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The effect of passages during Japanese BCG vaccine production on genetic stability and protective efficacy

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ABSTRACT

Many genetic differences have been found among currently available BCG vaccines. To avoid continued accumulation of phenotypic or genotypic changes in the strains, WHO and most national regulatory authorities request that the vaccine should not be prepared by more than 12 passages from the master seed lot. However, it has recently been reported that genetic changes occur even during the passage for vaccine production. In this study, the genetic stability of Japanese BCG vaccine production using currently available PCR methods and protective efficacy using a guinea-pig model during the passages were examined. The results showed that there were no significant differences between the seed lot, the product manufactured by normal procedures, and the 20th passage product. These results indicate that the maximum number of passages as currently required by WHO for BCG vaccine production is adequate for the Japanese vaccine, and that new genetic tools may help to examine the quality control of the BCG vaccine.

1. Introduction

Bacille Calmette-Guérin (BCG) is an attenuated strain originally developed by Albert Calmette and Camille Guérin, who repeated passage of a virulent *Mycobacterium bovis* strain 230 times on a glycerin-bile-potato medium from 1908 to 1921. BCG is the only live vaccine used to prevent tuberculosis, and it has been distributed to many countries in the world after 1924. The original strains were separately propagated as freshly prepared cultures through additional serial passages in different countries and different methods, and repeated subcultures produced many daughter strains (sub-strains), such as BCG Danish, Glaxo, Pasteur, Moreau, Russia, and Tokyo.

Many genetic differences have been found among the currently available BCG vaccines. The region of difference (RD) patterns vary among BCG sub-strains [1] and the multiplex PCR method based on RD patterns and number at *senX3-regX3* have successfully been used to identify BCG strains [2]. By using the multiplex PCR with commercial BCG vaccines, two *senX3-regX3* products were found in Copenhagen strain [2,3] and two RD16 products were found in Japan strain because the vaccine was a mixed culture with another strain [2]. However, further study indicated that the Japanese strain (Tokyo 172) contains two sub-populations with RD16 [4].

By using bacterial artificial chromosome libraries, tandem duplications (DU1, DU2) were identified in BCG Pasteur [5]. DU1 duplication was only present in the BCG Pasteur and DU2 varies between BCG sub-strains [5,6]. BCG Pasteur and Danish contain two sub-populations with DU2 [5,6]. Although the role of DU1 or DU2 in the attenuation and/or altered immunogenicity of BCG is yet unknown, knowledge of their existence will facilitate quality control of BCG vaccine lots [5].

To avoid continued accumulation of genotypic or phenotypic changes in the strains, the production of vaccine is based on the seed lot system. According to the present WHO requirements for dried BCG vaccine production, the vaccine should be prepared from a freeze-dried seed lot by as few cultural passages as possible, and by not more than 12 passages from the master seed lot [7]. Some BCG sub-strains contain different morphological colonies (spreading or non-spreading), and under certain conditions, almost total selection of a minority population can occur in the course of a single passage [8]. A sample grown directly from a vial used in immunization programs was found to contain either duplication or triplication of DU2-III coexisting in the population [6].

In this study, we examined the genetic stability and protective efficacy of BCG vaccine during the passages for vaccine production using currently available PCR methods and a guinea-pig model, respectively.

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2. Materials and methods

2.1. Seed lot and BCG vaccine product

The lyophilized seed lot (Tokyo 172-1) preparation was obtained from the National Institute of Infectious Diseases in Japan. We prepared lyophilized BCG vaccine products ourselves. Lyophilized BCG Pasteur and Connaught strains were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA, and lyophilized Danish strain was obtained from Statens Serum Institute, Copenhagen, Denmark.

2.2. Passage cultures of the seed lot

The seed lot was passaged by the method for vaccine production based on the Japanese Minimum Requirement for Biological Products. Briefly, the freeze-dried seed lot was reconstituted and inoculated on the surface of potato medium with glycerin solution, and cultivated at 37.5 ± 0.5 °C. After a further 17 passages on the surface of calf-bile potato medium at 37.5 ± 0.5 °C at intervals of about two weeks, the bacilli were transferred to the surface of Sauton medium and cultures was repeated 3 more times (total 20 passages). The 20th passage of BCG was freeze-dried, and used after reconstitution on the day of the experiment.

2.3. DNA extraction and genetic analysis

DNA was extracted with a DNA extraction kit (ISOPLANT, Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. Multiplex PCR was performed as described previously [2]. Briefly, five RDs (RD1, RD2, RD14, RD15 and RD16) or four RDs (RD1, RD2, RD14 and RD15) as well as senX3-regX3 were amplified. The sequence of primers, thermal profiles, and other conditions were the same as those reported by Bedwell et al. [2].

For single PCR of RD16 (RD16-PCR), the primer-pairs were employed as reported previously [9]. The bands of the *SenX3-regX3* product after 20 passages from the seed lot were cut out of the gel and sequenced with a DNA sequencer (ABI PRISM 310, Applied Biosystems Japan Ltd., Tokyo, Japan).

For tandem duplications, the primer-pairs that only produce a correctly sized amplification product when the strain was duplicated were used. The sequences of JDU1, JDU2-I, JDU2-III, and JDU2-IV primer pairs were proposed by Brosch et al. to improve the quality control of BCG vaccines [10] and we confirmed them by using Primer 3: WWW primer tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and Refs. [5,6] (Table 1). The PCR annealing temperature is 52 °C for JDU1, JDU2-I, and 50.5 °C for JDU2-III and JDU2-IV. The thermal profile was 1 cycle at 94 °C (10 min), and 30 cycles at 94 °C (1 min), 52 °C or 50.5 °C (1 min) and 72 °C (2 min) and 1 cycle at 72 °C (10 min).

PCR products were subjected to electrophoresis on 10–20% polyacrylamide gel and were stained with ethidium bromide.

2.4. Quality control tests on the 20th passage BCG

The quality control tests on the 20th passage BCG, including the viability, test of being free from virulent mycobacteria, test of total bacterial content, sterility test, skin reactions in guinea-pigs, stability and identity (microscopical examinations of stained specimens), etc. were carried out according to the WHO requirement test for dried BCG vaccines [7].

2.5. Isolation and passage cultures from two sub-populations, type I and type II colonies

After several selections of each colony type and confirmation of the genetic type, they were separately inoculated on the surface of potato medium. After 19 passages on the surface of calf-bile potato medium, each sub-population culture, type I or type II, was transferred to the surface of Sauton medium followed by 3 more passages (total 22 passages), respectively. The 22nd passage sub-population culture, type I or type II, was freeze-dried and used after reconstitution on the day of the experiment, respectively.

2.6. Experimental animals

Outbred (4-week-old) female Hartley guinea-pigs were purchased from SLE (Shizuoka, Japan) and held under barrier conditions in a biosafety level III animal laboratory. Animals were individually housed in polycarbonate caging with stainless steel grid covers and feeders, they were given commercial chow and sterilized water ad libitum, and temperature was maintained at 23 ± 0.5 °C in a 12 h light/dark cycle. All animal procedures were reviewed and approved by the Research Institute of the Tuberculosis Experimental Animal Committee.

2.7. BCG vaccination and PPD skin test

Hartley guinea-pigs were intradermally vaccinated with the Tokyo 172-1 seed lot, BCG vaccine product (8th passage from seed lot or 20th passage BCG vaccine from seed lot) (10^4 CFU/guinea-pig, 5 guinea-pigs/group). The viability of the BCG preparations was determined by plating dilutions on Ogawa medium. Seven weeks after vaccination, PPD (0.2 μg/0.1 mL/guinea-pig) was intradermally injected on the shaved back of the animals. To evaluate delayed-type hypersensitivity (DTH) responses, skin reactions were measured at 24 hr following PPD injection and were recorded as the mean of the two perpendicular measurements of the indurations (mm).

2.8. Aerosol challenge and necropsy

Eight weeks after vaccination, the vaccinated and unvaccinated guinea-pigs were challenged with *M. tuberculosis* H37Rv strain by the pulmonary route in the aerosol chamber. *M. tuberculosis* H37Rv suspension containing 2×10^6 CFU/5 mL was introduced into the nebulizer of a Glas-Col Aerosol Exposure Chamber. The concentration of the suspension was calculated for uptake of 100–200 viable bacilli by the lung after inhalation exposure. To determine the bacillary loads in tissues seven weeks after the virulent pulmonary challenge, the animals were humanely euthanized by an intraperitoneal injection of sodium pentobarbital, and the lungs and spleens of each guinea-pig were removed and homogenized separately. Appropriate dilutions were aseptically plated on Ogawa medium and incubated at 37 °C. Viable counts were determined using serial dilutions of the homogenates. Colony-forming units (CFU) were enumerated following incubation at 37 °C for 4 weeks and were expressed as the mean ± standard error of the means (SEM)/tissue. The minimal detectable limits were ($\log_{10} = 3.0$) and ($\log_{10} = 2.3$) in the lung and spleen, respectively.

2.9. Statistical analysis

Data were expressed as means ± SEM. Where appropriate, the data were analyzed using Student's *t*-test for assessment of significant differences between groups (5 animals/group). A probability of $p < 0.05$ was considered to be statistically significant.