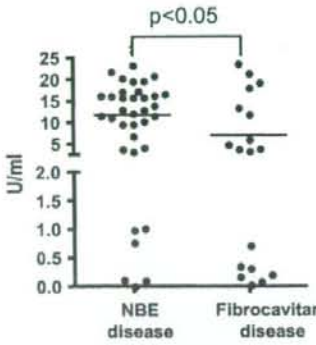

Figure 3. Levels of IgA antibody to glycopeptidolipid core antigens in nodular-bronchiectatic (NBE) and fibrocavitary subtypes of patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD). Significantly higher levels were found in patients with MAC-PD with NBE compared with fibrocavitary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

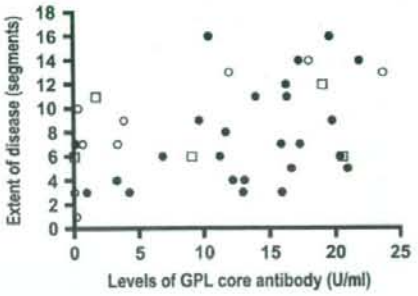

Figure 4. Correlation between antibody levels and radiographic severity using chest computed tomography in 41 patients with *Mycobacterium avium*-complex pulmonary disease. There was a positive correlation between the extent of disease and the levels of antibody ($r = 0.43$, $P < 0.05$). *Closed circles*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

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

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

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

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

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

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

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

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

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

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

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第83回総会ミニシンポジウム

I. ワクチン研究の現在と将来

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キーワーズ：改良BCG，弱毒結核菌，成分ワクチン，DNAワクチン，感染曝露前（予防）ワクチン，感染曝露後（治療）ワクチン

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2. BCG vaccine trials in South Africa
   Gregory HUSSEY (South African Tuberculosis Vaccine Initiative, University of Cape Town, Cape Town, South Africa)
3. Present and future of TB vaccine development research
   Peter ANDERSEN (Statens Serum Institute, Copenhagen, Denmark)
4. Comments and directions in research and development of TB vaccines
   Jerald C. SADOFF (Aeras Global TB Vaccine Foundation, Bethesda, Maryland, USA)

全世界で約20億人（全人口の3分の1）が結核菌（*Mycobacterium tuberculosis*）に既感染，すなわち，無症候性潜伏感染し，毎年920万人が結核を発病，170万人（後天性免疫不全症候群合併23万人を含む）が死亡している（http://www.who.int/tb/en/）。今後10年間に，少なくとも8000万人が結核を発病，2000万人が死亡することが推定されている。

日本（2006年）では年間2.6万人（罹患率人口10万対：20.6）が結核を発病し，2.3千人（死亡率：1.8）が死亡し，日本においても結核対策は重要な課題である。Robert Kochが1882年に「結核菌」を発見，爾来，120年余が経過した現在でも，国内外を問わず，結核は人類に甚大な健康被害を提供し続けている。

結核対策における世界的課題として，①薬剤耐性結核菌の出現や蔓延および②HIV-結核菌の重複感染がきわめて重要である。これらの課題を克服する科学的戦略は「安全で有効な結核ワクチン」である。現行結核ワクチンであるbacillus Calmette-Guérin（BCG）は乳幼児結核に有効であるが，潜在性結核菌感染を基盤とした多くの成人肺結核や内因性再燃結核に対するBCG接種の有効性は疑問視されている。

世界保健機関（WHO）は2015年までに現行BCGを凌駕する新規結核ワクチンの開発を目指している。新規結核ワクチンの開発戦略は「予防・治療：感染曝露前（予防的）や感染曝露後（治療的）ワクチン」，「ワクチン製剤：改良型BCG，弱毒結核菌，成分ワクチンやDNAなど遺伝子ワクチン」，「接種方法：PrimeやPrime-boostワクチン」などの視点から進捗しており，前臨床試験，さらに，第1相など臨床試験で評価され，有望なワクチン候補が開発されつつある。

第83回日本結核病学会総会（石川信克会長）において，ミニシンポジウム「ワクチン研究の現在と将来」を企画し，世界の第一線で活躍されている気鋭の結核ワクチン研究者が結核ワクチン開発の現況や将来展望を発表した。ミニシンポジウム「ワクチン研究の現在と将来」が会員諸氏に有用な情報を提供，そして，研究室から臨床に迅速・効率的に“橋渡し（Translation）”し，究極的に人類に甚大な健康被害を提供し続けている結核の制圧に寄与することを祈念している。

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1. 新しい結核DNAワクチン

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1998年，アメリカ合衆国疾病対策予防センター（Centers for Disease Control and Prevention：CDC）およびAdvisory Council for the Elimination of Tuberculosis（ACET）は新世代の結核ワクチン開発の必要性を発表した。しかしながら，BCGワクチンに代わる結核ワクチンは欧米でも臨床応用には至っていない。結核ワクチンは，DNAワクチン，リコンビナントBCGワクチン，サブユニットワクチンに大別される。DNAワクチンは予防ワクチン効果の切れ味ではほかより優れていることが多く，安定性・経済的にも優れている。われわれはBCGワクチンをはるかに凌駕する1万倍強力な結核予防ワクチン効果を示す新しいDNAワクチン（HVJ-エンベロープ/HSP 65＋IL-12 DNAワクチン）を開発した。

〔マウス〕の結核感染系ではBCGワクチンをはるかに凌駕する新しい結核ワクチンはきわめて少ない。われわれはプライム・ブースター法を用い，HSP 65 DNA＋IL-12 DNA（HVJ-エンベロープベクター）のワクチンはBCGワクチンよりも1万倍強力な結核予防ワクチンであることを世界に先駆けて明らかにした。このワクチンは，結核菌由来のHSP 65蛋白抗原特異的な，CD8陽性キラーT細胞およびinterferon：IFN-gamma産生T細胞の分化も増強した。肺の結核病理像の改善効果も示した。さらに生体内において，CD8陽性T細胞とCD4陽性T細胞の両者がこの結核予防ワクチンに必要であることを明らかにした。

〔治療ワクチン〕さらに，このワクチンは治療結核ワクチン効果も示した。すなわち結核菌をあらかじめ投与したマウスにおいてHVJ-エンベロープ/HSP 65 DNA＋IL-12 DNAワクチンを3回治療投与すると，コントロール群に比較して有意差をもって肺・肝・脾の結核菌数の減少を認めた。多剤耐性結核菌や超薬剤耐性結核（XDR-TB）に対しても治療ワクチン効果を示した。欧米では治療ワクチンは未開発である。モルモット（結核菌吸入感染系）の系でもこのワクチンはBCGより有効であった。〔新しいヒト生体内抗結核免疫解析モデルSCID-PBL/hu〕を用いてもワクチン効果を示した。

さらに，〔ヒト結核感染モデルに最も近いカニクイザル〕（Nature Med. 1996）を用い，HSP 65 DNA＋IL-12 DNAワクチンの強力な有効性を得た。カニクイザルに3回ワクチン接種後4週間後にヒト結核菌を経気道投与し，1年以上経過観察した。リンパ球増殖反応・サイトカイン（IFN-gamma，IL-2等）産生の増強および胸部X線所見・血沈，体重の改善効果が認められた。さらに，生存率改善・延命効果も認められた。DNAワクチン投与群は50%の生存率であり，コントロール群は生存率0%であった。さらに，サルの系でプライム-ブースター法を用いて，より強力なワクチン開発を行った。その結果，BCGワクチン・プライム-DNAワクチン・ブースター法を用いた群は100%の生存率を示した。一方，BCGワクチン単独群は33%の生存率であった。成人に対して切れ味の鋭い強力な新しい結核ワクチンが切望されているが，BCGワクチンは乳幼児でほぼ全員に実施されていることよりHSP65 DNA＋IL-12 DNAワクチンが強力な成人ワクチンとなることが示唆された。WHO STOP TB VACCINE Meetingでこのワクチンはきわめて高い評価を受けた。さらに，このワクチンを鼻粘膜または気道内ワクチンとして投与を試みつつある。さらに，カニクイザルの系で治療ワクチン効果およびプライムとブースターの期間を長期間とって，プライム-ブースター法を研究中である。（共同研究者：当臨床研究センター　喜多，井上，坂谷　各博士，金丸，橋元，西田，仲谷，高尾，栖原，岸上　各研究員，R.Gelber博士，B.Tan博士，中島俊洋博士，長澤鉄二博士，吉田栄人博士，松本真博士，金田安史博士，D.McMurray博士，厚生労働科学研究費補助金の支援による）

We have developed a novel tuberculosis (TB) vaccine; a combination of the DNA vaccines expressing mycobacterial heat shock protein 65 (HSP 65) and interleukin 12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-envelope and -liposome (HSP 65＋IL-12/HVJ). This vaccine provided remarkable protective efficacy in mouse and guinea pig models compared to the BCG vaccine on the basis of C.F.U of number of TB, survival, an induction of the CD8 positive CTL activity and improvement of the histopathological tuberculosis lesions. This vaccine provided therapeutic efficacy against multi-drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) (prolongation of survival time and the decrease in the number of TB in the lung) as well as protective efficacy in murine models. Furthermore, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. This novel vaccine provided a higher level of the protective efficacy than BCG based upon the assessment of mortality, the ESR, body weight, chest X-ray findings and immune responses (IFN-γ, IL-2, IL-6 produc-

tion, and lymphocyte proliferation of cynomolgus monkey). All monkeys in the control group (saline) died within 8 months, while 50% of monkeys in the HSP 65+IL-12/HVJ group survived more than 14 months post-infection (the termination period of the experiment). Furthermore, the combination of HSP 65+IL-12/HVJ and BCG by the priming-booster method showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). In contrast, 33% of monkeys from BCG Tokyo alone group were alive (33% survival). Furthermore, this vaccine exerted therapeutic efficacy in the TB-infected monkeys. These data indicate that our novel DNA vaccine might be useful against *Mycobacterium tuberculosis* for human clinical trials.

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2. BCG vaccine trials in South Africa

South African Tuberculosis Vaccine Initiative, University of Cape Town Gregory HUSSEY

The South African Tuberculosis Vaccine Initiative, located within the University of Cape Town, has been involved in a number of BCG vaccine trials over the last few years and in this presentation I will highlight results from some of our studies.

A randomized trial comparing the efficacy of percutaneous versus intradermal BCG in the prevention of tuberculosis disease in infants and young children

Intradermal BCG vaccine is currently recommended by the World Health Organization (WHO). Prior to this study, no randomized trial comparing the relative incidence of tuberculosis following intradermal as opposed to percutaneous BCG vaccination had been conducted. 11680 South African newborns were randomized to receive Tokyo 172 BCG vaccine via either the percutaneous (n=5775) or the intradermal (n=5905) route within 24 hours of birth and then followed up for 2 years to document and investigate adverse events and suspected tuberculosis (TB) disease. The cumulative incidence of tuberculosis over two years of follow up was 6.13% [95.5 %CI : 5.52−6.79%] in the intradermal group and 6.49% [5.86−7.18%] in the percutaneous group. No significant differences were found between the routes in the cumulative incidence of adverse events. Our results suggest that the WHO should consider revising its policy of preferential intradermal vaccination to allow national immunization programs to choose percutaneous vaccination if that is more practical.

Determining BCG-induced immune correlates of protection against childhood tuberculosis disease

This study aims to determine what we can measure in the blood of a BCG-vaccinated baby to tell us whether that infant has either been protected, or not protected, against future tuberculosis disease. Defining these "immune correlates" is critical for studies of new tuberculosis vaccines. 5675 infants, routinely vaccinated with BCG at birth were enrolled. Blood was collected, processed and cryopreserved at 10 weeks of age, and the infants were followed for at least 2 years. 45 infants developed culture-positive lung tuberculosis over this period (i.e., not protected by BCG). 91 infants did not develop tuberculosis disease despite exposure to adults with tuberculosis in the households (i.e., protected by BCG). We are now in the process of retrieving blood products stored at 10 weeks of age, to compare BCG-induced immunity in the 2 groups. Our comprehensive approach to analysis includes: determining cytokine levels in plasma, evaluating cytokine expression and the memory phenotype of specific T cells, determining specific T cell proliferative and cytokine-producing capacity, assessing the pattern of mRNA expression, and determining whether BCG-induced antibody production patterns may correlate with protection. Results will be presented.

The effect of BCG strain and route of administration on the immune responses caused by the vaccine in infants

At present, we do not know whether BCG strain or route of administration determine efficacy. We evaluated antigen-specific immunity after percutaneous or intradermal administration of Japanese BCG or intradermal administration of Danish BCG. Ten weeks after vaccination of neonates, percutaneous Japanese BCG had induced significantly higher frequencies of BCG-specific IFN-gamma (-producing CD4+ and CD8+ T cells in BCG-stimulated whole blood; significantly greater secretion of the T helper 1-type cytokines IFN-γ, tumor necrosis factor (TNF)-alpha and interleukin (IL) 2; and significantly lower secretion of the T helper 2-type cytokine IL-4; and greater CD4+ and CD8+ T cell proliferation than did intradermal Danish BCG. Thus, BCG strain and route of vaccination confer different levels of immune activation, which may affect the efficacy of the vaccine.

Immune response to BCG vaccination in HIV-infected newborns

We have evaluated the risks and benefits of BCG vaccination in HIV-infected infants. However, we do not know whether BCG does protect HIV-infected children against the disease; rather BCG may itself cause disease in this population. Sequential BCG-induced immune responses were determined in 22 HIV-positive infants compared with that in 25 healthy infants born to mothers not infected with HIV and in 25 HIV-negative infants born to HIV-positive mothers. Results will be presented in the near future.

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3. Present and future of TB vaccine development research

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Tuberculosis (TB) kills 2–3 million people every year. The current tuberculosis (TB) vaccine *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the most widely used vaccine worldwide, but it does not prevent the establishment of latent TB or reactivation of pulmonary disease in adults. The development of subunit vaccines has now reached the point where single antigens as well as poly-protein fusion molecules have been evaluated in animal models and found to provide efficient protection against tuberculosis. The most advanced of these vaccines such as the fusion between ESAT6/TB 10.4 and Ag85B are now in clinical trials. Currently the focus is on evaluating the influence of different adjuvants, live delivery systems, routes and prime-boost regimes for optimal expression of immunity in the lung, boosting of BCG and maintenance of immunological memory. Subunit vaccines can be used to boost BCG immunity either administered together (Tandem administration), shortly after BCG (early boost) or in adolescence when BCG immunity starts to wane (Late boost). A late BCG boost would frequently be administrated post-exposure to latently infected individuals and ongoing efforts are focused on understanding the impact this would have on existing vaccines and for the design of efficient booster vaccines.

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4. Comments and directions in research and development of TB vaccines

Aeras Global TB Vaccine Foundation, Bethesda, Maryland, USA Jerald C. SADOFF

The 83rd Annual Meeting Mini-symposium

RESEARCH AND DEVELOPMENT OF VACCINES AGAINST TUBERCULOSIS

Chairpersons: [1]Kazuo KOBAYASHI and [2]Isamu SUGAWARA

Speakers:

1. Novel DNA vaccines against tuberculosis : Masaji OKADA (Clinical Research Center, National Hospital Organization Kinki-chuo Chest Medical Center)
2. BCG vaccine trials in South Africa: Gregory HUSSEY (South African Tuberculosis Vaccine Initiative, University of Cape Town, Cape Town, South Africa)
3. Present and future of TB vaccine development research: Peter ANDERSEN (Statens Serum Institute, Copenhagen, Denmark)
4. Comments and directions in research and development of TB vaccines: Jerald C. SADOFF (Aeras Global TB Vaccine Foundation, Bethesda, Maryland, USA)

Mycobacterium tuberculosis is one of the most successful bacterial parasites of humans, infecting over one-third of the population of the world as latent infection without clinical manifestations. Over 9.2 million new cases and nearly 1.7 million deaths by tuberculosis (TB) occur annually (http://www.who.int/tb/en/). TB poses a significant health threat to the world population. Global tuberculosis control is facing major challenges today. In general, much effort is still required to make quality care accessible without barriers of gender, age, type of disease, social setting, and ability to pay. Coinfection with *M. tuberculosis* and human immunodeficiency virus (TB/HIV), and multidrug-resistant (MDR) and extensively drug-resistant (XDR)-TB in all regions, make control activities more complex and demanding. Treating and preventing TB is challenging, even in developed countries where there is a modern health care system and infrastructure. Current treatment regimens last six to nine months, and erratic or inconsistent treatment breeds MDR (490,000 new cases/year) and even XDR-TB (40,000 new cases/year), which means that this pandemic could become even more difficult to control throughout the world. TB is a leading cause of death among people who are also infected with HIV, according to the World Health Organization. One-third of the 33.2 million people living with HIV also suffer from TB. The coinfection causes 230,000 deaths annually worldwide. Without proper treatment, approximately 90 percent of people living with HIV die within two to three months of contracting TB (http://www.stoptb.org/wg/tb_hiv/default.asp). The goal of this symposium is to understand the current situation of research and development of novel TB vaccines and the future perspective.

To win the fight against TB, a comprehensive approach is needed that includes new and more effective vaccines as well as improved diagnostics and treatment. The bacillus Calmette-Guérin (BCG) vaccine, created in 1921, is the only existing vaccine against TB. Unfortunately, it is only partially effective. It provides some protection against severe forms of pediatric TB, namely disseminated and meningeal tuberculosis occurring in the first year of life, but is unreliable against adult pulmonary TB, which accounts for most of the disease burden worldwide. Although BCG is the most widely administered vaccine in the world, there have never been as many cases of TB on the planet. There is therefore an urgent need for a modern, safe and effective vaccine that would prevent all forms of TB, including the drug-resistant strains, in all age groups and among people with human immunodeficiency virus (HIV).

Strategies for the research and development (R&D) are included 1) pre-exposure (prophylactic) and 2) post-exposure (therapeutic) vaccines. Based on the preparation, there are 4 types, such as 1) improved BCG, 2) attenuated *M. tuberculosis*, 3) subunit/component vaccines, and 4) DNA vaccines. Speakers have presented and discussed "R&D of novel vaccines against TB" better than current BCG.

To control TB and overcome the issues, such as drug-resistant TB and HIV-TB coinfection, we hope the presentation in the Mini-symposium promotes a more adventurous approach to develop a novel, effective and safe TB vaccine.

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Key words: Improved BCG, Attenuated *Mycobacterium tuberculosis*, Subunit/component vaccines, DNA vaccines, Pre-exposure (prophylactic) vaccines, Post-exposure (therapeutic) vaccines

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RESEARCH ARTICLE

$CD4^+$ T-cell activation by antigen-presenting cells infected with urease-deficient recombinant *Mycobacterium bovis* bacillus Calmette-Guérin

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Keywords
BCG; urease; macrophage; dendritic cell.

Abstract

We constructed a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG-ΔUT) that lacks urease, providing acidic intraphagosomal conditions to drive an effective human immune T-cell response. BCG-ΔUT-infected macrophages stimulated autologous $CD4^+$ T cells more efficiently than parent BCG-infected macrophages. For further T-cell activation, BCG-ΔUT-infected macrophages required pretreatment with exogenous recombinant granulocyte-macrophage colony-stimulating factor or costimulation with either CD40 ligand or interferon-γ. By contrast, BCG-ΔUT-infected dendritic cells induced significant activation of naïve $CD4^+$ T cells without costimulating signals. C57BL/6 mice intradermally inoculated with BCG-ΔUT more efficiently produced memory T cells that responded to recall antigen. Therefore, the depletion of urease from BCG is useful for the activation of T cells.

Introduction

Mycobacteria, such as *Mycobacterium leprae* and *Mycobacterium tuberculosis*, are representative parasitic intracellular pathogens. *Mycobacterium leprae* is a causative agent of human leprosy, in cases of which skin lesions and chronic progressive peripheral nerve injury are usually observed (Stoner, 1979; Job, 1989). At present, around one-third of individuals are infected with *M. tuberculosis* and several millions die as result of tuberculosis each year (Dye *et al.*, 2005; World Health Organization, 2006). *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been used as a vaccine against leprosy, although its efficacy is quite limited (Andersen & Doherty, 2005; Setia *et al.*, 2006). The emergence of multidrug-resistant strains of these mycobacteria is of concern (Maeda *et al.*, 2001; Kai *et al.*, 2004; Kaufmann, 2005), and therefore the urgent development of a new vaccine, including a more efficacious recombinant BCG, is desired (Kaufmann, 2005).

Among various immunocompetent cells, $CD4^+$ T cells, especially IFN-γ-producing cells, play an extremely important role in inhibiting the multiplication of mycobacteria, killing them in the early stages of infection, and keeping the bacterial load at a stable level (Orme *et al.*, 1993; Dockrell *et al.*, 1996; Hashimoto *et al.*, 2002). $CD4^+$ T cells that can respond quickly to pathogenic mycobacteria and produce IFN-γ are known as memory T cells. The efficient production of such memory T cells needs pre-exposure to antigenic vaccinating molecules, which share their antigenicity with that of pathogenic mycobacteria (Kaufmann, 2006). BCG has been considered a good candidate for a vaccine against *M. leprae* in this respect, however its efficacy is limited in several aspects, including the ability to activate T cells (Kaufmann & McMichael, 2005). BCG resides in the phagosomes of macrophages and thus attenuates the trafficking of antigenic molecules to the macrophage cell surface (Grode *et al.*, 2005). One possible strategy for improving the ability of BCG to stimulate T cells is to enhance its ability to fuse with the lysosomes. To this end, we knocked out the *urease* gene from BCG. The urease-deficient recombinant BCG (BCG-ΔUT) is expected to allow phagosomal acidification in the host cells, and induce efficient phagosome maturation for cytolytic activity of the antigenic molecules of BCG (Schaible *et al.*, 1998; Honerzu Bentrup & Russell, 2001).

In the present study, we evaluated the ability of BCG-ΔUT to activate IFN-γ-producing type 1 $CD4^+$ T cells through

antigen-presenting cells (APCs), and to produce memory $CD4^+$ T cells. When used as a target of BCG-ΔUT, macrophages fully stimulated $CD4^+$ T cells in the presence of costimulatory agents such as CD40 ligand (L) and IFN-γ. In addition, BCG-ΔUT-infected monocyte-derived dendritic cells (DCs) activated type 1 $CD4^+$ T cells more efficiently than parent BCG-infected cells in the absence of these costimulators. Therefore, BCG-ΔUT was found to be a useful T-cell-stimulating agent.

Materials and methods

Preparation of blood cells

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. PPD-negative individuals provide more information, however, as healthy individuals are PPD-positive, due to compulsory BCG vaccination for children in Japan (0–4 years old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (Makino & Baba, 1997). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal antibody (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% fetal calf serum and recombinant (r) macrophage colony-stimulating factor (M-CSF) (R&D Systems, Abingdom, UK) (Makino *et al.*, 2007). Macrophages were pulsed with rBCGs on day 5 of culture, and were used as a stimulator of T cells on day 7 (Makino *et al.*, 2007). Monocyte-derived DCs were differentiated as described previously (Makino *et al.*, 1999). Briefly, monocytes were cultured in the presence of 50 ng recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Pepro Tech EC Ltd, London, UK) and 10 ng of recombinant interleukin (rIL)-4 (Pepro Tech) per millilitre (Makino *et al.*, 1999). On day 3 of culture, immature DCs were infected with rBCGs at the indicated multiplicity of infection (MOI), and on day 5 of culture, DCs were used for further analyses of surface antigens and for mixed-lymphocyte assays.

BCG culture and DNA manipulation

The mycobacterial strain, BCG substrain Tokyo, for DNA manipulation was grown in Middlebrook 7H9 broth (Difco Laboratories) with 0.05% Tween 80 or Middlebrook 7H10 agar (Difco) with 0.5% glycerol, each supplemented with 10% albumin–dextrose–catalase enrichment (Difco). DNA manipulations including isolation of DNA, transformation and PCR, were carried out as described previously (Miyamoto *et al.*, 2004). *Escherichia coli* strain DH5α was used for routine manipulation and the propagation of plasmid DNA. *Escherichia coli* strain STBL4 was used for the construction of plasmid vectors derived from phAE87. Antibiotics were added as required: hygromycin B, 150 μg mL^{-1} for *E. coli* and 75 μg mL^{-1} for *Mycobacterium smegmatis* (mc^2155) and *M. bovis* BCG. A recombinant BCG that lacks a *urease* gene was constructed. The sequence of the targeted gene, *ureC* (BCG 1886), was obtained from the BCG list (http://genolist.pasteur.fr/BCGList/). The *ureC* gene was inactivated by inserting a hygromycin-resistance cassette (*hyg*) using a specialized transducing phage system for homologous recombination (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). To construct the disrupted sequence, fragments of around 0.9 kb both upstream and downstream of *ureC* were amplified from BCG-Tokyo genomic DNA using the following two pairs of primers: F UureC and R UureC for upstream of *ureC*, and F DureC and R DureC for downstream of *ureC*. The PCR products were digested with each restriction enzyme and cloned into the corresponding site flanking *hyg* of pYUB854 to give pYUB854-ureC-UD. This plasmid was used for packaging into the phasmid vector phAE87 to construct a specialized transducing mycobacteriophage for gene disruption as described previously (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). BCG-Tokyo infected with the mycobacteriophage at an MOI of 50 was incubated at 37 °C for 3 h in 7H9 broth without Tween 80. Harvested bacterial cells were then plated and cultured on 7H10 agar containing hygromycin B (75 μg mL^{-1}) for 3 weeks. The hygromycin B-resistant colonies were selected and evaluated with a conventional urease assay. A change in the color of the assay medium from yellowish to red was scored as urease-positive. Furthermore, genomic DNA obtained from these colonies was subjected to PCR to confirm the disruption of the gene using primers F ureC and R ureC (Fig. 1). The colony which tested negative in the urease assay was named BCG-ΔUT, while the parental BCG substrain Tokyo is referred to as BCG-Tokyo.

Preparation of *M. leprae*

Mycobacterium leprae (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted by Shepard's method (Charles & Shepard, 1960). The MOI for infection to host cells was determined based on the assumption that macrophages and DCs were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002).

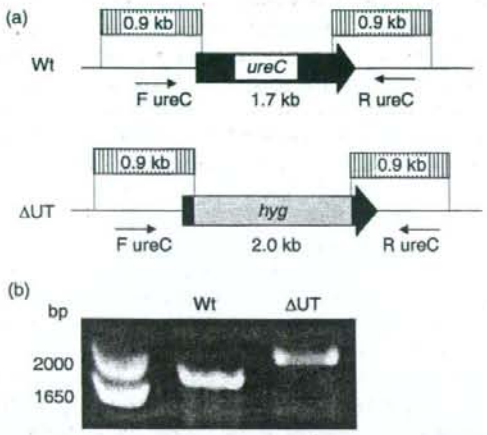

Fig. 1. Disruption of the *ureC* gene. (a) Schematic diagram of the *ureC* region on the chromosome of the wild-type *Mycobacterium bovis* BCG Tokyo strain and its gene disruptant, ΔUT. The shaded boxes indicate the regions included in the recombinant phage for gene disruption. The black arrow represents the coding region of the *ureC* gene. The gray box represents the hygromycin-resistace cassette (*hyg*). The primers used for PCR analysis are indicated by small arrows. (b) PCR analysis of the wild-type and the disruptant using the primers indicated above.

Preparation of mycobacterial antigen

The cytosolic fraction of BCG-Tokyo (BCC) was obtained as described previously (Maeda *et al.*, 2003). Briefly, the mycobacterial suspension containing the protease inhibitors was mixed with zirconium beads at a ratio of *c.* 1 : 1 (v/v) and homogenized using a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo). The suspension was centrifuged at 10 000 *g* to remove the cell-wall fractions. The supernatant was then ultracentrifuged at 100 000 *g* and the resulting supernatant was taken as the cytosolic fraction. For preparation of the *M. leprae* membrane (MLM) fraction, *M. leprae* was used instead of BCG and treated similarly. The pellet obtained by ultracentrifugation (100 000 *g* for 1 h) was used as a membrane fraction (MLM). The optimal concentration of BCC and MLM for stimulating T cells was determined in advance.

Analysis of cell surface antigens

The expression of cell surface antigens on macrophages and DCs, either untreated or treated with exogenous rIFN-γ (R&D Systems), was analyzed using a FACSCalibur flow cytometer. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and 1×10^4 live cells were analyzed. For the analysis of cell surface antigens, the following mAbs were used: fluorescein isothiocyanate (FITC)-conjugated mAbs against HLA-ABC (G46-2.6), HLA-DR (L243), CD14 (M5E2), CD40 (5C3) and CD86 (FUN-1). These mAbs were obtained from BD PharMingen (San Diego, CA).

APC function of rBCG-infected macrophages and DCs

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous mixed-lymphocyte assay as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder $CD4^+$ T cells were purified from freshly thawed PBMCs by using a CD4-negative isolation kit (Dynabeads 450; Dynal) (Wakamatsu *et al.*, 1999). The purity of $CD4^+$ T cells was more than 95% as assessed by fluorescence-activated cell sorting (FACS) analysis. Naïve $CD4^+$ T cells were produced by further treatment of $CD4^+$ T cells with an mAb to CD45RO antigen, followed by incubation with beads coated with goat antimouse IgG. Memory-type T cells were similarly produced by the treatment of cells with an mAb to CD45RA antigen. The purified responder cells (1×10^5 well^{-1}) were plated in 96-well round-bottom tissue culture plates and macrophages or DCs were added to give the indicated APC/$CD4^+$ T-cell ratio. Supernatants of the cocultures were collected on day 4 and the concentration of cytokines was determined. In some cases, macrophages were treated with the indicated dose of exogenous rGM-CSF (Pepro Tech) in advance of infection with rBCGs. Further, macrophages were infected with rBCGs in the presence of neutralizing mAb to IL-10 (JES3-9D7; Rat IgG, BD PharMingen) or control normal rat IgG. Macrophages infected with BCGs were further costimulated with either rCD40L (Pepro Tech) or rIFN-γ (R&D Systems), and in some cases, the macrophages were stimulated with rIFN-γ in the presence of anti-IFN-γ receptor α chain (CD119) (GIR-208, mouse IgG1, BD PharMingen) or control normal mouse IgG. In other cases, macrophages infected with BCG-ΔUT in the presence of exogenous rIFN-γ were treated with either mAb to HLA-DR (L243, mouse IgG2a), CD86 (IT2.2, mouse IgG2b, BD PharMingen) or control normal mouse IgG, and subsequently cocultured with responder $CD4^+$ T cells. The concentration of IFN-γ produced by $CD4^+$ T cells was quantified using an enzyme assay kit [OptEIA Human enzyme linked immunosorbent assay (ELISA) Set; BD Biosciences].

Production of IL-12p70 and IL-1β by DCs

The ability of DCs to produce IL-12p70 and IL-1β on stimulation with BCG-Tokyo or BCG-ΔUT was assessed. The DCs were stimulated with BCGs at the indicated MOI for 24 h, and the concentration of these cytokines was quantified using the Opt EIA Human ELISA Set.

Animal studies

For inoculation into mice, BCG-Tokyo and BCG-ΔUT were cultured in Middlebrook 7H9 to log phase and stored at 10^8 CFU mL^{-1} at −80 °C. Before aliquots were used for inoculation, the concentration of viable bacilli was determined by plating cells on the Middlebrook 7H10 agar plate. Three 5-week-old C57BL/6J mice per group were inoculated intradermally with 0.1 mL phosphate-buffered saline (PBS) containing 1×10^2 or 1×10^3 BCG-Tokyo or BCG-ΔUT. The animals were kept under specific pathogen-free conditions and were supplied with sterilized food and water. Four weeks after injection, the spleens were removed, and the splenocytes were suspended at a concentration of 2×10^6 cells mL^{-1} in culture medium, and stimulated with the indicated concentration of BCC or MLM in triplicate in 96-well round-bottomed microplates. The individual culture supernatants were collected 3 days after stimulation, and IFN-γ and IL-2 were measured using an OptEIA mouse ELISA set.

Statistical analysis

The Student's *t*-test was applied to determine statistical differences.

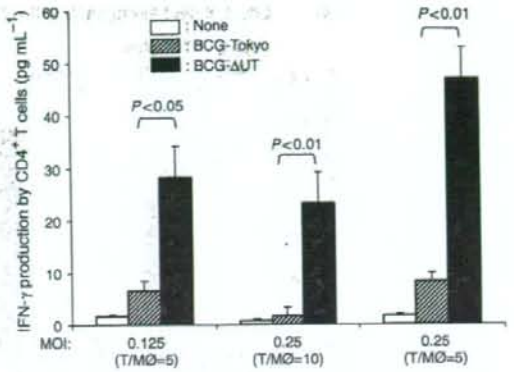

Fig. 2. Production of IFN-γ by CD4+ T cells. Macrophages, differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-Tokyo (parental BCG) or BCG-ΔUT at the indicated MOI, and cultured for another 2 days in the presence of rM-CSF. These macrophages were used as a stimulator of autologous CD4+ T cells (1×10^5 cells well^{-1}) at the indicated T-cell/macrophage ratio in a 4-day culture. A representative example of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

Results

Induction of the fusion of BCG-ΔUT-infected phagosomes with lysosomes

The efficacy with which BCG-ΔUT-infected phagosomes fused with lysosomes in macrophages was examined using confocal microscopy. Lysosomes were stained with anti-LAMP1 mAb after treatment of THP-1 cells with FITC-labeled BCG-Tokyo or BCG-ΔUT for 24 h. The parental BCG colocalized with lysosomes less efficiently than BCG-ΔUT (data not shown). Therefore, BCG-ΔUT may at least partially enhance the ability to induce phagosomal maturation.

T-cell-stimulating activity of BCG-ΔUT

The activity of BCG-ΔUT to stimulate IFN-γ-producing CD4+ T cells, when infected to macrophages, was assessed (Fig. 2). BCG-ΔUT-infected macrophages activated unseparated CD4+ T cells to release IFN-γ substantially more efficiently than parent BCG-infected macrophages. Although BCG-ΔUT-infected macrophages also induced production of IL-2 from CD4+ T cells (data not shown), the extent of IFN-γ (< 50 pg mL^{-1}) and IL-2 production was not as high as expected. Furthermore, BCG-ΔUT did not induce the activation of naïve CD4+ T cells (data not shown). As the activation of T cells is largely influenced by the cytokine milieu, in which T cells and their stimulators are present, we determined the level of cytokines produced from macrophages on stimulation with BCG-ΔUT. BCG-ΔUT produced significantly more cytokines, such as IL-10, GM-CSF, TNFα and IL-1β, than the parental BCG (data not shown). It has been reported that IL-10 inhibits the APC-mediated activation of T cells (Granelli-Piperno *et al.*, 2004) and GM-CSF regulates the function of macrophages (Makino *et al.*, 2007). To examine the role of IL-10 on T-cell activation, macrophages were infected with BCGs in the presence of a neutralizing mAb to IL-10 (Fig. 3a). The IFN-γ production by stimulated CD4+ T cells was not influenced by the treatment of macrophages with control IgG; however, a significantly higher level of IFN-γ was produced on treatment with the neutralizing mAb to IL-10. The upregulation by IL-10 mAb treatment was observed in both BCG-Tokyo and BCG-ΔUT in a similar fashion. Furthermore, the pretreatment of macrophages with exogenous GM-CSF also significantly upregulated the antigen-presenting function of macrophages, although the effect of GM-CSF was more pronounced in BCG-ΔUT-infected macrophages (Fig. 3b).

Next, we phenotypically assessed the effect of BCG-ΔUT on macrophages (Fig. 4a). BCG-ΔUT induced enhanced expression of both CD14 and CD40 on macrophages compared with BCG-Tokyo. Based on these results, we treated BCG-infected macrophages with CD40L to examine its role as a costimulator of macrophages (Fig. 4b). The CD40L treatment upregulated the T-cell activation by BCG-

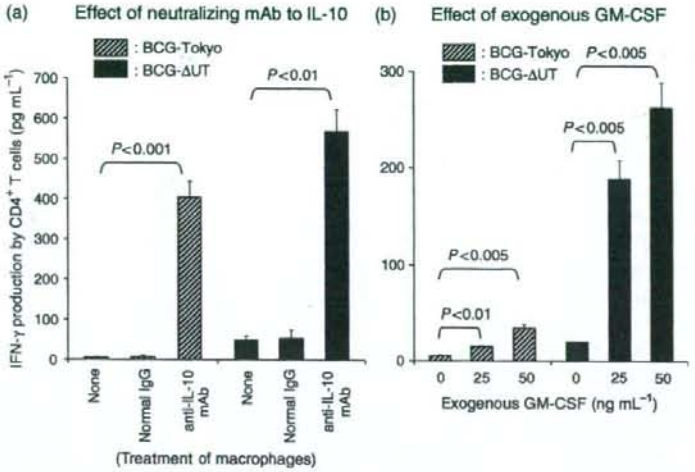

Fig. 3. Effect of IL-10 and GM-CSF on IFN-γ production. (a) Macrophages differentiated from monocytes by using rM-CSF were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 on day 5 of culture and cultured for another 2 days in the presence of rM-CSF. The BCG-infected macrophages were treated with neutralizing mAb to IL-10 or isotype-matched control IgG (10 μg mL^{-1}), and used as a stimulator of CD4$^+$ T cells, at a T-cell/macrophage ratio of 10 : 1, and cultured for another 4 days. The optimal concentration of mAb was determined in advance. (b) Macrophages obtained by 4 days of culture with rM-CSF were treated with the indicated dose of rGM-CSF. The macrophages pretreated with rGM-CSF were infected with BCG-Tokyo or BCG-ΔUT at an MOI of 0.25, cultured for another 2 days in the presence of rM-CSF used as a stimulator of CD4$^+$ T cells on day 8, at a T-cell/macrophage ratio of 10 : 1 (4 days of stimulation). A representative example of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

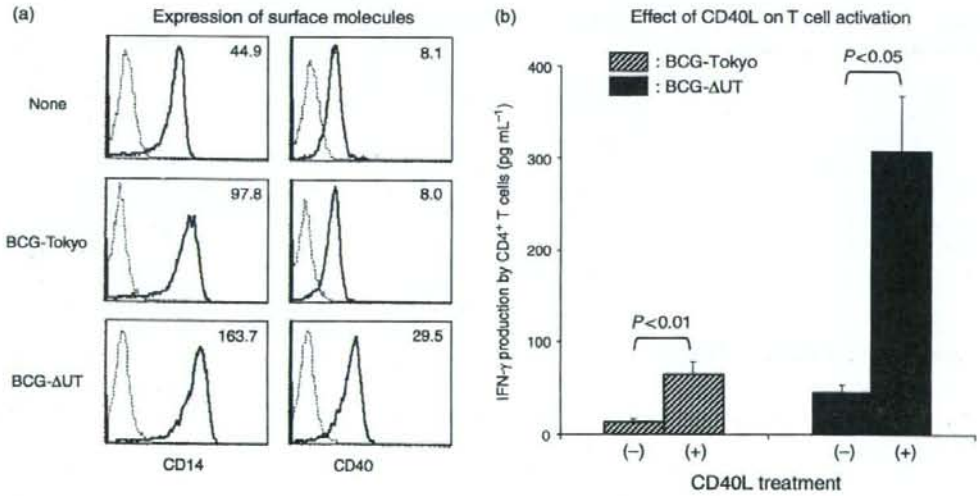

Fig. 4. (a) Expression of CD14 and CD40 molecules on macrophages. Macrophages produced by using rM-CSF were infected with BCGs at an MOI of 0.25, and cultured for another 2 days in the presence of rM-CSF. The macrophages on day 7 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (b) IFN-γ production by CD4$^+$ T cells stimulated with BCG-infected macrophages. Macrophages differentiated from monocytes using rM-CSF were infected with BCGs at an MOI of 0.25 on day 5 of culture, further treated with CD40L (1 μg mL^{-1}) on day 6, and used as a stimulator of CD4$^+$ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

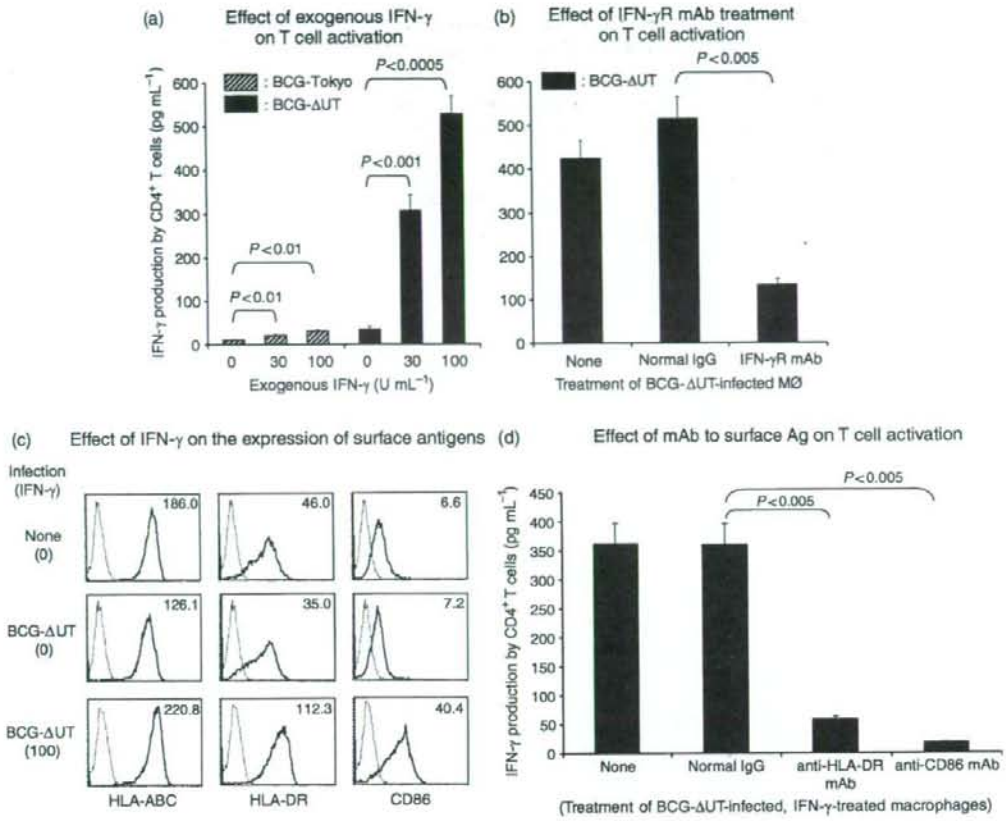

Fig. 5. (a) Effect of exogenous IFN-γ on CD4+ T-cell activation. Macrophages produced by 5 days of culture with rM-CSF from monocytes were infected with BCGs at an MOI of 0.25 and simultaneously treated with the indicated dose of exogenous IFN-γ. The macrophages were used as a stimulator of CD4+ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (b) Involvement of IFN-γ receptor in T-cell activation. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), stimulated with exogenous IFN-γ (100 U mL^{-1}) in the presence of mAb to IFN-γ receptor α-chain (CD119) or isotype matched control IgG (10 μg mL^{-1}), and cultured for another 2 days in the presence of rM-CSF. The macrophages were used as a stimulator of CD4+ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (c) Surface expression of various molecules on BCG-ΔUT-infected, IFN-γ-treated macrophages. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), stimulated with exogenous IFN-γ (100 U mL^{-1}) and cultured for another 2 days in the presence of rM-CSF. The macrophages on day 7 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (d) Involvement of surface antigens of BCG-ΔUT-infected, IFN-γ-stimulated macrophages in T-cell activation. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), treated with exogenous IFN-γ (100 U mL^{-1}) and cultured for another 2 days in the presence of rM-CSF. These macrophages were cocultured with autologous CD4+ T cells at a T-cell/macrophage ratio of 10 : 1 in a 4-day culture in the presence of the indicated mAb (10 μg mL^{-1}). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

infected macrophages, but it more efficiently affected BCG-ΔUT-infected macrophages. Similarly, there was a significant difference between parent BCG and BCG-ΔUT in sensitivity to IFN-γ (Fig. 5a). However, other cytokines such as TNFα and IL-1β did not enhance the T-cell-stimulating activity of rBCG-infected macrophages. The IFN-γ treatment was effective against both BCG-Tokyo- and BCG-ΔUT-infected macrophages; however, more than a 10-fold increase in the production of IFN-γ from T cells was achieved only when BCG-ΔUT-infected macrophages

were stimulated with exogenous IFN-γ. The optimal stimulation of T cells induced the production of more than 500 pg mL^{-1} IFN-γ. The exogenous IFN-γ seems to contribute directly to the enhancement of APC function, as the IFN-γ-mediated enhancement was cancelled out by the pretreatment of BCG-ΔUT-infected macrophages with mAb to IFN-γ receptor α-chain (Fig. 5b). Furthermore, IFN-γ significantly enhanced the expression of HLA-DR and CD86 on BCG-ΔUT-infected macrophages (Fig. 5c), while the phenotypic alteration of BCG-Tokyo-infected macrophages by IFN-γ was minimum (data not shown). When BCG-ΔUT-infected, IFN-γ-treated macrophages were treated with mAb to either HLA-DR or CD86 in advance of being cocultured with CD4$^+$ T cells, IFN-γ production by the T cells was significantly inhibited, while normal murine IgG treatment did not have any effect (Fig. 5d).

CD4$^+$ T-cell activation by BCG-ΔUT-infected DCs

As BCG-ΔUT significantly but less efficiently activated CD4$^+$ T cells through macrophages in the absence of costimulation, the potency of BCG-ΔUT-infected DCs as a T-cell activator was evaluated. Expression of surface molecules on DCs infected with either BCG-Tokyo or BCG-ΔUT was examined (Fig. 6a). Expression of HLA-ABC, HLA-DR, CD86 and CD83 was more significantly upregulated by the infection with BCG-ΔUT than with BCG-Tokyo. Higher levels of IL-12p70 and IL-1β were produced by BCG-ΔUT stimulation (Fig. 6b). Furthermore, we assessed whether BCG-ΔUT activated naïve and memory CD4$^+$ T cells through DCs by using various MOI titers and multiple T/DC ratios (Fig. 6c). IFN-γ levels were significantly higher following stimulation with BCG-ΔUT than with parent BCG in both naïve and memory CD4$^+$ T cells. Also, a higher level of CD40L was expressed on CD4$^+$ T cells after stimulation with BCG-ΔUT-infected DCs (data not shown). These results indicate that the infection of DCs with BCG-ΔUT alone was sufficient, as compared with macrophages which required costimulators to drive a strong T-cell response.

Memory T-cell production by BCG-ΔUT

Another important aspect of using BCG as a vaccine is the production of memory T cells *in vivo*. We examined the response of splenic T cells obtained from BCG-infected C57BL/6 mice to mycobacterial recall antigen (Fig. 7). We used BCC as a recall antigen. At 4 weeks following infection, splenic T cells from BCG-ΔUT-infected mice produced more IFN-γ than those from mice infected with BCG-Tokyo by responding to BCC. The lymphocyte population producing IFN-γ was found to be CD4$^+$ T cells by intracellular staining (data not shown). Furthermore, upon stimulation with MLM, which contains immunodominant antigens of *M. leprae*, CD4$^+$ T cells from BCG-ΔUT-infected mice produced significantly higher levels of IFN-γ than those from uninfected or BCG-Tokyo-infected mice (Fig. 7).

Discussion

To date, BCG is the only suitable vaccine against leprosy; however, its efficacy is quite limited. Overall efficacy in one meta-analysis was reported to be only 26% (Setia *et al.*, 2006). Several reasons might explain why BCG cannot block multiplication of *M. leprae* or inhibit the development of leprosy. The most important defect of BCG is that it is retained in phagosomes of macrophages, avoiding phagosomal acidification and hence interfering in the efficient fusion of BCG-containing phagosomes with lysosomes (Clements *et al.*, 1995; Reyrat *et al.*, 1995; Grode *et al.*, 2005). The lack of phagosome–lysosome fusion inhibits the trafficking of BCG-derived antigens through the major histocompatibility class (MHC) II pathway, which is enrolled for preferential stimulation of CD4$^+$ T cells, the most important cells involved in inhibition of *M. leprae* growth (Sendide *et al.*, 2004). Further, macrophages produce abundant amounts of IL-10 on infection with BCG, which, in turn, inhibits the activation of CD4$^+$ T cells (Mochida-Nishimura *et al.*, 2001; Granelli-Piperno *et al.*, 2004).

In the present study, we constructed a recombinant BCG (BCG-ΔUT) that lacks a *urease* gene through allelic exchange of chromosomal DNA. As urease is involved in the maintenance of intraphagosomal pH at neutral (Grode *et al.*, 2005) or slightly alkaline values (Sendide *et al.*, 2004), lack of this enzyme may contribute to the induction of phagosomal acidification (Sendide *et al.*, 2004), thereby promoting the fusion of BCG-containing phagosomes with lysosomes. The efficient colocalization of BCG-ΔUT with lysosome was observed, leading us to expect an efficient enhancement of T-cell activation by BCG-ΔUT-infected macrophages. Previously, rBCG deficient in urease C was produced by a similar system and found to be superior to parental BCG in producing acidic conditions (pH 4.5–5.5) in BCG-infected phagosomes in murine macrophages (Reyrat *et al.*, 1995; Grode *et al.*, 2005). However, it was not demonstrated whether the rBCG deficient in urease C promoted the MHC class II trafficking pathway and actually activated human CD4$^+$ T cells through APCs. The newly constructed BCG-ΔUT lacked urease activity and *in vitro* studies confirmed that it could not degrade urea to ammonia. When BCG-ΔUT was infected to macrophages, it activated human CD4$^+$ T cells more efficiently than the parental BCG. However, the amount of IFN-γ released from the T cells was not as high as expected (< 50 pg mL^{-1}). These results suggest that

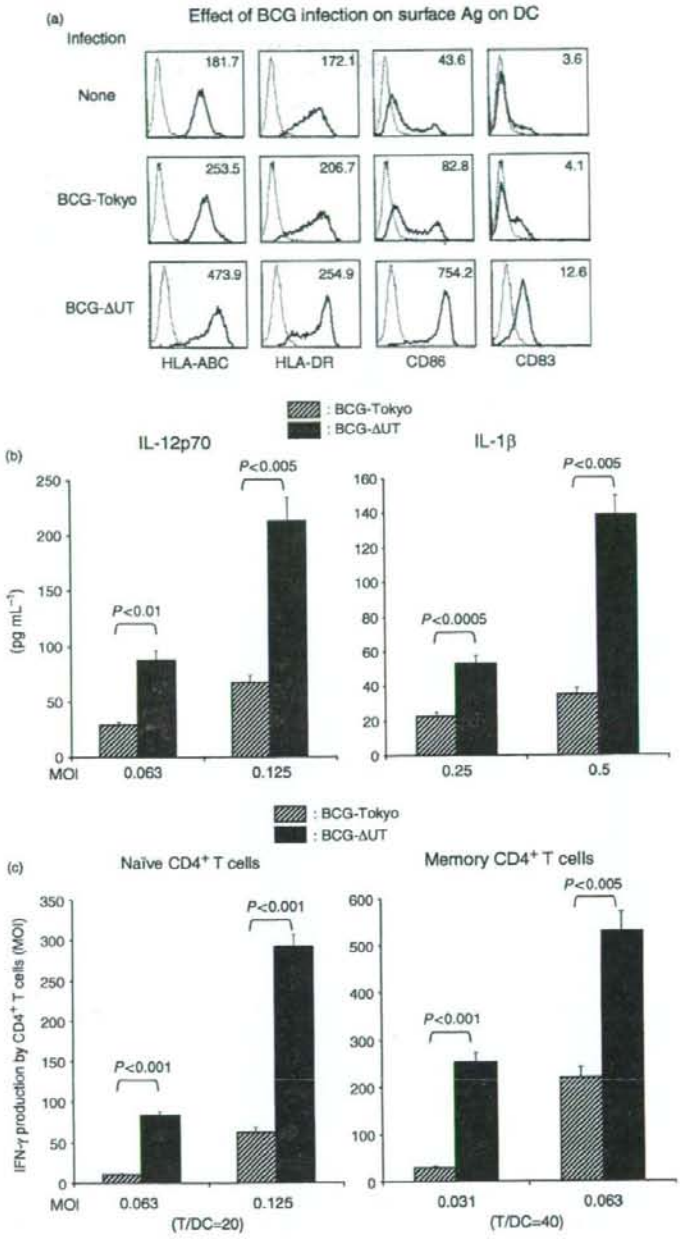

Fig. 6. (a) Expression of various molecules on BCG-infected DCs. Monocyte-derived immature DCs were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DCs from day 5 were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (b) Cytokine production from DCs stimulated by BCG. Monocyte-derived DCs from 4 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of either BCG-Tokyo or BCG-ΔUT for 24 h. The concentration of the indicated cytokine was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (c) IFN-γ production by naïve CD4+ T cells and memory CD4+ T cells. DCs obtained from monocytes infected with either BCG-Tokyo or BCG-ΔUT were used as a stimulator of naïve and memory CD4+ T cells in a 4-day culture. A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

improvement of intraphagosomal pH milieu for efficient phagosome–lysosome fusion was not sufficient for the induction of full T-cell activation as far as macrophages were concerned. Thus, we further searched for factors which might be helpful in inducing full activation of T cells. First, we examined the influence of endogenously produced IL-10, as abundant IL-10 was produced from macrophages by infection with BCG-ΔUT (data not shown). The neutralization of IL-10 from macrophages drastically enhanced T-cell activation (Fig. 3a). Furthermore, pretreatment of

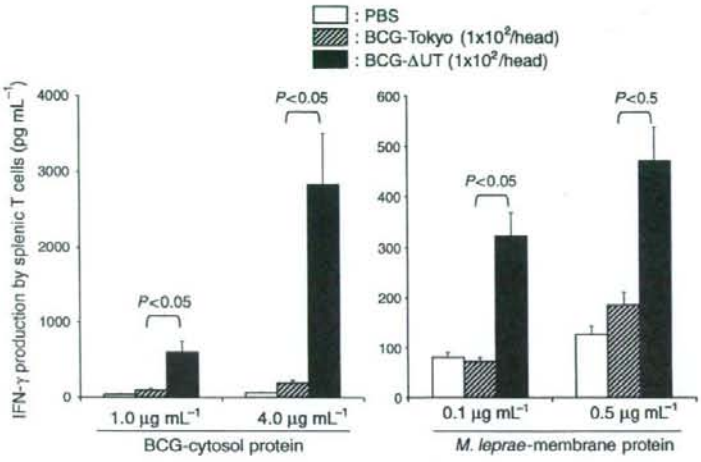

Fig. 7. IFN-γ production by splenic T cells obtained from C57BL/6 mice infected with BCG-Tokyo or BCG-ΔUT. Five-week-old C57BL/6 mice were infected with the indicated dose of BCG intradermally. Four weeks after the inoculation, splenocytes (2×10^5 cells well^{-1}) were stimulated with the indicated dose of either BCG-derived cytosol protein or *Mycobacterium leprae*-derived membrane protein for 4 days. Assays were performed in triplicate for each mouse, and the results for three mice per group are given, expressed as the means ± SD. Representative results for two separate experiments are shown. Titers were statistically compared using Student's *t*-test.

macrophages with GM-CSF, which is normally produced from activated $CD4^+$ T cells, monocytes and macrophages (data not shown), and inhibits IL-10 production (Makino *et al.*, 2007), was also quite efficient in enhancing the BCG-ΔUT-mediated T-cell activity. Therefore, the unexpectedly weak activation of $CD4^+$ T cells by BCG-ΔUT seemed to be at least partly due to the immunosuppressive effect of IL-10. Secondly, we focused on the costimulating factors capable of actively up-regulating the T-cell-stimulating function of macrophages, and found that both CD40L and IFN-γ were quite efficient. It was previously reported that both CD40L and IFN-γ were needed to costimulate macrophages infected with *M. leprae* (Makino *et al.*, 2007); however, in the present study, the sole treatment of BCG-ΔUT-infected macrophages with either CD40L or IFN-γ was enough to confer a sufficient effect (Figs 4 and 5). The high sensitivity of BCG-ΔUT-infected macrophages to CD40L may be due to the ability of rBCG to induce greater expression of CD40 (Fig. 4a). The exogenous IFN-γ may contribute to increased production of IFN-γ from T cells by activating macrophages, as it enhanced the surface expression of HLA-DR and CD86 on BCG-ΔUT-infected macrophages, which facilitated antigen-specific T-cell activation. As reported, *M. leprae* is less sensitive to IFN-γ (Makino *et al.*, 2007), and also parental BCG was found to be clearly less sensitive to IFN-γ than BCG-ΔUT. These results indicate that each mycobacterium may have differential sensitivity to IFN-γ (Verreck *et al.*, 2004). Although the molecular mechanism responsible for the difference in sensitivity remains unexplained, it is well known that IFN-γ facilitates the digestion of intracellular mycobacteria in macrophages, and thus the following speculation may be possible: in the present system, the alteration of the pH milieu of BCG-containing phagosomes caused by the depletion of urease activity may help to establish circumstances where cell activation as well as enhanced trafficking of mycobacterial antigens to the surface by the MHC class II pathway can be induced by IFN-γ treatment. The urease gene of pathogenic mycobacteria may be a good target for combination immunotherapy/chemotherapy as urease depletion downregulates the growth of mycobacteria (data not shown) and upregulates the immunoactivity of intracellular digestion of bacteria in host cells.

In contrast to macrophages, DCs were highly activated by the sole infection with BCG-ΔUT in terms of phenotype and cytokine production, and BCG-ΔUT-infected DCs efficiently activated both naïve and memory $CD4^+$ T cells in the absence of additional costimulation. The activated T cells produced abundant amounts of both IFN-γ (Fig. 5c) and GM-CSF, and induced CD40L expression (data not shown). Therefore, DCs can inherently provide the critical factors needed by BCG-ΔUT-infected macrophages. As BCG infects both macrophages and DCs *in vivo*, we evaluated the efficacy of BCG-ΔUT as a T-cell activator by using C57BL/6 mice. BCG-ΔUT was superior to BCG-Tokyo in the production of murine memory $CD4^+$ T cells, which can respond to BCG-derived recall antigen and also proteins derived from pathogenic *M. leprae*. Just 100 BCG-ΔUT bacilli were sufficient to produce such memory T cells. These findings indicate that BCG-ΔUT convincingly stimulated $CD4^+$ T cells *in vivo*. As the C57BL/6 strain is a T helper (Th)1 response-prone mouse, further study using Th2 response-prone mice would provide further insight into how memory T cells are generated by inoculation with BCG-ΔUT.

Taking our data together, BCG-ΔUT is more potent than the parental BCG in the activation of macrophages, DCs and $CD4^+$ T cells. The depletion of urease from BCG may be useful in upregulating the potency of BCG as an immunostimulator.

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今月の主題　結核

トピックス

結核ワクチン研究の現状と展望

松本　壮吉　　小林　和夫

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