Journal of Food and Drug Analysis, Vol. 15, No. 3, 2007, Pages 213-219 藥物食品分析 第十五卷 第三期

In Vitro and *In Vivo* Assessments for Developing an Oral BCG Vaccine Formulation

JIIN-LONG CHEN[1], CHIA-CHIA YU[1], JEN-RON CHIANG[2], TE-CHENG LEE[1], MING-KUNG YEH[3] AND CHIAO-HSI CHIANG[1]*

[1.] School of Pharmacy, National Defense Medical Center, 161 Minchuan E. Rd., Sec. 6, Taipei 114, Taiwan, R.O.C.
[2.] Center for Disease Control, National Institute of Preventive Medicine, Department of Health, 161 Kunyang St., Nangang District, Taipei 115, Taiwan, R.O.C.
[3.] Department of Clinical Pharmacy, Tri-Service General Hospital, National Defense Medical Center, Taipei 114, Taiwan, R.O.C.

(Received: June 6, 2006; Accepted: December 7, 2006)

ABSTRACT

The aim of this study was to establish *in vitro* analytical methods for assessing an investigated oral BCG vaccine formulation. In addition, *in vivo* immune response was also evaluated in guinea pigs by determining the potentially protective effect of the investigated formulation on tuberculosis. The bicinchoninic acid (BCA) method for BCG protein quantitation was developed for rapid measuring the content of BCG vaccine in the investigated formulation. The analytical method was conducted at 45°C to offer good precision (coefficient of variation <3.5%) and accuracy (error: -3.6~2.6%) in the range of 150~1000 μg/mL of BCG vaccine concentrations. In the simulation study of drug release from the investigated formulation in the gastrointestinal tract under physiological conditions, the investigated oral formulation could protect active component at lower pH (0.1 N HCl) and completely release active component at higher pH (phosphate buffer, pH 6.8). Although the investigated oral BCG vaccine formulation showed weak immune response to elicit IgG titer, the PPD (purified protein derivative) skin test in guinea pigs by oral dosing of 4 mg BCG vaccine formulation acquired about equal or higher response than that by 0.5 mg dose by subcutaneous vaccination. The analytical method and *in vivo* approach could be further applied in the development of oral BCG vaccine formulations.

Key words: oral BCG vaccine, BCA method, intestinal delivery, BCG potency

INTRODUCTION

Tuberculosis (TB) remains one of the major epidemic diseases in many countries. Two million deaths is estimated due to TB each year world wide[(1)]. In crowded environment associated with poor ventilation, such as in public transport vehicles, passengers or travelers are potentially infected by TB. Human TB has been treated by chemotherapy for many years. However, the treatment is lengthy and expensive. Therefore, the control of human TB is still an important public health issue in developing countries. Prophylactic vaccination may be a better option for controlling TB. Bacille Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, was obtained in 1919 after repeated cultures in bile medium. Various vaccinal substrains, derived from BCG, have been used since 1920s in an attempt to prevent human TB[(2,3)]. Recently, in a meta-analysis of human BCG vaccination trials, BCG was shown to reduce the risk of pulmonary TB by 50% and the number of death due to TB by 71%[(4)]. However, the efficacy of BCG is highly variable and the protection rates of human and animal trials range from 0 to 80%[(2,5,6)]. Oral immunization of mice with BCG has been shown to induce immune response. BCG has a high safe range of dose following oral administration. The complete form of Freund's adjuvant (FCA) is composed of inactivated and dried mycobacteria to significantly stimulate the cell-mediated immunity following the subcutaneous or intraperitoneal injections of FCA. Due to safety consideration, FCA is only used in animal study, but forbidden in humans. Mice immunized orally or intragastrically with high doses of BCG showed similar levels of protective immunity to mice immunized via the subcutaneous route and induced protection against intravenous challenge with *M. tuberculosis*[(7)]. In addition, oral administration of BCG vaccine preparation against TB has several advantages, including convenient administration, low cost, and avoidance of needles. A promising oral delivery system might protect BCG vaccine from the damage by gastric acid, which might provide an effective means to induce protective systemic immune responses from the mucosal immunization in gastrointestinal tract[(7)].

The classical method for measuring the potency of BCG vaccine involves enumerating colony forming unit (CFU) in solid medium via cultivation[(8)]. However,

* Author for correspondence. Tel: +886-2-87924838; Fax: +886-2-87924838; E-mail: cch@mail.ndmctsgh.edu.tw

the approach is cumbersome and time-consuming for more than 3 weeks. Thus, it is important to establish an analytical method for fast determination of the content of BCG vaccine during the development of oral BCG vaccine formulation. In this study, we attempted to establish a rapid analytical method to quantitate the content of BCG vaccine for quality control or formulation evaluation. The protein content of BCG vaccine in formulations can be determined by BCA (bicinchoninic acid) method[9]. Subsequently, the content of BCG vaccine was calculated using the calibration curve of pure BCG vaccine. In addition, we intended to develop an oral BCG vaccine preparation for controlling TB. The oral vaccine formulation was prepared and evaluated, including *in vitro* release characteristics in simulated gastrointestinal fluid and the stability of the BCG vaccine formulation during short-term storage. The immune response of the oral BCG vaccine formulation was also investigated in guinea pigs in comparison with subcutaneous vaccination. The purpose of the study was to evaluate some important physicochemical properties of the investigated oral BCG vaccine preparation and to determine the potential immune response related to TB protection. In addition, BCA method was established and evaluated for determining the content of BCG vaccine formulation.

MATERIALS AND METHODS

I. *Materials*

BCG vaccine was provided by Center for Disease Control, Taiwan. The vaccine has been freeze-dried, sealed in ampoules and stored at -20°C. The potency of the BCG vaccine is 3×10^7 CFU/mg. Guinea pigs were purchased from the National Laboratory Animal Center (Taipei, Taiwan), and housed at the Laboratory Animal Research Center, National Defense Medical Center, with free access to food and water. The vivarium was maintained on a 12 hr light/12 hr dark cycle, with room temperature of 22 ± 2°C and relative humidity of 50~70%. All studies were conducted in compliance with the rules set forth in the "Guide for the Care and Use of Laboratory Animals".

II. *Chemicals*

BCA reagents were purchased from Sigma (St. Louis, MO, USA), which included BCA solution and copper (II) sulfate pentahydrate 4% (w/v) solution. For protein determination, a test reagent was freshly prepared by mixing 1 part of copper sulfate pentahydrate 4% (w/v) solution with 50 parts of BCA solution. Phosphate buffered saline (PBS) tablet, bovine serum albumin (RIA grade), tween 20, goat anti-guinea pig IgG (whole molecule) peroxidase conjugate and o-phenylenediamine dihydrochloride were also from Sigma (St. Louis, MO, USA). Hydrogen peroxide and sodium phosphate dibasic were from Wako Chemical (Osaka, Japan). Sodium dodecyl sulfate (SDS) was from Mallinckrodt Baker (Paris, France). Citric acid was from Shimakyu's Pure (Osaka, Japan). Formaldehyde solution (36~38%) was ordered from Union Chemical Works Ltd. (Hsinchu, Taiwan). Other chemicals were of either reagent or pharmacopoeial grade.

III. *Methods*

(I) *Determination of BCG Content by BCA Method*

1. *Temperature Effect on BCA Method*

BCG vaccine aqueous solutions were prepared either in Milli-Q water alone, or in Milli-Q water combining with 8% SDS and 0.08 N NaOH, in a range of concentrations from 150 to 1000 μg/mL; the concentrations may not be equal for different lot samples due to various total content of BCG vaccine in different vials. Then, a series of BCG vaccine solutions in two different media were separately transferred into 10-mL test tubes, 2 mL per tube. Two sets of test BCG solutions were incubated at 37°C or 45°C for 1 hr. An aliquot of sample, 0.2 mL, was withdrawn (n = 3) and transferred into a new tube. Subsequently, 2 mL of BCA reagent was added in and the mixture was incubated at 37°C for 30 min. Finally, the concentration of purple complex in each tube was measured by absorbance at 562 nm using a spectrophotometer (UV-160, Shimadzu Co., Tokyo, Japan).

2. *Effect of Incubation Time on BCA Method*

Fifteen milliliter of BCG vaccine aqueous solution (1000 μg/mL) was prepared in 8% SDS and 0.08 N NaOH. And equally divided into three tubes and individually incubated in water bath at 25°C, 45°C, or 60°C. An aliquot of sample, 0.2 mL was taken (n = 3) at 0, 3, 6, 10, 20, 30, and 45 min, mixed with 2 mL of BCA reagent and incubated at 37°C for 30 min. Finally, the absorbance of formed purple complex in each tube was measured at 562 nm. Milli-Q water (0.2 mL) was used as blank.

3. *Data Analysis*

For each tube, the mean absorbance in duplicate measures was calculated. The calibration curves were made by plotting the absorbance (after blank correction) versus the BCG vaccine concentration.

(II) *ELISA Method*

1. *Tests and Controls*

Twelve guinea pigs were purchased 1 week prior to the experiments. Guinea pigs were randomly divided into 4 groups(A, B, C and D). Group A was used as control (n = 3) and vaccinated with 0.5 mL normal saline

subcutaneously. Group B, C, and D, as test groups, were subcutaneously vaccinated with 0.02 mL, 0.1 mL, or 0.5 mL of BCG vaccine solution (1 mg/mL in normal saline), respectively. Blood samples were collected from the ear central artery of the guinea pigs at 3, 4, 5, 6, 7, 8, and 9 weeks after vaccination. Sera were separated from blood samples immediately by centrifuging at 12,000 rpm for 5 min (FB-4000, Kurabo Industries Ltd., Osaka, Japan) and preserved at -80°C until assay.

For oral vaccination, 6 guinea pigs were separated into individual cages and taken off food for 12 hr prior to oral vaccination. Three guinea pigs, as oral control (group E), were administered with the oral formulation containing excipient only. Another three guinea pigs (group F) were also administered with the oral formulation with a dose of 4 mg BCG vaccine.

2. *BCG Preparations*

Twenty five milligrams BCG vaccine was suspended in 1 mL of 10% formaldehyde in PBS (pH 7.4). After mixing and standing for 30 min, the mixture was centrifuged at 12,000 rpm for 5 min and the supernatant was discarded. Then, the pellet was washed three times with Milli-Q water and resuspended in 2.5 mL of PBS, the final concentration was equivalent to 10 mg/mL BCG vaccine.

3. *ELISA*

The ELISA was performed by following the previously reported method[10]. Briefly, polystyrene plates (EIA/RIA plate, flat bottom treated for enhanced protein binding, Sigma) were loaded with 100 μL of the BCG preparations described above in each well. The plates were then placed in a laminar flow hood until dry. After washing twice with PBS, each well was blocked with 100 μL of 1% BSA in PBS, pH 7.4. Then, the supernatant was discarded, each well was filled with 100 μL of test or control sera (2.5% normal goat sera diluted 1:30 with 0.05% tween 20 in PBS, pH 7.4) and incubated at room temperature for 90 min. After removing the supernatant, each well was washed three times with PBS and subsequently incubated for 1 hr at room temperature with goat anti-guinea pig IgG coupled with horseradish peroxidase (1:1000 dilution). After 1 hr incubation, the plates were washed with PBS once. Then, 100 μL of freshly prepared 0.4 mg/mL o-phenylenediamine with 100 μL of 3% hydrogen peroxide was added as enzyme substrate. The reaction was stopped with 4 N sulfuric acid at 90 min, and the absorbances were read at 492 nm using an ELISA reader (Anthos 2010, Anthos Labtec Instrument, Austria)

4. *Data Analysis*

For each serum, the mean absorbance value for duplicate measures was calculated. The means and standard deviations of the absorbance at 492 nm for the control and the test groups were obtained. The IgG titer was expressed as the response ratio using the absorbance ratio of test to control groups.

(III) *Purified Protein Derivative (PPD) Skin Test*

At 8 weeks post vaccination, all guinea pigs were shaved in the region of right-side back and injected intracutaneously with 0.1 mL of the purified protein derivatives (PPD). The skin test reaction of the mean diameter of the induration was measured after 24 hr[11].

(IV) *Oral Vaccine Preparation*

Oral BCG preparation was prepared in the lab. Briefly, BCG vaccine was mixed with suitable excipients and encapsulated in a specially-designed polymeric membrane which was prepared following aseptic process in a cleaning room under 20°C. The oral delivery system was designed to resist the attack of gastric fluid, and to release the active component of BCG vaccine at neutral pH in intestinal tract. The delivery system was also prepared to carry fluorescein sodium as a releasing marker to substitute for BCG vaccine in assessing the release characteristics.

(V) *Release Studies*

The investigated oral delivery system, containing 1 mg of fluorescein sodium with the same excipients as oral BCG vaccine formulation, was used to investigate the release characteristics. USP disintegration apparatus with disc was also employed. For initial 2 hr, 1 L of 0.1 N HCl was used as the test medium and run at 37°C. Following, the test medium was switched to the phosphate buffer (pH 6.8) and run for another hour. At suitable time intervals, an aliquot of sample, 0.5 mL was withdrawn from the medium and diluted 200 times with alkaline borate buffer (pH 9.0). After mixing, the fluorescent intensitity of mixtures was measured using a fluorophotometer (RF-5301PC, Shimadzu Co., Kyoto, Japan) at 485 nm excitation and 515 nm emission.

(VI) *Stability of Oral BCG Vaccine Formulation*

The oral BCG vaccine formulation was stored at 5°C and -20°C to investigate the stability of BCG vaccine during short-term storage. At 0, 1 week, 1 and 2 month (two additional points, 2 and 3 week for 5°C), samples of the oral BCG vaccine formulation were withdrawn. The potency of BCG vaccine in the formulation was determined by cultivating the BCG vaccine in Lowenstein-Jensen medium after suitable dilution (20~60 CFU/0.1 mL in PBS) according to the WHO viability method[8]. The remained BCG potencies at different sampling times in oral vaccine formulation were calculated taking the CFU values of original BCG vaccine as 100%.

RESULTS

I. *BCG Content Determined by BCA Method*

The protein concentration in BCG vaccine solution was determined by BCA method. Proteins reduce Cu^{2+} to Cu^{+} to form a purple complex with BCA and display an absorbance maximum at 562 nm. The calibration curves of BCG vaccine is illustrated in Figure 1 by plotting the net absorbance (after deducting the blank absorbance) versus BCG concentration. The linear equations of calibration curves for determining BCG concentrations by BCA method at 37°C and 45°C with or without 8% SDS and 0.08 N NaOH were shown in Table 1. These results indicated that the BCA method at higher temperature (45°C) with SDS and NaOH presented better precision and accuracy (C.V. < 2.0% and error: -0.53~0.22%) for measuring BCG vaccine. The time course of the resultant absorbances in three different temperatures for BCG vaccine determination is shown in Figure 2. After 30 min, the absorbances of three temperatures were about equal, but incubation at 60°C associated with higher reaction rate displayed higher absorbances for initial 20 min as compared with 25°C and 45°C.

II. *Release Characteristics*

The release profiles of investigated delivery system were determined in simulated gastric fluid for 2 hr, thereafter in simulated intestinal fluid for another 1 hr as shown in Figure 3. For initial 2 hr, the marker (fluorescein sodium) was undetected, but it was completely released in

Table 1. Linear equations, precision and accuracy for using BCA method to determine BCG concentration in various conditions

Condition	Equation	C.V. (%)	Error (%)
37°C, (–)[a]	y = 0.000124x + 0.0589, R^2 = 0.9975	2.5	-2.40~1.39
37°C, (+)[b]	y = 0.000171x + 0.0712, R^2 = 0.9968	3.0	-3.62~2.51
45°C, (–)	y = 0.000158x + 0.0608, R^2 = 0.9944	3.5	-1.89~2.64
45°C, (+)	y = 0.000217x + 0.0693, R^2 = 0.9998	2.0	-0.53~0.22

The analytical BCG vaccine concentration ranged from 150 to 1000 μg/mL.

[a]BCG vaccine in the aqueous solution without 8% SDS and 0.08 N NaOH.

[b]BCG vaccine in the aqueous solution with 8% SDS and 0.08 N NaOH.

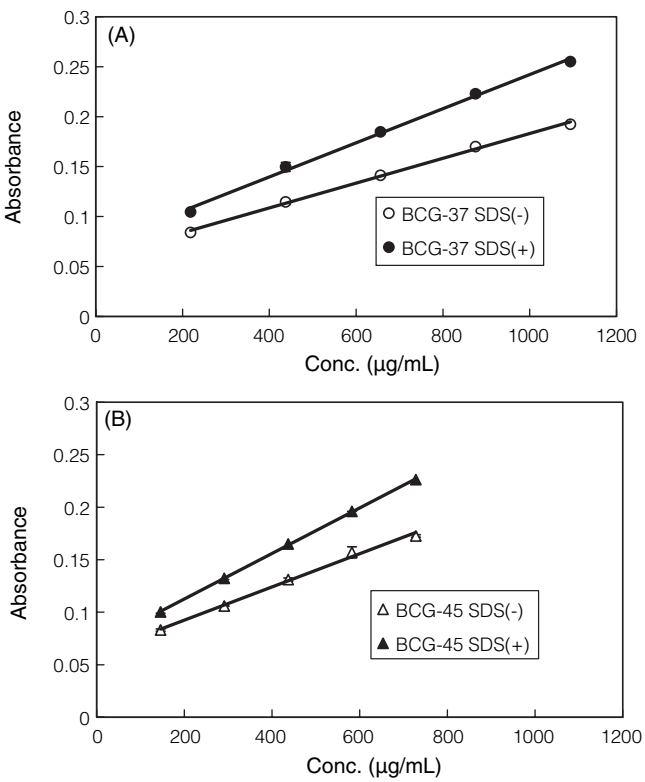

Figure 1. Calibration curves of BCG vaccine determined by BCA method at 37°C (A) and 45°C (B).

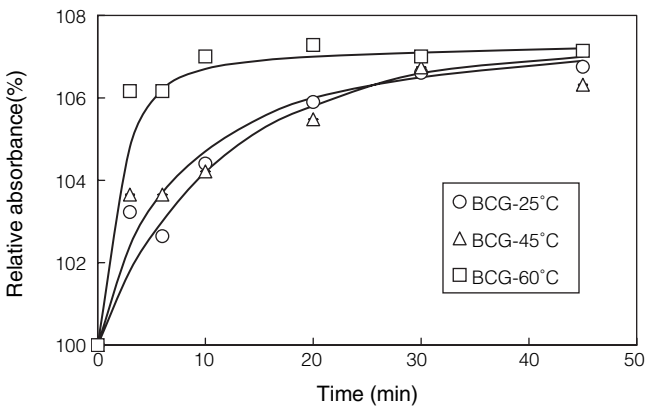

Figure 2. The effect of reacting temperature on the analysis of BCG vaccine determined by BCA method.

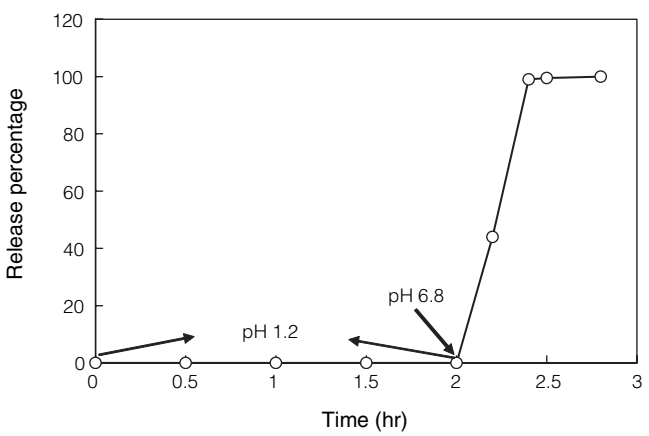

Figure 3. The release profile of the established oral delivery system in simulated physiological pH media by using sodium fluorescein as an active component.

Journal of Food and Drug Analysis, Vol. 15, No. 3, 2007

20 min at the medium of pH 6.8. *In vitro* release results indicated that the designed delivery system could provide an intestinal delivery to completely protect BCG vaccine from the attack of gastric fluid during the transit through stomach and release BCG exactly in intestinal tract.

III. *Stability of BCG in Oral Delivery System*

The change of potencies of BCG vaccine by enumerating the CFU value is shown in Figure 4 in investigated oral formulation during the storage of 8 weeks. The oral BCG vaccine formulation was stored at -20°C or 5°C and the potencies of BCG vaccine fell to the level of 10~20%. A higher potency of BCG vaccine is maintained under lower temperature. Our results were similar to the report of Aldwell *et al*.[12]. They prepared BCG vaccine in a lipid-based formulation. Formulated BCG vaccine could be stored for 8 weeks to maintain suitable potency at temperatures lower than 5°C.

IV. *IgG Titer*

The ELISA result is shown in Figure 5 for measuring the IgG titer in guinea pigs following subcutaneous injection with various doses of BCG vaccine. The IgG immune response followed biphasic change during 8 weeks of post-vaccination period. Two peaks appeared at 4th and 7th weeks with a trough at 5th week. The IgG response was enhanced as the dose of BCG vaccine increased. These results also indicated that oral vaccination only elicited weak IgG response (Figure 5). The immune responses in IgG titers including control (normal saline injection), oral BCG formulation and excipient (oral delivery) groups were very weak.

V. *Tuberculin Skin Testing*

To determine the dose response of BCG vaccine following subcutaneous vaccination in guinea pigs, we also measured the mean diameter of the induration after intradermal injection of tuberculin (PPD) at 8th week after vaccination. These results were compared with the investigated oral BCG vaccine formulation (Table 2). In two control groups, either subcutaneous or oral administrations did not elicit significant response to tuberculin. But the tuberculin test response for various doses of BCG vaccine in subcutaneous injection showed a linear relationship of the mean diameter and the logarithm of dosing volume (Figure 6). In addition, the oral vaccination group demonstrated a promising effect similar to the subcutaneous injection of 0.5 mg BCG vaccine.

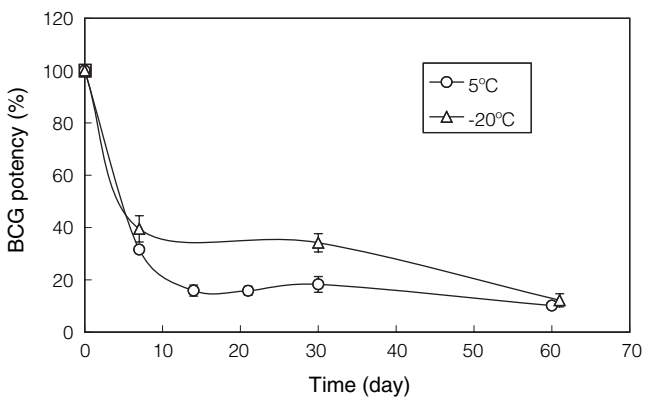

Figure 4. The degradation of BCG vaccine during storage at 5°C and -20°C.

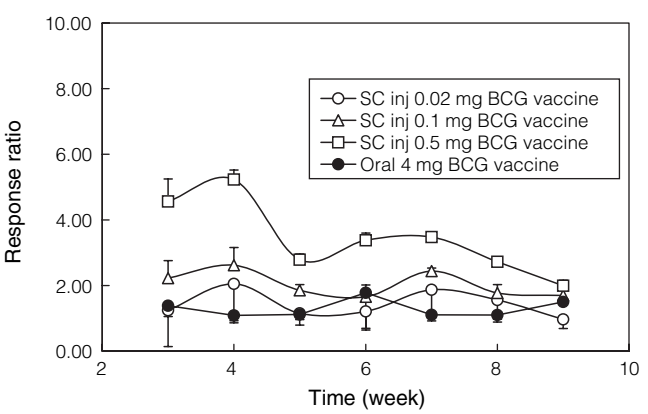

Figure 5. The humoral IgG immune response of BCG vaccine following subcutaneous (SC) injection or oral administration.

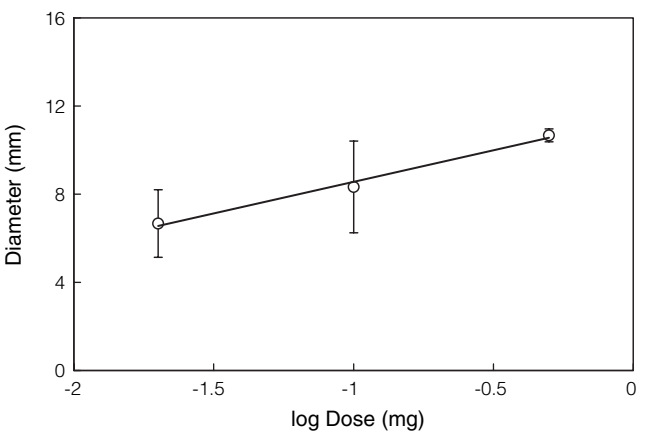

Figure 6. Dose dependent immune response of tuberculin skin test by subcutaneous (SC) injection of BCG vaccine.

Table 2. Results of the tuberculin skin test

Group	Delivery route	BCG dose (mg)	Diameter (mm) Mean ± S.D.
A (control)	SC inj	0	0
B	SC inj	0.02	6.7 ± 3.1
C	SC inj	0.1	8.3 ± 4.2
D	SC inj	0.5	10.7 ± 0.6
E (control)	Oral	0	0
F	Oral	4	12.0 ± 2.0

DISCUSSION

In this study, we intended to develop an oral BCG vaccine formulation. From the preliminary animal studies, the developed oral BCG vaccine preparation seemed to elicit immune response from oral vaccination. The investigated BCG vaccine formulation might be further optimized to fabricate an oral BCG vaccine preparation. BCG is a live attenuated vaccine and the acquired resistance against tuberculosis depends on the survival, multiplication and persistence of BCG in the host organs. The viability and stabilization of BCG vaccine in a delivery system are important factors to achieve good immunogenicity[13]. Because live or dead BCA vaccines can not be differentiated in BCA method, the short-term viability preservation was also investigated by traditional solid medium cultivation method in the study. In addition, BCA method was established to measure the protein content and determine the concentration of BCG vaccine. BCA method could be employed to quickly determine the content of BCG vaccine as long as the oral BCG vaccine preparation did not contain protein, copper or other interfering components. Content uniformity is also an essential requirement for low dose preparations. Since the investigated oral formulation only contained few milligrams of BCG vaccine, it is necessary to analyze its content uniformity. Viability and content uniformity are two important items to optimize the oral BCG formulation. In this study, BCG vaccine incubated with 8% SDS and 0.08 N NaOH has higher absorbance (Figure 1) in BCA method. It could be the lipophilic cell wall of BCG vaccine to be efficiently disrupted by surfactant SDS in alkaline condition to achieve better protein recovery.

Many analytical methods have been reported for measuring BCG vaccine potency. The tetrazolium salt, 3-(4,5-dimethy thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis-(2-methoxy-4-nitro-sulphonyl)-(2H)-tetrazolium-5-carboxanilide (XTT), are used as alternative substrates in the assay of BCG[14]. The method may achieve reproducible results, but the assay needs incubation of at least 2 days, which is not suitable for the investigation of oral BCG vaccine formulation. In addition, Kuan and Lin reported a method using capillary electrophoresis to analyze and detect the PCR products of BCG vaccine genomic DNA. The method offers advantages of short running time and reliable identification[15], however, the analysis might be devoid of quantitative determination of BCG vaccine.

Estimation of adenosine triphosphate (ATP) using the firefly bioluminescence reaction was also applied to quantitate living bacteria in BCG vaccine[16]. The bioluminescence method is sensitive and time saving. However, the ATP assay method has a potential variation of more than 20% due to significant ATP degradation during the extraction process. The bioluminescence method might be combined with the established BCA method for rapid assessment of the potency of investigated BCG formulation.

In the study, fluorescein sodium was used as a marker to study the release characteristics of investigated oral formulation. The approach provided a simple, safe and fast determination for assessing the oral delivery system. Fluorescein sodium with strong fluorescent intensity is easily measured by fluorometry. In addition, the small molecule is safe and thus avoiding any possible contamination or infection due to BCG vaccine.

The short-term stability study indicated that BCG vaccine is a heat liable microgranism. At higher temperature, the BCG vaccine lost potency quickly. The viability of BCG vaccine only maintained for few hours at 37°C after opening. It might explain the large variation of protection rates in BCG vaccination when BCG vaccine is not appropriately stored or it is exposed long time to hot weather by vaccinators.

Many studies assessed the immune response of BCG vaccine by measuring IgG titer which is also used in the diagnosis of tuberculosis[11, 17]. In this study, ELISA was employed to determine the specific BCG vaccine antibody using the formaldehyde-treated BCG vaccine as a primary antigen. Although the determined IgG titers were low, the immune response was proportional to the dose of BCG vaccine in subcutaneous vaccination (Figure 5). However, the IgG titer in oral vaccination was poor and similar to the control group. The result indicated that the BCG vaccine of oral formulation through mucosal immunosystem only elicit a lower immune response in IgG. It is consistent with the mucosal immunosystem to secrete predominantly IgA[18]. For PPD skin test, we found that the mean diameters of induction in subcutaneous vaccination increased as the vaccinated dose increased. Guinea pigs by oral dosing 4 mg of BCG vaccine formulation acquired about equal or slightly higher response than that by 0.5 mg dose in subcutaneous vaccination. The response in PPD skin test have been reported to correlate with IFN-γ assay and lymphocyte proliferation assay which are used to evaluate the cell-mediated immune response of BCG vaccine[19]. In addition, the mycobacteria of BCG, as facultative intracellular parasites, can survive within macrophage to initiate a specific T cell-mediated immune response for providing protective immunity.

Overall, we developed an oral BCG vaccine formulation to protect the viability of BCG vaccine for eliciting immune response in gut-associated lymphoid tissue in oral vaccination. The oral formulation could maintain live bacilli in short-term storage below 5°C. In addition, we established an analytical method for measuring BCG vaccine content by BCA method, which provided a simple and rapid approach for further determination of the content uniformity in the development of BCG vaccinal formulation.

Journal of Food and Drug Analysis, Vol. 15, No. 3, 2007

ACKNOWLEDGEMENTS

This research was financially supported by the Department of Health, Taiwan, ROC (DOH92-DC-1060, DOH93-DC-1109). In addition, we deeply appreciated Center for Disease Control, Taiwan, ROC for providing BCG vaccine.

REFERENCES

1. Dye, C., Scheele, S., Dolin, P., Pathania, V. and Raviglione, M. C. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. TAMA 282: 677-686.
2. Fine, P. E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 346: 1339-1345.
3. Lagranderie, M. R., Balazuc, A. M., Deriaud, E., Leclerc, C. D. and Gheorghiu, M. 1996. Comparison of immune responses of mice immunized with five different *Mycobacterium bovis* BCG vaccine strains. Infect. Immun. 64: 1-9.
4. Brewer, T. F. 2000. Preventing tuberculosis with Bacillus Calmette-Guerin vaccine: a meta-analysis of the literature. Clin. Infect. Dis. 31 (Suppl. 3): S64-S67.
5. Golditz, G. A., Berkey, C. S., Mosteller, F., Brewer, T. F., Wilson, M. E., Burdick, E. and Fineberg, H. V. 1995. The efficacy of Bacillus Calmette-Guerin vaccination of newborns and infants in the prevention of tuberculosis: meta-analysis of the published literature. Pediatrics 96: 29-35.
6. Golditz, G. A., Brewer, T. F., Berkey, C. S., Wilson, M. E., Burdick, E., Fineberg, H. V. and Mosteller, F. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 271: 698-702.
7. Lagranderie, M., Chavarot, P., Balazuc, A. M. and Marchal, G. 2000. Immunogenicity and protective capacity of *Mycobacterium bovis* BCG after oral or intragastric administration in mice. Vaccine 18: 1186-1195.
8. Donikian, R., Gheorghiu, M. and Jablokova, T. B. 1987. Requirments for dried BCG vaccine. WHO Tech. Rep. Ser. 745: 126-159.
9. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Hujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76-85.
10. Escobar-Gutierrez, A., Amezcua-Chavarria, M. E., Pasten-Sanchez, S., Ramirez-Casanova, E., Cazares, J. V., Granados, G., Loo-Mendez, E. and Cicero, R. 1996. Enzyme-linked immunosorbent assay (ELISA) with mycobacterial crude antigens for the sero-epidemiological diagnosis of active tuberculosis. Int. J. Lepr Other Mycobact. Dis. 64: 417-427.
11. Bartow, R. A. and McMurray, D. N. 1989. Vaccination with *Mycobacterium bovis* BCG affects the distribution of Fc receptor-bearing T lymphocytes in experimental pulmonary tuberculosis. Infect. Immun. 57: 1374-1379.
12. Aldwell, F. E., Tucker, I. G., deLisle, G. W. and Buddle, B. M. 2003. Oral delivery of *Mycobacterium bovis* BCG in a lipid formulation induces resistance to pulmonary tuberculosis in mice. Infect. Immun. 71: 101-108.
13. Gheorghiu, M., Lagranderie, M. and Balazuc, A. M. 1996. Stabilisation of BCG vaccines. Dev. Biol. Stand. 87: 251-261.
14. Kairo, S. K., Bedwell, J., Tyler, P. C., Carter, A. and Corbel, M. J. 1999. Development of a tetrazolium salt assay for rapid determination of viability of BCG vaccines. Vaccine 17: 2423-2428.
15. Kuan, C. P. and Lin, C. P. 2003. Detection of BCG vaccine by capillary electrophoresis. J. Food Drug Anal. 11: 92-95.
16. Askgard, D. S., Gottschau, A. Knudsen, K. and Bennedsen, J. 1995. Firefly luciferase assay of adenosine triphosphate as a tool of quantitation of the viability of BCG vaccines. Biologicals 23: 55-60.
17. Umadevi, K. R. and Ramalingam, B. 2002. Qualitative and quantitative analysis of antibody response in childhood tuberculosis against antigens of *Mycobacterium tuberculosis*. Indian J. Med. Microbio. 20: 145-149.
18. McGhee, J. R., Mestecky, J., Dertzbaugh, M. T., Eldridge, J. H., Hirasawa, M. and Kiyono, H. 1992. The mucosal immune system from fundamental concepts to vaccine development. Vaccine 10: 75-88.
19. Buddle, B. M., deLisle, G. W., Pfeffer, A. and Aldwell, F. E. 1995. Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with a low dose of BCG. Vaccine 13: 1123-1130.
20. Broccolo, F., Scarpellini, P., Locatelli, G., Zingale, A., Brambilla, A. M., Cichero, P., Sechi, L. A., Lazzarin, A., Lusso, P. and Malnati, M. S. 2003. Rapid diagnosis of mycobacterial infections and quantitation of *Mycobacterium tuberculosis* load by two real-time calibrated PCR assays. J. Clin. Microbiol. 41: 4565-4572.
21. Hellyer, T. J., DesJardin, L. E., Teixeira, L., Perkins, M. D., Cave, M. D. and Eisenach, K. D. 1999. Detection of viable *Mycobacterium tuberculosis* by reverse transcriptase-strand displacement amplification of mRNA. J. Clin. Microbiol. 37: 518-523.