MDRTB can occur on various situations. These results underscore the importance of placing MDRTB patients separately from drug-sensitive TB patients.

2. Reviews of the exogenous re-infection in tuberculosis: Hideo OGATA (Fukujuji Hospital, JATA)

In Japan, they have thought that a tubercular relapse is based on endogenous reactivation in almost all cases. However, there are many studies which prove exogenous re-infection using tuberculin test or drug susceptibility test. The technique of developed strain typing contributed exogenous re-infection to clarifying greatly in a real proof and its frequency in recent years.

3. Multiple and repeated polyclonal infections in patients with *Mycobacterium avium* lung diseases: Katsuhiro KUWABARA (NHO Nishi-Niigata Chuo National Hospital)

The routes of transmission and environmental reservoirs of *Mycobacterium avium* infections have been unclear. IS*1245* based RFLP analysis showed genetic diversity of *Mycobacterium avium* clinical isolates and the relation between clinical subtype and polyclonal infection. Our study demonstrates that polyclonal infections are common in *Mycobacterium avium* lung diseases, especially nodular bronchiectasis type. In addition, not only simultaneous polyclonal infections but also repeated polyclonal infections were observed in some patients. The knowledge of polyclonal infection will lead to better understanding of *Mycobacterium avium* pathogenesis and epidemiology.

Special commentaries: Consideration of exogenous re-infection of tuberculosis in Osaka. Japan, by using molecular epidemiologic tools: Tomoshige MATSUMOTO (Osaka Prefectural Medical Center for Respiratory and Allergic Diseases)

By using IS*6110* RFLP, we showed that 9.5% of TB recurrence was caused by re-infection in the middle-eastern area of Osaka Prefecture, Japan. The molecular typing tools are now being applicable not only to epidemiological but also to clinical fields by an introduction of PCR-based method, such as Variable Numbers of Tandem Repeats (VNTR) typing. We showed some examples about usefulness of the clinical application of molecular epidemiology, using VNTR.

Key words: Exogenous re-infection, Endogenous reactivation, Multidrug-resistant tuberculosis, Nosocomial infection, *Mycobacterium avium* infection, Molecular epidemiology.

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小児科

Q インフルエンザ罹患に伴うライ様症候群・急性壊死性脳症

インフルエンザ罹患に伴うライ様症候群、急性壊死性脳症の発生機序と予防策について。 (神奈川県　Y)

A ライ様症候群とは「ライ症候群に臨床生化学的所見は酷似しているが、肝所見で病理組織所見がライ症候群とは異なるもの」と定義される。脳浮腫と肝障害がみられるという点がライ症候群に共通している。ライ様症候群の肝所見は必ずしも非炎症性ではなく、細胞浸潤、壊死など多様性がある。実際の医療現場では、最近では発生が減少しているライ症候群よりもライ様症候群のほうが遭遇する機会が多く、重要である。

ライ様症候群の原因はインフルエンザ、水痘、ヘルペスなどのウイルス感染症、脂肪酸酸化障害などの代謝異常、バルプロ酸などの薬剤など、多岐にわたっている。発症機序はミトコンドリア機能を一次的もしくは二次的に障害することが想定されている。すなわち、ホパンテン酸カルシウム、バルプロ酸、マルゴサ油などの薬剤や化学物質ではミトコンドリア機能を障害して発症に至ることが知られているし、メフェナム酸やジクロフェナクなどもライ様症候群の発症に関与している可能性がある。

また、脂肪酸代謝異常で絶食により低血糖を引き起こし、容易に意識障害に陥り、急性脳症を発症することが知られている。この場合は絶食によりグリコーゲンの枯渇化が進展するにもかかわらず、脂肪酸の酸化障害のために、容易にエネルギーの供給不足状態に陥りやすいことと関連している。

予防としては、これらのライ様症候群と関連があると想定されている薬剤の使用を中止もしくはできるだけ控えることであり、脂肪酸代謝異常などでは絶食時間を短くすることや、炭水化物の摂取を早めに心がけることであろう。ウイルス感染症では有効なワクチンが利用できる場合には、積極的に予防接種を受けることであろう。

急性壊死性脳症はわが国で提唱された疾患概念で、日本を含む東アジアに多発し、小児の急性脳症のサブタイプといえる。六カ月～一歳六カ月の乳幼児が罹患しやすく、発症前には発熱を伴うウイルス感染が必発である。中でもインフルエンザが最も多く、その他、突発性発疹症、ロタウイルス等が知られている。脳の病理所見では浮腫性壊死が観察される。

発症機序については、サイトカインの関与が想定されており、事実、各種サイトカインの増加が知られている。ウイルス感染後、半日～三日で発症することが多く、意識障害、痙攣、嘔吐で発症する場合が大半である。ライ症候群とは異なり、高アンモニア血症、低血糖、高ピルビン酸および乳酸血症の頻度が低いことから、急性壊死性脳症の発症機序にミトコンドリア障害が関与している可能性は低いと考えられる。いずれにしても、本症はインフルエンザ流行に関連し、多発することが知られており、インフルエンザ対策が何よりの予防であろう。

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Q STAT1欠損症による免疫不全の病態

免疫不全症の一つSTAT(signal tranducer and activator of transcription)1欠損症について。インターフェロン(IFN)-α, β, γの反応を悪くし、非定型抗酸菌症の感染を起こしやすくするようであるが。 (東京都　F)

A まず、STAT1の生理学的意義を解説する。STAT1はシグナル伝達と転写活性化の二

つの機能を有する蛋白で、IFN-α/β, IFN-γ, 上皮成長因子（EGF）、血小板由来成長因子（PDGF）、インターロイキン6（IL-6）などによりチロシンの部分がリン酸化されることにより活性化され、二量体、三量体などを形成して転写を促進する[1]。

図1に示すように、IFN-γからの刺激の場合、STAT1は主にホモダイマーであるGAF（IFN-γ-activated factor）を形成し、GAS（IFN-γ-activated site）に結合し、転写を促進する。また、IFN-α/βからの刺激の場合、STAT1は主にSTAT2, IRF9（IFN regulatory factor 9）と結合し、ISGF3（IFN-stimulated gene factor 3）を形成する。ISGF3はISRE（IFN-stimulated gene response element）に結合し転写を活性化する。

また、図2のように、STAT1はIFN-γの刺激により活性化され、腫瘍壊死因子α（TNF-α）、IL-12などの遺伝子発現を促進し、抗酸菌の排除に重要な役割を果たす。

図1 IFN-α/βとγのシグナル伝達経路の概略

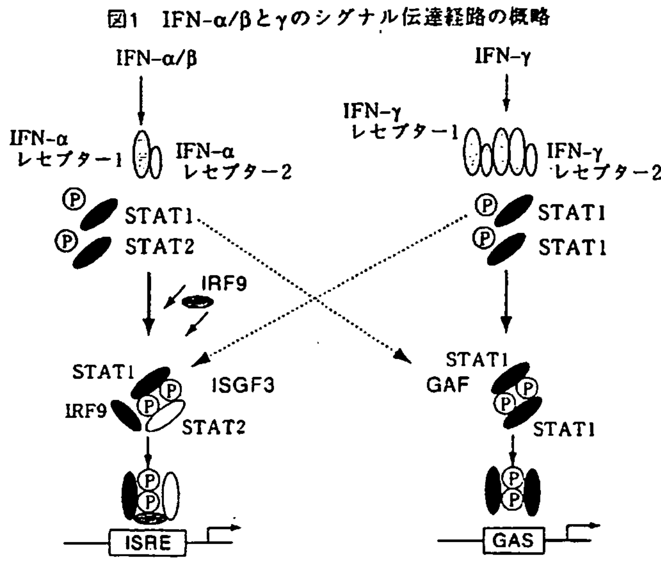
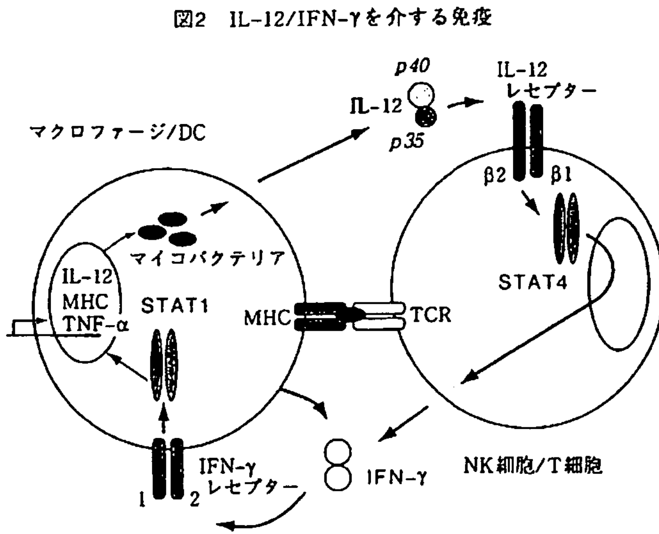


図2 IL-12/IFN-γを介する免疫



①STAT1部分欠損症

片側のアリルのみのSTAT1の七六一番目のアミノ酸がリジンからセリンに変化した変異の場合、異常なSTAT1のため七〇一番目のチロシンのリン酸化が起きず、転写が促進されない。しかし、この場合、三量体のISGF3は正常に形成され、IFN-α/βのシグナル伝達には異常が生じないため、マイコバクテリア易感染性（BCG播種、非定型抗酸菌症）は起きるが、ウイルスに対する易感染性は生じていない[2]。

②STAT1完全欠損症

両側のアリル変異の場合、GAFとISGF3両方の異常が生じ、マイコバクテリアとウイルス両方に易感染性が生じている。STAT1完全欠損症の患者では、一例がBCGの播種と再発性単純ヘルペスI型脳炎を起こし死亡、もう一例もBCG播種の後、ウイルス様疾患で死亡している[3]。

STAT1はEGF, PDGFなど他のサイトカインのシグナル伝達にも働くが、他のサイトカイン欠損に関連する症状は出現していない。また、STAT1は細胞周期、アポトーシスとも関連し、腫瘍抑制因子としての可能性が考えられているが[1]、ヒトSTAT1完全欠損症と癌との関連は明らかではない。

以上のようにSTAT1欠損症

は、IFN-γ経路の異常により抗酸菌への易感染性を示す疾患群（IL-12, IL-12レセプター、IFN-γレセプター1&2欠損症）に新しい疾患として加わった。これらの疾患は、一般には抗酸菌のみならず、リステリア、サルモネラ、レジオネラなど細胞内寄生性細菌に対しても易感染性を示す[4]。

動物モデルの研究で、STAT1完全欠損マウスでは、ヒトと同様に、IFN-αやIFN-γに反応せず、ウイルス、細胞内寄生性細菌に対して易感染性を示した。しかし、他のサイトカインに対する反応性は正常で、成長・発達には異常を来していない。また、STAT1完全欠損マウスでは悪性腫瘍も発生している[5]。

以上のように、ヒトの原発性免疫不全症の研究により、さまざまな免疫分子の生理学的意義が明らかとなった。

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Novel roles of osteopontin and CXC chemokine ligand 7 in the defence against mycobacterial infection

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Summary

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced human monocyte-derived macrophage (GM-Mϕ) or macrophage CSF (M-CSF)-induced human monocyte-derived Mϕ (M-Mϕ) are distinct in terms of the resistance to *Mycobacterium tuberculosis*. To elucidate the role of molecules involved in the functional differences between these Mϕs, we investigated the gene expression profiles using microarray. After culture of $CD14^+$ monocytes with CSFs, Mϕs were cultured with or without bacillus Calmette–Guérin (BCG) (GM-Mϕ-BCG and M-Mϕ-BCG). The gene expression profiles from these cells were compared. Chemokines highly expressed in M-Mϕs were selected and evaluated for anti-mycobacterial activity and superoxide production. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕ and M-Mϕ, respectively. After stimulation with BCG, three chemokine genes (*Osteopontin* (*SPP1*), *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were highly expressed in M-Mϕ-BCG when compared to those in GM-Mϕ-BCG. A significantly increased resistance to *M. tuberculosis* H37Ra was observed after the stimulation of GM-Mϕ with SPP1 or CXCL7. Superoxide production levels of SPP1- or CXCL7-stimulated GM-Mϕs were higher than those of GM-Mϕs without stimulation. These results indicate that both SPP1 and CXCL7 might have a role in the resistance against mycobacteria, at least in part, through augmenting reactive oxygen intermediate production in Mϕs.

Keywords: GM-CSF, M-CSF, macrophage, microarray

Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, is one of the most important burdens on human health [1]. Both environmental and genetic factors contribute to the development of the disease, which approximates 10% of the infected subjects [2]. Twin studies provided the evidence that human genetic factors could influence the development of tuberculosis [3]. The genetic basis of susceptibility to mycobacteria has been clarified partly by the recent identification of defects in the molecules of the interferon (IFN)-γ-mediated immune pathway, such as IFN-γ receptors 1 and 2 [4,5], interleukin (IL)-12 receptor-β1 [6], IL-12p40 [7] and STAT1 [8]. In addition, linkage and/or association studies have demonstrated many susceptibility genes, such as *HLA* [9], *NRAMP1* [10], *IFN-G* [11], *TNF-A* [12], *IL-10* [12], *IL-1RA* [13], *MBL* [14], *VDR* [15] and *TLR2* [16].

The immune response against mycobacteria is mounted in a complex process. In the host, mycobacteria dwell chiefly within macrophages (Mϕs). Following activation, Mϕs produce a wide range of cytokines/chemokines and activate T cells. IFN-γ secreted by activated T cells and natural killer (NK) cells is one of the principal Mϕ activating factors, and acts as the central cytokine in the control of mycobacterial infection. Activated T cells stimulate anti-mycobacterial machinery in Mϕs, which includes reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) [17].

Mϕs that play a pivotal role in the mycobacterial infections are heterogeneous in nature, with different phenotypes and functions. They are derived predominantly from peripheral blood monocytes, and differentiate to specific cells in target tissues. Peripheral blood monocytes need colony-stimulating factors (CSFs) such as granulocyte-macrophage (GM)-CSF or macrophage (M)-CSF for their survival and

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differentiation *in vitro*. GM-CSF-induced monocyte-derived macrophage (GM-Mϕ) and M-CSF-induced monocyte-derived macrophage (M-Mϕ) are distinct in their morphology, cell surface antigen expression and function. GM-Mϕ and M-Mϕ show susceptibility and resistance to mycobacteria, respectively [18,19].

To determine novel host resistance or susceptibility genes in mycobacteria infection, we investigated the differences in the gene expression profiles between GM-Mϕ and M-Mϕ with a high-density oligonucleotide microarray containing approximately 30 000 human genes. The expression profiles of each Mϕ subset were analysed with and without the stimulation of bacillus Calmette–Guérin (BCG) (GM- and M-Mϕ-BCG). Our results enlarged the views in the immunological mechanisms against mycobacteria, especially in the roles of several chemokines.

Materials and methods

Mϕ culture

Peripheral blood mononuclear cells (PBMC) were prepared from blood buffy coats of eight different healthy donors separately by density gradient centrifugation using lymphocyte separation medium (Cappel, Aurora, OH, USA). $CD14^+$ monocytes were purified (> 95%) from PBMC using a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), with anti-CD14 monoclonal antibody (mAb)-coated microbeads and an FcR blocking reagent (Miltenyi Biotec). $CD14^+$ monocytes were cultured at a concentration of 5×10^4 cells/100 µl in 96-well tissue culture plates or at a concentration of 5×10^5 cells/2 ml in 6-well tissue culture plates with RPMI-1640 (Invitrogen Japan KK, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD, USA), and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml; Sigma-Aldrich, St Louis, MO, USA) in an incubator containing 5% CO_2 at 37°C. Cells were stimulated with GM-CSF (100 ng/ml, PeproTech, London, UK) (GM-Mϕ) or M-CSF (75 ng/ml, PeproTech) (M-Mϕ). Optimal conditions were maintained by refreshing the medium and cytokines every 3 days. After 7 days of culture, a fraction of the cells were stimulated with BCG (10 mg/ml, BCG Tokyo 172; Japan BCG Laboratory, Japan) for 3 h (GM- and M-Mϕ-BCG). During BCG stimulation, a culture medium without antibiotics was used. For the analysis of anti-mycobacterial function and superoxide production, GM-Mϕs were stimulated with or without different concentrations of a chemokine: osteopontin (SPP1) (Biogenesis, Poole, UK: 0·02, 0·25 and 2·5 µg/ml), CXCL7 (Sigma-Aldrich: 0·05, 0·15 and 0·5 µg/ml) or CCL11 (Wako, Osaka, Japan: 0·5, 5 and 50 ng/ml) for another 6 days.

Bacterial preparation and infection to Mϕs

M. tuberculosis H37Ra was grown for 1 week in Middlebrook 7H9 liquid medium (Difco, Detroit, USA) at 37°C and aliquots were frozen at −80°C. In each experiment, an aliquot was thawed and grown in 7H9 medium to mid-exponential growth phase. The culture was sonicated (time: 10 s, output: 1, duty: 80%) (Branson Sonifier 250, CT, USA) to disperse bacilli before the infection. Both types of Mϕ layers were exposed to H37Ra for 3 h in a multiplicity of infection ratio of 1 : 1 in triplicate, washed three times and reincubated in the culture medium (RPMI-1640 plus 10% FBS) with antibiotics. After culture, the medium was removed and sterile phosphate-buffered saline was added to each well. The cells in the bottoms of the wells were scraped with a sterile scraper (Techno Plastic Products AG, Transadingen, Switzerland) and then sonicated as mentioned previously. Serial dilutions of the bacterial suspensions were plated on Middlebrook 7H10 agar plates (Difco). Colonies on the agar plates were counted 3 weeks after inoculation.

RNA isolation

Mϕs were harvested at 7 days after culture with CSF, and after further 24 h with BCG stimulation. Total RNA was extracted using RNA Extraction Kit, Isogen (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. All experiments were performed according to the guidelines of the ethics committee of Kyushu University.

Microarray processing

mRNA was amplified linearly using an Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX, USA). In brief, mRNA (1·5 µg) was reverse transcribed to synthesize complementary DNA (cDNA) using an oligo(dT) primer bearing a T7 RNA polymerase promoter. Second-strand synthesis was carried out to make a transcription template. *In vitro* transcription of the cDNA with incorporation of amino allyl UTP was performed to produce multiple copies of amino allyl-labelled anti-sense RNA (aRNA). After purification, amino allyl-labelled aRNA was reacted with N-hydroxy succinimide esters of Cy3 and Cy5 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the Mϕ samples and a standard control, respectively. Uncoupled dye molecules were removed using Micro Bio-Spin P-30 Tris chromatography columns (Bio-rad, Hercales, CA, USA). Cy3- and Cy5-labelled products were mixed together in the same amounts. After the aRNA was fragmented in a buffer containing 40 mM Tris-acetate, 100 mM CH_3COOK and 30 mM $(CH_3COO)_2Mg.4H_2O$ at 94°C for 15 min, the hybridization buffer (5 × SSC, 0·5% SDS, 4 × Denhardt's solution, 100 µg/ml salmon sperm DNA, 10% formamide) was added. The hybridization was performed by incubating 60 µl of the product into three Acegene Human oligo chip 30K slides (Hitachi Software Engineering, Yokohama, Japan). Each slide was rinsed with a solution provided by the manufacturer (Hitachi Software Engineering). Two microarray experiments for each Mϕ subset were conducted, using two

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RNA mixtures, each one equally combined from four independent cell cultures.

Signal detection and data analysis

Fluorescence signals for approximately 30 000 spots in slides were detected separately by fluorescent image analyser FLA-8000 (Fuji Film, Tokyo, Japan) for Cy3 and Cy5. Hybridization intensities were processed using Arrayvision software version 6·0 (Imaging Research, Ontario, Canada). Signal and background intensities were determined by the median pixel values. Local background values were determined as the average of four background spots around each gene spot. All spots in the image (for both Cy3 and Cy5 signals) were evaluated for a possibility of dusts, to lower the probability of false data in all experiments. GeneSpring version 6·2. (Silicon genetics, Redwood City, CA, USA) was used for data analysis. According to the GeneSpring instruction, normalization of the data was performed using the 'Lowess method' [20]. Spots with dust, or with signal values of which the Cy5 or Cy3 channels were less than three times of background, were excluded.

*Taq*Man real-time quantitative reverse transcriptase-PCR (qRT-PCR)

The same RNA used in the microarray analysis was employed for qRT-PCR. The cDNA was synthesized from the RNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA), as described previously [21]. PCR primers and the target probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from ABI (Applied Biosystems, Foster City, CA, USA) as a *Taq*Man GAPDH control reagent kit. PCR primers and *Taq*Man probes for *FN1* and *FCGR2B* genes were purchased from ABI as assay reagents (Assays-on-Demand™, Gene Expression Products) with the following numbers: Hs00415006_m1 for *FN1* and Hs00414000_m1 for *FCGR2B*, and used according to the instructions of the manufacturer. The qRT-PCR was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) [22]. To calculate the relative amount of gene expression, the value of each gene expression was divided by that of the internal control, GAPDH. The analysis was carried out in duplicate samples.

Flow cytometry

Flow cytometric analysis was performed using an EPICS XL (Beckman Coulter, Miami, FL, USA). Multi-colour staining was performed by fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or PE-cyanin 5·1 (PC5)-conjugated mAbs against the following markers: HLA-DR, CD14, CD71, CD44, CXCR2 and appropriate controls (Immunotech, Marseille, France).

Superoxide production assay

Superoxide production by Mϕs was determined as described previously [23]. GM-Mϕs were cultured with or without a chemokine for 7 days. After treatment with trypsin (Invitrogen), cells were harvested, washed and resuspended in Hanks's balance salt solution (HBSS) (Invitrogen) (5×10^4/0·5 ml). They were stimulated with antibody-opsonized zymosan (1 mg/ml, Sigma-Aldrich) at 37°C, and the reaction was terminated by the addition of SOD (50 μg/ml, Sigma-Aldrich). The chemiluminescence was counted for 35 min with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics) using a luminometer (Auto Lumat LB953; EG & G Berthold).

Statistical analysis

Data in qRT-PCR, colony forming unit (CFU) counting and superoxide production assays were assessed by Student's *t*-test using SPSS software version 11.

Online supplemental material

The microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series Accession number GSE3408.

Results

Characterization of GM- and M-Mϕ

After culture with GM- and M-CSF for 1 week, peripheral blood monocytes differentiated into GM-Mϕ and M-Mϕ, respectively. These two types of Mϕs showed distinct features in their phenotypes and functions. Although both Mϕs expressed HLA-DR, GM-Mϕs strongly expressed CD71 and M-Mϕs were strongly positive for CD14 (Fig. 1a). M-Mϕs showed a higher resistance to *M. tuberculosis* H37Ra and a higher superoxide production than GM-Mϕs (Figs 2 and 3B), as reported by Akagawa [18].

Comparison of the constitutive gene expression levels between GM- and M-Mϕ, by microarray and quantitative PCR

To identify the molecules involved in the functional differences between GM- and M-Mϕ, we compared the constitutive gene expression profiles in each Mϕ using microarray (Fig. 1b). The 10 most up-regulated genes, which are a result of comparison between these Mϕ, are listed in Table 1. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕs and M-Mϕs, respectively, both of which encode proteins that potentially interact with *M. tuberculosis*. These microarray data were confirmed by qRT-PCR. As shown in Fig. 1c, the

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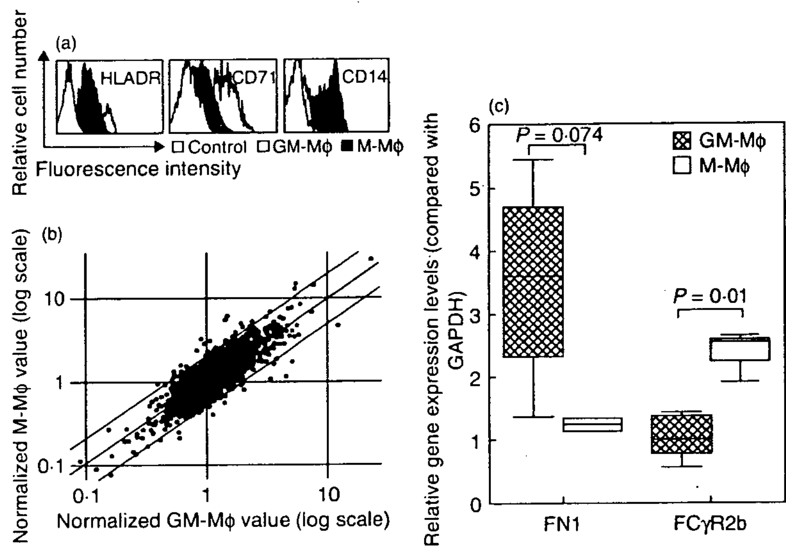

Fig. 1. (a) Phenotypic characteristics of GM-Mϕ and M-Mϕ, generated from human CD14⁺ monocyte. (b) The scatter-plot between two types of Mϕs in their constitutive states. Each spot is the representative of normalized data in logarithmal scale from the average of two values from each cell type. (c) The qRT-PCR analysis for *FN1* and *FCGR2b* gene expression levels, which were the most up-regulated genes in each Mϕ (Table 1).

expression levels of *FN1* and *FCGR2B* genes were increased in GM-Mϕs and M-Mϕs, respectively, although the difference of *FN1* expression levels did not reach the statistical significance.

Comparison of the gene expression levels between GM- and M-Mϕ with and without BCG exposure by microarray

When we compared the gene expression profiles between GM- and M-Mϕ with and without BCG, *IL-1B* showed the highest expression among BCG-stimulated genes in both Mϕs (Table 2). Also, *SOD2* gene was listed among highly expressed genes in both Mϕs after BCG stimulation (Table 2). Then, we compared the gene expression profiles between GM-Mϕ-BCG and M-Mϕ-BCG (Table 3). *Osteopontin* (*SPP1*) was the most up-regulated gene in M-Mϕ-BCG compared with GM-Mϕ-BCG, suggesting the protective role of *SPP1* in M-Mϕ against mycobacteria. Analysis of genes according to the gene ontology (GeneSpring software) revealed that four HLA-related genes were included in the 10 most up-regulated genes in GM-Mϕ-BCG compared with M-Mϕ-BCG, while three chemokine genes (*SPP1*, *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG.

Effects of 3 chemokines on the growth of *M. tuberculosis* H37Ra in GM-Mϕ

We selected three chemokine genes (*SPP1*, *CXCL7* and *CCL11*) as the candidate genes that potentially contribute to the protective function of M-Mϕs. To clarify the possible effects of these chemokines on the resistance of M-Mϕs against *M. tuberculosis*, GM-Mϕs were cultured in the presence of different concentrations of one of these chemokines for 6 days, and their protective abilities against *M. tuberculosis* H37Ra were evaluated (Fig. 2). SPP1 or CXCL7-stimulated GM-Mϕs significantly inhibited the growth of H37Ra 6 days after the infection with the organism, while CCL11 stimulation had no effects on it (Fig. 2).

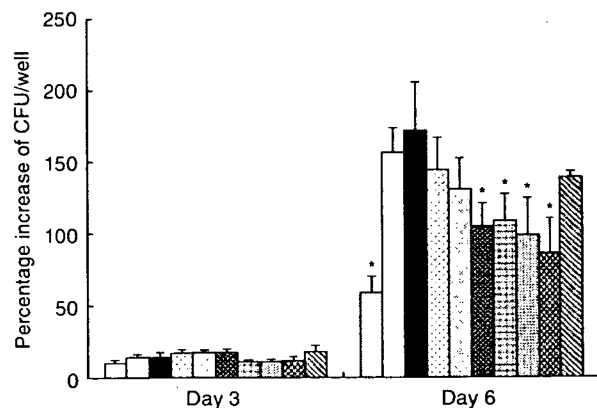

Fig. 2. Inhibition of *M. tuberculosis* H37Ra growth in Mϕs by SPP1 and CXCL7. Mtb colony-forming unit (CFU) assay was performed on days 3 and 6 after H37Ra-Mϕ exposure. M-Mϕ □, GM-Mϕ with granulocyte macrophage-colony stimulating factor (GM-CSF) only □ and GM-Mϕ without any cytokines ■ were cultured as controls. GM-Mϕs were stimulated with three different chemokines: for SPP1, 0.02 □, 0.25 □ and 2.5 ■ μg/ml; for CXCL7, 0.05 ▦, 0.15 ▦ and 0.5 ▦ μg/ml. Data for CCL11 ▧ are shown only for the results using the highest concentration in the experiments (see Methods for details). Mean values and standard deviations of triplicates are shown. *Indicates that *P* value was <0.05 in comparison with GM-Mϕ.

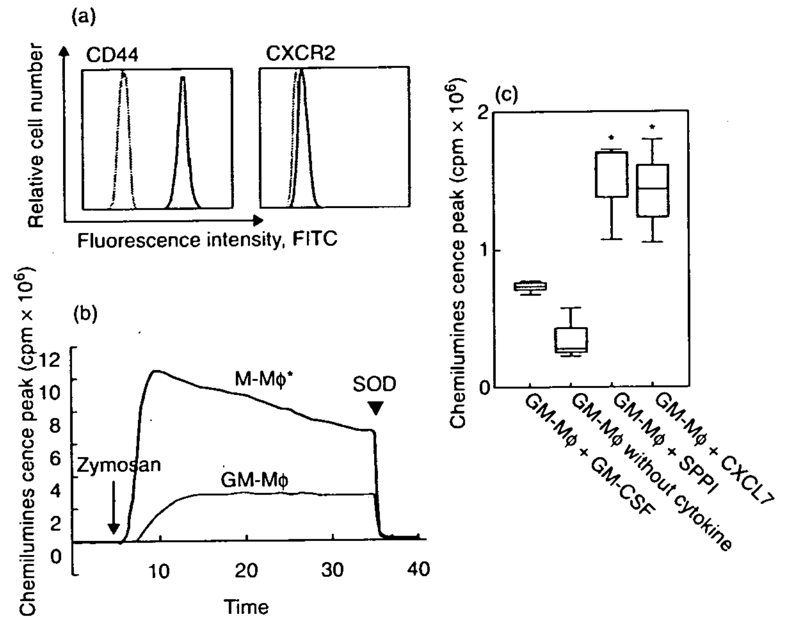

Fig. 3. Increased superoxide production from GM-Mϕs after the stimulation with SPP1 and CXCL7. (a) Receptor expression for SPP1 (CD44) and CXCL7 (CXCR2) on the surface of GM-Mϕ, determined by flow cytometry. (b) Superoxide production by the Mϕs measured by a change of chemiluminescence. The Mϕs (5.0×10^4 cells) were stimulated with antibody-opsonized zymosan (arrow), and the chemiluminescence change was monitored continuously for 40 min with DIOGENES. SOD was added to terminate the reaction (arrowhead). (c) Superoxide production from GM-Mϕs with or without chemokine stimulation. Representatives of three independent experiments are shown. *Indicates that P value was <0.05 in comparison with GM-Mϕ.

Table 1. Gene expression profiles of GM-Mϕ and M-Mϕ. (a) Genes which up-regulated in GM-Mϕ (top 10) compared to those in M-Mϕ; (b) genes which up-regulated in M-Mϕ (top 10) compared to those in GM-Mϕ.

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	FN1	ENSG00000115414	Fibronectin 1	3·81
2	Unknown	ENSG00000079310	ensembl prediction	3·12
3	Unknown	ENSG00000085063	ensembl prediction	2·93
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor; scya7	2·86
5	AD 158	AL136919–1	Hypothetical protein; dkfzp586j1119	2·83
6	ARPC2	AF116702–1	pro2446	2·56
7	KIAA1838	XM_035688–1	Hypothetical protein xp_035688; loc94580	2·53
8	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	2·53
9	HBD	NM_000519–1	Haemoglobin, delta	2·35
10	ABCC3	AF085692–1	Multidrug resistance-associated protein 3b; mrp3	2·17
(b)				
1	FCGR2B	NM_004001–1	fc fragment of igg, low affinity iib, receptor for (cd32)	4·14
2	Unknown	ENSG00000024862	ensembl prediction	3·90
3	MHC Ag	L34093–1	MHC class ii hla-dq-alpha chain	3·64
4	Unknown	ENSG00000126461	ensembl prediction	3·01
5	C15orf12	AK001830–1	cDNA flj10968 fis clone place1000863 moderately similar to putative mitochondrial 40 s ribosomal protein yhr148w	2·90
6	Unknown	AC069384·3.87217·105230·1	ensembl genscan prediction	2·85
7	Unknown	AP002767·1.52387·73825·2	ensembl genscan prediction	2·84
8	MMP9	NM_004994–1	Matrix metalloproteinase 9 preproprotein	2·82
9	TM7SF1	NM_003272–1	Transmembrane 7 superfamily member 1 (up-regulated in kidney)	2·70
10	Unknown	AC003958·1.1·127834·1	ensembl genscan prediction	2·50

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 2. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in GM-Mϕ (a), and in M-Mϕ-BCG compared to those in M-Mϕ (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	IL1B	NM_000576–1	Interleukin 1, beta	46·66
2	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	13·62
3	MT1G	XM_048213–1	Metallothionein 1 g	10·71
4	CLECSF9	AB024718–1	Macrophage c-type lectin mincle; mincle	6·28
5	CCL1	M57502–1	Secreted protein i-309; scya1	5·96
6	BCL2A1	NM_004049–1	bcl2-related protein a1	5·85
7	AKR1C3	L43839–1	3-alpha-hydroxysteroid dehydrogenase; 3alpha-hsd	5·72
8	Unknown	AC005027·2.1·157073·2	ensembl genscan prediction	5·62
9	GRO1	NM_001511–1	Gro1 oncogene (melanoma growth stimulating activity, alpha)	5·61
10	MT1H	NM_005951–1	Metallothionein 1 h	5·38
(b)				
1	IL1B	NM_000576–1	Interleukin 1, beta	74·62
2	CCL20	NM_004591–1	Small inducible cytokine subfamily a (cys-cys), member 20	59·71
3	ARHGEF1	NM_004706–1	Rho guanine nucleotide exchange factor 1	56·29
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor	27·49
5	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	25·94
6	IL8	NM_000584–1	Interleukin 8	17·98
7	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	16·33
8	SERPINB2	NM_002575–1	Serine (or cysteine) proteinase inhibitor, clade b (ovalbumin), member 2	15·81
9	TNFAIP6	NM_007115–1	Tumour necrosis factor, alpha-induced protein 6	15·29
10	H1F2	NM_005319–1	h1 histone family, member 2	14·06

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 3. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in M-Mϕ-BCG (a) and in M-Mϕ-BCG compared to those in GM-Mϕ-BCG (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	HLA-DRA	NM_019111–1	Major histocompatibility complex, class ii	6·86
2	HLA-DMA	NM_006120	Major histocompatibility complex	6·79
3	ID2	NM_002166–1	Inhibitor of dna binding 2, dominant negativ ehelix-loop-helix protein	6·06
4	HLA-DP	S66883–1	Major histocompatibility complex class ii antigen beta chain	5·81
5	HLA-DQA	L34093–1	MHC class II hla-dq-alpha chain	5·30
6	PRG1	NM_002727–1	Proteoglycan 1, secretory granule	4·88
7	RGC32	NM_014059–1	rgc32 protein	4·72
8	TNFSF13B	NM_006573–1	Tumour necrosis factor (ligand) superfamily, member 13b	4·44
9	Unknown	AC026785·3.13728·33112·2	ensembl genscan prediction	4·31
10	Unknown	XM_016170–1	Hypothetical protein xp_016170; loc88021	4·17
(b)				
1	SPP1	NM_000582–1	Secreted phosphoprotein 1 (osteopontin)	22·37
2	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	19·20
3	CXCL7	NM_002704–1	Pro-platelet basic protein (NAP2, SCYB7, CTAP3, PPBP)	15·08
4	FLJ20033	NM_017629–1	Hypothetical protein flj20033	9·43
5	LOC64182	NM_022359–1	Similar to rat myomegalin	8·82
6	Unknown	BC000845–1	Unknown (protein for image:3457769)	7·01
7	C8B	NM_000066–1	Complement component 8, beta polypeptide	6·78
8	Unknown	BC006174–1	Unknown (protein for image:4053618)	6·73
9	STK4	NM_006282–1	Serine/threonine kinase 4	6·62
10	CCL11	NM_002986–1	Small inducible cytokine subfamily a (cys-cys), member 11 (eotaxin)	6·60

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Effects of SPP1 and CXCL7 on GM-Mϕs

The expression of cell surface receptors for SPP1 (CD44) and CXCL7 (CXCR2) on GM-Mϕ were confirmed (Fig. 3a). To investigate further the mechanism of increased resistance of SPP1- or CXCL7-stimulated GM-Mϕs against *M. tuberculosis*, superoxide production by Mϕs was investigated. After the stimulation with antibody-opsonized zymosan, M-Mϕs produced a higher amount of superoxide than GM-Mϕ (Fig. 3b). Superoxide production by GM-Mϕs was significantly enhanced after the stimulation with SPP1 or CXCL7 (Fig. 3c). The reaction was terminated by SOD, which inhibits cytochrome *c* reduction (Fig. 3b). These results suggested that increased superoxide production was one of the mechanisms of increased resistance of SPP1- or CXCL7-stimulated Mϕs against *M. tuberculosis*.

Discussion

GM-Mϕs and M-Mϕs show distinct features, although both Mϕs come from the same origin ($CD14^+$ PMNC). It has been reported that GM-Mϕs show a susceptibility to *M. tuberculosis*, while M-Mϕs have a resistance to *M. tuberculosis* with a greater FcγR-mediated phagocytic capacity and a higher capability of ROI production [18].

In our experiment, *FN1* that encode fibronectin (FN) was expressed predominantly in GM-Mϕs compared with M-Mϕs (Table 1a). FN is expressed constitutively in the lung [24]. *M. tuberculosis* binds to the FN by FN attachment proteins on the surface of *M. tuberculosis*. After fibronectin opsonization, *M. tuberculosis* can be phagocytosed easily via complement receptors and integrin receptors [25,26]. Therefore, it is possible that increased FN production led to the enhanced *M. tuberculosis* load into the cells. On the other hand, *FCGR2B* was highly expressed in M-Mϕs compared with GM-Mϕs (Table 1b). It was reported that *FCGR2B* expression levels were increased in peripheral blood monocytes in patients with tuberculosis compared with healthy controls by microarray analysis [27]. In contrast to FcγR1, FcγR2a and FcγR3, FcγR2b is an inhibitory receptor that does not contain immunoreceptor tyrosine-based activation motifs (ITAM) [28]. Therefore, FcγR2b seems to modulate inflammatory responses and inhibits phagocytosis of Mϕs [25]. Further analysis for the role of FcγR2b in *M. tuberculosis* infection would be necessary.

IL-1B and *SOD2* expression levels were up-regulated in both types of Mϕs after the stimulation with BCG (Table 2), which was consistent with the previous report [29]. IL-1β is produced by activated Mϕs following *M. tuberculosis* infections, and is an important mediator of cellular anti-mycobacterial activities [30]. The importance of IL-1 for the generation of protective immunity against mycobacterial infection was clarified using *IL-1*-knock-out mice [31]. Cell wall components of *M. tuberculosis* are known to induce *IL-1B* expression in human monocytes and macrophages [32]. In addition, increased *IL-1B* gene expression was observed in bronchoalveolar lavage cells from tuberculosis patients compared with those from healthy individuals [33]. Mϕ stimulation triggers an oxidative burst and the generation of superoxide anions (O_2^-) and other ROI in Mϕs [34]. SOD2 may play a role in protection of Mϕs against ROI in *M. tuberculosis*-infected Mϕs. None the less, the protective function of M-Mϕs against *M. tuberculosis* in contrast to GM-Mϕs do not seem to be obtained solely by the increased expression of these molecules, because these molecules were also highly expressed in GM-Mϕs.

Three chemokines were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG (Table 3b). In *M. tuberculosis* infections, chemokines contribute to the recruitment of other immune cells, especially of T cells, and the formation and maintenance of granuloma [35]. We also found that GM-Mϕs, which were stimulated with SPP1 and CXCL7, were more bacteriostatic to *M. tuberculosis* than unstimulated GM-Mϕs (Fig. 2). This is the first description that these two chemokines played protective roles against *M. tuberculosis* in humans. After BCG stimulation, the ratio of *SPP1* expression was highest in M-Mϕ compared with GM-Mϕ (Table 3b). SPP1 is a multi-functional protein that is expressed in both alveolar and peritoneal Mϕs [36]. *SPP1* knock-out mice were more susceptible to *M. tuberculosis* with small and immature granuloma formation in their lungs [37]. *M. tuberculosis* infection of primary human alveolar macrophages causes a substantial increase in *SPP1* gene expression [38]. Many investigators recognize SPP1 as a proinflammatory cytokine, which causes cellular adhesion of inflammatory leucocytes. Furthermore, SPP1 promotes chemotaxis and adhesion of human peripheral blood T cells [39] and enhances their IFN-γ production [40]. It is worthy of notice that its expression can be used as a prognostic marker in patients with *M. tuberculosis* infection [41].

CXCL7 is a cleavage product of platelet basic protein with a length of 70 amino acids [42]. In neutrophils, CXCL7 induces an increase of cytosolic calcium concentration, chemotaxis, exocytosis, production of ROI, degranulation and elastase release [42,43]. Although there is a recent report showing that CXCL7 can modulate the synthesis of IL-12 in Mϕs [44], the role of CXCL7 in Mϕs has not been well determined.

In addition, we demonstrated that SPP1 and CXCL7 facilitated the production of superoxide in GM-Mϕs after the stimulation with antibody-opsonized zymosan particles. The high production of ROI in Mϕs following *M. tuberculosis* infections may be compatible with the high expression of SOD2 in microarray results from BCG-stimulated Mϕs, which may play an important role in preventing Mϕ damages induced by ROI. On the other hand, RNI production in GM-Mϕs was not increased after the stimulation with SPP1 or CXCL7 (data not shown). Immunologically activated Mϕs can generate superoxide anion and other ROI [34]. Mice

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deficient in the NADPH oxidase complex have a susceptibility to *M. tuberculosis* infection [45]. In humans, patients with chronic granulomatous disease are more susceptible to *M. tuberculosis* [46]. Although our data showed that SPP1 and CXCL7 may play an important role against *M. tuberculosis*, possibly through the up-regulation of superoxide production in Mϕs, it is possible that their anti-mycobacterial effect could be based on other mechanisms.

In summary, our data showed that Mϕs can secret a large array of molecules that induce host defence after the exposure to BCG. Among them, we found new roles of SPP1 and CXCL7 against *M. tuberculosis* in Mϕs. Further analysis of these molecules using iRNA will confirm the role of these chemokines in *M. tuberculosis* infection more clearly.

Acknowledgements

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Rapid Detection of *Mycobacterium tuberculosis* in Respiratory Samples by Transcription-Reverse Transcription Concerted Reaction with an Automated System

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The aim of this study was to evaluate the performance of the transcription-reverse transcription concerted (TRC) method for the detection of *Mycobacterium tuberculosis* complex (MTC) 16S rRNA in clinical respiratory samples for the diagnosis of pulmonary tuberculosis. TRC is a novel method that enables the rapid and the completely homogeneous real-time monitoring of isothermal sequence RNA amplification without any post-amplification procedure. The detection limit of the TRC method for MTC was one organism per 100 μl of sputum. The specificity of the method was confirmed by the absence of positive signals for sputum containing 10^6 *M. avium* or *M. kansasii* organisms per 100 μl. A total of 201 respiratory samples from patients diagnosed with or suspected of having tuberculosis were tested. Of the 72 MTC culture-positive samples, the TRC method was positive for 52 (sensitivity, 72.2%), whereas the Roche COBAS AMPLICOR PCR was positive for 58 (sensitivity, 80.6%). Both the TRC method and the COBAS AMPLICOR PCR showed no positive identification for any of the 129 culture-negative samples. The percent agreement between the two methods was 95% (191 of 201 samples). The high sensitivity and specificity together with shorter detection time (within 1 h) of the TRC method allow it to be proposed as a useful method for the rapid detection of MTC in respiratory samples.

The rapid detection and identification of *Mycobacterium tuberculosis* complex (MTC) in respiratory samples are extremely important for optimal diagnosis and effective treatment, as well as for prevention and control of tuberculosis transmission. Various molecular tests based on amplification and detection techniques have been devised for the detection of MTC in clinical samples (2, 14), such as the PCR-based COBAS AMPLICOR Mycobacterium system (Roche Diagnostics, Basel, Switzerland) (4–7), the transcription-mediated amplification-based Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test system (Gen-Probe Inc., San Diego, CA) (5, 15, 16), the strand displacement amplification-based BDProbeTec ET system (Becton Dickinson, Franklin Lakes, NJ) (6, 8), and the ligase chain reaction-based Abbott LCx Mycobacterium tuberculosis assay system (Abbott Laboratories, North Chicago, IL) (1). While they are much more rapid than any culture-based method, these systems still require several hours to get results and involve some complicated procedures (14). Therefore, a faster molecular test with greater ease of manipulation as well as high sensitivity and specificity is desirable.

Recently, we have reported on a novel method designated the transcription-reverse transcription concerted (TRC) method (9). This method, a schematic of which is shown in Fig. 1, is based on isothermal RNA amplification at 43°C with transcriptase and reverse transcriptase in the presence of the intercalation activating fluorescence (INAF) probe (10). Measurement of the fluorescence intensity of the reaction mixture with a dedicated multicolor detector enables completely homogeneous real-time monitoring of the amplification of specific RNA, while it requires only 30 min for simultaneous amplification and detection. We have used the TRC method to establish a system for the detection of specific mRNA transcripts: *tdh* and *trh* of *Vibrio parahaemolyticus* (13), *mecA* of methicillin-resistant *Staphylococcus aureus* (11), and *pab* of *M. tuberculosis* (9, 19).

This report concerns the establishment and evaluation of the TRC method-based targeting of MTC 16S rRNA (hereinafter abbreviated the "TRC method") for the direct detection of MTC in clinical respiratory samples. rRNA was chosen as the target to enable highly selective and sensitive detection because of its multicopy nature in a single cell. The sensitivity and specificity of the TRC method were compared with those of the COBAS AMPLICOR PCR for the direct detection of MTC in sputum samples.

MATERIALS AND METHODS

Preparation of standard RNAs for calibration. Standard RNA containing the target region for TRC amplification was prepared by the in vitro transcription of the SP6 promoter-bearing double-stranded DNA as the template for SP6 RNA

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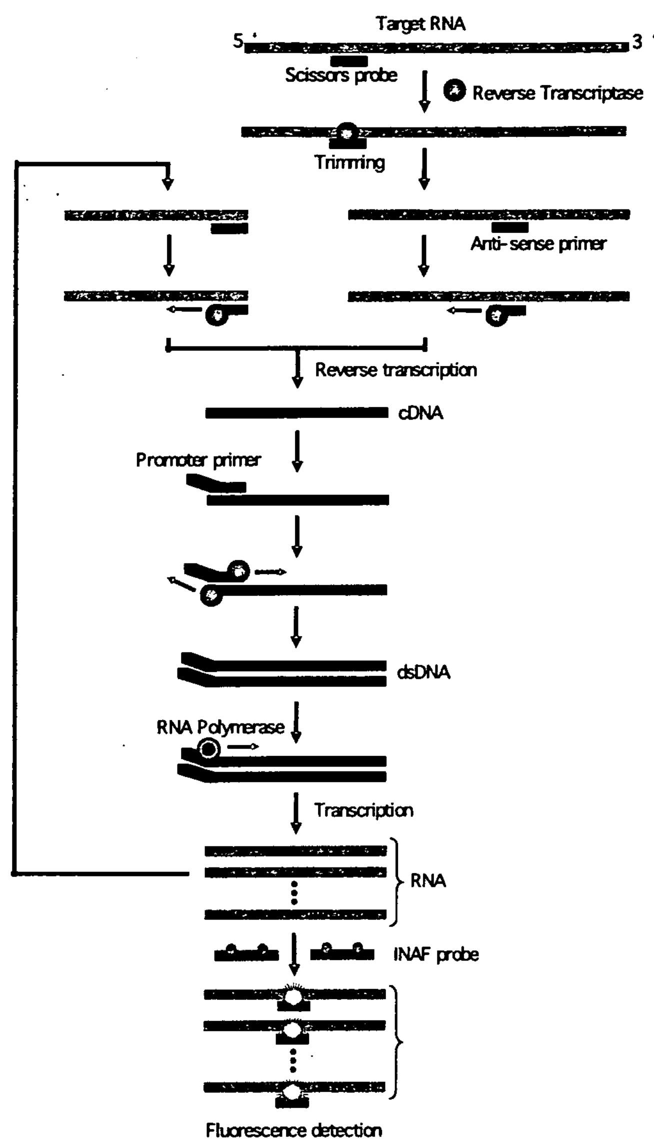

FIG. 1. Schematic description of the elementary steps of the TRC method. The progress of the reaction is monitored by measuring the fluorescence intensity of the reaction mixture. dsRNA, double-stranded RNA.

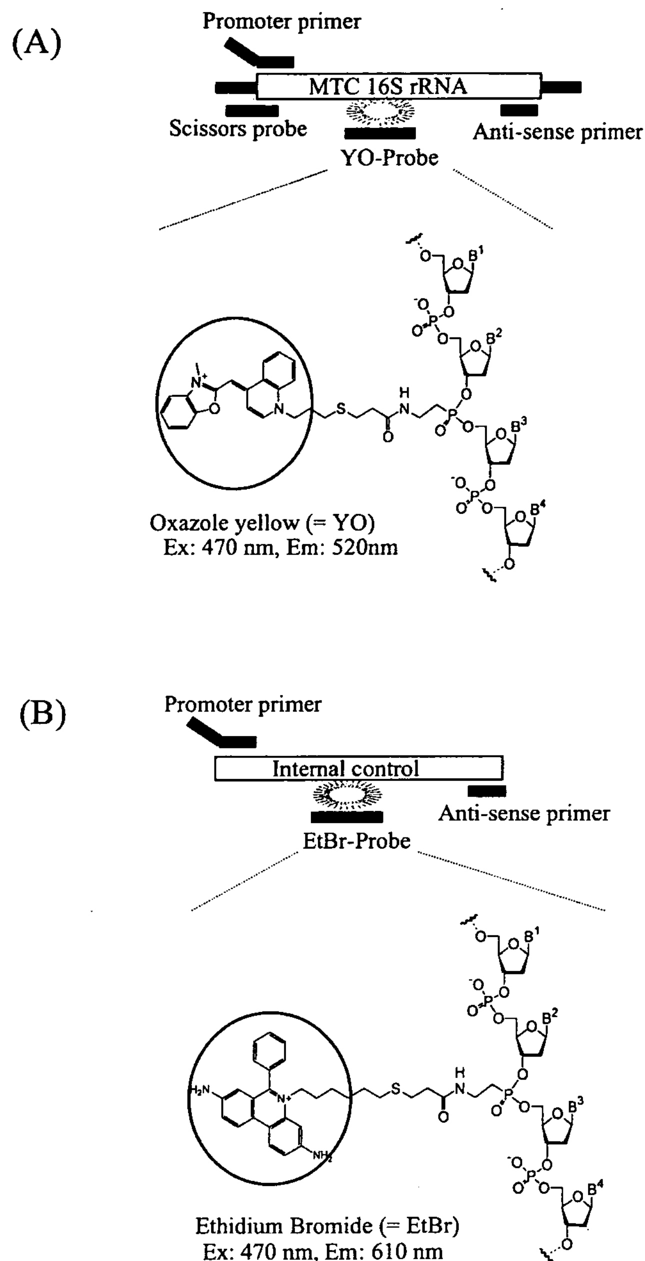

FIG. 2. Primers and probes used in the TRC method designed for amplification and detection of MTC 16S rRNA (A) and internal control (B). Ex, excitation wavelength; Em, emission wavelength.

polymerase. The DNA templates were synthesized from the total DNA extracted from the *Mycobacterium bovis* BCG strain (TOKYO 172; purchased from the National Institute of Infectious Diseases, Tokyo, Japan) by means of PCR (30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 3 min) with a pair of synthetic oligonucleotide primers, 5'-CGG TAC CCA TTT AGG TGA CAC TAT AGA ATA CAA GTT TTG TTT GGA GAG TTT GAT CC-3' and 5'-CGG TAC CCC TAC AGA CAA GAA CCC CTC A-3'. The long primer has the SP6 RNA polymerase-binding sequence at its 5' end (underlined) to provide the preferred transcription initiation site.

The amplified gene sequence was cloned into the EcoRI site of pUC19. The plasmid DNAs were then digested with EcoRI to yield linear DNAs containing the SP6 promoter, followed by in vitro transcription in a reaction mixture composed of 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM nucleoside triphosphates, 0.1 mg/ml of bovine serum albumin, 1 U/ml of RNase inhibitor, 2.5 U/ml of SP6 RNA polymerase (TaKaRa Bio, Otsu, Shiga, Japan), and 0.025 mg/ml of template DNA. The resultant RNAs were purified by gel filtration with Chromaspin-100 columns (BD Biosciences, Palo Alto, CA). The concentration of the purified RNA was determined spectrophotometrically at an optical density at 260 nm and adjusted to 10^2 to 10^7 copies/5 ml with TE (Tris-EDTA) buffer containing 0.25 U/ml of RNase inhibitor and 5 mM dithiothreitol. The RNAs were then stored at −20°C until use.

Primers, probe, and internal control for the detection of *M. tuberculosis* complex by TRC method. Synthetic oligonucleotides used for the TRC reaction included a pair of amplification primers (designated the promoter primer [5'-AAT TCT AAT ACG ACT CAC TAT AGG GAG ACG GAA AGG TCT CTT CGG AGA TAC-3'] and the antisense primer [5'-ACA AGA CAT GCA TCC CGT-3']), a scissors probe (5'-TTT CCG TTC GAC TTG CAT GTG TTA-3') to initiate the TRC reaction, and an INAF probe (5'-CGA AGT GCA GGG C*AG ATC, where the asterisk indicates the base position linked by oxazole yellow) to detect the RNA amplicons. For the specific detection of MTC, primers were designed to amplify base positions 313 to 443 of *M. tuberculosis* 16S rRNA (GenBank accession no. Z83862). As shown in Fig. 2, oxazole yellow-linked and ethidium bromide-linked INAF probes were synthesized for the homogeneous and simultaneous detection of the target 16S rRNA and the internal amplifica-

tion control in the same tube by means of two-color fluorescence monitoring of the reaction mixture at 520 nm and 610 nm, respectively. For the experiments to be able to evaluate the species specificity of the assay, the 16S rRNAs of 16 mycobacterial species other than MTC were prepared in the same manner described above for MTC 16S rRNA. These species comprised *M. avium*, *M. intracellulare*, *M. kansasii*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *M. szulgai*, *M. gastri*, *M. xenopi*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, and *M. peregrinum*.

TRC method for detection of *M. tuberculosis* complex. The principles of amplification and fluorescence-based detection have been described elsewhere (9). Briefly, 20 μl of the TRC buffer, consisting of a mixture of the substrate solution and the primer solution at a 1:1 (vol/vol) ratio, was added to 5 μl of the nucleic acid extract in a thin-walled PCR tube (Applied Biosystems, Foster City, CA). The basic composition of the reaction mixture was the same as that described previously (9), with the exception of a slight modification consisting of the omission of RNase H. The tube was then set in the dedicated instrument, a TRCR-160 real-time monitor (Tosoh Co., Tokyo, Japan), for 5 min while being preheated to 43°C, followed by the addition of 5 μl of the enzyme solution. The TRCR-160 real-time monitor consists of a square incubator block maintained at 43°C and a sliding fluorescence scanning unit. The latter comprises a light-emitting diode light source to irradiate the excitation light (470 nm) from below into the tube from the bottom and a light guide to collect the fluorescence from the bottom of the reaction tube in two photomultiplier tubes (520 nm and 610 nm) at 1-min intervals. The reaction time required for fluorescence enhancement to reach a cutoff value of 1.2 was adopted as the detection time for the TRC assay. The samples for which the detection time was ≤30 min were considered to have a positive signal, and those with positive signals at 520 nm were judged to be positive. Samples without a positive signal at 520 nm and with a positive signal at 610 nm were considered negative, and those with negative signals at both 520 nm and 610 nm were considered indeterminate.

Sample processing for detection of MTC in experimental sputum samples by TRC method. *Mycobacterium* culture-negative, clinical sputum specimens were obtained from patients diagnosed as not having mycobacterial infection at Kyoto University Hospital, Kyoto, Japan. The samples were mixed and homogenized by vigorous vortexing and were decontaminated by treatment with N-acetyl-L-cysteine (NALC)–NaOH. Various amounts of *M. bovis* BCG (10^0 to 10^4 cells per 100 μl), *M. avium* (10^2 to 10^6 cells per 100 μl), and *M. kansasii* (10^2 to 10^6 cells per 100 μl) were added to these culture-negative sputum specimens. Pretreatment consisted of mixing of 100 μl of the decontaminated samples with 500 μl of 67 mM phosphate buffer (pH 8.6) containing glass beads. After centrifugation at 3,000 × *g* for 5 min at room temperature, the supernatant was removed, followed by the addition of 300 μl of extraction buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25 U/μl RNase inhibitor, and 1 mM dithiothreitol. After sonication for 5 min, followed by centrifugation at 3,000 × *g* for 5 min, 5 μl of the supernatant was used as the template.

Sample processing for detection of MTC in clinical sputum samples by culture, TRC method, and COBAS AMPLICOR PCR. Clinical samples were obtained from patients diagnosed with or suspected of having a mycobacterial infection at the National Central Hospital for Chest Disease, Osaka, Japan, and the National Tokyo Hospital, Tokyo, Japan. All samples were decontaminated by treating them by a commercially available NALC-NaOH-based method, CC-E Nichibi (Japan BCG Laboratory, Tokyo, Japan) or BBL Micoprep (Becton Dickinson, Franklin Lakes, NJ). After centrifugation at 3,000 × *g* for 15 min at room temperature, the sediment was resuspended in 1.0 ml of phosphate buffer (pH 7.0). A smear of the decontaminated suspension was stained with the Ziehl-Neelsen stain, and a 500-μl aliquot of the suspension was cultivated in the BACTEC MGIT 960 liquid culture system (Becton Dickinson) for 6 weeks, in accordance with the manufacturer's recommendations. The remaining decontaminated suspension of the samples was immediately used or was stored at −20°C until use. The TRC method was applied to 100 μl of the suspension; the Roche COBAS AMPLICOR PCR was applied to 200 μl of the suspension, which had been treated with the Amplicor Mycobacterium Specimen Pretreatment Set II (Roche Diagnostics). The isolated mycobacteria were identified by an immunochromatographic assay with the anti-MPB64 antibody (Capillia; Becton Dickinson), a chemiluminescent DNA hybridization probe assay (AccuProbe; Gen-Probe Inc.), or a DNA hybridization assay (DDH Mycobacteria; Kyokuto Pharmaceutical Co., Tokyo, Japan).

RESULTS

Sensitivity and specificity of TRC assay with experimental samples. In the experiment with the standard RNA calibrator for the TRC assay (Fig. 3), the time to detection depended on the initial copy number, which ranged from 10^2 to 10^5. For the internal control, the time to detection at 610 nm remained approximately constant at about 10 min, irrespective of the initial copy number. The results for MTC detection in experimental sputum samples containing BCG by the TRC method are shown in Table 1. The detection limit for BCG was one organism per 100 μl of sputum, and the time to detection became shorter for samples with a lower number of BCG organisms. All of the sputum samples containing $\geq 10^2$ *M. avium* or *M. kansasii* organisms per 100 μl were judged to be negative by the TRC method (Table 1). The species specificity of the TRC method was examined by testing the 16S rRNA solution with 16 mycobacterial species other than MTC as the templates. The TRC method did not show positivity for any of the 16S rRNAs

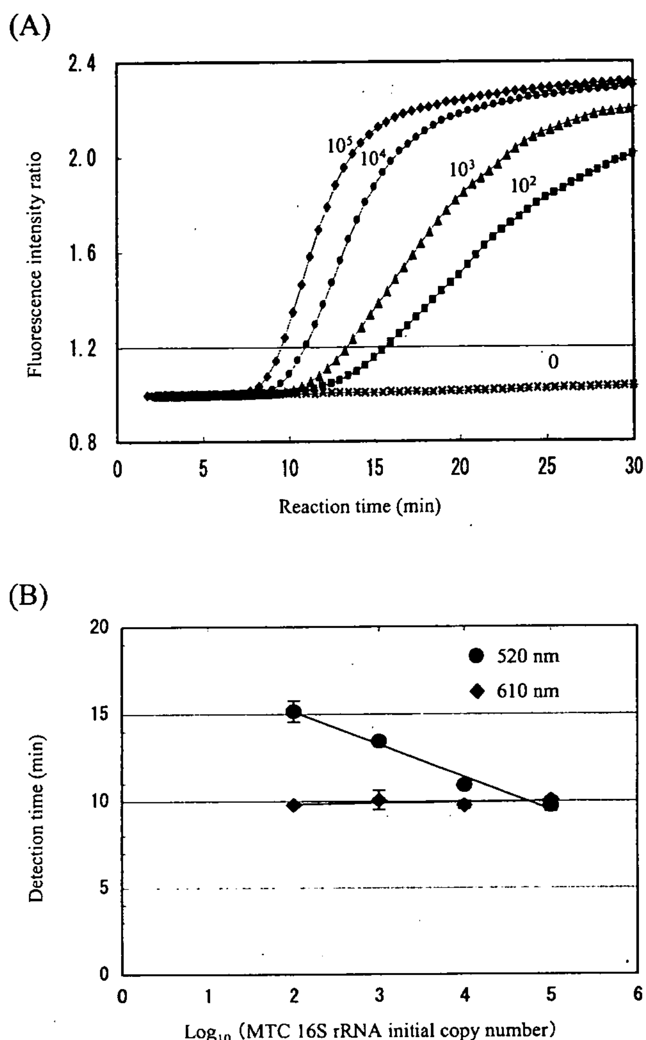

FIG. 3. (A) Fluorescence monitoring of the TRC reaction of the MTC 16S rRNA calibrator. The initial copy numbers of the calibrator are indicated as follows: diamonds, 10^5; circles, 10^4; triangles, 10^3; squares, 10^2; crosses, 0. (B) Initial copy numbers of the calibrator on a logarithmic scale plotted against the time needed to reach the cutoff value of 1.2. Circles indicate the detection time at 520 nm, and diamonds indicate that at 610 nm. The average of the values obtained with triplicates of each sample is plotted.

TABLE 1. Detection of *Mycobacterium tuberculosis* complex-specific 16S rRNA sequence by TRC method in sputum containing BCG (10^0 to 10^4 cells/100 μl), *Mycobacterium avium*, and *M. kansasii* (10^2 to 10^6 cells/100 μl)

No. of organisms/ 100 μl of sputum	Detection time (min)		
	BCG	*M. avium*	*M. kansasii*
10^6	NE[a]	ND[b]	ND
10^5	NE	ND	ND
10^4	10.0	ND	ND
10^3	10.4	ND	ND
10^2	12.5	ND	ND
10^1	15.6	NE	NE
10^0	21.7	NE	NE

[a] NE, not evaluated.
[b] ND, not detected.

(10^6 copies, equivalent to an rRNA content of 10^3 cells, per 100 μl) of the species examined (data not shown).

Evaluation of performance of TRC assay for detection of MTC in clinical sputum samples. Table 2 presents the results of the clinical evaluation of the TRC method and the COBAS AMPLICOR PCR with 201 sputum samples from 173 patients. The TRC method detected as positive 39 of 43 smear-positive, culture-positive samples (sensitivity, 90.7%) and 13 of 29 smear-negative, culture-positive samples (sensitivity, 44.8%), for a total of 52 of 72 culture-positive samples (sensitivity, 72.2%). The COBAS AMPLICOR PCR detected as positive 41 of 43 smear-positive, culture-positive samples (sensitivity, 95.3%) and 17 of 29 smear-negative, culture-positive samples (sensitivity, 58.6%), for a total of 58 of 72 culture-positive samples (sensitivity, 80.6%). The 129 culture-negative samples (Table 2) contained 40 samples culture positive for nontuberculous mycobacteria, such as 13 *M. avium*, 15 *M. intracellulare*, 6 *M kansasii*, 3 *M. abscessus*, and 2 *M. gordonae* isolates and 1 *M. fortuitum* isolate, as well as 89 mycobacteria culture-negative samples. The TRC method did not produce positive results for any of the 129 culture-negative samples (specificity, 100%), none of which were kept suspended, as in the case of in the TRC method, because the fluorescence intensity ratios at 610 nm reached ≥1.2.

Comparison of TRC assay and COBAS AMPLICOR PCR for detection of MTC in clinical sputum samples. As shown in Table 2, the COBAS AMPLICOR PCR detected as positive 41 of 43 smear-positive, culture-positive samples (sensitivity, 95.3%) and 17 of 29 smear-negative, culture-positive samples (sensitivity, 58.6%), for a total of 58 of 72 culture-positive samples (sensitivity, 80.6%). The COBAS AMPLICOR PCR produced no positive results for any of the 129 culture-negative samples (specificity, 100%). There were thus no significant differences in sensitivity and specificity between TRC and PCR, although the sample size was comparatively small for the detection of small differences in sensitivity between the two methods. The correlation of the results of the TRC method and the COBAS AMPLICOR PCR are shown in Table 3, with the overall agreement between TRC and PCR being 95.0% (191 of 201 samples).

TABLE 2. Detection of *Mycobacterium tuberculosis* complex in 201 clinical sputum samples from 173 patients with diagnosed or suspected mycobacterial infection by TRC method, the COBAS AMPLICOR PCR, MGIT[a] culture for MTC, and smear test

Smear result (*n*)	Culture result (*n*)	No. of samples with the indicated result by:			
		TRC method		COBAS AMPLICOR-PCR	
		Positive	Negative	Positive	Negative
Positive (43)	Positive (43)	39	4	41	2
Negative (158)	Positive (29)	13	16	17	12
	Negative (129)	0	129	0	129

[a] MGIT, mycobacterial growth indicator tube.

TABLE 3. Agreement between of results of TRC method and COBAS AMPLICOR PCR for detection of *Mycobacterium tuberculosis* complex in 201 respiratory samples from 173 patients

COBAS AMPLICOR PCR result	No. of samples with the following result by TRC method:	
	Positive	Negative
Positive	50	8
Negative	2	141

DISCUSSION

In this study, we were able to demonstrate that the performance of the TRC method for the detection of MTC from respiratory samples was comparable to that of the COBAS AMPLICOR PCR in terms of sensitivity and specificity. The TRC method detected as little as one BCG organism per 100 μl of sputum. The selection of rRNA as the amplification target, of which a single cell contains multiple copies, may be the primary reason for this favorable result.

In the case of MTC smear-negative and culture-positive samples, the TRC method showed substantially lower rates of positivity than it did for smear- and culture-positive samples, as did the COBAS AMPLICOR PCR. This less favorable result was, however, comparable to the results of previous studies that evaluated nucleic acid amplification assays in similar settings (18). As for the specificity, the TRC method did not show positivity for 10^6 *M. avium* or 10^6 *M. kansasii* organisms per 100 μl of sputum, nor did it show positivity for 10^6 initial copies of 16S rRNA of 16 non-MTC mycobacterial species. The 129 MTC culture-negative samples comprised 40 samples which were culture positive for nontuberculous mycobacterial isolates as well as 89 mycobacterial culture-negative samples. These results clearly demonstrate the excellent specificity of the TRC method for the detection of MTC.

As to sensitivity, the difference between the two methods was not significant, although the comparatively small sample size provided this study with insufficient power to detect small differences in sensitivity. The slightly smaller number of MTC culture-positive samples detected by the TRC method than by the COBAS AMPLICOR PCR may have been due to a reduction in the numbers of viable cells during treatment, since this study included samples from patients undergoing antituberculosis chemotherapy. The levels of mycobacterial rRNA may therefore have decreased faster than the levels of DNA in proportion to the decrease in viable cells. This finding may, rather, indicate some advantage of the TRC method over DNA-based amplification methods in terms of greater correct-

ness in the diagnosis of active disease. Actually, Moore et al. has reported that elimination of MTC rRNA from sputum samples may indicate successful antituberculosis therapy (12).

We found unaccountable false-negative results, four by the TRC method and two by the COBAS AMPLICOR PCR, among the 43 smear- and culture-positive samples. The reason could not be that no viable cells remained in the samples, since MTC was indeed identified by smear as well as culture of these samples. The promptly appearing positive signal of the internal control with the use of either method excluded the possibility that inhibitors were present in the samples. The slightly greater number of false-negative results obtained by the TRC method could be explained by a reduction in the number of viable cells during treatment, although the exact reason is difficult to identify. The fact that these false-negative results were found with the use of both methods together, however, makes it less likely that there are any critical problems specific to the TRC method in terms of false-negative results.

The samples examined in this study showed a prevalence of MTC-positive samples considerably higher than the prevalence in most clinical laboratories in developed nations. This is not surprising, since Japan is characterized by a substantially higher prevalence of tuberculosis compared with that in other developed countries: 24.8 new cases per million population in 2003 (11). In addition, public health policies in Japan have been promoting the concentration of tuberculosis patients in core referral centers specializing in tuberculosis, which include the Kinki-Chuo Chest Medical Center and National Tokyo Hospital.

Of special importance are the several advantages that the TRC method has over the other nucleic acid amplification tests. First, an internal control is amplified to avoid false-negative results; second, the entire procedure is conducted in a completely homogeneous and isothermal format, thus eliminating contamination by postamplification analysis; and third, the results from the time that the samples are decontaminated are available within an hour because amplification and detection require only 30 min. The TRC method also proved to be clinically useful for the rapid identification of MTC in respiratory samples, at least for those smear-positive ones, as is the case for COBAS AMPLICOR PCR.

Pulmonary tuberculosis and nontuberculous mycobacterial infection are sometimes difficult to differentiate based on clinical findings. The nucleic acid amplification test, which could detect MTC only, may add little information in such cases if the result was negative. Development of the TRC method for the detection of *M. avium* complex and *M. kansasii*, which is now in progress in the laboratory at Tosoh Corporation, could further enhance the clinical usefulness of this method for the management of patients with suspected mycobacterial infection especially when the smear result is positive.

In summary, the sensitivity of the TRC method was found to be comparable to that of the COBAS AMPLICOR PCR, and its specificity was excellent. Moreover, less than an hour was required to obtain results after the specimens had been treated with NALC-NaOH. This novel method can thus be expected to be suitable for routine use for the rapid diagnosis of tuberculosis.

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Bedside Teaching

肺結核を見落とさないために

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肺結核を見落とさないために*

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はじめに

結核罹患率の低下に伴い，医療従事者の結核に対する関心は院内感染対策に絞られてきた．この点で臨床医に求められる最も重要な事項が，診断の遅れをなくすことであることは言うまでもない．一般病棟に肺炎として，喘息として，または高齢者の衰弱として入院していた患者が，後に喀痰塗抹(ガフキー)陽性結核と判明した場合，同室者や医療従事者の接触者検診に膨大なエネルギーを費やすことになる．

本稿では，一般臨床医が肺結核の診断の遅れをなくすための注意点を，当院に紹介されてくる症例をとおして概説する．注意点の理解に必要な結核の基礎知識と肺結核診断の基本プロセスについても述べる．

結核の基礎知識

結核は結核菌による慢性の感染症であり，全身全ての臓器に病変が生じうる．しかし，空気感染(後述)という伝染様式から，肺結核が全体の85%を占める．肺以外の結核も肺からリンパ行性または血行性に広がったものである．胸膜，リンパ節，骨・関節，腎，全身播種型(粟粒結核)が比較的頻度が高い肺外結核である．

戦後順調に低下した結核罹患率であるが，1980年代より低下率の鈍化傾向が顕著となり，97年から99年まで微増に転じたことは記憶に新しい．幸い各方面の努力により2000年以降は再度低下している．現在，結核は高齢者の病気であり，70歳以上が40%以上を占めている．罹患率の地域格差が大きいのも近年の特徴である．2003年罹患率ワースト3は大阪，東京，兵庫である．現在結核は東京地区以西の大都市の病気といっても過言ではない[1]．

結核の感染様式は空気(飛沫核)感染である．喀痰塗抹陽性の呼吸器系の結核患者が咳やくしゃみをした時に2～3個の結核菌を中心に含む飛沫が放出され，その一部が裸の飛沫核となり，空気中を長時間浮遊する(図1)．この飛沫核を肺胞内に直接吸い込むことが感染の必要条件である．体内には各種非特異的防御機序が存在し，結核菌の分裂速度が遅い(15～24時間)こともあり，大部分の菌は増殖する前に体外に排出されるか殺される．頻回・多量に吸い込んだ場合にのみ，結核菌が体内で増殖を開始し感染が成立する．この後6～8週間かけて結核菌に対する特異細胞性免疫が成立し，菌は肉芽腫中に封じ込められて増殖を停止する[2]．感染の成立は結核菌に対する特異細胞性免疫の存在を通して間接的に判断される．従来ツベルクリン反応が唯一の方法であったが，最近QuantiFERON TB-2G(QFT2G)が開発された(後述)．

感染者の9割は菌の封じ込めが継続し生涯発病せずに過ごす．しかし，約6～7%の感染者は2年以内に封じ込めが破綻し，結核菌が再増殖を開始し発病に至る[2]．この場合には免疫低下を示唆する基礎疾患が特にない例も多い．感染2年以内

* Prevention of Delay in Diagnosis of Pulmonary Tuberculosis

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