14:40 **Dr. David S. STEPHENS** (Emory University)
The genetic basis and emergence of macrolide resistance in pneumococci

15:00 **Dr. Kathryn M. EDWARDS** (Vanderbilt University)
Have antibiotic resistant Streptococcus pneumoniae been reduced by conjugate pneumococcal vaccine (PCV)?

15:20 **Dr. Randall S. SINGER** (University of Minnesota)
Potential impacts of antibiotic use in poultry production

15:40 **Dr. Lawrence J. GEITER** (Otsuka Pharmaceutical)
Clinical trials of OPC-67683: a potential new drug for the treatment of tuberculosis

16:00 **COFFEE/TEA BREAK**

SESSION IV: Strategies for optimizing use of available drugs and those in the pipeline
Moderators: Kazunori OISHI and Clifton BARRY

16:20 **Dr. Clifton E. BARRY, III** (NIAID, NIH)
Molecular imaging as a surrogate endpoint in clinical trials of new anti-tuberculars

16:40 **Dr. Norio DOI** (Research Institute of TB, Japan Anti-Tuberculosis Association)
Novel Drug Delivery System: Possibility of Inhalation Drugs for TB/HIV

17:00 **Dr. Seung-Kyu PARK** (International TB Research Center, Korea)
Clinical perspective of MDR-TB treatment strategies

17:30-19:00 **POSTER VIEWING (Remove posters at end of this session)**

DINNER – On Your Own

DAY 3 **THURSDAY, 6 DECEMBER 2007**

SESSION V: Future directions and next steps: new technologies

Moderators: Yichen LU and David MCMURRAY

8:00 **Dr. Daniel P. CHIN** (Bill & Melinda Gates Foundation)
Moving new TB diagnostics and drugs into use to tackle the problem of MDR/XDR-TB

8:20 **Dr. Thelma E. TUPASI** (Tropical Disease Foundation, Philippines)
DOTS-Plus for MDR-TB management: Experience in the Philippines

8:40 **Dr. Tomotada IWAMOTO** (Kobe Institute of Health)
Rapid and simple detection of M. Tuberculosis complex from sputum samples using Loop-Mediated Amplification (LAMP) with simplified manual DNA extraction

9:00 **Dr. Yasuhiko SUZUKI** (Hokkaido University)
Perspectives to wider applications: Molecular diagnostic tools for tuberculosis

9:20 **Dr. Richard M. KRAUSE** (NIAID)
Enzybiotics: A novel new alternative to antibiotic therapy

MEETING SUMMARY AND CLOSING PRESENTATIONS

Moderators: Toru MORI and Patrick BRENNAN

9:30 **MEETING SUMMARY**

Dr. Patrick BRENNAN (Colorado State University)

Dr. Toru MORI (National Institute of Infectious Diseases, Japan)

10:00 **CLOSING REMARKS**

US Delegation: Dr. Ashley HAASE

Japanese Delegation: Dr. Takehiko SASAZUKI

Local Committee: **Mr. Ci CHEN**, Secretary of Haikou Municipal Committee

10:20 **COFFEE/TEA BREAK**

10:30 – 13:00 **WORKSHOPS SESSION 1**

13:00 – 14:00 **LUNCH** (Breeze Restaurant)

14:00 – 16:30 **WORKSHOPS SESSION 2** (Each workshop will be conducted 2 times – once in the morning and once in the afternoon; participants can choose one morning and one afternoon workshop)

16:30 – 19:30 **BEACH PARTY HOSTED BY VTI** (Sheraton Resort)

WORKSHOP TOPICS:

WORKSHOP I: Safe Laboratory Practices - Respiratory Pathogens: Laboratory work with respiratory pathogens, especially potentially drug-resistant strains, requires safe handling to protect personnel. Strategies for implementation of good laboratory and clinical practices from initial collection of field samples through clinical and research laboratory testing.

[Format: approx. 30 min talks each on biosafety, laboratory procedures, and laboratory standards, followed by discussion]

Leads: Polly Sager NIH; Janet Robinson FHI

WORKSHOP II: Sequencing, Bioinformatics: Publicly available genomic, proteomic and bioinformatics resources for *Mycobacterium tuberculosis* and other respiratory pathogens supported by the National Institute of Allergy and Infectious Diseases, NIH for the scientific community will be discussed and include hands on bioinformatics demonstrations.

[Format: approx. 30 min overviews on each of the following: Sequencing/Comparative Genomics, Functional Genomics, Structural Genomics & Bioinformatics databases]

Leads: Maria Giovanni, NIH; Valentina Francesco, NIH

WORKSHOP III: Protecting those in the field: Strategies to protect those collecting and handling specimens; protect sample integrity during transport, and update on portable assays – relevant to antimicrobial resistance detection/diagnosis.

[Format: 1.5 hr comprehensive overview, followed by three (15min) talks]

Leads: Farukh Khambaty, NIH; Douglas Abbott, USDA

WORKSHOP IV: Introduction to building and operating a biocontainment laboratory in developing countries: Topics covered include descriptions of BSL2, 3/3e labs; resources available when planning to set-up new or renovate existing labs to the desired biosafety level; and information on portable laboratory facilities that can be customized and deployed in emergency situations where a significant amount of specialized field testing is needed.

[Format: 1.5 hr comprehensive overview followed by three (15min) talks]

Leads: Farukh Khambaty, NIH; Chris Peter, San Diego County Health & Human

会議報告：12th International Conference on Emerging Infectious Diseases in the Pacific Rim…Antimicrobial resistance in respiratory infections

日米医学協力計画結核・ハンセン病専門部会　部会長　菅原　勇
同　ARI 専門部会　　部会長　鈴木　宏

1．　はじめに

第 12 回汎太平洋感染症国際会議が、中国海南島海口海口シェラトン　リゾートで、2007 年 12 月 4 日から 6 日まで、3 日間開催された。今回、日米の結核・ハンセン病専門部会、ARI 専門部会、親委員会が中心になって、会議の内容が、決められた。免疫専門部会会員も演者として加わった。現地の海口 VTI 生物学研究所が、会議開催に協力してくれた。 日米のみならず、中国をはじめ他のアジアの研究者も加わり、150 名以上の参加者があった。会議の詳細は、添付したプログラムを見ていただくとして、簡単に会議の内容を紹介したい。

2．12 月 4 日

歓迎の挨拶の後、plenary symposia が 3 題あった。順天堂大学医学部平松教授は、薬剤耐性 *Staphylococcus aureus* が病院の内外に生ずることを、実例をもって示した。またその耐性メカニズムにも言及した。中国 CDC Dr. Liang Li は、中国における多剤耐性結核の現状と対策を、疫学的見地から話された。ペンシルバニア大学 D. Joshua Metlay は、薬剤耐性呼吸器感染をどのように追跡し治療するかを、実例で示した。

3．12 月 5 日

1）セッション　1．抗細菌抵抗性（AMR）と関連した地球規模の問題

大阪大学大石先生は、アジアにおける S. pneumonia, H. influenza, P. aeruginosa 等の細菌に対する薬剤耐性について述べた。とくにベトナムでは、*S. pneumoniae* の *mefA* 遺伝子の７３%に変異が見つかった。タイでは、*Acinetobacter baumannii* に変異が見つかった。結核研究所御手洗先生は、2002 年の療研で 3,025 分離株のうち、一次抗結核剤耐性は、8.4%、いわゆる XDR-TB に該当する株は、17 株あったと報告した。香港衛生部の Kai Man Kam 先生は、アジアの多剤耐性結核についてリビューした。新患での MDR-TB は、黒竜江省、河南省、内蒙古で多く、XDR-TB は、全世界で、30-40,000 と見積もられると述べた。Beijing W 株が、最も多い。

2）セッション 2.　クローンの伝播、フィットネスおよびモデル研究

Broad Institute の Galagan 先生は、DNA シークエンス法を用いて、結核菌ゲノムの repetitive sequence, unique sequence を調べた。低レベルストレプトマイシンが多かったが、生物学的意味は不明である。NIAID microbial sequence center があるので、他の研究者が、今日研究で利用できる。結核に対する Chip-Seq という方法をも紹介した。

英国 MRC の Gagneux 先生は、薬剤耐性結核菌のフィットネスに及ぼす細菌遺伝学のイン

パクトについて述べた。この研究では、数学モデル、疫学、実験の３つが大切である。しかしながら、彼の研究から、MDR-TB が将来どうなるかは、はっきりわからない。和歌山県立医科大学山中教授は、薬剤耐性 *S. pneumonia, H. influenzae* のクローン伝播についてのべ、急性中耳炎の 70%は、薬剤耐性だと述べた。2 歳以下の子供で多く、家族、同胞内、デイケアセンターでの感染が多い。エモリー大学 Levin 先生は、薬剤耐性のフィトネス　コスト（対価）と薬剤耐性呼吸系病原菌の疫学及ぼす影響について述べた。Compartment model を使って、薬剤耐性のフィトネス　コストを調べた。次いでマウスを使って、ストレプトマイシンとその耐性大腸菌の関係を調べた。新潟大学医学部斉藤先生は、日本と他のアジアにおけるアマンタジン耐性A型インフルエンザの頻度について報告した。彼らは、Clade N株を見つけ、この株が、迅速に伝播していることを報告した。東京大学Hatakeyama先生は、ニューラミニデース阻害剤耐性インフルエンザウイルスについてリビューした。これらの阻害剤は、ニューラミニデース上のシアル酸結合部位を阻害する。B 型インフルエンザウイルスを持つ子供の 1.4%が、oseltamivir 耐性であった。この阻害剤耐性 H5N1 ウイルスは、ヒトに由来する。エモリー大学 Shafer 先生は、薬剤耐性と細菌フィットネスを研究するための感染モデルの使用を紹介した。Efflux pump の重要性を説き、例として MtrCDE と MTRF を紹介した。京都大学稲葉先生は、免疫学専門部会からの参加である。*Candida albicans* を用いた抗細菌応答における SIGNR1 の役割について述べた。SIGNR1 は、TNF-α産生を亢進し、TLR とは無関係な oxidative burst を高める。

3)　セッション　３．抗細菌抵抗性(AMR)出現の機序

国際医療センター切替先生は、薬剤耐性結核菌の遺伝子機序について述べ、次いで新しい検査法（line probe assay）について解説した。中国河南省胸科病院石先生は、変性 HPLC 法を用いて、多剤耐性結核菌の遺伝学的検査を紹介した。エタンブトール、オフロキサシン、ストレプトマイシン耐性について報告した。

エモリー大学 Stephens 先生は、肺炎球菌におけるエリスロマイシン耐性について述べた。*mefE, ermAM* における突然変異を報告した。バンダービルト大学 Edwards 先生は、薬剤耐性 *S. pneumoniae* が、肺炎球菌ワクチンで減少するかについて興味ある報告を行った。このワクチンは、2000 年に、2 歳以下の子供を対象に、認可された。テネシー州で広範な臨床研究を行った。答えは「否」である。ミネソタ大学 Singer 先生は、家禽生産に及ぼす抗生物質の影響について述べた。牛糞やミネソタ川で少量の抗生物質が、認められる。家禽で薬剤耐性（たとえば Florfenicol）colibacillosis, mycoplasmosis が、報告されている。もう少し、家禽に対して抗生物質の使用を工夫する必要がある。大塚製薬 Geiter 先生は、新しい抗結核剤 OPC-67683 の臨床トライアルについて述べた。今のところ QT 時間の延長は、あるものの目立った副作用はない。200mg　単回使用で行っている。多剤耐性結核菌を用いて臨床トライアルも行っている（phase 2)。将来、phase 3 の臨床研究も行う予定である。

4）セッション　４．　現用している薬物の適正化使用のための戦略

NIAID, NIH Barry, III は、韓国の結核病院で行われた metronidazole 20mg/kg, 一日二回服用で、結核治療効果を、PETScan, HRCT を用いた画像処理で、経過を観察した。現在、phase 2 で、治療効果は認められる。結核研究所　土井先生は、TB/HIV のための吸入薬物使用の可能性を述べた。Lipid microsphere に包んだリファンピシン、caprazamycin B, CPZEN-45 を用いてマウスモデルで、その有用性を確認した。　韓国馬山病院　Park 先生は、外科医の立場から、MDR-TB の治療について講演した。昨年、800 人の結核患者が入院し、142 人が MDR-TB だった。治療成功が 43%、治療失敗 13%。少数ながら、外科治療も行った。Drug susceptibility testing(DST)の結果を勘案して、治療しているが、薬剤耐性度と治療効果に乖離がある。現在、DST に代わるよりよい DST はない。

4．　12 月 6 日

1）セッション　5．未来の方向と次のステップ：新しい技術

ビル&メリンダ　ゲイツ財団の Chin 先生は、多剤耐性結核を克服するための新しい診断薬と新薬の開発の可能性を述べた。いままで、有望な製品が出ているが、結核が蔓延している国々での利用が、十分なされていない。ここでの問題点は、ライセンスの問題、国特有の規制があり、多剤耐性結核への道筋は、十分ついていない。フィリピン熱帯病財団の Tupasi 先生は、フィリピンにおける MDR-TB 治療で用いられる DOTS plus について、やや詳しく述べた。結核蔓延国で、フィリピンは、第 9 位である。スメア陽性結核の頻度は、人口 10 万対 124 である。2004 年、MDR-TB の頻度は、再治療例で 21%を占める。フィリピンでは、1999 年から DOTS plus を採用している。悪心、眠気、不眠が主な、副作用で、HRES, CS, capreomycin 等を用いて MDR-TB を治療すると、治癒 74%、治療失敗 3%、死亡 8%であった。彼女は、自治体に基礎を置く患者のケアを提唱しており、総費用は、一人あたり、400,000 ドル以上かかる。ある 1 つの臨床研究で、1082 人の MDR-TB 結核患者で、459 人が、fluorquinolone に耐性であり、37 人が、XDR-TB 患者であった。彼女の臨床経験から、DOTS は、確実に MDR-TB の発生を抑える。神戸健康研究所の岩本先生は、Loop-mediated amplification (LAMP)という新しい結核菌診断法を紹介した。また DNA を捕捉する microweb chip を開発し、タンザニア、ペルー、バングラデシュで feasibility study を行った。特異度は、97.7%で、設備のない地方では、さらなる改良が望まれる。北海道大学の鈴木先生は、広範な応用が可能な結核の遺伝子検査法を紹介した。彼の開発した技術は、microarray の一種で、SpoligoArray97, ISOligoArray55 という。操作が煩雑であるが、特異性は高い。米国 NIAID の Krause 先生は、「Enzybiotics」について、広範に述べた。1954 年に、バクテリオファージを発見し、それから、Streptococus group C phage-associated lysin を取り出し、lysin 毒素治療を提唱した。すなわち、lysin 毒素治療とは、ファージ無きファージ治療である。菌特異的に菌を phage lysin (50　unit を用いる)で殺す。8 つの菌が、特異的 phage lysin を持つことが知られている。彼の研究は、ロックフェラー大学 V. Fischetti 教授に引き継がれている。

2）会議の総括

会議の総括が、Brennan（コロラド州立大学），森先生（ハンセン病研究センター）によってなされた。続いて、親委員会の委員長である Haase, 笹月両先生による閉会の挨拶がなされた。海口市政府の秘書長の Chen 先生の挨拶もあった。

3）ワークショップ

引き続いてワークショップがあった。全部で 4 つ有り、(1) 呼吸器系病原体に対する安全な実験室プラクテイス、(2) シークエンシングと bioinformatics, (3) フィールドでこれら病原体から防護するには、(4) 開発途上国で生物封じ込め実験室を作り、運営するには、である。ワークショップの内容が、いいのにもかかわらず、参加者が、少なく残念だった。別に、講義の内容を納めた CD-ROM が、参加者に配られた。

4）ポスター発表

全部で 50 以上のポスター発表があった。詳細は、割愛する。

5．終わりに

AMR 全体の講演内容はすばらしかったと思う。しかしながら、内容は、薬剤耐性細菌、薬剤耐性ウイルスなどバラバラで、興味本位で、統一がとれていない。特に MDR-TB の発表が半分以上を占めた。また、数年後に、焦点（テーマ）を絞って、同種の国際会議を開くべきである。

社会保障国際協力推進研究推進事業（国際共同研究事業）
研究報告書

上海における多剤耐性結核の頻度と現状

主任研究者　菅原　勇　（財）結核予防会結核研究所
共同研究者　Xiao Heping　上海市肺科医院

研究要旨

２００７年４月から２００８年３月末までに 4,000 人の結核患者が、上海市肺科医院結核病棟に入院した。171 名が、多剤耐性結核患者で、そのうち 29 名が、XDR-TB 患者であった。XDR-TB 患者の治療は、困難で、平均 24 か月以上治療している。方策としては、XDR-TB にならないように、入院時、完全に治療せねばならない。

A. 研究目的

これまで中国の多剤耐性結核の報告は、部分的ではあるが、存在する。しかしながら、正確な統計は存在しない。本研究は、期間を、調査場所を限定して、正確に行うものである。頻度、社会背景が、判明することにより、多剤耐性結核に対する問題点が、はっきりして、中国の結核対策に基本的情報を与えることができるだろう。

同時に、日中の人的交流が盛んになっており、輸入感染症としての結核の研究の一環として、実態も把握する必要がある。 この研究は、小規模ではあるが、日本の結核対策にも基本的情報を与える。

A. 研究方法

2007年4月から2008年3月まで上海市肺科病院に入院している結核患者から分離された結核菌を用いて比率法によりINH, RFP耐性の頻度を調べる。同時にこれら患者の社会背景を調べる。

B. 研究結果

２００７年４月から２００８年３月末までに4,000人の結核患者が、上海市肺科医院結核病棟に入院した。171（4.3%）名が、多剤耐性結核患者で、そのうち 29 名（17%）が、XDR-TB患者であった。MDR-TB患者の治療には、平均２１ヶ月を要した。XDR-TB 患者の治療は、困難で、平均24か月以上治療している。方策としては、XDR-TB にならないように、入院時、完全に治療せねばならない。

C. 考察

上海付近の結核患者を集めた。95%以上が農民であった。この病院は、結核治療で有名で、診療レベルも高い。その成果、予期した割合より、MDR-TB, XDR-TBの率が低い。この研究を基礎にして、他の省の結核患者の実態を調べられたらと思う。我々は、つい最近報告したが、結核治療に失敗した MDR-TB 患者の40%以上が、XDR-TB 患者であった。今回の結果は、この値より有意に低い。日中の人的交流が盛んになっており、輸入感染症としての結核の研究の一環として、実態も把握する必要がある。 この研究は、小規模ではあるが、日本の結核対策にも基本的情報を与える。

MDR-TB, XDR-TB 患者の治療は、現在、有効な治療薬がない。今後、中国でやるべき方策としては、薬剤感受性結核を、完全に治療して、MDR-TB, XDR-TB 患者を生み出さないことである。DOTSは有効な手段である。

（倫理面への配慮）結核患者の名前が表面に出ないように、患者名を使わずに、番号を付し、プライバシーが漏れないように留意した。

D. 結論

今回の研究では、MDR-TBは、入院患者の 4.3%を占め、そのうち XDR-TB 患者は、17%であった。国の方策としては、XDR-TB にならないように、入院時、完全に治療せねばならない。

E. 研究発表

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Tuberculosis

Recombinant BCG Tokyo (Ag85A) protects cynomolgus monkeys (*Macaca fascicularis*) infected with H37Rv *Mycobacterium tuberculosis*

I. Sugawara[a,*], Z. Li[b], L. Sun[c], T. Udagawa[a], T. Taniyama[d]

[a]*Mycobacterial Reference Center, The Research Institute of Tuberculosis, 3-1-24 Matsuyama, Kiyose, Tokyo 204-0022, Japan*
[b]*Shanghai H&G Biotechnology Co., Shanghai, China*
[c]*Animal Biosafety Level 3 Laboratory, The Center of Animal Experiments, Wuhan University, Wuhan, China*
[d]*National Institute of Infectious Diseases, Tokyo 162-8640, Japan*

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Summary
One tuberculosis vaccine candidate that has shown a promising degree of protective efficacy in guinea pigs is recombinant BCG Tokyo (Ag85A)(rBCG-Ag85A[Tokyo]). As a next step, cynomolgus monkeys were utilized because they are susceptible to *Mycobacterium tuberculosis* and develop a continuous course of infection that resembles that in humans both clinically and pathologically. The recombinant BCG vaccine was administered once intradermally in the back skin to three groups of cynomolgus monkeys, and its protective efficacy was compared for 4 months with that of its parental BCG Tokyo strain. Vaccination of the monkeys with the rBCG-Ag85A[Tokyo] resulted in a reduction of tubercle bacilli CFU ($p<0.01$) and lung pathology in animals challenged intratracheally with 3000 CFU H37Rv *M. tuberculosis*. Vaccination prevented an increase in the old tuberculin test after challenge with *M. tuberculosis* and reaction of *M. tuberculosis*-derived antigen. Thus, it was shown in monkeys that rBCG-Ag85A[Tokyo] induced higher protective efficacy than BCG Tokyo. This warrants further clinical evaluation.

Introduction

Tuberculosis (TB) still remains a major health threat affecting millions of people worldwide. The only TB vaccine currently available is *Mycobacterium bovis* BCG. However, the efficacy of BCG against adult pulmonary TB still remains controversial.[1–4] Thus, development of a better TB vaccine is urgently required to counteract the global threat of TB.

We have previously reported the protective efficacy of a TB DNA vaccine (Ag85A) and a recombinant strain BCG Tokyo (Ag85A) in small-animal models challenged with *M. tuberculosis* Kurono strain.[5,6] We found that recombinant BCG

*Corresponding author. Tel.: +81 424935075; fax: +81 424924600.
E-mail address: sugawara@jata.or.jp (I. Sugawara).

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Tokyo was better than Ag85A DNA in terms of protective efficacy against *M. tuberculosis*.[2] The spleen tissues from guinea pigs vaccinated with rBCG-Ag85A[Tokyo] or Ag85A DNA expressed IFN-γ and IL-2 mRNA at significantly high levels.[6] 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.[7] Previous studies have shown that whereas the rhesus macaque is highly susceptible to *M. tuberculosis*, the closely related cynomolgus macaque is more resistant.[8–10] 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[11,16–19] and several recent reviews on TB vaccines have been published.[12–15] 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.[20] DNA vaccine (HSP65+IL-12/HVJ) as well as 72f recombinant BCG provide better protective efficacy in cynomolgus monkeys.[21] 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.[6]

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 °C. The culture strain was filtered through a membrane filter (4-μm 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 2×10^6 CFU/ml rBCG-Ag85A[Tokyo]. Group 2 received one intradermal injection of 2×10^6 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.[22] 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^3 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 pulmonary 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.

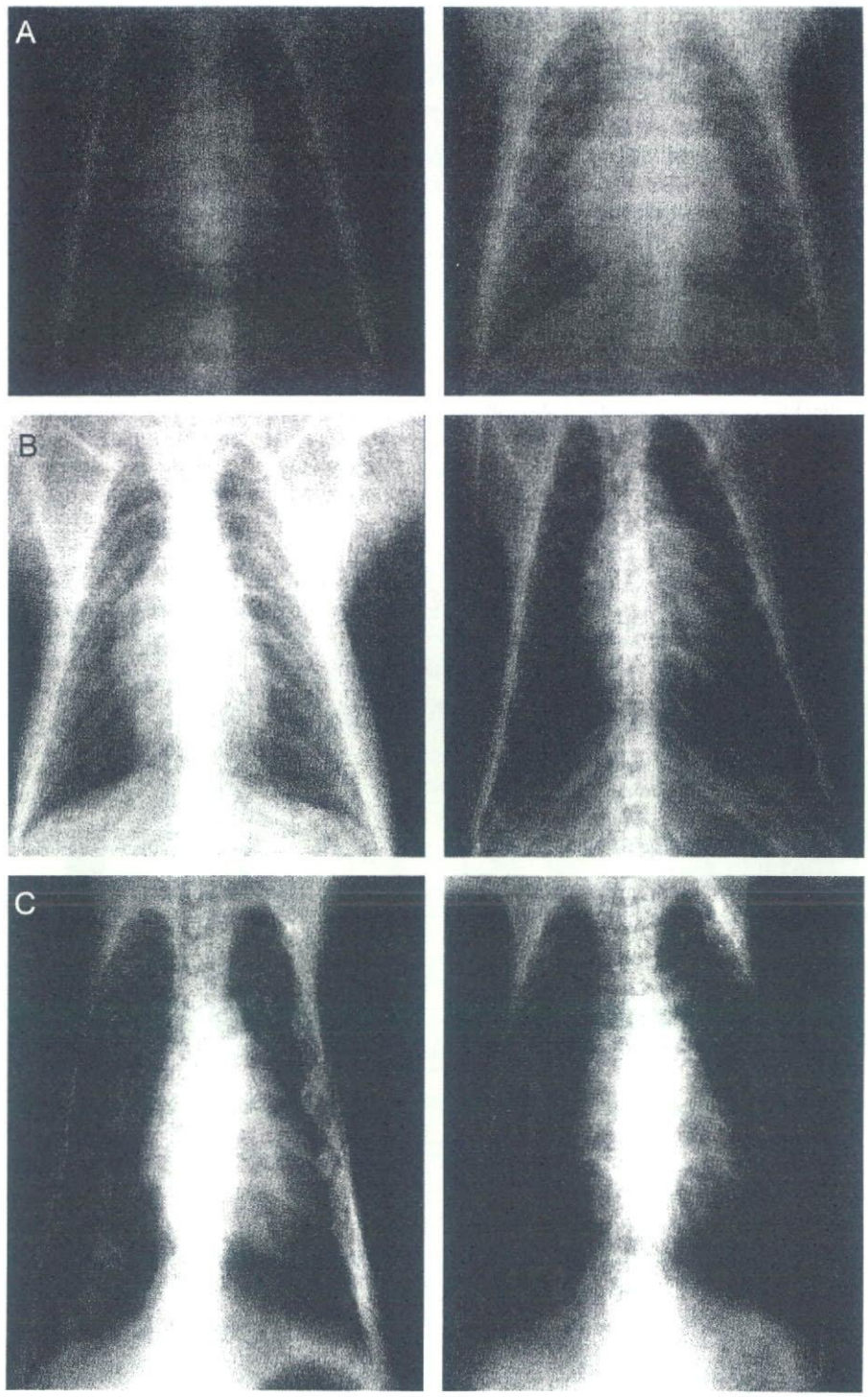

Figure 1 Chest radiologic examinations after challenge with H37Rv *M. tuberculosis*. (A) The monkey (No. 2) vaccinated with rBCG-Ag86A[Tokyo]. The chest X-ray picture was taken 1 day before necropsy. (B) The monkey (No. 9) vaccinated with parental BCG Tokyo. The chest X-ray picture was taken 1 day before 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 2×10^6 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 (1000 CFU or more) for optimal intratracheal infection.[10,16,17] We chose 3000 CFU 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 2×10^6 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

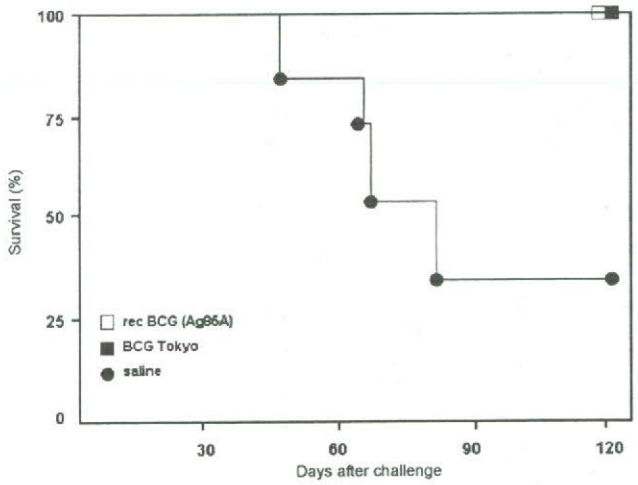

Figure 2 Mortality curve after challenge. Animals in groups of six were challenged by intratracheal inoculation with 3000 CFU of H37Rv. Non-vaccinated animals (six per group) were inoculated with saline.

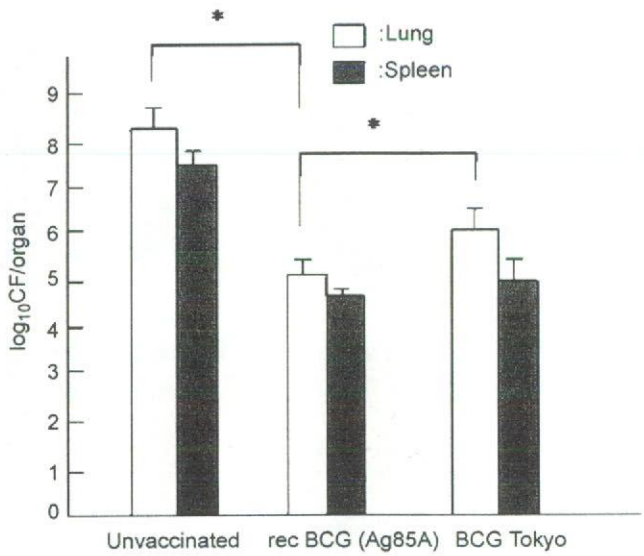

Figure 4 CFU counts in lung and spleen tissues of H37Rv *M. tuberculosis*-infected cynomolgus monkeys vaccinated with recombinant BCG Tokyo (Ag85A), and in non-vaccinated controls. *Statistical difference at $p<0.01$.

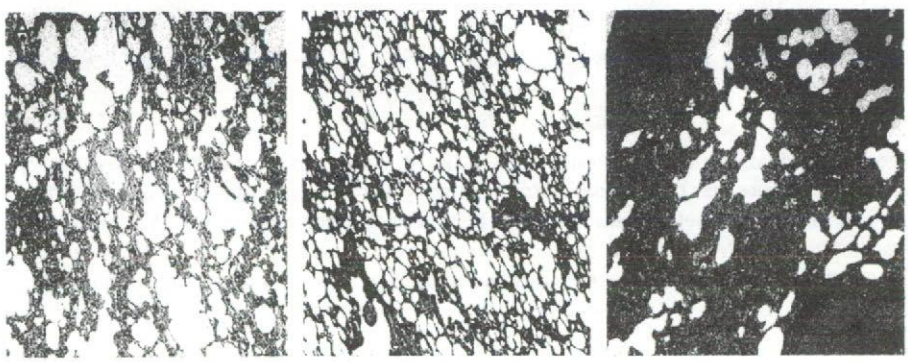

Figure 3 Histopathology of lung tissues from *M. tuberculosis*-infected monkeys vaccinated with rBCG-Ag85A[Tokyo] (A), BCG Tokyo (B) or non-vaccinated controls (C) at necropsy. The miliary granulomas with caseating necrosis are surrounded peripherally by a dense infiltrate of epithelioid cells and lymphocytes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

and cell-mediated immune responses.[23,24] The expression levels of IFN-γ 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-γ and IL-2 mRNAs were the highest in rBCG-Ag85A[Tokyo].[6] 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.[5,6] 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.[6] Although a single intradermal inoculation of 2×10^6 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.[6,18]

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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