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Meeting Report

WHO Informal Consultation on Standardization and Evaluation of BCG Vaccines

Geneva, Switzerland

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Abstract

The current World Health Organization Requirements for BCG vaccine are in need of revision to address the diversity of sub-strains used for production, potential improvements of quality control assays for lot release, and the establishment of sub-strain specific Reference Reagents. A consultation meeting was organised to discuss issues regarding the standardization and evaluation of BCG vaccines in the forum of regulators, BCG vaccine manufacturers, developers of selected new live tuberculosis (TB) vaccines and researchers. The development of new recombinant BCG and live attenuated TB vaccines and the characterization of different BCG sub-strains using state-of-the-art technologies were also reviewed. The objective of the meeting was to revise and update the current recommendations focused on the scope, terminology, manufacturing issues, and the incorporation of new reference reagents and new quality control tests.

1. Introduction

Tuberculosis (TB) is at crisis levels among the poor communities of many countries. Where human immunodeficiency virus (HIV) infection is common, TB spreads and kills rapidly and is responsible for increasing fatalities each year. The development and spread of multidrug resistant (MDR) strains of *Mycobacterium tuberculosis* is becoming an increasing cause for concern. The BCG vaccines have been in use since the 1920s and while these protect very young children from the more invasive forms of TB, adolescents and adults are variably protected and remain susceptible to pulmonary diseases caused by *M. tuberculosis*.

As part of the revision of the WHO Requirements for production and control of BCG vaccines (WHO TRS 745, annex 2; WHO TRS 771, annex 12), a WHO working group on BCG vaccines has been established and several consultation meetings on the evaluation of BCG vaccines were conducted [1-3]. Activities in past years were focused on the genetic characterisation of BCG sub-strains, development of the sub-strain specific Reference Reagents for quality control of BCG vaccines as well as on the investigations of potential improvements of the quality control tests for lot release of BCG vaccines. A two-day meeting was organised by WHO and the participants (Appendix I) in this consultation meeting included a wide range of temporary advisors from National Regulatory Authorities (NRAs), National Control Laboratories (NCLs) of countries where BCG vaccines are manufactured and/or extensively used, experts from organisations sponsoring research in new live TB vaccines, representatives of classical BCG vaccine manufacturers, and researchers in the biology of mycobacteria and the clinical effects of BCG immunization.

Dr. Ivana Knezevic opened the meeting and outlined the need for revising the WHO recommendations for BCG vaccines. The objectives of this consultation meeting were to revise and update the existing Requirements for dried BCG Vaccine (1987) [4] with regard to

current knowledge, practice of manufacture and quality control of BCG vaccines. Dr. James Southern and Dr. Mei M. Ho were appointed chairman and rapporteur respectively. Presentations (speakers are marked in the Appendix I) on the development of selected new TB vaccines, genetic characterisation, quality control and review of recent clinical studies of BCG vaccines were followed by the discussion on the revision of the Requirements for dried BCG vaccines. The consultation also aimed to review the results of international collaborative studies on new alternative assays for both viability and identity of BCG vaccine; to discuss the intended use of the proposed WHO Reference Reagents for BCG vaccines; and to reach consensus on key issues in the revision of the recommendations and set up plan for submission to WHO Expert Committee on Biological Standardisation (ECBS).

2. Development of selected new TB vaccines

A number of live TB vaccines based on recombinant BCG (rBCG) or attenuated *M. tuberculosis* are currently under development as replacement vaccines for the currently licensed BCG vaccines. Examples such as AERAS rBCG AFRO-1 and 401 [5], *M. tuberculosis* $mc^2$6020 [6] and $mc^2$6030 [7], and *M. tuberculosis phoP* [8, 9] are approaching Phase I clinical trial. Others have already been approved for evaluation in first-in-human trials, such as the rBCGΔureC::Hly [10] and the rBCG30 [11]. Since these new vaccines have been constructed using molecular techniques and have, in some cases, been manufactured using methods that differ from the traditional methods used to produce BCG vaccines, recommendations will be required for general manufacturing and vaccine testing methods as well as for non-clinical and clinical testing. Vaccine developers have been seeking guidance on the regulatory safety and quality requirements for entry to the clinic. Although the current requirements provide comprehensive information on assessing classical BCG vaccines [4, 12], these requirements do not formally apply to the new rBCG and other live TB vaccines.

Nevertheless these requirements should be considered during development of relevant product specifications, as regulators are likely to use these BCG vaccine requirements to form the basis of the requirements for new live TB vaccines.

Unlike current BCG vaccines, certain new rBCG vaccines are being produced by improved fermentation techniques including submersed fermentation processes and disposable systems, and in facilities not dedicated solely to rBCG production. Latest analytical and molecular biology techniques are used for characterisation, in-process and release controls of new live TB vaccine candidates. The acid fast staining method is no longer considered adequate and molecular biology techniques, such as PCR assays targeting specific genetically modified regions are used as identity tests for these new candidates. The genetically modified mycobacteria strains used for vaccine production may require tests for attenuation and possible reversion, persistence, plasmid retention, antibiotic sensitivity and the issues raised by the possible presence of antibiotic resistance markers will need to be addressed.

Potential safety issues associated with new recombinant and attenuated mycobacteria strains have been raised and additional safety evaluation using various animal models will be required for these types of products to demonstrate their genetic and phenotypic stability, and improved safety profiles for use in immune-compromised individuals if targeting HIV infected population. Such immune-suppressed animal models including severe combined immunodeficiency (SCID) and interferon gamma knock-out mouse models may be used to predict the safety of these vaccine candidates.

The new TB vaccines may also use new immunisation strategies, such as the ‘prime-boost strategy’ in which a live mycobacterial vaccine is administered as ‘prime’ followed by another novel subunit TB vaccine for boosting. This new strategy has been shown to be more protective in animal models [13-15], and also induce higher immune responses in vaccinated

individuals in recent clinical trials [16, 17]. Tuberculin skin test (TST), also known as PPD conversion, serves as a marker for cell-mediated immune responses to mycobacteria but is no longer considered as a useful clinical indicator of the effectiveness of vaccination for TB or protective immunity. New assays for correlates of protection are urgently required for the rapidly progressing new TB vaccines development. Such assays including biomarker panels and mycobacterial growth inhibition bioassay are under investigation [18]. In addition, recommendations are required for monitoring clinical safety and efficacy of new live TB vaccines. As the new TB vaccines are likely to be distributed to many developing countries, the WHO recommendations will set stringent standards for measuring the quality of these products during the pre-qualification process.

3. Genetic characterization of BCG sub-strains used for vaccine production

Since BCG was first used in the 1920s many different sub-strains have emerged. The various sub-strains used as vaccines are divided into two broad groups known as 'early' and 'late' strains [19]. The entire genome sequences of BCG Pasteur 1173 P2 and Tokyo 172-1 have been published and compared [19, 20] as 'late' and 'early' strains of BCG respectively. The BCG Pasteur 1173 P2 contains two separate genetic populations with double and triple tandem duplications in the DU2 region (a protein encoding region with 58 genes) [21]. The BCG Tokyo 172-1 contains two separate genetic populations which differ in the protein encoding RD16 region, one variant having the full RD16 region the other with a 22 bp deletion in this region. The variants also have phenotypic differences such as colony morphologies and growth characteristics [22, 23]. BCG Danish 1331 also contains two separate genetic populations, which differ in the SenX3-RegX3 region, one variant having two repeats of a 77 bp mycobacterial interspersed repetitive unit while the other has three copies of this unit [24, 25]. In fact, both Danish 1331 and Tokyo 172-1 sub-strains have been

subjected to twenty repeated passages on culture medium and did not show significant differences in mRNA expression patterns when compared with the first culture (personal communications from Dr K. Haslov and Dr S. Yamamoto respectively). There are also many other specific insertions and deletions in the genomes of the different BCG sub-strains [19, 26, 27].

The BCG vaccine was originally used worldwide by oral administration which was gradually replaced by intradermal or percutaneous route in most countries, while the BCG Moreau RDJ had been continually used as an oral vaccine in the National Programme in Brazil until 1974 and remained commercially available until 2005 [28]. Oral BCG was used in recent clinical studies and it was found that re-vaccination orally induced circulating cell mediated immune responses but does not induce a positive TST in responsive individuals. It is also able to induce modulation in humoral immunological responses (switch from IgG to IgA) [29]. A 975 bp deletion that eliminates the distal end of *fad*D26 and the start of *ppsA* genes were identified in the BCG Moreau sub-strain [27]. These genes are part of the biosynthetic locus of phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs) which are cell wall lipids known to be important for the virulence of *M. tuberculosis* and *M. bovis* [30-32]. Another BCG sub-strain that had been used in Central and South America until 1997 was BCG Mexico which was derived from two different seed lots, assigned as 1931 and 1988. The genetic profile of BCG Mexico seed lot used in 1931 resembled BCG Phipps but the genetic characteristics of the seed lot used in 1988 are similar to BCG Danish 1331 which was introduced in Mexico in 1970s (personal communication from Prof. Y. López Vidal).

All these studies provide a better understanding of the genetic differences and characteristics of various BCG sub-strains, and they may also be useful for identification and monitoring genetic stability during vaccine production. Recent report of different BCG sub-strains at proteome level contributes to phenotype characterisation of these products [33].

There is no conclusive evidence available to show which BCG sub-strain offers the best protection from TB disease in human populations. A recent study demonstrated that in a guinea pig model of pulmonary TB, no substantial differences in efficacy were observed between the early (BCG Tokyo 172-1) and late strains (BCG Danish 1331, BCG Pasteur 1173 P2, BCG Connaught and BCG Tice) and they all gave comparable protection from *M. tuberculosis* aerosol challenge, except BCG Glaxo (late strain) which had relatively poor protection [34]. The cytokine production (IL-1 β, IL-6, IL-8, IL-12 and TNF-α) stimulated by various BCG sub-strains using the human myelomonocytic cell line, THP-1 was studied and stronger induction of inflammatory cytokines was observed from 'early' strains of BCG, such as the Russian, Moreau and Tokyo than the 'late' strains which lack the methoxymycolate residue [35]. A direct comparison of effectiveness of different BCG sub-strains was also studied using a mouse intra-tracheal challenge model indicating both BCG Birkhaug and Phipps are the relatively more efficient sub-strains when live bacilli load determinations in lung is used as principal parameter to define the effectiveness [36].

4. Quality control and safety assessment of BCG vaccines

The current quality control test methods for BCG vaccine are limited as they were developed many years ago. The clinical response to BCG vaccination involves the local skin reactogenicity and tuberculin skin test conversion which may not necessarily be a predictor of protective immunity. Relevant and effective safety testing of live attenuated vaccine products is of fundamental importance. With BCG, the guinea pig model has been used for testing excessive dermal reactivity and detection of virulent mycobacteria in licensed BCG vaccines. As the more comprehensive genetic information about mycobacteria becomes available, molecular biology techniques can be used to detect the presence of virulent mycobacteria in a vaccine preparation. However, the sensitivity and specificity of this new technique should be

thoroughly assessed before implementation as an alternative assay to the guinea pig testing currently required. Also the necessity of testing for absence of virulent mycobacteria in final lots of BCG vaccine may be considered redundant as operation within the modern Good Manufacturing Practice (GMP) environment in vaccine production, contamination with virulent mycobacteria may be considered practically impossible. However the GMP standards may vary considerably in different countries, thus the requirement for a relevant safety test should remain. Antibiotic sensitivity requirements for BCG vaccine such as the type of antibiotics, assays used and the acceptable level of sensitivity are not well defined in the current requirements.

The cultural viable count method which estimates colony forming units (CFU) per container has been considered as the 'gold' standard for testing viability of BCG vaccine and as a surrogate marker for potency for this live product. A rapid alternative method, the ATP assay was modified and developed by Staten Serum Institut (SSI) [37] and has been evaluated by collaborative study for its suitability for viability testing of BCG vaccine, including for use in the temperature stability test [38]. This rapid assay was also included in the evaluation of WHO Reference Reagents for BCG vaccines [39], with the results of these studies being reviewed in this meeting. It was agreed that there are remaining issues on calculation and reporting of results of the ATP assay and if CFU equivalents or content of ATP per container still need to be defined.

The current required identity test for BCG vaccine is acid fast staining together with a characteristic appearance of colonies grown on solid medium. The monograph for BCG vaccine, freeze-dried in European Pharmacopoeia (PhEur) has stated that molecular biological techniques may be used as alternatives for identification [12]. A multiplex PCR (mPCR) assay, which was developed and used routinely in National Institute for Biological Standards and Control (NIBSC), uses six different target regions (five regions of deletion in

BCG and the SenX3-RegX3 mycobacterial two component system) to distinguish different BCG sub-strains and also differentiate BCG from virulent mycobacterial strains [24]. This method has recently been evaluated in an international collaborative study to assess its accuracy, robustness and reproducibility for use as an identity test for BCG vaccine [25]. The results from this study was discussed in this meeting and demonstrated that the mPCR assay is highly specific and able to identify and differentiate among BCG sub-strains and to distinguish between both these strains and *M. tuberculosis*. The assay has demonstrated itself to be robust with consistent and reproducible results across a number of laboratories.

4.1. The establishment of WHO Reference Reagents for BCG vaccines

The First International Reference Preparation (IRP) for BCG vaccine was established in 1965 and its replacement was recommended in earlier WHO consultation meetings [1-3]. The First WHO Reference Reagents for BCG vaccines of three different sub-strains (Danish 1331, Tokyo 172-1 and Russian BCG-I) have been developed. Two of them were adopted by the WHO ECBS in 2009, while the Russian BCG-I is a subject of further review. These preparations cover all sub-strains used for production of BCG vaccines currently pre-qualified by the WHO. They were evaluated in a collaborative study using two viability assays (cultural viable count and modified ATP assays) [39] and mPCR as identity assay [25]. The results were discussed in this meeting and the unitage of these Reference Reagents were assigned and agreed [39]. The intended uses of these WHO Reference Reagents for BCG vaccine are as comparators or references for validity and consistency monitoring in viability assays (such as cultural viable count and modified ATP assays); and for identity assay using molecular biology techniques. These purposes were evaluated in the collaborative studies [25, 39]. In addition, these Reference Reagents may also be considered as potential references for comparison purposes for *in vivo* assays (such as absence of virulent mycobacteria, dermal reactivity and protection assays) used in non-clinical studies for the

evaluation of new TB vaccines. These three Reference Reagents for BCG vaccines will be available and distributed by NIBSC-HPA.

5. Review of recent BCG vaccine clinical research studies

Some clinical data on BCG vaccine trials were reviewed in the consultation meeting. The efficacy of current BCG vaccines against pulmonary TB disease is highly variable and is approximately 50% on average worldwide, while the efficacy against meningeal and miliary TB in infants is relatively higher at about 75% in average [40]. An efficacy trial in infants in South African indicated that there is no significant difference in TB incidence rates between intradermal and percutaneous BCG administration following vaccination at birth and with two years follow up [41]. A recent efficacy study in Brazil demonstrated that revaccination of children aged 7-14 years does not provide substantial additional protection [42]. Recent studies continue to show that BCG vaccination can reduce risk of TB diseases in children [43, 44]. In addition, infants who received delayed BCG vaccination demonstrated higher frequencies of BCG-specific CD4 T cells, particularly polyfunctional T cells co-expressing IFN-gamma, TNF-alpha and IL-2. The response was most striking at one year of age [45]. On the other hand, infection with HIV severely impairs the BCG-specific T cell response during the first year of life. Thus BCG provides little, if any, vaccine-induced benefit in HIV-infected infants [46]. Considering the risk of disseminated BCG disease in HIV-infected children, these data strongly support not giving BCG to HIV-infected infants [47]. This highlights the need for the development of safer alternative TB vaccine approaches for HIV-infected individuals.

6. Discussion on the revision of the Requirement for dried BCG vaccine

The current requirements for freeze-dried BCG vaccine in WHO Technical Report Series (TRS) 745, 1987 [4] continue to provide a solid basis for evaluation of BCG vaccines and may be referred to when assessing new BCG vaccines for licensure and pre-qualification. It is important to consider the possibility of inclusion of new live TB vaccines within the scope of the document as this provides guidance for developers, manufacturers and NRAs. There are several issues related to the development of new live TB vaccines, such as persistence of attenuation and reversion to virulence, genetic and phenotypic stability, plasmid retention, presence of antibiotic resistance markers, and the requirement for dedicated manufacturing facilities. However, it is recognized that the new vaccines are of a diverse nature and at an early stage of development. So far no new vaccines are sufficiently developed to have a defined final formulation. Thus it is not possible to formulate the recommendations until clear clinical outcomes (such as evidence of safety and efficacy) with a defined formulation have been obtained. The scope of the recommendations does not cover rBCG and other mycobacteria strains modified by molecular biology techniques. Many principles of production and quality control assays for lyophilised BCG preparations used for the purpose of immunotherapy are the same as or similar to those for classic BCG vaccines, so the recommendations may find relevant application to such products. However, issues on clinical evaluation should be considered on a case by case basis.

During the consultation meeting several suggestions for amendment of the current requirements were made in order to re-affirm, clarify and provide further guidance on specific issues. These are summarised in Table 1. The revised recommendations will cover both BCG vaccines intended for intradermal and percutaneous administration. Although the intradermal route of administration is used for the majority of these vaccines, vaccines for percutaneous route are still currently in use, *e.g.*, in U.S. and the prequalified BCG vaccines of Tokyo sub-strain are used for both administration routes. In addition, this consultation

reaffirmed the inclusion of "Percutaneum" as a part of descriptive name of the BCG vaccines intended for percutaneous vaccination.

The diverse use of different BCG sub-strains should be reflected in the general considerations section. Also, description on the existence of different sub-strains of BCG used in vaccines is needed. However, there are insufficient data for recommendation on superiority of any particular BCG sub-strains. There are different levels of antimicrobial sensitivity among BCG sub-strains and it has been agreed as a part of the ongoing characterisation of BCG sub-strains that the antimicrobial status should be included on the product labels and/or packaging inserts.

The First WHO Reference Reagents for BCG vaccines of three different sub-strains (Danish 1331, Tokyo 172-1, and Russian BCG-I) will be included in the revised requirements with the exact terminology and the intended uses of these preparations. For the purpose of consistency with other WHO recommendations, the use of 'master' and 'working' seed lots to replace 'primary and 'secondary' ones respectively has been agreed. The definition of vaccine lot was deleted as the concept is no longer used.

For manufacturing recommendations, all current BCG vaccine products are manufactured in dedicated facilities and this recommendation will remain unchanged. However, views from selected new TB vaccine developers were discussed in the meeting indicated that production facilities for new live TB vaccines in a non-dedicated area should be permitted and be subjected to the approval of NRAs giving due regard to improvements in manufacture control as a consequence of current GMPs. This practice has been accepted in some countries, such as USA and India, but it still requires further discussion. It was agreed that the total number of passages from master seed to final lot should not exceed 12. The use of material originating from animals in the production process should be strongly

discouraged, and consideration of current TSE policy remains subject to NRA approval in case constituents derived from animal origin are necessary.

The classical TST is no longer used as a relevant marker of vaccine immunogenicity, but only as a diagnostic tool for TB disease. Therefore, the vaccine produced from a master seed strain should show in field trials in man clinical efficacy and safety rather than induction of adequate sensitivity to tuberculin. Methodological considerations for testing of seed lots were also discussed. It was agreed that guinea pigs injected with a quantity of vaccine equivalent to 50 single human doses should be observed for at least 6 weeks. The vaccine is free from virulent mycobacteria if none of the guinea pigs shows signs of TB and if there is greater than 90% survival of animals during this period. The test for skin reactions in guinea pigs is considered to provide good indication for the absence of excessive dead BCG in the final lot and also to monitor batch-to-batch consistency. However, the value of this test is under question and its removal as a requirement has been proposed. Further discussion whether to keep the test for absence of virulent mycobacteria at the final bulk stage and the test for skin reactions in guinea pigs on final lot will be required. It was recognised that antimicrobial sensitivity test at the final bulk stage may not be needed on a routine basis.

The mPCR assay is proposed to replace the acid fast staining method which provides no specificity in identification apart from confirming the presence of mycobacteria. The necessity of implementation of this improved identity method for BCG vaccine production has been questioned by vaccine manufacturers as only one vaccine strain is typically produced in each vaccine manufacturing site and also in some countries only one BCG vaccine strain is being used. It is obvious that no identity assay for biological products will be accepted in new applications by NRAs and NCLs if the assay does not give specific identity information of a product. The PCR technique is widely used by new vaccine developers as

necessary identity assay for live bacterial products. This technique also enables NCLs to check that the vaccine strain has not changed.

No additional collaborative studies were required for ATP assay used as alternative viability assay for BCG vaccines. Results from this assay should be expressed as CFU equivalents for the purpose of manufacturing consistency, and other assay methods should also be considered as alternative tests for viability. The inclusion of the ATP method, which was used in WHO collaborating studies, was requested as an appendix. The value of the thermal stability test using viability assay is uncertain as data from one BCG vaccine manufacturer showed that this does not correlate with long-term stability of the product. Although there was a consensus on thermal stability testing as a good indicator for batch-to-batch consistency, it was suggested that this test should be omitted from the final lot and subjected to approval from NRAs if production consistency is demonstrated.

In relation to the new section on clinical evaluation, recommendations should focus on i) the issues on prime-boost strategy and bridging studies; ii) comparing efficacy or safety between vaccines with intradermal and percutaneous route of administration; iii) the clinical trial results using different BCG sub-strains; iv) application of general principles of clinical evaluation until product specific recommendations are required; v) post-marketing surveillance including the HIV positive population.

7. Conclusion

The revised WHO Requirements for dried BCG vaccine is designated as 'Recommendations to assure the quality, safety and efficacy of BCG vaccines'. It will cover both BCG vaccines intended for intradermal and percutaneous administration. Although most requirements are in common for BCG for immunotherapy, some issues related to usage of the products will require specific considerations. Also the revised document will not cover

mycobacteria strains modified by molecular biology techniques. The discussion of this consultation meeting was focused in the revision of the current requirements focused on the scope, terminology, manufacturing issues, and the incorporation of new reference reagents and new quality control tests for the classical BCG vaccines. It was agreed that there was no need to include recommendations for clinical evaluation given that there are no new classical BCG vaccine in development. New live TB vaccines are in development and will build on the experience of BCG vaccine production, control and clinical evaluation. The new recommendations may form the basic guidance for new vaccine developers, however, it was concluded that it is premature to formulate recommendations for these vaccines. However, progress in this area will be monitored and WHO working group will re-visit the issue in coming years. The consultation process will continue through 2010 and the final document will be submitted to the ECBS for endorsement.

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Appendix I. List of participants

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Key: *speakers with oral presentations in the consultation meeting

Table 1

The summary of major proposed changes in the revision of the Requirement for dried BCG vaccine.

Targets for revision	**Proposed changes**
Title of the document – Requirement for dried BCG vaccine	Recommendations to assure the quality, safety and efficacy of BCG vaccines
In general consideration section	The diverse use of different BCG sub-strains should be reflected.
The 1st International Reference Preparation for BCG vaccine	This has been replaced by sub-strain specific WHO Reference Reagents for BCG vaccines.
BCG seed lot should show induction of adequate sensitivity to tuberculin.	Master seed strain should show in field trials in man clinical efficacy and safety.
Test for absence of virulent mycobacteria in seed lot – inject one vaccine lot in guinea pigs with at least 6 months observation and 60% survival	Inject with a quantity of vaccine equivalent to 50 single human doses in guinea pigs with at least 6 weeks observation and 90% survival.
Test for skin reactions in guinea pigs	It is proposed to be deleted for the final lot products.
Identity test – final lot of vaccine shall be verified by the morphological appearance of the bacilli in stained smears	Preferably sub-strain specific nucleic acid amplification techniques (such as PCR) should be used.
Rapid test for viability	As an alternative to the colony counting method, a bioluminescense or other biochemical method can be used.
Thermal stability test	This test should be omitted from the final lot and subjected to approval from NRAs if production consistency is demonstrated.
Information on product labels and /or packaging inserts	The antimicrobial status should be included.
New addition in the control of source materials	The use of constituents of animal origin in production should be discouraged or subjected to NRA approval.
Oxygen uptake test in final bulk	This section should be deleted.

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