

Figure 12.7. Development of clinical grade HVJ envelope vector. HVJ infects human cell from working cell bank (WCB), and human cell-derived HVJ is prepared using animal product-free medium in a pilot plant based on good manufacturing practice (GMP) standard. The envelope vector prepared from human cell-derived HVJ is more efficient in gene transfection compared with that from egg-derived HVJ. (See Color Insert.)

observation). We recently succeeded in producing HVJ in human cells. We cloned HEK 293 cells that produce a high titer of HVJ after infection, and determined the growth conditions for these cells in an animal product-free medium using a bioreactor. We are able to culture these cells in 10 liters of serum-free medium at a density of more than 10^6 cells/ml, and we can obtain HVJ at the efficiency of more than 3×10^9 particles/ml (Fig. 12.7). The production in a 10 liter culture is comparable to that in 500 chick eggs. A pilot plant to commercially produce clinical grade HVJ envelope vector was established in a venture company named GenomIdea, Inc. (7-7-15 Saito-Asagi, Ibaragi, Osaka 567-0085, Japan; <http://www.anges-mg.com/news/030324.htm>). GenomIdea created a working cell bank, master cell bank and master virus bank for producing clinical grade HVJ for Phase I and IIa trials. They also set up an apparatus to purify inactivated HVJ using two different column systems. The human cell-derived HVJ envelope vector was more effective for gene transfection than

the egg-derived HVJ envelope vector (Fig. 12.7). Human cell-derived HVJ envelope vector is also available for drug delivery both *in vitro* and *in vivo*. This vector will soon be available for human gene therapy.

VIII. MAIN ABBREVIATIONS

HVJ	hemagglutinating virus of Japan
HN	hemagglutinin-neuraminidase
F	fusion
FITC	fluorescein isothiocyanate
ODN	oligodeoxynucleotide
HA	hemagglutinin
CTLs	cytotoxic T cells
ES	embryonal stem
GFP	green fluorescent protein
siRNA	small interfering RNA
CSF	cerebrospinal fluid
HGF	hepatocyte growth factor
SGCs	spiral ganglion cells
KM	kanamycin
DCs	dendritic cells
MAA	melanoma-associated antigen
TAA	tumor-associated antigen
YFP	yellow fluorescent protein
PE	phycoerythrin
gp100	glycoprotein 100
TRP2	tyrosine-related protein 2
DTH	delayed-type hypersensitivity
WCB	working cell bank
GMP	good manufacturing practice
HVJ-E	HVJ envelope

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Further Reading

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BCG を用いた抗酸菌の抗原性および病原性に関する研究

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BCG ワクチンは結核に対する安全なワクチンとして、1948 年以降延べ 25 億人以上の人々に使用されてきた。その効果については疑問が持たれており、現行の BCG にかわる著効なワクチンの開発が望まれている。また、抗酸菌による感染症の中で 2 番目に患者数の多いハンセン病についてもワクチンの開発が望まれている。本研究では、BCG の産生する感染防御抗原である Ag85 complex を過剰発現させることで BCG の免疫効果を高めることができることを明らかにした。また、結核菌の特徴である宿主内における休眠状態に同菌の持つ 2 種類の低分子ストレス蛋白質 HspX (Acr) と HrpA が関係し、菌の休眠および再活性化と相関があることを明らかにした。HrpA は一度酸素飢餓状態に陥った後に再度酸素に触れることにより発現することから、菌の再活性化に関与することが示唆された。さらに、骨結核および BCG 接種の副作用である骨炎に関連し、BCG 感染骨芽細胞が、TNF- α 受容体ファミリーの 4-1BB (CD137) を産生すること、4-1BB は破骨細胞前駆細胞上の 4-1BB L (CD137L) を介して逆向きのシグナルを細胞内に伝達することにより、破骨細胞への分化・成熟を抑制することを明らかにした。TNF- α 受容体ファミリーの、いわゆるリバースシグナルの伝達経路は全く不明であったが、Akt の経路を介すること、NFAT2 の転写を抑制することが明らかになった。

はじめに

ヒトと抗酸菌とのつきあいは長く、一般には 1 万年から 1 万 5 千年前くらい前からとされている。さらに 10 万年前にはすでに関わり合いを持っていたとする説もある。Robert Koch が 1882 年に結核菌を発見してから 120 年が過ぎ、そのゲノム配列が解明されてから、すでに 7 年が経つ。らい菌が Gerhard Amauer Hansen により発見されたのは結核菌よりも 8 年前で、そのゲノムが解明されたのは 2001 年のことである。しかし、未だに両菌が原因である結核およびハンセン病に悩まされている人の数は多い。WHO の統計によれば、世界人口の 3 分の 1 が結核菌に感染しており、全世界で新たに発生する結核患者は年間 850 万人で、約 180 万人が結核で死亡している。結核による死亡者の 98% 以上は発展途上国であり、特にエイズの合併が拍車をかけている。また多剤耐性結核菌が蔓延しており、その感染者数は約 5000 万人にのぼると考えられている。

結核菌は呼吸器を介して感染するが、感染後すぐに発症

するのは感染者の約 10% であり、ほとんどは持続生残菌 (persister) として冬眠状態 (dormancy) のまま数十年にわたって潜伏している。その中で二次結核 (内因性再燃) として発症するのは 5% 程度のみで、残りの大半のケースはそのまま発症することもなく、宿主の死とともに死滅する。

一方、ハンセン病の日本における最近の年間新規患者数は、日本人では 5 名前後、在日外国人では 10 名前後であり、きわめて少ない。しかし世界的に見れば、現在治療を受けているハンセン病患者は 110 カ国 46 万人であり、また、新規患者数は 2002 年 1 年間に 62 万人と報告されている。2002 年の国外における新患としては、インド (473,658)、ブラジル (38,365)、ネパール (13,830)、タンザニア (6,492)、モザンビーク (5,830)、マダガスカル (5,482) に多数の発生が見られ、これら 6 カ国で世界の 88% を占める。このように結核およびハンセン病は今なお重要な感染症であり、新たな治療法の開発とともに予防法の確立、特に新規ワクチンの開発が強く望まれている。

結核に対しては生菌ワクチンである BCG が広く使用されている。これはパスツール研究所の Albert Calmette と Carnille Guérin によって、強毒菌である牛型結核菌 (*Mycobacterium bovis*) を 15 年間、231 代にわたってグリセリン胆汁馬鈴薯培地に継代培養して得られた病原性のない弱毒菌である。これまでに 30 億人以上に接種され、持続

性が長く、安価であり、しかも安全性についても十分に証明されているワクチンである。当初は経口投与であったが、すぐに経皮投与に切り替えられ現在に至っている。また、結核だけでなく、他の抗酸菌による感染症に対しても有効と考えられている。しかし、さまざまな疫学調査の結果等からすると、BCG ワクチンの効果については疑問視されており、乳幼児に対しては有効であるが、成人の肺結核に対しては効果があまり無いというのが一般的な見解となっている。

現在の抗酸菌をめぐる問題点として、今述べた、著効なワクチンがないこと、薬剤耐性菌が蔓延していること、他の疾患、特に AIDS との合併による重篤化がある。またどのようにして、どのような状態で長期間ヒト体内に潜伏しているかについては謎の部分が多く、ヒト体内での長期にわたる冬眠状態のメカニズムの解明が重要な課題である。

1. 抗酸菌の α 抗原

BCG による感染防御免疫は生菌の接種によって得られるが、死菌では得られないことがよく知られている。生菌と死菌の違いとして、菌体外へ産生分泌される成分の有無があげられる。すなわち、生菌では分泌蛋白質と総称される種々の蛋白質が持続的に菌体外に産生・分泌されるが、死菌ではそれらの分泌はほとんど無い。Pal と Horwitz は結核菌の分泌蛋白質で免疫することによりある程度の結核菌の感染防御ができたことを報告している (30)。分泌蛋白質によって誘導される感染防御免疫はらい菌の感染に対しても有効である (16)。

結核菌、BCG の培養上清中にはさまざまな蛋白質が含まれる。この中で α 抗原は最もよく研究されてきた分泌蛋白質である。 α 抗原は約 40 年前に大阪大学微生物病研究所の米田博士と福井博士によって精製、同定された (49)。その結果、結核菌の主要蛋白質抗原であることが明確に示された。 α 抗原は抗酸菌に広く分布する蛋白質であり、それぞれの菌種の α 抗原は血清学的に区別することができる (44)。その後、1989 年に当時同研究所にいた山田毅博士と味の素株式会社中央研究所によって BCG の α 抗原遺伝子がクローニングされた (15)。さらにその後海外のグループにより構造の類似した蛋白質抗原が複数あることがわかり、Ag85 complex と総称されている。Ag85 complex は 85A, 85B= α 抗原, 85C の 3 種類の蛋白質からなり、結核菌では全分泌蛋白質の約 30% を占める。*M. kansasii* や *M. avium*, *M. intracellulare* では 85B= α 抗原の分泌量は結核菌に比し極度に多く、逆に 85A と 85C の産生は僅かである (27)。Nagai らは MPT51 の N 末端アミノ酸配列が Ag85 complex に類似していることを報告したが、その遺伝子クローニングの結果、蛋白質全体にわたり相同性を有することから、Ag85 complex に準ずる蛋白質であることが明らかとなった (17, 22)。

ところで、非結核性抗酸菌の数菌種について α 抗原遺伝

子のクローニングをおこなってみると、 α 抗原 C 末端部位に種特異的エピトープが存在していた (10, 11, 21, 23, 41)。菌にとっても Ag85 complex は重要な蛋白質であり、ミコール酸の合成に携わる酵素であることが明らかとなり (3)、また、フィブロネクチン結合能が強いことから感染に重要な役割をしていることが示唆されてきた (20)。Ag85 complex は IFN- γ の産生を誘導し、感染を予防する防御抗原であることもわかってきた (7)。Ag85 complex の感染防御免疫誘導能は高く、マウスの感染実験では 85A, α 抗原, 85C のいずれの蛋白質も 2 mg 前後の免疫でらい菌の増殖を抑制した (19)。

2. 組換え BCG ワクチン

この約 20 年間に様々なタイプの結核ワクチンの開発が世界中でおこなわれてきた。主要なものとして、DNA ワクチン、菌体蛋白質あるいは細胞壁脂質等を使用したサブユニットワクチン、免疫惹起能の強い蛋白質あるいはサイトカインを産生する組換え BCG ワクチン、弱毒 (栄養要求株) 化したマイコバクテリア、非定型抗酸菌の利用がある。我々が後述の組換え BCG ワクチンを作製した段階で現行の BCG ワクチンを凌駕するものはなかった。

組換え BCG ワクチンの作製は 1980 年後半、抗酸菌に遺伝子組み換え技術が導入されたことによって可能となった。まず米国 Bloom のグループが BCG に形質転換可能な抗酸菌—大腸菌シャトルベクターを開発した (36)。ほぼ同時にフランスパスツール研究所でも同様のベクターが開発された (31)。1991 年、Bloom のグループと Young のグループによって、これらのベクターを利用して外来遺伝子を導入した BCG を作成し、宿主に免疫応答を誘導させた報告がなされた (1, 37)。前述の山田毅博士と味の素株式会社中央研究所のグループは α 抗原の分泌シグナルを応用し、外来抗原を BCG 菌体外に分泌する系を開発した (14)。Stover らは 1993 年に *Borrelia burgdorferi* の表層リポ蛋白質を発現する BCG を動物に投与し、感染を防御できたと報告したが、これが組換え BCG による感染防御の最初の報告である (38)。これまでにさまざまな病原体および疾患を対象に組換え BCG の作製が行われている (29)。

BCG ワクチンの特徴に長期にわたる免疫の持続性があるが、組換え BCG でもその特徴が受け継がれている。HIV-1 の gag p17 の B 細胞エピトープと α 抗原のキメラ蛋白質を発現する組換え BCG を接種したマウスでは最終免疫後このエピトープを認識する抗体の産生が 14 ヶ月以上持続した (47)。またマラリアのエピトープに対しても 7 ヶ月以上にわたり抗体の産生が持続した (13)。

ところで、菌体成分の中で感染防御抗原が明らかとなれば、それを多く産生させることで、BCG のワクチンとしての効果を上昇できる可能性がある。Ag85 complex が感染防御抗原であることから、Ag85 complex 過剰発現株は現行の BCG を上回るワクチン効果を有することが期待できる。Ag85 complex の遺伝子をつないだプラスミドで BCG を形質

転換することによって Ag85 complex 過剰産生株を作製した。BCG の Ag85 complex 遺伝子を使用したが、自身のプロモータを使用した場合には過剰発現するものの、その発現量は十分ではない (26)。そこで、強力なプロモータである *M. kansasii* と *M. avium* の α 抗原プロモータを用いたところ、発現量は増加した (25)。Ag85A 過剰発現株 rBCG/85A あるいは Ag85A, α 抗原 (=85B) および MPB51 の 3 抗原を過剰発現する rBCG/BA51 について感染防御実験をおこなった。rBCG/85A と rBCG/BA51 の免疫効果は C57BL/6 マウスのフットパッドに感染させたい菌の増殖抑制効果で判定した。これらの組換え BCG を 5.0×10^7 個接種し、1 カ月後フットパッドに 5.0×10^3 個のらい菌を感染させる。そして 25 週後の抗酸菌数を数える。らい菌の増殖速度が遅いため、実験には長期間必要とする。その結果、親株である BCG Tokyo 株を越える効果があった (図 1) (24)。さらにその効果は初回免疫 5 カ月後に同じ rBCG/85A を追加免疫することで増強された。抗酸菌に対する感染防御には Th1 タイプの免疫が重要であるが、rBCG/85A 接種マウス脾細胞の IFN- γ および NO 産生能は長期間保たれており、BCG 接種マウスよりも強力であった。それでは 1 つの菌から複数の感染防御抗原を過剰発現すればより高い効果が得られるのか。rBCG/BA51 は上述のように 3 種類の抗原を過剰発現しているが、rBCG/BA51 による免疫応答は rBCG/85A によるものよりも高い結果となった (図 2) (25)。なお Horwitz らはほぼ同時期に異なる作製法により Ag85B を過剰発現する組換え BCG を作製し、結核菌に対する感染防御効果が強いことを報告している (6)。

3. 低分子ストレス蛋白質

結核菌に感染したほとんどの人の体内では結核菌は持続生残菌 (persister) として冬眠状態 (dormancy) のまま数十年にわたって潜伏している。どのようにして、どのような状態で長期間ヒト体内に潜伏しているか、また何がきっかけで冬眠状態の菌が目覚め、増殖を始めるのか、未だに解明されていない部分が多い。冬眠状態につながる環境として、酸素飢餓状態が言われている (32, 35, 48)。結核菌は好気性菌であるが、徐々に酸素分圧が低下した場合は嫌気状態下でも死滅することなく長期間生存する。他に栄養物の枯渇 (4)、低 pH (5)、酸化窒素の低下 (46) 等の環境との関係が示されている。

ストレス蛋白質は種々の環境の変化、適応に必要であるが、低分子ストレス蛋白質である α -crystallin (HspX) は酸素飢餓にしたがい発現してくる (39, 50)。 α -crystallin はマクロファージ内での菌の生存に必要であることが示されている (51)。さらに、蛋白質合成の場であるリボゾームと結合する (39)。これらのことから α -crystallin は結核菌の dormancy と密接に関係していると考えられている。ところで、BCG に熱ストレスを与えると様々なストレス蛋白質が誘導される。そのひとつ HrpA は熱ストレスで発現するとともにリボゾームへ集積してくる (28)。N 末端のアミノ酸

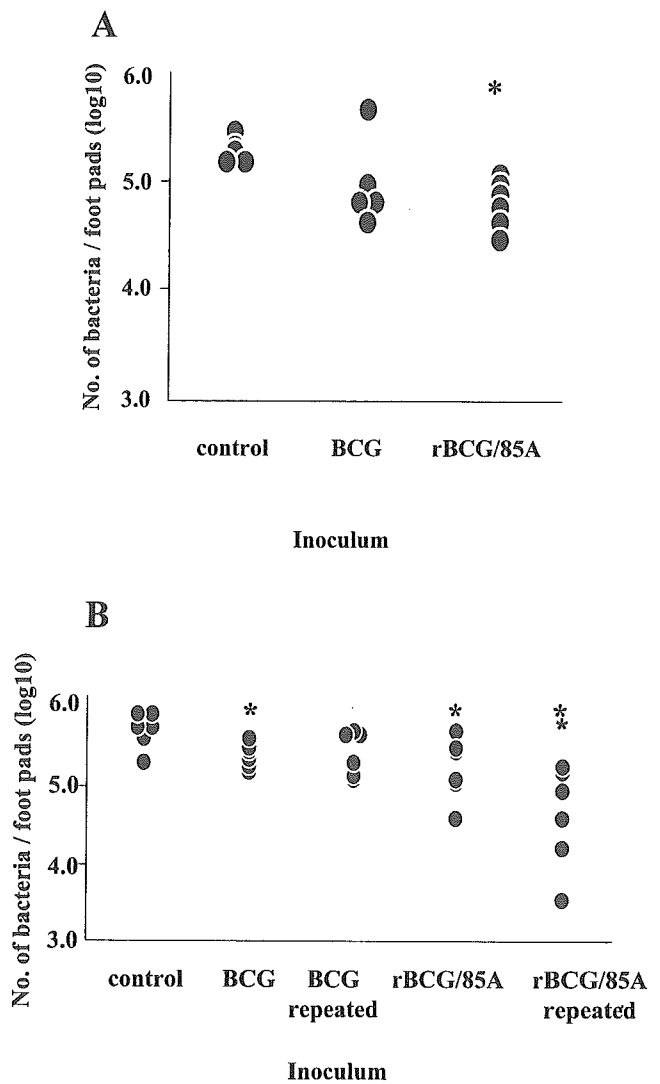


図 1. らい菌感染に対する rBCG/85A 免疫による感染防御効果。(A) 短期間における免疫効果。親株である BCG あるいは rBCG/85A を 6 週齢の C57BL/6 マウスの胸部皮内に 1 個体につき 5.0×10^7 個接種した。1 カ月後、各群のマウスのフットパッドに 5.0×10^3 個の *M. leprae* 生菌を接種し、25 週後にフットパッドから検出された抗酸菌数を測定した。rBCG/85A 接種群では対照群と比較して検出される菌数が有意に ($P < 0.01$) 減少した。(B) BCG あるいは rBCG/85A を接種した 5 カ月後、各群の半数のマウスに同量の BCG あるいは rBCG/85A を接種した。さらに 1 ヶ月のちに (最初の接種から 6 ヶ月後)、各群のマウスのフットパッドに 5.0×10^3 個の *M. leprae* 生菌を接種し、30 週後にフットパッドから検出された抗酸菌数を測定した。rBCG/85A 接種群では対照群と比較して検出される菌数が有意に (* $P < 0.05$, ** $P < 0.01$) 減少した。黒丸はフットパッド中の抗酸菌数を示す。文献 25 より引用、改変。

配列から HrpA は α -crystallin のホモログであることがわかった。 α -crystallin とは異なり酸素飢餓によって誘導されることはなかったが、興味深いことに BCG を一定期間酸素飢餓状態にした後に、再度酸素に暴露することで発現量が増加した (図 3) (40)。このことは菌の再燃化と関係があることを示唆するが、具体的な役割については不明である。

4. 抗酸菌の感染と骨代謝の関係

ところで結核菌は肺以外にも感染し、ほぼすべての臓器

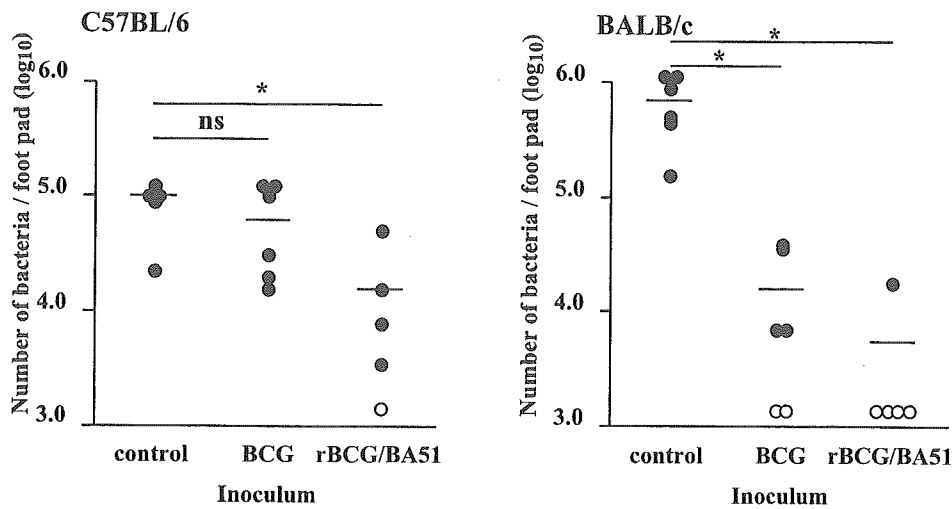


図2. らい菌感染に対する rBCG/BA51 免疫による感染防御効果。黒丸はフットパッド中の抗酸菌数を示す。白丸はフットパッド中に抗酸菌が認められなかったことを示す。文献 25 より引用。

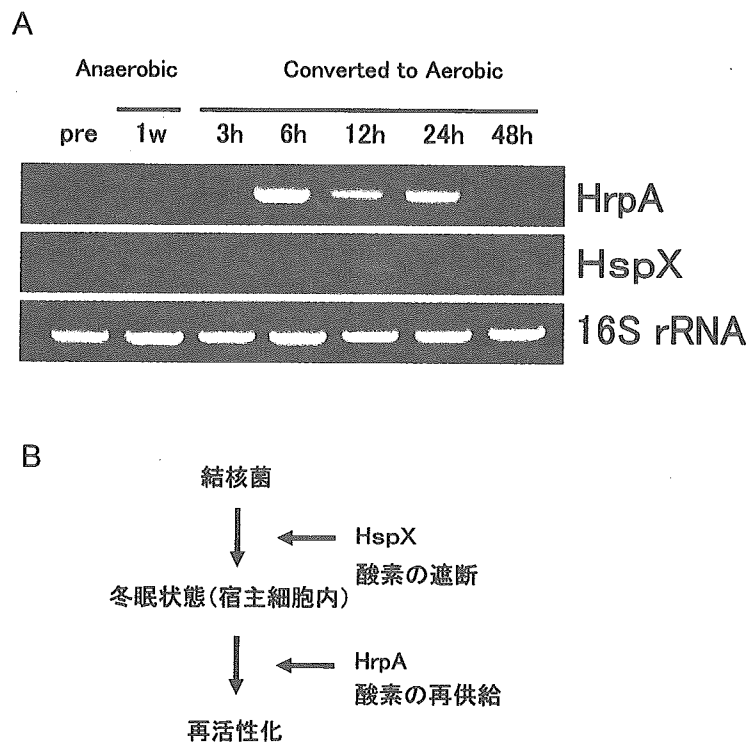


図3. 酸素分圧の変化に対する BCG 低分子ストレス蛋白質の変化。(A) BCG より RNA を抽出し、RT-PCR をおこなった。Sauton 培地で好氣的に 2 週間培養した BCG を嫌気ジャーの中に移動させると HspX が発現し、一週間後においてもメッセージ RNA は存在している (Anaerobic 1W)。その後好気状態に戻しても変化しない (converted to aerobic)。一方 HrpA は嫌気条件下では発現しないが、好気状態に戻すと一時的に発現する。(B) HspX と HrpA の働きについての模式図。HspX と HrpA は同じ低分子ストレス蛋白質ファミリーであるが、菌の冬眠状態に対しては異なる働きをしている可能性がある。

に感染しうる。特に骨組織に感染した場合には脊椎カリエス等の特徴的な病理像を呈する。また BCG ワクチンの副作用として骨炎を生じる。このような病理像がどのようにして生じてくるのかは不明である。このことへの興味がきっかけで骨関連細胞の研究を始めた。骨組織の中で骨芽細胞は骨添加に、破骨細胞が骨吸収を担う中心的かつ直接的な細胞であり、健全な骨は両者がバランスよく働いている。このバランスが崩れると骨の過形成あるいは過吸収

となる。破骨細胞を主にコントロールしているのは骨芽細胞である。破骨細胞は骨芽細胞から産生される M-CSF と RANKL の刺激により前駆細胞である単球・マクロファージ系の前駆細胞から単核の破骨細胞に分化し、その後破骨細胞相互に融合を繰り返す、多核の成熟破骨細胞となる。破骨細胞分化のマーカーの 1 つに酒石酸抵抗性酸フォスファターゼ (tartrate-resistant acid phosphatase, TRAP) が知られている。

ところで、BCG は骨芽細胞に効率よく感染する (9)。感染骨芽細胞ではその分化マーカーの発現が抑制されるとともに、急性の炎症に関わる蛋白質の発現が顕著に増加した (33)。また TNF 受容体ファミリーである 4-1BB (CD137) が産生されるようになる。4-1BB は抗原提示の際の補助刺激分子として知られている。*in vitro* の M-CSF/RANKL 刺激による破骨細胞形成系において 4-1BB は濃度依存的に破骨細胞の形成を顕著に抑制した。(図 4)

4-1BB の作用は単球・マクロファージ系細胞の細胞膜上にあるリガンド (4-1BBL (CD137L)) を介するリバースシグナルにより、破骨細胞分化に必須の転写因子である NFAT2 の産生が抑制されることによる。TNF スーパーファミリーのリバースシグナルの伝達経路は不明であった。4-1BBL の場合、TRAF6, NFκB シグナリング, MAP キナーゼカスケードに非依存性であるが、Akt を抑制することがわかった。また、4-1BBL 分子内のカゼインキナーゼドメインの活性化を介してフォスファターゼが活性化することが必要であることが明らかになった。しかし、Akt のカスケー

ドが実際に 4-1BB による破骨細胞形成抑制に働いているのかを含め、シグナル伝達経路は虫食い状態であり、今後明らかにしていく必要がある。またリアルタイムに生細胞内における菌の動きをダイナミックに追うことも必要であろう (12, 34)。ところで、細菌感染により骨芽細胞から 4-1BB が産生されるという現象は抗酸菌に特異的ではなかった。また、一般に細菌感染による炎症では骨吸収の方向に進むと考えられている。今回の破骨細胞の形成抑制は逆の方向性を示している。さらに、これまでに INF-β, INF-γ, IL-12, IL-18 等のサイトカインが同様に破骨細胞の形成を抑制することが示されている (8, 18, 42, 43, 45)。推測の域を出ないが、骨への感染が起きた場合にこれらの破骨細胞分化抑制性のサイトカインが産生されることにより、骨代謝の平衡状態が保たれる。しかし、炎症が進みサイトカインのバランスが崩れると骨吸収が促進されることになる。また結核による特異的な病態形成については長期にわたる慢性感染の間に繰り返される急性期に吸収が進み、それが積み重なることによってできあがると考えられる。このことを

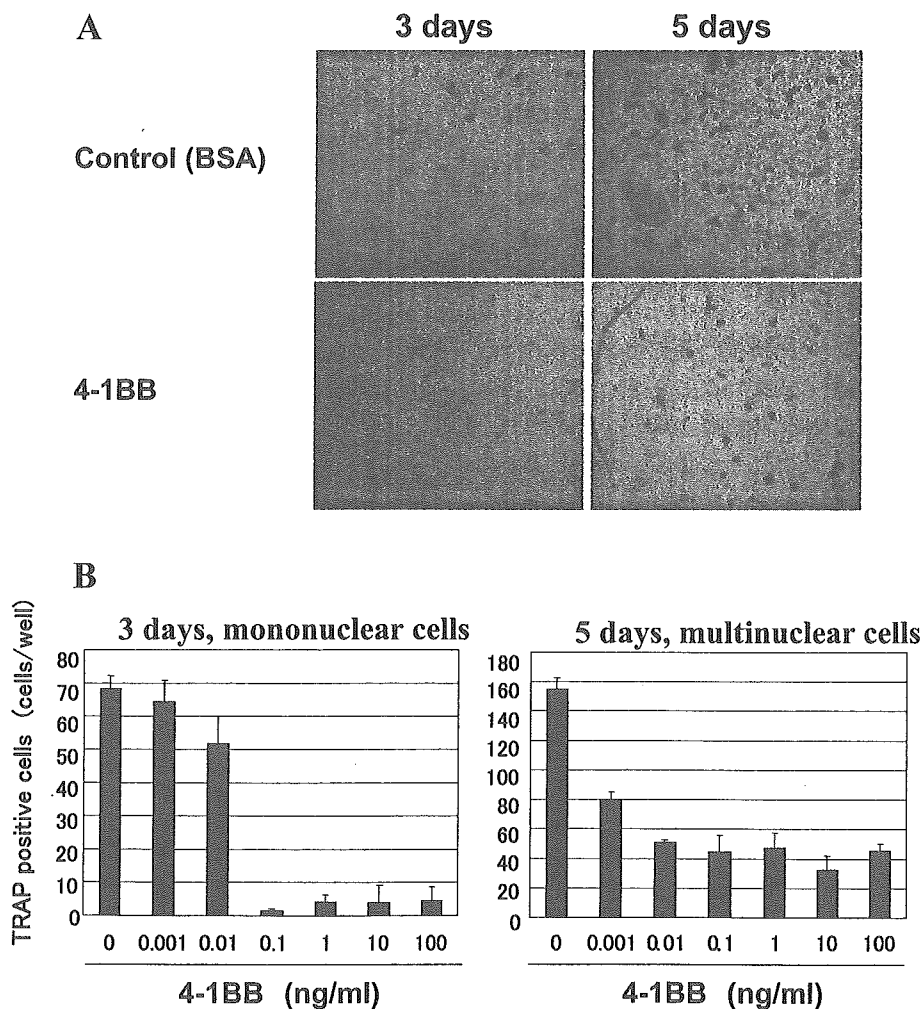


図 4. 4-1BB の破骨細胞分化に対する抑制効果。G-10 カラムを通過したマウス骨髄培養を BSA あるいは 4-1BB でコートしたプレート上で M-CSF (10 ng/ml) と RANKL (20 ng/ml) 存在下に 3 日間あるいは 5 日間培養した。(A) BSA でコートしたプレート上では 3 日目に TRAP 陽性の単核細胞が、5 日目に TRAP 陽性の多核細胞が多数観察されるが、4-1BB でコートしたプレート上では 3 日目における TRAP 陽性単核細胞、および 5 日目における TRAP 陽性多核細胞の形成が著しく阻害される。(B) 4-1BB による単核および多核の TRAP 陽性細胞の形成阻害は濃度依存的である。文献 33 より引用。

証明できる動物モデルを構築して示していきたい。

おわりに

冒頭にも述べたように Gerhard Amauer Hansen が 1874 年にらい菌を発見し、Robert Koch が 1882 年に結核菌を発見してから今日まで多くのことが解明されてきた。しかし、基本的なことがまだ解明されていないことが多いことも事実である。たとえば、抗酸菌は発育速度が遅い。特に病原性の強い菌にこの傾向が強い。なぜ遅発育性になったのか、遅発育性を担っているメカニズムは何か。これは細菌学的には基本的な疑問であろう。Matsumoto らによって発見された MDP1 が重要な鍵を握っているようであるが (2)、未だに明確な解答は得られていない。らい菌は未だ培養が成功していない菌である。逆に結核菌は Sauton 培地というアスパラギン、クエン酸ナトリウム、リン酸水素二カリウム、硫酸マグネシウム、クエン酸鉄アンモニウム、グリセリンのみからなる培地で十分に増殖する。この違いは何に依存するのか。両菌ともにヒトの体内に住み着くことに高度に適応した菌である。らい菌がその生活を宿主に完全に依存するが如く、ゲノム上の遺伝子をそぎ落としていった結果だと言い切ってしまうのかもしれない。本研究に関連したことでは、どのようなかたちで長期間ヒトの体内に姿をくらまして生きているのか、未だに謎である。また特異的な骨病変ができあがる過程も不明である。今挙げた以外にも基本的なことではあるが、いまだに解明されていないことは多い。今後もこれらの謎解きに挑戦していきたい。

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Diverse humoral immune responses and changes in IgG antibody levels against mycobacterial lipid antigens in active tuberculosis

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Humoral immune responses of active TB patients against six mycobacterial lipid antigens [trehalose 6,6'-dimycolate (TDM) from *Mycobacterium bovis* BCG (TDM-T) and *Mycobacterium avium* complex (TDM-M), trehalose 6-monomycolate (TMM) from *M. bovis* BCG (TMM-T) and *M. avium* complex (TMM-M), triacyl (PL-2) and tetraacyl (PL-1) phosphatidylinositol dimannosides] were examined by ELISA. IgG antibodies of TB patients with active disease reacted against the six lipid antigens distinctively, but heterogeneously. If tests were combined and an overall positive was scored cumulatively when any one of the six tests was positive, a good discrimination between patient and normal subject was obtained. A positive result in any one of the six tests was obtained in 91.5% of all 924 hospitalized patients and 93.3% of 210 patients at their first visit to the outpatient clinic. The IgG antibody response differed considerably from patient to patient, and the response patterns were grouped into several types. IgG antibody levels paralleled the bacterial burden; however, the smear-negative (culture-positive) patient group also showed high positive rates and mean ELISA ΔA values against the six lipid antigens. There were also marked differences in positive rate and mean ΔA values between cavity-positive and -negative groups, the former being higher than the latter. After anti-TB chemotherapy was initiated, IgG antibody levels decreased dramatically, paralleling the decrease in the amount of excretion of bacteria. Since multiple-antigen ELISA using particular lipid antigens was highly sensitive, and IgG antibody levels vary greatly at different stages of the disease, this technique is applicable for early diagnosis of smear-negative (and -positive) active TB and the prognosis for completion of anti-TB chemotherapy.

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INTRODUCTION

Tuberculosis (TB) has long been one of the most important infectious diseases of humans. During the 20th century the incidence of TB decreased, owing to the development of anti-TB chemotherapy. Nevertheless, TB remains globally uncontrolled, especially in developing countries, owing to co-infection with HIV or environmental factors such as malnutrition and poverty. The diagnosis of active TB has been done based on the detection of acid-fast bacilli by smear test or cultivation; recently, the PCR or nucleic acid amplification (NAA) technique has been introduced to diagnose early stages of the disease. However, sensitivity of

the smear test is low, cultivation takes a long time and NAA techniques are expensive. The tuberculin skin test (TST) is highly sensitive and useful as a criterion for delayed-type hypersensitivity based on the cellular immune response due to the *Mycobacterium tuberculosis* infection. However, this test is not applicable for differential diagnosis of active disease versus latent infection or the differential diagnosis of TB versus other mycobacterial infection, including BCG vaccination. Serological testing based on the humoral immune response is simple, economical and non-invasive and it is applicable to smear-negative (and -positive), culture-negative and -positive patients diagnosed clinically. To date, many investigations or trials on the serodiagnostics of TB using crude cell sonicate (Grange *et al.*, 1980; Kardjito *et al.*, 1982), culture filtrates (Benjamin *et al.*, 1984; Kiran *et al.*, 1985), purified protein antigens (Benjamin & Daniel, 1982; Zeiss *et al.*, 1982) and cell wall lipids (Reggiardo *et al.*, 1980; Sada *et al.*, 1990) as antigens have been reported (for reviews see Chan *et al.*, 2000; Daniel & Debanne, 1987). However, the practical use of

Abbreviations: DOTS, directly observed treatment, short course; MAC, *M. avium* complex; NAA, nucleic acid amplification; PL-1, tetraacyl phosphatidylinositol dimannoside; PL-2, triacyl phosphatidylinositol dimannoside; TDM-M, TDM-T, trehalose 6,6'-dimycolate from *M. bovis* and *M. avium* complex, respectively; TMM-M, TMM-T, trehalose 6-monomycolate from *M. bovis* and *M. avium* complex, respectively; TB, tuberculosis; TST, tuberculin skin test.

serodiagnosis for active TB has not been widely introduced, apparently because the reliability of the current tests using protein antigen is not satisfactory (Chan *et al.*, 2000; Daniel & Debanne, 1987). If these tools are to facilitate the diagnostic process and to increase case findings, a DOTS strategy would be more effective and a decrease in transmission would be expected (WHO, 2001). We have previously reported that IgG antibody against cord factor (trehalose 6,6'-dimycolate), the most characteristic cell surface glycolipid antigen, can be detected by ELISA with 81 % sensitivity and 96 % specificity for active TB patients and 70 % sensitivity in smear-negative TB patients (He *et al.*, 1991; Maekura *et al.*, 1993). For a clinically reliable test to diagnose active TB, sensitivity and specificity are necessary. Recent studies show that the sensitivity of serodiagnostic testing in smear-negative TB cases is equal or rather higher than that of NAA tests (Iinuma *et al.*, 2002; Maekura *et al.*, 2003).

In this study we examined humoral immune responses of patients with active TB against various mycobacterial lipid antigens by ELISA. The purpose of this investigation was to determine the humoral immune responses of TB patients to representative mycobacterial lipid antigens and to improve the sensitivity of IgG antibody detection system for simple and precise diagnosis of active TB and other mycobacterioses. We found that the IgG antibody responses of the individual TB patient sera were extremely heterogeneous depending on the structure of the antigens. However, the multiple combination of selected lipid antigens gave a high sensitivity, and the IgG antibody levels against lipid antigens varied greatly according to the progression of the disease. Therefore, this test is useful as a criterion for the completion of anti-TB chemotherapy.

METHODS

Serum samples and patients. (a) *Samples from hospitalized patients.* A total of 924 serum samples were obtained from patients attending the National Sanatorium Tokyo Hospital, Kiyose, Tokyo. These patients were diagnosed to be suffering from pulmonary TB, based on clinical symptoms such as long-lasting coughing and sputum over 2 months, and chest X-ray examination. Smear test with acid-fast stain and culture examination with 2 % Ogawa medium were performed 1 month after admission to the hospital. The patients of this group stayed in the hospital for a further 1 month or longer during 1995 and 1996. (b) *Samples from patients at their first visit to the outpatient clinic.* A total of 210 patients before hospitalization were diagnosed to be suffering from TB clinically based on the typical symptoms and chest X-ray examination between 1995 and 1996 at the outpatient clinic of the National Sanatorium Tokyo Hospital. Among them, 157 were smear-positive, 22 were smear-negative, culture-positive and 31 were unknown.

Selection of healthy control subjects and determination of cut-off points for ELISA. To select the healthy control subjects, we carefully chose individuals who had received a health examination including a chest X-ray. Among these, 70 % of individuals were BCG vaccinated when younger than 12 years old. However, the IgG antibody titres against mycobacterial lipid antigens were not elevated after BCG vaccination, although TST results were positive; hence a co-relationship between IgG antibody titre elevation and the TST

results was not observed. Therefore, we simply determined that the normal range for IgG antibody titres was lower than the mean ΔA value + 2SD of the healthy control ΔA .

The control subjects consisted of 85 sera from HIV-negative healthy adult individuals with no history of TB in their families, and included both TST-positive and TST-negative individuals.

Antigens. Lipid antigens were isolated from heat-killed *M. tuberculosis* Aoyama B, *Mycobacterium bovis* BCG and *Mycobacterium avium* complex (MAC) serotype 16 strain. Lipids were extracted from packed cells with chloroform/methanol (2:1, v/v) and the solvent was evaporated off with a rotary evaporator. The lipids were first partially separated by solvent fractionation and then the acetone-soluble or chloroform-soluble, or tetrahydrofuran-soluble or -insoluble fractions obtained were further separated by thin-layer chromatography on silica gel (Uniplate, Analtech) with the solvent system chloroform/methanol/water (65:25:4, by vol.), chloroform/methanol/acetone/acetic acid (90:10:6:1, by vol.) or chloroform/methanol/water (90:10:1, by vol.). Antigenic lipids were purified until a single spot was obtained by repeating thin-layer chromatography, and these were identified by mass spectrometric analysis. Each component such as carbohydrates or fatty acids was analysed by gas-chromatography/mass spectrometry (GC/MS) after hydrolysis or methanolysis of the original lipids, and the molecular mass of the intact lipids was determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

In a preliminary study, we tested the reactivity of 40 active TB patient sera (IgG antibodies) against 12 lipid antigens [TDM from *M. tuberculosis* Aoyama B, *M. bovis* BCG Tokyo 172 and BCG Connaught and MAC serotype 16 strain, sulfolipid I, triacyl (PL-2) and tetraacyl (PL-1) phosphatidylinositol dimannosides from *M. tuberculosis* Aoyama B, mycoside B and cardiolipin from *M. bovis* BCG Tokyo 172, TMM from *M. bovis* BCG Tokyo 172 and BCG Connaught and MAC serotype 16 strain]. None of the single antigens gave a sufficiently high positive rate to diagnose active TB, although up to 70 % of the 40 active TB patient sera tested positive against a single lipid antigen (data not shown). However, if an overall positive was scored when any one or more tests among the 12 antigens were positive, the positive rate increased to a much higher level than that obtained with a single antigen. Based on the antigenic reactivity, the stability in ELISA plates, the species specificity in mycobacteria and the availability of lipid antigens, we selected the following six antigens as most appropriate: TDM-T, TDM-M, TMM-T, TMM-M, PL-1 and PL-2.

MALDI-TOF mass analysis showed that TMM-T derived from *M. bovis* BCG Tokyo was a trehalose monomycolate possessing one molecule of α -, methoxy- or keto-mycolic acid ranging from C₇₆ to C₉₁ (the methoxy- and keto-mycolic acid subclasses predominated), while TDM-T from the same strain was a trehalose dimycolate possessing two molecules of mycolic acid from among the above subclasses or molecular species, thus showing a highly complex mixture of trehalose diesters with molecular masses ranging from 2660 to 2920 Da. TMM-M from MAC serotype 16 strain showed a different mass spectrometric pattern, showing the existence of C₈₂ or longer wax ester-mycolic acids characteristic of the MAC, instead of the methoxy-mycolic acids of *M. bovis* BCG Tokyo. TDM-M from MAC serotype 16 strain also showed multiplex molecular ions in mass spectra, due to the combined α -, keto- and wax ester-mycolic acids. On the other hand, both PL-1 and PL-2 derived from *M. tuberculosis* Aoyama B were major components of membrane mannophospholipids possessing C_{16:0} and/or branched-chain C₁₉ (tuberculostearic) fatty acids. Thus, TDM (-T and -M) and TMM (-T and -M) were species-specific mycobacterial cell wall antigens, although they shared part of their molecular structure and were therefore cross-reactive. PL-1 and PL-2 were essentially common antigens in the mycobacterial strains. By the

combination of above six antigens, the multiple-antigen ELISA gave a highly positive rate, reliable for the diagnosis of active TB and other mycobacterioses.

Multiple-antigen ELISA microplate system. TDM-T and TDM-M were dissolved in n-hexane (0.2 µg TDM in 50 µl n-hexane per well), while TMM-T, TMM-M, PL-1 and PL-2 were dissolved in ethanol (1.0 µg TMM, PL-1 and 2.0 µg PL-2 in 50 µl ethanol per well). One antigen was deposited in a polystyrene microplate well (Nunc-Immuplate; Nalge Nunc International) and the plates were allowed to dry at room temperature overnight. Plates were either used immediately or sealed in aluminium foil and stored at 4 °C. We confirmed that no difference was observed in results between plates used immediately after preparation and those stored for a month (data not shown). All chemicals used were purchased from Wako Pure Chemical Industries. For multiple-antigen ELISA, non-specific binding was blocked with 150 µl per well 0.05% Tween 20 in phosphate-buffered saline (PBS-T) adjusted to pH 7.4 with 5 M NaOH and incubated for 10 min at room temperature. The plates were then washed three times with 250 µl PBS-T per well. Serum samples tested for determination of the titre of IgG antibodies were diluted 1:201 with PBS-T. The diluted serum samples (50 µl per well) were added to each well, and the plate was incubated for 1 h. Horseradish-peroxidase-conjugated goat anti-human IgG (H+L) (Zymed) diluted 1:500 in PBS-T was used as a secondary antibody. After incubation for 1 h, the substrate, *o*-phenylenediamine (Sigma; 1 mg ml⁻¹) in citrate buffer containing 0.06% H₂O₂ was added. The reaction was stopped with 0.5 M H₂SO₄, and absorbance was measured in a microplate reader (NPR-A4i; Tosoh) at 492–600 nm. Each incubation was performed at room temperature, and after each step of the procedure the plates were washed three times with PBS-T.

Classification of types of pathological lesion based on chest X-ray examination. The extent and severity of the pathological lesion, with or without cavity, were classified based on chest X-ray examination, according to the 1963 'Gakken' classification of pulmonary TB. Extent of disease or the basic lesion including exudative, caseous, fibrotic, disseminated or far-advanced mixed was classified as follows: I, largest (larger than one side of the lung lobe); II, middle (intermediate between I and III); and III, small (less than one third of one side of the lung lobe). Side was classified as r (right lobe only), l (left lobe only) or b (both lung lobes). Severity was classified as 1 (unstable lesion without cavity confined in one third of one side of the lobe), 2 (lesion limited to one side of the lobe with or without cavity) or 3 (larger lesion with cavity in more than

one side of the lobe). For example, 'b13' indicates a large caseous lesion with large cavities over both lung lobes, and 'rIII1' indicates a small but unstable caseous and fibrotic lesion without cavity confined to one third of right lobe.

Data analysis. The titre of serum IgG antibodies against TDM-T, TDM-M, TMM-T, TMM-M, PL-1 and PL-2 was expressed as the absorption index (ΔA), equal to the absorption value of the test serum sample minus that of the same serum sample in wells without coated antigen. Statistical analysis was done according to the Mann-Whitney U test.

RESULTS

IgG antibody responses against single or multiple lipid antigens

We tested the reactivities of sera of 924 hospitalized patients with TB and of 210 active TB patients on their first visit to the outpatient clinic. The data for sensitivity, specificity and positive predictive values (PPV) of all these patient sera against each single antigen and six multiple antigens are summarized in Table 1. Among these six antigens, the positive rate (sensitivity) to PL-2 was the highest, at 71.8%, and that to TDM-M was the lowest, at 53.5%, in the total hospitalized TB patients. When the tests were combined and an overall positive was scored cumulatively, a positive result in any of the six tests was obtained for 91.5% of all 924 hospitalized patients and 93.3% of the 210 patients at their first visit to the outpatient clinic, including smear-positive and -negative (culture-positive) cases. In both groups, the multiple-antigen ELISA results were superior to any single-antigen tests for the clinical diagnosis of TB and other mycobacterioses.

Diverse responsiveness of IgG antibody against lipid antigens

To clarify the reason for the superiority of multiple-antigen ELISA, we carefully compared the IgG antibody response patterns of each patient serum against each of the six lipid antigens. The patterns of IgG antibody responsiveness

Table 1. Sensitivities of single- and multiple-antigen ELISA in the two groups of TB patients

Antigen	Hospitalized patients		Outpatients		Healthy controls Negative/total specificity (%)
	Sensitivity (%)*	PPV†	Sensitivity (%)*	PPV†	
TDM-T	617/924 (66.8)	0.994	153/210 (72.9)	0.975	81/85 (95.3)
TDM-M	494/924 (53.5)	0.992	125/210 (59.5)	0.969	81/85 (95.3)
TMM-T	634/924 (68.6)	0.994	143/210 (68.1)	0.973	81/85 (95.3)
TMM-M	519/924 (56.2)	0.994	110/210 (52.4)	0.973	82/85 (96.5)
PL-1	638/924 (69.0)	0.991	148/210 (70.5)	0.961	79/85 (92.9)
PL-2	663/924 (71.8)	0.994	155/210 (73.8)	0.975	81/85 (95.3)
Total	845/924 (91.5)	0.980	196/210 (93.3)	0.920	68/85 (80.0)

*Sensitivity is shown as positive/total, with the percentage in parentheses.

†PPV, positive predictive value.

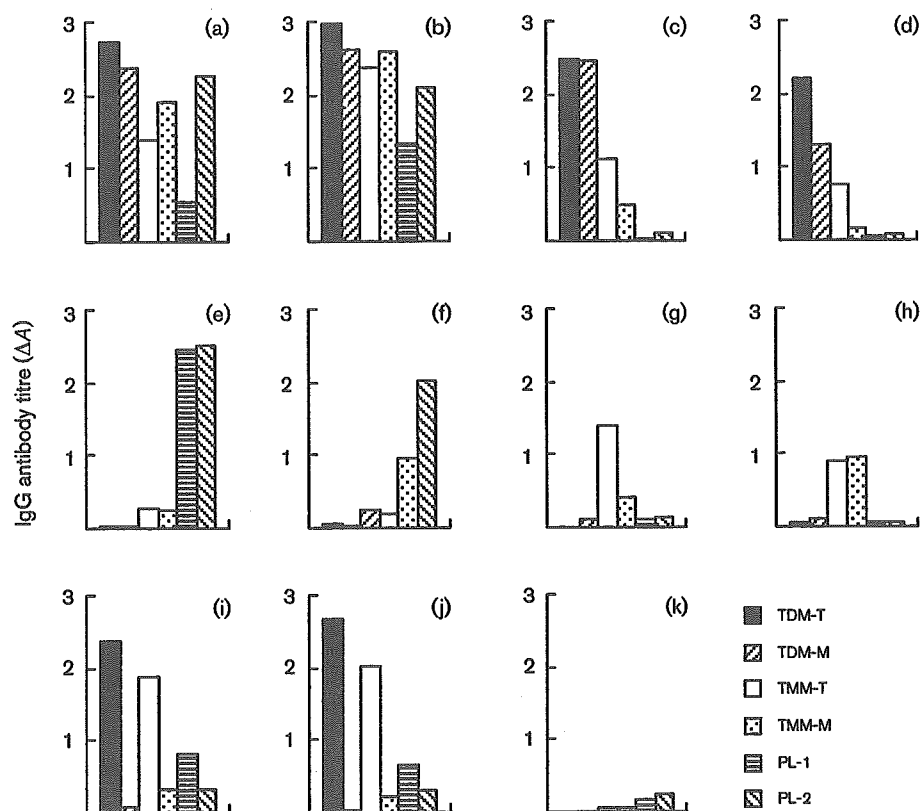


Fig. 1. Heterogeneity and diversity of IgG antibody responses at their first visit to the outpatient clinic. (a) Male, 51 years, smear (+), culture (+), multidrug resistant, relapse TB; (b) male, 45 years, smear (+), culture (+), relapse TB; (c) male, 61 years, smear (+), culture (+), primary TB; (d) male, 47 years, smear (paucibacillary), culture (+), primary TB; (e) female, 78 years, smear (+), culture (-), primary TB; (f) male, 67 years, smear (+), culture (-), primary TB; (g) male, 23 years, smear (paucibacillary), culture (+), primary TB (pleuritis); (h) male, 67 years, smear (+), culture (-), primary TB (pleuritis); (i) male, 44 years, smear (+), culture (+), primary TB (pleuritis); (j) male, 37 years, smear (+), culture (+), primary TB; (k) male, 51 years, smear (+), culture (+), primary TB (pleuritis). There are several types of IgG antibody response to lipid antigens: (a, b) reactive to all six lipid antigens; (c, d) reactive to TDM mainly and TMM weakly, but not to PL; (e, f) reactive to only PL markedly; (g, h) reactive to only TMM; (i, j) reactive to TDM-T and TMM-T, but not or slightly to TDM-M and TMM-M; (k) low-reactive to all six lipid antigens (slightly reactive to PL-2). All ELISA conditions were the same and are described in the text.

against each lipid antigen were extremely diverse in the TB patient sera (Fig. 1). Most of the smear-positive TB patient sera were more or less reactive against at least one of the six antigens, although some were only poorly reactive. The cases of Fig. 1(c) and (d) were reactive against only mycoloyl glycolipids (TDM-T, TDM-M, TMM-T and TMM-M), not against mannophospholipids (PL-1 and PL-2). In contrast, the cases of Fig. 1(e) and (f) were reactive against mannophospholipids, but not against mycoloyl glycolipids. Further, some were reactive against only TMM, but not TDM (Fig. 1g and h) and some were reactive against only TDM, but not TMM. The cases of Fig. 1(i) and (j) were highly reactive against TDM-T and TMM-T, but not against TDM-M and TMM-M, indicating recognition of the difference of the mycoloyl moiety of trehalose esters. The majority of IgG antibody titres of active TB patient sera were highly elevated, and reactive against all the six antigens (Fig. 1a and b). Some patient sera had negative IgG antibody titres at

their first visit to the outpatient clinic (Fig. 1k), but after 2 or 3 weeks, the IgG antibody titres against TDM-T and PL-2 were distinctly elevated (data not shown). From the IgG antibody response patterns, we suggest that such diverse reactivity of active TB patient sera against particular lipid antigens may be partially due to the clinical background, such as the amount of excreted bacilli or the stage after onset or progression of the disease, but mainly due to the difference of individual genetic background of the patient. To reveal the immunological and clinical characteristics of each six lipid antigens, we followed up the clinical documents of each active TB patient in as much detail as we could.

Time-course changes in IgG antibody titres after initiation of anti-TB chemotherapy

The time-course changes of the IgG antibody titres against each lipid antigen were examined in active TB patient sera.

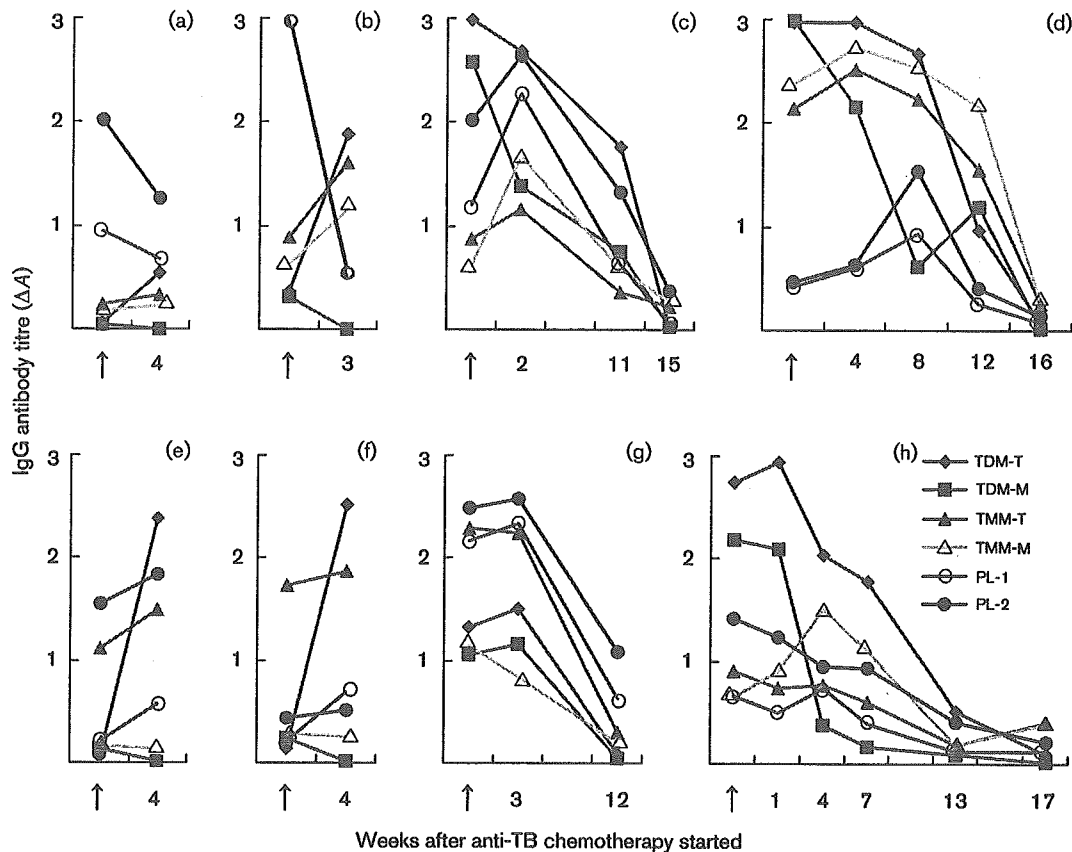


Fig. 2. Changes in IgG antibody levels after initiation of anti-TB chemotherapy. (a) Male, 67 years, smear (+), culture (+), primary TB (bll3); (b) male, 37 years, smear (+), culture (-), primary TB (bll2) (results for PL-1 and PL-2 almost identical); (c) male, 51 years, smear (+), culture (+), relapsed TB (rl12); (d) male, 38 years, smear (+), culture (+), primary TB (pleuritis, bll2); (e) male, 39 years, smear (-), culture (+), primary TB (rl13); (f) male, 46 years, smear (+), culture (+), relapsed TB (bll2); (g) male, 67 years, smear (+), culture (+), primary TB (bll3); (h) male, 43 years, smear (+), culture (+), primary TB (pleuritis, bll1). IgG antibody titres of eight active TB patient sera were estimated against six mycobacterial lipid antigens at different time points after initiation of anti-TB chemotherapy. The arrow shows the initiation point of anti-TB chemotherapy. In (a), (b), (e) and (f), IgG antibody titres were estimated during the first month after anti-TB chemotherapy was started. The final IgG antibody titre estimation of (c), (d), (g) and (h) was carried out when the smear tests were shown to be negative. IgG antibody titres against PL-2 and/or TDM-T were elevated initially, and decreased dramatically after initiation of the anti-TB chemotherapy. In general, 16–17 weeks after the initiation of anti-TB chemotherapy, all the IgG antibody titres were decreased to near the healthy control level. In cases (b), (e) and (f), IgG antibody titres against TDM-T were low at their first visit, but those were distinctly elevated 3–4 weeks after initiation of anti-TB chemotherapy.

Fig. 2(a, b, e and f) shows the initial changes in IgG antibody titres against the six mycobacterial lipid antigens during the first month after anti-TB chemotherapy was started. In the cases of Fig. 2(a, b and e), IgG antibody titres against PL-1 and/or PL-2 were already elevated when they first visited the outpatient clinic at the initial stage of the disease, whereas in case Fig. 2(f), IgG antibody titres against TMM-T, but not TDM, were elevated. After anti-TB chemotherapy was initiated, the elevated IgG antibody titres against PL-1 and/or -2 decreased immediately (Fig. 2a and b), whereas those against TDM were sharply elevated (Fig. 2a, b, e and f). Fig. 2(c, d, g and h) shows four representative active TB cases whose smear tests were all positive (Gaffky +8 to +10) and IgG antibody titres were fully

elevated against six lipid antigens at the first examination. In these cases, the initially elevated IgG antibody titres decreased altogether at once, or increased for about a month and then declined sharply until the smear test became negative. Thus, the decline in IgG antibody titres against lipid antigens paralleled the decrease in the amount of excreted acid-fast bacilli, and IgG antibody titres had settled down near to the normal level at 3–4 months after the anti-TB chemotherapy was initiated and the smear or culture test became negative. In cases with prolonged infection, a tendency for a long-lasting high level of IgG antibody titres was observed. In the chemotherapy-failed or relapsed cases also, declining of IgG antibody titres retarded longer (for 5–6 months) or the titres were elevated again.

Table 2. Effect of numbers of excreted mycobacteria on the IgG antibody seropositive rate in active TB patients at their first visit to the outpatient clinic

Antigen		Bacterial burden (Gaffky group):					Total
		G0	G1-3	G4-7	G8-10	G unknown	
TDM-T	Sensitivity, positive/total (%)	14/22 (63.6)	15/29 (51.7)	44/61 (72.1)	53/67 (79.1)	27/31 (87.1)	153/210 (72.9)
	ΔA , mean \pm SD	0.946 \pm 1.02	0.657 \pm 0.910	1.139 \pm 1.036	1.644 \pm 1.128		
TDM-M	Sensitivity, positive/total (%)	14/22 (63.6)	11/29 (37.9)	31/61 (50.8)	48/67 (71.6)	21/31 (67.7)	125/210 (59.5)
	ΔA , mean \pm SD	0.592 \pm 0.729	0.281 \pm 0.631	0.563 \pm 0.812	1.023 \pm 1.008		
TMM-T	Sensitivity, positive/total (%)	11/22 (50.0)	10/29 (34.5)	44/61 (72.1)	54/67 (80.6)	24/31 (77.4)	143/210 (68.1)
	ΔA , mean \pm SD	0.605 \pm 0.586	0.421 \pm 0.415	0.941 \pm 0.655	1.147 \pm 0.690		
TMM-M	Sensitivity, positive/total (%)	10/22 (45.5)	8/29 (27.6)	29/61 (47.5)	46/67 (68.7)	17/31 (54.8)	110/210 (52.4)
	ΔA , mean \pm SD	0.690 \pm 0.760	0.450 \pm 0.547	0.689 \pm 0.673	0.966 \pm 0.820		
PL-1	Sensitivity, positive/total (%)	14/22 (63.6)	16/29 (55.2)	43/61 (70.5)	52/67 (77.6)	23/31 (74.2)	148/210 (70.5)
	ΔA , mean \pm SD	0.392 \pm 0.481	0.454 \pm 0.730	0.620 \pm 0.791	0.804 \pm 0.867		
PL-2	Sensitivity, positive/total (%)	15/22 (68.2)	17/29 (58.6)	43/61 (70.5)	57/67 (85.1)	23/31 (74.2)	155/210 (73.8)
	ΔA , mean \pm SD	0.745 \pm 0.693	0.545 \pm 0.745	0.892 \pm 0.880	1.213 \pm 0.967		
Total	Sensitivity, positive/total (%)	21/22 (95.5)	24/29 (82.8)	57/61 (93.4)	64/67 (95.5)	30/31 (96.8)	196/210 (93.3)

Therefore, it was concluded that even in the multibacillary active TB cases with cavity, fully elevated IgG antibody titres against lipid antigens had settled down near to the normal level, when the anti-TB chemotherapy was completed successfully.

Effect of amount of excreted acid-fast bacilli on the IgG antibody responses

Since the IgG antibody levels of active TB patient sera varied considerably, we compared the positive rate and IgG antibody level expressed as mean ΔA values for each lipid antigen, according to the bacterial burden. As shown in Table 2, at the first examination the multibacillary group, who excreted more acid-fast bacilli (Gaffky +8 to +10), showed significantly higher IgG antibody levels to all six lipid antigens, expressed as mean ΔA values, in ELISA than the paucibacillary group (Gaffky +1 to +3) or moderate bacillary group (Gaffky +4 to +7). However, unexpectedly, the lowest positive rates and IgG antibody levels were found in the paucibacillary group, but not in the smear-negative group, to TDM, TMM and PL-2. When we take scores for the total six antigens cumulatively, the positive rate ranged between 82.8% in the paucibacillary cases and 95.5% in multibacillary cases, and the smear-negative group showed a 95.5% positive rate, indicating that the multiple-antigen ELISA gave satisfactory results reliable for the initial diagnosis of active TB, irrespective of whether smear-positive or -negative.

Effect of the severity (or extent) of the pathological lesions (cavity-positive or -negative) on the IgG antibody responses

Since the IgG antibody levels of active TB patient sera varied greatly according to the stage of the disease, we compared the positive rate for each single and total (multiple) antigens

of various patient groups with the different degrees of pathological lesion as determined by chest X-ray examination. Generally, the patients with more extensive or more severe pathological lesions showed a higher positive rate (Table 3). However, the highest positive rate varied among the antigens. TDM-M, TMM and PL-2 showed the highest positive rate for b, l or rI (2 or 3) cases, while TDM-T and PL-1 showed the highest for bII (1, 2 or 3) cases. The TB patients with cavitory lesion showed a higher positive rate than those without cavity, for all six mycobacterial lipid antigens (Table 4). These results indicate that the elevation of IgG antibody titres against mycobacterial lipid antigens is not homogeneous, but varies greatly with the progression or stages of active TB, related directly or indirectly to changes in the amount of acid-fast bacilli excreted and the extent and severity of pathological lesion.

DISCUSSION

Studies on the humoral immune response in human patients infected with *M. tuberculosis* have been done since Arloing (1898) first reported the agglutination test for diagnosis of pulmonary TB. A serological test would have been expected to be the 'gold standard' or 'Holy Grail' of TB diagnostics worldwide due to low cost, simplicity and rapidity, as has been already used in other fields of infectious disease diagnostics. However, no satisfactory test has been available due to the low sensitivity and specificity of the antibody response against mycobacterial antigens. The World Health Organization recommended that TB diagnostic tools for wide use should have sensitivities of over 80% and specificities of over 95% (WHO, 2001). Although the recent development of serodiagnosis using multiple protein antigens gives a considerable improvement in the sensitivity and specificity for the early diagnosis of TB (Lyashchenko *et al.*, 2000), it takes much work to isolate the