

Tokyo was better than Ag85A DNA in terms of protective efficacy against *M. tuberculosis*.<sup>2</sup> The spleen tissues from guinea pigs vaccinated with rBCG-Ag85A[Tokyo] or Ag85A DNA expressed IFN- $\gamma$  and IL-2 mRNA at significantly high levels.<sup>6</sup> This finding prompted us to explore further the efficacy of rBCG-Ag85A[Tokyo] in cynomolgus monkeys. We chose cynomolgus monkeys because this animal is reportedly protected more efficiently than rhesus monkeys by BCG vaccination.<sup>7</sup> Previous studies have shown that whereas the rhesus macaque is highly susceptible to *M. tuberculosis*, the closely related cynomolgus macaque is more resistant.<sup>8-10</sup> Cynomolgus monkeys are more efficiently protected by BCG vaccination than rhesus monkeys and therefore afford a good experimental model for the evaluation of new TB vaccine candidates.

Several TB vaccines are currently being tested using various models<sup>11,16-19</sup> and several recent reviews on TB vaccines have been published.<sup>12-15</sup> These include recombinant BCG vaccine expressing Ag85B, recombinant-modified vaccinia virus Ankara expressing Ag85A, TB polyprotein vaccine, Mtb72f, ESAT-6 subunit vaccine, auxotrophic vaccines for TB, and recombinant BCG overexpressing major extracellular proteins (rBCG30). However, there have been few reports on the efficacy of TB vaccine candidates in cynomolgus monkey models due to lower availability of monkey P3 facilities. Vaccination of cynomolgus monkeys with Ag85B-ESAT-6 reportedly induces protective immune responses.<sup>20</sup> DNA vaccine (HSP65+IL-12/HVJ) as well as 72f recombinant BCG provide better protective efficacy in cynomolgus monkeys.<sup>21</sup> In order to find a better TB vaccine, it is progression to the primate model after positive results in the small animal models. In the present investigation, we examined the efficacy of rBCG-Ag85A[Tokyo] in cynomolgus monkeys, and found that it induced higher protective efficacy than BCG Tokyo.

## Materials and methods

### Construction of recombinant BCG Tokyo (rBCG-Ag85A[Tokyo])

The Ag85A gene was amplified by PCR and subcloned into the pCR4 vector. The presence of the Ag85A gene was then confirmed by DNA sequencing. The gene was inserted into the pBBN vector (Ag85A-HA) possessing a hemagglutinin (HA) tag at its 5' end. At this stage, the Ag85A-HA was expressed in *Escherichia coli*, and then the Ag85A-HA gene was introduced into the downstream region of the pHPS integration vector. The vector was then electroporated into BCG Tokyo. The resulting transformants (rBCG-Ag85A[Tokyo]) were cultured individually and the content of the extracted lysate that contains Ag85 protein was confirmed by western blotting.<sup>6</sup>

### Bacterial strain

*M. tuberculosis* H37Rv (ATCC 25618) was passed through mice and grown once in 7H9 liquid medium before titration and storage in aliquots at  $-85^{\circ}\text{C}$ . The culture strain was filtered through a membrane filter (4- $\mu\text{m}$  pore size;

Millipore, Bedford, MA, USA) before use to ensure even dispersal.

### Monkeys

A total of 18 cynomolgus male monkeys (*Macaca fascicularis*) (5-7 kg, 6-8 years old) were used. All animals were housed at the animal biosafety level (ABSL) 3 facility of Wuhan University, Wuhan, China. The animals were studied in groups of six. Before the start of the studies, all animals were examined clinically and radiologically, and tuberculin skin-tested. For intratracheal challenge, animals were anesthetized with ketamine. Prior to commencement, experiments were reviewed and approved by the Wuhan University ethics committee.

### Inoculation of monkeys

The monkeys were randomly assigned to three groups. Group 1 (6 monkeys) received one intradermal injection of  $2 \times 10^6$  CFU/ml rBCG-Ag85A[Tokyo]. Group 2 received one intradermal injection of  $2 \times 10^6$  CFU/ml BCG Tokyo. Group 3 comprised 6 unvaccinated monkeys that received physiological saline as a control.

### Intratracheal infection of monkeys

Eight weeks after vaccination, the animals were challenged by intratracheal instillation of 1 ml (3000 CFU) of H37Rv *M. tuberculosis*. All animals were challenged on the same day with the same preparation, and were then observed for 4 months after infection. As PPD did not give better positive results to the monkeys, old tuberculin was used. The old tuberculin test (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was carried out 1, 2 and 3 months after infection. Briefly, 0.1 ml of old tuberculin solution was injected intradermally into the left palpebral skin and 0.1 ml saline was injected intradermally into the right palpebral skin. Two days later, swelling and redness on both sides were compared. When the diameter of redness is more than 10 mm, it was judged as +, and when more than 11 mm, it was judged as 2+. All animals were housed in animal biosafety level (ABSL) 3 facilities.

### Animal care

After infection, animals were observed daily by the animal caretakers for changes in behavior, eating and coughing. Weight, erythrocyte sedimentation rate (ESR) and temperature were recorded at times of blood sampling. Body temperature was measured rectally.

### Immunological examination

Blood from the femoral vein was used to obtain serum. TB Dot assay (Shanghai Upper Biotech and Pharma Co., Shanghai, China) was carried out according to the instruction sheet provided by the manufacturer. Briefly, two drops of blocking buffer were spotted on a TB blot membrane previously coated with 38-kDa *M. tuberculosis*-derived

antigen.<sup>22</sup> Then, 40 µl serum was added and semi-dried. Thereafter, six drops of washing buffer were added and semi-dried. Then, two drops of gold-labeled anti-human antibody solution were added and semi-dried. Finally, six drops of washing buffer were added and allowed to dry completely. When a reddish spot appeared, it was judged as positive and when no reddish spot appeared, it was judged as negative.

### Bacterial enumeration

Just after death of the unvaccinated monkeys, 10 pieces from the upper and lower lobes of lungs, and also spleen tissue, about 0.5 cm<sup>3</sup> in size were taken randomly. For the vaccinated monkeys, similar samples were taken randomly at necropsy. After being weighed, the samples were combined, homogenized and diluted with physiological saline. For the vaccinated groups, pyrazinamide (200 µg/20 µl) was added to determine BCG Tokyo-derived colonies (background count). Pyrazinamide kills *M. tuberculosis*, but does not kill BCG Tokyo. Triplicate 10-fold dilutions were incubated for 4 weeks in 1% Ogawa solid slant agar and the number of colonies was counted. To examine *M. tuberculosis*-derived colonies, the background count was subtracted from the number of colonies. The lung and spleen tissues were weighed and the results were expressed as CFU ± SD/whole organ.

### Histopathology

Necropsies were undertaken on unvaccinated monkeys just after death and on vaccinated monkeys after euthanasia. The removed organs were fixed with 15% formalin for 10 days. Tissue sections from paraffin blocks containing lung, spleen, hilar lymph nodes and liver were stained with hematoxylin and eosin or the Ziehl-Neelsen method for acid-fast bacilli. The severity of pulmonary lesions was judged independently by two experts (I.S. and T.U.).

### Statistical analysis

We performed analysis of variance (ANOVA) for repeated measurements using the baseline results at screening as a covariate on log-transformed data to compare between groups.

## Results

### Clinical course

The monkeys vaccinated with rBCG-Ag85A[Tokyo] or parental BCG Tokyo and their non-vaccinated controls were infected intratracheally with H37Rv *M. tuberculosis*. No coughing was observed in the animals after challenge. None of the vaccinated animals gained weight during the infection period. Their ESR was within the normal range (1–2 mm/h). None of the non-vaccinated animals showed an appreciable increase in body weight. Two of the monkeys (17 and 18) showed a gradual decrease in weight (50 g). The other four non-vaccinated control animals showed a severe decrease in

weight of 550–800 g, and their ESR was higher than the reference value (55, 10, 8 and 10 mm/h)(Table 1).

On radiographs of the chest, the non-vaccinated animals exhibited early development of multilobar pneumonia in the right lung and rapid progression to bilateral pneumonia. Lobar consolidation and atelectasis in the involved lungs and hilar lymphadenopathy were observed frequently in the non-vaccinated groups (Table 1).

In the vaccinated groups, pneumonia was slight to mild (Figure 1).

### Immunological responses

Two immunological methods (the old tuberculin test and serum TB diagnosis) were utilized to clarify the severity of *M. tuberculosis* infection. In the vaccinated monkeys 2 months after infection, the old tuberculin test gave a positive result (+). In the non-vaccinated animals, the reaction was strongly positive (2+) and marked palpebral reddish skin swelling was observed.

The TB dot assay gave a negative result in all vaccinated and non-vaccinated animals 1 month after infection. However, 2 months after infection, the result was positive in the vaccinated monkeys, and strongly positive (2+) in the non-vaccinated monkeys (Table 1).

### Gross pathology and histopathology of the vaccinated and non-vaccinated monkeys

At necropsy, all unvaccinated animals showed extensive bilateral lung pathology characterized by the presence of multiple granulomas. These granulomas showed conglomeration to larger caseous areas, especially in the hilar region. Granulomas were also present in the liver and spleen. In the vaccinated animals, a few small granulomas were evident, but these showed no caseous changes. Small liver granulomas were noted in two of the BCG Tokyo-vaccinated monkeys, but such granulomas were not observed in the recombinant BCG-vaccinated monkeys. Four of the non-vaccinated monkeys died of advanced TB 50, 67, 70 and 84 days after infection (Figure 2). These were necropsied just after death for further examination.

On microscopic examination, the non-vaccinated animals showed multifocal, coalescing granulomas with central necrosis and pronounced cellular infiltrates in the periphery (Figure 3). The vaccinated animals showed markedly less severe histopathology. In particular, the peripheral inflammatory cell infiltration was notably more pronounced in the unvaccinated than in the vaccinated animals. Histological examination of the animals that had received the recombinant BCG (Ag85A) showed almost normal lung tissue without granulomas in five of them. The remaining vaccinated animal showed a solitary small granuloma without central necrosis. Two BCG Tokyo-vaccinated animals showed a single small granuloma (Table 1).

### Replication of tubercle bacilli in the lung and spleen tissues of vaccinated and non-vaccinated monkeys

At autopsy, 10 different pieces of lung and spleen tissue were taken for determination of CFU. Background culture

**Table 1** Summary of the monkey experiments.

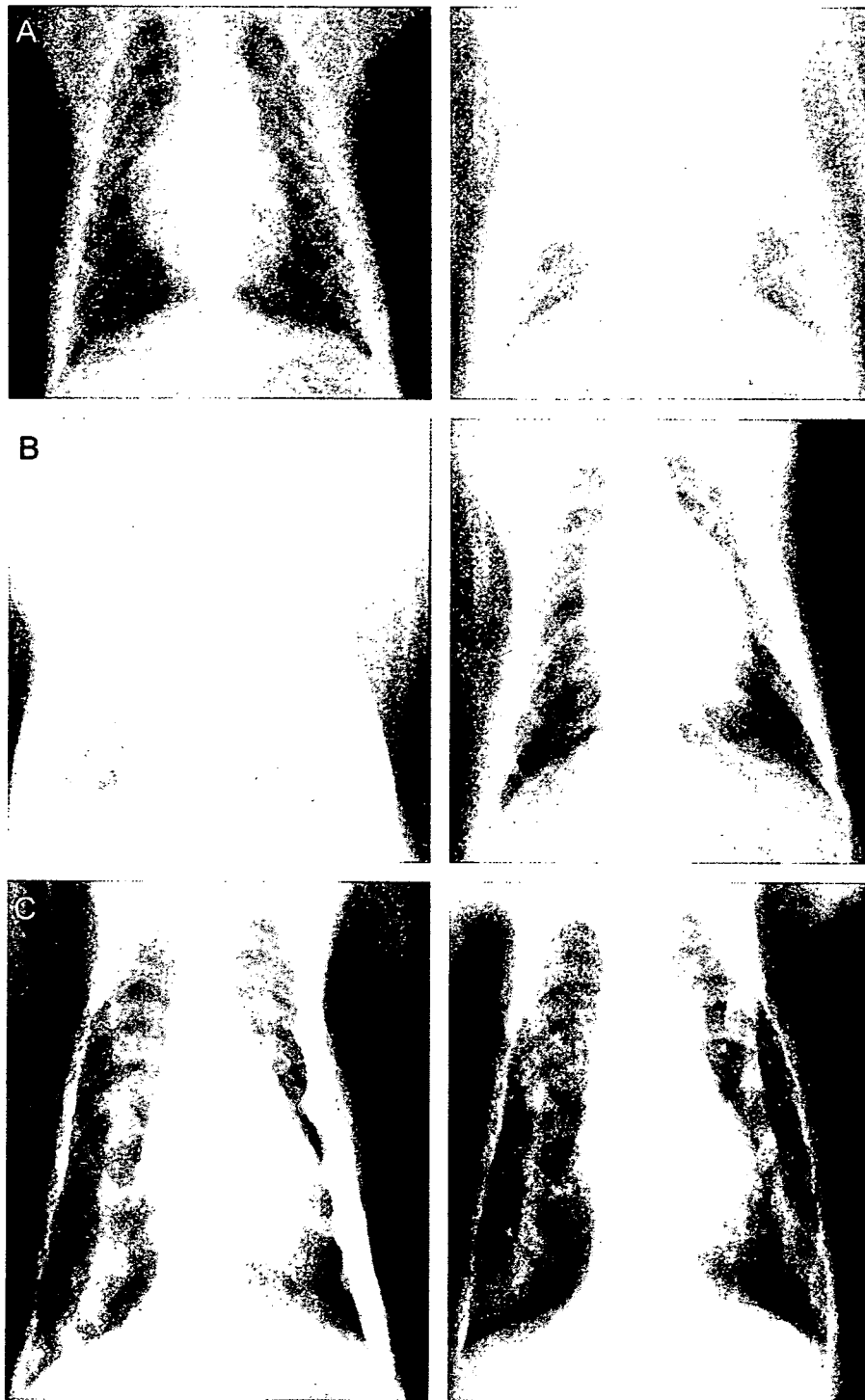
Monkey no.	Vaccination	Gross pathology	Lung histopathology	ESR (mm/h, 2 months)	Old tuberculin (2 months)	Serum diagnosis (1 month, 2 months)	Chest X-ray before death or necropsy
1	rec BCG	Lung, spleen, LN	Solitary granuloma	2	+	-, +	Slight pneumonia
2	rec BCG	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
3	rec BCG	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
4	rec BCG	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
5	rec BCG	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
6	rec BCG	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
7	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
8	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
9	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
10	BCG Tokyo	Lung, spleen, LN, liver	Solitary granuloma	3	+	-, +	Slight pneumonia
11	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	-, +	Slight pneumonia
12	BCG Tokyo	Lung, spleen, LN, liver	Solitary granuloma	2	+	-, +	Slight pneumonia
13	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	55	2+	-, 2+	Severe pneumonia, consolidation
14	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	10	2+	-, 2+	Severe pneumonia, consolidation
15	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	8	2+	-, 2+	Severe pneumonia, consolidation
16	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	10	2+	-, 2+	Severe pneumonia, consolidation
17	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	5	2+	-, 2+	Severe pneumonia, consolidation
18	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	6	2+	-, 2+	Severe pneumonia, consolidation

Criteria of old tuberculin test and serum diagnosis: +, positive; and 2+, strongly positive. rec BCG; recombinant BCG.

CFU of BCG Tokyo or rBCG-Ag85A[Tokyo] after pyrazinamide was added to the tissue homogenates was one or two. The lung tissue of animals vaccinated with recombinant BCG showed a significant 1000-fold decrease in the number of bacteria compared to the non-vaccinated animals ( $p < 0.01$ ). The number of CFU in BCG Tokyo-vaccinated animals after 16 weeks of infection was reduced 100-fold relative to that in the non-vaccinated animals ( $p < 0.01$ ). There was a statistically significant difference in the number of pulmon-

ary CFU between recombinant BCG-vaccinated and BCG Tokyo-vaccinated animals ( $p < 0.01$ ) (Figure 4).

A similar tendency was also observed in the number of splenic CFU. The spleen tissues of animals vaccinated with recombinant BCG or BCG Tokyo showed a significant 1000-fold decrease in the number of bacteria compared to the non-vaccinated animals ( $p < 0.01$ ). However, there was no significant difference in the number of splenic CFU between recombinant BCG-vaccinated and BCG-vaccinated animals.



**Figure 1** Chest radiologic examinations after challenge with H37Rv *M. tuberculosis*. (A) The monkey (No. 2) vaccinated with rBCG-Ag86A[Tokyo]. The chest X-ray picture was taken 1 day before necropsy. (B) The monkey (No. 9) vaccinated with parental BCG Tokyo. The chest X-ray picture was taken 1 day before necropsy. (C) The non-vaccinated monkey (No. 13) 2 days before death. After H37Rv challenge, the non-vaccinated monkeys rapidly developed extensive bronchopneumonia. Many nodular shadows (→) were recognized, but the vaccinated monkeys had negative chest X-ray findings 3 months after H37Rv challenge.

## Discussion

In the present study, we have demonstrated that vaccination of cynomolgus monkeys with recombinant BCG (Ag85A)(rBCG-Ag85A[Tokyo]) induces protection against

infection with H37Rv *M. tuberculosis*. In addition to measurement of protection in terms of reduction in bacterial number and/or lung pathology, we have also shown that recombinant BCG vaccination prevented the development of a number of important clinical and

immunological changes during infection. These changes included an increase of the ESR and the development of strong immune responses to a wide spectrum of mycobacterial antigens (old tuberculin). When we inoculated monkeys once with  $2 \times 10^6$  CFU rBCG-Ag85A[Tokyo], there was a significant reduction of CFU in lung and spleen tissues compared to that in BCG Tokyo-inoculated monkeys. We showed for the first time that the H37Rv strain could also be used for intratracheal infection instead of the Erdman strain. Many researchers use the Erdman strain (1000CFU or more) for optimal intratracheal infection.<sup>10,16,17</sup> We chose 3000CFU as the dose for the H37Rv strain because it is less virulent than Erdman strain, and we were recommended to use H37Rv instead of the Erdman strain at the Animal Biosafety Level 3 Facility of Wuhan University.

When parental BCG Tokyo was used for vaccination, we found several grayish tubercles in the liver in two of six monkeys, but no such tubercles were evident in monkeys

vaccinated with recombinant BCG. Moreover, there was a significantly lower number of CFU in lung tissues of monkeys vaccinated with recombinant BCG than in monkeys vaccinated with BCG Tokyo ( $p < 0.01$ ). Taken together, the results suggest that the recombinant BCG bearing the introduced Ag85A gene gives better protective efficacy than BCG Tokyo. However, to evaluate the efficacy of the Ag85A antigen carefully, it will be necessary to lower the dose of recombinant BCG because  $2 \times 10^6$  CFU BCG Tokyo alone is still sufficiently effective.

TB dot assay, which targets the 38-kDa antigen from *M. tuberculosis*, gave a negative result 1 month after infection, but a positive one 2 months after infection. Therefore, care is needed when diagnosing TB in the early phase. The old tuberculin test may be more useful for early-phase TB diagnosis.

We selected Ag85A as a promising immunogen because the protein from *M. tuberculosis* induces significant humoral

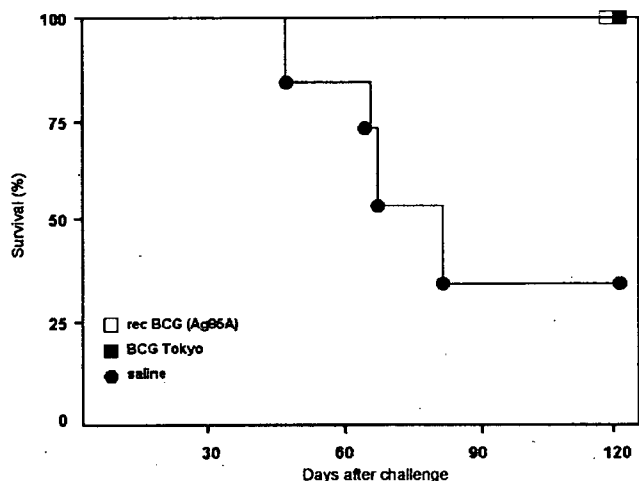


Figure 2 Mortality curve after challenge. Animals in groups of six were challenged by intratracheal inoculation with 3000 CFU of H37Rv. Non-vaccinated animals (six per group) were inoculated with saline.

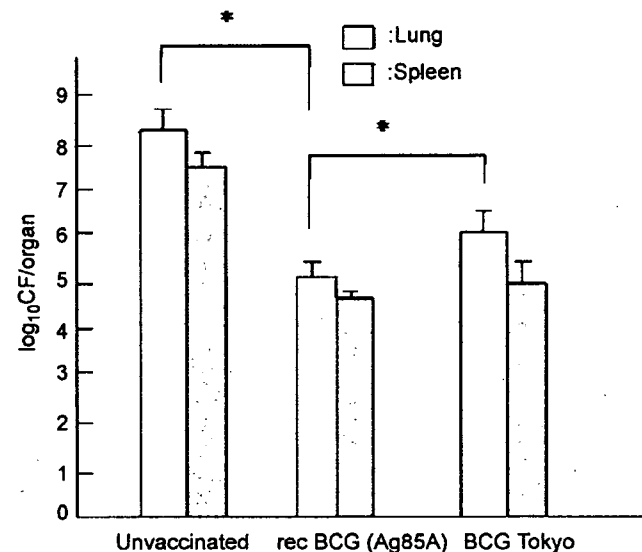


Figure 4 CFU counts in lung and spleen tissues of H37Rv *M. tuberculosis*-infected cynomolgus monkeys vaccinated with recombinant BCG Tokyo (Ag85A), and in non-vaccinated controls. \*Statistical difference at  $p < 0.01$ .

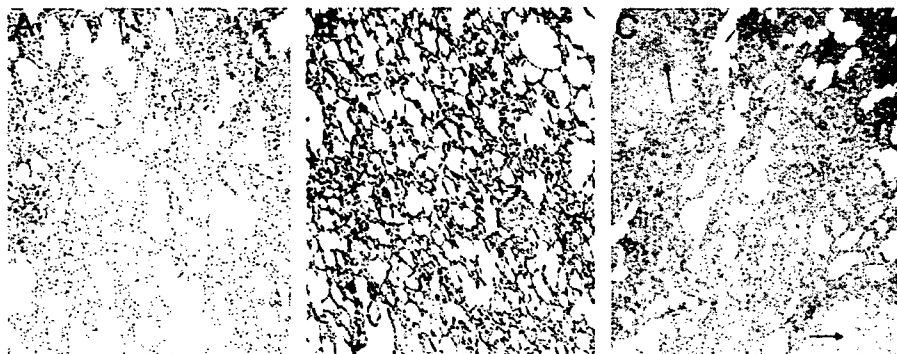


Figure 3 Histopathology of lung tissues from *M. tuberculosis*-infected monkeys vaccinated with rBCG-Ag85A[Tokyo] (A), BCG Tokyo (B) or non-vaccinated controls (C) at necropsy. The miliary granulomas with caseating necrosis are surrounded peripherally by a dense infiltrate of epithelioid cells and lymphocytes (C), but in the vaccinated monkeys interstitial pneumonia is evident (A and B)  $\times 100$ . Hematoxylin and eosin stain.

and cell-mediated immune responses.<sup>23,24</sup> The expression levels of IFN- $\gamma$  and IL-2 mRNAs were increased in spleen tissues from guinea pigs that had been vaccinated with parental BCG Tokyo, rBCG-Ag85A[Tokyo], and Ag85A DNA vaccine. Among them, the expression levels of IFN- $\gamma$  and IL-2 mRNAs were the highest in rBCG-Ag85A[Tokyo].<sup>6</sup> Furthermore, the sera from the rBCG-Ag85A[Tokyo]-vaccinated guinea pigs reacted with Ag85A peptide we used in our previous study significantly (data not shown). We have shown previously that vaccination with Ag85A DNA twice by gene gun bombardment or with rBCG-Ag85A[Tokyo] once significantly reduced the severity of pulmonary pathology and the number of CFU in guinea pigs.<sup>5,6</sup> When the immunogenic synthetic Ag85A peptide was further used as a booster together with recombinant BCG (Ag85A), lung pathology was improved significantly, together with a significant reduction in the number of pulmonary CFU.<sup>6</sup> Although a single intradermal inoculation of  $2 \times 10^6$  CFU BCG (Ag85A) was enough to induce protective efficacy in the present study, it would be desirable to use Ag85A peptide as a booster, Ag85B-ESAT-6 fusion protein and 72f fusion protein in combination with recombinant BCG Tokyo (Ag85A) to achieve much better protective efficacy.<sup>6,18</sup>

In summary, we have shown that vaccination of primates with rBCG-Ag85A[Tokyo] induces good protective immune responses. Using the macaque challenge model, further optimization of the dose and timing, and use of a booster, may well lead to levels of protection that are better than those achieved with BCG.

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**Ethical Approval:** Not required

## References

- Clemens JD, Chuong JJ, Feinstein AR. The BCG controversy. A methodological and statistical reappraisal. *JAMA* 1983;249:2362–9.
- Zodpey SP. The BCG controversy: a reappraisal of the protective effect against tuberculosis and leprosy. *Indian J Public Health* 2004;48:70–7.
- Haile M, Kallenius G. Recent developments in tuberculosis vaccines. *Curr Opin Infect Dis* 2005;18:211–5.
- Andersen P, Doherty TM. The success and failure of BCG—implications for a novel tuberculosis vaccine. *Nat Rev Microbiol* 2005;3:656–62.
- Sugawara I, Yamada H, Udarawa T, Huygen K. Vaccination of guinea pigs with DNA encoding Ag85A by gene gun bombardment. *Tuberculosis* 2003;83:331–7.
- Sugawara I, Udagawa T, Taniyama T. Protective efficacy of recombinant (Ag85A) BCG Tokyo with Ag85A peptide boosting against *Mycobacterium tuberculosis*-infected guinea pigs in comparison with that of DNA vaccine encoding Ag85A. *Tuberculosis* 2007;87:94–101.
- Langermans JA, Andersen P, van Soolingen D, Vervenne RA, Frost PA, vander Laan T, et al. Divergent effect of bacillus Calmette–Guerin (BCG) vaccination on *Mycobacterium tuberculosis* infection in highly related macaque species: implications for primate models in tuberculosis vaccine research. *Proc Natl Acad Sci USA* 2001;98:11497–502.
- Good RC. Biology of the mycobacterioses Simian tuberculosis: immunologic aspects. *Ann NY Acad Sci* 1968;154:200–13.
- Ribi E, Anacker RL, Barclay WR, Brehmer W, Harris SC, Leif WR, et al. Efficacy of mycobacterial cell walls as a vaccine against airborne tuberculosis in the Rhesus monkey. *J Infect Dis* 1971;123:527–38.
- Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, et al. The Philippine cynomolgus monkey (*Macaca fascicularis*) provides a new nonhuman primate model of tuberculosis that resembles human disease. *Nat Med* 1996;2:430–6.
- Chambers MA, Williams A, Hatch G, Gavier-Widen D, Hall G, Huygen K, et al. Vaccination of guinea pigs with DNA encoding the mycobacterial antigen MPB83 influences pulmonary pathology but not hematogenous spread following aerogenic infection with *Mycobacterium bovis*. *Infect Immun* 2002;70:2159–65.
- Reed S, Lobet Y. Tuberculosis vaccine development; from mouse to man. *Microbes Infect* 2005;7:922–31.
- Dietrich J, Lundberg CV, Andersen P. TB vaccine strategies—what is needed to solve a complex problem? *Tuberculosis* 2006;86:163–8.
- Skeiky YA, Sadoff JC. Advances in tuberculosis vaccine strategies. *Nat Rev Microbiol* 2006;4:469–76.
- Kaufmann SH, Baumann S, Nasser Eddine A. Exploiting immunology and molecular genetics for rational vaccine design against tuberculosis. *Int J Tuberc Lung Dis* 2006;10:1068–79.
- Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. Recombinant bacillus Calmette–Guerin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc Natl Acad Sci USA* 2000;97:13853–8.
- Skeiky YAW, Alderson MR, Ovendale PJ, Guderian JA, Brandt L, Dillon DC, 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* 2004;172:7618–28.
- Brandt L, Skeiky YA, Alderson MR, Lobet Y, Dalemans W, Turner OC, et al. The protective effect of the *Mycobacterium bovis* BCG vaccine is increased by coadministration with the *Mycobacterium tuberculosis* 72-kilodalton fusion polyprotein Mtb72F in *M. tuberculosis*-infected guinea pigs. *Infect Immun* 2004;72:6622–32.
- Guleria I, Teitelbaum R, McAdams RA, Kaplan G, Jacobs Jr WR, Bloom BR. Auxotrophic vaccines for tuberculosis. *Nat Med* 1996;2:334–7.
- Langermans JAM, Doherty TM, Vervenne RAW, van der Laan T, Lyashchenko K, Greenwald R, et al. Protection of macaques against *Mycobacterium tuberculosis* infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. *Vaccine* 2005;23:2740–50.
- Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, et al. Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. *Vaccine* 2005;23:2132–5.

22. Anderson AB, Brennan P. Proteins and antigens of *Mycobacterium tuberculosis*. In: Bloom BR, editor. *Tuberculosis. Pathogenesis, protection, and control*. ASM Press; 1994. p. 307–32.
23. Kamath AT, Feng CG, MacDonald M, Briscoe H, Britton WJ. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. *Infect Immun* 1999;67:1702–7.
24. Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, Deck RR, et al. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat Med* 1996;2:893–8.



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## Anti-tuberculosis drug susceptibility testing of *Mycobacterium bovis* BCG Tokyo strain

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### SUMMARY

**SETTING:** The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine is the only vaccine against tuberculosis (TB), owing to its valuable protective effects and low virulence. However, it can occasionally cause systemic infection in immunocompromised hosts. Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are known to be effective anti-tuberculosis drugs and are used for the treatment of BCG infections. Unfortunately, there are few studies of the susceptibility of BCG vaccine strains to these drugs.

**OBJECTIVE:** To measure the minimum inhibitory concentrations (MICs) of BCG Tokyo vaccine products for anti-tuberculosis drugs and assess vaccine safety in terms of drug susceptibility.

**DESIGN:** We measured the MIC for one seed and five product lots of BCG Tokyo strain for INH, RMP, SM and EMB using Middlebrook 7H11 agar plates.

**RESULTS:** The MIC results for INH were 0.06 and 0.125 µg/ml for the product and seed lots, respectively. The MIC results for RMP, SM and EMB were 0.25–0.5, 0.25 and 2–4 µg/ml, respectively.

**CONCLUSION:** Our results indicate that the BCG Tokyo strain was susceptible to the major anti-tuberculosis drugs and treatable even in cases of severe adverse events, including systemic infection.

**KEY WORDS:** BCG; minimum inhibitory concentration; drug susceptibility

TUBERCULOSIS (TB) is an infectious disease of international importance that remains a major life-threatening disease worldwide. It is estimated that approximately one third of the world's population is infected with *Mycobacterium tuberculosis*. Every year, approximately 9 million people develop active disease and 1.7 million die of TB.<sup>1</sup>

Bacille Calmette-Guérin (BCG) vaccines are safe, attenuated live bacteria and have been shown to have valuable protective effects against TB. The BCG Tokyo strain is recognised as a low virulence strain among all BCGs,<sup>2</sup> and is widely used in several countries as a vaccine strain. If used properly, it protects against the development of TB and the dissemination of TB bacilli. Few severe complications have been reported.<sup>3</sup> However, systemic BCG infection may occur frequently when it is administered to immunocompromised hosts with congenital or acquired immunodeficiency such as human immunodeficiency virus (HIV) infection.<sup>4,5</sup> BCG is contraindicated in symptomatic HIV diseases. When general BCG infection occurs, patients are treated empirically using anti-tuberculosis drugs because there is limited information about the

drug susceptibility of BCG strains. It is therefore very important to evaluate the drug susceptibility of BCG Tokyo strain to ensure the safety of the vaccine.

Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are the first-line anti-tuberculosis drugs most commonly used in standard TB treatment regimens. These drugs are currently available even in developing countries. The present study aimed at measuring the minimum inhibitory concentrations (MICs) of these drugs against the BCG Tokyo strain to estimate the effect of clinical treatment in case of infection by the BCG Tokyo strain.

### MATERIALS AND METHODS

#### *BCG Tokyo strain*

Five lots of vaccine product (number 1003 as 'Lot A', 1960 as 'Lot B', 1036 as 'Lot C', 1061 as 'Lot D', 1998 as 'Lot E') and one seed lot were provided by the Japan BCG Laboratory (Tokyo, Japan) and used in this study. These vaccines were produced by Japan BCG Laboratory for vaccination from the seed lot in 2004. The experiment was carried out in a type II-B

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#### Minimum inhibitory concentrations

The MICs were measured modifying the proportion method described in M24-A of the Clinical and Laboratory Standards Institute (CLSI, former National Committee for Clinical Laboratory Standards) and in previous reports.<sup>6,7</sup> The following procedure was used: lyophilised BCG Tokyo products were suspended in 1 ml of distilled water and were cultured on Middlebrook 7H10 agar (DIFCO, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC: BBL Prepared Culture Media, Becton Dickinson) at 37°C until sufficient growth was observed. After harvesting colonies from culture media, each lot strain of BCG Tokyo was dispersed by vortex mixing with glass beads (dispenser tube: Nichibi, BCG Laboratory, Tokyo, Japan) and two drops of 10% Tween 80 (LC-MS, Santa Fe, CA, USA). After vortex mixing for 30 s, 1 ml of distilled water was added to each sample and they were vortexed again for 10 s. The supernatant of each bacterial suspension was transferred to 10 ml of Middlebrook 7H9 broth supplemented with albumin, dextrose and catalase (BBL Prepared Culture Media, Becton Dickinson), and the suspension density was adjusted to an optical density (OD) of 0.05 at 530 nm. These culture tubes were incubated at 37°C with daily mixing and OD checking. When the OD reached 0.2, they were used as the original bacterial suspension.

To prepare 10<sup>-2</sup> dilutions, a 100 µl aliquot was transferred into 10 ml of distilled water. In a similar way, 100 µl of the 10<sup>-2</sup> dilution was added to 10 ml of distilled water for 10<sup>-4</sup> dilutions. One hundred microlitres of the 10<sup>-2</sup> dilution were inoculated onto Middlebrook 7H11 agar plates with anti-tuberculosis drugs at the designated concentrations. Final INH concentrations were 0.03, 0.06, 0.125, 0.5, 1.0 and 2.0 µg/ml. RMP (0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 µg/ml), SM (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) and EMB concentrations (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) were adjusted accordingly. The 10<sup>-2</sup>

and 10<sup>-4</sup> suspensions were inoculated onto Middlebrook 7H11 medium containing no drugs for growth control and 1% proportion measurements. These plates were incubated at 37°C. When the 10<sup>-2</sup> dilution control showed sufficient growth (>100 visible colonies), the MICs were measured as the lowest concentration of drug that inhibited more than 99% of the bacterial population compared with the number of colonies on drug-containing media and the 10<sup>-4</sup> growth control. Each test was performed in triplicate.

#### RESULTS

The MICs of one seed and five product lots were measured in triplicate. The MICs of the anti-tuberculosis drugs varied slightly with the lots tested, but were identical among the triplicate tests. The MICs for all tested drugs are shown in the Table. The MICs of INH were 0.06 µg/ml and the seed lot MIC was 0.125 µg/ml. The MIC in test 3 of lot A was not determined due to contamination. For RMP, the MICs for lots A, B and C were 0.25 µg/ml; those for lots D and E were 0.5 µg/ml. It was considered that the MICs of RMP were between 0.25 and 0.5 µg/ml. For SM, the MICs were determined to be 0.25 µg/ml in all tests. For EMB, the MICs were 4 µg/ml for lots A, B and C, while the MICs for lots D and E were 2 µg/ml. The MIC of EMB was 2–4 µg/ml.

#### DISCUSSION

The BCG vaccine was developed by Calmette and Guérin in 1921. All BCG vaccines consist of live attenuated *Mycobacterium bovis* bacteria. BCG vaccination is commonly performed on neonates and infants once or twice in middle to high tuberculosis prevalence countries, and more than 100 million children have received BCG in recent years.<sup>8</sup> Its safety is therefore a priority issue.

BCG vaccination may sometimes cause complications as a pathogen. Local adverse effects of BCG vaccination have at times been observed and usually improve spontaneously, although severe complications in immunocompromised patients have been reported. McKenzie et al. reported systemic haematological dis-

**Table** MIC values of four first-line drugs for the BCG Tokyo strain

Samples	MIC (µg/ml)											
	INH			RMP			SM			EMB		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Lot A	0.06	0.06	cont	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot B	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot C	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot D	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Lot E	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Seed lot	0.125	0.125	0.125	ND	ND	ND	ND	ND	ND	ND	ND	ND

MIC = minimum inhibitory concentration; BCG = bacille Calmette-Guérin; INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol; cont = contaminated; ND = not done.

semination of BCG in a child with X-linked severe combined immunodeficiency.<sup>9</sup> Puthanakit et al. reported four cases of BCG infection in HIV-positive children receiving BCG vaccinations at birth; the strain was not indicated.<sup>10</sup>

BCG strains have also been utilised for immunotherapy in addition to TB prevention. BCG is injected into the urinary bladder for intravesical instillation therapy in the early stages of bladder carcinoma.<sup>11,12</sup> The BCG Tokyo strain is popular for such adjuvant therapy in Japan,<sup>13</sup> whereas the Connaught strain is popular in other parts of the world. In a recent study, Mugiyā et al. described good, complete response rates of 84% with BCG Tokyo (40 mg administered every 6 weeks) against bladder carcinoma in situ.<sup>14</sup> However, adverse reactions can also occur after instillation therapy. Eichel et al. reported INH-resistant BCG cystitis successfully treated with RMP and EMB.<sup>15</sup>

There is at present no recommended treatment regimen for BCG infection. Anti-tuberculosis drugs are the most potent agents for treating BCG infection. Drug susceptibility testing (DST) of BCG strains has been reported using different methods. Durek et al. evaluated the Connaught BCG strain using a BACTEC 460TB system (Becton Dickinson).<sup>16,17</sup> DST was performed for 31 drugs, including INH, RMP, SM, EMB and rifabutin. The BCG Connaught strain was susceptible to all of the anti-tuberculosis drugs except pyrazinamide (PZA) (BCG has natural/intrinsic resistance to PZA) and some other drugs used for general bacterial infections. The BACTEC 460 TB system employs critical drug concentrations of 0.1, 1.0, 2.0 and 2.5 for INH, RMP, SM and EMB, respectively. Rousseau and Dupuis reported the DST for a seed lot of the BCG Montreal strain by using solid Dubos medium.<sup>18</sup> They showed that this strain was sensitive to INH (0.2 µg/ml), RMP (1.0 µg/ml), SM (2.0 µg/ml) and EMB (5.0 µg/ml). These reports are not, however, comparable because of the differences in testing methods. There is no standard method for the DST of BCG; however, they may be equivalent to each other in the concept of detecting 1% resistance in the strain population. The proportion method with Middlebrook 7H11/OADC media, which is commonly used for the DST of *M. tuberculosis*, was used for this study.

The MICs indicated in the present study were lower than the critical concentrations employed in the previous studies, except for EMB with MIC close to the critical concentration of BACTEC. In the previous studies, the MICs of EMB to *M. tuberculosis* vary between 0.5 µg/ml and 2.0 µg/ml,<sup>19,20</sup> in 7H12 BACTEC broth MIC varies between 0.95 and 3.8 µg/ml and on 7H10 agar between 1.9 and 7.5 µg/ml.<sup>21</sup> Heifets proposed possible guidelines for the interpretation of MIC to *M. tuberculosis* determined in Middlebrook 7H12 broth (radiometric), and MIC 4.0 µg/ml of EMB as moderately susceptible.<sup>22</sup> It is possible that the MIC of BCG Tokyo strain for EMB was higher than wild

type *M. tuberculosis*. However, these reports show the tendency of lower MIC in liquid media than solid media. The plasma concentration ( $C_{max}$ ) of EMB reaches 2.0–5.0 µg/ml<sup>23</sup> and EMB generally works in a time-dependent manner. For this reason it is suggested that EMB could be effective. Although BCG and *M. tuberculosis* are different species, these MICs and pharmacokinetic data would support the potentials of EMB for the treatment of BCG infection. It was therefore considered that, like the BCG Montreal and Connaught strains, the BCG Tokyo strain is susceptible to the four major anti-tuberculosis drugs.

Hesseling et al. reported that BCG in an HIV co-infected infant who received a BCG Danish 1331 strain vaccination developed INH and RMP resistance following treatment with INH and RMP.<sup>24</sup> The MICs of the original strain were 0.15 and <0.4 µg/ml for INH and RMP, respectively. However, they had risen above 0.3 and 32 µg/ml after treatment. These results suggest that the strain was already clinically resistant to INH (MIC 0.15 µg/ml for INH), and monotherapy with RMP against BCG resulted in RMP resistance. Su et al. reported two general disseminated cases of the BCG Tokyo vaccine strains.<sup>5</sup> One of them was treated using anti-tuberculosis drugs (INH, RMP, SM and EMB) based on the susceptible DST results, and the patient recovered. Another case died following one month's treatment with INH, RMP and EMB. However, no DST data were shown in the mortality case and the infant seemed to have died from severe combined immunodeficiency. The MICs of the BCG Tokyo strain indicated in this study were considered less than or equivalent to those of the previous cases, so it was estimated that BCG Tokyo could be treated successfully even in severe adverse events such as systemic dissemination.

The reason why BCG strains have different phenotypic characteristics with respect to drug susceptibility is not clear. BCG has lost several regions of difference (RD) compared to *M. bovis* as the ancestral strain. In particular, the RD1 deletion made a significant contribution to the attenuation of BCG.<sup>24–26</sup> RD1 encodes a 6 kDa early secreted antigenic target protein (ESAT-6)<sup>27</sup> and a 10 kDa culture filtrate protein (CFP-10)<sup>28</sup> associated with virulence in *M. tuberculosis* complex. The BCG vaccine therefore has attenuated virulence compared to wild *M. bovis* strains. The loss of virulence apparently occurred through repeated passages.

The BCG strains were originally donated by the Pasteur Institute (Paris, France), and have been subcultured by several tuberculosis institutes around the world (Russia, Brazil, Sweden, Denmark, Japan, etc.) since 1924. The donated BCG strains differ from the original BCG strains due to differences in passage cultivation, culture medium and storage conditions. In 1972, Hesselberg found that a Swedish/Norwegian BCG strain became resistant to INH during the period 1953–1964, which was the reason why the serial sub-

culture system was discontinued and a seed lot system was adopted.<sup>29</sup> However, in 2003, low-grade INH-resistant (MIC >0.5 µg/ml) Danish 1331 strains were reported again to the World Health Organization (WHO). The WHO therefore recognises the necessity of a new quality assurance method for BCG vaccines.<sup>30,31</sup>

The BCG Tokyo strain was obtained from Calmette in the Pasteur Institute in 1924. Passage cultivation of BCG Tokyo strain has been performed strictly according to Calmette's original instructions, while some of the other BCG strain passages were tailored to each institute's needs. The BCG Tokyo 172 strain, which has undergone 172 passages since the Second World War II, has been used as the seed lot for lyophilised BCG Tokyo vaccines. In this study, the BCG Tokyo strain proved to be susceptible to the major anti-tuberculosis drugs; however, the results of this study do not apply to all BCG substrains. It will be necessary to ensure the safety of BCG vaccine by checking susceptibility to other antimicrobial agents.

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#### References

- World Health Organization. WHO report 2005. Global tuberculosis control: surveillance, planning, financing. WHO/HTM/TB/2005.349. Geneva, Switzerland: WHO, 2005.
- Landi S, Barbara C, Przykuta K, Held H R. Comparison of freeze-dried vaccines prepared from four different strains of BCG. *Dev Biol Stand* 1977; 38: 19–28.
- Okazaki T, Ebihara S, Takahashi H, Asada M, Sato A, Seki M. Multiplex PCR-identified cutaneous tuberculosis evoked by *Mycobacterium bovis* BCG vaccination in a healthy baby. *J Clin Microbiol* 2005; 43: 523–525.
- Su W J, Huang C Y, Huang C Y, Perng R P. Utility of PCR assays for rapid diagnosis of BCG infection in children. *Int J Tuberc Lung Dis* 2001; 5: 380–384.
- Albrecht H, Stellbrink H J, Eggers C, Rusch-Gerdes S, Greten H. A case of disseminated *Mycobacterium bovis* infection in an AIDS patient. *Eur J Clin Microbiol Infect Dis* 1995; 14: 226–229.
- Heifets L B. Drug susceptibility in the chemotherapy of mycobacterial infections. London, UK: CRC Press, 1991.
- National Committee for Clinical Laboratory Standards (NCCLS). Susceptibility testing of *Mycobacterium tuberculosis*, *Nocardia*, and other aerobic *Actinomycetes*: approved standard. NCCLS document M-24A. Vol 23, no 18. Wayne, PA, USA: NCCLS, 2003.
- Trunz B B, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 2006; 367: 1173–1180.
- McKenzie R H, Roux P. Disseminated BCG infection following bone marrow transplantation for X-linked severe combined immunodeficiency. *Pediatr Dermatol* 2000; 17: 208–212.
- Puthanakit T, Oberdorfer P, Punjaisee S, et al. Immune reconstitution syndrome due to bacillus Calmette-Guérin after initiation of antiretroviral therapy in children with HIV infection. *Clin Infect Dis* 2005; 41: 1049–1052.
- Morales A, Eiding D, Bruce A W. Intracavitary bacillus Calmette-Guérin in the treatment of superficial bladder tumors. *J Urol* 1976; 116: 180–183.
- Debois H, Loupi E, Saliou P. Surveillance of the safety of intravesical BCG therapy in France: quantitative analysis of serious adverse events notified over a period of five years. *Prog Urol* 2002; 12: 604–608. (in French)
- Ikeda N, Honda I, Yano I, Koyama A, Toida I. Bacillus Calmette-Guérin Tokyo 172 substrain for superficial bladder cancer: characterization and antitumor effect. *J Urol* 2005; 173: 1507–1512.
- Mugiya S, Ozono S, Nagata M, et al. Long-term outcome of a low-dose intravesical bacillus Calmette-Guérin therapy for carcinoma in situ of the bladder: results after six successive instillations of 40 mg BCG. *Jpn J Clin Oncol* 2005; 35: 395–399.
- Eichel L, Erturk E, Disant'Agnes A. Drug resistant *Mycobacterium bovis* cystitis following intravesical bacillus Calmette-Guérin treatment. *J Urol* 1999; 162: 2096.
- Durek C, Rusch-Gerdes S, Jocham D, Bohle A. Sensitivity of BCG to modern antibiotics. *Eur Urol* 2000; 37 (Suppl): S21–S25.
- Durek C, Rusch-Gerdes S, Jocham D, Bohle A. Interference of modern antibacterials with bacillus Calmette-Guérin viability. *J Urol* 1999; 162: 1959–1962.
- Rousseau P, Dupuis M. Antituberculous drug susceptibility testing of *Mycobacterium bovis* BCG strain Montreal. *Can J Microbiol* 1990; 36: 735–737.
- Otten H. Ethambutol (EMB). In: Bartmann K, ed. Anti-tuberculosis drugs. Berlin, Germany: Springer-Verlag 1988: pp 197–204.
- Rastogi N, Labrousse V, Goh K S. In vitro activities of fourteen antimicrobial agents against drug susceptible and resistant clinical isolates of *Mycobacterium tuberculosis* and comparative intracellular activities against the virulent H37Rv strain in human macrophages. *Curr Microbiol* 1996; 33: 167–175.
- Suo J, Chang C E, Lin T P, Heifets L B. Minimal inhibitory concentrations of isoniazid, rifampin, ethambutol and streptomycin against *Mycobacterium tuberculosis* strains isolated before treatment of patients in Taiwan. *Am Rev Respir Dis* 1988; 138: 999–1001.
- Heifets L. Qualitative and quantitative drug-susceptibility tests in mycobacteriology. *Am Rev Respir Dis* 1988; 137: 1217–1222.
- Holdiness M R. Clinical pharmacokinetics of the antituberculosis drugs. *Clin Pharmacokinet* 1984; 9: 511–544.
- Hesseling A C, Schaaf H S, Victor T, et al. Resistant *Mycobacterium bovis* bacillus Calmette-Guérin disease: implications for management of bacillus Calmette-Guérin disease in human immunodeficiency virus-infected children. *Pediatr Infect Dis J* 2004; 23: 476–479.
- Mahairas G G, Sabo P J, Hickey M J, Singh D C, Stover C K. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J Bacteriol* 1996; 178: 1274–1282.
- Pym A S, Brodin P, Brosch R, Huerre M, Cole S T. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol Microbiol* 2002; 46: 709–717.
- Behr M A, Wilson M A, Gill W P, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 1999; 284: 1520–1523.
- Sørensen A L, Nagai S, Houen G, et al. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. *Infect Immun* 1995; 63: 1710–1717.
- Berthet F X, Rasmussen P B, Rosenkrands I, Andersen P, Gicquel B. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). *Microbiology* 1998; 144: 3195–3203.
- Hesselberg I. Drug resistance in the Swedish/Norwegian BCG strain. *Bull World Health Organ* 1972; 46: 503–507.
- Knezevic I, Corbel M J. WHO discussion on the improvement of the quality control of BCG vaccines. Pasteur Institute, Paris, France, 7 June 2005. *Vaccine* 2006; 24: 3874–3877.

## RÉSUMÉ

**CONTEXTE :** Le bacille de Calmette et Guérin (BCG) à base de *Mycobacterium bovis* est un vaccin unique contre la tuberculose (TB) en raison de ses effets protecteurs valables et de sa faible virulence. Toutefois, il peut causer occasionnellement une infection systémique chez les sujets en état d'immunodépression. L'isoniazide (INH), la rifampicine (RMP), la streptomycine (SM) et l'éthambutol (EMB) sont des médicaments antituberculeux reconnus comme efficaces et peuvent être utilisés dans le traitement des infections par le BCG. Il n'y a malheureusement que peu d'études concernant la sensibilité des souches de vaccin BCG à l'égard de ces médicaments.

**OBJECTIF :** Mesurer les concentrations minimales inhibitrices (CMI) du vaccin BCG Tokyo pour les médicaments antituberculeux et évaluer la sécurité du vaccin en ce qui concerne la sensibilité aux médicaments.

**SCHEMA :** Nous avons mesuré les CMI sur plaques d'agar Middlebrook 7H11 pour la souche-mère et pour cinq lots de vaccin de la souche BCG Tokyo à la fois pour l'INH, la RMP, la SM et l'EMB.

**RÉSULTATS :** Les résultats des CMI pour l'INH ont été respectivement de 0,06 et de 0,125 µg/ml pour la souche-mère et pour les lots de vaccin. Les résultats des CMI pour la RMP, la SM et l'EMB ont été respectivement de 0,25–0,5, 0,25 et 2–4 µg/ml.

**CONCLUSION :** Nos résultats indiquent que la souche BCG Tokyo est sensible à l'égard des médicaments antituberculeux majeurs qui sont efficaces même en cas d'effets indésirables graves, y compris des infections systémiques.

## RESUMEN

**MARCO DE REFERENCIA :** *Mycobacterium bovis*, el bacilo de Calmette y Guérin (BCG), es la única vacuna contra la tuberculosis (TB), debido a su valioso efecto de protección y a su baja virulencia. Sin embargo, esta vacuna puede causar en ocasiones infecciones generalizadas en individuos inmunodeprimidos. Isoniazida (INH), rifampicina (RMP), estreptomycina (SM) y etambutol (EMB) son medicamentos antituberculosos eficaces y se emplean en el tratamiento de las infecciones por BCG. Desafortunadamente, existen pocos estudios sobre la sensibilidad de la cepa de la vacuna antituberculosa a estos medicamentos.

**OBJETIVO :** Medir las concentraciones mínimas inhibitorias (CMI) de los medicamentos antituberculosos contra el BCG de Tokio contenido en las vacunas y evaluar

su seguridad toxicológica en la concentración de sensibilidad al medicamento.

**MÉTODOS :** Se midieron las concentraciones inhibitorias mínimas de INH, RMP, SM y EMB para un lote de siembra y cinco lotes de vacuna de la cepa BCG de Tokio usando cultivos en placas de agar con Middlebrook 7H11.

**RESULTADOS :** La CMI para INH fue 0,06 con los lotes de siembra y 0,125 µg/ml con los lotes de vacuna. La CMI para los lotes de vacuna con RMP fue de 0,25 a 0,5 ; con SM fue 0,25 ; y con EMB fue de 2 a 4 µg/ml.

**CONCLUSIÓN :** Estos resultados indican que la cepa BCG de Tokio es sensible a los principales medicamentos antituberculosos y que es posible tratar los casos de reacciones adversas graves, incluida la infección generalizada.

## Method for efficient storage and transportation of sputum specimens for molecular testing of tuberculosis

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### SUMMARY

**SETTING:** The polymerase chain reaction (PCR) is a highly sensitive method for the detection of *Mycobacterium tuberculosis* and is available in most countries, though to a lesser extent in rural areas.

**OBJECTIVE:** To amplify *M. tuberculosis* DNA sequences of sputum spotted on FTA<sup>®</sup> cards and compare them with the results of microscopic examination among culture-positive samples.

**DESIGN:** A total of 102 sputum specimens of TB patients in treatment were spotted on FTA cards and stored at room temperature until DNA analysis. We assessed the IS6110 region of *M. tuberculosis*. The efficacy of the PCR assay for the direct detection of *M. tuberculosis* was evaluated and compared with the results of cultures

(Middlebrook 7H9 broth) and smears of fresh sputum specimens.

**RESULTS:** We were able to detect 10 fg/ $\mu$ l of mycobacterial DNA even after 6 months in storage. The PCR sensitivity and specificity using the FTA card system were 82% and 96%, while microscopic examination showed 41% and 95%, respectively.

**CONCLUSION:** The FTA<sup>®</sup> card system for the storage of bacterial DNA from sputum samples should be considered for the molecular diagnosis of tuberculosis. Samples can easily be obtained from geographically isolated populations and shipped by mail for accurate molecular diagnosis.

**KEY WORDS:** tuberculosis; sputum; PCR; FTA<sup>®</sup> card

CURRENT GLOBAL TUBERCULOSIS (TB) control efforts are based on the diagnosis of cases followed by adequate treatment. Difficulties involved in the collection, transport and processing of samples in clinical practice have also been a major issue. An initial error made by microscopic diagnosis will not be known until weeks later, when the clinical signs are more evident (in false negatives). Microscopic examination of sputum acid-fast bacilli (AFB) smear is still the most widely available diagnostic tool for TB. Unfortunately, smear microscopy is neither specific for *Mycobacterium tuberculosis*, nor is it very sensitive. Depending on the number of specimens examined, smear microscopy detects 30–60% of culture-positive TB suspects.<sup>1</sup> In most situations in which TB is diagnosed by AFB microscopy, it should be assumed to be *M. tuberculosis* until proven otherwise.<sup>2</sup> Detection of AFB smear-negative patients, who make up a significant proportion of all TB patients, is even more problematic.<sup>3,4</sup> As *M. tuberculosis* grows very slowly, diagnosis by culture is a long process, requiring 3–8 weeks in solid media and 1–4 weeks in liquid media.<sup>5</sup>

The development of rapid and accurate procedures for the diagnosis of TB has been a long-standing goal for two main reasons—to improve case finding and case management, and to improve disease surveillance.<sup>5</sup> Molecular amplification assays such as polymerase chain reaction (PCR), which can specifically amplify large quantities of DNA from small starting quantities (10–100 mycobacteria/ml), have been shown to be a promising alternative even for developing countries.<sup>6,7</sup> Microscopic examination requires  $>10^3$  to  $10^4$  mycobacteria/ml, and it is necessary for the diagnostic laboratory to either process the specimen shortly after collection or store it at 4°C to inhibit the growth of contaminating micro-organisms. The latter procedure entails additional labour costs for the processing and conservation of specimens, and reductions in sensitivity.<sup>8</sup>

The FTA<sup>®</sup> card system (Whatman International Ltd, Abingdon, Cambridge, UK) was originally developed for storing blood samples for DNA testing; its matrix binds and lyses cells, resulting in amplifiable DNA being immobilised on the paper sections, which can

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be punched out directly for use. The card's size allows storage of several hundred samples at room temperature. The filter is impregnated with a chaotropic agent that denatures infectious agents, and thus, as the samples no longer represent a biohazard, their storage and transport can be managed without burdensome precautions.

In this study, we set out to investigate the sensitivity and specificity of a PCR system for the detection of *M. tuberculosis* in sputum samples spotted on FTA® cards and to evaluate the potential of using this method to overcome the difficulties of transporting and storing sputum samples during the TB diagnosis process.

## INDIVIDUALS, MATERIALS AND METHODS

### Patient and clinical specimens

A total of 102 sputum specimens from 35 TB patients at the Japanese Red Cross Sendai Hospital were collected prospectively. The clinical diagnosis of TB was established by patient histories and clinical and radiological findings, as recommended by the American Thoracic Society.<sup>9</sup> All the patients provided written informed consent. The study was approved by the Tohoku University Committee on Clinical Investigation and by the Ethics Committee of the Red Cross Hospital.

### Evaluation and application of the sputum onto the FTA® cards

Spontaneously produced sputum was the specimen of choice. No patient was assisted by respiratory therapy technicians nor stimulated with hypertonic saline aerosol to produce acceptable sputum. The gross appearance of the sputum was evaluated according to Miller & Jones' classification: M1 (pure mucus), M2 (little purulent content), P1 (purulent sputum less than one third of the volume), P2 (purulent sputum between one third and two thirds of the volume) and P3 (purulent sputum more than two thirds of the volume).<sup>10</sup> Standard precautions, such as the use of gloves and a mask, were taken when manipulating sputum specimens.

To improve the chance of detecting *M. tuberculosis*, we chose the thicker (purulent) particles of the sputum and applied them directly onto a FTA® card using a foam-tipped applicator (Whatman®, Tokyo, Japan) that was squeezed over an area of 2.5 cm in diameter. The card was then allowed to dry for 1 h at room temperature. Heat was not used during the drying period. The cards were then put into storage desiccant packets (Whatman®, Japan) and stored at room temperature until DNA analysis.

### Culture and hybridisation

Equal volumes of N-acetyl-L-cysteine/NaOH (4%) were mixed with the specimens for digestion and decontamination. The resulting mixtures were allowed to stand for 15 min at room temperature, then centrifuged at 3000 × g for 15 min. The sediment was re-suspended in 2 ml of phosphate buffered saline

(PBS) and 0.5 ml was inoculated into Middlebrook 7H9 broth (Middlebrook, Becton Dickinson, Cockeysville, MD, USA). DNA was extracted in all the culture-positive samples to identify and confirm *M. tuberculosis* using a DNA-DNA calorimetric microdilution plate hybridisation kit (DDH Mycobacteria; Kyokuto Pharmaceuticals, Tokyo, Japan).

### Preparation of isolated DNA from FTA® cards

Four discs of 1.2 mm from the spotted area of the filters were cut out using a sterile hole puncher (Harris Micro punch 1.2 mm, Whatman®, Japan) and placed in 1.5 ml PCR tubes. The punch was cleaned by placing the end of the punch in the flame of a Bunsen burner and by sterilisation with 70% ethanol between cuts from different samples. The discs were washed three times for 5 min with 800 µl FTA® purification reagent (Whatman®, Japan), which removes PCR inhibitors and other potential contaminants to ensure the quality of the DNA for downstream analysis. The discs were also rinsed twice with 800 µl TE buffer (10 mM Tris HCL, 0.1 mM EDTA, pH 8.0) (Promega, Tokyo, Japan) for 5 min each time. The discs were then dried at room temperature for 1 h and used directly as templates in the PCR reaction mix in the same tube.

### PCR procedure

The primers were synthesised using an Automated Multiplex Oligonucleotide Synthesizer (Roche Diagnostics, Tokyo, Japan) (Table 1). Two different PCR assays were performed. First, as an internal control for monitoring successful DNA extraction, β-globin was amplified by using primers GH21 and PCO3 to yield a 250-bp product.<sup>11</sup> The total reaction volume was 50 µl and the reaction mixture contained each primer (10 pmol each), 2U taq DNA polymerase (Invitrogen Cat N°10342-020), 5 µl 10x PCR buffer (200 mM Tris pH 8.4, 500 mM KCl), 1 µl 10 mM dNTP mixture (Invitrogen Cat N°18427-013), 1.5 µl 50 mM MgCl<sub>2</sub> and 38.1 µl distilled water. PCR was performed in an MJ Research PTC-200 DNA Engine Thermal Cycler (MJ Research, Waltham, MA, USA) using the following amplification conditions: 95°C for 5 min, 35 cycles at 95°C for 30 s, 55°C for 45 s, 72°C for 30 s and one cycle at 72°C for 10 min. Finally, a 123-bp sequence of insertion element IS6110 was amplified using T4-T5 primers.<sup>12</sup> This insertion sequence is present in different numbers and locations in the genomes of most clinical isolates of *M. tuber-*

**Table 1** PCR primers used in this study

Target	Sequencing	bp	Ref.
β-globin	5'-GGA-AAA-TAG-ACC-AAT-AGG-CAG-3'	250	10
GH21-PCO3	5'-ACA-CAA-CTG-TGT-TCA-CTA-GC-3'		
<i>M. tuberculosis</i>	5'-CCT-GCG-AGC-GTA-GGC-GTC-GG-3'	123	11
IS6110	5'-CTC-GTC-CAG-CGC-CGC-TTC-GG-3'		
T4-T5			

*culosis*, which makes it a useful probe for diagnostic and epidemiological purposes. The final composition of the PCR mix was the same as in the previous case. The PCR conditions were preheating at 94°C for 5 min, then 40 cycles at 94°C for 2 min, 68°C for 2 min, 72°C for 2 min, and then 72°C for 10 min.

#### Determination of sensitivity

To determine the sensitivity of *M. tuberculosis* detection, 10-fold serial dilutions (1 ng to 10 fg) of H37Rv DNA (kindly provided by Dr I Sugawara, Research Institute of Tuberculosis, Tokyo, Japan) were performed using T4-T5 primers. The final composition and PCR conditions were the same as for amplifying IS6110.

All amplification products were detected on 1.5% agarose gel in 1 × TAE buffer stained with ethidium bromide and visualised by ultraviolet transillumination.

#### Control procedures

A positive control tube containing 0.1 ng H37Rv DNA and a negative control tube containing no DNA were included with each set of reactions. To evaluate cross-contamination during sampling, we performed control punches using unspotted cards.

#### Statistical methods

The sensitivity and specificity of each pair of primers for the detection of *M. tuberculosis* were calculated on the basis of the study reference standards, with the liquid culture method taken as a gold standard. In addition, the results of individual PCR were employed for the analysis of smear-positive and -negative samples according to the culture results.

## RESULTS

#### Patient characteristics

We evaluated by PCR 102 sputum samples from 35 TB patients (23 male, 12 female), all of whom were receiving anti-tuberculosis treatment for periods ranging from 2 weeks to 1 year. The average age was 51.4 years.

#### Gold standard

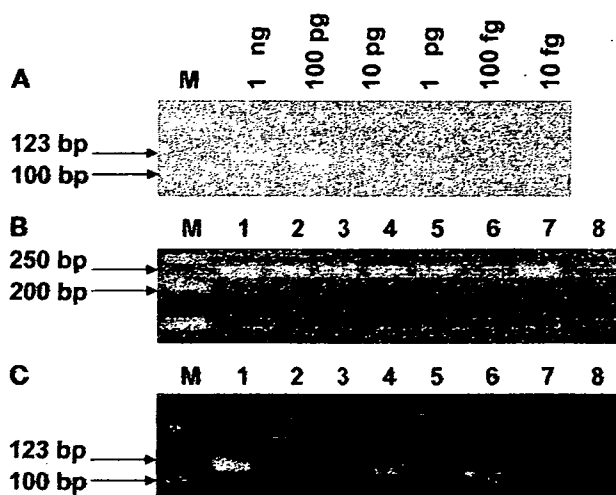
Twenty-two positive culture samples were taken as gold standard. In all of these samples, *M. tuberculosis* was identified by hybridisation assay.

#### Appearance of the specimens

Eighty-five per cent of smear positives, 68% of culture positives and 81% of PCR positives contained >30% of purulent sputum (P2 or P3 in Miller & Jones' classification<sup>10</sup>).

#### Sensitivity detection

Four small discs of the FTA® card system were used as templates for the PCR processing. The criterion for using four discs was based on the assumption that the mycobacteria were scanty and heterogeneously dis-



**Figure 1** PCR results. **A.** Sensitivity of detection of *M. tuberculosis*: ten-fold serial dilution of H37Rv DNA was amplified. **B.** PCR amplicons from  $\beta$ -globin (250 bp): M: marker; lanes 1–7: results using FTA® cards from TB patients; lane 8: negative control. **C.** PCR amplicons from *M. tuberculosis* (123 bp) stored on FTA® cards: M: marker; lane 1: positive control (H37Rv); lanes 2, 4 and 6: cards from TB patients; lanes 3, 5 and 7: no spotted cards (control punch); lane 8: negative control. In each case, 10  $\mu$ l samples were electrophoresed through a 1.5% agarose gel and photographed under UV illumination. PCR = polymerase chain reaction; bp = base pairs; TB = tuberculosis; UV = ultraviolet.

tributed during the absorption process; in addition, in a pilot study we determined that using four disks increased the degree of amplification (data not shown). The detection limit of the PCR assay for the amplification of IS6110 was 10 fg/ $\mu$ l of purified *M. tuberculosis* H37Rv (Figure 1A). The amplification of the 123 bp fragments by PCR using the FTA® card system is depicted in Figure 1C.

#### Effect of PCR inhibitors

The ability to detect *M. tuberculosis* by PCR can be impaired by the presence of substances inhibitory to Taq DNA polymerase. The  $\beta$ -globin PCR assay generated the expected 250-bp band (Figure 1B) in 90 (89%) of the samples. All the culture-positive samples were also positive in the amplification of  $\beta$ -globin. This finding may suggest that the PCR-negative, culture-positive samples contained low concentrations of TB bacteria rather than PCR inhibitors, which would tend to rule out inhibition as a cause.

#### PCR and smear results compared with culture

Among the 22 culture-positive samples, 18 (82%) were PCR-positive and 9 (41%) smear-positive (Table 2). The remaining four culture-positive samples were both PCR- and smear-negative. All 9 smear- and culture-positive samples were also positive by PCR. The sensitivity and specificity of PCR were 82% and 96%, compared to 41% and 95%, respectively, for smear examination. Differences were observed on comparing the sensitivity of smear microscopy with that of

**Table 2** Comparison of PCR with smear and culture for detection of *M. tuberculosis*

Culture	Positive <i>n</i> (%)	Negative <i>n</i>
PCR*		
Positive	18 (82) <sup>†</sup>	3
Negative	4	77
Smear		
Positive	9 (41) <sup>†</sup>	4
Negative	13	76

\* PCR using the FTA® card system.

<sup>†</sup> (%) sensitivity.

PCR = polymerase chain reaction.

PCR ( $P < 0.05$ ), but there was no observed difference in specificity (Figure 2).

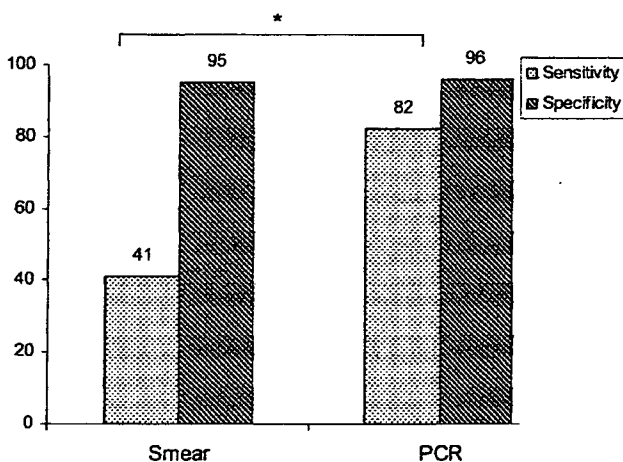
We repeated the PCR procedure for all culture- and smear-positive samples. The second experiment showed the same results as the first.

#### Sensitivity of PCR by smear result

The sensitivity of PCR for smear-positive, culture-positive samples was 9/9 (100%), whereas that for smear-negative, culture-positive samples was 9/13 (69%). These results show that, even in the paucibacillary form of TB resulting from treatment, this PCR system could provide rapid and sensitive detection of *M. tuberculosis* DNA impregnated on the FTA® card.

#### Stability and control procedures

All of the 102 samples were analysed by PCR at two time points—at the time of sample collection and after 6 months of storage—obtaining positive amplifications in both cases, clearly showing that storage for 6 months did not affect the amplification. PCR products were not detected in negative controls or control punch cards, confirming the absence of contamination during the procedure (Figure 1C). The total assay time was 9 h.



**Figure 2** Accuracy of the methods. Sputum smear examination (smear) and PCR assay using the FTA® card system (PCR) among the 22 positive culture samples. \*  $P < 0.05$ . PCR = polymerase chain reaction.

## DISCUSSION

The present study demonstrated that the DNA of *M. tuberculosis* can be amplified using sputum spotted on an FTA® card. We found that the *M. tuberculosis* DNA stability with this card at room temperature was up to 6 months. However, care must be taken to avoid cross-contamination between specimens during sampling and handling. The present findings are relevant to patient care and clinical trials and suggest that sputum stored on FTA® cards could provide a simple, economical method for the collection, storage and transport of suspected TB specimens for later testing.

In addition, samples can easily be obtained from geographically isolated populations where access to and/or availability of TB diagnostic testing may be limited. Samples collected may be shipped by mail to a central laboratory for molecular diagnosis without the triple packing system otherwise required for transport.<sup>13</sup>

Depending on the gold standard and other methodological factors, studies have shown PCR sensitivities ranging from 77% to >95% and PCR specificities of >95% in TB patients before treatment.<sup>4,5</sup> Regarding patients under treatment, Kennedy et al. found 76% agreement between culture and PCR.<sup>14</sup> In our study, three culture-negative samples (one smear-positive and two smear-negative) were detected by our method, which may be explained by the limited quantity of TB DNA.<sup>15</sup> While the sensitivity of smear is dependent on the type and quality of the specimen, our method could be employed with accuracy even 6 months after obtaining the sample. However, as we recommend taking the purulent part of the sputum, it will be necessary to help patients understand that secretions from deep in the lung are required.

More sensitive methods exist, including the use of automated culture systems, but the best tests are not always available for the people who most need them.<sup>16</sup>

In summary, the present system appears to be a promising method for transporting and storing sputum samples. Other advantages are the simplicity of the sample preparation and the use of the small disc as a template during the PCR process, using specific targets, without the need for extensive nucleic acid purification.

#### Acknowledgements

The authors thank Drs J C Agapito and M Rabie for excellent technical assistance and B Bell for reading the manuscript. They also appreciate Dr P Escalante's thoughtful review of and comments on the manuscript.

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#### References

- Perkins M D. New diagnostic tools for tuberculosis. *Int J Tuberc Lung Dis* 2000; 4 (Suppl): 182–188.
- Pio A, Chaulet P. Tuberculosis handbook. 2nd ed. WHO/CDS/



- TB 2003.320. Geneva, Switzerland: World Health Organization, 2003.
- 3 American Thoracic Society. Rapid diagnostic test for tuberculosis. What is the appropriate use? *Am J Respir Crit Care Med* 1997; 155: 1804–1814.
  - 4 Sarmiento O, Weigle K, Alexander J, et al. Assessment by meta-analysis of PCR for diagnosis of smear-negative pulmonary tuberculosis. *J Clin Microbiol* 2003; 41: 3233–3240.
  - 5 Eichbaum Q, Rubin E. Tuberculosis: advances in laboratory diagnosis and drug susceptibility testing. *Am J Clin Pathol* 2002; 118 (Suppl 1): 3–17.
  - 6 Barnes P, Cave M. Molecular epidemiology of tuberculosis. *N Engl J Med* 2003; 349: 1149–1156.
  - 7 Daar A, Thorsteinsdottir D, Martin A, et al. Top ten biotechnologies for improving health in developing countries. *Nature Genetics* 2002; 32: 229–232.
  - 8 Paramasivan C, Narayana A, Prabhakar R. Effect of storage of sputum specimens at room temperature on smear and culture results. *Tubercle* 1983; 64: 119–124.
  - 9 American Thoracic Society. Diagnostic standards and classification of tuberculosis in adults and children. *Am J Respir Crit Care Med* 2000; 61: 1376–1395.
  - 10 Miller D L, Jones R. The bacterial flora of the upper respiratory tract and sputum of working men. *J Pathol Bacteriol* 1964; 87: 182–186.
  - 11 Popper H H, Winter E, Hofer G, et al. DNA of *Mycobacterium tuberculosis* in formalin-fixed, paraffin-embedded tissue in tuberculosis and sarcoidosis detected by polymerase chain reaction. *Am J Clin Pathol* 1994; 101: 738–741.
  - 12 Eisenach K, Cave D, Bates J, et al. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis* 1990; 161: 977–981.
  - 13 World Health Organization. Guidelines for the safe transport of infectious substances and diagnostic specimens. WHO/EMC/97.3. Geneva, Switzerland: WHO, 1997. [http://www.who.int/csr/resources/publications/biosafety/WHO\\_EMC\\_97\\_3/en/index.html](http://www.who.int/csr/resources/publications/biosafety/WHO_EMC_97_3/en/index.html) Accessed May 2006.
  - 14 Kennedy N, Gillespie S H, Saruni A O S, et al. Polymerase chain reaction for assessing treatment response in patients with pulmonary tuberculosis. *J Infect Dis* 1994; 170: 713–716.
  - 15 Kent PT, Kubica G P. Isolation procedures. In: Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, USA: Centers for Disease Control and Prevention, 1985.
  - 16 Mabey D, Peeling R, Ustianowski A, et al. Diagnostics for the developing world. *Nat Rev Microbiol* 2004; 2: 231–240.

## RÉSUMÉ

**CADRE :** La réaction polymérase en chaîne (PCR) est sensible pour la détection de *Mycobacterium tuberculosis*, et est disponible dans la plupart des pays, mais dans une moindre mesure dans les zones rurales.

**OBJECTIFS :** Amplifier les séquences d'ADN de *M. tuberculosis* des crachats repérées sur les cartes FTA et les comparer avec les résultats des examens microscopiques dans les échantillons de cultures positives.

**MÉTHODES :** Au total, 102 échantillons de crachats de patients tuberculeux ont été marqués sur les cartes FTA et stockés à l'air ambiant. La spécificité et la sensibilité de deux amorces de PCR qui amplifient la région IS6110 de *M. tuberculosis* ont été évaluées et comparées à celles de cultures (milieu liquide 7H9), et de l'examen microscopique d'échantillons frais de crachats.

**RÉSULTATS :** Il a été possible de détecter 10 fg/μl de DNA mycobactérienne même après 6 mois de stockage. L'analyse PCR des deux paires d'amorces révèle une sensibilité et une spécificité respectivement de 82% et 96%, alors que celles de l'examen microscopique sont respectivement de 41% et de 95%.

**CONCLUSION :** Le système de carte FTA permettant le stockage de l'ADN bactérien issu d'un échantillon de crachats devrait être envisagé pour le diagnostic moléculaire de la tuberculose. Les échantillons de crachats peuvent être facilement obtenus dans des populations géographiquement isolées, stockés et adressés par courrier afin d'établir à distance un diagnostic moléculaire précis.

## RESUMEN

**CONTEXTO :** La reacción en cadena de la polimerasa (PCR) es un método sensible para la detección de *Mycobacterium tuberculosis* y se encuentra al alcance en la mayoría de los países, aunque en menor medida en zonas rurales.

**OBJETIVO :** Amplificar secuencias del ADN de *M. tuberculosis* a partir de manchas de esputo en papel de filtro (FTA cards®) y comparar estos resultados con los resultados de la baciloscopia, en muestras con cultivo positivo para micobacterias.

**MÉTODOS :** Se recogió un total de 102 muestras de esputo de pacientes con tuberculosis en curso de tratamiento, las cuales se almacenaron como manchas en papel de filtro a temperatura ambiente hasta el momento del análisis. Con la PCR se amplificó un fragmento de 123 pares de bases de la secuencia de inserción IS6110 de *M. tuberculosis*. Se evaluó la eficacia de la PCR en la detección de *M. tuberculosis* y los resultados se com-

pararon con los resultados de los cultivos en medio líquido 7H9 (método de referencia) y de la baciloscopia, de muestras frescas de esputo.

**RESULTADOS :** El método permitió detectar hasta 10 fg/μl de ADN micobacteriano en muestras almacenadas durante más de 6 meses. La PCR a partir de las manchas de esputo en tarjetas FTA® mostró una sensibilidad del 82% y una especificidad del 96%, comparada con una sensibilidad del 41% y una especificidad del 95% de la baciloscopia.

**CONCLUSIÓN :** El sistema con tarjetas FTA® debería tenerse en cuenta como método de conservación del ADN micobacteriano presente en las muestras de esputo, para el diagnóstico molecular de la tuberculosis. Así, en poblaciones geográficamente aisladas, de manera sencilla podrían obtenerse las muestras de esputo, almacenarlas y expedirlas por correo con el fin de establecer un diagnóstico molecular exacto.

## Low antibody response against tuberculous glycolipid (TBGL) in elderly gastrectomised tuberculosis patients

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### SUMMARY

To evaluate differences in anti-tuberculous glycolipid (TBGL) antibody titers in patients who developed tuberculosis (TB) with and without gastrectomy, 11 gastrectomised patients who developed TB after surgery (GS-TB), 19 TB patients without any other complications (TB), 12 gastrectomised patients who did not develop TB after surgery (GS) and 27 healthy subjects (H) with

normal findings on chest X-ray were evaluated, although there were no differences in the clinical findings at admission between the TB and GS-TB groups. The assay used here allowed us to find low anti-TBGL antibody titers in GS-TB patients.

**KEY WORDS:** TBGL; gastrectomy; tuberculosis

GASTRECTOMY is known as an associated co-factor in the development of tuberculosis (TB). Although the reasons for the association have not been clarified,<sup>1,2</sup> previous studies with a significant number of gastrectomised patients have reported prevalences of 1.7% to 2.5%. In addition, immunodeficiency and/or malnutrition contribute to the development of TB.<sup>3</sup> We therefore evaluated the clinical and laboratory findings, including purified protein derivative (PPD) reaction, in TB and/or gastrectomised patients and healthy controls. In the gastrectomised patients, group 2 lymph nodes were dissected, which could have affected the host immune responses. The glycolipid antigen trehalose 6, 6'-dimycolate (TDM) purified from *Mycobacterium tuberculosis* H37Rv has recently been reported as a useful diagnostic antigen.<sup>4-6</sup> We thus investigated specific immune responses against TDM by measuring anti-tuberculous glycolipid (TBGL) antibody titers.

Between 1999 and 2001, a total of 60 patients from Tohoku University Hospital enrolled in the study were divided into four groups: 1) gastrectomised patients who developed TB after surgery (GS-TB,  $n = 11$ ); 2) TB patients without any other complications (TB,  $n = 19$ ); 3) gastrectomised patients who did not develop TB after surgery (GS,  $n = 12$ ); and 4) healthy subjects ( $n = 27$ ) with normal findings on chest X-ray. For both TB and GS-TB patients, blood samples were taken before anti-tuberculosis drugs were given.

The study was approved by the ethics committee of the Tohoku University School of Medicine. Informed consent was obtained from patients and volunteers to participate in the study. For the diagnosis

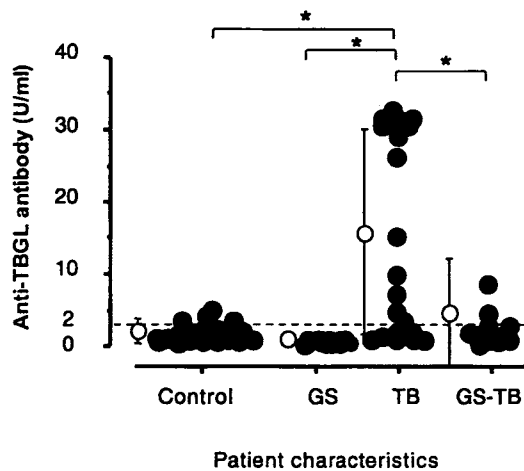
of TB, in addition to clinical features, acid-fast smear, culture and polymerase chain reaction tests (Roche Amplicor Mycobacterium Kit, Branchburg, NJ, USA) were performed using sputum or gastric fluid. Gastrectomised patients with recurring cancer, those treated with anticancer drugs and those with other diseases were excluded. Immunoglobulin G (IgG) antibodies against TBGL antigen was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Kyowa Medex Co, Tokyo, Japan). A cut-off value  $\geq 2$  U/ml was considered positive.<sup>4,5</sup> Statistical analyses were performed using conventional methods.

Mean age and postoperative duration were  $74.5 \pm 9.2$  years and  $24.3 \pm 6.3$  months in the GS-TB group and  $74.8 \pm 3.0$  years and  $20.6 \pm 9.2$  months in the GS group, respectively. There were no significant differences between the two groups ( $P > 0.05$ ). The mean ages in the TB patients and healthy controls were respectively  $73.0 \pm 9.4$  and  $73.2 \pm 8.2$  years. There were no significant differences in positive tuberculin test (6/11 vs. 10/19), lung infiltration shadow ( $\geq 2$  lobes) (5/11 vs. 14/19) or acid-fast smear ( $\geq 1$ /field) (6/11 vs. 10/19) between the GS-TB and TB groups.

In this study, 55% of the GS-TB patients were positive for the anti-TBGL antibody titers by ELISA; this result was comparable to that obtained for the TB patients (68%). Maekura et al. found a significant number (17%) of TBGL-positive serum samples in healthy individuals.<sup>5</sup> In our healthy subjects, 22% of serum samples showed positive responses, but curiously none of the GS group was positive. Furthermore, the anti-TBGL antibody titers in the GS-TB patients were

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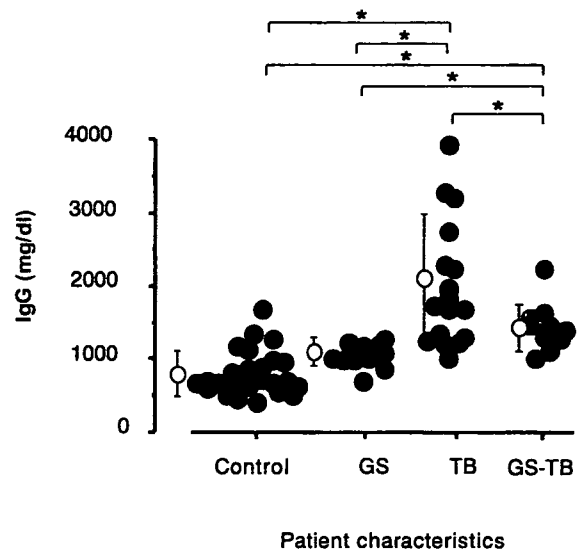
**Figure 1** Individual anti-TBGL values in healthy controls and GS, TB and GS-TB patients. Circles = individual anti-TBGL values; bars = standard deviation; dotted line = cut-off; anti-TBGL = anti-tuberculous glycolipid; GS = gastrectomised; TB = tuberculosis. \*  $P < 0.05$ .

significantly lower than those of the TB patients ( $P < 0.05$ ) (Figure 1). The serum IgG in both TB and GS-TB patients was significantly higher than in the GS patients and healthy controls (Figure 2), confirming that the decrease of anti-TBGL antibody in GS-TB patients could not be explained by the low IgG in their serum. It should also be noted that both anti-TBGL antibody and total IgG values were lower among GS-TB patients than in the TB group.

The reason for the lack of anti-TBGL antibody in GS patients and the low anti-TBGL antibody titers in GS-TB patients is not clear, but both suggest that the stomach may play a role in the production of anti-TBGL antibodies.

#### Acknowledgements

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**Figure 2** Individual IgG values in healthy controls and GS, TB and GS-TB patients. Circles = individual IgG values; bars = standard deviation; IgG = immunoglobulin G; GS = gastrectomised; TB = tuberculosis. \*  $P < 0.05$ .

from gastrectomised patients. We are also grateful to Dr Yano at Japan BCG Laboratory for critical reading of the manuscript.

#### References

- Snider D E. Tuberculosis and gastrectomy. *Chest* 1985; 87: 414–415. [Editorial]
- Steiger Z, Nickel W O, Shannon G J, Nedwicki E G, Higgins R F. Pulmonary tuberculosis after gastric resection. *Am J Surg* 1976; 131: 668–671.
- Dai G, Phalen S, McMurray D. Nutritional modulation of host responses to mycobacteria. *Front Biosci* 1998; 3: e110–122. [Review]
- Maekura R, Nakagawa M, Nakamura Y, et al. Clinical evaluation of rapid serodiagnosis of pulmonary tuberculosis by ELISA with cord factor (Trehalose—6, 6'-dimycolate) as antigen purified from *Mycobacterium tuberculosis*. *Am Rev Respir Dis* 1990; 148: 997–1001.
- Maekura R, Okuda Y, Nakagawa M, et al. Clinical evaluation of anti-tuberculous glycolipid immunoglobulin G antibody assay for rapid serodiagnosis of pulmonary tuberculosis. *J Clin Microbiol* 2001; 39: 3603–3608.
- De Smet K A, Weston A, Brown I N, Young D B, Robertson B D. Three pathways for trehalose biosynthesis in mycobacteria. *Microbiology* 2000; 146: 199–208.

#### RÉSUMÉ

Afin d'évaluer les différences entre les titres d'anticorps glycolipides anti-tuberculeux (TBGL) chez des patients ayant développé TB avec ou sans gastrectomie, ont été évalués 11 patients gastrectomisés ayant développé une TB après chirurgie (GS-TB), 19 patients TB sans autre complication (TB), 12 patients gastrectomisés n'ayant

pas développé de TB après chirurgie (GS), et 27 sujets sains (H) présentant des radiographies thoraciques normales, bien qu'il n'y a eu aucune différence dans les investigations cliniques à l'admission entre les groupes TB et GS-TB. L'essai utilisé nous a permis de trouver de faibles titres d'anticorps d'anti-TBGL chez les patients GS-TB.

#### RESUMEN

Para evaluar la diferencia de títulos de anticuerpos para un glicolípido antituberculoso (TBGL), en pacientes infectados con TB con y sin antecedente de gastrectomía, se evaluaron 11 pacientes gastrectomizados (GS-TB), 19 pacientes infectados con TB sin otra complicación (TB),

12 pacientes gastrectomizados que no desarrollaron TB (GS) y 27 sanos (H) con exámenes radiológicos normales. No hubo diferencias significativas entre los grupos TB y GS-TB al ingreso. Los resultados mostraron bajos títulos anti-TBGL en pacientes GS-TB.

## Enhanced TLR-mediated NF-IL6-dependent gene expression by Trib1 deficiency

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Toll-like receptors (TLRs) recognize a variety of microbial components and mediate downstream signal transduction pathways that culminate in the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein (MAP) kinases. Trib1 is reportedly involved in the regulation of NF- $\kappa$ B and MAP kinases, as well as gene expression *in vitro*. To clarify the physiological function of Trib1 in TLR-mediated responses, we generated Trib1-deficient mice by gene targeting. Microarray analysis showed that Trib1-deficient macrophages exhibited a dysregulated expression pattern of lipopolysaccharide-inducible genes, whereas TLR-mediated activation of MAP kinases and NF- $\kappa$ B was normal. Trib1 was found to associate with NF-IL6 (also known as CCAAT/enhancer-binding protein  $\beta$ ). NF-IL6-deficient cells showed opposite phenotypes to those in Trib1-deficient cells in terms of TLR-mediated responses. Moreover, overexpression of Trib1 inhibited NF-IL6-dependent gene expression by down-regulating NF-IL6 protein expression. In contrast, Trib1-deficient cells exhibited augmented NF-IL6 DNA-binding activities with increased amounts of NF-IL6 proteins. These results demonstrate that Trib1 is a negative regulator of NF-IL6 protein expression and modulates NF-IL6-dependent gene expression in TLR-mediated signaling.

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Abbreviations used: 24p3, lipocalin-2; BLP, bacterial lipoprotein; C/EBP, CCAAT/enhancer-binding protein; Jnk, c-Jun N-terminal kinase; MALP-2, macrophage-activating lipopeptide-2; MAP, mitogen-activated protein; mPGES, prostaglandin E synthase; TLR, Toll-like receptor.

Innate immunity is promptly activated after the invasion of microbes through recognition of pathogen-associated molecular patterns by pattern-recognition receptors, including Toll-like receptors (TLRs) (1). The recognition of microbial components by TLRs effectively stimulates host immune responses such as proinflammatory cytokine production, cellular proliferation, and up-regulation of co-stimulatory molecules, accompanied by the activation of NF- $\kappa$ B and mitogen-activated protein (MAP) kinases (2, 3). Although the inhibitory protein I $\kappa$ B family members sequester NF- $\kappa$ B in the cytoplasm of unstimulated cells, TLR-dependent I $\kappa$ B phosphorylation by the I $\kappa$ B kinase complex and degradation by the ubiquitin-proteasome pathway permit translocation of NF- $\kappa$ B to the nucleus (4). MAP kinases such as c-Jun N-terminal kinase (Jnk) and p38 are also rapidly phosphorylated

and activated by upstream kinases in response to TLR stimulation (5). Moreover, TLR-mediated activity of NF- $\kappa$ B and MAP kinases is shown to be regulated at multiple steps regarding the strength and the duration of the activation (6).

Recent extensive experiments have identified a variety of modulators that have positive and negative effects on the activation of NF- $\kappa$ B and MAP kinases, including a family of serine/threonine kinase-like proteins called Trib (7). Trib consists of three family members: Trib1 (also known as c8fw, GIG2, or SKIP1), Trib2 (also known as c5fw), and Trib3 (also known as NIPK, SINK, or SKIP3) (7–12). Trib3 has been shown to interact with the p65 subunit of NF- $\kappa$ B and to inhibit NF- $\kappa$ B-dependent gene expression *in vitro* (11). In terms of MAP kinases, Trib1, Trib2, and Trib3 reportedly bind to Jnk and p38, and affect the activity of MAP kinases and IL-8 production in response to PMA or

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