developed an EIA kit detecting serum IgA antibody specific for GPL core and investigated its usefulness in a multicenter study.

METHODS

See the online supplement for additional methodologic details.

Patients and Serum Samples

Six institutions participated in this study. Between June 2003 and December 2005, serum samples were collected from 70 patients with MAC-PD, 18 with MAC contamination, 36 with pulmonary TB, 45 with other lung diseases, and 76 healthy subjects. All patients with MAC-PD met the ATS guidelines (1). Of the 70 patients with MAC-PD, 64 had previously received combination chemotherapy for mycobacterial diseases recommended by the ATS guidelines, but had MAC-positive cultures at the time of serum collection. Pulmonary TB was confirmed by culture positivity for *M. tuberculosis*. Patients with pulmonary TB who had an underlying pulmonary disease or past history of treatment for pulmonary TB were excluded. Individuals with MAC contamination showed a single culture positive for MAC in small amounts, but were asymptomatic and had no significant chest computed tomography (CCT) findings indicating active mycobacterial disease. The other lung diseases included chronic obstructive pulmonary disease (n = 15), idiopathic interstitial pneumonia (n = 11), lung cancer (n = 11), bacterial pneumonia (n = 4), pulmonary sarcoidosis (n = 2), and bronchiectasis (n = 2). All sera were stored at −20°C until assayed for IgA GPL core antibody. None of the patients was seropositive for HIV type 1 or 2. The patients with MAC-PD were classified into two groups on the basis of the chest radiography: fibrocavitary disease and nodular-bronchiectatic (NBE) disease (1).

Fibrocavitary disease was defined as the presence of cavitary forms in upper lobes. NBE disease was defined as the presence of bronchiectasis and multiple nodular shadows on CCT. Disease conforming to neither of these types was considered unclassifiable. Forty-five patients underwent CCT and serodiagnosis at the same time. A correlation between the extent of disease and antibody levels was investigated. The extent of disease was expressed as the number of MAC-involved CCT segments, as described in the previous study (9).

The studies in human subjects were approved by the research and ethical committees of the NHO National Toneyama Hospital, and written, informed consent was obtained from all subjects.

EIA Kit

The EIA kit was developed by Tauns Laboratories, Inc. (Shizuoka, Japan), with a slight modification of the method described previously (8). Results are given as arbitrary U/ml in relation to a standard curve that was constructed by mixing sera from three patients with MAC-PD as a reference. The intra- and interplate coefficients of variation were 2.27–9.29% and 0.57–8.86%, respectively, which indicated good reproducibility. The linearity of measurement was confirmed. The influence of blood elements and temperature was examined, and revealed good stability. The assay was performed by a technologist with no prior knowledge of the clinical data.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA). Antibody levels in patient groups are expressed as means ± SD. For comparison of the mean values of multiple groups, data were compared by analysis of variance and nonparametric analysis. A probability value of less than 0.05 was regarded as significant.

RESULTS

Study Subjects

The characteristics of the subjects are shown in Table 1. Patients with pulmonary TB and healthy subjects were younger than patients with MAC-PD ($P < 0.001$), and there was a larger proportion of females in the latter group ($P < 0.001$). Of the 70 patients with MAC-PD, 15 had underlying pulmonary disease, all of which were the sequelae of pulmonary TB. Of the 18 individuals with MAC contamination, 15 had underlying pulmonary diseases (8 patients with the sequelae of pulmonary TB, 2 with lung cancer, 2 with chronic obstructive pulmonary disease, 1 with emphysema, 1 with pneumoconiosis, and 1 with sarcoidosis. Of the patients with MAC-PD, 19 were classified as having fibrocavitary disease, and 35 as having NBE disease, with 16 patients unclassifiable. The MAC-PD group included infections with *M. avium* (n = 56), *Mycobacterium intracellulare* (n = 12), or both (n = 2). The MAC contamination group included *M. avium* (n = 16) and *M. intracellulare* (n = 2).

Level of GPL Core IgA Antibody

The level of serum IgA antibody to GPL core was quantified using the EIA kit (Figure 1). As expected, patients with MAC-PD had significantly higher levels than patients with MAC contamination, those with pulmonary TB, those with other lung diseases, and healthy subjects—namely, 10.7 ± 7.9, 0.2 ± 0.1, 0.1 ± 0.1, 0.0 ± 0.1, and 0.0 ± 0.0 U/ml, respectively ($P < 0.0001$). A receiver operating characteristic (ROC) curve was constructed for MAC-PD and the other groups to establish the best cutoff value (Figure 2). Setting the cutoff value at 0.7 U/ml resulted in 100% specificity, at a sensitivity of 84.3% (Table E1). Using the EIA kit allowed clear discrimination between patients with MAC-PD and MAC contamination, pulmonary TB, and other lung diseases, as well as healthy subjects.

Next, we compared levels of serum IgA antibody to GPL core in fibrocavitary disease and NBE disease of MAC-PD. Significantly higher levels were found in NBE ($P < 0.05$) (Figure 3). With the cutoff value set at 0.7 U/ml, positivity in NBE and fibrocavitary disease was 91.4 and 63.2%, respectively. In contrast, in patients with MAC-PD, no significant differences between *M. avium* and *M. intracellulare* as causative agents were observed ($P = 0.403$). The erythrocyte sedimentation rate in MAC-PD was 32.6 ± 28.6 mm/hour and there was a significant positive correlation between the erythrocyte sedimentation rate and antibody levels in patients with MAC-PD ($r = 0.294$, $P < 0.05$).

Radiographic Severity and the Level of GPL Core Antibody

Forty-five patients with MAC-PD (10 with fibrocavitary disease, 26 with NBE disease, and 9 with unclassifiable type disease) underwent CCT and serodiagnosis at the same time. Four patients with unclassifiable type disease were excluded from the investigation because it was hard to discriminate between MAC lesions and underlying pulmonary disease. There was a positive correlation between the extent of disease and the

TABLE 1. CHARACTERISTICS OF STUDY SUBJECTS

	MAC-PD	MAC Contamination	Pulmonary TB	Other Lung Disease	Healthy Subjects
Number	70	18	36	45	76
Age, mean yr ± SD	68.0 ± 9.6	64.6 ± 11.6	52.9 ± 16.6*	66.3 ± 10.9	38.1 ± 12.0*
Age range, yr	50–90	28–78	24–76	29–82	20–65
Sex, no. male/no. female	25/45	10/8	26/10*	34/11*	41/35*
Duration of disease, mean yr ± SD	4.8 ± 4.6		0.3 ± 0.2	2.2 ± 2.4	

* $P < 0.001$.

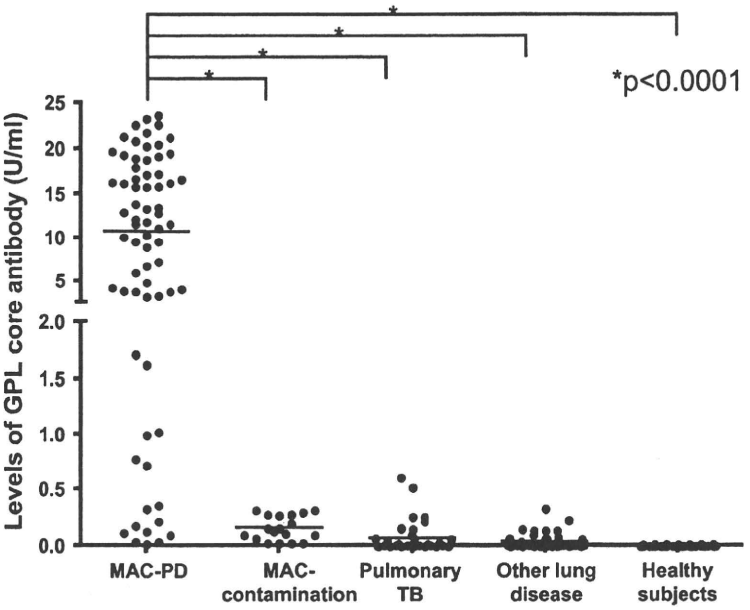

Figure 1. The level of serum IgA antibody to glycopeptidolipid (GPL) core antigen. Serum samples from six different institutions included 70 patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD), 18 with MAC contamination, 37 with pulmonary tuberculosis (TB), 45 with other lung diseases, and 76 healthy subjects. Antibody levels in MAC-PD were significantly higher than in the other groups ($P < 0.0001$). All results are expressed as individual data, and *horizontal bars* indicate geometric means.

levels of the antibody ($r = 0.43$, $P < 0.05$) (Figure 4). The total numbers of involved segments were not different (7.8 ± 4.9 and 7.9 ± 4.2 in fibrocavitary and NBE disease, respectively). Of 26 patients with NBE disease, 9 had small thin wall cavities. A tendency toward elevated GPL core antibody levels was found in NBE patients with cavities compared with those without, but this trend was not statistically significant ($P = 0.08$).

DISCUSSION

We previously established a serologic test for MAC-PD using a mixture of GPLs and GPL core antigen, and reported the clinical application of the EIA method for quantifying antibody levels (7, 8). GPL is an antigen located on the surface of the MAC cell wall and determines the serotype. At present, 31 distinct serotype-specific GPLs have been identified, of which the complete structures of 14 have been identified (10–12). GPL consists of a core common to all MAC serotypes and a serotype-specific oligosaccharide. In the initial study to establish the serodiagnosis of MAC-PD, we used the whole GPL antigen, a mixture of 11 serotype-specific GPLs (7). We then found that the GPL core was the dominant antigenic epitope of GPL, and subsequently developed a serologic test using GPL core antigen (8). In the previous study, GPL core antibody (IgG, IgA, and IgM) levels were found to be elevated in sera of patients with MAC-PD, but not pulmonary TB, *M. kansasii*–PD, MAC colonization/contamination, and healthy subjects. The study showed that this serologic test was useful for diagnosing MAC-PD and for differentiating it from pulmonary TB and *M. kansasii*–PD. Consistent with this, Fujita and colleagues (13) reported elevated levels of antibody against the GPL core antigen in patients with MAC-PD but not in those with pulmonary TB. In our previous study (8), of the different Ig classes, best results were obtained by IgA, including an association with CCT findings. Thus, a higher level of serum IgA antibody to GPL core indicated a wider extent of MAC disease and larger nodule formation on CCT (9). Therefore, we have attempted to develop and to assess an EIA kit for quantifying serum IgA antibody to GPL core in the present study. Optical density levels were converted to U/ml using standard serum samples, which provided reliable and reproducible results. In this multicenter study, using the EIA kit, it was confirmed that patients with MAC-PD could be clearly differentiated from those with pulmonary TB, those with MAC contamination, those with other lung diseases, and healthy subjects. Similar to our previous studies (7–9), the sensitivity and specificity for diagnosing MAC-PD by the kit was high and the level of the antibody correlated with the extent of MAC-PD assessed using CCT.

Distinguishing pulmonary TB from MAC-PD in clinical practice using the EIA kit has proven useful. Differentiating TB from MAC is difficult because symptoms and radiographic findings are often similar among patients with pulmonary mycobacterial diseases. Patients with pulmonary TB require immediate treatment and isolation, whereas the diagnosis of MAC-PD does not necessitate rapidly starting antimicrobial therapy (1), and isolation is not required. GPL antigens, which are major cell surface antigens of MAC, are not present in the cell wall of *M. tuberculosis* complex (11). On the basis of this observation, patients with TB do not produce anti-GPL antibody. Indeed most patients with TB did not possess serum antibodies against GPLs (Figure 1) (7, 8). However, we cannot exclude the possibility that disease in patients with TB was of too short duration (MAC-PD, 4.8 ± 4.6 yr, vs. TB, 0.3 ± 0.2 yr) to have allowed immune responses and shed mycobacterial antigen. In this present study, with a cutoff level of 0.7 U/ml, all patients with TB were classified as seronegative. The levels of GPL core antibody in patients with pulmonary TB were very low or absent

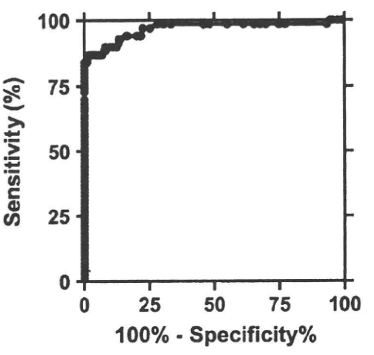

Figure 2. Receiver operating characteristic curve constructed for patients with *Mycobacterium avium*–complex pulmonary disease and the other groups.

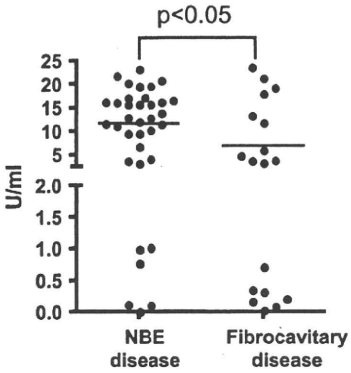

Figure 3. Levels of IgA antibody to glycopeptidolipid core antigens in nodular-bronchiectatic (NBE) and fibrocavitary subtypes of patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD). Significantly higher levels were found in patients with MAC-PD with NBE compared with fibrocavitary disease ($P < 0.05$).

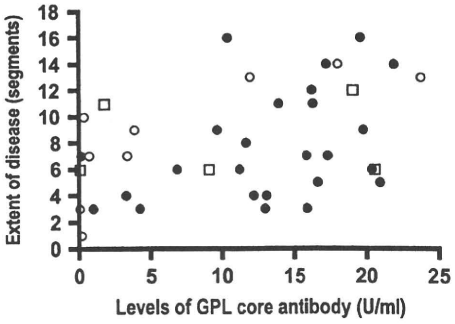

Figure 4. Correlation between antibody levels and radiographic severity using chest computed tomography in 41 patients with *Mycobacterium avium*–complex pulmonary disease. There was a positive correlation between the extent of disease and the levels of antibody ($r = 0.43$, $P < 0.05$). *Closed circles* represent patients with nodular-bronchiectatic disease, *open circles* represent patients with fibrocavitary disease, and *open squares* represent patients with unclassifiable type disease.

(0.1 ± 0.1 U/ml). In contrast, in previous studies (7, 8, 13), GPL seropositivity in patients with pulmonary TB ranged between 5.2 and 25%. One possible explanation for this previously reported lack of specificity may be that there was latent coinfection of MAC in patients with pulmonary TB. In the present study, however, we attempted to exclude patients with such latent coinfection because the entry criteria precluded patients having underlying lung disease or past history of pulmonary TB. Patients with lung diseases such as chronic obstructive pulmonary disease associated with smoking, bronchiectasis, previous mycobacterial disease, cystic fibrosis, and pneumoconiosis are prone to have MAC coinfection (1). In addition, future studies are needed to verify the cutoff value obtained from the ROC analysis using another sample of cases and controls on a much larger scale.

MAC-PD has recently been classified into two distinct subtypes: fibrocavitary disease and NBE disease (1). Fibrocavitary disease, the most common manifestation of MAC-PD, is usually seen in middle-aged or elderly men predisposed to lung disease due to smoking and alcohol drinking. This subtype of disease, generally progressive, is similar to pulmonary TB on chest radiography. If left untreated, it can lead to extensive lung destruction and death. In contrast, NBE disease is mostly seen in nonsmoking middle-aged or elderly women without predisposing lung disease. The clinical course is usually slower and less dramatic. Patients with NBE are presumed to have had a long subclinical period before appearance of disease manifestations. Significantly higher levels of GPL core antibody were seen in NBE than in fibrocavitary disease ($P < 0.05$) and higher seropositivity was found in patients with the former (91.4% compared with 63.2%). There were no significant differences of extent of disease between the two groups in patients who underwent CCT and serodiagnosis at the same time. Therefore, the results suggested the possibility that the antibody levels tend not to elevate in patients with fibrocavitary disease. This may reduce the utility of serodiagnosis for discriminating cavitary MAC from cavitary TB. However, the antibody would probably be present at high levels in patients with extensive lesions in fibrocavitary disease as was indeed found in three patients (17.9 ± 5.9 U/ml) who had extensive lesions (more than 13 segments) (Figure 4). Further investigations are required for confirmation of this notion in a larger study.

Of the 70 patients with MAC-PD, 64 had previously received combination chemotherapy, as recommended by the ATS guidelines (1). However, all had MAC-positive cultures at the time of serum collection, and were considered to have active MAC-PD. Thus, antibody levels were not changed by the failure of chemotherapy—that is, there was no conversion to seronegative from seropositive status (8); therefore, effects of the previous treatment on antibody levels were limited. Obviously, it would nonetheless be better to enroll chemotherapy-naive patients from diverse ethnic and racial populations and different geographic areas in future studies.

At present, the diagnosis of MAC-PD is usually made according to the ATS guidelines, which include clinical, radiographic, and microbiological criteria (1). The latter requires multiple positive cultures for MAC from sputum, a positive culture from bronchial lavage or a lung biopsy specimen, together with the other diagnostic features. Although it is easy to meet the criteria in advanced-stage MAC-PD, it is often difficult in early-stage disease. In clinical routine, it is impractical to obtain multiple sputum samples or perform bronchoscopy to obtain bronchial washings or lung tissue in all patients. It is also time consuming, because a long duration is required before the results of multiple cultures are available. There are several rapid methods for identification of MAC, but they have some limitations. The liquid culture–based system using radiometry and fluorometry allows the detection of mycobacterial growth at an early stage, fewer than 7 days for nontuberculous mycobacteria. However, limitations of this system include the inability to observe colony morphology, difficulty in recognizing mixed cultures, overgrowth by contaminations, cost, and radioisotope disposal. Rapid identification of MAC is also possible using DNA hybridization, nucleic acid amplification, or high-pressure liquid chromatography (1). The use of molecular biological technology has shortened the time required to identify mycobacteria from several weeks to as little as 1 day. The overall sensitivity for detecting MAC varies between 70 and 100%, with a specificity greater than 98%. However, the inability to distinguish live and dead organisms precludes nucleic acid amplification for definite diagnosis of active disease (14).

The EIA kit is a rapid (within a few hours) and noninvasive assay with high sensitivity (84.3%) and specificity (100%) for diagnosing MAC-PD. Using the EIA kit, as reported here, MAC-PD could be efficiently differentiated from MAC contamination. "MAC contamination" defined in the present study was considered to represent contamination from the environment, because patients were asymptomatic and revealed no significant CCT findings indicating active mycobacterial disease. Most of those people classified into the MAC contamination group were so categorized based on a single positive MAC culture by chance during the follow-up period after completion of chemotherapy for pulmonary TB or at routine examination on admission for other diseases. It is difficult to be certain that MAC contamina-

tion, as defined here, does not indicate subclinical infection because no confirmatory pathology was obtained. However, if MAC contamination does reflect subclinical infection, it is of little clinical importance and does not mandate therapy.

There were 15.7% false-negative EIA determinations in patients with MAC-PD. In such cases, diagnosis of MAC-PD should be made according to the ATS guidelines, as previously described. There are several possible explanations for these false-negative results, including the following: (*1*) recently diagnosed disease; (*2*) change of GPL core antigenicity after chemotherapy; or (*3*) diversity of immune responses to GPL core in individual patients, potentially governed by HLA genes (15). Therefore, it might be expected that not all patients with MAC-PD are capable of producing antibody to GPL core. Although the specificity determined here for the EIA kit was high, there remains also the possibility of false-positive results in patients with disease due to other mycobacteria, such as *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium abscessus*, and *Mycobacterium scrofulaceum*, because these organisms also possess GPL on their cell wall surface (10, 11, 16). Indeed, we have detected seropositivity in several patients with culture-positive *M. fortuitum* (data not shown). The incidence of pulmonary disease due to these other mycobacteria is relatively low (<5%) in Japan and the United States (6, 17), but a report from South Korea documented a high incidence of pulmonary infection by *M. abscessus* or *M. fortuitum* (33 and 11%, respectively (18). Therefore, caution is necessary when interpreting the results of the EIA kit in locations where other mycobacterial infections are endemic.

A recent study using high-resolution CT documented that characteristic findings with multiple small nodular shadows combined with bronchiectasis are predictive for culture-positive MAC with a relatively high probability. Swenson and colleagues (19) reported that, of 15 patients with these characteristic findings, 8 (53%) had cultures positive for MAC. Tanaka and coworkers (20) reported that, of 26 similar patients, 13 (50%) had positive cultures for MAC in bronchial washings. Therefore, combining positive results obtained by the EIA and the characteristic findings of high-resolution CT should yield a definitive diagnosis of MAC-PD even in patients with sputum culture–negative results for MAC. This approach may be useful especially in elderly patients with complications, in whom bronchoscopy cannot be performed.

In summary, the EIA kit for detection of serum IgA antibody specific for GPL core antigen is useful for rapid and accurate serodiagnosis of MAC-PD. Taken together with clinical, radiographic, and microbiological criteria, the kit may be a valuable tool for the diagnosis of MAC-PD. Validation of the EIA kit in the diagnosis of MAC-PD requires a larger controlled study in diverse populations.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, *et al.*; ATS Mycobacterial Diseases Subcommittee. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* 2007;175:367–416.
2. Subcommittee of the Joint Tuberculosis Committee of the British Thoracic Society. Management of opportunist mycobacterial infections: Joint Tuberculosis Committee guidelines 1999. *Thorax* 2000;55:210–218.
3. Field SK, Fisher D, Cowie RL. *Mycobacterium avium* complex pulmonary disease in patients without HIV infection. *Chest* 2004;126:566–581.
4. Field SK, Cowie RL. Lung disease due to the more common nontuberculous mycobacteria. *Chest* 2006;129:1653–1672.
5. Khan K, Wang J, Marras TK. Nontuberculous mycobacterial sensitization in the United States: national trends over three decades. *Am J Respir Crit Care Med* 2007;176:306–313.
6. Sakatani M. Nontuberculous mycobacteriosis: the present status of epidemiology and clinical studies. *Kekkaku* 1999;74:377–384.
7. Kitada S, Maekura R, Toyoshima N, Fujiwara N, Yano I, Ogura T, Ito M, Kobayashi K. Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. *Clin Infect Dis* 2002;35:1328–1335.
8. Kitada S, Maekura R, Toyoshima N, Naka T, Fujiwara N, Kobayashi M, Yano I, Ito M, Kobayashi K. Use of glycopeptidolipid core antigen for serodiagnosis of *Mycobacterium avium* complex pulmonary disease in immunocompetent patients. *Clin Diagn Lab Immunol* 2005;12:44–51.
9. Kitada S, Nishiuchi Y, Hiraga T, Naka N, Hashimoto H, Yoshimura K, Miki K, Miki M, Motone M, Fujikawa T, *et al.* Serological test and chest computed tomography findings in patients with *Mycobacterium avium* complex lung disease. *Eur Respir J* 2007;29:1217–1223.
10. Aspinall GO, Chatterjee D, Brennan PJ. The variable surface glycolipids of mycobacteria: structures, synthesis of epitopes, and biological properties. *Adv Carbohydr Chem Biochem* 1995;51:169–242.
11. Brennan PJ, Nikaido H. The envelope of mycobacteria. *Annu Rev Biochem* 1995;64:29–63.
12. Fujiwara N, Nakata N, Maeda S, Naka T, Doe M, Yano I, Kobayashi K. Structural characterization of a specific glycopeptidolipid containing a novel N-acyl-deoxy sugar from mycobacterium intracellulare serotype 7 and genetic analysis of its glycosylation pathway. *J Bacteriol* 2007;189:1099–1108.
13. Fujita Y, Doi T, Maekura R, Ito M, Yano I. Differences in serological responses to specific glycopeptidolipid-core and common lipid antigens in patients with pulmonary disease due to *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *J Med Microbiol* 2006;55:189–199.
14. Hellyer TJ, Fletcher TW, Bates JH, Stead WW, Templeton GL, Cave MD, Eisenach KD. Strand displacement amplification and the polymerase chain reaction for monitoring response to treatment in patients with pulmonary tuberculosis. *J Infect Dis* 1996;173:934–941.
15. Arend SM, Geluk A, van Meijgaarden KE, van Dissel JT, Theisen M, Andersen P, Ottenhoff TH. Antigenic equivalence of human T-cell responses to mycobacterium tuberculosis-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. *Infect Immun* 2000;68:3314–3321.
16. Chatterjee D, Khoo KH. The surface glycopeptidolipids of mycobacteria: structures and biological properties. *Cell Mol Life Sci* 2001;58:2018–2042.
17. O'Brien RJ, Geiter LJ, Snider DE Jr. The epidemiology of nontuberculous mycobacterial diseases in the United States: results from a national survey. *Am Rev Respir Dis* 1987;135:1007–1014.
18. Koh WJ, Kwon OJ, Jeon K, Kim TS, Lee KS, Park YK, Bai GH. Clinical significance of nontuberculous mycobacteria isolated from respiratory specimens in Korea. *Chest* 2006;129:341–348.
19. Swensen SJ, Hartman TE, Williams DE. Computed tomographic diagnosis of *Mycobacterium avium-intracellulare* complex in patients with bronchiectasis. *Chest* 1994;105:49–52.
20. Tanaka E, Amitani R, Niimi A, Suzuki K, Murayama T, Kuze F. Yield of computed tomography and bronchoscopy for the diagnosis of *Mycobacterium avium* complex pulmonary disease. *Am J Respir Crit Care Med* 1997;155:2041–2046.

トピックス

結核ワクチン研究の現状と展望

松本壮吉[1)]/小林和夫[2)]

〔KEYWORDS〕 結核，細菌感染症，ワクチン

1. 結核(症)の現状

世界の年間死亡総数の約1/4を占める感染症において，結核は感染症の死因で後天性免疫不全症候群(AIDS)に次ぐ第二位で，全感染症による死亡者数の約1/7を占める．世界保健機関の統計(2008年5月23日現在)によると2005年の結核患者発生数は881.1万人，死亡者数が157.7万人である．AIDS患者における結核死亡を考慮した場合，毎年約200万人が結核によって死亡している．このように現在でも結核は甚大な健康被害を招来している．

結核には菌の感染後即発症する一次結核と，潜伏期を経て発症する二次結核がある(**図1**)．わが国を含め，結核の低―中蔓延地域における成人肺結核の多くは二次結核である．結核菌は現在人類の1/3(20億人)に潜伏感染しており，既感染者の5～10%が終生の間に二次結核を発症する．ヒト免疫不全ウイルス(HIV)感染は内因性再燃を加速し，HIV-結核菌重複感染者の約10%が毎年結核を発症する．したがって，結核ワクチン開発においては，感染暴露前(pre-exposure vaccine)のみならず，感染暴露後(治療的)ワクチン(post-exposure vaccine)の開発が希求される．

2. 結核ワクチンの歴史とBCG

結核菌は，1882年にRobert Kochによって同定されたグラム陽性桿菌である．当時，Kochは結核菌の培養濾液に予防効果があると信じた．こ

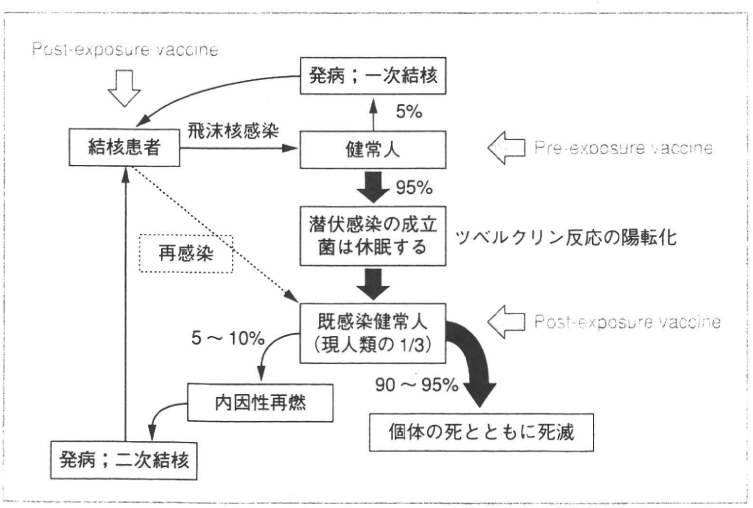

図1　結核菌の伝播と結核の発症
結核患者由来の飛沫が空中にて乾燥し菌を含んだ飛沫核となり，健常人もしくは既感染者(再感染)の肺胞に届いて感染が成立する．この時，感染者の5%未満が一時結核を発症する．残る95%は発症しないが，菌は生体から排除されずに潜伏感染が成立する．既感染者は現人類の1/3にのぼる．既感染者の5～10%が終生の間に結核を発病する(二次結核)．また，HIVの感染は二次結核発症率を顕著に上昇させる．初感染時の感染や発病を抑制するpre-exposure vaccineと既感染者の発症を予防する．もしくは免疫介入療法に用いるpost-exposure vaccineの両方が結核ワクチンに開発において求められる．

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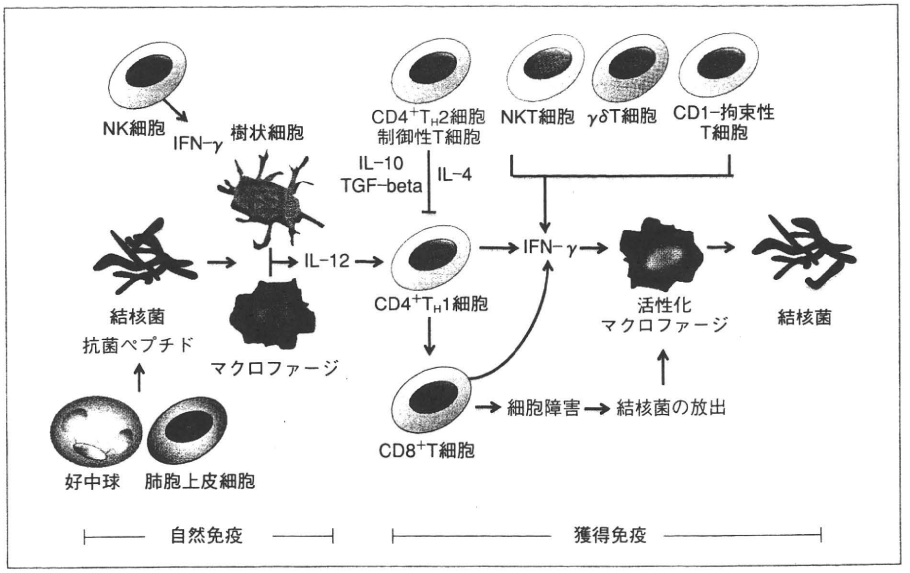

図2　結核菌感染と宿主応答

肺に侵入した結核菌は，肺胞マクロファージに貪食されるか，Ⅱ型肺胞上皮細胞に感染する．感染初期には好中球の浸潤があり，Ⅱ型肺胞上皮細胞とともに抗菌ペプチドによって結核菌を攻撃する．またNK細胞はIFN-γを生産し，細胞性免疫の誘導を促す．マクロファージや樹状細胞が結核菌抗原をIL-12の存在下において提示することで，CD4陽性T細胞はT_H1細胞に分化する．T_H1細胞はIFN-γを生産し，マクロファージを活性化することで結核菌の増殖停止や殺傷を促す．T_H1細胞以外にも，CD8陽性T細胞，NKT細胞，γδT細胞，CD1-拘束性T細胞もIFN-γを生産しマクロファージを活性化する．これを抑制するのが，T_H2細胞や制御性T細胞である．CD8陽性T細胞は細胞傷害性を有するキラー細胞でもあり，活性化マクロファージによる結核菌の再貪食を誘導する．

れが現在，結核菌など抗酸菌感染の診断に用いられているツベルクリンの起源である．

一方，Louis Pasteurらが当時確立したワクチン開発法(すなわち，自然発生的な弱毒病原体の取得)をPasteur研究所のCalmetteとGuérinが実践し，牛型結核菌Nocard株を13年間230代に渡って継代培養を行った結果，弱毒菌株を得た．これが現行の結核ワクチンbacillus Calmette-Guérin(BCG)の原型である．日本で接種されているBCG Tokyo 172は，1924年に志賀潔がPasteur研究所から持ち帰った菌株に由来する[1]．結核に対する予防効果は，BCG接種で得られるのに対し，ツベルクリン接種では得られないことから防御免疫は生菌免疫でのみ獲得されるとの考えが定着する．

現行ワクチンBCGは乳幼児結核(全身播種性結核や髄膜結核)の予防に効果(70～80%)が認められている．しかしながら，成人型肺結核の予防効果は疑問視されている．また，HIV感染者にBCGを含む生ワクチンの接種は有害事象を惹起する可能性があり，原則禁忌である．このような現状は，肺結核に有効，かつ，安全な新規ワクチンの必要性を示唆している

3. 自然免疫と結核

免疫賦活物質であるアジュバントは自然免疫の活性化物質であり，特に成分ワクチンの開発に欠かせない．自然免疫はマクロファージや樹状細胞のパターン認識受容体の活性化を介して活性化され，T細胞への円滑な抗原提示を促すことで獲得免疫の発動と免疫記憶を誘導する(**図2**)．しかしながら，結核菌は樹状細胞の活性化をC型レクチンを介して抑制する機構を有している．また，結核菌菌体成分は自然免疫の賦活化において最も主要なレセプターToll-like receptor(TLR)4をほとんど活性化しない．これらは結核菌の巧妙な寄生戦略の一端を示すものであり，ワクチン開発において憂慮すべき問題である．一方，結核菌の菌体成分は，TLR2やTLR9(それぞれリポ蛋

白質とCpG-DNA)を刺激し，これらの受容体は結核の防御に重要な役割を果たすことが明らかとなっている．

4. 獲得免疫と結核

結核菌は感染後対数的に増殖するが，健常宿主においては獲得免疫(特に，マクロファージとT細胞から構成される細胞性免疫)の発動により増殖は阻止される．この防御免疫の主役を担う細胞がCD4陽性の1型ヘルパーT(T_H1)細胞である(**図2**)．活性化されたT_H1細胞はエフェクターT細胞に分化してinterferon-γ(IFN-γ)[3]を産生し菌の増殖抑制や殺菌を促す．ワクチン効果の主体はこのエフェクターT細胞が病原体の駆逐による抗原消失後，記憶T細胞に分化し長期間の免疫記憶が成立することで形成される．しかしながら，結核菌は潜伏感染して宿主から排除されることがないため，多くのT_H1細胞がエフェクター細胞のまま次第に死滅してしまう．BCGも生体内に持続感染するため記憶T細胞の誘導能に乏しく，この機構が成人接種者における効果の減衰にかかわっている．

T_H1細胞以外にも，免疫記憶を担うCD8陽性細胞傷害性T細胞や結核菌糖脂質を認識するCD1拘束性T細胞も感染防御に重要な役割を果たす．他方，interleukin 4(IL-4)を産生するT_H2細胞や制御性T細胞(Treg)は防御免疫の抑制にかかわる記憶T細胞である．細胞内寄生菌である結核菌に対し，抗体など液性免疫の防御的役割はマイナーとされる．

5. 結核ワクチン開発の現状

成人型肺結核に対する有効で安全な結核ワクチンの成功は未了であるが，①遺伝子組み換えBCG，②組み換え弱毒結核菌，③成分ワクチン，④DNAワクチンやウイルスベクター組み換えワクチンなど，世界的に結核ワクチン研究・開発が進行中である[4]．以下に抜粋して紹介する(**表**)．

1) 組み換えBCG

BCGに特定の防御抗原や免疫賦活分子を発現させる，もしくはBCGが欠失した結核菌抗原を再度入れ戻すことでBCGを改良する試みである．Antigen 85Bなどの抗原や，IL-2, IFN-γなどのサイトカインを発現させたBCGが作成されているが，特に注目すべきはIL-15を組み入れたBCGであろう[5]．IL-15は記憶CD8陽性T細胞の維持にかかわり，防御免疫の持続を可能にするかもしれない．

BCGは，region of deleted 1(RD1)領域を欠いているため，抗原提示細胞内でほとんどの菌体抗原はファゴゾーム内にとどまっている．結果として十分なCD8陽性T細胞を活性化することができない．Kaufmannらは，低pHでファゴゾーム膜を障害するリステリアの毒素をウレアーゼの欠失したBCGに発現させた組み換えBCG, rBCG Δ UreC：Hly＋を作成した．rBCG Δ UreC：Hly＋はCD4陽性細胞とともにCD8T細胞の活性化を促し，BCG親株を超える効果のあることが判明している[6]．

2) 組み換え弱毒結核菌

結核菌の弱毒株を作成して，より病原体そのものに近い抗原で免疫することが効果的なワクチンの作成に繋がるとの考えがある．結核菌そのものを使用するため少なくともゲノム上離れた二種の遺伝子を欠失させ病原性の回帰を阻止している．結核菌H37Ra株の病原性の消失に強くかかわる二成分制御系分子のPhoP[7]やビタミンB5の合成酵素(PanC, PanD)[8]を欠失させた結核菌株の臨床試験が始まっている．

3) 成分ワクチン

成分ワクチンは生ワクチンに比べ安全性に優れ，HIV感染者にも対応可能である．加えて，抗原は投与後しばらくして消失するために，記憶T細胞を誘導しやすい利点がある．一方，免疫原性は生菌ワクチンに劣るため一般的にアジュバントや追加免疫を必要とする．現行のアジュバントの多くが体液性免疫の賦活を念頭に開発されてきたため，細胞性免疫の誘導に優れるアジュバントの開発も必要である．

ワクチン抗原は当初，分泌蛋白質を標的として行われた．これは生菌免疫の効果が，分泌する蛋白質に依存するとの考えによる．防御免疫を誘導する結核菌分泌蛋白質は，Antigen 85B(α抗原)をさきがけとして，Antigen 85 complex, ESAT6, MPT51, MPT64, HBHA, Mtb32などが同定されている．しかしながら，Dnak, Mtb39, HSP65, MDP1[9]など，非分泌性蛋白質にも防御免疫を誘導する抗原が多数同定されている．蛋白質成分ワ

表　現在開発中の主な結核ワクチン

ワクチン	施設・施行者	備考
組み換え BCG		
rBCG-Ag85B-IL15	九州大学・吉開ら	Antigen 85B と IL-15 を BCG より発現.
rBCG30	カリフォルニア大学・Horwitz ら	Antigen 85B を BCG より発現. Phase1 済み.
BCG::RD1	パスツール研究所・Cole ら	結核菌の RD1 領域を BCG に入れ戻したもの.
rBCGΔUreC：Hly＋	マックスプランク研究所・Kaufmann ら	本文参照. Phase1 済み.
組み換え結核菌		
M. tuberculosis mc^{2}6030	ニューヨーク大学・Jacobs ら	panCD と RD1 領域を欠失させた結核菌.
M. tuberculosis PhoP	ニューヨーク大学・Jacobs ら	PhoP を欠失させた結核菌.
その他の生菌ワクチン		
組み換えリステリア	浜松医科大学・小出ら	リステリアに Antigen 85A, 85B, MPT51 を発現させたもの.
成分ワクチン		
Mtb72f	Corixa 社・Reed ら	Mtb39 と Mtb32 の融合蛋白質. Phase1 済み.
Hybrid-1	Statens Serum Institutes Andersen ら	Antigen 85B と ESAT6 の融合蛋白質. Phase1 済み.
HyVac-4	Statens Serum Institutes Andersen ら	Antigen 85B と TB10.4 の融合蛋白質.
DNA やウィルスベクターを利用したワクチン		
HSP65DNA	英国国立医学研究所・Lowrie ら	ライ菌由来 *HSP60* 遺伝子を用いた DNA ワクチン.
HVJ-liposome/HSP65 DNA＋IL-12 DNA	近畿中央病院・岡田ら	結核菌由来 HSP65 と *IL-12* 遺伝子をリポソームに封入した DNA ワクチン.
MVA85A	オックスフォード大学・Hill ら	ワクシニアウィルスを用いて Antigen 85A を発現させたもの.
Aeras-402	Aeras 社	アデノウイルスベクターを用いて Antigen 85A, 85B, TB10.4 を発現させたもの.

クチン開発においては，Mtb72f[10], Hybrid1, HyVac-4 など，複数の抗原をハイブリッドさせることでより強い免疫応答を惹起できる融合蛋白質ワクチンも作成され試験中である．

一方，脂質抗原が CD1 分子拘束性の T 細胞の分化を促すことが判明している[11]．結核菌細胞壁の 40% は脂質であり，結核菌感染においては脂質抗原に対する免疫応答が活発である．脂質抗原は蛋白質に比べ生産効率や操作性に劣ることから，ワクチンへの応用は現在のところ低調であるが，特にアジュバントとしての利用価値は高い．将来のワクチン設計において脂質抗原も加えて検討すべきと考えられる．

4）DNA ワクチンやウイルスベクター組み換えワクチン

抗原そのものを接種するのでなく，蛋白質抗原の遺伝子を発現ベクターやウイルスベクターに導入し生体内で発現させる手法である．これらのワクチンは，これまでヒトでの実績がなく安全性を慎重に検討しなければならないが，内在性抗原として蛋白質を提示するため CD8 陽性細胞傷害性 T 細胞の誘導に優れる．また，DNA 取り扱い技術の発達により簡便かつ安価にワクチンを作成できる．一方，生体内における DNA の分解を防ぐ必要があり，DNA ワクチンをリポソームに封入したり[12]，病原性を失活させたウイルス粒子を用いることで対応している．現在，結核菌の主要防御抗原やサイトカインを発現するワクチンが作成され検討されている(**表**)．

6. 今後の展望

人類の 1/3 に結核菌が潜伏感染している．天然ワクチンの接種者である結核菌既感染者に再感染が生じるように，結核は“二度がかり有り”の慢性疾患である．これまでのワクチンが著効を示してきたのは，天然痘や麻疹に代表されるような“二度がかり無し”の急性疾患のみである．従来

のワクチン開発戦略のみでは結核ワクチンの開発は困難であり，安易な抗原の組み合わせや一時的な免疫応答の惹起のみでは最終的な成功に至らぬことは明白である．加えて，ワクチンの評価は成人の肺結核に効果の乏しい native BCG を実験対照として用いているため，評価系自体にも問題がある．ヒトの一次結核と二次結核，それぞれの病態を表現するモデルを確立し，検討することが重要と考えられる．

一方，“ヒト”に立ち返れば，結核菌に感染しても終生発病を免れるヒトが約 90% である事実は，優れたワクチンの開発が可能であることを示している．結核菌既感染者における“菌の増殖を制御する機構”の解明はワクチン開発に寄与するであろう．“二度がかり有り”の慢性疾患に対して有効・安全なワクチンを作成することは，これまでに人類が成しえていない大きな挑戦である．今後，免疫理論と実践の蓄積により，結核ワクチン開発は成し遂げられるものであろう．

文　献

1) Yamamoto S, Yamamoto T : Historical review of BCG vaccine in Japan. Jpn J Infect Dis 60 : 331-336, 2007
2) Yamamoto S, Yamamoto T, Kataoka T, et al : Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN [correction of INF] and augment IFN-mediated [correction of INF] natural killer activity. J Immunol 148 : 4072-4076, 1992
3) Kawamura I, Tsukada H, Yoshikawa H, et al : IFN-gamma-producing ability as a possible marker for the protective T cells against *Mycobacterium bovis* BCG in mice. J Immunol 148 : 2887-2893, 1992
4) Young DB, Perkins MD, Duncan K, et al : Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest 118 : 1255-1265, 2008
5) Tang C, Yamada H, Shibata K, et al : Efficacy of recombinant bacille Calmette-Guérin vaccine secreting interleukin-15/antigen 85B fusion protein in providing protection against *Mycobacterium tuberculosis*. J Infect Dis 197 : 1263-1274, 2008
6) Grode L, Seiler P, Baumann S, et al : Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin. J Clin Invest 115 : 2472-2479, 2005
7) Lee JS, Krause R, Schreiber J, et al : Mutation in the transcriptional regulator PhoP contributes to avirulence of *Mycobacterium tuberculosis* H37Ra strain. Cell Host Microbe 3 : 97-103, 2008
8) Sambandamurthy VK, Wang X, Chen B, et al : A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. Nat Med 8 : 1171-1174, 2002
9) Matsumoto S, Matsumoto M, Umemori K, et al : DNA augments antigenicity of mycobacterial DNA-binding protein 1 and confers protection against *Mycobacterium tuberculosis* infection in mice. J Immunol 175 : 441-449, 2005
10) Skeiky YA, Alderson MR, Ovendale PJ, et al : Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J Immunol 172 : 7618-7628, 2004
11) Beckman EM, Porcelli SA, Morita CT, et al : Recognition of a lipid antigen by CD1-restricted alpha beta + T cells. Nature 372 : 691-694, 1994
12) Okada M, Kita Y, Nakajima T, et al : Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA + IL-12 DNA) against tuberculosis using the cynomolgus monkey model of TB. Vaccine 25 : 2990-2993, 2007

Biophilia special

再興した感染症「結核」の診断・治療・予防法

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はじめに—結核とは—

結核とは、結核菌感染によっておもに肺に炎症を起こす疾患である。結核患者が咳やくしゃみをした時に飛散する「しぶき(飛沫核)」に存在する結核菌を吸入することにより感染・発病する(図1)。結核は人類に甚大な健康被害を及ぼしている。世界の感染症による年間死亡者数(2005年)は1,400万人(総死亡者数:5,800万人の4分の1弱)を占める(表1)。

痰に結核菌を排出していない結核の場合、他人に伝播することはほとんどない。結核菌を吸い込んでも、免疫防御機能により、結核菌の活動が抑制され、発病は感染者の約10%である。結核は、6か月間毎日確実に薬を服用すれば、ほとんど治癒する。

世界保健機関(WHO)やG8頂上会議は、1)ヒト免疫不全ウイルス(HIV)感染/後天性免疫不全症候群(エイズ)、2)

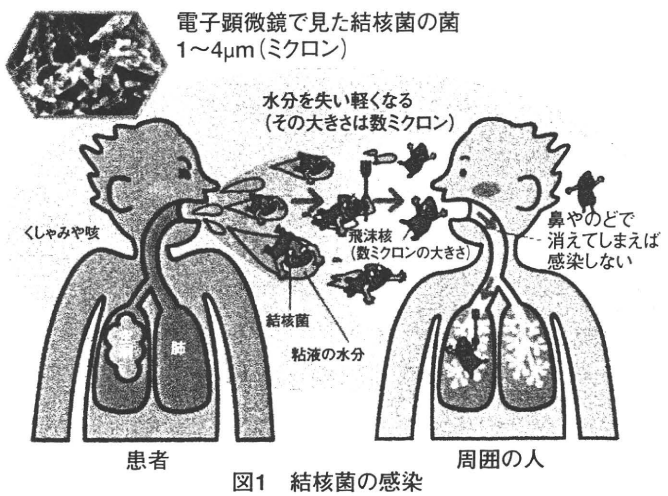

図1 結核菌の感染
財団法人結核予防会:結核の常識 2006(http://www.jatahq.org/aboutTB/2006/joshiki2006-2.html)

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(厚生労働技官)

昭和大学医学部第一内科学講師、大阪市立大学大学院医学研究科感染防御学分野教授を経て、2006年より現職。専門は、感染症学、臨床免疫学、結核病学。結核など抗酸菌感染症の分子医学、基礎−臨床医学の橋渡し研究に従事。サイトカインの病因的役割、抗酸菌病原因子の解明、抗酸菌感染症の血清診断の開発などで成果を挙げている。持田記念医学薬学振興財団研究奨励賞受賞。著書に、「標準微生物学 第9版」(分担、医学書院、2005年)など。

表1　世界における感染症による死亡者数（2006）

感　染　症	死亡者数（万人）
全感染症	1,400
肺炎など急性呼吸器感染症	376
後天性免疫不全症候群（結核合併を含む）	210
結核	170
下痢性疾患	168
マラリア	89

結核、および3）マラリアによる死亡が年間約500万人、患者発生が3億人であることから、これら3大疾患を最重要感染症に認定し、世界が協調して対策を構築することを宣言している。

結核の発生動向

世界では約20億人（全人口の3分の1）が結核菌（*Mycobacterium tuberculosis*）に既感染（ほとんどは潜在性）、毎年920万人（ヒト免疫不全ウイルス感染合併：71万人を含む）が結核を発病、170万人（後天性免疫不全症候群合併：20万人を含む）が死亡している。1人の無治療結核患者が年間10～15名の感染者を生じさせている。なお、結核菌感染後の発病率は10%である。世界保健機関は、今後20年間に10億人の新規感染者が発生、1億5,000万人が結核を発病、そして、3,600万人の結核死亡を予測している。

1951（昭和26）年、結核予防法（2007年「感染症の予防及び感染症患者に対する医療に関する法律」に統廃合）は施行されたが、当時の日本における結核罹患率は人口10万人対698.4、死亡率は111.1であり、結核は甚大な健康被害であった。その後、抗結核化学療法や検診の発達・普及、また、衛生行政の整備により、結核は減少した。しかし、1997年、罹患率や発生患者数ともに38年ぶりに増加し、結核は「再興感染症」として注目されている。日本における増加要因は、1）70歳以上の高齢患者の増加、2）集団感染、さらに、3）貧困など経済的弱者の結核の増加が挙げられる。加えて、国際的には、4）薬剤耐性結核や5）ヒト免疫不全ウイルス感染症／後天性免疫不全症候群の合併も増加要因である。

表2　世界および日本の結核発生動向

	結核菌 既感染者数	年間死亡数 （死亡率）	新規登録患者数 （罹患率）
世界	20億人	170万人（2.5）	920万人（139）
日本	0.25億人	0.22万人（1.7）	2.5万人（19.8）

表3　結核の増加要因

社会的	人口の集中／都市化、国際化／移動・移民、貧困、感染症対策の行政的不備
宿主的	易感染性宿主の増加（高齢者、糖尿病、慢性腎不全、ヒト免疫不全ウイルス感染、免疫抑制薬／臓器移植、免疫疾患、抗サイトカイン療法：関節リウマチやクローン病治療薬）
微生物学的	薬剤耐性抗酸菌の出現、病原性の変化

日本では、2007年に年間2万5,000人（罹患率人口10万対：19.8）が結核を発病、2,200人（死亡率：1.7）が死亡している（平成19年結核登録者情報調査年報集計結果―概況，厚生労働省健康局結核感染症課【http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou03/07.html】）（表2）。

日本における結核対策の課題として、1）急速な人口の高齢化に伴う高齢者結核の増加（70歳以上の占める割合：48%）、2）国内地域格差の拡大（最高罹患率は大阪市：52.9、最低は長野県：10.3）、3）薬剤（とくに、多剤や超多剤）耐性結核菌の出現、4）特異的、迅速かつ簡便な結核菌感染の検査法の開発や5）潜在性結核菌感染対策などがある。

結核の増加要因は、社会要因、宿主要因および病原体要因に大別される。社会的要因として、都市化による過密、貧困、交通機関の発達による高速移動、国際化や感染症対策の軽視などが寄与している。宿主要因として、感染抵抗力の減弱（高齢化、糖尿病、慢性腎不全、ヒト免疫不全ウイルス感染症／後天性免疫不全症候群、免疫抑制薬/臓器移植や免疫疾患など）が易感染性を招来している。また、病原体要因として、薬剤耐性結核菌の出現および病原性の変化などが結核の増加に関与している（表3）。

とくに、世界と共通した重要な要因や課題は、1）多剤耐性（MDR）結核菌（抗結核薬であるイソニアジドおよびリファンピシンに同時耐性）の出現、2）ヒト免疫不全ウイルス感染／後天性免疫不全症候群、および3）潜在性結核菌感染対策である。最近、超多剤耐性（XDR）結核菌（多剤耐性に加え、フルオロキノン耐性＋カナマイシン、カプレオマイシン、アミカシンの1剤以上に耐性）も出現している。薬剤耐性結核の出現を防止する効果的な戦略は、薬剤感受性結核を確実に治療、そして、治癒させることであり、世界保健機関は直接監視下短期抗結核化学療法（directly observed treatment, short course：DOTS）を推奨している。

世界のヒト免疫不全ウイルス感染者（後天性免疫不全症候群を含む）は3,320万人、結核菌とヒト免疫不全ウイルスの重複感染は約71万人、結核を発症した患者（920万

人)でヒト免疫不全ウイルス陽性は約8%を占めている。結核菌感染に対する防御は細胞性免疫に依存しているが、ヒト免疫不全ウイルス感染症／後天性免疫不全症候群は細胞性免疫を破壊するため、結核菌感染や発病を惹起しやすくする。実際、ヒト免疫不全ウイルス感染陽性者における発病の相対危険度はヒト免疫不全ウイルス感染陰性者の約10倍である。また後天性免疫不全症候群死亡の約10%が結核を直接原因としている。世界の人口の3分の1が結核菌に既感染、ほとんどは無症候性潜在性感染であり、大部分の結核は潜在性結核菌感染から発病に至る。したがって潜在性結核菌感染対策は結核の制圧に重要である。

結核菌の生物学的特徴や病原性

結核菌の生物学的特徴として、1)細胞内寄生性、2)脂質成分に富む細胞壁、3)好気(酸素)性、4)遅発育性、5)飛沫核(空気)感染、6)慢性炎症、および7)遺伝子の解読などがある。分裂倍加時間は約12～15時間(参考：大腸菌は約20分間)の遅発育菌であり、感染伝播は、飛沫核(空気)感染による。結核菌感染により、菌は消失することなく、一生涯、体内に残存する。一般的に、活動性結核患者と接触した者の約30%が感染する。宿主防御機構では細胞性免疫が役割を演じ、その結果、結核菌初感染者の10%が結核を発病、潜在性結核菌感染者の20%が免疫力の低下に伴い発病する(図2)。

病変は、慢性炎症、肉芽腫、乾酪壊死(結核病変の中心部が壊死し、黄色乳成分凝固物[チーズ状塊]を形成すること。乾酪壊死巣内の結核菌は減少し、生菌として残存する)、空洞形成や線維化などが特徴的である。結核菌の遺伝子、全遺伝子塩基配列が解明された。今後、遺伝子解析を基盤とした科学的戦略が推進され、分子／遺伝子標的を視点とした新規診断法、抗結核薬の開発、薬剤耐性獲得機構の解明や新規ワクチン開発が展開されるであろう。

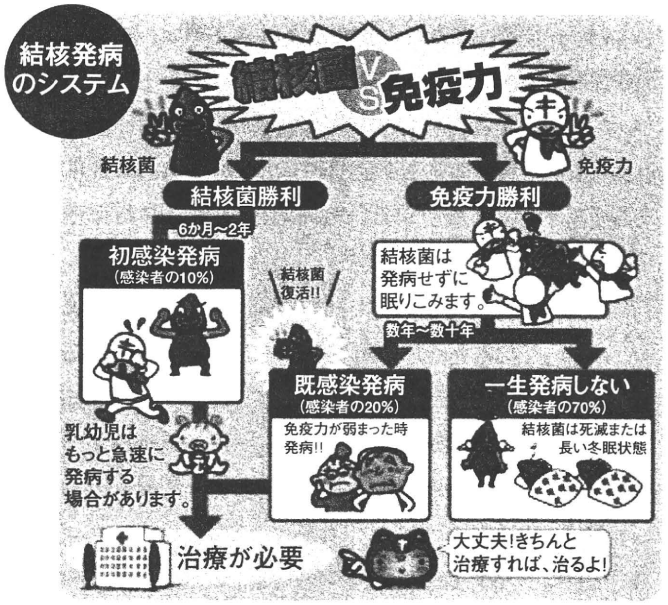

図2　結核の発病
財団法人結核予防会：結核の常識 2006
(http://www.jatahq.org/aboutTB/2006/joshiki2006-2.html)

結核の診断

結核は、肺結核と肺外結核(肺あるいは気管支以外の臓器を主要罹患臓器とする結核および播種性結核)に分類されるが、85%以上は肺結核である。肺結核の症状として、咳(咳嗽)や痰(喀痰)(持続性、2週間以上)、血痰、胸痛、軽度発熱、体重減少、とくに、持続性咳嗽と喀痰は重要である(表4)。

肺外結核部位として、喉頭、リンパ節、胸膜、泌尿生殖器、骨・関節、髄膜・中枢神経系、腹膜・消化管や心外膜などがある。喉頭結核は、肺結核に続発することが多く、症状として、嚥下痛、しわがれ声(嗄声)や呼吸困難が見られる。全身播種性(粟粒性)結核は少なくとも2臓器以上に活動性病変があり、全身散布性病巣が形成されるものをいう。乳幼児や免疫不全者(副腎皮質ステロイド薬や免疫抑制薬の投与、慢性腎不全：血液・腹膜透析、ヒト免疫不全ウイルス感染症／後天性免疫不全症候群)などに起こりやすい。発熱、全身倦怠、衰弱、咳、胸痛、息切れ、頭痛などの症状があり、全身性に小結節病変が出現する。腸結核は結核菌を含んだ痰を嚥下することにより発症し、症状として下痢、腹痛、腹部膨満、悪心や嘔吐がある。

診断には、病原体および補助診断がある(表4)。病原体の検出は診断に確定的であるが、痰塗抹検査陽性(図3a)の場合、結核菌のみならず、類縁の非結核性抗酸菌(非結核性抗酸菌感染症は結核を含む抗酸菌感染症の約20%を占める)を考慮する必要がある。現在、最も信頼性の高い検査は培養法(図3b)であるが、欠点として、長期間を要することである(10～14日間以上)。核酸増幅による遺伝子診断は迅速性、感度や特異性に優れるが、生死菌の識別や技術的問題(熟練、偽陽性／偽陰性)がある(図3c)。

胸部X線所見では、浸潤影(水様物質が肺胞腔に蓄積することで呈する境界不鮮明な陰影)、結節(境界明瞭な円形状陰影)、空洞(病変部に穴がある陰影)、線維化、肺

表4　結核の症状や診断

症状	持続性咳嗽や喀痰(2週間以上)　その他:発熱、血痰、胸痛、体重減少など	
病原体診断	塗抹検査	抗酸菌染色、蛍光染色
	培養検査	10〜14日以上、薬剤感受性試験
	遺伝子検出	核酸増幅法:ポリメラーゼ連鎖反応(PCR) デオキシリボ核酸(DNA)—デオキシリボ核酸交雑形成
補助診断	胸部X線	中および上肺野病変(浸潤、結節や空洞) リンパ節腫大や石灰化 胸膜炎／胸水貯留
	病理学的検査	乾酪壊死を伴う肉芽腫
	ツベルクリン皮内反応	48時間後判定 陽性:結核菌感染、BCG陽転、非結核性抗酸菌感染 陰性:未感染、BCG未接種、免疫不全 (ヒト免疫不全ウイルス感染症／後天性免疫不全症候群、重症結核、薬物性)
	クオンティフェロン:QFT	末梢血細胞インターフェロンガンマ産生・遊離試験(クオンティフェロン®)

門リンパ節腫大や石灰化、無気肺(気管支などが閉塞され、一部の肺の空気が消失している状態)、胸膜肥厚・癒着(胸膜炎が治癒し、胸膜が肥厚・癒着した状態)、胸水貯留など、多彩である(図3d)。好発部位は、酸素濃度の高い上肺や中肺野である。多発性びまん性結節陰影は播種(粟粒)性結核で見られる。これらの所見は他の炎症性や腫瘍性肺疾患(肺がんなど)にも認められる所見であり、結核特異的でなく、注意を要し、胸部X線所見は結核の補助的診断法である。

ツベルクリン皮内反応の陽性(日本:紅斑≧直径10mm以上、欧米:硬結≧直径5mm以上)は結核菌感染のみならず、弱毒ウシ型結核菌ワクチン(bacillus Calmette-Guérin:BCG)接種や非結核性抗酸菌感染でも見られ、逆に、活動性結核の約25%は陰性である。陰性は真の陰性(結核菌未感染)や偽陰性(結核菌既感染にもかかわらず、陰性)を包含する。偽陰性として、栄養障害、高齢者、免疫疾患、リンパ系悪性腫瘍、副腎皮質ステロイド薬療法、慢性腎不全、サルコイドーシス、ヒト免疫不全ウイルス感染者(後天性免疫不全症候群を含む)や重症結核(播種性)などがある。したがって、ツベルクリン皮内反応も結核の補助診断である。ツベルクリン皮内反応陽性は感染防御の指標とならないことも留意する。

近年、結核菌特異的タンパク質抗原を用いた免疫学的診断法が開発され、臨床応用されている(インターフェロンガンマ遊離試験:クオンティフェロン®:QFT)。これらの抗原は、結核ワクチンであるBCGや多くの非結核性抗酸菌に存在しないため、結核菌感染を特異的に検出できる。原理は、末梢血に特異的タンパク質抗原を加え、培養後、産生・遊離されるインターフェロンガンマを定量する(陽性:0.35 IU/mL以上)。検査対象として、1)潜在性結核菌感染や、2)活動性結核の補助診断に応用されている。

治療法

結核は、薬をきちんと服用すれば治る。痰の中に結核菌が出なくなれば外来通院治療も可能である。

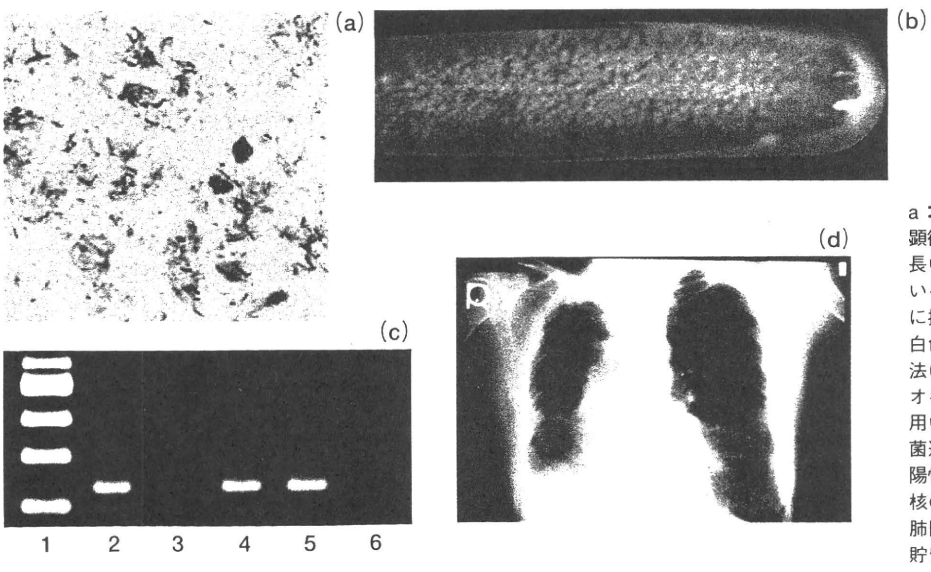

図3　結核の診断[1)]
a:痰の結核菌塗抹検査(特殊な染色をし、顕微鏡で観察)。結核菌は赤染されている細長い(桿)菌であり、ヒト組織は青染されている。b:結核菌の培養所見。喀痰を卵培地に接種し、6週間後に多数の結核菌集落(乳白色一薄黄色)形成を認めた。c:核酸増幅法による結核菌遺伝子の検出。結核菌のデオキシリボ核酸(DNAに特異的DNA断片)を用い、核酸重合酵素連鎖反応で喀痰の結核菌遺伝子を検出した。分子基準標識:列1、陽性:列2、4、5、陰性:列3、6。d:肺結核の胸部X線所見。浸潤影、結節、線維化、肺門リンパ節腫大、胸膜肥厚・癒着や胸水貯留など、多彩な所見を認めた。

治療の原則は、確実な多剤併用抗結核化学療法(服薬期間は約6か月[毎日]、最初の2か月が4剤：イソニアジド、リファンピシン、エタンブトール、ピラジナミド、その後4か月が2剤：イソニアジドおよびリファンピシン)である。1薬剤当たりの耐性菌出現頻度は$1/10^6$～10^9であるため、薬剤を併用することにより、耐性菌の出現頻度を低下させることが可能となる。ただし、確実な服用は絶対条件であり、世界保健機関は直接監視下短期抗結核化学療法(DOTS)を推進している。直接監視下短期抗結核化学療法とは、結核患者を見つけて治すために利用されている保健福祉の包括的計画の名称で、世界保健機関が提唱した結核対策戦略である。そのおもな5要素は、1)政府が結核を重要課題と認識し適切な指導性を発揮すること、2)結核菌検査による診断、病状経過観察の推進、3)結核患者が薬を飲み忘れないよう医療従事者の前で内服すること、4)薬の安定供給、5)菌検査結果の記録監視・調査である。

薬剤耐性結核の原因は、不適切な結核医療、すなわち、抗結核化学療法薬の不適切な選択や使用、治療中断や脱落であり、医療関係者や患者の対応に起因する人原病(man-made disease)である。全世界で5,000万人以上が多剤耐性結核菌(イソニアジドおよびリファンピシンに同時耐性)に既感染し、医療費は薬剤感受性結核に比し、3～100倍を要し、再発率(28%)がきわめて高く、結核制圧対策の大きな課題である。加えて、超多剤耐性結核菌の出現は抗結核化学療法をきわめて困難にしている。

予防

予防は、感染源対策として患者の早期発見・治療、接触者(家族、学校、会社など)の調査、さらに、予防接種や潜在性結核菌感染の治療(化学予防)がある。予防接種はBCGが汎用されている。現行の結核発病予防ワクチンであるBCGは乳幼児結核(結核性髄膜炎など播種性結核)に有効であるが、成人肺結核に対するBCG接種の効果は疑問視されている。BCGは乳幼児期(原則として、生後6か月までにツベルクリン皮内反応を省略したBCGの初回接種)のみに限定している。

潜在性結核菌感染の治療(化学予防)は、抗結核化学療法薬(イソニアジドなど)を服用し、発症を防止する(効果：70～80%)。ただし、感染結核菌が化学療法薬に感受性であることが不可欠である。

結核予防法の統廃合

2007年4月の改正感染症法の施行に伴い、結核予防法は「感染症の予防及び感染症の患者に対する医療に関する法律」に統廃合された。結核は二類感染症に位置づけられ、結核を診断した場合、医師は直ちに最寄りの保健所長を経由し都道府県知事に届け出なければならない。また、生物テロ対策として、2007年6月から、「特定病原体等(一～四種)の管理規制」が施行されている。結核菌は空気感染病原体、かつ、個体に対する高い危険度を示すため、施設や保管の基準が定められた。多剤耐性結核菌は三種病原体であり、施設や保管の基準に加え、所持に際し、厚生労働大臣へ届出、また、運搬に際し、都道府県公安委員会へ届出が必要である。結核菌(多剤耐性結核菌を除く)は四種病原体であり、施設や保管の基準の遵守が必要である。

おわりに

結核は、代表的な再興感染症であり、現在でも、人類に甚大な健康被害を及ぼしている。結核対策には多くの課題が山積しているが、科学的根拠に基づいた「感染源、感染経路、感受性宿主」対策や「診断、治療、予防」が実施され、結核が制圧されることを期待している。

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[参考文献]

1) 小林和夫：標準微生物学 第9版, 山西弘一監修, 平松啓一, 中込治編, 医学書院, 279-292, 2005.
2) 厚生労働省：結核・感染症に関する情報 (http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansen-shou.html).
3) 厚生労働省：平成19年結核発生動向調査年報集計結果 (http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansen-shou03/07.html).
4) ストップ結核パートナーシップ日本 (http://www.stoptb.jp/index.html).
5) 財団法人結核予防会 結核研究所：結核の常識；新結核用語辞典(http://www.stoptb.jp/index.html).
6) 国立感染症研究所感染症情報センター：感染症の話「結核」 (http://idsc.nih.go.jp/idwr/kansen/k03/k03-07/k03_07.html).

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NON-TUBERCULOUS MYCOBACTERIA: GENERAL

High transmissibility of the modern Beijing *Mycobacterium tuberculosis* in homeless patients of Japan

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SUMMARY

A population-based study of *Mycobacterium tuberculosis* isolated from homeless tuberculosis patients was performed during 2002–2004 in Osaka City, Japan. The data show that the ancient Beijing subfamily was predominant, whereas clustered isolates based on refined variable number of tandem repeats genotyping (19 loci) mainly belonged to the modern Beijing subfamily, suggesting its increased transmissibility.

In Japan, situated in the far eastern end of Eurasia, strains of *Mycobacterium tuberculosis*—an etiologic agent of tuberculosis (TB)—belonging to the Beijing family have been highly prevalent (approximately 75%), as in other eastern Asian countries.[1] It is well known that the Beijing family can be divided into the ancient (atypical) and the modern (typical) subfamilies.[2–5] It is presumed that the modern subfamily is more virulent and has a higher fitness to human hosts than the ancient subfamily.[2,3,6–8] Moreover, it has been speculated that the modern subfamily has been positively selected by BCG-induced immunity,[9,10] which could be attributable to the antigenic properties of the subfamily.[11,12] In a previous study, it was found that although the strains of the modern subfamily are disseminated worldwide, those of the ancient subfamily are mainly prevalent in Japan.[5]

The TB case rate in Japan has declined gradually from 25.8 per 100,000 to 19.8 during 2002–2007.[13] Osaka City, Japan, has had the highest TB case rate in Japan (about three times higher than the average: from 74.4 to 52.9 during 2002–2007). The prominent case rate observed among homeless people in the city has been considered to be the salient cause. The Airin area, in which reside about 30,000 homeless or day-laboring residents (both are strictly indistinguishable because of their fluidity), has consistently reported over 500 per 100,000 TB patients annually.[14] Generally, homelessness is regarded as a risk factor for TB incidence.[15–18] Hence, it is important to elucidate the population structure of *M. tuberculosis* in this area to control further diffusion of the infection.

To elucidate the population structure of *M. tuberculosis* isolated from TB patients among the homeless person group in Osaka City, we obtained 274 *M. tuberculosis* isolates from TB patients residing in the Airin area between January 2002 and December 2004. They were all isolates obtained from the homeless TB patients at three hospitals and two public health facilities. They covered 64.5% of the total culture-positive homeless patients in Osaka City over three years. The characteristics of patients and the drug susceptibility of the isolates are presented in Table 1. It is likely that the extremely high number of middle-aged male patients in our study reflects the general trend of the human population in that area (data not shown).

All 274 isolates were subjected to genotypic classification including the identification of Beijing family strains and the subdivision of the ancient and modern Beijing subfamilies by PCR, as described in previous reports.[5,19] Consequently, they were

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Table 1
Characteristics of 274 homeless tuberculosis (TB) patients analyzed in this study.

Characteristics	Total (%)	Year		
		2002	2003	2004
Total	274 (100.0)	71 (100.0)	97 (100.0)	106 (100.0)
New cases	223 (81.4)	55 (77.5)	86 (88.7)	82 (77.4)
Median age [range]	57.2 [29–85]	56.9 [34–76]	57.5 [29–83]	56.8 [35–85]
Age group, y				
<35	2 (0.7)	1 (1.4)	1 (1.0)	0 (0.0)
35–44	22 (8.0)	5 (7.0)	4 (4.1)	13 (12.3)
45–54	83 (30.3)	23 (32.4)	31 (32.0)	29 (27.4)
55–64	116 (42.3)	27 (38.0)	45 (46.4)	44 (41.5)
65–74	42 (15.3)	13 (18.3)	12 (12.4)	17 (16.0)
>74	9 (3.3)	2 (2.8)	4 (4.1)	3 (2.8)
Sex				
Female	2 (7.3)	0 (0.0)	1 (1.0)	1 (0.9)
Male	272 (92.7)	71 (100.0)	96 (99.0)	105 (99.1)
Disease site				
Any pulmonary	270 (98.5)	71 (100.0)	97 (100.0)	102 (96.2)
Extrapulmonary only	4 (1.5)	0 (0.0)	0 (0.0)	4 (3.8)
Respiratory acid fast bacilli smear test results*				
Positive	218 (79.6)	60 (84.5)	78 (80.4)	80 (75.5)
Negative	52 (19.0)	11 (15.5)	19 (19.6)	22 (20.8)
Drug resistance†				
Only INH	4 (1.5)	1 (1.4)	3 (3.1)	0 (0.0)
Only RFP	3 (1.1)	2 (2.8)	0 (0.0)	1 (0.9)
MDRTB	1 (0.4)	1 (1.4)	0 (0.0)	0 (0.0)

* The results of extrapulmonary TB patients were excluded.
† INH, isoniazid; RFP, rifampin.

classified into three genetic groups according to their types: non-Beijing, ancient Beijing, and modern Beijing (Table 2). Of all isolates, Beijing family isolates were 213 (77.7%). They were further classified into 137 (50.0%; 64.3% of Beijing family) ancient subfamily isolates and 76 (27.7%; 35.7% of Beijing family) modern subfamily isolates. This population structure was consistent with the predominance of the ancient subfamily in Japan reported previously.[5]

We performed clustering analysis for all 274 isolates using variable number of tandem repeats (VNTR) genotyping methods[20] to investigate the putative direct transmission of bacilli within the population. Supply et al. reported an international set of VNTR comprising 15 genomic loci of short tandem repeats for epidemiological use (15-MIRU-VNTR).[21] Although a promising genotypic tool for global comparison, it has provided insufficient discrimination in Japanese populations of *M. tuberculosis*.[18–20] The reliable discriminatory power for epidemiological observation could be conferred by the additional hypervariable VNTR loci.[22,24,25] Therefore, we added four hypervariable loci, QUB-2163a, QUB-3232, VNTR 3820, and VNTR 4120 to the global standard. The addition of these four loci provided high discriminatory power even for Beijing family strains,[22] although they have been excluded from standard sets because of their genotypic instability and technical difficulty for comparison among different laboratories.[21,23] They were analyzed carefully by using capillary electrophoresis system, SV1210 (Hitachi Electronics).[26] All allelic profiles are listed in Table S1. Clusters were defined as two or more than two isolates with 19 identical VNTR alleles. In a total of 274 isolates, we found 114 (41.6%) clustered isolates (Table 2). The clustering rate was significantly higher in the modern Beijing subfamily (61.8%) than in the non-Beijing strains (27.9%; $P < 0.0001$, χ^2 test) and the ancient subfamily (36.5%; $P = 0.0004$, χ^2 test). We calculated the clustering rates after excluding the largest cluster(s) in order to examine whether occasional outbreaks of modern Beijing strains might be responsible for the results. On excluding one of the largest clusters (composed of 10 modern Beijing isolates) from the total population, the clustering rate was significantly higher in the modern Beijing subfamily (56.1%) than in the non-Beijing strains ($P = 0.0013$,

Table 2
Classification of Beijing family/subfamilies and clustering analysis based on VNTR* genotypings of 274 *M. tuberculosis* isolates from homeless patients in Osaka City.

	Total	Non-Beijing	Beijing	
			Ancient	Modern
No. of isolates (%)	274 (100.0)	61 (22.3)	137 (50.0)	76 (27.7)
Clustering analysis (19 loci VNTR)				
No. of type patterns	195	50	106	39
No. of unique types	160	44	87	29
No. of clusters	35	6	19	10
No. of clustered isolates	114 (41.6%)	17 (27.9%)	50 (36.5%)	47 (61.8%)
Maximum no. of isolates in a cluster	10	4	5	10
Average size of clusters	3.25	2.83	2.63	4.70
Recent transmission rate (RTI_{n-1})	0.288	0.180	0.226	0.487

* Variable number of tandem repeats.

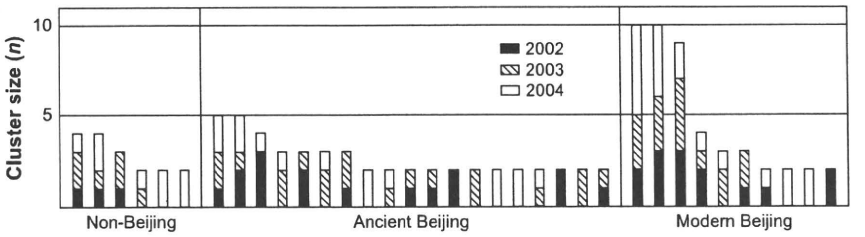

Figure 1. Distribution of all 35 clusters (114 isolates) by VNTR genotyping including 19 loci.

χ^2 test) and ancient subfamily ($P = 0.0083$, χ^2 test). On excluding the two largest clusters (20 isolates), the clustering rate in the modern Beijing family was 48.2%, which was higher than that in the non-Beijing strains ($P = 0.0232$, χ^2 test) and ancient subfamily ($P = 0.13$, χ^2 test), although the differences were not significant. When the 114 clustered isolates were analyzed by IS*6110* restriction fragment length polymorphism (RFLP) genotyping, 33 (94.3%) of 35 VNTR clusters were found to exhibit identical or less than 3 different types of band patterns (data not shown). The two exceptional clusters were found in non-Beijing strains and in the ancient Beijing subfamily; this result indicated robust cluster formation in the modern Beijing subfamily.

We introduced a recent transmission index RTI_{n-1}[27,28] (calculated by the following equation: $RTI_{n-1} = (n_c - c)/n$, where n is the total number of isolates, n_c is the number of clustered isolates, and c is the number of clusters) to quantify the transmissibility of each genotypic group. This index indicates that there is a higher likelihood of recent transmission of the modern Beijing subfamily (0.487) than of the other two groups (non-Beijing strains, 0.180; ancient Beijing subfamily, 0.226; Table 2). Previous population genetic structure analyses have revealed that the hypervariable VNTR loci exhibited high diversity (variation).[21–24] Therefore, the accordance of VNTR genotypes, including these loci, strongly supports the identification of the isolates. It is suggested that the clusters were probably formed because of active transmission in circulation, although its epidemiological links were not certified. As shown in Figure 1, the clusters by VNTR genotyping (19 loci) were classified into three groups—non-Beijing, and the ancient and modern Beijing. In our setting, we observed that large clusters were mainly observed in the modern Beijing subfamily. This result suggests that *M. tuberculosis* strains of the modern Beijing subfamily was likely to spread among homeless people to a greater extent than the other groups. Although the ancient Beijing subfamily is predominant, the population structure may be altered to resemble the worldwide typical population structure exhibiting the superiority of the modern Beijing subfamily.

Finally, we compared the clinical characterization of the homeless TB patients across the three genotypic groups (Table 3). Although higher transmissibility of the modern Beijing subfamily was speculated on the basis of the results of the clustering analysis, this subfamily did not exhibit a significant difference from other groups in the age of infected patients (vs non-Beijing, $P = 0.94$; vs ancient-Beijing, $P = 0.83$, Welch's t test) and the smear-positive rate of pulmonary TB patients (vs non-Beijing, OR = 0.51 [95% CI: 0.22–1.20]; vs ancient-Beijing, OR = 0.52 [95% CI: 0.26–1.03]). These results suggest that both the incidence of TB among younger people and the number of smear-positive cases were not associated with the higher clustering rate in the modern Beijing subfamily. This observation may lead us to understand reasons underlying the higher putative transmissibility of the modern Beijing subfamily among homeless patients. The high transmission rate of the subfamily may be associated with homelessness. It is important to analyze the population structure of

Table 3
Distribution of Characteristics of 274 homeless tuberculosis (TB) patients among Beijing family/subfamilies of causal *M. tuberculosis* isolates.

Characteristics	Total (%)	Non-Beijing (%)	Beijing	
			Ancient (%)	Modern (%)
Total	274	61	137	76
New cases	223 (81.4)	52 (85.2)	110 (80.3)	61 (80.3)
Median age [range]	57.2 [29–85]	56.8 [38–83]	57.0 [29–85]	57.4 [38–83]
Age group, y				
<35	2 (0.7)	0 (0.0)	1 (0.7)	1 (1.3)
35–44	22 (8.0)	5 (8.2)	12 (8.8)	5 (6.6)
45–54	83 (30.3)	19 (31.1)	41 (29.9)	23 (30.3)
55–64	116 (42.3)	25 (40.1)	57 (41.6)	34 (44.7)
65–74	42 (15.3)	10 (16.4)	21 (15.3)	11 (14.5)
>74	9 (3.3)	2 (3.3)	5 (3.6)	2 (2.6)
Disease site				
Any pulmonary	270 (98.5)	60 (98.3)	135 (98.5)	75 (98.7)
Extrapulmonary only	4 (1.5)	1 (1.7)	2 (1.5)	1 (1.3)
Respiratory acid fast bacilli smear test results*				
Positive	218 (79.6)	50 (83.3)	112 (81.8)	54 (71.1)
Negative	52 (19.0)	10 (16.7)	23 (16.8)	21 (27.6)
Drug resistance†				
Only INH	4 (1.5)	0 (0.0)	4 (2.9)	0 (0.0)
Only RFP	3 (1.1)	0 (0.0)	1 (0.7)	2 (2.6)
MDRTB	1 (0.4)	0 (0.0)	0 (0.0)	1 (1.3)

* The results of extrapulmonary TB patients were excluded.
† INH, isoniazid; RFP, rifampin.

TB patients other than homeless patients in order to elucidate the possibility.

In summary, the population genetic structure analysis of *M. tuberculosis* revealed that the transmission of the modern Beijing subfamily strains may be more frequent than that of other strains. The vicissitudes of population structure must be observed on the basis of up-to-date genotyping data to devise precautionary measures against the epidemic expansion of this subfamily. Our results may be linked to the dynamic observation of the process of the predominance of the modern Beijing subfamily that had occurred around Japan in the past. Further, it is also important to uncover the nature of the modern Beijing subfamily in order to ascertain the causes of its worldwide prevalence and transmission.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tube.2009.05.007.

References

1. van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. *J Clin Microbiol* 1995;**33**:3234–8.
2. Dou HY, Tseng FC, Lu JJ, Jou R, Tsai SF, Chang JR, et al. Associations of *Mycobacterium tuberculosis* genotypes with different ethnic and migratory populations in Taiwan. *Infect Genet Evol* 2008;**8**:323–30.
3. Mokrousov I, Ly HM, Otten T, Lan NN, Vyshnevskyi B, Hoffner S, et al. Origin and primary dispersal of the *Mycobacterium tuberculosis* Beijing genotype: clues from human phylogeography. *Genome Res* 2005;**15**:1357–64.
4. Mokrousov I, Narvskaya O, Otten T, Vyazovaya A, Limeschenko E, Steklova L, et al. Phylogenetic reconstruction within *Mycobacterium tuberculosis* Beijing genotype in northwestern Russia. *Res Microbiol* 2002;**153**:629–37.
5. Wada T, Iwamoto T, Maeda S. Genetic diversity of the *Mycobacterium tuberculosis* Beijing family in East Asia revealed through refined population structure analysis. *FEMS Microbiol Lett* 2009;**291**:35–43.
6. Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. *Trends Microbiol* 2002;**10**:45–52.
7. Hanekom M, van der Spuy GD, Streicher E, Ndabambi SL, McEvoy CR, Kidd M, et al. A recently evolved sublineage of the *Mycobacterium tuberculosis* Beijing strain family is associated with an increased ability to spread and cause disease. *J Clin Microbiol* 2007;**45**:1483–90.
8. Mokrousov I, Jiao WW, Sun GZ, Liu JW, Valcheva V, Li M, et al. Evolution of drug resistance in different sublineages of *Mycobacterium tuberculosis* Beijing genotype. *Antimicrob Agents Chemother* 2006;**50**:2820–3.
9. Abebe F, Bjune G. The emergence of Beijing family genotypes of *Mycobacterium tuberculosis* and low-level protection by bacille Calmette–Guérin (BCG) vaccines: is there a link? *Clin Exp Immunol* 2006;**145**:389–97.
10. Kremer K, van der Werf MJ, Au BK, Anh DD, Kam KM, van Doorn HR, et al. Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. *Emerg Infect Dis* 2009;**15**:335–9.
11. Reed MB, Domenech P, Manca C, Su H, Barczak AK, Kreiswirth BN, et al. A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature* 2004;**431**:84–7.
12. López B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. *Clin Exp Immunol* 2003;**133**:30–7.
13. The Research Institute of Tuberculosis JATA. Welcome to the Research Project for Surveillance, <http://www.jata.or.jp/rit/re/eprol_top.htm>; 2007.
14. McCurry J. Drinking too much sake in Osaka. *Lancet* 2005;**365**:375–6.
15. Barnes PF, el-Hajj H, Preston-Martin S, Cave MD, Jones BE, Otaya M, et al. Transmission of tuberculosis among the urban homeless. *Jama* 1996;**275**:305–7.
16. Nardell EA. Tuberculosis in homeless, residential care facilities, prisons, nursing homes, and other close communities. *Semin Respir Infect* 1989;**4**:206–15.
17. Potter B, Rindfleisch K, Kraus CK. Management of active tuberculosis. *Am Fam Physician* 2005;**72**:2225–32.
18. Raoult D, Foucault C, Brouqui P. Infections in the homeless. *Lancet Infect Dis* 2001;**1**:77–84.
19. Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN, et al. Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. *J Clin Microbiol* 2004;**42**:4040–9.
20. Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. *Mol Microbiol* 2000;**36**:762–71.
21. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006;**44**:4498–510.
22. Iwamoto T, Yoshida S, Suzuki K, Tomita M, Fujiyama R, Tanaka N, et al. Hypervariable loci that enhance the discriminatory ability of newly proposed 15-loci and 24-loci variable-number tandem repeat typing method on *Mycobacterium tuberculosis* strains predominated by the Beijing family. *FEMS Microbiol Lett* 2007;**270**:67–74.
23. Murase Y, Mitarai S, Sugawara I, Kato S, Maeda S. Promising loci of variable numbers of tandem repeats for typing Beijing family *Mycobacterium tuberculosis*. *J Med Microbiol* 2008;**57**:873–80.
24. Yokoyama E, Kishida K, Uchimura M, Ichinohe S. Improved differentiation of *Mycobacterium tuberculosis* strains, including many Beijing genotype strains, using a new combination of variable number of tandem repeats loci. *Infect Genet Evol* 2007;**7**:499–508.
25. Kremer K, Au BK, Yip PC, Skuce R, Supply P, Kam KM, et al. Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS *6110* restriction fragment length polymorphism typing and spoligotyping. *J Clin Microbiol* 2005;**43**:314–20.
26. Wada T, Maeda S, Hase A, Kobayashi K. Evaluation of variable numbers of tandem repeat as molecular epidemiological markers of *Mycobacterium tuberculosis* in Japan. *J Med Microbiol* 2007;**56**:1052–7.
27. Small PM, Hopewell PC, Singh SP, Paz A, Parsonnet J, Ruston DC, et al. The epidemiology of tuberculosis in San Francisco: a population-based study using conventional and molecular methods. *N Engl J Med* 1994;**330**:1703–9.
28. Durmaz R, Zozio T, Gunal S, Allix C, Fauville-Dufaux M, Rastogi N. Population-based molecular epidemiological study of tuberculosis in Malatya, Turkey. *J Clin Microbiol* 2007;**45**:4027–35.

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Virulence of *Mycobacterium avium* complex strains isolated from immunocompetent patients

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Pulmonary disease

ABSTRACT

Mycobacterium avium complex (MAC) disease has been increasing worldwide not only in immunocompromised but also in immunocompetent humans. However, the relationship between mycobacterial strain virulence and disease progression in immunocompetent humans is unclear. In this study, we isolated 6 strains from patients with pulmonary MAC disease. To explore the virulence, we examined the growth in human THP-1 macrophages and pathogenicity in C57BL/6 mice. We found that one strain, designated 198, which was isolated from a patient showing the most progressive disease, persisted in THP-1 cells. In addition, strain 198 grew to a high bacterial load with strong inflammation in mouse lungs and spleens 16 weeks after infection. To our knowledge, strain 198 is the first isolated MAC strain that exhibits hypervirulence consistently for the human patient, human macrophages *in vitro*, and even for immunocompetent mice. Other strains showed limited survival and weak virulence both in macrophages and in mice, uncorrelated to disease progression in human patients. We demonstrated that there is a hypervirulent clinical MAC strain whose experimental virulence corresponds to the serious disease progression in the patients. The existence of such strain suggests the involvement of bacterial virulence in the pathogenesis of pulmonary MAC disease in immunocompetent status.

1. Introduction

Mycobacterium avium complex (MAC) is the most common cause of human infection due to nontuberculous mycobacteria. Initially MAC was regarded as only an opportunistic pathogen, primarily in acquired immunodeficiency syndrome (AIDS) patients [1]; however, it has now been shown to cause progressive pulmonary disease even in immunocompetent humans [2]. The American Thoracic Society indicates a wide range of clinical manifestation in patients with non-AIDS MAC disease; some patients keep a stable condition for years, whereas others progress their illness rapidly [3]. Furthermore, MAC infection can be more difficult to treat than *M. tuberculosis* due to even fewer available anti-microbial agents [3].

The pathogenesis of MAC infection has been recently investigated with respect to the host immune response. Interferon-gamma (IFN-γ) activates macrophages to produce proteolytic enzymes and other metabolites, which exhibit mycobactericidal effects. Tumor necrosis factor-alpha (TNF-α), of which production is also stimulated by IFN-γ, augments the bactericidal capacity of macrophages and plays a key role in the induction of the acquired immune response against mycobacteria [4]. A defective IFN-γ response has been shown recently to cause disseminated MAC disease in IFN-γ knock out mice and in humans with genetic mutations of IFN-γ receptor [5,6] or autoantibodies to IFN-γ in some young non-AIDS patients [7,8]. In addition to that, the activity of interleukin-10 (IL-10), which is known to inhibit cytokine synthesis by IFN-γ-producing type1 helper T cells (Th1 cells), has been shown to increase susceptibility to MAC infection in immunocompetent mice [9].

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Besides genetic factors of the host, bacterial virulence should play an important role for the development of MAC disease. While isolates of *M. tuberculosis* are genetically homogeneous at the nucleotide level [10], MAC has high genetic diversity, including the presence of multiple plasmids [11], and thus likely to have a large corresponding diversity in virulence. In the most complete study examining virulence, forty-one MAC isolates from the environment as well as infected humans and animals were compared for virulence in C57BL/6 mice by intravenous injection [12]. Monitoring of the virulence by CFU counts in lungs, livers, and spleens over 4 months revealed three virulence phenotypes; high (logarithmically increasing load), intermediate (chronic infection at a constant load), and low (initial load increase followed by a decrease until clearance). In addition, clinical studies have suggested severe disease outcome in patients infected with some specific strain type of MAC. For example, MAC serovars 1, 4, and 8 *Mycobacterium avium* are associated with disease severity in AIDS patients [13], and a serovar 4 *M. avium* isolate from an AIDS patient was more invasive and proliferative in blood mononuclear cell-derived human macrophages than a serovar 2 strain from chickens [14]. In non-AIDS MAC disease, *Mycobacterium intracellulare* is associated with greater disease progression [15], and moreover, our previous prospective study on 68 non-AIDS patients suggests that serovar 4 *M. avium* is linked to greater disease progression with a pulmonary MAC infection [16]. Taking these previous data into consideration, we hypothesize that relatively hypervirulent MAC strains exist and may be associated with serious disease progression in immunocompetent patients. In order to elucidate the involvement of mycobacterial virulence in the pathogenesis of human pulmonary MAC disease, in this study we examined the difference of mycobacterial virulence of clinical isolates from patients with different disease types using human macrophages and immunocompetent mice.

2. Results

2.1. Characteristics of mycobacterial strains

Six clinical isolates of MAC were isolated from sputum of non-AIDS patients with pulmonary MAC disease, and designated 27, 33, 36, 198, 288, and 347 (Table 1). Strains 33, 198 and 288 were derived from patients with progressive disease against combination chemotherapy recommended by the American Thoracic Society guideline (progressive type) [3]. The patients with progressive disease exhibited higher levels of erythrocyte sedimentation rate (ESR), diffuse and severe pulmonary lesions in chest X-ray findings, and numerous bacteria in the sputum. The patient infected with strain 198 exhibited the most serious disease outcome among study patients in that a right pneumonectomy was needed to prevent disease progression. Strains 27, 36, and 347 were derived from patients with little progression of disease without chemotherapy (silent type). They exhibited lower levels of ESR, segmental pulmonary lesions in chest X-ray findings, and fewer bacteria in the sputum. The isolates belonging to the progressive type consisted of *M. intracellulare* unclassified serovar similar to serovar 12 (strain 198) and *M. avium* apolar type (strains 33 and 288). The isolates belonging to the silent type consisted of *M. intracellulare* serovar 1 (strain 27) and *M. avium* apolar type (strains 36 and 347). For comparison, we employed 2 veterinary strains of *M. avium* ATCC 25291 (serovar 2) as a highly virulent strain in mice [12] and ATCC 35767 (serovar 4) as a low virulent strain. Four clinical isolates other than strains 33 and 347, and ATCC 25291 formed the transparent colony morphology. Strain 33 produced both transparent and rough colony morphologies. Strain 347 and ATCC 35767 displayed smooth opaque colony morphology.

2.2. Growth of clinical isolates in 7H9 broth

All strains showed logarithmic growth from 3 days after culture in 7H9 broth (Table 2). At day 5, two isolates from progressive type (strains 198 and 288) and one isolate from silent type (strain 36) grew significantly slower than ATCC 25291 ($P < 0.005$), and all clinical strains grew significantly slower than ATCC 35767 ($P < 0.0001$). The growth of strain 198 at day 5 was significantly slower than that of strain 27 ($P = 0.001$), and was not significantly different from that of other clinical isolates.

2.3. Virulence of clinical isolates in THP-1 monocyte-derived macrophages

We next studied intracellular survival of the isolates. THP-1 cells, a human monocytic cell line, were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) and infected with MAC strains. Strain 198 grew in THP-1 cells significantly higher than any other strains during 7 days of infection ($P < 0.0001$) (Table 3). Strain 198 grew to approximately 20-fold during 2 days of infection ($P = 0.005$), and even at day 7, it kept the same level of bacterial load as day 0. Strain 36 also grew to approximately 2-fold during 2 days of infection ($P = 0.008$); however, it was rapidly eliminated at day 7, similar to the other strains except for strain 198. There was no significant difference in

Table 1
Characteristics of isolated strains and clinical findings.

Isolates	Species and serovar	Age	Sex	Duration of illness (years)	Erythrocyte sedimentation rate (mm/h)	Chest X-ray findings[a]	Sputum[b]	
							Smear	Culture
Progressive type								
33	*M. avium* apolar type	58	M	17	62	Advanced	2+	3+
198	*M. intracellulare* unclassified serovar[c]	62	F	3	108	Advanced	2+	2+
288	*M. avium* apolar type	56	F	12	78	Advanced	2+	2+
Silent type								
27	*M. intracellulare* serovar 1	67	F	17	50	Moderate	–	1+
36	*M. avium* apolar type	54	F	9	29	Moderate	–	1+
347	*M. avium* apolar type	79	F	14	50	Moderate	1+	1+

Data and sputum samples were collected at the enrollment of the study in 2003.

[a] Advanced chest X-ray findings were defined as bilateral cavities, giant cavities, or bilateral bronchiectasis, and moderate findings were defined as focal inflammation, small or fewer cavities, or mild bronchiectasis.

[b] Smear findings of sputum were defined as follows in high performance fields of microscopy; -: no bacteria in all fields, 1+: less than one bacteria in several fields, 2+: approximately 1–12 bacteria in one field. Culture findings were defined as follows using Ogawa egg agar; 1+: colonies less than 200, 2+: colonies more than 200 and less than 500, 3+: colonies more than 500 and less than 2000.

[c] The serovar of strain 198 was identified as a new type similar to serovar 12 determined by the liquid chromatography/mass spectrometry.