patients belong to the Beijing family. 'K strain' (Park *et al.*, 2000), one of the Beijing family strains, has been reported as the cause of a severe outbreak of TB in South Korea (Kim *et al.*, 2001). Although their phylogenetic position in the Beijing family lineage has been unclear, Shamputa *et al.* (2010) reported recently that K strains show genetic diversity by some genotyping methods, even in isolates obtained from a single hospital.

The detailed phylogenetic variation of the Beijing family has been unveiled by various genetic markers such as single nucleotide polymorphisms, regions of difference and variable number of tandem repeat (VNTR) loci (Filliol *et al.*, 2006; Tsolaki *et al.*, 2005; Wada & Iwamoto, 2009). A notable characteristic of the lineage is the insertion of IS*6110* in a genomic region named NTF (Mokrousov *et al.*, 2005; Plikaytis *et al.*, 1994). This phylogenetic marker can classify the Beijing family into two distinct sublineages: ancient and modern. The modern Beijing sublineage has been more predominant than the ancient sublineage throughout the world, including countries surrounding South Korea (Bifani *et al.*, 2002; Dou *et al.*, 2008; Kremer *et al.*, 2009; Mokrousov *et al.*, 2005, 2006; van Soolingen & Kremer, 2009). However, Wada *et al.* (2009) reported that the ancient Beijing sublineage has been observed to be exceptionally predominant in Japan.

Abbreviations: MIRU-VNTR, variable number of tandem repeats of mycobacterial interspersed repetitive units; MST, minimum spanning tree; TB, tuberculosis.

Supplementary tables giving information on and genotypic profiles of the isolates analysed in this study are available with the online version of this paper.

The goal of this study was to elucidate the phylogenetic distinctiveness and the genetic diversity of Beijing family strains around the Republic of Korea, including K family strains, for international comparison with strains from surrounding countries. For this purpose, we analysed a collection of *M. tuberculosis* isolates obtained from public health centres in the country using well-known phylogenetic markers and an international standard set of VNTR loci (Supply *et al.*, 2006).

METHODS

Bacterial isolates. Eighty *M. tuberculosis* clinical isolates were analysed in this study. They were randomly selected from smear-positive and culture-positive pulmonary TB patients who were under 46 years of age with no epidemiological links during 2006. All patients were primary cases. Information such as geographical origin, age and sex of the patients, date of isolation and drug susceptibility for each of the 80 isolates is presented in Supplementary Table S1 in JMM Online. The geographical distribution of the population is presented in Fig. 1.

Identification of Beijing family strains and their phylogenetic subdivision. The Beijing family strains were defined by the deletion of RD207 in this study (Tsolaki *et al.*, 2004). The deletion of RD207 corresponds with the absence of signals 1–34 in spacer oligonucleotide (spoligo) genotyping, which is the most standard definition of the lineage (van Soolingen *et al.*, 1995). The presence or absence of RD207 was analysed using PCR according to a previous report (Warren *et al.*, 2004).

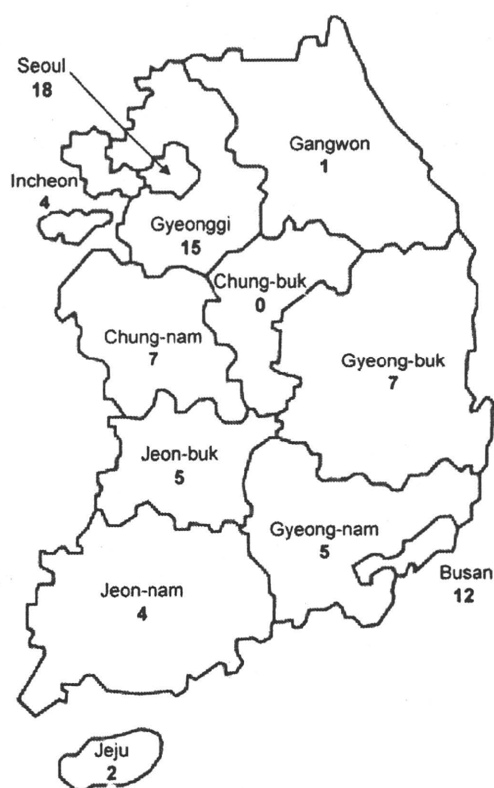

Fig. 1. Nationwide distribution of the *M. tuberculosis* strains used for this study. The 80 *M. tuberculosis* isolates were obtained from new pulmonary TB patients registered in public health centres across South Korea in 2006.

The classification of the ancient and modern subfamilies of the 64 Beijing family isolates was determined according to a previous report (Wada *et al.*, 2009). The presence or absence of RD181 was also verified as described previously (Tsolaki *et al.*, 2004). The genomic deletion of RD181 has been considered to have occurred during evolution of the ancient Beijing sublineage (Tsolaki *et al.*, 2004; Maeda *et al.*, 2010). The sets of primer sequences were described in these previous reports. They were designed to detect insertions or deletions of regions by the difference in sizes of amplified DNA fragments.

***IS6110* DNA fingerprinting.** For all 80 isolates, DNA isolation and IS*6110* RFLP typing were performed as described previously (Park *et al.*, 2000). The IS*6110* RFLP patterns were compared with UPGMA using the Dice coefficient to find RFLP clusters (GelCompar v. 5.1; Applied Maths). An RFLP cluster was defined by the identification of two or more isolates with identical band patterns. K family strains were identified according to the previously reported definition (Kim *et al.*, 2001; Park *et al.*, 2000).

VNTRs. The standard 15 VNTR loci for routine epidemiological discrimination (hereinafter, 15-MIRU-VNTR) were also analysed for all 80 isolates (Supply *et al.*, 2006). Their copy number was calculated from their size and assigned according to the number of repeats for each locus, and in agreement with published allelic tables (Iwamoto *et al.*, 2007). The accuracy of the size of amplified PCR fragments was confirmed using a capillary electrophoresis system (SV1210; Hitachi High Technologies). A VNTR cluster was defined by the identification of all 15 loci in two or more isolates.

Minimum spanning tree (MST). A MST was generated based on the 15-MIRU-VNTR types using software (Bionumerics v. 4.6; Applied Maths) for clustering analysis. We used the reconstruction rules as follows. A categorical coefficient was selected. The priority rule was set such that the type that had the highest number of single-locus variants would be linked first. Creation of hypothetical types was not allowed. The VNTR types of Beijing family strains from neighbouring countries were retrieved from previous reports (Jiao *et al.*, 2008; Wada *et al.*, 2009). All 15-MIRU-VNTR types published in these reports were incorporated into the construction of the MST tree.

RESULTS

Phylogeographical specificity of the Beijing family in South Korea

To elucidate the population structure of *M. tuberculosis* in South Korea, we identified 64 (80.0 %) Beijing family strains from 80 isolates obtained from various geographical origins (Fig. 1) and subdivided them into three phylogenetic sublineages [ancient (RD181+), ancient (RD181−) and modern]. A high proportion of the lineage is concordant with previous reports from South Korea and surrounding East Asian countries (Mokrousov *et al.*, 2006; Park *et al.*, 2000, 2005; van Soolingen *et al.*, 1995; Yun *et al.*, 2009). Phylogenetic subdivision of the 64 Beijing isolates revealed a higher proportion of the ancient Beijing sublineage (46 strains; 71.9 %) than the modern sublineage

(18 strains; 28.1 %). The ancient (RD181 +) sublineage, having diverged from the evolutionary process towards the modern Beijing sublineage before the deletion of RD181, was observed to be predominant (29 strains; 45.3 % of the Beijing strains) in the population. This study identified 13 K family strains (16.3 % of all; Fig. 2): all were found to belong to the ancient (RD181 +) sublineage.

Genotypic diversity of isolates in South Korea

We analysed all 80 isolates by 15-MIRU-VNTR to investigate the detailed genotypic diversity of the population. This genotyping method has been used as a standard discrimination tool for *M. tuberculosis* because of its high resolution among isolates from cosmopolitan origins (Supply *et al.*, 2006). The VNTR profiles and other genotypic characteristics analysed in this study are combined and listed in Supplementary Table S2. The genotyping was able to classify our 80 isolates into 71 distinct genotypes. There were four clusters, which comprised 16 (20.0 %) isolates. The clustering rate was slightly higher than that of RFLP genotyping (10 isolates; 12.5 %), which was concordant with the previous reports on the Beijing family (Iwamoto *et al.*, 2007; Yokoyama *et al.*, 2007). Six of 13 K family strains belonging to two RFLP clusters were separated further into unique genotypes using 15-MIRU-VNTR (Fig. 3).

Recently, it was reported that cluster modelling of 15-MIRU-VNTR genotypes of the *M. tuberculosis* Beijing strains was highly concordant with their phylogenetic subdivision into some sublineages (Wada & Iwamoto, 2009). MST clustering analysis was performed for the 64 Beijing strains of our population (Fig. 4). The tree topology

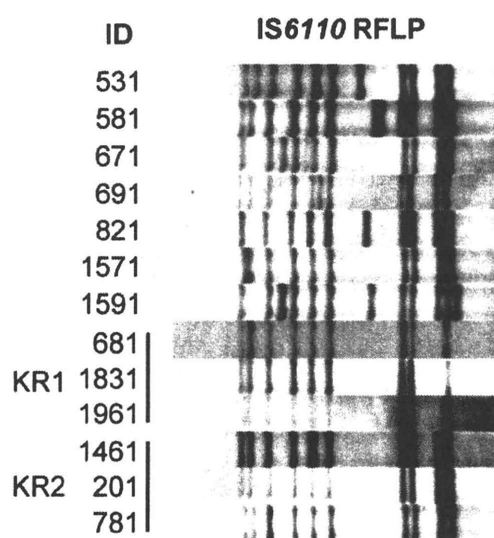

Fig. 2. IS*6110* RFLP patterns of the K family isolates identified in this study. Two clusters were detected in these 13 isolates (KR1, 681, 1831 and 1961; KR2, 1461, 201 and 781).

was observed to be highly associated with the sublineage classification.

Comparison of VNTR profiles with those of neighbouring countries

The MST clustering of 15-MIRU-VNTR genotypes was sufficient to visualize the phylogenetic differences among the Beijing family successfully in our study (Fig. 4), which prompted us to make an international comparison of the 15-MIRU-VNTR types with those of strains from the neighbouring countries (China and Japan) reported previously. The 202 15-MIRU-VNTR types of Beijing strains from Japan (Wada *et al.*, 2009) and the 64 types from Beijing City, China (Jiao *et al.*, 2008), were combined with our current data to construct a mixed clustering tree using MST (Fig. 5). Results showed that three branches including types of strains from South Korea were readily apparent, two of which included mostly South Korean types (indicated by arrowheads). Both these branches comprised strains belonging to the ancient (RD181 +) sublineage, isolated in Japan and South Korea. A remaining branch included types from all three countries (indicated by an arrow). The results of the ancient/modern classification in the previous report by Wada *et al.* (2009) and this study showed that this branch was occupied by modern Beijing strains.

DISCUSSION

In general, the ancient Beijing sublineage has been considered to be an atypical Beijing genotype which has been only rarely observed (Bifani *et al.*, 2002; Milan *et al.*, 2004; Strauss *et al.*, 2008). The sole exception has been the population study of Beijing family strains from Japan (Wada *et al.*, 2009). In this study, we found that the ancient sublineage was predominant in South Korea. Contrary to the situation in Japan, it is unique that the ancient (RD181 +) sublineage was the most prevalent in our 80 isolates. This sublineage has been reported to be a minor component of the Beijing family population, even in Japan (Maeda *et al.*, 2010). Therefore, our data also revealed the particularity of the population structure of the *M. tuberculosis* Beijing family in South Korea. These observations suggest that the distribution of the Beijing family sublineages is broadly variable in different regions. It still remains unclear whether the higher prevalence of the sublineages in certain areas has been caused by the difference of fitness or by occasional clonal expansion in the past.

K family strains, derivatives of the Beijing family, have been isolated frequently in both a population-based study and outbreaks of TB in South Korea (Kim *et al.*, 2001; Shamputa *et al.*, 2010). In this study, they were also detected with a high clustering rate (46.2 %) using RFLP genotyping (Fig. 2) despite no epidemiological link. They belonged to the ancient (RD181 +) sublineage. Therefore,

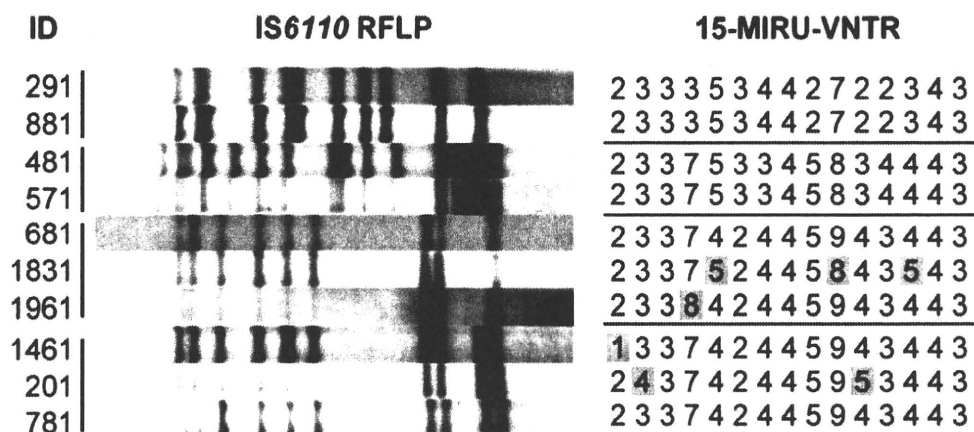

Fig. 3. 15-MIRU-VNTR profiles of 10 isolates belonging to four clusters by IS*6110* RFLP genotyping. The order of the VNTR loci is: MIRU 04, MIRU 10, MIRU 16, MIRU 26, MIRU 31, MIRU 40, ETR A, ETR C, QUB-2163b, QUB-26, QUB-4156, Mtub04, Mtub21, Mtub30 and Mtub39. The alleles that differed from those of other isolates in the respective clusters are shaded.

the domestic prevalence of K family strains may be related to the predominance of the sublineage in the country. The 15-MIRU-VNTR genotyping was able to classify the RFLP clusters as single-locus to three-locus variants comprising the K family strains (Fig. 3). This result suggests that frequent isolation of K family strains in South Korea may be caused not by a recent expansion of a single strain but by endemic fixation in the past. The genetic diversity within the K family strains should be analysed in more detail to uncover the history of their prevalence.

Combining our data with the reported VNTR types of strains from the surrounding countries for MST clustering analysis revealed that the tree topology (the branch formation) was consistent with the phylogenetic classification of strains, irrespective of their origins (Fig. 5). The types of strains originating in China (Beijing) reported by Jiao *et al.* (2008) were concentrated in the branch of the modern Beijing sublineage of South Korea and Japan. Although their data did not include information on sublineages of strains, the result is consistent with those

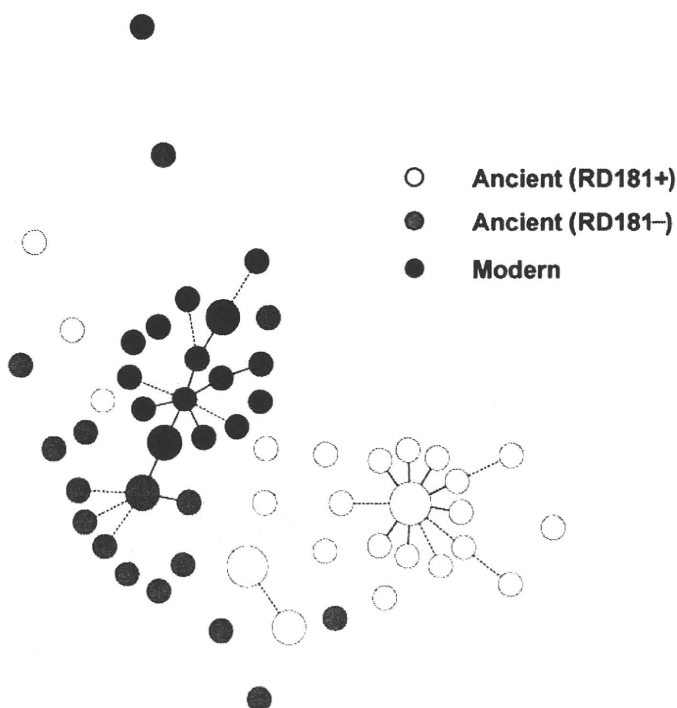

Fig. 4. Minimum spanning tree based on the 15-MIRU-VNTR genotypes of the 64 *M. tuberculosis* Beijing isolates. The 56 circles depicted correspond to the different types discriminated by the 15-MIRU-VNTR genotyping. Their sizes correspond to the number of isolates with a particular genotype. They were coloured according to the phylogenetic sublineages: ancient (RD181 +), ancient (RD181 −) and modern. Heavy lines connecting two types denote single-locus variants; thin lines connect double-locus variants; and dotted lines (black) connect triple-locus variants. The grey dotted lines represent the most likely connection between two types differing by more than three VNTR loci.

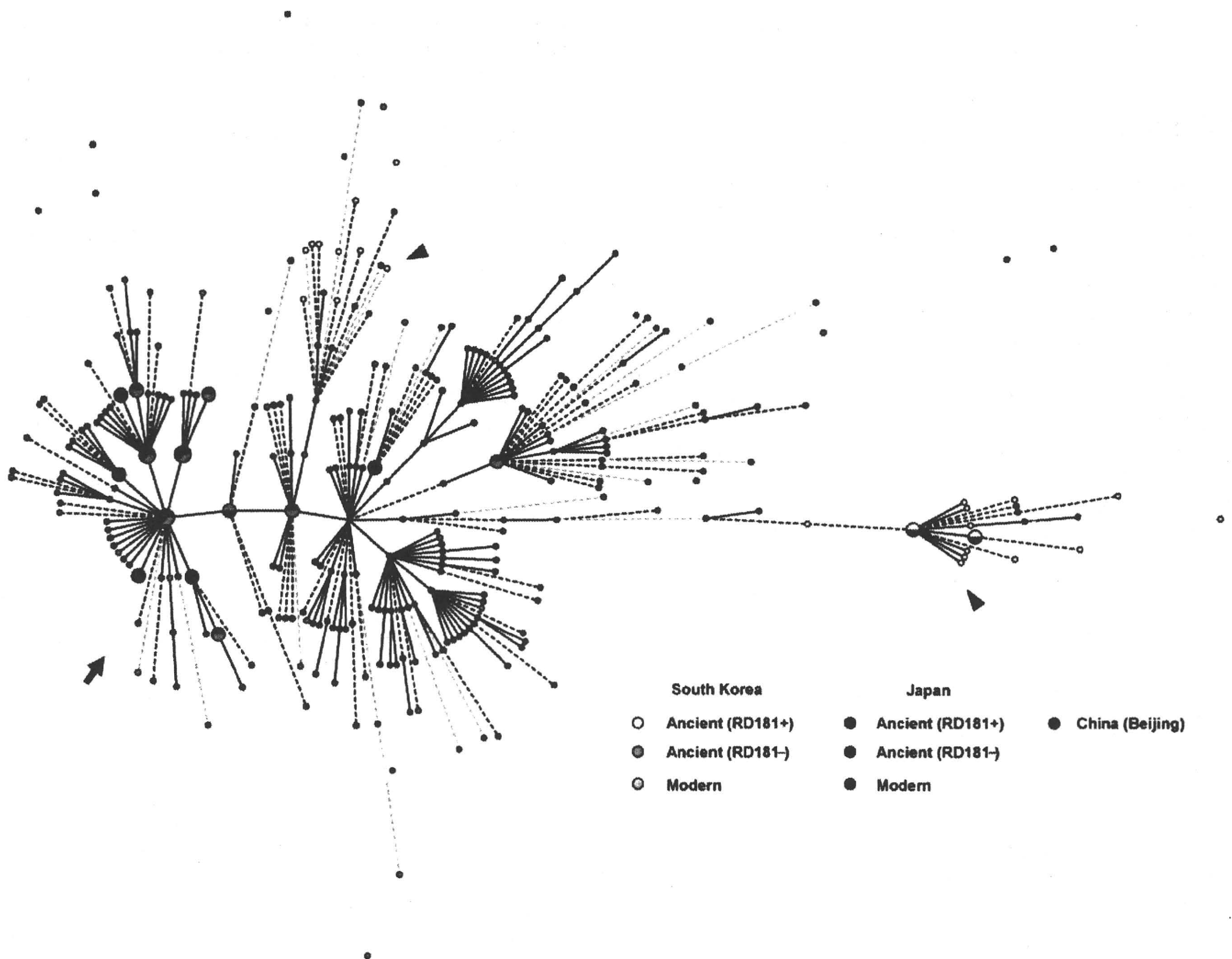

Fig. 5. Minimum spanning tree based on 15-MIRU-VNTR genotypes of the *M. tuberculosis* Beijing family from three East Asian countries. The 297 circles depicted correspond to the different types discriminated by the 15-MIRU-VNTR genotyping. The origin of each genotype is represented by different colours. The phylogenetic information of strains belonging to each genotype (from Japan and South Korea) is also indicated in colour. The designation of lines in the tree is the same as in Fig. 4. Two arrowheads point to clustering branches where genotypes originating from South Korea gather tightly. An arrow indicates a branch including genotypes of strains from all three countries.

of previous reports indicating that most of the isolated *M. tuberculosis* strains in the Beijing area (76–93 %) belonged to the modern Beijing sublineage (Mokrousov *et al.*, 2006; van Soolingen & Kremer, 2009). These results mean that similarity of 15-MIRU-VNTR types has been preserved in the ancient/modern sublineages of the Beijing family over the three East Asian countries. The phylogenetic validity of similar VNTR types must be verified in *M. tuberculosis* lineages because these genotypes are generally highly homoplasic. However, it is notable that similarity of VNTR types of the Beijing family has been observed based not on their geographical origins but in a phylogenetic manner. One merit of VNTR genotyping is its convenience for comparison of data from different countries (Allix-Béguec *et al.*, 2008; Mokrousov, 2008). Worldwide data accumulation of VNTR types of *M. tuberculosis* may shed light on the microevolution and genetic diversity of the species. Such global characterization of the phylogeographical distribution of the Beijing family may be useful in providing fundamental information about the ongoing worldwide expansion of the lineage.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Health, Labour and Welfare, Japan (Research on Emerging and Re-emerging

Infectious Diseases, Health Sciences Research Grants) and the Korean Centers for Disease Control & Prevention.

REFERENCES

Allix-Béguec, C., Harmsen, D., Weniger, T., Supply, P. & Niemann, S. (2008). Evaluation and strategy for use of MIRU-VNTR*plus*, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* **46**, 2692–2699.

Bifani, P. J., Mathema, B., Kurepina, N. E. & Kreiswirth, B. N. (2002). Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. *Trends Microbiol* **10**, 45–52.

Dou, H. Y., Tseng, F. C., Lin, C. W., Chang, J. R., Sun, J. R., Tsai, W. S., Lee, S. Y., Su, I. J. & Lu, J. J. (2008). Molecular epidemiology and evolutionary genetics of *Mycobacterium tuberculosis* in Taipei. *BMC Infect Dis* **8**, 170.

Filliol, I., Motiwala, A. S., Cavatore, M., Qi, W., Hazbon, M. H., Bobadilla del Valle, M., Fyfe, J., Garcia-Garcia, L., Rastogi, N. & other authors (2006). Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. *J Bacteriol* **188**, 759–772.

Iwamoto, T., Yoshida, S., Suzuki, K., Tomita, M., Fujiyama, R., Tanaka, N., Kawakami, Y. & Ito, M. (2007). Hypervariable loci that enhance the discriminatory ability of newly proposed 15-loci and 24-loci variable-number tandem repeat typing method on *Mycobacterium tuberculosis* strains predominated by the Beijing family. *FEMS Microbiol Lett* **270**, 67–74.

Jiao, W. W., Mokrousov, I., Sun, G. Z., Guo, Y. J., Vyazovaya, A., Narvskaya, O. & Shen, A. D. (2008). Evaluation of new variable-number tandem-repeat systems for typing *Mycobacterium tuberculosis* with Beijing genotype isolates from Beijing, China. *J Clin Microbiol* **46**, 1045–1049.

Kim, S. J., Bai, G. H., Lee, H., Kim, H. J., Lew, W. J., Park, Y. K. & Kim, Y. (2001). Transmission of *Mycobacterium tuberculosis* among high school students in Korea. *Int J Tuberc Lung Dis* **5**, 824–830.

Korea Centers for Disease Control & Prevention (2008). *Annual Report on the Notified Tuberculosis Patients in Korea.* Seoul: Korea Centers for Disease Control & Prevention.

Kremer, K., van der Werf, M. J., Au, B. K., Anh, D. D., Kam, K. M., van Doorn, H. R., Borgdorff, M. W. & van Soolingen, D. (2009). Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. *Emerg Infect Dis* **15**, 335–339.

Maeda, S., Wada, T., Iwamoto, T., Murase, Y., Mitarai, S., Sugawara, I. & Kato, S. (2010). The Beijing family *Mycobacterium tuberculosis* isolated from throughout Japan: phylogeny and genetic features. *Int J Tuberc Lung Dis* **14**, 1201–1204.

Milan, S. J., Hauge, K. A., Kurepina, N. E., Lofy, K. H., Goldberg, S. V., Narita, M., Nolan, C. M., McElroy, P. D., Kreiswirth, B. N. & Cangelosi, G. A. (2004). Expanded geographical distribution of the N family of *Mycobacterium tuberculosis* strains within the United States. *J Clin Microbiol* **42**, 1064–1068.

Mokrousov, I. (2008). Genetic geography of *Mycobacterium tuberculosis* Beijing genotype: a multifacet mirror of human history? *Infect Genet Evol* **8**, 777–785.

Mokrousov, I., Ly, H. M., Otten, T., Lan, N. N., Vyshnevskyi, B., Hoffner, S. & Narvskaya, O. (2005). Origin and primary dispersal of the *Mycobacterium tuberculosis* Beijing genotype: clues from human phylogeography. *Genome Res* **15**, 1357–1364.

Mokrousov, I., Jiao, W. W., Sun, G. Z., Liu, J. W., Valcheva, V., Li, M., Narvskaya, O. & Shen, A. D. (2006). Evolution of drug resistance in different sublineages of *Mycobacterium tuberculosis* Beijing genotype. *Antimicrob Agents Chemother* **50**, 2820–2823.

Park, Y. K., Bai, G. H. & Kim, S. J. (2000). Restriction fragment length polymorphism analysis of *Mycobacterium tuberculosis* isolated from countries in the western pacific region. *J Clin Microbiol* **38**, 191–197.

Park, Y. K., Shin, S., Ryu, S., Cho, S. N., Koh, W. J., Kwon, O. J., Shim, Y. S., Lew, W. J. & Bai, G. H. (2005). Comparison of drug resistance genotypes between Beijing and non-Beijing family strains of *Mycobacterium tuberculosis* in Korea. *J Microbiol Methods* **63**, 165–172.

Plikaytis, B. B., Marden, J. L., Crawford, J. T., Woodley, C. L., Butler, W. R. & Shinnick, T. M. (1994). Multiplex PCR assay specific for the multidrug-resistant strain W of *Mycobacterium tuberculosis. J Clin Microbiol* **32**, 1542–1546.

Shamputa, I. C., Lee, J., Allix-Beguec, C., Cho, E. J., Lee, J. I., Rajan, V., Lee, E. G., Min, J. H., Carroll, M. W. & other authors (2010). Genetic diversity of *Mycobacterium tuberculosis* isolates from a tertiary care tuberculosis hospital in South Korea. *J Clin Microbiol* **48**, 387–394.

Strauss, O. J., Warren, R. M., Jordaan, A., Streicher, E. M., Hanekom, M., Falmer, A. A., Albert, H., Trollip, A., Hoosain, E. & other authors (2008). Spread of a low-fitness drug-resistant *Mycobacterium tuberculosis* strain in a setting of high human immunodeficiency virus prevalence. *J Clin Microbiol* **46**, 1514–1516.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H. & other authors (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis. J Clin Microbiol* **44**, 4498–4510.

Tsolaki, A. G., Hirsh, A. E., DeRiemer, K., Enciso, J. A., Wong, M. Z., Hannan, M., Goguet de la Salmoniere, Y. O., Aman, K., Kato-Maeda, M. & other authors (2004). Functional and evolutionary genomics of *Mycobacterium tuberculosis*: insights from genomic deletions in 100 strains. *Proc Natl Acad Sci U S A* **101**, 4865–4870.

Tsolaki, A. G., Gagneux, S., Pym, A. S., Goguet de la Salmoniere, Y. O., Kreiswirth, B. N., Van Soolingen, D. & Small, P. M. (2005). Genomic deletions classify the Beijing/W strains as a distinct genetic lineage of *Mycobacterium tuberculosis. J Clin Microbiol* **43**, 3185–3191.

van Soolingen, D. & Kremer, K. (2009). Findings and ongoing research in the molecular epidemiology of tuberculosis. *Kekkaku* **84**, 83–89.

van Soolingen, D., Qian, L., de Haas, P. E., Douglas, J. T., Traore, H., Portaels, F., Qing, H. Z., Enkhsaikan, D., Nymadawa, P. & other authors (1995). Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. *J Clin Microbiol* **33**, 3234–3238.

Wada, T. & Iwamoto, T. (2009). Allelic diversity of variable number of tandem repeats provides phylogenetic clues regarding the *Mycobacterium tuberculosis* Beijing family. *Infect Genet Evol* **9**, 921–926.

Wada, T., Iwamoto, T. & Maeda, S. (2009). Genetic diversity of the *Mycobacterium tuberculosis* Beijing family in East Asia revealed through refined population structure analysis. *FEMS Microbiol Lett* **291**, 35–43.

Warren, R. M., Victor, T. C., Streicher, E. M., Richardson, M., Beyers, N., Gey van Pittius, N. C. & van Helden, P. D. (2004). Patients with active tuberculosis often have different strains in the same sputum specimen. *Am J Respir Crit Care Med* **169**, 610–614.

Yokoyama, E., Kishida, K., Uchimura, M. & Ichinohe, S. (2007). Improved differentiation of *Mycobacterium tuberculosis* strains, including many Beijing genotype strains, using a new combination of variable number of tandem repeats loci. *Infect Genet Evol* 7, 499–508.

Yun, K. W., Song, E. J., Choi, G. E., Hwang, I. K., Lee, E. Y. & Chang, C. L. (2009). Strain typing of *Mycobacterium tuberculosis* isolates from Korea by mycobacterial interspersed repetitive units-variable number of tandem repeats. *Korean J Lab Med* **29**, 314–319.

Tohoku J. Exp. Med., 2010, **220**, 21-25

High Numbers of Interferon-γ-Producing T Cells and Low Titers of Anti-Tuberculous Glycolipid Antibody in Individuals with Latent Tuberculosis

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Latent tuberculosis infection (LTBI) is defined as an infection with *Mycobacterium tuberculosis* (MTB) without clinical, bacteriological, or radiological findings, and its early diagnosis is essential for eradication of tuberculosis. To identify LTBI, we measured the numbers of interferon-γ producing T cells, based on the ELISPOT assay, and the antibody titers in the sera to tuberculous glycolipid antigen (TBGL-Ab). Seventeen culture-confirmed TB patients, 13 controls from TB endemic areas (EC) and 13 controls from TB non-endemic areas (NEC) were enrolled. Peripheral blood mononuclear cells (2.5×10^5 per well) were cultured on plates precoated with antibody against interferon-γ. ELISPOT response was defined as positive when the MTB-specific antigen-containing wells showed at least 6 spots and twice numbers of spots than negative control wells. ELISPOT responses were positive in 15 (88%), 8 (62%) and 4 (31%) subjects of TB, EC and NEC groups, respectively. The ELISPOT data differ between TB and NEC groups ($p < 0.01$) but not between TB and EC groups. In contrast, TBGL-Ab titers were elevated (> 2.0 U/ml) in 12 TB patients (71%), but only in one subject (8%) each from EC and NEC groups. These results indicate the high prevalence of LTBI in EC. In conclusion, LTBI is associated with positive ELISPOT assay and the low titer of TBGL-Ab, while positive results both in ELISPOT and TBGL-Ab assays indicate active TB. The low titer of TBGL-Ab is a helpful marker to identify LTBI in ELISPOT-positive individuals in TB endemic areas.

Keywords: interferon-γ producing T cells/TBGL antibody/active tuberculosis/latent tuberculosis
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An estimated 9 million new cases of tuberculosis (TB) and 2-3 million deaths are reported globally every year, making TB the leading cause of death from a single infectious pathogen (World Health Organization 2008). A major challenge in tuberculosis control is the diagnosis of latent tuberculosis infection that is defined as an infection with *Mycobacterium tuberculosis (MTB)* without clinical, bacteriological, or radiological findings. However, latent TB infected individuals may develop TB disease in the future (American Thoracic Society 2000a). Until recently, the tuberculin skin test (TST) has been used to identify persons infected by *Mycobacterium tuberculosis* who are at high risk for the progression to active disease. This method has several limitations especially in immunosupressed individuals such as HIV infected people who may reveal false negative TST response. In addition, the immune-reconstitution in HIV patients after the initiation of anti retroviral therapy makes this problem even more complicated (Okada et al. 2002; Changan-Yasutan et al. 2009). Furthermore, false positive TST responses are also possible in cases of BCG vaccination and/or non-tuberculosis mycobacteria (NTM). (American Thoracic Society 2000b; Jasmer et al. 2002; Barnes et al. 2004).

Recently, the region of difference 1 (RD1), that encodes two highly antigenic proteins ESAT-6 and CFP-10 (Mahairas et al. 1996; Behr et al. 1999; Gordon et al. 1999), was found to be present in all pathogenic strains of mycobacterium TB but not in most NTM species and also deleted from attenuated BCG strains. The ELISPOT method based on the numbers of spots made by interferon-γ producing T cells stimulated by CFP-10 or ESAT-6 (T-Spot.TB; Oxford Immunotec, Oxford, UK) and the ELISA based QuantiFERON-TB test (Cellestis Limited, Carnegie, Australia) were developed using these antigens to overcome the false positive result by TST (Codecasa et al. 2006; van Leeuwen et al. 2007), and both were approved by FDA. ELISPOT assay may offer greater sensitivity than the ELISA-based method (Pai et al. 2004; Ferrara et al. 2006).

Received August 13, 2009; revision accepted for publication November 14, 2009. doi:10.1620/tjem.220.21
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However, these T cell-based diagnostic approaches failed to differentiate active TB from LTBI.

The detection of anti-tuberculous glycolipid IgG antibody (TBGL-Ab) in serum, produced in response to the glycolipid antigen trehalose 6, 6′-dimycolate (TDM) has also been found to be useful for the diagnosis of active TB infection (Maekura et al. 1993, 2001; Ashino et al. 2005). It is generally accepted that cell-mediated immunity plays an important role in controlling MTB infection. In contrast, antibody response is believed to have no protective role, although recent studies in B cell-deficient mice showed that antibody responses are essential to contain mycobacterial infection (de Valliere et al. 2005; Abebe and Bjune 2009). We also believe that the antibody responses in AIDS patients could be detected although their T cell numbers were very low.

Until now, no comparison has been made between ELISPOT assay and TBGL-Ab method for human tuberculosis infection. Therefore, we compared both methods in patients with active pulmonary tuberculosis and in healthy adults from high and low endemic areas for TB to characterize LTBI.

Material and Methods

Subjects and specimens

All participants were recruited prospectively in Tohoku University Hospital and the Metropolitan Tokyo Fuchu Hospital over a 20-month period from November 2004 through June 2006. The diagnosis of pulmonary TB patients was based on the growing of *M. tuberculosis* from a sputum sample. The BCG vaccination status in the healthy BCG-vaccinated controls was confirmed by the presence of a typical BCG scar, which is a good indicator of BCG vaccination (World Health Organization. 1999; Pereira et al. 2001). In addition, chest X-rays abnormalities, history of TB or TB treatment were the exclusion criteria for this group. All the subjects were underwent voluntary counseling and testing for HIV-1 infection and a positive HIV result was an exclusion criterion to be a study subject. All of the participants gave informed written consent according to the protocols of the Tohoku University Ethical Committee on clinical investigations. Sample size was based on an estimated positivity of 80% in TB patients and 20% in control subjects with an alpha error of 5% and a power of 90%. The required sample size was 16 patients per arm.

ELISPOT assay

Freshly isolated peripheral blood mononuclear cells (PBMCs) (2.5×10^5 per well) were cultured on plates precoated with antibody against IFN-γ (T-Spot.TB Oxford Immunotec, Oxford, UK). After stimulation of the cells for 16 hours with ESAT-6 and CFP-10, spots were developed according to the manufacturer's instructions. Spot-forming units (SFUs) were counted with an automated reader (KS ELISPOT Carl Zeiss Microimaging-Germany). The test was considered as positive only when the MTB-specific antigen-containing wells had at least 6 spots more than the negative control wells and this number was at least twice that in negative control wells (Lalvani et al. 2001).

TBGL-Ab assay

The serum specimens were assayed without knowledge of the clinical status of the patients. We used TBGL assay kits manufactured using TBGLs consisting of TDM and a minor glycolipid (trehalose monomycolate, diacyltrehalose, phenolic glycolipid 2,3,6,6-tetraacyl-trehalose-2-sulfate, and 2,3,6-trialcyl-trehalose) (Kyowa Medex Co.,Ltd. Tokyo, Japan). Details of this assay were reported previously (Maekura et al. 2001). A value greater than or equal to 2 U/mL was considered to be positive.

Statistical analyses

We compared sensitivity and specificity between ELISPOT and TBGL-Ab assays using the chi squared test. We also compared the titers of TBGL-Ab and the ELISPOT between TB patients with and those without active tuberculosis (NEC and EC) using the Mann-Whitney *U* test. Correlations were evaluated using the Spearman test. A p value of less than 0.05 was considered significant. All the calculations were done using the statistical package STATA version 8.2 (StataCorp LP, College Station, Texas).

Results

We recruited 43 individuals: 25 males (M) and 18 females (F). Seventeen of them were culture-proven pulmonary TB patients (10M/7F). Twenty-six participants were healthy BCG-vaccinated controls. The non-endemic control (NEC) comprised 13 (7M/6F) Japanese, and the 13 endemic controls (EC) comprised immigrants who were born and had lived in countries with a high incidence of TB. Among the 13 EC, 9 (5M/4F) were from Peru, 2 (1M/1F) from China, 1 (1M) from Zambia, and 1 (1M) from Bolivia. No one was less than 16 years old. The mean ± S.D. ages were 54.3 ± 20.9, 56.7 ± 3.17 and 26 ± 9.59 years in the TB, NEC and EC subjects, respectively. Endemic controls were clearly younger ($p < 0.05$) than others. All the 43 subjects recruited in this study had a BCG scar.

ELISPOT and TBGL-Ab assays were positive in 15/17 (88%) and 12/17 (71%) of TB cases, in 4/13 (31%) and 1/13 (8%) of NEC cases and in 8/13 (62%) and 1/13 (8%) of EC cases, respectively. ELISPOT and TBGL-Ab assays had comparable sensitivity ($p = 0.2$) to diagnose active TB, but TBGL-Ab had greater specificity than ELISPOT assay to distinguish active tuberculosis (specificity: 92% versus 54% for TBGL-Ab and ELISPOT assays, respectively; $p < 0.01$). In addition, it should be noted that both of two TB patients who were negative in the ELISPOT assay, were TBGL Ab positive (19.5 and 6.7 U/mL)

ELISPOT assay positive subjects in NEC (31%) and EC (62%) groups were considered as latent TB infected individuals, as they had neither any TB specific symptom nor any abnormality on chest X-ray. Accordingly, there was a significant difference between percentage of positive results by ELISPOT assay and TBGL-Ab titers only in EC group ($p = 0.01$) (Fig. 1). Among the ELISPOT assay positive cases, 10/15 (67%), 1/4 (25%) and 1/8 (12.5%) were also TBGL-Ab positive in the TB, NEC and EC groups, respectively.

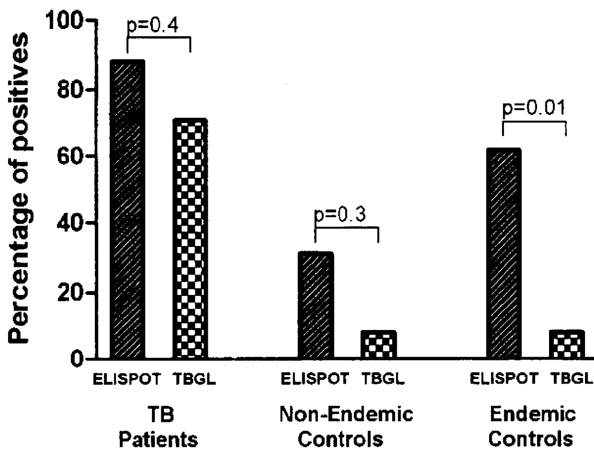

Fig. 1. Comparison between percentage of positive ELISPOT and TBGL-Ab assays. The percentages of positive results with ELISPOT and TBGL-Ab tests among patients with active tuberculosis (n = 17), non endemic control (n = 13) and endemic control (n = 13) groups were shown. Only in endemic control group, the percentage of ELISPOT positivity differ significantly from the TBGL-Ab positives rates (p = 0.01).
Non endemic controls: Volunteers from Japan
Endemic controls: Volunteers from high TB incidence countries.

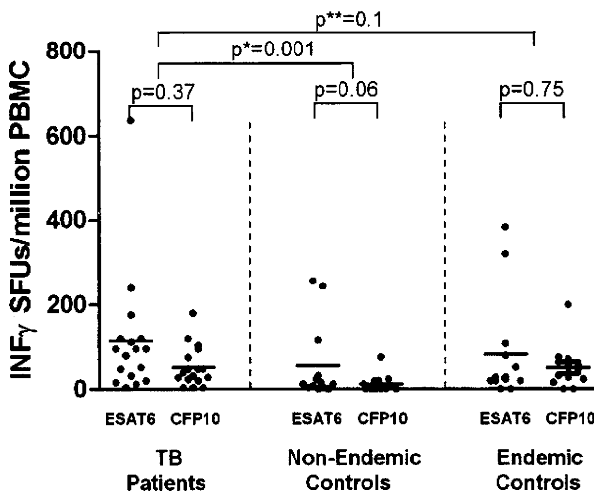

Fig. 2. Comparison of interferon-γ-producing T cells. Dot plot of individual response to ESAT-6 and CFP-10 expressed in numbers of interferon-γ spot forming units (IFN-γ SFUs) per million of peripheral blood mononuclear cells (PBMC). SFUs were compared between ESAT-6 and CFP-10 in TB patients (n = 17), non-endemic control (n = 13) and endemic control (n = 13) groups. Comparisons were also made between TB patients and both non-endemic control and endemic control groups as well. IFN-γ SFUs only differ significantly between TB patients and non-endemic control group (p = 0.001).
p*, p value between TB patients and Non-Endemic Controls
p**, p value between TB patients and Endemic Controls

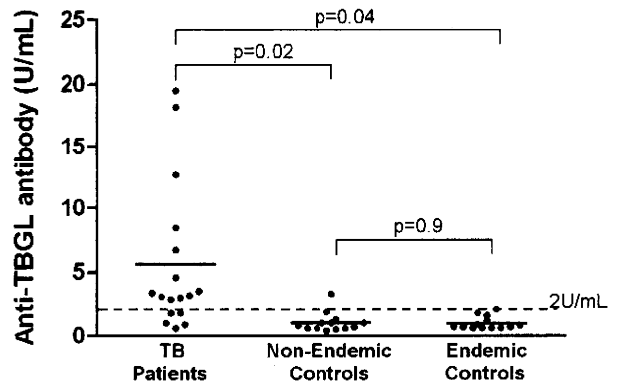

Fig. 3. Comparison of anti-TBGL antibody results. Dot plot of individual responses to anti-TBGL antibody (TBGL) for active TB patients (n = 17), non-endemic controls (n = 13) and endemic controls (n = 13). Significant differences were observed between TB patients and both non-endemic control and endemic control groups (p = 0.02 and 0.04, respectively).
The dashed line represents the cutoff value of 2 U/mL for TBGL antibody.

In our study, ELISPOT assay showed significant differences between TB and NEC (p = 0.001) but not between TB and EC (p = 0.1) (Fig. 2). There were no statistical differences between spot forming units (SFUs) against ESAT-6 and CFP-10 in all groups. But, the anti-TBGL-Ab titers, depicted in Fig. 3, were significantly lower in subjects with LTBI from both EC (0.9 ± 0.26 U/mL) and NEC (0.7 ± 0.49) groups than active TB group (p = 0.04 and 0.02 respectively). In addition, there was no correlation between anti-TBGL-Ab titers and the SFUs after stimulation either with ESAT-6 (correlation coefficient = 0.11, $p > 0.48$) or CFP10 (correlation coefficient = 0.10, $p > 0.51$).

Discussion

The present study suggests that both positive in ELISPOT and TBGL-Ab assays indicate active TB disease. On the other hand, positive ELISPOT assay with the low titers of TBGL-Ab indicates LTBI.

The incidence of TB per 100,000 inhabitants in Japan is 25, in Peru 206, in China 206, in Zambia 618 and in Bolivia 280 (World Health Organization 2007). Immigrants from TB endemic countries constitute a high-risk population because of the high rates of reactivation (Codecasa et al. 1999; Das et al. 2006). In Japan infection is heavily concentrated in the age group of more than 60 years and 82% of the active TB patients are in the age of more than 40 years (Mori 2000).

In our study, the rate of high ELISPOT positivity in EC group (62%) than that of NEC (31%) revealed elevated incidence of LTBI with increased exposure to TB that is also in concordance with a previous report in Japan (Harada et al. 2006). Using QuantiFERON-TB Gold assay, Harada et al. (2006) reported that in those more than 50 years old, latent TB infection was found in 23%, which was compara-

ble to the age of our NEC population (56.7 ± 3.17 years old), in which it was found in 31%.

We hypothesize that IFN-γ production (TH1-dependent) in response to antigenic challenge may be long-lasting in memory cells, while antibody production against mycobacterium glycolipid (TH2-dependent) may be more related to the active replication of mycobacteria, or inflammation that predisposes TH2. In fact the decrease of anti-TBGL antibody after therapy has already been reported (Maekura et al. 2001), but the effect of active TB treatment on IFN-γ responses has shown declining responses in some studies, whereas others have shown unchanging, fluctuating or increasing responses during treatment (Pai et al. 2004; Dheda et al. 2005). This variation was also observed in case of other cytokines (Turgut et al. 2006; Djoba et al. 2009).

TBGL-Ab titers were significantly different between active TB and both NEC and EC. In preliminary studies, ELISPOT (Dosanjh et al. 2008) and TBGL-Ab (Maekura et al. 2001) assays showed sensitivities of 85% and 81% respectively, in diagnosis of active TB. In our study, the sensitivity was 88% for the ELISPOT assay and 71% for the TBGL-Ab assay, confirming the high rates of detection in active TB patients. Moreover, the combined sensitivity for the two methods reached nearly 100%.

The reasons for false-negative ELISPOT assay results in active tuberculosis have not been clarified. Previous studies based on IFN-γ production reported a frequency of 4 to 38% of false negatives (Pai et al. 2004). Kobashi in Japan, using QuantiFERON TB-2G, a method based on a whole blood assay, found 7.1% negative result (Mean age: 66 years old) in TB patient suggesting due to lymphocytopenia caused by aging or no production of IFN-γ for MTB specific antigens (Kobashi et al. 2009). In our study we found 12% (2/17) of false negatives. We think that lymphocytopenia by aging cannot explain this phenomenon since our method is based on the number of IFN-γ producer cells instead of whole blood stimulation, Moreover, these two patients possessed high titers of anti-TBGL-Ab (19.5 and 6.7 U/mL). The mechanism of immune protection provided by humoral immunity is not understood fully. When and how it cooperates with the cell mediated immunity and works in synergy is a topic of future investigations. Recently, in Thailand, we do not have enough evidence that the levels of anti-TBGL IgG are associated with tuberculosis infection in children (unpublished data).

The originality of this study, performed in a country with a low incidence of TB, is that a positive TBGL-Ab can be quite specific for active TB infection in both non-endemic and endemic populations. TDM (the main component of the TBGL-Ab assay) is present in large amounts on the surface of virulent but not avirulent MTB (Bloch and Noll 1953) and is also one of the important factors for the granuloma formation among the lipids in the mycobacterial cell-wall fraction (Behling et al. 1993; Geisel et al. 2005). Hunter et al. described that virulent MTB releases large amounts of TDM during growth as a pellicle within cavities, and such growth results in the activation of the toxicity and antigenicity of TDM (Hunter et al. 2006). Recently, we reported the increased synthesis of anti-TBGL IgG in relation to cavity formation in pulmonary tuberculosis and elevated titers of C-reactive protein, which suggest that TBGL IgG could reflect inflammation in active pulmonary TB patients. (Mizusawa et al. 2008). Additionally, the anti-TBGL antibody is not influenced by prior BCG vaccination (Nabeshima et al. 2005). These findings may explain the high titers of TBGL antibody in active TB but not in the control groups.

Therefore, anti-TBGL-Ab could be suitable for screening serum for detection of active TB patients especially in high endemic countries. One of the main limitations in this study is the low number of patients. Therefore, more extensive studies based on the immune status of patients and people at high risk to develop TB should be done in order to confirm our findings.

Acknowledgments

We thank Ms. Yuko Sato for her technical assistance. This work was supported by Grant-in-Aid for Special Educational Grant from Ministry of Education, Culture Sports, Science and Technology and Grant-in-Aid from the Scientific Expenses for Health and Welfare Program from the Ministry.

References

Abebe, F. & Bjune, G. (2009) The protective role of antibody responses during *Mycobacterium tuberculosis* infection. *Clin. Exp. Immunol.*, **157**, 235-243.

American Thoracic Society (ATS). (2000a) Diagnostic standards and classification of tuberculosis in adults and children. *Am. J. Respir. Crit. Care Med.*, **161**, 1376-1395.

American Thoracic Society (ATS). (2000b) Targeted tuberculin testing and treatment of latent tuberculosis infection. *Am. J. Respir. Crit. Care Med.*, **161**, S221-247.

Ashino, J., Ashino, Y., Guio, H., Saitoh, H., Mizusawa, M. & Hattori, T. (2005) Low antibody response against tuberculous glycolipid (TBGL) in elderly gastrectomised tuberculosis patients. *Int. J. Tuberc. Lung Dis.*, **9**, 1052-1053.

Barnes, P.F. (2004) Diagnosing latent tuberculosis infection: turning glitter to gold. *Am. J. Respir. Crit. Care Med.*, **170**, 5-6.

Behling, C.A., Perez, R.L., Kidd, M.R., Staton, G.W. Jr. & Hunter, R.L. (1993) Induction of pulmonary granulomas, macrophage procoagulant activity, and tumor necrosis factor-alpha by trehalose glycolipids. *Ann. Clin. Lab. Sci.*, **23**, 256-266.

Behr, M.A., Wilson, M.A., Gill, W.P., Salamon, H., Schoolnik, G.K., Rane, S. & Small, P.M. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science*, **284**, 1520-1523.

Bloch, H. & Noll, H. (1953) Studies on the virulence of tubercle bacilli; variations in virulence affected by tween 80 and thiosemicarbazone. *J. Exp. Med.*, **97**, 1-16.

Chagan-Yasutan, H., Saitoh, H., Ashino, Y., Arikawa, T., Hirashima, M., Li, S., Usuzawa, M., Oguma, S., Telan, E., Obi, C.L. & Hattori, T. (2009) Persistent elevation of plasma osteopontin levels in HIV patients despite highly active antiretroviral therapy. *Tohoku J. Exp. Med.*, **218**, 285-292.

Codecasa, L.R., Porretta, A.D., Gori, A., Franzetti, F., Degli Espositi, A., Lizioli, A., Carreri, V., Di Proietto, M.C.,