Kaneda, Y. (2002b). *In* "Gene Therapy Protocol – The second edition" (J. R. Morgan, ed.), pp. 63–72. Humana Press, New Jersey.

Kaplan, J. M., Yu, Q., Piraino, S. T., Pennington, S. E., Shankara, S., Woodworth, L. A., and Roberts, B. L. (1999). Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. *J. Immunol.* **163,** 699–707.

Kawakami, Y., and Rosenberg, S. A. (1997). Human tumor antigens recognized by T-cells. *Immunol. Res.* **16,** 313–339.

Lam, P. Y. P., and Brakefield, X. O. (2000). Hybrid vector designs to control the delivery, fate and expression of transgenes. *J. Gene Med.* **2,** 395–408.

Ledley, F. D. (1995). Non-viral gene therapy: The promise of genes as pharmaceutical products. *Hum. Gene Ther.* **6,** 1129–1144.

Li, S., and Huang, L. (2000). Non-viral gene therapy: Promises and challenges. *Gene Ther.* **7,** 31–34.

Marshall, E. (1995). Gene therapy's growing pains. *Science* **269,** 1052–1055.

Miyagishi, M., Hayashi, M., and Taira, K. (2003). Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev.* **13,** 1–7.

Miyazawa, T., Matsumoto, K., Ohmichi, H., Katoh, H., Yamashima, T., and Nakamura, T. (1998). Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor: A novel neurotrophic factor. *J. Cereb. Blood Flow Metab.* **18,** 345–348.

Mulligan, R. C. (1993). The basic science of gene therapy. *Science* **260,** 926–932.

Nakamura, H., Kimura, T., Ikegami, H., Ogita, K., Kohyama, S., Shimoya, K., Tsujie, T., Koyama, M., Kaneda, Y., and Murata, Y. (2003). Highly-efficient and minimally invasive *in vivo* gene transfer to the mouse uterus by Hemagglutinating Virus of Japan (HVJ) envelope vector. *Mol. Hum. Reprod.* **9,** 603–609.

Nakamura, N., Hart, D. A., Frank, C. B., Marchuk, L. L., Shrive, N. G., Ota, N., Taira, T., Yoshikawa, H., and Kaneda, Y. (2001). Efficient transfer of intact oligonucleotides into the nucleus of ligament scar fibroblasts by HVJ-cationic liposomes is correlated with effective antisense gene inhibition. *J. Biochem.* **129,** 755–759.

Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. (1998). Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.* **4,** 328–332.

Okada, Y. (1993). *In* "Methods in Enzymology" (N. Duzgunes, ed.), Vol. 221, pp. 18–41. Academic Press, Inc., San Diego.

Oshima, K., Shimamura, M., Mizuno, S., Tamai, K., Doi, K., Morishita, R., Nakamura, T., Kubo, T., and Kaneda, Y. (2004). Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats. *The FASEB J.* **18,** 212–214.

Pecher, G., Spahn, G., Schirrmann, T., Kulbe, H., Ziegner, M., Schenk, J. A., and Sandig, V. (2001). Mucin gene (MUC1) transfer into human dendritic cells by cationic liposomes and recombinant adenovirus. *Anticancer Res.* **21,** 2591–2596.

Prabakaran, I., Menon, C., Xu, S., Gomez-Yafal, A., Czerniecki, B. J., and Fraker, D. L. (2002). Mature CD83(+) dendritic cells infected with recombinant gp100 vaccinia virus stimulate potent antimelanoma T cells. *Ann. Surg. Oncol.* **9,** 411–418.

Race, E., Stein, C. A., Wigg, M. D., Baksh, A., Addawe, M., Frezza, P., and Oxford, J. S. (1995). A multistep procedure for the chemical inactivation of human immunodeficiency virus for use as an experimental vaccine. *Vaccine* **13,** 1567–1575.

Ramani, K., Bora, R. S., Kumar, M., Tyagi, S. K., and Sarkar, D. P. (1997). Novel gene delivery to liver cells using engineered virosomes. *FEBS Lett.* **404,** 164–168.

Ramani, K., Hassan, O., Venkaiah, B., Hasnain, S. E., and Sarkar, D. P. (1998). Site-specific gene delivery *in vivo* through engineered Sendai virus envelopes. *Proc. Natl. Acad. Sci. USA* **95,** 11886–11890.

Shimamura, M., Morishita, R., Endoh, M., Oshima, K., Aoki, M., Waguri, S., Uchiyama, Y., and Kaneda, Y. (2003). HVJ-envelope vector for gene transfer into central nervous system. *Biochem. Biophys. Res. Comm.* **300,** 464–471.

Shimamura, M., Sato, N., Oshima, K., Aoki, M., Kurinami, H., Waguri, S., Uchiyama, Y., Ogihara, T., Kaneda, Y., and Morishita, R. (2004). A novel therapeutic strategy to treat brain ischemia: Over-expression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. *Circulation* **109,** 424–431.

Sioud, M. (2004). Ribozyme- and siRNA-Mediated mRNA Degradation: A General Introduction. *Methods Mol. Biol.* **252,** 1–8.

Staecker, H., Kopke, R., Malgrange, B., Lefebvre, P., and Van de Water, T. R. (1996). NT-3 and/or BDNF therapy prevents loss of auditory neurons following loss of hair cells. *Neuroreport* **7,** 889–894.

Suzuki, K., Nakashima, H., Sawa, Y., Morishita, R., Matsuda, H., and Kaneda, Y. (2000). Reconstituted fusion liposomes for gene transfer *in vitro* and *in vivo*. *Gene Ther. Regulat.* **1,** 65–77.

Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E. B., Steinman, R. M., Enk, A., Kampgen, E., and Schuler, G. (1999). Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J. Exp. Med.* **190,** 1669–1678.

Tijsterman, M., and Plasterk, R. H. (2004). Dicers at RISC; the mechanism of RNAi. *Cell* **117,** 1–3.

Tomita, N., Morishita, R., Taniyama, Y., Koike, H., Aoki, M., Shimizu, H., Mastumoto, K., Nakamura, T., Kaneda, Y., and Ogihara, T. (2003). Angiogenic property of hepatocyte growth factor is dependent on upregulation of essential transcription factor for angiogenesis, ets-1. *Circulation* **107,** 141–147.

Vilella, R., Benitez, D., Mila, J., Vilalta, A., Rull, R., Cuellar, F., Conill, C., Vidal-Sicart, S., Costa, J., Yachi, E., Palou, J., Malvehy, J., Puig, S., Marti, R., Mellado, B., and Castel, T. (2003). Treatment of patients with progressive unresectable metastatic melanoma with a heterologous polyvalent melanoma whole cell vaccine. *Int. J. Cancer* **106,** 626–631.

Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M. L. (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. USA* **89,** 7934–7938.

Wang, J., Saffold, S., Cao, X., Krauss, J., and Chen, W. (1998). Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines. *J. Immunol.* **161,** 5516–5524.

Wu, G. Y., and Wu, C. H. (1988). Receptor-mediated gene delivery and expression *in vivo*. *J. Biol. Chem.* **263,** 14621–14624.

Xu, S., Koski, G. K., Faries, M., Bedrosian, I., Mick, R., Maeurer, M., Cheever, M. A., Cohen, P. A., and Czerniecki, B. J. (2003). Rapid high efficiency sensitization of CD8+ T cells to tumor antigens by dendritic cells leads to enhanced functional avidity and direct tumor recognition through an IL-12-dependent mechanism. *J. Immunol.* **171,** 2251–2261.

Yagi, M., Kanzaki, S., Kawamoto, K., Shin, B., Shah, P. P., Magal, E., Sheng, J., and Raphael, Y. (2000). Spiral ganglion neurons are protected from degeneration by GDNF gene therapy. *J. Assoc. Res. Otolaryngol.* **1,** 315–325.

Yang, S., Kittlesen, D., Slingluff, C. L., Jr., Vervaert, C. E., Seigler, H. F., and Darrow, T. L. (2000). Dendritic cells infected with a vaccinia vector carrying the human gp100 gene simultaneously present multiple specificities and elicit high-affinity T cells reactive to multiple epitopes and restricted by HLA-A2 and -A3. *J. Immunol.* **164,** 4204–4211.

Yeagle, P. L. (1993). The fusion of Sendai virus. *In* "Viral fusion mechanisms" (J. Benty, ed.), pp. 313–334. CRC Press, Inc., London.

Yokota, T., Miyagishi, M., Hino, T., Matsumura, R., Tasinato, A., Urushitani, M., Rao, R. V., Takahashi, R., Bredesen, D. E., Taira, K., and Mizusawa, H. (2004). siRNA-based inhibition specific for mutant SOD1 with single nucleotide alternation in familial ALS, compared with ribozyme and DNA enzyme. *Biochem. Biophys. Res. Commun.* **314,** 283–291.

Yoshimura, S., Morishita, R., Hayashi, K., Kokuzawa, J., Aoki, M., Matsumoto, K., Nakamura, T., Ogihara, T., Sakai, N., and Kaneda, Y. (2002). Gene transfer of hepatocyte growth factor to subarachnoid space in cerebral hypoperfusion model. *Hypertension* **39,** 1028–1034.

Yonemitsu, Y., Kitson, C., Ferrari, S., Farley, R., Griesenbach, U., Juda, D., Steel, R., Scheid, P., Zhu, J., Jeffery, P., Kato, A., Hasan, M. K., Nagai, Y., Masaki, I., Fukumura, M., Hasegawa, M., Geddes, D. M., and Alton, E. F. W. (2000). Efficient gene transfer to airway epithelium using recombinant Sendai virus. *Nature Biotech.* **18,** 970–973.

Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M. L. (1990). Receptor-mediated endocytosis of transferrin-polycation conjugates: An efficient way to introduce DNA into hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **87,** 3655–3659.

Further Reading

Kaneda, Y. (1994). Virus (Sendai virus envelopes) mediated gene transfer. *In* "Cell Biology A Laboratory Handbook" (J. E. Celis, ed.), pp. 50–57. Academic Press, Orlando, FL.

日本細菌学雑誌
60（2）：349-356，2005

平成17年黒屋奨学賞受賞論文

BCGを用いた抗酸菌の抗原性および病原性に関する研究

大　原　直　也

長崎大学大学院医歯薬学総合研究科　口腔病原微生物学分野
〒852-8588　長崎県長崎市坂本1-7-1

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

Naoya OHARA
Study on antigenicity and pathogenicity of mycobacteria
Division of Microbiology and Oral Infection, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588

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大きく，ヒト体内での長期にわたる冬眠用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ヵ月以上持続した(47)。またマラリアのエピトープに対しても 7ヵ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ら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ヵ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ヵ月後に同じ 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)。

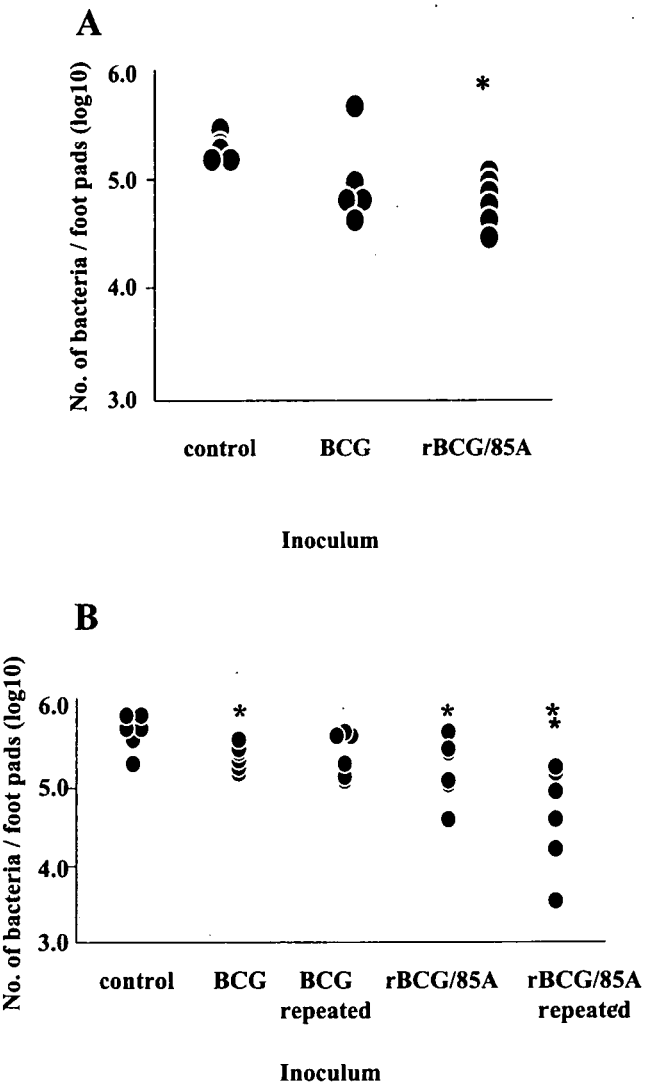

図 1. らい菌感染に対する rBCG/85A 免疫による感染防御効果。(A) 短期間における免疫効果。親株である BCG あるいは rBCG/85A を 6 週齢の C57BL/6 マウスの胸部皮内に 1 個体につき 5.0×10^7 個接種した。1ヵ月後，各群のマウスのフットパッドに 5.0×10^3 個の *M. leplae* 生菌を接種し，25 週後にフットパッドから検出された抗酸菌数を測定した。rBCG/85A 接種群では対照群と比較して検出される菌数が有意に（$P<0.01$）減少した。(B) BCG あるいは rBCG/85A を接種した 5ヵ月後，各群の半数のマウスに同量の BCG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

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で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

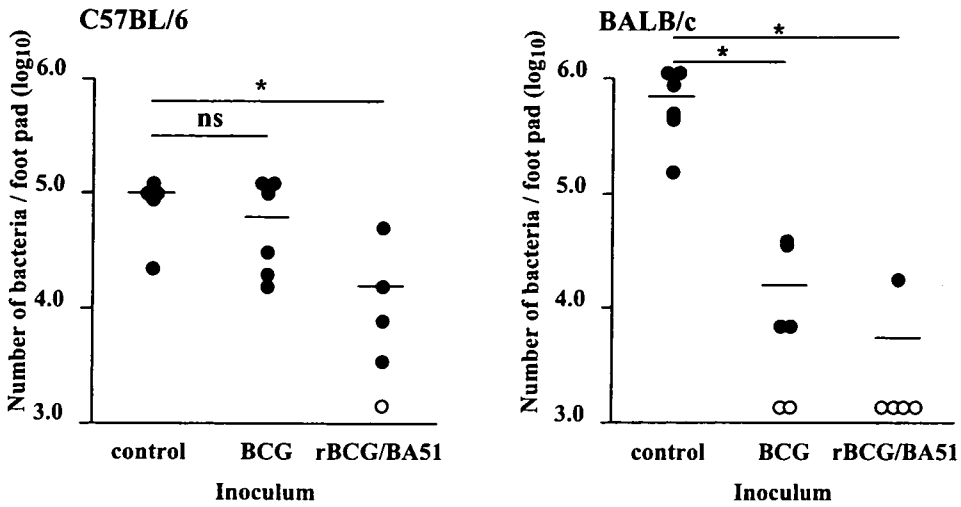

図 2. らい菌感染に対する rBCG/BA51 免疫による感染防御効果。黒丸はフットパッド中の抗酸菌数を示す。白丸はフットパッド中に抗酸菌が認められなかったことを示す。文献 25 より引用。

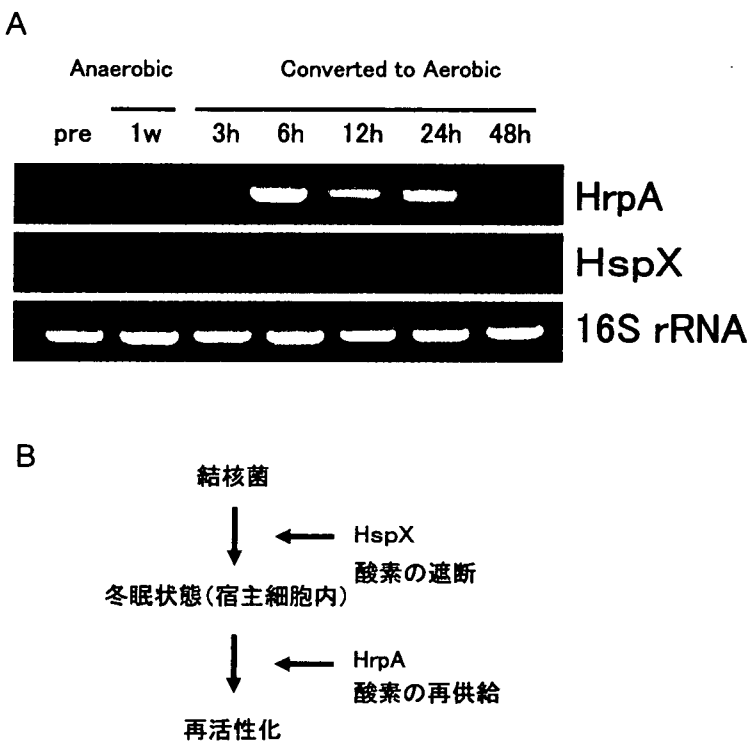

図 3. 酸素分圧の変化に対する BCG 低分子ストレス蛋白質の変化。(A) BCG より RNA を抽出し，RT-PCR をおこなった。Sauton 培地で好気的に 2 週間培養した BCG を嫌気ジャーの中に移動させると HspX が発現し，一週間後においてもメッセージ RNA は存在している (Anaerobic 1W)。その後好気状態に戻しても変化しない (converted to aerobic)。一方 HrpA は嫌気条件下では発現しないが，好気状態に戻すと一時的に発現する。(B) HspX と HrpA の働きについての模式図。HspX と HrpA は同じ低分子ストレス蛋白質フ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

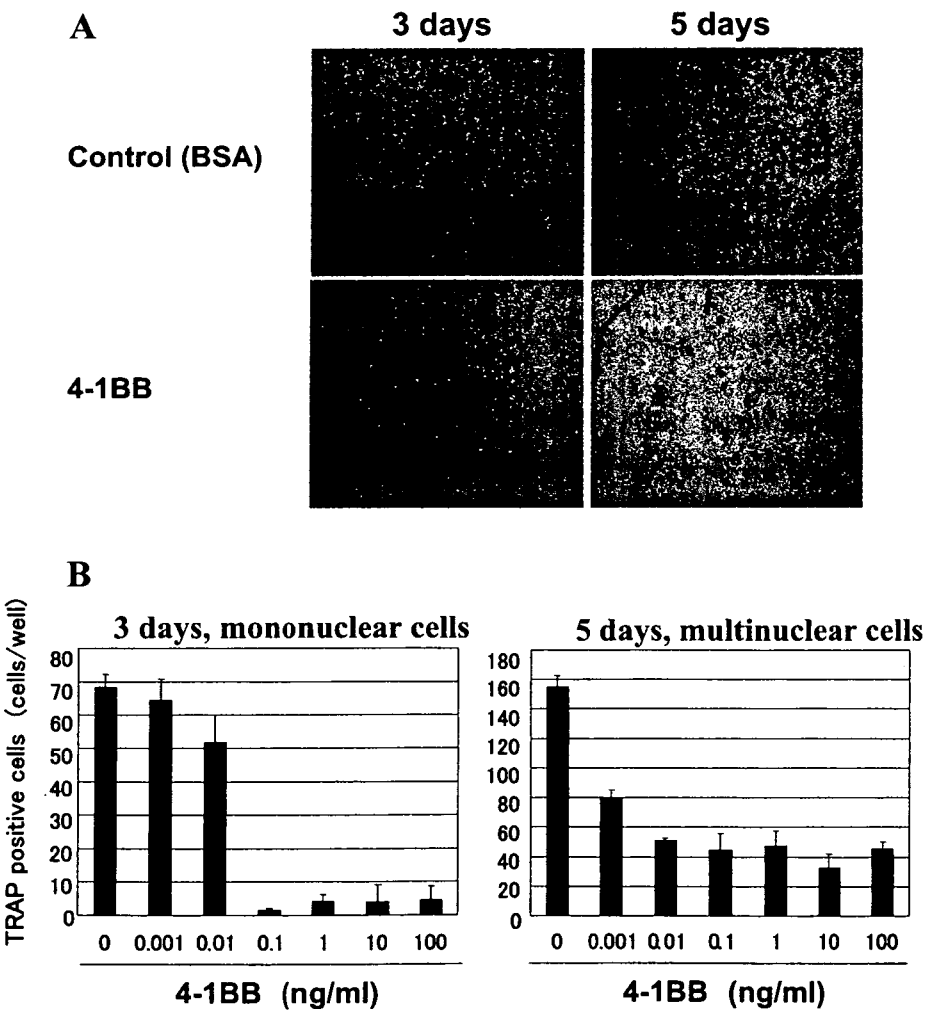

図 4. 4-1BB の破骨細胞分化に対する抑制効果。G-10 カラムを通過したマウス骨髄培養を BSA あるいは 4-1BB でコートしたプレート上で M-CSF (10 ng/ml) と RANKL (20 ng/ml) 存在下に 3 日間あるいは 5 日間培養した。(A) BSA でコートしたプレート上では 3 日目に TRAP 陽性の単核細胞が，5 日目に TRAP 陽性の多核細胞が多数観察されるが，4-1BB でコートしたプレート上では 3 日目における TRAP 陽性単核細胞，および 5 日目における TRAP 陽性多核細胞の形成が著しく阻害される。(B) 4-1BB による単核お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

謝辞

本研究を遂行するにあたり，御指導いただきました山田毅名誉教授（長崎大学）に深く感謝いたします。また，本賞に御推薦いただきました小林和夫教授（大阪市立大学大学院医学研究科）に厚く御礼を申し上げます。最後に本研究に協力してくださいました中山浩次教授をはじめ教室員の方々および共同研究で御協力いただきました先生方に感謝の意を捧げます。

文献

1) Aldovini, A., Young, R.A. (1991): Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature **351**, 479–482.
2) Aoki, K., Matsumoto, S., Hirayama, Y., Wada, T., Ozeki, Y., Niki, M., Domenech, P., Umemori, K., Yamamoto, S., Mineda, A., Matsumoto, M., Kobayashi, K. (2004): Extracellular mycobacterial DNA-binding protein 1 participates in mycobacterium-lung epithelial cell interaction through hyaluronic acid. J. Biol. Chem. **279**, 39798–39806.
3) Belisle, J.T., Vissa, V.D., Sievert, T., Takayama, K., Brennan, P.J., Besra, G.S. (1997): Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science **276**, 1420–1422.
4) Betts, J.C., Lukey, P.T., Robb, L.C., McAdam, R.A., Duncan, K. (2002): Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Mol. Microbiol. **43**, 717–731.
5) Fisher, M.A., Plikaytis, B.B., Shinnick, T.M. (2002): Microarray analysis of the *Mycobacterium tuberculosis* transcriptional response to the acidic conditions found in phagosomes. J. Bacteriol. **184**, 4025–4032.
6) Horwitz, M.A., Harth, G., Dillon, B.J., Maslesa-Galic', S. (2000): Recombinant bacillus Calmette-Guérin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. Proc. Natl. Acad. Sci. U.S.A. **97**, 13853–13858.
7) Horwitz, M.A., Lee, B.W., Dillon, B.J., Harth, G. (1995): Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. U.S.A. **92**, 1530–1534.
8) Horwood, N.J., Elliott, J., Martin, T.J., Gillespie, M.T. (2001): IL-12 alone and in synergy with IL-18 inhibits osteoclast formation *in vitro*. J. Immunol. **166**, 4915–4921.
9) Hotokezaka, H., Kitamura, A., Matsumoto, S., Hanazawa, S., Amano, S., Yamada, T. (1998): Internalization of *Mycobacterium bovis* Bacillus Calmette-Guérin into osteoblast-like MC3T3-E1 cells and bone resorptive responses of the cells against the infection. Scand. J. Immunol. **47**, 453–458.
10) Kitaura, H., Ohara, N., Matsuo, T., Tasaka, H., Kobayashi, K., Yamada, T. (1993): Cloning, sequencing and expression of the gene for α antigen from *Mycobacterium intracellulare* and use of PCR for the rapid identification of *Mycobacterium intracellulare*. Biochem. Biophys. Res. Commun. **196**, 1466–1473.
11) Kitaura, H., Ohara, N., Naito, M., Kobayashi, K., Yamada, T. (1998): Serological analysis of C-terminal region of α antigen from *Mycobacterium avium-intracellulare* complex and *Mycobacterium tuberculosis*. APMIS **106**, 893–900.
12) Lee, J.-S., Kamijo, K., Ohara, N., Kitamura, T., Miki, T. (2004): MgcRacGAP regulates membrane blebbing through RhoA during cytokinesis. Exp. Cell. Res. **293**, 275–282.
13) Matsumoto, S., Yanagi, T., Ohara, N., Wada, N., Kanbara, H., Yamada, T. (1996): Stable expression and secretion of the B-cell epitope of rodent malaria from *Mycobacterium bovis* BCG and induction of long-lasting humoral response in mouse. Vaccine **14**, 54–60.
14) Matsuo, K., Yamaguchi, R., Yamazaki, A., Tasaka, H., Terasaka, K., Totsuka, M., Kobayashi, K., Yukitake, H., Yamada, T. (1990): Establishment of a foreign antigen secretion system in mycobacteria. Infect. Immun. **58**, 4049–4054.
15) Matsuo, K., Yamaguchi, R., Yamazaki, A., Tasaka, H., Yamada, T. (1988): Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α antigen. J. Bacteriol. **170**, 3847–3854.
16) Matsuoka, M., Nomaguchi, H., Yukitake, H., Ohara, N., Matsumoto, S., Mise, K., Yamada, T. (1997): Inhibition of multiplication of *Mycobacterium leprae* in mouse foot pads by immunization with ribosomal fraction and culture filtrate from *Mycobacterium bovis* BCG. Vaccine **15**, 1214–1217.
17) Nagai, S., Wiker, H.G., Harboe, M., Kinomoto, M. (1991): Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. Infect. Immun. **59**, 372–382.
18) Nagata, N., Kitaura, H., Yoshida, N., Nakayama, K. (2003): Inhibition of RANKL-induced osteoclast formation in mouse bone marrow cells by IL-12: involvement of IFN-γ possibly induced from non-T cell population. Bone **33**, 721–732.
19) Naito, M., Matsuoka, M., Ohara, N., Nomaguchi, H., Yamada, T. (1999): The antigen 85 complex vaccine against experimental

Mycobacterium leprae infection in mice. Vaccine **18**, 795–798.
20) Naito, M., Ohara, N., Matsumoto, S., Yamada, T. (1998): The novel fibronectin-binding motif and key residues of mycobacteria. J. Biol. Chem. **273**, 2905–2909.
21) Naito, M., Ohara, N., Matsumoto, S., Yamada, T. (1998): Immunological characterization of α antigen of *Mycobacterium kansasii*: B-cell epitope mapping. Scand. J. Immunol. **48**, 73–78.
22) Ohara, N., Kitaura, H., Hotokezaka, H., Nishiyama, T., Wada, N., Matsumoto, S., Matsuo, T., Naito, M., Yamada, T. (1995): Characterization of the gene encoding the MPB51, one of the major secreted protein antigens of *Mycobacterium bovis* BCG, and identification of the secreted protein closely related to the fibronectin binding 85 complex. Scand. J. Immunol. **41**, 433–442.
23) Ohara, N., Matsuo, K., Yamaguchi, R., Yamazaki, A., Tasaka, H., Yamada, T. (1993): Cloning and sequencing of the gene for α antigen from *Mycobacterium avium* and mapping of B-cell epitopes. Infect. Immun. **61**, 1173–1179.
24) Ohara, N., Matsuoka, M., Nomaguchi, H., Naito, M., Yamada, T. (2000): Inhibition of multiplication of *Mycobacterium leprae* in mouse foot pads by recombinant Bacillus Catmette-Guérin (BCG). Vaccine **18**, 1294–1297.
25) Ohara, N., Matsuoka, M., Nomaguchi, H., Naito, M., Yamada, T. (2001): Protective responses against experimental *Mycobacterium leprae* infection in mice induced by recombinant Bacillus Calmette-Guérin over-producing three putative protective antigen candidates. Vaccine **19**, 1906–1910.
26) Ohara, N., Nishiyama, T., Ohara-Wada, N., Matsumoto, S., Matsuo, T., Yamada, T. (1997): Characterization of the transcriptional initiation regions of genes for the major secreted protein antigens 85C and MPB51 of *Mycobacterium bovis* BCG. Microb. Pathog. **23**, 303–310.
27) Ohara, N., Ohara-Wada, N., Kitaura, H., Nishiyama, T., Matsumoto, S., Yamada, T. (1997): Analysis of the genes encoding the antigen 85 complex and MPT51 from *Mycobacterium avium*. Infect. Immun. **65**, 3680–3685.
28) Ohara, N., Ohara, N., Naito, M., Miyazaki, C., Matsumoto, S., Tabira, Y., Yamada, T. (1997): HrpA, a new ribosome-associated protein which appears in heat-stressed *Mycobacterium bovis* bacillus Calmette-Guérin. J. Bacteriol. **179**, 6495–6498.
29) Ohara, N., Yamada, T. (2001): Recombinant BCG vaccines. Vaccine **19**, 4089–4098.
30) Pal, P.G., Horwitz, M.A. (1992): Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. Infect. Immun. **60**, 4781–4792.
31) Ranes, M.G., Rauzier, J., Lagranderie, M., Gheorghiu, M., Gicquel, B. (1990): Functional analysis of pAL5000, a plasmid from *Mycobacterium fortuitum*: construction of a "mini" *mycobacterium-Escherichia coli* shuttle vector. J. Bacteriol. **172**, 2793–2797.
32) Rosenkrands, I., Slayden, R.A., Crawford, J., Aagaard, C., Barry, C.E.III, Andersen, P. (2002): Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. J. Bacteriol. **184**, 3485–3491.
33) Saito, K., Ohara, N., Hotokezaka, H., Fukumoto, S., Yuasa, K., Naito, M., Fujiwara, T., Nakayama, K. (2004): Infection-induced up-regulation of the costimulatory molecule 4-1BB in osteoblastic cells and its inhibitory effect on M-CSF/RANKL-induced *in vitro* osteoclastogenesis. J. Biol. Chem. **279**, 13555–13563.
34) Saito, S.I., Liu, X.-F., Kamijo, K., Razziudin, R., Tatsumoto, T., Okamoto, I., Chen, X., Lee, C.-C., Lorenzi, M.V., Ohara, N., Miki, T. (2004): Deregulation and mislocalization of the cytokinesis regulator ECT2 activate the Rho signaling pathways leading to malignant transformation. J. Biol. Chem. **279**, 7169–7179.
35) Sherman, D.R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M.I., Schoolnik, G.K. (2001): Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding α-crystallin. Proc. Natl. Acad. Sci. U.S.A. **98**, 7534–7539.
36) Snapper, S.B., Lugosi, L., Jekkel, A., Melton, R.E., Kieser, T., Bloom, B.R., Jacobs, W.R.Jr. (1988): Lysogeny and transformation in mycobacteria: stable expression of foreign genes. Proc. Natl. Acad. Sci. U.S.A. **85**, 6987–6991.
37) Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., *et al.* (1991): New use of BCG for recombinant vaccines. Nature **351**, 456–460.
38) Stover, C.K., Bansal, G.P., Hanson, M.S., Burlein, J.E., Palaszynski, S.R., Young, J.F., Koenig, S., Young, D.B., Sadziene, A., Barbour, A.G. (1993): Protective immunity elicited by recombinant bacille Calmette-Guérin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. J. Exp. Med. **178**, 197–209.
39) Tabira, Y., Ohara, N., Ohara, N., Kitaura, H., Matsumoto, S., Naito, M., Yamada, T. (1998): The 16-kDa α-crystallin-like protein of *Mycobacterium bovis* BCG is produced under conditions of oxygen deficiency and is associated with ribosomes. Res. Microbiol. **149**, 255–264.
40) Tabira, Y., Ohara, N., Yamada, T. (2000): Identification and characterization of the ribosome-associated protein, HrpA, of Bacillus Calmette-Guérin. Microb. Pathog. **29**, 213–222.
41) Takano, M., Ohara, N., Mizuno, A., Yamada, T. (1994): Cloning, sequencing and expression in *Escherichia coli* of the gene for α antigen from *Mycobacterium scrofulaceum*. Scand. J. Immunol. **40**, 165–170.
42) Takayanagi, H., Kim, S., Matsuo, K., Suzuki, H., Suzuki, T., Sato, K., Yokochi, T., Oda, H., Nakamura, K., Ida, N., Wagner, E.F., Taniguchi, T. (2002): RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-β. Nature **416**, 744–749.
43) Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., Taniguchi, T. (2000): T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ. Nature **408**, 600–605.
44) Tasaka, H., Nomura, T., Matsuo, Y. (1985): Specificity and distribution of α antigens of *Mycobacterium avium-intracellulare*, *Mycobacterium scrofulaceum*, and related species of mycobacteria. Am. Rev. Respir. Dis. **132**, 173–174.
45) Udagawa, N., Horwood, N.J., Elliott, J., Mackay, A., Owens, J., Okamura, H., Kurimoto, M., Chambers, T.J., Martin, T.J., Gillespie, M.T. (1997): Interleukin-18 (interferon-gamma-inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-gamma to inhibit osteoclast formation. J. Exp. Med. **185**, 1005–1012.
46) Voskuil, M.I., Schnappinger, D., Visconti, K.C., Harrell, M.I., Dolganov, G.M., Sherman, D.R., Schoolnik, G.K. (2002): Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. J. Exp. Med. **198**, 705–713.
47) Wada, N., Ohara, N., Kameoka, M., Nishino, Y., Matsumoto, S.,

Nishiyama, T., Naito, M., Yukitake, H., Okada, Y., Ikuta, K., Yamada, T. (1996): Long-lasting immune response induced by recombinant bacillus Calmette-Guérin (BCG) secretion system. Scand. J. Immunol. **43**, 202–209.

48) Wayne, L.G., Hayes, L.G. (1996): An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect. Immun. **64**, 2062–2069.

49) Yoneda, M., Fukui, Y. (1965): Isolation, purification, and characterization of extracellular antigens of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **92**, 361–370.

50) Yuan, Y., Crane, D.D., Barry, C.E.III. (1996): Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial α-crystallin homolog. J. Bacteriol. **178**, 4484–4492.

51) Yuan, Y., Crane, D.D., Simpson, R.M., Zhu, Y.Q., Hickey, M.J., Sherman, D.R., Barry, C.E.III. (1998): The 16-kDa α-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. Proc. Natl. Acad. Sci. U.S.A. **95**, 9578–9583.

Microbiology (2005), 151, 2065–2074 DOI 10.1099/mic.0.27790-0

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

Yukiko Fujita,[1] Takeshi Doi,[1] Koji Sato[2] and Ikuya Yano[1]

[1]Japan BCG Central Laboratory, 3-1-5 Matsuyama, Kiyose-shi, Tokyo 204-0022, Japan

[2]National Sanatorium Kumamotominami Hospital, 2338 Toyofuku Matsubase-machi, Shimomashiki-gun, Kumamoto 869-0593, Japan

Correspondence
Yukiko Fujita
y-fujita@bcg.gr.jp

Received 26 November 2004
Revised 16 February 2005
Accepted 17 February 2005

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.

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 C_{76} to C_{91} (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 C_{82} 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 C_{19} (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-Immunoplate; 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 % H_2O_2 was added. The reaction was stopped with 0·5 M H_2SO_4, 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, 'bI3' 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
	Sensitivity (%)*	PPV†	Sensitivity (%)*	PPV†	Negative/total specificity (%)
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.

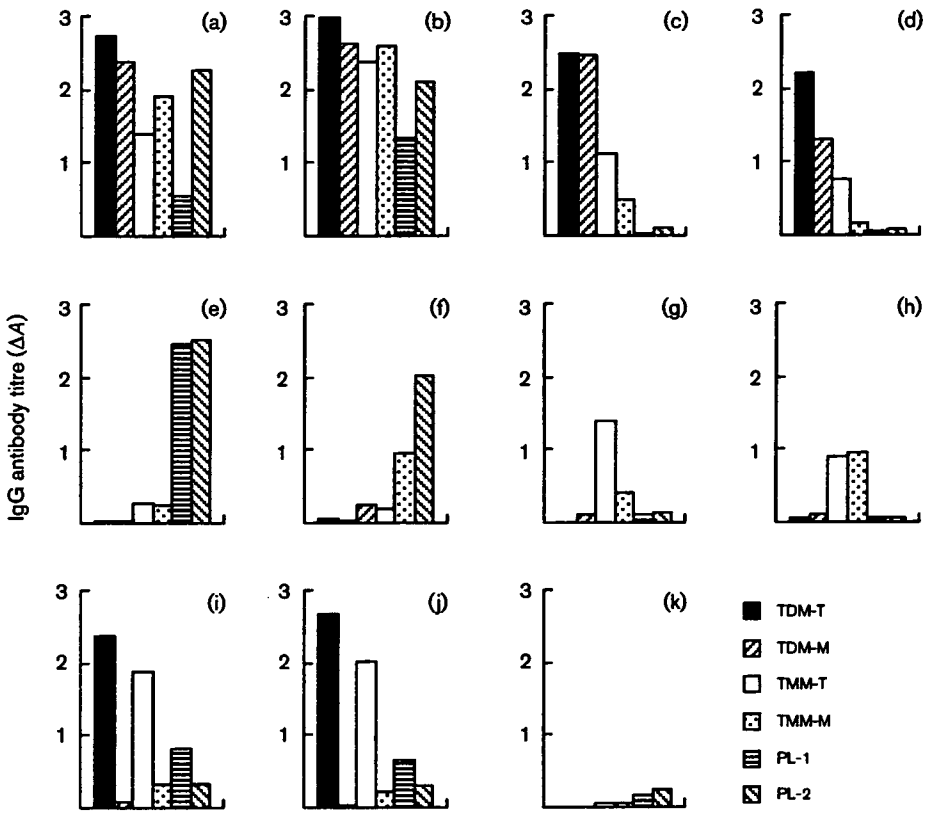

Fig. 1. Heterogeneity and diversity of IgG antibody responses at their first visit to the outpatient clinic. (a) Male, 51 years, smear (+), culture (+), multidrug resistant, relapse TB; (b) male, 45 years, smear (+), culture (+), relapse TB; (c) male, 61 years, smear (+), culture (+), primary TB; (d) 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.

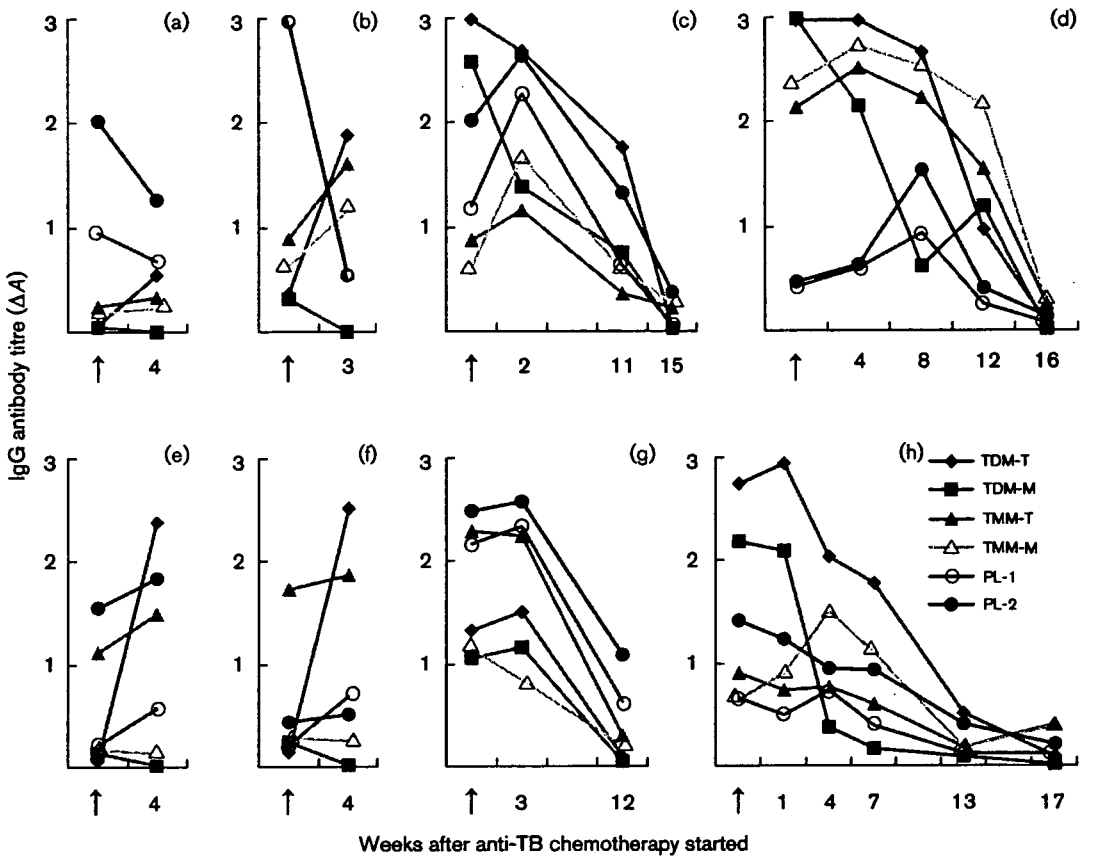

Fig. 2. Changes in IgG antibody levels after initiation of anti-TB chemotherapy. (a) Male, 67 years, smear (+), culture (+), primary TB (bII3); (b) male, 37 years, smear (+), culture (+), primary TB (bII2) (results for PL-1 and PL-2 almost identical); (c) male, 51 years, smear (+), culture (+), relapsed TB (rII2); (d) male, 38 years, smear (+), culture (+), primary TB (pleuritis, bII2); (e) male, 39 years, smear (−), culture (+), primary TB (rII3); (f) male, 46 years, smear (+), culture (+), relapsed TB (bII2); (g) male, 67 years, smear (+), culture (+), primary TB (bII3); (h) male, 43 years, smear (+), culture (+), primary TB (pleuritis, bII1). 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):					
		G0	G1–3	G4–7	G8–10	G unknown	Total
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 ± SD	0·946 ± 1·02	0·657 ± 0·910	1·139 ± 1·036	1·644 ± 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 ± SD	0·592 ± 0·729	0·281 ± 0·631	0·563 ± 0·812	1·023 ± 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 ± SD	0·605 ± 0·586	0·421 ± 0·415	0·941 ± 0·655	1·147 ± 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 ± SD	0·690 ± 0·760	0·450 ± 0·547	0·689 ± 0·673	0·966 ± 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 ± SD	0·392 ± 0·481	0·454 ± 0·730	0·620 ± 0·791	0·804 ± 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 ± SD	0·745 ± 0·693	0·545 ± 0·745	0·892 ± 0·880	1·213 ± 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 cavitary 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

Table 3. Effect of severity or extent of pathological lesions of active TB patients at their first visit to the outpatient clinic on the IgG antibody seropositive rate

Antigen	Sensitivity* with pathological lesion type† shown:					
	(b,l,r)I(2,3)	bII(1,2,3)	l or rII(1,2,3)	(b,l,r)III(1,2,3)	Unknown	Total
TDM-T	15/20 (75·0)	61/73 (83·6)	21/33 (63·6)	29/53 (54·7)	27/31 (87·1)	153/210 (72·9)
TDM-M	15/20 (75·0)	52/73 (71·2)	18/33 (54·5)	19/53 (35·8)	21/31 (67·7)	125/210 (59·5)
TMM-T	16/20 (80·0)	54/73 (74·0)	23/33 (69·7)	26/53 (49·1)	24/31 (77·4)	143/210 (68·1)
TMM-M	14/20 (70·0)	45/73 (61·6)	18/33 (54·5)	16/53 (30·2)	17/31 (54·8)	110/210 (52·4)
PL-1	14/20 (70·0)	57/73 (78·1)	23/33 (69·7)	31/53 (58·5)	23/31 (74·2)	148/210 (70·5)
PL-2	17/20 (85·0)	57/73 (78·1)	25/33 (75·8)	33/53 (62·3)	23/31 (74·2)	155/210 (73·8)
Total	18/20 (90·0)	71/73 (97·3)	31/33 (93·9)	46/53 (86·8)	30/31 (96·8)	196/210 (93·3)

*Sensitivity is shown as positive/total, with the percentage in parentheses.
†Types of pathological lesion are described in Methods.

single-protein antigen and the test gives low sensitivity and high cross-reactivity. The overall sensitivity of multiple-antigen ELISA with 10 protein antigens has been reported to be at best 70 % (Lyashchenko *et al.*, 1998).

Previously, we reported that anti-cord-factor IgG antibody titres were elevated significantly in active TB patient sera (He *et al.*, 1991) and demonstrated that the detection of anti-cord factor antibody was applicable for the early diagnosis of active pulmonary TB (Maekura *et al.*, 1993), colonic TB (Kashima *et al.*, 1995) and later tuberculous uveitis (Sakai *et al.*, 2001). Cord factor is one of the most characteristic and ubiquitous cell wall glycolipids and the structure of the mycoloyl moiety varies widely among mycobacterial species. It has also been reported that glycolipids are extremely stable physico-chemically on microplate ELISA (Julian *et al.*, 2001). A TBGL (tuberculous glycolipid) antibody detection system using mainly cord factor with minor glycolipid antigens derived from *M. tuberculosis* $H_{37}Rv$ has been developed (Kawamura *et al.*, 1997) and evaluated clinically to be useful for the diagnosis of TB at early stages of the disease by a multiple-centre study (Maekura *et al.*, 2001). Compared to protein antigens, ELISA using lipid antigens shows better stability and reproducibility, and low cross-reactivity, if we choose appropriate antigens and antibody detection system. Chan *et al.* (1990) reported haemagglutination tests with three glycolipid antigens and ELISA with antigen-5 on pulmonary TB patient sera; tests with all four antigens were of similar efficiency, with 30–52 % of smear-positive patients. More recently, it has also been reported that the detection of immune complexes and IgG antibodies against the three glycolipid antigens is useful for the serodiagnosis of children with a high probability of pulmonary TB (Simonney *et al.*, 2000). Furthermore, such immunecomplex multiple-antigen ELISA has been described to serve as a fast, cheap and easy method for the diagnosis of TB (Harrington *et al.*, 2000). Julian *et al.* (2004) reported antibody responses against a combination of specific glycolipids and proteins for test sensitivity improvement in TB serodiagnosis.

In our present study, we carefully selected six antigens from

Table 4. Effect of existence of cavity in the pathological lesion of active TB patients at their first visit to the outpatient clinic on the IgG antibody seropositive rate

Antigen	Sensitivity*			
	Cavity (+)	Cavity (−)	Unknown	Total
TDM-T	97/126 (77·0)	29/53 (54·7)	27/31 (87·1)	153/210 (72·9)
TDM-M	85/126 (67·5)	19/53 (35·8)	21/31 (67·7)	125/210 (59·5)
TMM-T	93/126 (73·8)	26/53 (49·1)	24/31 (77·4)	143/210 (68·1)
TMM-M	77/126 (61·1)	16/53 (30·2)	17/31 (54·8)	110/210 (52·4)
PL-1	94/126 (74·6)	31/53 (58·5)	23/31 (74·2)	148/210 (70·5)
PL-2	99/126 (78·6)	33/53 (62·3)	23/31 (74·2)	155/210 (73·8)
Total	120/126 (95·2)	46/53 (86·8)	30/31 (96·8)	196/210 (93·3)

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

among 12 lipid components obtained from mycobacterial cell walls and membranes, and evaluated the reactivities of IgG antibody in active-TB patients and healthy control subjects. A diverse patient-to-patient variation of IgG antibody responses against each of the six lipid antigens was observed; when we combined the six antigens and an overall positive was scored when any one of the six tests was positive, a markedly higher sensitivity was obtained. Some patients gave a highly positive result against all six antigens generally. However, some patients scored highly positive against only mycoloyl glycolipids derived from cell walls, whereas others were positive against only mannophospholipids from cell membranes. Some were positive to TMM, but not to TDM. Some were positive to TDM-T but not to TDM-M, or positive to TMM-T but not to TMM-M, indicating that the mycolyl moiety of the trehalose esters was strictly recognized by IgG antibody in TB patient sera, as we have already reported (Pan *et al.*, 1999).

Recently, the lipid antigen presenting molecules group 1 CD1 (such as CD1a, CD1b, CD1c) and group 2 CD1 (CD1d and CD1e) in human dendritic cells or macrophages have been investigated (Beckman *et al.*, 1994; Moody *et al.*, 1997; Porcelli *et al.*, 1992), and T-cell responses in humans infected with *M. tuberculosis* to CD-1 restricted lipid antigens were reported (Ulrichs *et al.*, 2003), in which the T cell response in TB patients was the most active against the glycolipid fraction by proliferation assay. However, the lipid antigen presentation to B cells in humoral immune responses has not yet been reported. Mycobacterial lipid antigen epitopes may be presented by novel mechanisms different from the classical MHC class I or class II restricted protein antigens. Although in the case of protein antigens the diversity of T cell responses has been reported to be related to HLA type exclusively, a diversity mechanism of humoral response in the human system against lipid antigens has not yet been reported. To date, serodiagnostics have been designed for early diagnosis of TB or other mycobacterial diseases, and therefore the changes or variation of antibody titres according to the progression or stage of the disease have not been focused on. To reveal the diversity mechanism of antibody responses, we examined the time-course changes in IgG antibody titres. We found that IgG antibody levels against lipid antigens in TB patients' sera varied greatly depending on the stages of the disease. This differs from TST results, which show long-lasting positive scores, once changed to positive from negative. In most cases after the initiation of anti-TB chemotherapy, the IgG antibody titres of active TB patient sera against mycobacterial lipid antigen decreased immediately or were first elevated for a few weeks, and then decreased distinctly near to the normal healthy control level after 3–4 months, when the excretion of bacilli had ceased. The above results and our previous reports (Maekura *et al.*, 1993, 2001), strongly suggest that in parallel with the bacterial burden, IgG antibody levels changed dramatically. To demonstrate the relationship between positive rate and/or mean ΔA values and loading amount of bacteria, we carefully compared the IgG antibody responses of smear-negative (Gaffky 0), paucibacillary (Gaffky +1 to +3), moderate bacillary (Gaffky +4 to +7) and multibacillary (Gaffky +8 to +10) groups. Unexpectedly, the smear-negative group did not necessarily show the lowest positive rate and IgG antibody levels, and the paucibacillary group showed the lowest. This may reflect the incompleteness of the detection technique of the smear test for acid-fast bacteria. IgG antibody responses were also related to the severity or extent of the pathological lesions determined by chest X-ray examination. In general, patients with the larger and more severe pathological lesions showed a higher positive rate against all antigens, while patients with smaller or less severe lesions showed a lower rate. As the chemotherapy for TB proceeded, excretion of bacilli was eradicated and the pathological lesions were minimized, IgG antibody levels against the lipid antigens decreased distinctly. Therefore, the serodiagnostic test can be useful for the diagnosis of the progression of the disease or as a criterion to determine whether the patient should end or continue the anti-TB chemotherapy. Although the pathological role of the humoral antibody response in TB is not known, marked elevation of IgG antibody against cell-surface lipid antigens may promote the generation of granulomatous lesions or induction of cytokine production by the cellular immune responses, as reported previously (Fujiwara *et al.*, 1999).

Recently, the incidence of non-TB mycobacteriosis such as MAC infection has increased in TB patients being treated with anti-TB chemotherapy and in immunosuppressed hosts such as AIDS. Therefore, a differential diagnostic test is required. In the present study, we observed that TB patient sera were more reactive against TDM-T and TMM-T, while MAC patient sera were more reactive against TDM-M and TMM-M, indicating that IgG antibody recognized the mycoloyl residue of TDM and/or TMM, as reported previously (Enomoto *et al.*, 1998; Pan *et al.*, 1999). However, in the present study, IgG antibody responses against species-specific TDM and TMM were cross-reactive, and 100% sensitivity or specificity was not obtained. This is probably due to the fact that natural trehalose esters consist of a mixture of mycolic acid subclasses. In contrast, phosphoinositomannnosides are widely distributed in mycobacterial species; thus PL-1 and PL-2 are essentially mycobacterial common antigens. Therefore, with the present combination of six antigens we cannot strictly differentiate between TB and MAC disease. However, if we use species-specific antigens, more precise differential diagnosis of TB and MAC disease would be possible. Our recent papers have described highly sensitive discrimination between TB and MAC disease, using ELISA with serotype-specific glycopeptidolipid (GPL) cocktail antigens (Kitada *et al.*, 2002) or GPL core (Kitada *et al.*, 2005) from MAC. A more comprehensive serological analysis of MAC infection using mycobacterial multi-lipid antigens including MAC-specific GPL core will be reported in a later paper.

Another use of multiple-antigen ELISA is for serological

testing of high-risk contacts who might have been infected with *M. tuberculosis* recently and may develop active disease if chemoprophylaxis is not applied. Silva *et al.* (2003), using multivariate analysis, reported that the humoral response to 38 kDa protein was associated with active disease, while those to 14 kDa and ESAT-6 antigens were associated with inactive TB, namely recent *M. tuberculosis* infection or latent TB. Since in our study also, there were probably two types of antigens – early-responsive or infection-specific antigens such as PL-1, PL-2, TMM-T and TMM-M, and late-responsive or disease-specific antigens such as TDM-T and TDM-M – it would be possible for serological screening to use serodiagnostics with appropriate antigens. In contrast to TST, which is an infection-associated criterion based on the cellular immune response, humoral IgG antibody responses well reflected the loading amount of bacilli and were more disease specific. Therefore, TST-negative active-TB patients who have been excreting bacilli and are immunocompromised due to steroid use, diabetes mellitus or anti-TNF-α antibody treatment, and old-aged or low-protein nutritional cases, may yet give a positive IgG antibody response (data not shown). To establish whether a serological tool using a particular single antigen or multiple antigens is useful for the screening of latent infection of *M. tuberculosis*, it is essential to perform clinical tests on a population from an area of high TB incidence. Overall, multiple-antigen ELISA, based on the humoral immune responses, may be the most useful for the rapid diagnosis of active disease due to mycobacterial infection, diagnosis of progression of the disease and differential diagnosis of the mycobacterial species responsible for the infection.

The future interest will be to investigate the possible immunological role of IgG antibodies against each lipid antigen and the relationship between the humoral and cellular immune responses.

REFERENCES

Arloing, S. (1898). Agglutination de bacille de la tuberculose vraie. *C R Acad Sci* **126**, 1398–1400.

Beckman, E. M., Porcelli, S. A., Morita, C. T., Behar, S. M., Furlong, S. T. & Brenner, M. B. (1994). Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. *Nature* **372**, 691–694.

Benjamin, R. G. & Daniel, T. M. (1982). Serodiagnosis of tuberculosis using the enzyme-linked immunoabsorbent assay (ELISA) of antibody to *Mycobacterium tuberculosis* antigen 5. *Am Rev Respir Dis* **126**, 1013–1016.

Benjamin, R. G., Debanne, S. M., Ma, Y. & Daniel, T. M. (1984). Evaluation of mycobacterial antigens in an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of tuberculosis. *J Med Microbiol* **18**, 309–318.

Chan, S. L., Reggiardo, Z., Daniel, T. M., Girling, D. J. & Mitchison, D. A. (1990). Serodiagnosis of tuberculosis using an ELISA with antigen 5 and a hemagglutination assay with glycolipid antigens. Results in patients with newly diagnosed pulmonary tuberculosis ranging in extent of disease from minimal to extensive. *Am Rev Respir Dis* **142**, 385–389.

Chan, E. D., Heifets, L. & Iseman, M. D. (2000). Immunologic diagnosis of tuberculosis. *Tuber Lung Dis* **80**, 131–140.

Daniel, T. M. & Debanne, S. M. (1987). The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. *Am Rev Respir Dis* **135**, 1137–1151.

Enomoto, K., Oka, S., Fujiwara, N., Okamoto, T., Okuda, Y., Maekura, R., Kuroki, T. & Yano, I. (1998). Rapid serodiagnosis of *Mycobacterium avium-intracellulare* complex infection by ELISA with cord factor (trehalose 6,6′-dimycolate), and serotyping using the glycopeptidolipid antigen. *Microbiol Immunol* **42**, 689–696.

Fujiwara, N., Oka, S., Ide, M., Kashima, K., Honda, T. & Yano, I. (1999). Production and partial characterization of antibody to cord factor (trehalose 6,6′-dimycolate) in mice. *Microbiol Immunol* **43**, 785–793.

Grange, J. M., Gibson, J., Nassau, E. & Kardjito, T. (1980). Enzyme-linked immunosorbent assay (ELISA): a study of antibodies to *Mycobacterium tuberculosis* in the IgG, IgA and IgM classes in tuberculosis, sarcoidosis and Crohn's disease. *Tubercle* **61**, 145–152.

Harrington, J. J., 3rd, Ho, J. L., Lapa e Silva, J. R., Conde, M. B., Kritski, A. L., Fonseca, L. S. & Saad, M. H. (2000). *Mycobacterium tuberculosis* lipid antigens: use of multi-antigen based enzyme immunoassay for free and complex dissociated antibodies. *Int J Tuberc Lung Dis* **4**, 161–167.

He, H., Oka, S., Han, Y. K., Yamamura, Y., Kusunose, E., Kusunose, M. & Yano, I. (1991). Rapid serodiagnosis of human mycobacteriosis by ELISA using cord factor (trehalose-6,6′-dimycolate) purified from *Mycobacterium tuberculosis* as antigen. *FEMS Microbiol Immunol* **3**, 201–204.

Iinuma, Y., Senda, K., Takakura, S. & 8 other authors (2002). Evaluation of a commercially available serologic assay for antibodies against tuberculosis-associated glycolipid antigen. *Clin Chem Lab Med* **40**, 832–836.

Julian, E., Cama, M., Martinez, P. & Luquin, M. (2001). An ELISA for five glycolipids from the cell wall of *Mycobacterium tuberculosis*: Tween 20 interference in the assay. *J Immunol Methods* **251**, 21–30.

Julian, E., Matas, L., Alcaide, J. & Luquin, M. (2004). Comparison of antibody responses to a potential combination of specific glycolipids and proteins for test sensitivity improvement in tuberculosis serodiagnosis. *Clin Diagn Lab Immunol* **11**, 70–76.

Kardjito, T., Handoyo, I. & Grange, J. M. (1982). Diagnosis of active tuberculosis by immunological methods. 1. The effect of tuberculin reactivity and previous BCG vaccination on the antibody levels determined by ELISA. *Tubercle* **63**, 269–274.

Kashima, K., Oka, S., Tabata, A., Yasuda, K., Kitano, A., Kobayashi, K. & Yano, I. (1995). Detection of anti-cord factor antibodies in intestinal tuberculosis for its differential diagnosis from Crohn's disease and ulcerative colitis. *Dig Dis Sci* **40**, 2630–2634.

Kawamura, M., Sueshige, N., Imayoshi, K., Yano, I., Maekura, R. & Kohno, H. (1997). Enzyme immunoassay to detect antituberculous glycolipid antigen (anti-TBGL antigen) antibodies in serum for diagnosis of tuberculosis. *J Clin Lab Anal* **11**, 140–145.

Kiran, U., Shriniwas, Kumar, R. & Sharma, A. (1985). Efficacy of three mycobacterial antigens in the serodiagnosis of tuberculosis. *Eur J Respir Dis* **66**, 187–195.

Kitada, S., Maekura, R., Toyoshima, N., Fujiwara, N., Yano, I., Ogura, T., Ito, M. & Kobayashi, K. (2002). Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. *Clin Infect Dis* **35**, 1328–1335.

Kitada, S., Maekura, R., Toyoshima, N., Naka, T., Fujiwara, N., Kobayashi, M., Yano, I., Ito, M. & Kobayashi, K. (2005). Use of