tors such as TLRs.[6] Through sensing microbe patterns, dendritic cells mature to present extrinsic Ag and release lymphocytes from an anergic state.[2,6] The cell-mediated immune system is thereafter activated to target Ag-bearing cells. This concept was demonstrated only recently, and it is now apparent that TLR agonists were being used as popular adjuvants for therapeutic purposes without knowledge of their mechanistic function.

For more than 6 years, clinical trials of many TLR-directed adjuvants have been conducted, aiming at adjuvant-augmented immunotherapy.[6,7] Most of these trials are still in progress with fruitful or anticipated results. Our earlier studies suggested that Bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) has the potential to activate human antigen-presenting dendritic cells and induce interleukin 6 (IL-6), IL-12, tumor necrosis factor alpha (TNF-α),[8] and possibly, CTL.[9] Interferon-gamma (IFN-γ) levels increase in response to BCG-CWS.[10] A sole BCG-CWS without peptides was used in these studies since cancer patients are usually exposed to their own TAAs.[10] Studies on mouse tumor implant models suggest that BCG-CWS induces cross-priming facilitating class I presentation of exogenous antigens.[9] An efficient CTL response against Ag-bearing cells appears evident. These immune responses are attributed to TLR2 and TLR4 in antigen-presenting dendritic cells.[8,11]

The current clinical study was designed to investigate these basic findings on BCG-CWS adjuvant. We anticipated that BCG-CWS alone has the ability to evoke an antitumor immune response because patients with cancer postoperatively still possess TAAs.[10,12] To find out if patients who undergo radical surgery followed by adjuvant BCG-CWS immunotherapy for NSCLC are more likely to have a favorable outcome, we conducted a case–control study.

Materials and Methods

Preparation of BCG-CWS and Its Inoculation Schedule

BCG-CWS, donated by Dr. Azuma,[13] was used as an immunotherapeutic agent in the form of an oil-in-water emulsion, using either mineral oil (Drakeol 6VR) or a metabolizable oil such as squalene or squalane. After sterilizing by heating for 30 min at 60°C, the oil-attached BCG-CWS suspension was inoculated intracutaneously at a final concentration of 1 mg/ml in the upper arm according to the schedule described by Hayashi et al.[12,14] In the sensitization phase, 200 μg was inoculated four times weekly, whereas in the therapeutic phase, the amount inoculated, at 4-week intervals, ranged between 10 and 200 μg, depending on the patient's biological responses, including IFN-γ induction, local skin reaction at the inoculation site, various physical conditions (fever or general malaise), and indicators of laboratory tests showing liver function or inflammatory reactions.

Interferon-γ Induction Test

To evaluate the effect of immunotherapy on BCG-CWS, an IFN-γ induction test was performed at the time of the fourth inoculation in the sensitization phase, and at the time of the first and sixth inoculations in the therapeutic phase. The level of IFN-γ in the peripheral blood was measured before inoculation and 18 h after inoculation. Interferon-γ levels were detected with an enzyme-linked immunosorbent assay at the laboratory of Otsuka Assay (Tokushima, Japan), with the lower limit of sensitivity for detecting human serum IFN-γ being 7.8 pg/ml.

Case–Control Study

In May 1994, the protocol of a pilot study on BCG-CWS immunotherapy for patients with various malignant neoplasms was approved by the Ethical Review Board of Osaka Medical Center for Cancer and Cardiovascular Diseases. At the time of informed consent, we explained to patients about the expected effectiveness and side effects based on previous reports on immunotherapy,[15] chemotherapy,[16] and our survival data of surgery alone. In the 1990s, with the exception of one article published in 1995 from the NSCLC Collaborative Group,[16] there was no clear evidence of the survival benefit of adjuvant chemotherapy for NSCLC.

Written informed consent was obtained from all patients who chose to be treated with immunotherapy, which was started 4–6 weeks after the operation. Among the patients who received BCG-CWS, we recruited 83 NSCLC patients. Between 1994 and 2000, these patients received immunotherapy with BCG-CWS alone as adjuvant therapy after radical surgery for NSCLC. Since the clinical records of 12 patients were unavailable because their operations had been performed at other hospitals, they were excluded from the final analysis. Thus, 71 patients with both clinical records and follow-up data were enrolled in this study as the case group. They did not receive any other adjuvant therapy until recurrence was confirmed.

The case–control study was designed with one control selected for each patient. The control was matched to the patient by pathological stage and year of birth (±5 years). The matched control was recruited from among patients who underwent radical surgery for NSCLC, regardless of adjuvant chemo- and/or radiotherapy, with the shortest interval between the operation from our medical history data file.

Adverse effects of immunotherapy were graded from 0 (none) to 5 (fatal) according to the Common Terminology Criteria for Adverse Events; version 3 (CTCAE) of the National Cancer Institute.

Statistical Analysis

Continuous variables were analyzed by the *t*-test, and categorical variables were evaluated using χ^2 analysis. Overall survival rates and survival rates at each stage were compared between the patients and controls. We performed survival analysis with StatView version 5 (Abacus Computer, Berkeley, CA, USA), and survival curves were calculated with the Kaplan–Meier method.[17] Differences in survival were evaluated with the log-rank test. A P value of less than 0.05 was considered significant.

Results

The case-group patients were inoculated with 45 ± 22.6 (average ± SD) cycles of BCG-CWS over a range of 6–94 cycles (Fig. 1). Table 1 compares the patient characteristics of the case group with the control group. There were no significant differences between the groups in matched criteria, pathological stage (P = 1.000), or histology (P = 0.913). The mean age of the case-group patients was slightly less than that of the control patients (P = 0.087). The male to female ratio of the case-group patients was slightly lower than that of the control patients (P = 0.217). The types of lung resection and the pathological T and N factors were similar in the two groups (P = 0.967, 0.986, and 0.980, respectively). Among 40 control patients with stage II or III disease, 5 had received adjuvant chemo- and/or radiation therapy. The median follow-up was longer than 5 years and was similar in the two groups.

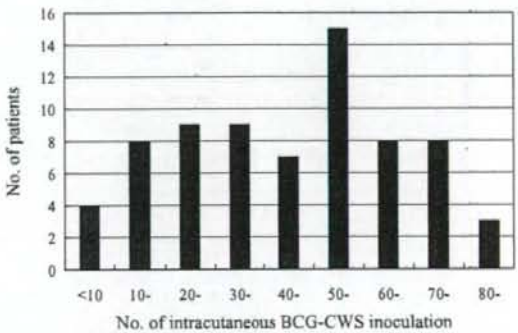

Fig. 1. Postoperative adjuvant immunotherapy with Bacillus Calmette-Guérin cell wall skeleton (*BCG-CWS*) for non-small cell lung cancer. Patient distribution according to the number of treatment courses

Table 1. Case–control study: patient backgrounds

Characteristic	Case patients	Control patients	*P* value
No. of patients	71	71	
Age, years (mean, range)	59 (37–78)	62 (38–78)	0.087
Sex			0.217
Male	43	50	
Female	28	21	
Surgery			0.967
Lesser resection	8	9	
Lobectomy	58	57	
Pneumonectomy	5	5	
Pathological T factor			0.986
T1	36	35	
T2	22	24	
T3	12	11	
T4	1	1	
Pathological N factor			0.980
N0	38	37	
N1	18	19	
N2	15	15	
Pathological stage			1.000
I	31	31	
II	21	21	
III	19	19	
Histology			0.913
Adenocarcinoma	51	49	
Squamous cell carcinoma	16	17	
Large cell carcinoma	4	5	
Median follow-up period (months)	68	66	

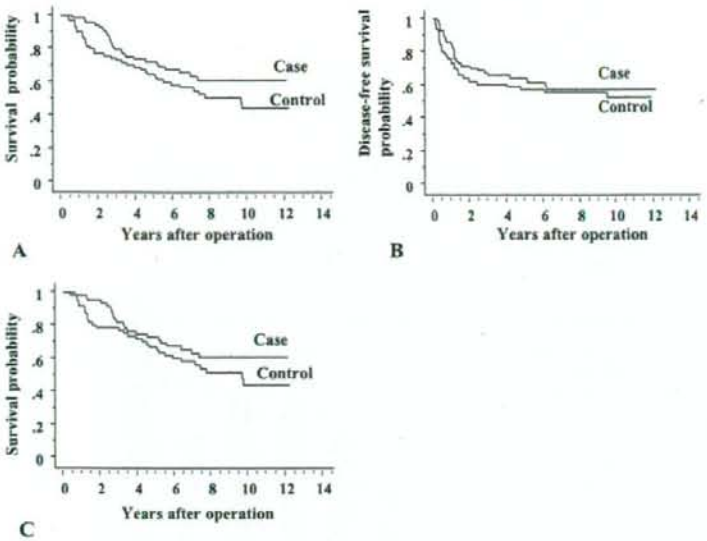

Fig. 2A–C. Kaplan–Meier overall survival estimates. **A** Survival curves. Case-group patients ($n = 71$) versus controls ($n = 71$). $P = 0.114$. **B** Disease-free survival curves. Case-group patients ($n = 71$) versus controls ($n = 71$). $P = 0.473$. **C** Survival curves of 62 gender-matched pairs. Case-group patients ($n = 62$) versus controls ($n = 62$). $P = 0.190$

When patients who had undergone radical surgery for NSCLC hoped to receive adjuvant immunotherapy in spite of a weak IFN-γ induction, the immunotherapy was continued if they had a local skin reaction at the injection site of BCG-CWS, such as an area of erythema greater than 20 mm in diameter, or an induration with an ulcer.[18] As a result, none of the patients given immunotherapy as postoperative adjuvant therapy stopped receiving it. The 5-year and 10-year survival rates were 74% and 62%, respectively, for 20 case-group patients given IFN-γ induction of 35 pg/ml or more, and 70% and 60%, respectively, for 39 given IFN-γ induction of less than 35 pg/ml ($P = 0.700$). The IFN-γ assay was not done for 12 patients.

The overall 5-year and 10-year survival rates were 71% and 61%, respectively, for the case-group patients, and 63% and 43%, respectively, for the control patients group (Fig. 2A). Although the difference was not significant, the survival rate of the case-group patients was better than that of the controls over the observation period ($P = 0.114$). The same trend was observed in disease-free survival between these two groups (Fig. 2B).

To exclude the influence of gender heterogeneity (Table 1) on survival, we selected 62 gender-matched pairs and compared their survival curves. The 5-year and 10-year survival rates were 73% and 61%, respectively, for the 62 case-group patients, and 67% and 44%, respectively, for the 62 control patients ($P = 0.190$; Fig. 2C).

According to the pathological stages, there were no significant differences in survival between the case and control group patients with stage I or II disease (Fig. 3A and 3B). The survival rate of the case-group patients with stage III disease was better than that of the control group patients with stage III disease, although the difference did not reach significance ($P = 0.114$; Fig. 3C). Ten multi-station N2 patients were included in the case group and 8 in the control group. When the survival of pathologically N+ patients was analyzed, both groups showed the same tendency ($P = 0.168$; Fig. 3D).

Table 2. Adverse effects of Bacillus Calmette-Guérin cell wall skeleton treatment

No. of patients	71 (100%)
Nonadverse effect	52 (73)
Adverse effect	19 (27)
Nonmalignant axillary and/or cervical lymph node swelling	9 (13)
ALT and AST elevation (≤grade 2)	6 (8)
Nonmalignant pleural effusion (grade 1)	2 (3)
Infection	1 (1.5)
Neuropathy (grade 2)	1 (1.5)

ALT, alanine aminotransferase; AST, aspartate aminotransferase

Adverse effects were seen in 19 (27%) of the 71 case-group patients (Table 2). These included mild or moderate elevation of alanine aminotransferase and aspartate aminotransferase in six patients, mild nonmalignant pleural effusion in two, and moderate focal infection at the BCG-CWS inoculation site and neuropathy in one patient each. Although there is no grade that refers to the severity of CTCAE, nonmalignant axillary and/or cervical lymph node swelling was observed in nine

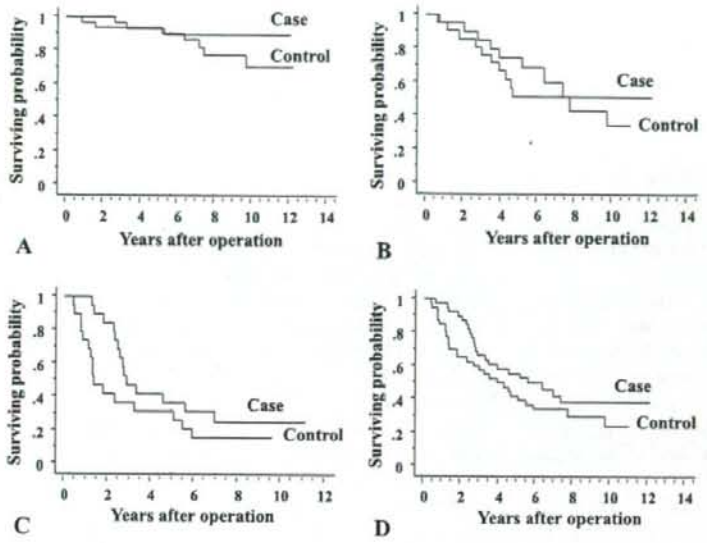

Fig. 3A–D. Kaplan–Meier survival estimates. **A** Stage I. Case-group patients (n = 31) versus controls (n = 31). P = 0.207. **B** Stage II. Case-group patients (n = 21) versus controls (n = 21). P = 0.420. **C** Stage III. Case-group patients (n = 19) versus controls (n = 19). P = 0.114. **D** N(+). Case-group patients (n = 33) versus controls (n = 34). P = 0.168

patients. There was no life-threatening or fatal event. Thirteen of the 19 patients were managed with a temporary dose reduction or discontinuation of BCG-CWS.

Discussion

Immune control of tumor growth can be mediated by the antigen-specific activity of CTLs or by the innate immune response of NK cells. Cytotoxic T cells recognize malignant cells in the context of antigen presentation via major histocompatibility complex (MHC) molecules.[19] Tumor cell recognition by NK cells is antigen-independent and MHC-unrestricted.[20]

Cancer cells share the same MHC as host cells and barely express pathogen-associated molecular patterns (PAMPs). We hypothesized that the inability of tumors to evoke the host immune response is due to the lack of PAMPs in patients with cancer. Supplementation with PAMPs as adjuvants may increase the efficacy of immune responses against tumor antigens (tumor vaccine). Adjuvants are materials added to a vaccine preparation to enhance its immunogenicity. One of the most powerful adjuvants is complete Freund's adjuvant; a suspension of killed mycobacteria in mineral oil. We used BCG-CWS as an adjuvant for this purpose.

Tsuji et al.[8] demonstrated that BCG-CWS can activate immature human dendritic cells (iDC). Antigen presentation and T-cell stimulation are enhanced by BCG-CWS, which also induces up-regulation of the DC maturation marker CD83, and the secretion of inflammatory cytokines such as IL-6, IL-12, and TNF-α. These responses and the increase in antigen-presenting ability indicate that the activation and maturation of DC is induced by CWS-containing mycobacterial peptidoglycan. This suggests that BCG-CWS induces TNF-α secretion from myeloid DC via Toll-like receptor (TLR) 2 and TLR4 and that the secreted TNF-α induces the maturation of DC.

Using a murine subcutaneous and lung metastatic sarcoma treatment model, Mason et al.[21] showed that a local injection of synthetic oligodeoxynucleotides (ODN) containing an unmethylated CpG motif (characteristic of bacterial DNA) could be given with conventional radiation therapy to augment therapeutic efficacy via an apparently immune-mediated mechanism. Combination cancer vaccines with TLR9 agonists such as ODN may induce tumor-specific CD4+ and CD8+ T cells, whose migration and killing activity would be enhanced by radiation therapy. Toll-like receptor expression differences exist between mice and humans; mouse plasmatoid and myeloid DC express TLR9, whereas only human plasmatoid DC does.[22] Mason et al.[21] hypothesized that when radiotherapy is given after TLR agonist injection, the tumor antigens released by dying tumor cells are taken up by activated DC, inducing a tumor-specific T-cell response.

The injection of BCG-CWS sometimes causes lymphadenopathy of the draining lymph nodes (Table 2). We demonstrated the uptake of fluorodeoxyglucose (FDG) into the enlarged lymph nodes not only in the axillary lymph nodes, but also in the cervical and mediastinal lymph nodes by positron emission tomography (PET)

during immunotherapy (data not shown). Lipford et al.[23] reported that among cells within enlarging lymph nodes are many DCs that express increased levels of costimulatory molecules and MHC. Interferon-γ is the final output produced either by the direct stimulation of lymphocytes or by the stimulation of lymphocytes secondary to activation of antigen-presenting cells such as DC. In vitro BCG-CWS induces IL-12 p40 production in peripheral blood culture. Interleukin-12 p40 is an inducible element of IL-12, and in humans may represent IFN-γ-inducing activity.[24]

According to a previous report,[18] BCG-CWS can induce IFN-γ when administered intracutaneously in a patient's upper arm. An elevated serum IFN-γ level is regarded as evidence of a systematic immune response. Interferon-γ is an important immune regulator that performs a wide spectrum of physiologic functions, such as activation of macrophages, NK cells, and CTL, regulation of antigen presentation in many cells, and generation of Type 1 helper T cells (Th1 cells).[25] Our data showed no significant difference in survival between the case-group patients with, and those without IFN-γ induction in the peripheral blood. In this study, we performed IFN-γ assay within 7 months after immunotherapy was started. Matsumoto et al.[24] reported that the levels of production of IFN-γ and IL-10 by lymphocytes were lower in patients with lung cancer than in healthy subjects. In our study, BCG-CWS was repeatedly injected into the skin of both shoulders, for more than 1 year in most patients. When patients receive long-term, repeated inoculations of BCG-CWS, the serum IFN-γ level may increase, especially in those with a good outcome. Our literature search found no report clearly stating an association between increased serum IFN-γ levels and survival benefit in patients receiving BCG-CWS immunotherapy. A recent study of NSCLC patients by Trojan et al.[26] suggested that peritumoral CD8+ T cells exhibit locally higher expression of IFN-γ mRNA; a finding indicative of sustained T-cell reactivity, compared with tumor-infiltrating T lymphocytes (TILs); however, they failed to demonstrate the influence of IFN-γ/CD8 mRNA ratio on overall survival in these patients.

We hypothesized that activation of the innate immune system with BCG-CWS after curative resection for lung cancer may have a survival benefit and conducted a case–control study. Although the difference was not significant, survival of the case-group patients was better than that of the control patients over a long-term follow-up period (Fig. 2). This trend was seen in the subgroups of pathological stage III or lymph node metastasis (Fig. 3C and D). However, there was no difference in survival between the subgroups of p-stage I or II (Fig. 3A and B). These results suggest that monotherapy using BCG-CWS may improve survival without major complications after curative surgery for lung cancer. Patients with advanced lung cancer, especially those with lymph node metastasis, seem to be good candidates for this innate immunotherapy. When patients have micrometastasis to distant lymph nodes, specific cancer antigens may be expressed by the cancer cells and recognized by mature myeloid DC activated with BCG-CWS. The survival benefit of BCG-CWS adjuvant therapy in this series was 17% at 10 years after surgery (Fig. 2A). Tanaka[27] reported a single-institute phase II trial of adjuvant chemotherapy with carboplatin/paclitaxel followed by tegafur and uracil (UFT) for completely resected node-positive (p-stage II-N1 or IIIA-N2) NSCLC. His interim analysis revealed favorable overall and recurrence-free survival of 73% and 49%, respectively, at 3 years, with minimal toxicity. These results suggest that chemotherapy followed by BCG-CWS immunotherapy should be prescribed in a postoperative adjuvant setting after NSCLC resection.

Regarding histological differences between the case and control groups, it was very difficult to completely match three factors at the time of control recruitment. Thus, we gave priority to pathological stage and year of birth. Histology was considered as much as possible but perfectly matched pairing was impossible. According to the survival analysis of BCG-CWS and historical control groups by Yasumoto et al.,[28] all types of lung cancer including squamous cell carcinoma, adenocarcinoma and anaplastic carcinoma were sensitive to treatment with BCG-CWS, and there was no significant difference in survival among those histological types. Their results suggest that the histological differences between case and control group are not of great consequence.

To achieve more effective control of cancer, two modalities should be used with BCG-CWS. The first is the coadministration of a peptide vaccine with BCG-CWS as the adjuvant. The Wilms' tumor gene, *WT1*, is overexpressed in leukemia and a variety of solid tumors, and the WT1 protein has been identified as a tumor-associated antigen.[29] Thus, WT1 products may provide the basis for the development of a new peptide-based anti-cancer immunotherapy. It was demonstrated that 3.0 mg of WT1 therapy can induce a generation of WT1-specific T lymphocytes without damaging normal tissues.[30] Nakajima et al.[31] demonstrated for the first time that a WT1 peptide vaccination combined with BCG-CWS effectively eradicated WT1-expressing tumor cells implanted in mice before vaccination; as a "therapeutic" model, not a "prophylactic" model. Vermorken et al.[32] reported that adjuvant active specific immunotherapy with an autologous tumor cell; namely, BCG (but not CWS) vaccine following surgical resection was more beneficial than resection alone against stage II and III colon cancer. The second modality is the stimulation of NK cells to lyse MHC-unrestricted cancer

cells. A high concentration of IL-10 in the tumor microenvironment may stimulate NK cells to lyse cancer cells, leading to increased availability of tumor-associated antigens and delivery of biologically active molecules, such as heat-shock proteins, needed for the activation of DCs and for effective priming of CTLs against tumor-associated antigens.[33]

We need good manufacturing practices to purify adjuvants such as BCG-CWS for translational research and to coadministrate with more personalized peptide vaccines as a future challenge. In conclusion, our results suggest that BCG-CWS immunotherapy following radical surgery for NSCLC improves overall survival without compromising quality of life.

Acknowledgments. We thank Dr. Ichiro Azuma for kindly providing BCG-CWS. This work was supported in part by a Grant from the Foundation for Promotion of Cancer Research.

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Vaccine 27 (2009) 1710–1716

Contents lists available at ScienceDirect

Vaccine

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Whole genome sequence analysis of *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) Tokyo 172: A comparative study of BCG vaccine substrains

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ARTICLE INFO

Article history:
Received 13 May 2008
Received in revised form 7 October 2008
Accepted 13 January 2009
Available online 4 February 2009

Keywords:
BCG Tokyo
Complete genomic sequence
Genetic differences

ABSTRACT

To investigate the molecular characteristics of bacillus Calmette–Guérin (BCG) vaccines, the complete genomic sequence of *Mycobacterium bovis* BCG Tokyo 172 was determined, and the results were compared with those for BCG Pasteur and other *M. tuberculosis* complex. The genome of BCG Tokyo had a length of 4,371,711 bp and contained 4033 genes, including 3950 genes coding for proteins (CDS). There were 18 regions of difference (showing differences of more than 20 bp), 20 insertion or deletion (ins/del) mutations of less than 20 bp, and 68 SNPs between the two BCG substrains. These findings are useful for better understanding of the genetic differences in BCG substrains due to in vitro evolution of BCG.

1. Introduction

A virulent strain of *Mycobacterium bovis* lost its virulence after 13 years and 230 passages by Calmette and Guérin, and the attenuated derivative was designated as bacillus Calmette–Guérin (BCG). BCG is the only viable bacterial vaccine used to prevent tuberculosis and it has been distributed to many countries around the world. The original strain of BCG has produced many daughter strains (substrains), which are the progenitors of the commonly used vaccines. The major daughter strains of BCG used for vaccine production are BCG Danish, Glaxo, Pasteur, Moreau, Tokyo, and Russia.

These strains have been propagated separately as freshly prepared cultures through serial passages in different countries, and repeated subculture has yielded BCG strains that differ markedly from each other.

Differences of various characteristics, such as colony morphology [1–3], biochemical properties [4–6], drug resistance [7,8], immunogenicity in animals [9,10] or humans [11,12] and virulence in animals [13], have been found among currently available vaccines.

In order to prevent continued accumulation of genetic changes in the strains, the WHO recommended in 1966 that vaccine should not be prepared from any culture that had gone more than 12 passages starting from a defined freeze-dried seed lot.

In addition to differences of colony morphology, differences in the copy number of IS6110 [14], production of several antigenic proteins (MPB64, MPB70, and MPB83) [4,5], and a point mutation of the *mma3* gene which encodes fatty acyl methyltransferase [6] were also known as differences between the early BCG strains distributed by the Pasteur Institute between 1924 and 1926 (such as BCG Russia, Tokyo, and Moreau), and the later strains distributed after 1931 (such as BCG Danish, Glaxo, and Pasteur).

After the complete genomic sequences of *M. tuberculosis* and *M. bovis* were determined [15–17] and studies were done with the DNA microarray method [18,19], many genetic differences among BCG substrains have become clearer.

Recently, the genomic sequence of BCG Pasteur was determined [20] and the authors suggested that early BCG vaccines may be superior to the later ones based on stability of tandem duplication DU2, changes of genes encoding sigma-factors and transcriptional regulators, and comparison of the immune response elicited in babies [11]. However, the entire sequence of BCG has only been analyzed for BCG Pasteur, which is one of the later strains. To further understand differences among BCG substrains, analysis of the complete genome of an early strain is necessary. Therefore, we analyzed the genome of BCG Tokyo 172 (Tokyo), a representative early strain, and compared the findings with those for the later strain, BCG Pasteur.

2. Materials and methods

2.1. Bacteria and preparation of genomic DNA

A seed lot (Tokyo 172-1), which is used for the production of BCG vaccine in Japan, was cultured in Sauton medium at 37 °C. Genomic

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0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.01.034

DNA was isolated by using Qiagen Genomic-tip (QIAGEN K.K., Japan) or then phenol chloroform method.

2.2. Library construction and DNA sequencing

Construction of a library and sequencing were performed by Agencourt Bioscience Corporation, USA.

Libraries with insert lengths of about 4 kb and 800 bp were constructed in the high-copy pAGEN vector system (Agencourt Bioscience Corporation, USA), and a fosmid insert library (about 40 kb) was constructed in the CopyControl™ pCCFOS™ vector system (Epicentre Technologies, USA). DNA templates were sequenced in a 384-well format, with the forward and reverse reactions (pair ends) being performed by using BigDye® Version 3.1 base calling software and with constant monitoring of quality using Phred Q20.

2.3. Assembly

Assembly was performed by Agencourt Bioscience Corporation, USA. A passing read was defined as an average high quality PHRED score ≥20 for at least 100 bases. Only passing reads were used. The Paracel Genome Assembler™ (TIGER) was used with Agencourt's LIMS system to assemble the genome, while both Paracel's scaffold viewer and Consed (University of Washington) were employed to view the assembled genome.

2.4. Comparative genomic analysis

The sequences of bacteria closely related to *Mycobacterium bovis* BCG Tokyo (including *M. bovis* BCG Pasteur, *M. bovis* AF2122/97, *M. tuberculosis* H37Rv, and *M. tuberculosis* CDC1551) were obtained from ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/Mycobacterium_bovis_BCG_Pasteur_1173P2, ftp://ftp.sanger.ac.uk/pub/pathogens/mb/, ftp://ftp.sanger.ac.uk/pub/tb/sequences, and ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/Mycobacterium_tuberculosis_CDC1551/. To find regions of similarity between BCG Tokyo and the other related bacteria, the following software programs were used: in silico Molecular Cloning(R) software, Genomic Edition (In Silico Biology Inc, Japan); "BioEdit" (http://www.mbio.ncsu.edu/BioEdit/bioedit.html); and Basic Local Alignment and Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

3. Results

3.1. Genomic sequence of BCG Tokyo

The genome of *M. bovis* BCG Tokyo had a size of 4,371,711 bp. When the genomic sequence of BCG Tokyo was compared with those of related bacteria (*M. bovis* AF2122/97, *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551, and BCG Pasteur), the highest homology was found with BCG Pasteur. Therefore, comparison with BCG Pasteur was mainly employed to identify each gene of BCG Tokyo.

A total of 4033 genes were identified, including 3950 genes encoding for proteins (CDS), 3 genes for rRNA, 45 genes for tRNA, and 30 pseudogenes [supplementary data (SD) Table 1; genome sequence deposited in DDBJ (accession no. AP 010918)].

3.2. Comparison of BCG Tokyo and Pasteur (differences more than 20-bp)

Regions of difference (RDs), which had differences of more than 20 bp between the two BCG substrains, are shown in Fig. 1. A total of 18 RDs were discovered. Among them, 7 RDs were resulted from different copy numbers of sequence repeats in the two substrains, and were found to be variable-number tandem repeats (VNTRs)

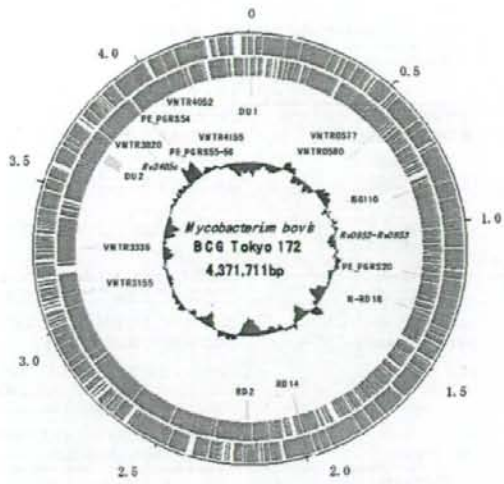

Fig. 1. The entire genomic sequence of BCG Tokyo 172. The scale is shown in megabases on the outer black circle. The outer blue circle shows forward strand CDS and the inner blue circle shows reverse strand CDS. Yellow = 18 RDs that differed between BCG Tokyo and Pasteur. The central wave-shaped circle represents the G+C content.

that had already been identified in *M. tuberculosis* complex [21,22]. Other 3 RDs were in PE family genes [15]. A tandem duplication (DU1) only existed in BCG Pasteur and was not seen BCG Tokyo. DU2 differed between the two BCG substrains with regard to copy number and length; it is a polymorphic tandem duplication that varies among BCG substrains [23]. Four previously reported RDs (IS6110, n-RD18, RD14, and RD2) were deleted in BCG Pasteur [18,19]. There was a 22-bp deletion in *Rv3405c* of BCG Tokyo, as reported previously [24,25]. The remaining one RD was within the intergenic region (length: 62 bp) between *Rv0952* (*sucD*) and *Rv0953* (possible oxido-reductase). *SucD* was reported to be an essential gene by TraSH analysis of the H37Rv strain [26]. In the intergenic region, BCG Tokyo showed a 53-bp deletion from the end of *Rv0953c* to *Rv0952*, which meant that the distance between the two genes decreased to 9 bp. As there is no predicted promoter-like sequence in this 62-bp region (http://fruitfly.org:9005/seq_tools/promoter.html), the deletion might not affect the expression of either gene.

3.3. Variable-number tandem repeats (VNTRs)

Seven of the RDs were found at the VNTR locus, which is known to show polymorphism in *M. tuberculosis* complex [23]. Five VNTR polymorphisms were located in intergenic regions, while 2 were in CDS (Table 1).

VNTR0577 (also called ETR-C) is located between *Rv0487* and *Rv0488*, and the number of copies found in *M. bovis* (including both BCG substrains) is larger than in *M. tuberculosis*. The copy number was reported to be 5 in BCG Tokyo and 6 in BCG Pasteur by Frothingham and Meeker-O'Connell [21], which differed from our results. When we used the same PCR primers described by Frothingham and Meeker-O'Connell and predicted the PCR products for BCG Tokyo and Pasteur based on the same relationship between copy number and the size of repeating units as shown by them, we obtained the same results as they reported if we employed a particular 58-bp unit, but not if we used another 58-bp unit. Therefore, the copy numbers may not have changed with recent in vitro passaging and microevolution of the BCG Tokyo or Pasteur variants in different laboratories. Instead, the use of different 58-bp units and

Table 1
7 RDs in VNTR locus between BCG Tokyo and Pasteur.

RD in VNTR locus	Sequence (from 5' to 3') in both BCG substrains	Number of repeats				
		BT[a]	BP[b]	Mb[c]	Rv[d]	CDC[e]
0577/intergenic (*Rv0487–Rv0488*) (ETR-C)	GTCGAGCCCGACGACGATGCAGAGCGCGCAGCGCGATG AGAA GGAGTTGGGCGGTTAG (58 bp)	3	4	3	2	1
0580/intergenic (*Rv0490–Rv0491*) (*senX3-regX3*)	TGCGCCGACGACGATGCAGAGCGTAGCGATGAGGTGGGGGCA CCACCCGCTTGCGGGGGGAGAGTGGCGCTGATGACC (77 bp)	3	2	4	3	2
3155/intergenic (*Rv2847c–Rv2848c*)	CGACCCGCGGCGCCCGGTCCCCGCGCTTGCGATCGCCACTGGC CCTGATGGTGG (54 bp)	4	3	2	3	3
3336/intergenic (*Rv2980–Rv2981c*)	TGCGCCGGGCGCGGCGGGTCGGCACCATCGGGCTAAGTGCCG ATCGCAAGCGCGGCGCT (177 bp = 59 bp × 3)	6	9	2	4	5[f]
3820/intergenic (*Rv3401–Rv3402c*)	CGATGCGGGCCGCGTAGCGGCCCGAGGAGGAGCCGGGCAATC CAGCCTGAGCCCGGTGA (59 bp)	3	4	4	1	0
4052 (*Rv3611*)	CCATCAGCCCCGTGGCGATCGCAAACCCCGCGCCTGGCGACAA TGCGGCCCGCAAAACGGGCCGAGGAGGAGCCAGGCAATCACC CCAGAGCCGGGTGCAGCGGGTCGCCA (111 bp)	4	5	4	5	5
4155 (*Rv3710:leuA*)	TCGCGAGCCCGGCGCAGCCGGGCGAAGCGGGTCGGCACGCAT CGGACCCCGTGACGA (57 bp)	11	12	5	2	2

[a] BCG Tokyo
[b] BCG Pasteur.
[c] *Mycobacterium bovis* AF2122/97.
[d] *M. tuberculosis* H37Rv.
[e] CDC: *M. tuberculosis* CDC1551.
[f] Into 2 genes (MT3058.1, MT3058.2)

methods of determining the copy number may have led to this discrepancy. VNTR loci contain several units with different sequences, so it is necessary to clarify the unit sequence and the method of determining the copy number.

VNTR0580 (alternatively, *senX3-regX3*) is located between *senX3* and *regX3*. BCG Tokyo had three repeats of 77 bp, while BCG Pasteur had two repeats. This VNTR is known to show polymorphism among BCG substrains (from 1 to 3 copies) [27]. Although *senX3* and *regX3* form a two-component system that is considered to be related to the virulence of *M. tuberculosis* [28], the relationship between copy number and virulence is unknown.

VNTR3336 (also called QUB-3336) is located between *Rv2980* (possible conserved secreted protein) and *Rv2981c* (D-alanyl-alanine synthetase A), and had 59-bp repeats, with a copy number of 6 in BCG Tokyo and 9 in BCG Pasteur. The copy number was previously found to be five in the intergenic region of CDC 1551 strain and two CDSs were identified [MT3058.1 (177 bp) and MT3058.2 (177 bp)], by using glimmer version 2 (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/Mycobacterium_tuberculosis_CDC1551/).

VNTR3820 is located between *Rv3401* (probably involved in cellular metabolism) and *Rv3402c* (probably involved cell process). Although *M. tuberculosis* has none or only one 59-bp sequences, BCG Tokyo had three 59-bp repeats and BCG Pasteur had four 59-bp repeats at this locus, so the copy number of both BCG strains was increased over that of *M. tuberculosis*. In clinical isolates of *M. tuberculosis*, 16 different copy numbers have been found at this locus, which is the greatest variety among 48 VNYR loci in *M. tuberculosis* [22]. Both BCG strains and *M. bovis* had one SNP of *Rv3402c*, which resulted in tyrosine being changed to a stop codon (C1053G), so that this gene was shortened from 1239 bp to 1053 bp. In mycobacteria, the iron-regulated transcriptional repressor IdeR (iron-dependent repressor) is a homologue of DtxR (the diphtheria toxin repressor) and it is essential for *M. tuberculosis*. *Rv3402c* is one of the predicted IdeR-regulated gene promoter and there is a growing body of evidence to support a role of IdeR in both in vivo and in vitro survival of *M. tuberculosis* [29]. Thus, the difference of 59-bp copy numbers between the BCG strains and the SNP mutations of *Rv3402c* in both strains might affect their phenotypes.

Two VNTR polymorphisms existed in *Rv3611* and *Rv3710* (*leuA*, 2-isopropylmalate synthase) of BCG Tokyo and Pasteur. *Rv3611* had four 111-bp repeats in BCG Tokyo and *M. bovis*, while five repeats were seen in BCG Pasteur and *M. tuberculosis*. The function of this gene is unknown, but it was indicated to be essential by TraSH analysis of the H37Rv strain [26]. At *leuA*, there were eleven 57-bp repeats in BCG Tokyo and 12 repeats in BCG Pasteur. The copy number of both BCG strains was markedly increased compared with *M. bovis* and *M. tuberculosis*. The length of the encoded protein was 815 aa in BCG Tokyo and 834 aa in BCG Pasteur. This protein is considered to be related to the biosynthesis of leucine and to pyruvate metabolism. Changes in the length of the protein may modify its function, although the phenotypic effect is not clear [30].

3.4. PE_PGRS

The PE_PGRS20 gene (1870 bp) of BCG Tokyo was 323 bp shorter than that of BCG Pasteur (2193 bp).

As the sequence homology of this gene of BCG Pasteur was 100% with that of *M. bovis*, the gene of BCG Tokyo might have undergone mutation to become shorter than that of BCG Pasteur. Compared with *M. tuberculosis* H37Rv, there was a 717-bp insert in the gene of *M. bovis* and BCG Pasteur. Since the sequence of BCG Tokyo showed 81% homology with that of the initial part (394 bp) of this insert, it is likely that the 717-bp insert in the gene of BCG Tokyo had mutated to become shorter than that of BCG Pasteur. As a result, the gene of BCG Tokyo divided into two genes (Fig. 2A). The PE_PGRS20 genes show polymorphism among *M. tuberculosis* complex.

The PE_PGRS54 gene of BCG Tokyo (6153 bp) was 132 bp shorter than that of BCG Pastuer (6285 bp).

There were two repeats of 465 bp (A) in BCG Tokyo and one such repeat in BCG Pasteur, as well as three repeats of 552 bp (B) in BCG Tokyo and four such repeats in BCG Pasteur (Fig. 2B). The PE_PGRS54 gene also shows polymorphism among *M. tuberculosis* complex.

The PE_PGRS55 gene of BCG Tokyo (5088 bp) was 345 bp shorter than that of BCG Pasteur (5433 bp).

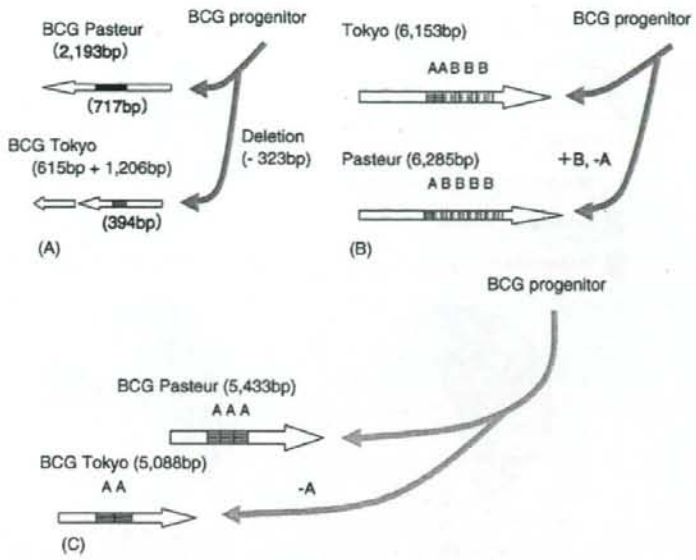

Fig. 2. Possible process of PE_PGRS mutation. (A) PE_PGRS20. (B) PE_PGRS54, repeat of A (465-bp), repeat of B (552-bp). (C) PE_PGRS55, repeat of A (345-bp).

As 345 bp is the length of a repeat in the gene, the difference reflected the copy number of both BCG strains (Fig. 2C). The PE_PGRS55 gene also shows polymorphism among *M. tuberculosis* complex.

3.5. Tandem duplications (DU1 and DU2)

There was no tandem duplication of DU1 in BCG Tokyo like that present in BCG Pasteur. However, DU2 (20,704 bp) existed in BCG Tokyo with a different length and position from that of BCG Pasteur, as reported previously [14]. BCG Tokyo had three copies of the region from the *astB* gene (*Rv3299c*) to the *sdhC* (*Rv3316*) and *sdhD* genes (*Rv3317*), including 20 genes. In the copied region, the *astB* gene was truncated.

3.6. RD2

RD2 (including 11 genes) existed in BCG Tokyo, as it was previously reported to be present in early substrains and absent in later substrains [18]. The sequence homology of the 11 genes (*Rv1978–Rv1988*) were 100% with those of *M. bovis* or *M. tuberculosis*. The 11 genes included those encoding for the transcriptional regulator (*Rv1985c*) and immunogenic protein MPT64 (*Rv1980c*). The sequence of *Rv1978* in BCG Tokyo was same as that of *M. tuberculosis*, but the sequences of BCG Tokyo and *M. tuberculosis* had a SNP compared with that of *M. bovis*. The other sequences of 10 genes were same as those of *M. bovis*.

3.7. RD14

Although RD14 (including 10 genes) existed in BCG Tokyo, this region was deleted in BCG Pasteur. The sequences of 10 genes (*Rv1765c–Rv1773c*) in BCG Tokyo showed 100% homology with those of *M. bovis* or *M. tuberculosis*. These 10 genes included one coding for the transcriptional regulator (*Rv1773c*). The sequence homology of PE_PGRS31 (*Rv1768*) was 100% with that of *M. bovis*, but there was an 18-bp insertion and a SNP in *M. tuberculosis* H37Rv. As a result of the 18-bp insertion, 6 aa were inserted into the protein and one amino acid (N207H) was replaced by the SNP. The sequence homology of BCG Tokyo (*Rv1773c*) was 100% with that of *M. tuberculosis*, but showed a SNP compared with *M. bovis*. The other sequences of 8 genes showed 100% homology with those of *M. bovis*.

3.8. N-RD18

N-RD18 contained three intact genes (*sigI*, *Rv1190*, and *Rv1191*) in BCG Tokyo. *Rv1190* was deleted and *sigI* and *Rv1191* were fused in BCG Pasteur (N-RD20), so the function of *sigI* seemed to be lost [20]. The *sigI* gene was reported to have a role in adaptation to cold shock [31]. The sequence of BCG Tokyo (*Rv1190*) showed 100% homology with that of *M. tuberculosis*, but showed a SNP compared with *M. bovis*. The other 2 sequences of BCG Tokyo (*sigI*, *Rv1191*) showed 100% homology with those of *M. tuberculosis* and *M. bovis*.

3.9. Rv3405c

BCG Tokyo had a 22-bp deletion in *Rv3405c* (possible transcriptional regulatory protein) of RD16. There were two different types of colonies (S: smooth and R: rough) when BCG Tokyo was cultured on Middlebrook 7H10 medium. PCR of each type of colony showed a strong relationship between colony morphology and genotype, since 98.7% of S-colonies had the 22-bp deletion (type I) and 95.9% of R-colonies did not (type II) [25]. In every Tokyo 172 preparation studied, S-colonies (type I) exceeded 90% of the total. Accordingly, it is probable that Tokyo 172 predominantly has the type I genotype [25].

3.10. Comparison of BCGs Tokyo and Pasteur (differences of less than 20 bp)

There were 19 genes and one intergenic region that differed between BCG Tokyo and Pasteur due to insertion or deletion (ins/del) of less than 20 bp. These 20 genes were compared between *M. tuberculosis* and BCG Tokyo or Pasteur. As a result, 15 genes had ins/del mutations between *M. tuberculosis* and BCG Tokyo, and 12 genes had ins/del mutations between *M. tuberculosis* and BCG Pasteur (Fig. 3, SD Table 2).

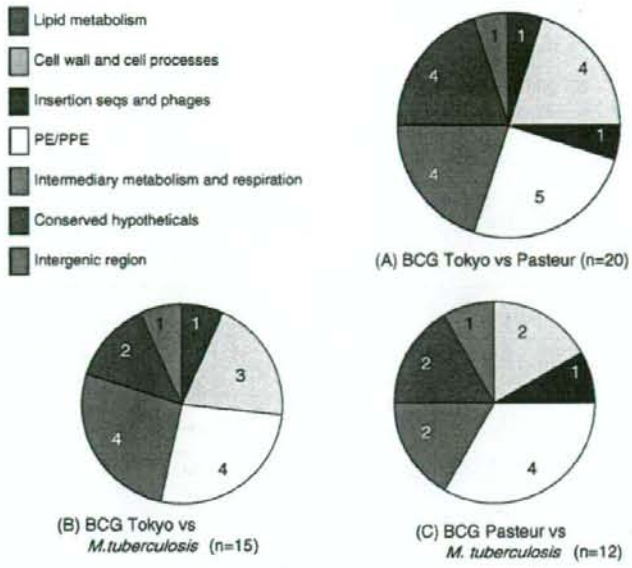

Fig. 3. Functional classification of 20 ins/del (<20 bp) mutations between BCG Tokyo and Pasteur (A), as well as between BCG Tokyo and *M. tuberculosis* (B), and between BCG Pasteur and *M. tuberculosis* (C).

Compared with other genomes, ins/del mutations of 8 genes [*acs* (−1-bp), *ftsW* (−1-bp), *rpfE* (−1-bp), PE_PGRS24 (−9-bp), *sdhA* (+1-bp), *Rv3814c* (+3-bp), *Mb3263c* and *Mb3359c* (*RvD5*)] were found in BCG Tokyo only, and those of 5 genes [*Rv3835* (+1-bp), *Rv1313* (IS1557), *PE_PGRS7* (−9-bp), *Rv1486c* (+1-bp), and *Rv3433c* (−3-bp)] were found in BCG Pasteur only.

When the sequences near ins/del mutations of BCG Tokyo and Pasteur were analyzed, 13 microsatellite polymorphisms (also known as simple sequence repeats comprising tandem repeat motifs of 1–6 bp in length) were found between the two BCG substrains (SD Table 3), as previously reported between *M. tuberculosis* and *M. bovis* [32].

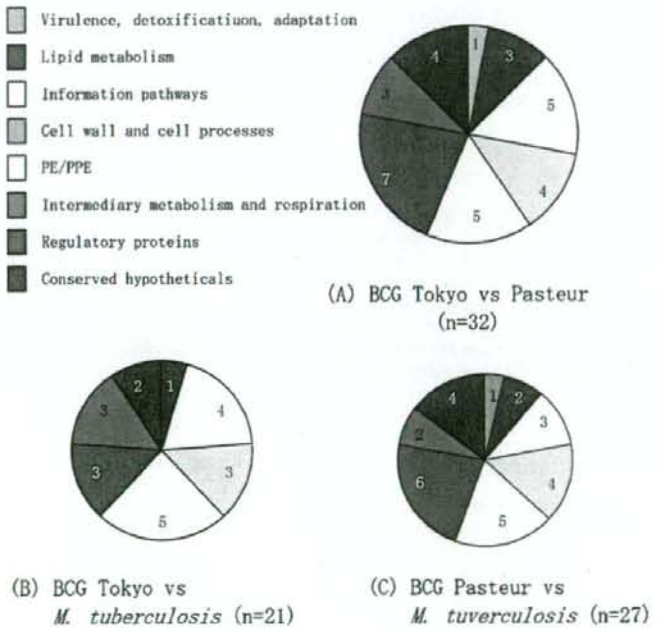

Fig. 4. Functional classification of 32 SNPs mutations between BCG Tokyo and Pasteur (A), as well as SNPs or ins/del mutations between BCG Tokyo and *M. tuberculosis* (B), and SNPs or ins/del mutations between BCG Pasteur and *M. tuberculosis* (C).

When the function of genes with ins/del was assessed, the PE or PPE families were over-represented in each BCG substrain relative to the frequency in the individual genome (BCG Tokyo: 4/15 versus PE/PPE in BCG Tokyo genome of 170/3996, odds ratio = 8.1, $P < 0.01$; Fisher's exact, BCG Pasteur: 4/12 versus PE/PPE in BCG Pasteur of 168/4002, odds ratio = 11.4, $P < 0.01$; Fisher's exact). These results show that the PE_PGRS genes are unstable and ins/del mutation occurs more easily than in other genes of these BCG substrains.

There were 68 SNPs between BCG Tokyo and Pasteur. A total of 56 SNPs were found in 43 genes, in which 37 genes (86%) had nonsynonymous mutations (leading to amino acid substitution) and 6 genes had synonymous mutations, so the majority of these mutations related to SNPs were nonsynonymous. As 32 of the 37 genes with nonsynonymous mutations had no ins/del mutations, these genes were compared. Of the 32 genes with nonsynonymous mutations, 21 of BCG Tokyo and 27 of BCG Pasteur had SNPs or ins/del mutations as compared to the genome with SNPs of *M. tuberculosis* (Fig. 4, SD Table 4).

When the genes with SNPs were assessed on the basis of function, the PE or PPE families were over-represented in each BCG substrain relative to the frequency in the individual genome (BCG Tokyo: 5/21 versus the PE/PPE in BCG Tokyo genome of 170/3996, odds ratio = 6.9, $P < 0.01$; Fisher's exact, BCG Pasteur: 5/27 versus PE/PPE in the BCG Pasteur genome of 168/4002, odds ratio = 5.2, $P < 0.01$; Fisher's exact).

Compared with other genomes, SNP mutations of 5 genes [*rplE, pcaA, typA, Rv3401* and *Rv3583*] were found in BCG Tokyo only, and SNP mutations of 11 genes [*hrcA, echA3, mmaA3, sigK, Rv2571, narJ, pepN, ilvN, lcd1, Rv0552* and *Rv3258*] were found in BCG Pasteur only.

4. Discussion

The BCG substrains are considered to have evolved mainly by gene deletion and gene amplification (DU1 and DU2). The results of this study demonstrated several new VNTRs and PE_PGRS polymorphisms. As polymorphisms are known to exist among other *M. tuberculosis* complex, not only BCG Tokyo and Pasteur but also other BCG substrains might have such polymorphisms frequently.

The PE_PGRS gene family also shows variation among clinical isolates of *M. tuberculosis*, and it might play a role in the variability of antigenicity and persistency of infection [33,34]. Certain proteins are localized on the surface of BCG and may influence interactions between BCG cells and host macrophages [35]. When the genes showing ins/del mutations or SNPs in BCG Tokyo and Pasteur were compared with those of their individual genomes, the PE or PPE families were over-represented in both BCG substrains relative to their genomic frequency. Differences of PE_PGRS gene expression between BCG Tokyo and Pasteur have also been reported [20]. Therefore, mutations of these genes and differences in the level of expression might play a role in the different protective effects of the BCG substrains [36,37].

To examine the genetic characteristics of BCG Sofia from seed lots and commercial batches, VNTR typing was done with six alleles, and a profile identical to that of other BCG substrains was obtained [38]. The 5 alleles that were newly shown to vary between BCG Tokyo and Pasteur in this study may be useful to identify BCG substrains and to examine genetic stability during vaccine production.

The entire genomic sequence of BCG Pasteur was determined previously, but the sequences of RD2 and RD14 were unknown because these regions were absent in BCG Pasteur. In the present study, it was revealed that the genes of RD2 and RD14 in BCG Tokyo were almost 100% identical with those of *M. bovis* AF2122/97. The two regions contained a total of 21 genes, including two genes encoding regulatory proteins. Five genes were classified into functional category 3 (cell wall and cell processes) and an immunogenic protein MPB64 was included in RD2. Therefore, the existence or deletion of these regions might affect the phenotype of BCG, but further study will be necessary to clarify this issue.

Only a few nonsynonymous SNPs are known to affect the phenotype of BCG substrains, such as SNPs of the *mma3, sigK* [39], and *crp* [40,41] genes. In the present study, there were no nonsynonymous SNPs in the start or stop codons among the new SNPs, but 32 nonsynonymous SNPs were found between BCGs Tokyo and Pasteur, including those of the *mma3, sigK*, and *crp* genes. The effect of other SNPs on the phenotype is unknown.

There have been no reports about ins/del mutations (<20 bp) of BCG substrains, except for a 10-bp deletion of the PhoR gene in DU-2-III substrains [20]. However, 19 genes with ins/del mutations were detected between BCG Tokyo and Pasteur, with 13 being located in microsatellite regions. Eight genes (*acs, ftsW, rpfE*, PE_PGRS24, *sdhA, Rv3814c, Mb3263c* and *Rv3583*) showed ins/del in BCG Tokyo. The *acs* gene, which encodes acetyl-CoA synthetase, had a 1 bp deletion in codon 209 and this mutation causes frame shift in the region of the putative AMP-binding domain signature (from codon 260 to codon 271). Acetyl-CoA synthetase is involved in the pyruvate pathway and pyruvate is an initial substrate of the TCA cycle. The *sdhA* gene encodes a subunit of the succinate dehydrogenase complex and is related to the TCA cycle which *sdhB, sdhC* and *sdhD*. The *sdhB* gene of BCG Tokyo had 2 SNPs compared with that of *M. tuberculosis*, while *sdhC* and *sdhD* were present in triplicate at the DU2 region. Such these mutations might affect the TCA cycle, but further study is necessary to clarify this issue.

BCG Tokyo had a specific 22-bp deletion within *Rv3405c*, and a strong relationship has been demonstrated between colony morphology and genotype [25]. Therefore, *Rv3405c* might have an important influence on colony morphology. Chen et al. recently showed that BCG Tokyo does not produce phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs), two cell wall lipids that are known to be important for the virulence of *Mycobacterium tuberculosis*. They reported that *Rv3405c* is involved in PGL biosynthesis and that deletion of this gene is partially responsible for defective PGL synthesis in BCG Tokyo [42]. Although they did not describe colony morphology, there might be a relationship between PGL biosynthesis and colony morphology. The morphology of BCG colonies has been suggested to be related to cell wall components such as mycoside B [43]. An investigation is now in progress to determine the relationship between colony morphology and cell wall glycolipids (PDIMs/PGLs) in the two genotypes of BCG Tokyo.

The genomic and phenotypic differences demonstrated between early and later BCG substrains by our comparative study possibly also exist between other early and later BCG substrains. These findings may be useful with the respect to the standardization of BCG vaccine substrains and for more the precise understanding of the genotypic and phenotypic differences between early and later substrains.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.01.034.

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BJUI BJU INTERNATIONAL

Immunoprotection against murine bladder carcinoma by octaarginine-modified liposomes incorporating cell wall of *Mycobacterium bovis* bacillus Calmette-Guérin

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Accepted for publication 8 August 2008

OBJECTIVE

To develop a prototype of a non-live bacterial agent that consists of a cell wall (CW) preparation from heat-killed bacillus Calmette-Guérin (BCG-CW) incorporated into octaarginine-modified cationized liposomes as a vector (R8-liposome-BCG-CW), and to evaluate its immunoprotective potentiation in mice, as although BCG is an established effective immunotherapy for nonmuscle-invasive bladder cancer, more active and less toxic treatments are needed.

MATERIALS AND METHODS

The cellular interaction of R8-liposome-BCG-CW co-cultured with mouse bladder cancer cell line (MBT-2) was examined by confocal laser scanning microscopy. MBT-2 cells (7×10^5) were subcutaneously inoculated with 1 mg BCG, 0.1 mg or 1 mg BCG-CW, 0.1 mg or 1 mg R8-liposome-BCG-CW in female C3H/HeN mice. The MBT-2 cells pretreated with BCG or R8-liposome-BCG-CW were re-challenged at 6 weeks. The sizes of the primary and re-challenged tumours were evaluated at 4 and 10 weeks, respectively.

RESULTS

Confocal laser scanning microscopy showed the enhanced incorporation of R8-liposome-BCG-CW into MBT-2 cells after 1 h of co-incubation. 0.1 mg R8-liposome-BCG-CW completely inhibited the growth of MBT-2 tumours while 0.1 mg BCG-CW alone did not ($P = 0.002$). Mice vaccinated with a mixture of MBT-2 cells and R8-liposome-BCG-CW inhibited the growth of re-challenged tumour of MBT-2 cells pretreated with BCG or R8-liposome-BCG-CW but did not inhibit that of MBT-2 cells with no pretreatment at 10 weeks, with mean (SD) tumours sizes of 54 (60) mm^2 ($P < 0.001$) or 69 (43) mm^2 ($P = 0.003$) compared with 309 (125) mm^2, respectively.

CONCLUSION

The immunotherapeutic potential of BCG-CW was enhanced by improving cellular association using the R8-liposomes delivery system. Development of this non-live bacterial agent may contribute to providing a more active and less toxic tool as a substitute for live BCG as immunotherapy against nonmuscle-invasive bladder cancer in the future.

KEYWORDS

mycobacterial cell wall, octaarginine-modified liposomes, bladder neoplasm, BCG, immunotherapy, drug delivery system

INTRODUCTION

BCG immunotherapy for nonmuscle-invasive bladder cancer has been successfully established for decades and intravesical instillation of *Mycobacterium bovis* BCG has been used to prevent recurrence of high-risk nonmuscle-invasive bladder cancer and to treat carcinoma *in situ*. Although the detailed mechanism has not been fully elucidated, live BCG has been established as a tuberculosis vaccine since 1921 and recognised to stimulate both innate and specific immunity to tuberculosis in human hosts. In cases of nonmuscle-invasive bladder cancer, BCG has been thought to act directly and/or indirectly on the anticancer immune system as a potent adjuvant, and to provide long lasting immune protection that prevents recurrence of nonmuscle-invasive bladder cancer [1,2]. Although immunotherapy using live BCG has been recognised as an effective tool, fatal adverse events such as systemic dissemination of BCG cannot be precluded [3]. To avoid such unfavourable events, it is necessary to develop a more active and less toxic immunotherapeutic agent.

Mycobacterial cell walls (CWs) consist of highly characteristic hydrophobic molecules, such as mycoloyl glycolipids, mannose containing lipoglycans and CW skeleton, most of which stimulate Th-1 type immune responses. BCG survives within the cytoplasm of dendritic cell and plays a crucial role as an immunostimulant for months to years, due to its hydrophobic and unique molecular properties that contribute to preventing BCG from being digested by phagosome–lysosome fusion in dendritic cells. As the CW components of mycobacteria have been reported to stimulate antitumour responses

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through production of TNF-α, interleukin 12, and interferon γ in experimental animal systems [4–6], we have examined the effect of a BCG-CW preparation on bladder tumour generation with MBT-2 cells in mice. However, despite that the immunotherapeutic potential of BCG-CW by initiating the inside-outside signalling pathway through membrane-bound CD14/Toll-like receptor (TLR) 2 has been reported [7–10], this application is hampered by the unfavourable physicochemical characteristics of BCG-CW [11]. Both negative surface charge and highly hydrophobic properties cause poor cellular association [12,13], which obstructs the critical step for evoking immune protection.

To overcome these unfavourable physicochemical properties of the BCG-CW preparation, octaarginine-modified liposomes (R8-liposomes) were applied as a vector to transport BCG-CW into the cytoplasm effectively. R8-liposomes were developed to transfer highly negative charged DNA molecules into the cytoplasm by macropinocytosis [14–16]. R8-liposomes resemble an envelope-type virus and their surface-modification by anchored R8, a characteristic and efficient cell-penetrating peptide [17]. The enhanced ability of cellular association of stearyl R8-liposomes incorporating BCG-CW (R8-liposome-BCG-CW) was shown using a bladder cancer cell line (MBT-2) derived from C3H/HeN mouse *in vitro*. We also investigated whether R8-liposome-BCG-CW induced antitumour effects and long-term immunoprotection using a s.c. bladder cancer model that we have previously used to show the adjuvant effects of BCG [18,19].

MATERIALS AND METHODS

For the *in vitro* study, BCG-CW was prepared. The CW fraction was prepared from the heat-killed cells of *Mycobacterium bovis* BCG Tokyo 172. Briefly, bacteria were cultivated on the surface of Sauton medium for 9 days at 37 °C and autoclaved at 121 °C for 15 min and centrifuged. The cells were re-suspended in deionized water and passed three times through a French Pressure Cells (5501-MF, Ohtake Works, Tokyo, Japan) at a pressure of 180 MPa to break the cells into small fragments. The unbroken cells were removed by centrifugation twice at 6760*g* for 20 min at 25 °C. In addition, the CW fraction was separated from the supernatant by ultra-centrifugation at 18 000*g* for 1 h at 25 °C, and then lyophilized for storage. The CW fraction thus obtained contained mainly the CW skeleton, arabinogalactan peptidoglycan mycolate complex, cord factor (trahalose 6,6'-dimycolate), lipomannan, phosphatidyl inositol di- and hexamannosides, and CW-bound proteins. The crude cell wall fraction (BCG-CW) showed sizes with 500–700 nm at median diameter.

For the preparation and characterization of R8-liposomes incorporating BCG-CW, egg phosphatidylcholine and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sulforhodamine B, fluorescein-5-thiosemicarbazide (FTSC), Lysotracker Red, and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR, USA). Stearylated-octaarginine (STR-R8) was synthesized and purified as described previously [20]. R8-liposomes incorporating varying amounts of BCG-CW were prepared of egg phosphatidylcholine/cholesterol/STR-R8 (7:3:0.08, molar ratio). The liposomes were prepared by the lipid-film hydration method described previously [21]. Briefly, each lipid was dissolved in chloroform/methanol (2:1, v/v) in a round-bottom flask, and BCG-CW suspended in the same solvent was added. The thin lipid film containing BCG-CW was then obtained by evaporating the solvent with a rotary evaporator under reduced pressure. The lipid film was hydrated by adding 1 mL of 10 mM PBS and shaking the flask for 20 min at 65 °C. The liposomes prepared were extruded through a polycarbonate membrane filter with a pore size of 0.4 μm using a hand extruder (Avanti Polar Lipids, Alabaster, AL, USA). Unincorporated components, which were dissociated from BCG-CW, were removed from the liposomes by ultracentrifugation at 91 000*g* for 30 min at 4 °C (Himac CS150 GX, Hitachi, Japan). Finally, the liposomes were resuspended in PBS and kept at 4 °C until use.

For the uptake and intracellular localization studies, fluorescent-labelled liposomes were prepared. The terminal oxidizable carbohydrate moieties exposed on the surface of BCG-CW were labelled with FTSC after periodate oxidation as described previously [22]. Liposome formulations were prepared as described above using FTSC-labelled BCG-CW. The aqueous phase of liposomes was also labelled with sulforhodamine B. The diameter, polydispersity index, and ζ-potential of the prepared liposomes were measured by dynamic light scattering at 25 °C with a Malvern Nano Zetasizer (Malvern, Worcester, UK).

Bladder tumour cells (MBT-2) derived from C3H/HeN mice were obtained from RIKEN, Tsukuba, Japan, and maintained at 37 °C in air with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum.

To investigate the cellular association of R8-liposomes containing BCG-CW, MBT-2 cells were incubated in serum-free RPMI 1640 medium containing either unmodified liposomes or R8-liposomes (both liposomes incorporating BCG-CW) at a final concentration of 0.1 mM lipids for 1 h at 37 °C, followed by three washes with ice-cold PBS. Cells were trypsinized, washed, and then suspended in 0.5 mL of FACS buffer. After being passed through a nylon mesh, the cells were analysed by flow cytometry.

To investigate the cellular interaction and uptake of R8-liposome-BCG-CW, the MBT-2 cells were treated with double-labelled R8-liposome-BCG-CW (FTSC-labelled glycolipid moiety and rhodamine-labelled aqueous phase of R8-liposome, with a final concentration of 0.1 mM lipid) in serum-free RPMI 1640 medium at 37 °C for 1 h. The cells were then washed three times with ice-cold PBS and analysed by confocal laser scanning microscopy (LSM510 Meta, Carl Zeiss). To investigate the intracellular fate of the BCG-CW incorporated into the R8-liposomes, the MBT-2 cells were treated with R8-liposome containing FTSC-labelled BCG-CW at 37 °C for 1 h. At 30 min before observation, the endosome/lysosome compartments were stained with 75 nM Lysotracker Red. Nuclei were stained with Hoechst 33342 in the last 10 min of incubation. After incubation, the cells were washed three times with ice-cold PBS and were directly analysed.

For the *in vivo* study, female C3H/HeN mice (7-week-old) were used. The mice were housed in plastic cages and maintained under standard conditions of temperature, humidity, and a 12:12-h light-dark cycle daily. Mice had free access to a standard diet and water. The Guide for the Care and Use of Laboratory Animals of Tsukuba University was followed at all times.

MBT-2 cells were trypsinized and washed twice with PBS. MBT-2 cells (7×10^5) were inoculated into the right side back of each mouse with 100 μL of PBS alone (group A, six

TABLE 1 The study design, mice were inoculated at 0 week, evaluated at 4 weeks, re-inoculated at 6 weeks and then evaluated again at 10

Group	N	Inoculation at 0 week	Re-inoculation at 6 weeks after initial inoculation
A	6	MBT-2	MBT-2 pretreated with BCG
B	6	MBT-2 + 1 mg BCG	MBT-2 pretreated with BCG
C	6	MBT-2 + 1 mg BCG-CW	MBT-2
C	6	MBT-2 + 1 mg BCG-CW	MBT-2 pretreated with BCG
C	6	MBT-2 + 1 mg BCG-CW	MBT-2 pretreated with R8-liposome-BCG-CW
D	6	MBT-2 + 0.1 mg BCG-CW	MBT-2 pretreated with BCG
E	6	MBT-2 + 1 mg R8-liposome-BCG-CW	MBT-2
E	6	MBT-2 + 1 mg R8-liposome-BCG-CW	MBT-2 pretreated with BCG
E	6	MBT-2 + 1 mg R8-liposome-BCG-CW	MBT-2 pretreated with R8-liposome-BCG-CW
F	6	MBT-2 + 0.1 mg R8-liposome-BCG-CW	MBT-2 pretreated with BCG
G	6	MBT-2 + R8-liposomes	MBT-2 pretreated with BCG
H	6	none	MBT-2 pretreated with BCG

7×10^6 MBT-2 (syngeneic bladder cancer cell line) cells alone or mixed with BCG, BCG-CW, R8-liposome-BCG-CW or R8-liposomes vehicle alone were s.c. inoculated to the right side back of 7-weeks-old female C3H/HeN mice. Tumours were surgically removed from mice bearing tumours at 4 weeks. Pretreated MBT-2 cells were prepared by co-culturing with BCG or R8-liposome-BCG-CW at 0.1 mg/mL with serum free medium for 24 h before re-inoculation. Pretreated or un-pretreated MBT-2 cells were s.c. re-inoculated to the left side back of mice at 6 weeks. The number of mice bearing tumours and the size of the tumours was recorded weekly. All experiments were terminated at 10 weeks.

mice), PBS containing 1 mg BCG (group B, six mice), 1 mg BCG-CW (group C, 18 mice), 0.1 mg BCG-CW (group D, six mice), 1 mg R8-liposome-BCG-CW (group E, 18 mice), 0.1 mg R8-liposome-BCG-CW (group F, six mice), or R8-liposomes vehicle alone (group G, six mice). The six mice in group H were not treated (Table 1). The size of the growing tumour was recorded weekly. After 4 weeks, mice bearing tumours were anaesthetized, and the tumours were removed surgically.

TABLE 2 The properties of the prepared R8-liposomes. The polydispersity index (P.D.) reflects the distribution of particle sizes, ranging from 0.0 for entirely monodispersed particles up to 1.0 for heterogeneous particles

Liposome formulation	Mean (SD) particle size, nm	P.D.	Mean (SD) ζ-potential, mV
R8-liposome + 1 mg BCG-CW	233 (35)	0.27	26.9 (4.3)
R8-liposome + 0.1 mg BCG-CW	232 (43)	0.29	19.9 (9.1)
R8-liposome (with no BCG-CW)	270 (48)	0.31	29.7 (4.6)

For pretreatment of MBT-2 cells for re-inoculation, MBT-2 cells growing on culture dishes were washed twice with PBS, and then cultured for 24 h before re-inoculation in serum-free RPMI 1640 medium alone, containing BCG or R8-liposome-BCG-CW (0.1 mg/mL). Just before use for re-inoculation, pretreated MBT-2 cells were trypsinized and washed with PBS three times.

At 6 weeks after the primary inoculation, all mice except those in groups C and E were re-inoculated with 7×10^5 of BCG-pretreated MBT-2 cells in 100 μL of PBS to the left side back of the mice (Table 1). Mice that had been vaccinated with MBT-2 cells with 1 mg BCG-CW (group C) or 1 mg R8-liposome-BCG-CW (group E) were divided into three subgroups. Mice in these subgroups were s.c. re-inoculated with 7×10^5 of BCG or R8-liposome-BCG-CW pretreated or untreated MBT-2 cells in 100 μL of PBS to left side of the back. The size of the growing tumour was recorded at 4 weeks after re-inoculation.

Tumour size was estimated by calculating the area using the lengths of the two axes of the tumour with the formula $S(mm^2) = \pi ab/4$. The antitumour effect was assessed by comparing the size of the tumours in each group or subgroup of mice by using the Student's *t*-test.

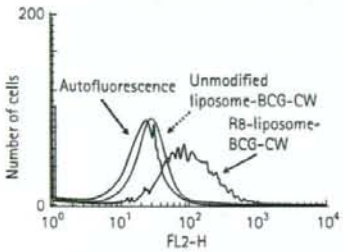
RESULTS

The R8-liposomes incorporated with CWs derived from *Mycobacterium bovis* BCG Tokyo 172 (BCG-CW) used in this study were uniform of ≈230 nm in particle size with a positively charged surface (Table 2).

Flow cytometric analysis of MBT-2 cells incubated with either sulforhodamine-labelled liposomes containing BCG-CW (without R8) or sulforhodamine-labelled R8-liposome-BCG-CW for 1 h at 37 °C confirmed enhanced cellular interaction with the R8-liposomes (Fig. 1). There was fluorescently double-labelled R8-liposome-BCG-CW in the cellular membrane and cytoplasm after co-incubation with MBT-2 cell for 1 h at 37 °C, as the carbohydrate moieties exposed on the surface of BCG-CW were labelled with FTSC (green), while the aqueous phase of the liposomes were labelled with sulforhodamine (red), that indicated that BCG-CW was internalized into the cytoplasm of MBT-2 cell as a R8-liposome (Fig. 2a). Intracellular BCG-CW (green) was eventually co-localized with the endosome/lysosome compartment (red) (Fig. 2b).

The growth of tumours was initially determined at 2 weeks, but then tumours of MBT-2 cells mixed with 1 mg BCG (group B), 1 mg BCG-CW (group C) and 0.1 mg R8-

FIG. 1. Cellular association of R8-liposomes or unmodified liposomes incorporating BCG-CW with mouse bladder tumour (MBT-2) cells was analysed by flow cytometry. The MBT-2 cells were incubated with sulforhodamine-labelled liposomes containing BCG-CW (with no R8) or sulforhodamine-labelled R8-liposome-BCG-CW for 1 h at 37 °C. Flow cytometric analysis showed that more of the MBT-2 cells were associated with labelled R8-liposome-BCG-CW (red solid line) than that of MBT-2 cells associated with fluorescent-labelled unmodified liposomes containing BCG-CW (green dotted line), which overlapped the distribution of MBT-2 cells alone (autofluorescence).



liposome-BCG-CW (group D) regressed or vanished by 4 weeks. The numbers of mice bearing tumours at 4 weeks were all six in group A (PBS alone), three of six in group B (1 mg BCG), eight of 18 in group C (1 mg BCG-CW), all six in group D (0.1 mg BCG-CW), 15 of 18 in group E (1 mg R8-liposome-BCG-CW), none in group F (0.1 mg R8-liposome-BCG-CW,), and five in group G (R8-liposomes) at 4 weeks (Fig. 3a). The tumours that developed from MBT-2 cells mixed with 1 mg BCG (group B), 1 mg BCG-CW (group C), 1 mg R8-liposome-BCG-CW (group E) and 0.1 mg R8-liposome-BCG-CW (group F) were significantly smaller than those developed from MBT-2 cells alone (group A) as a control, at a mean (SD) size of 6 (7) mm^2, 26 (50) mm^2, 22 (21) mm^2, and 0 mm^2 compared with 290 (193) mm^2, respectively ($P < 0.001$). There was no significant effect against tumour growth in the 0.1 mg BCG-CW (group D) and R8-liposomes with no BCG-CW (group G), with tumour sizes of 201 (136) mm^2 and 359 (268) mm^2, respectively. The 0.1 mg R8-liposome-BCG-CW (Group F) completely inhibited the growth of all tumours, while 0.1 mg BCG-CW with no R8-liposomes (Group D) did not ($P = 0.002$).

MBT-2 cells pretreated *in vitro* with BCG were re-inoculated at 6 weeks in to the mice. The number of mice bearing re-challenged tumours of BCG-pretreated MBT-2 cells was evaluated at 10 weeks (Table 3). Only one of six mice that had been vaccinated with a mixture of MBT-2 cells and either 1 mg BCG (group B) or 0.1 mg R8-liposome-BCG-CW (group F) developed a re-challenged tumour at 10 weeks, while all six mice with no vaccination (group H) developed re-challenged tumours ($P = 0.002$). Five of six mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg R8-liposome-BCG-CW (group E) developed re-challenged tumours. Four of five mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg BCG-CW developed re-challenged tumours.

TABLE 3 BCG-pretreated MBT-2 cells were re-inoculated in the vaccinated mice. The number of mice bearing re-challenged tumours of BCG-pretreated MBT-2 cells was recorded after 4 weeks from re-inoculation (at 10 weeks)

Vaccination	Number of mice, *n*/*N*
MBT-2 + 1 mg BCG	1/5*
MBT-2 + 1 mg BCG-CW	4/5
MBT-2 + 1 mg R8-liposome-BCG-CW	5/6
MBT-2 + 0.1 mg R8-liposome-BCG-CW	1/6*
With no vaccination	6/6

**P = 0.002 vs mice with no vaccination.*

The numbers of mice bearing re-challenged tumours of BCG-, R8-liposome-BCG-CW-pretreated MBT-2 cells or MBT-2 cells with no pretreatment were four of five, three of three or five of five after 4 weeks from the re-inoculation, respectively, in mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg BCG-CW. The growth of the re-challenged tumours of BCG-pretreated MBT-2 cells was suppressed, while that of MBT-2 cells with no pretreatment was not, with a mean (SD) size of 96 (156) mm^2 compared with 396 (98) mm^2, respectively ($P = 0.004$, Fig. 3b). The growth of the re-challenged tumours of R8-liposome-BCG-CW-pretreated MBT-2 cells was moderately suppressed, with a mean size of 207 (108) mm^2.

The numbers of mice bearing re-challenged tumours of BCG-, R8-liposome-BCG-CW-pretreated MBT-2 cells or MBT-2 cells with no pretreatment after 4 weeks from the re-inoculation were five of six, three of three or six of six, respectively, in mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg R8-liposome-BCG-CW. The growth of the re-challenged tumours of MBT-2 cells pretreated with 1 mg or 0.1 mg R8-liposome-BCG-CW was suppressed while that of MBT-2 cells with no pretreatment was not (Fig. 3C). The mean (SD) sizes of the tumours in each group were 54 (60) mm^2 ($P < 0.001$), and 69 (44) mm^2 ($P = 0.003$), respectively, compared with 309 (125) mm^2.

DISCUSSION

The antitumour mechanism of BCG and the role of its CW components have been gradually unveiled recently. Most of immunopotentiative activities are associated with the CW lipids of BCG [23], and which involve inflammation, innate and acquired immune responses. Intravesical instillation of BCG induces durable remission of nonmuscle-invasive bladder cancer and prevents recurrence.

Our previous study showed that mice that had been vaccinated with a mixture of MBT-2 cells and BCG, rejected re-challenged tumour of BCG-pretreated MBT-2 cells, but did not reject that of MBT-2 cells with no pretreatment. This suggests that the bladder cancer cell, which is usually under the condition of immune tolerance from the host, can be recognised through the presence of BCG-related molecules associated with the cancer cell by the immune system that was activated by the previous BCG therapy.

The aim of the present study was to develop a non-live bacterial agent using a CW extract of BCG (BCG-CW), which consisted mainly of essential molecules to induce immune responses, such as mycoloyl arabinogalactan peptideglycan complex (CWS), mycoloyl glycolipids and lipomannan [24–26]. However, the BCG-CW preparation itself has difficulty associating with the bladder cancer cells due to its highly hydrophobic properties, the crude

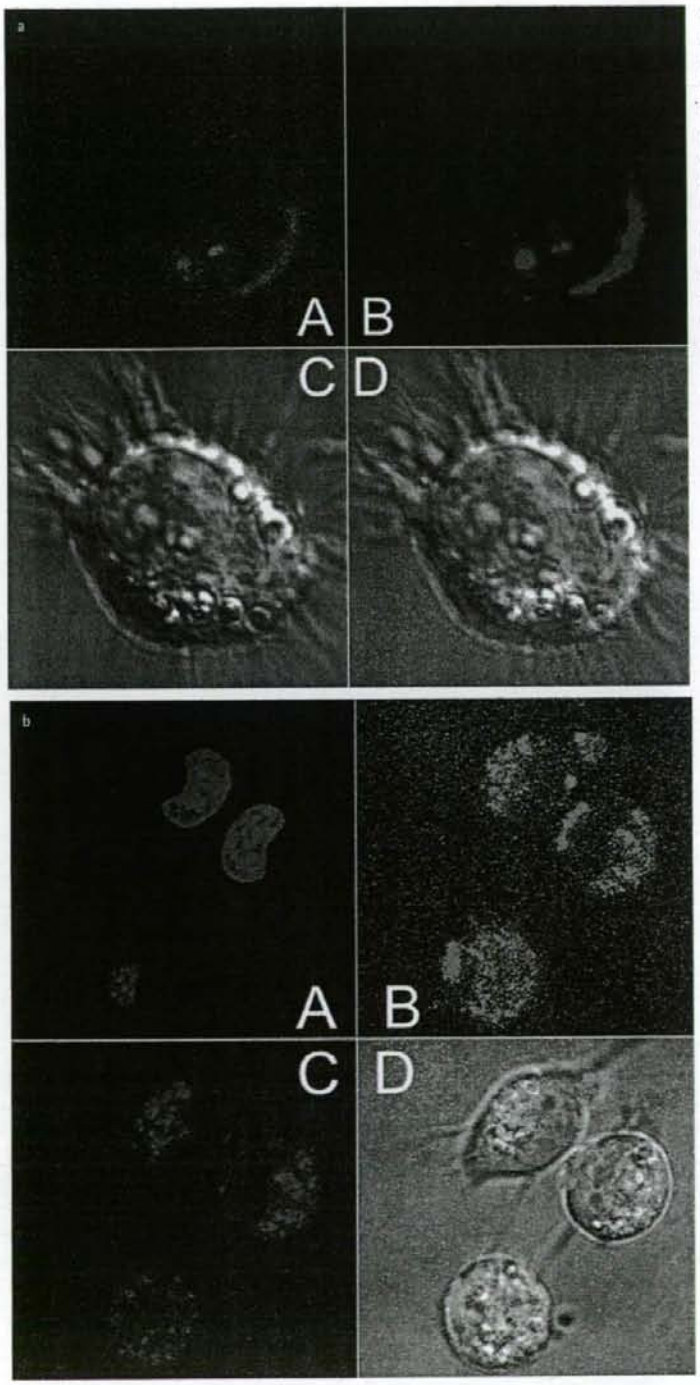
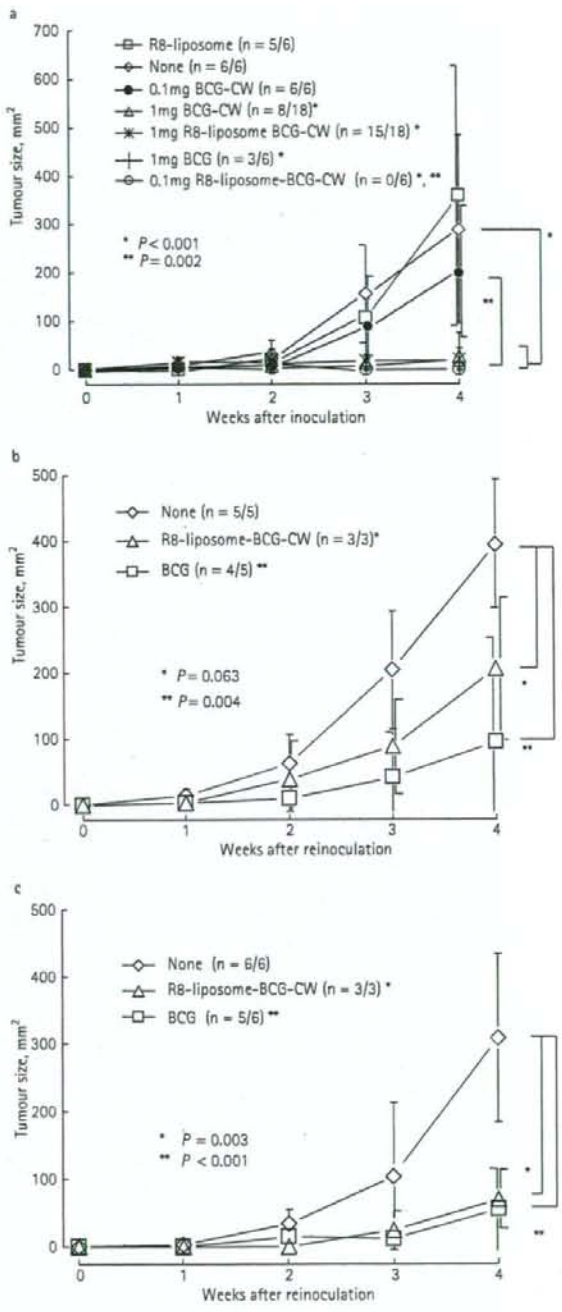


FIG. 2.
***a**, Fluorescently double-labelled R8-liposome-BCG-CW was incubated with MBT-2 cell for 1 h at 37 °C. The carbohydrate moieties exposed on the surface of BCG-CW were labelled with FTSC (A), while the aqueous phase of liposomes were labelled with sulforhodamine (B). Visible light microscopic view of MBT-2 cell (C). BCG-CW and R8-liposomes were together distributed to the cellular membrane and cytoplasm of MBT-2 cell (D). **b**, The fate of internalized BCG-CW was tracked. The cell nuclei were stained with Hoechst 33342 (A). MBT-2 cells were incubated with R8-liposome incorporating single-labelled BCG-CW (B) at 37 °C for 1 h. Endosomes and lysosomes of MBT-2 cells were stained for 30 min before visualization with 75 nM Lysotracker Red (C). BCG-CW in the cytoplasm was co-localized with endosome/lysosome compartments (D).*

FIG. 3

*a, The growth of primary tumour was recorded weekly. The growth of MBT-2 tumours was suppressed when MBT-2 cells were inoculated with mixture of 1 mg BCG, 1 mg BCG-CW, 0.1 mg or 1 mg R8-liposome-BCG-CW compared with that of MBT-2 cells alone (*P < 0.001). The 0.1 mg R8-liposome-BCG-CW inhibited the growth of all tumours of MBT-2 cells by 4 weeks, while 0.1 mg BCG-CW with no R8-liposomes vector did not (**P = 0.002). b, The growth of re-challenged tumour was recorded weekly in mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg BCG-CW. The growth of re-challenged tumours of BCG-pretreated MBT-2 cells was suppressed compared with that of MBT-2 cells with no pretreatment (**P = 0.004). c, The growth of the re-challenged tumours was recorded weekly in mice that had been vaccinated with a mixture of MBT-2 cells and 1 mg R8-liposome-BCG-CW. The growth of the re-challenged tumours of R8-liposome-BCG-CW- and BCG-pretreated MBT-2 cells was suppressed compared with that of MBT-2 cells with no pretreatment (*P = 0.003, **P < 0.001, respectively).*



CW fraction contains more hydrophobic molecules such as lipoglycan and glycophospholipids [12]. Furthermore, because usually urothelial (cancer) cells are not phagocytic, transportation of a non-infectious molecule such as BCG-CW across the cell membrane is passively performed by endocytosis. Due to its hydrophobic properties, BCG-CW tends to form relatively large agglutinated clumps in an aqueous environment, which also obstructs its transportation by endocytosis. BCG-CW and cell membrane also electronically repel each other because both of their surfaces are negatively charged. Therefore, we hypothesized that the immune response would be evoked if we could successfully deliver BCG-CW into the cytoplasm of murine bladder cancer cells by using an appropriate vector.

R8-liposomes vector was originally developed as an efficient non-viral vehicle to transfer gene plasmids into cells by macropinocytosis [15,16]. Experiments using this delivery system *in vivo* confirmed that they can reach various organs [27] and cancer tissues [28]. The incorporation of BCG-CW into R8-liposomes circumvented the unfavourable physicochemical properties of BCG-CW; the positively charged surface of the R8-liposome-BCG-CW allowing better contact to the negatively charged cell surface. It is as small as 230 nm in diameter, therefore of a molecular size that can be transported across the cellular membrane by macropinocytosis. The present *in vitro* study showed that R8-liposome-BCG-CW successfully attached to the surface of MBT-2 cell and was efficiently internalized into the cytoplasm within an hour of co-incubation. Internalized BCG-CW was then distributed to the lysosome of the MBT-2 cell. Although urothelial cells do not have full antigen stimulating features because they lack the second co-stimulatory signal, antigen exposure may be feasible using gradually digested external molecules with the lysosomal enzymes, as MHC class I molecule is expressed on the surface of MBT-2 cells (data is not shown).

Thus, the R8-liposome encapsulated BCG-CW had enhanced cellular association and internalization of BCG-CW into bladder cancer cells and therefore was able to induce an antitumour immune response in the host mouse. The present experiment showed that 0.1 mg BCG-CW incorporated into R8-liposomes completely inhibited the growth of MBT-2 bladder tumour at 4 weeks, while 0.1 mg BCG-CW with no R8-liposomes did not evoke highly effective antitumour activity. The BCG-induced antitumour effect involves both innate and acquired immune responses, and therefore we expected both immunostimulatory activities. As we have previously shown that the 'normal' urothelial cell, which is not a professional immune cell, expressed TLR-2, -3, -4 and -9 and was able to respond directly to BCG [29], BCG-CW incorporated with R8-liposomes contacted the cellular surface and might induced innate immune responses through the stimulation of TLR on the bladder cancer cell. During the 4 weeks of the primary inoculation experiment, the tumours regressed or vanished in the mice that were inoculated with a mixture of MBT-2 cells and 1 mg BCG (group B), 1 mg BCG-CW (group C), or 0.1 mg R8-liposome-BCG-CW (group F). As it takes longer for immunostimulation to establish acquired immune protection such as cell-mediated immunity in the host, this phenomenon might suggest that acquired-specific immune protection plays an important role in BCG immunotherapy in later stages, which is similar to the immune response against infection by the host.

As it is known that BCG immunotherapy provides long lasting immune protection against recurrence of nonmuscle-invasive bladder cancer, the results of the re-challenge experiment produced important findings related to immune memory induced by BCG (Fig. 3b,c). The suppressive growth of re-challenged tumour was seen only if BCG- or R8-liposome-BCG-CW-pretreated MBT-2 cells were re-inoculated in to the vaccinated mice. As $CD4^+$ T cell lines from BCG-treated mice showed a specific immune response against bladder cancer cells that were co-cultured with BCG [30], immune memory induced in vaccinated mice must recognise the MBT-2 cancer cell through the BCG-CW antigen exposure on it. Thus, immunization using BCG-CW was effective due to the modification with R8-liposomes vector, which overcomes the unfavourable physicochemical properties of BCG-CW and improves cellular association.

In the present study, antitumour activity was induced better by 0.1 mg R8-liposome-BCG-CW than by 1 mg R8-liposome-BCG-CW. In the primary challenge experiments, although both 0.1 mg and 1 mg R8-liposome-BCG-CW clearly suppressed tumour growth, 0.1 mg R8-lipsome-BCG-CW inhibited tumour growth completely by 4 weeks (Fig. 3a). In the re-challenge experiments showing the impact of vaccination, mice vaccinated with 0.1 mg R8-liposome-BCG-CW had a better effect on tumour growth than those vaccinated with 1 mg R8-liposome-BCG-CW. The phenomenon of nonlinear dose–response is known in immune responses. One of the reasons that might explain this in the present experiments may be the incidence of oversaturated BCG-CW in the cytoplasm. Because transportation of BCG-CW into the cytoplasm is performed efficiently by R8-liposomes vector as shown *in vitro*, the amount of BCG-CW in the cytoplasm may easily exceed the appropriate concentration to activate the immunological responses of the cell. A dependency study of R8-liposome-BCG-CW is planned to elucidate the optimum concentration for BCG-CW incorporation.

In conclusion, the immunotherapeutic potential of BCG-CW was enhanced by improving cellular association using an R8-liposomes delivery system. Development of this non-live bacterial agent may contribute to providing a more active and less toxic tool as a substitute for live BCG in immunotherapy against nonmuscle-invasive bladder cancer in the future.

CONFLICT OF INTEREST

None declared.

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