Report

WHO discussion on the improvement of the quality control of BCG vaccines

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WORLD HEALTH ORGANIZATION
Quality Assurance and Safety of Biologicals
Introduction

The evolution of BCG strains and the diversity of strains used for production of currently licensed vaccines as well as a need to explore their potential impact on efficacy and safety of BCG vaccines in humans have been considered at previous WHO meetings, held over the last few years (1,2). As recommended by a WHO working group in December 2004, a meeting to review assays for 1) genetic characterization of BCG strains and 2) quality control of BCG vaccines, established at the Institute Pasteur, was convened. The meeting was opened by Dr I Knezevic, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, who summarized the background of the meeting and its objectives in the context of the revision of WHO requirements for production and control of BCG vaccines. The meeting was hosted by Dr Gilles Marchal, Reference Laboratory for Mycobacteria of the Institute Pasteur. The list of participants is included in the annex of this report.

Background

Due to the fact that the use of BCG vaccines will continue, either as stand alone vaccine or as a prime vaccine in a prime-boost immunization strategy in conjunction with new vaccines against tuberculosis, the necessity for review of the quality control of BCG vaccines has been identified as a WHO priority. Given that novel vaccines against tuberculosis are being developed, comparability studies in terms of quality, nonclinical and clinical testing with currently licensed BCG vaccines are foreseen as part of their evaluation. There is also a need to evaluate BCG vaccine in the context of a prime/boost strategy.

A number of unresolved issues in the quality assessment of BCG vaccines have been identified. These include immunological mechanisms of efficacy and protection induced by different BCG vaccines; genetic differences of sub-strains and their implications to phenotypic characteristics, immunogenicity and efficacy in animal models and in humans; and the impact of vaccine characteristics on the safety profile of different products. In terms of quality control, a key issue is lack of correlates of protection and therefore the absence of a tool to distinguish ‘protective’ from ‘non-protective’ vaccines in the laboratory. In addition, recently reported findings on antimicrobial resistance of some strains raised an issue of their relevance in the quality assessment of BCG vaccines.

A key target is to reconcile new developments in methodology for assessing the quality of BCG vaccines with the procedures that had been in use for many years. Quality control issues that need to be addressed have already been discussed and reported (2). A better understanding of vaccine characteristics relevant to production and control as well as to the safety and efficacy in humans should lead to improved control tests. For this purpose, a WHO repository of BCG strains had now been established at the WHO International Laboratory for Biological Standards at National Institute for Biological Standards and Control, UK. The repository is intended to be used, in the first instance, for
defining differences between sub-strains and for the investigation of new methods for application to production and control of vaccines.

A revision of current WHO recommendations for production and quality control should result in improved methods for identity testing and determination of viable counts. New reference preparation(s) have been identified as essential tools in these improvements. In addition, a consideration should be given to the scientific basis for the establishment of a potency assay. Ideally, potency test may be based on established correlates between in vitro properties and in vivo markers such as γ- interferon, CD4/CD8 responses. It would be beneficial for the assessment of already licensed vaccines and essential for the development of new BCG vaccines. More guidance should be given with respect to the monitoring consistency of production and clinical testing of new BCG vaccines. The latter is intended to provide a basis for better assessment of efficacy and safety of BCG vaccines in pre-licensing clinical trials as well as in post-marketing surveillance.

The main objectives of the current discussions were to:

1. Review methods for genetic characterization of BCG strains, established at the Institute Pasteur, and explore their applicability for characterization of BCG strains in the WHO repository
2. Discuss potential improvements of the test for viable counts for BCG vaccines
3. Develop a proposal for the replacement of current international reference preparation through a collaborative study
4. Consider test methods for determining antimicrobial sensitivity of BCG sub-strains
5. Develop an action plan for global consultation on recommendations for production, control and evaluation of BCG vaccines in 2006.

Summary of the discussion

The discussion was structured in following sessions:

1. Genetic characterization
2. Evaluation of rapid assays for monitoring viable counts
3. International reference material - potential use and design of a study on suitability of the material
4. Antibiotic susceptibility of BCG strains
5. Conclusions and action plan

1. Genetic characterization of BCG vaccines: methods developed at the Institute Pasteur
1.1. Differentiation of strains by variable number tandem repeat sequences (VNTRs)

Dr C Gutierrez (Pasteur Institute, Paris) summarized the genetic characterization of *M. bovis* BCG vaccines and a proposal for testing BCG sub-strains. The *M. bovis* genome contains repeat sequences that can vary in the number of repeats at a single position and which can be differentiated by the size of the PCR products. These variable number tandem repeat sequences (VNTRs) can be used for strain differentiation. About 40 mycobacterial interspersed repetitive units (MIRUs) occur in the *M. tuberculosis* genome at positions where tandem repeats occur but in only about 12 positions does the number of repeats vary. Tandem repeats at the *senX3-regX3* locus (MIRU locus 4) of BCG can contain 1, 2 or 3 repeats and this is the only locus in which the number of repeats can vary between sub-strains. There is evidence that sub-strains vary in VNTR number at this position e.g., Danish BCG contains 2 repeats, Glaxo BCG 2 repeats, Merieux BCG 3 repeats and Prague BCG 1 repeat, even though of the same lineage. Other loci have proved remarkably stable.

The *M. tuberculosis* genome also contains regions of difference (RDs), many of which are absent from *M. bovis*, with further deletions from BCG. Within the BCG group, RD patterns can vary between sub-strains. Thus RD2, RD14, RD15 and RD16 distribution differentiates sub-strains as does the copy number of IS6110. Tandem duplications could also emerge during sub-culture even within a sub-strain. It was suggested that a genetic typing pattern for BCG sub-strains could be based on VNTR number at MIRU 4, RD pattern, IS6110 copy number and location/number of tandem duplications. This scheme had been used successfully to characterize BCG strains recovered from a patient with complications of vaccination. It could be used for monitoring consistency of production of BCG vaccines, if applied at appropriate time intervals.

1.2. Testing tandem duplications of specific genes

Dr R. Brosch (Pasteur Institute, Paris) discussed the evolution and genetic characterization of BCG strains. Regions of difference are defined relative to the *M. tuberculosis* genome. The study conducted at Pasteur Institute included deletion analysis of 100 strains from various hosts and countries. The study provided a basis for differentiation in terms of deletion regions (RD) between the strains within the same lineage. Restoration of RD1 partially restores virulence to BCG and improves protective efficacy. BCG:RD1 gives better protection against splenic but not pulmonary infection in guinea-pigs. A perfect correlation between deletion and point mutations were found in the analysis of the results. In conclusion, the use of deletion analysis in conjunction with molecular typing and analysis of specific mutations was shown to represent a very powerful approach for the study of the evolution of the tubercle bacilli (4). BCG strains also could develop tandem duplications of specific genes. The effects of these on biological properties were not clear but they could be used as markers to monitor the evolution of sub-strains. The DU1 duplication was present in the BCG Pasteur 1133 sub-strain but not in other sub-strains. DU2 varies between sub-strains. A
duplicate/triplicate was detected in Danish 1311 but its presence was also influenced by passage. Dr Gheorghiu emphasized a need for testing a hypothesis generated on the basis of her experience with the Pasteur strain that the number of passages from the working seed to the final product should not exceed 3 passages. Currently available methods for testing genetic variability during the production process should be employed to provide scientific basis for a future requirement on passage level for production.

2. Evaluation of rapid assays for monitoring viable counts

Dr K. Haslov (SSI, Denmark) described the development of an improved ATP luminescence assay for estimating the viable count of BCG vaccine. This was designed to measure intracellular ATP of live BCG bacteria. Its key features were that the vaccine was re-constituted in a growth medium (Dubos) and incubated at 37°C for 24 h to have the BCG in an active growth phase before extraction and measurement of intracellular ATP. The intracellular ATP was then extracted by heat treatment in buffer; this treatment also served to denature ATP degrading enzymes. The ATP of non-viable cells is rapidly degraded on cell death. The ATP extracted was then assayed by a bioluminescence reaction and light emission measured in a luminometer; the amount of ATP extracted was calculated from an ATP standard curve. For samples of lyophilised BCG, the correlation between ATP content and CFU was good and showed a linear relationship between log ATP content and log CFU. The method was undergoing validation and would be submitted to the Danish regulatory authority for approval. The aspects that needed to be assessed included robustness, precision, accuracy, transferability and linear and non-linear aspects of the response curve. The issue of validity criteria and internal reference was discussed. Dr Gheorghiu suggested to explore possibility of reconstituted BCG vaccine to multiply in Middlebrook medium during the incubation period. This could be considered as one of the criteria of the assay validity.

3. International reference material - potential use and design of a study on suitability of the material

Dr M Corbel (NIBSC, UK) discussed the proposed evaluation of the replacement WHO Reference Preparation for BCG vaccine. The First International Reference Preparation for BCG Vaccine was established in 1965 and held initially by International Standards Laboratory (ISL), SSI, Copenhagen but was transferred to NIBSC when SSI ceased ISL activity. WHO re-visited the 1st IRP in 1979 and concluded that it was ‘no longer representative of strains used for production of currently licensed vaccines. SSI was requested to develop a 2nd IRP. SSI reported in 1982 that four candidate vaccines had been obtained and in vitro/in vivo properties had been assessed by six laboratories. Three of the candidates were considered similar in properties and one was to be selected as candidate 2nd IRP. This did not progress. Studies over the years have indicated a steady decline in the viable count of the 1st IRP and this reinforces the case for replacement. However, the application and mode of use of the replacement needs to be clarified. Potential applications included calibration of methods for viable counts, as a reference for control of diluent, medium, cultural conditions, as an internal reference for checking test to test variation in viable counts, as a reference for residual virulence/local reactogenicity
assays, as a reference for protection assays, as a calibrant and for inter-laboratory control/validation of new test methods. It was considered that the main application would be in relation to controlling variation in viable counts. The current colony counting methods were inherently problematic because of the nature of the organism and inter-laboratory variation was wide causing problems for manufacturers and official control laboratories. Other methods had been developed based on ATP content, tetrazolium reduction, esterase activity, vital staining (Chemscan RDI), propidium iodide staining but none had been fully validated and all seemed to show a difference between ‘vital activity’ and culturability. A Reference Preparation would be essential to resolve these issues. A candidate preparation had been offered by SSI but it was not clear if this would be suitable as a reference for vaccines based on other sub-strains. Following discussion, it was decided that candidate references based on the Danish, Japan and Moscow strains should be obtained and evaluated in parallel in a collaborative study. Both colony counts and alternative methods would be used.

4. Antibiotic susceptibility of BCG strains

Dr G Marchal (Pasteur Institute, Paris) described the methods used to assess antimicrobial sensitivity in mycobacteria. The emphasis had been on *M. tuberculosis* and methods had not been developed specifically with BCG in mind. However, both proportion methods and resistance ratio method were suitable for BCG strains if validated. Comparison of growth on Lowenstein Jensen medium at pH 7 and pH 5.1 in the presence or absence of serial dilutions of antimicrobials allowed determination of sensitivity. Colony counts were usually greatest at pH 5.1. For isoniazid, the sensitivity limit was 0.2 \( \mu g/ml \). Dr Haslov reported that the Danish strain was sensitive to 0.4\( \mu g/ml \) but resistant to 0.1\( \mu g/ml \). The Glaxo strain was less sensitive. Dr V. Vincent reported that the Connaught strain was resistant to 0.1\( \mu g/ml \), the Japan strain sensitive to 0.2\( \mu g/ml \). Dr Knezevic noted the need to investigate the antimicrobial sensitivity of BCG sub-strains more thoroughly aiming for defined levels of sensitivity for sub-strains currently used for production. It was agreed that the determination of antimicrobial sensitivity would be part of the ongoing characterization of BCG sub-strains. It would be appropriate to test this property at the level of both master and working seed for licensing purposes and to monitor this periodically. However, there was insufficient scientific evidence to define a specification for this characteristic. The definition of ‘resistant’ and ‘sensitive’ as applied to vaccine strains was discussed. It was proposed that for BCG strains a sensitivity of specified level determined by defined methodology should be reported in future. A term "resistant" reflect resistance of the Mycobacterium tuberculosis to the treatment and is not appropriate for defining biological characteristic of the vaccine strains. Moreover, the clinical relevance of the antimicrobial sensitivity of BCG strains was unclear. Cases of severe complications were rare and controlled trials of therapeutic regimens had not been reported. The WHO Global Advisory Committee on Vaccine Safety considered the implications of the recent isolation of BCG, Danish 1331 strain, "resistant" to isoniazid from five patients with lymphadenitis in the Netherlands. The committee concluded that this report does not justify a change in standard policy for the use of BCG vaccines (6). Dr M. Gheorghiu considered that there was a need for WHO to
develop more extensive recommendations on the treatment of complications of vaccination.

5. Conclusions and action plan

1. Genetic characterization

The group appreciated work done at the Pasteur Institute and provided some suggestions for further testing. Although the established methods for genetic testing of tubercle bacilli are promising tools in better understanding of the evolution of Mycobacterium tuberculosis complex, the research has to be streamlined to provide the information relevant for production and control of BCG vaccines. For this purpose, the group requested Dr Brosch and Dr Gutierrez to develop a proposal for testing with the details of the methods, samples and the time frame, by the end of 2005. It is essential to perform testing on the vaccine strains currently used for production of BCG vaccines collected at the WHO repository. As soon as proposal for testing is submitted to WHO, Dr Knezevic would coordinate realization of this project.

2. Evaluation of rapid assays for monitoring viable counts

Review of the results of the study conducted at the Staten Serum Institute provided a solid basis for further considerations of the ATP assays as one of rapid methods for determination of the number of culturable particles. However, it was concluded that the repeatability of the improved assay should be assessed initially in 3-4 additional laboratories and results reported back to the working group. The validation of the assay at the Staten Serum Institute would be completed by the end of 2005. The group agreed that Drs Haslov and Corbel would prepare a protocol for the first phase of the collaborative study to assess the method in a small number of independent laboratories. The protocol would be submitted to WHO by 31 October 2005. Dr Knezevic would arrange provision of samples for this purpose and all other necessary steps towards the completion of the study.

3. International reference material - a study on suitability of the material

It was clearly stated that the replacement of the 1st International Reference Preparation is needed. However, the appropriate use of this material in the context of potential improvement of the test for determination of the number of culturable particles is to be resolved after completion of the study. Dr Corbel accepted to develop an outline of the collaborative study for testing suitability of the different candidates (Tokyo 172-1, Russian BCG - I and Danish 1331 strains) for an international standard. The study would be supported by WHO.

4. Antibiotic susceptibility of BCG strains

Data generated at the Pasteur Institute clearly indicated that the method for testing antibacterial sensitivity of BCG vaccine strains could be used for an independent testing
of the strains collected at the WHO repository. A proposal for testing would be submitted by Dr Marchal, by end 2005. Dr Knezevic would collect results obtained by the manufacturers with the details of the methodology used. This would be provided to Dr Marchal to help designing the testing accordingly. Provision of the results would be a subject of the agreement by the manufacturers of the vaccines in question.

5. WHO consultation

The group suggested that WHO consultation be held when results were available.

References


Annex

List of participants

Dr M J Corbel (NIBSC, UK); Dr M Gheorghiu (independent advisor, Paris); Dr C Gutierrez, (Pasteur Institute, Paris); Dr R Brosch (Pasteur Institute, Paris), Dr K Haslov (Staten Serum Institute, Denmark); Dr M Lagranderie (Pasteur Institute, Paris); Dr G Marchal (Pasteur Institute, Paris); Dr M Roumiantzeff (independent advisor, Lyon); Dr V Vincent (Pasteur Institute, Paris), Dr U Fruth (WHO, Geneva); Dr I Knezevic (WHO, Geneva).

Knezevic Ivana 12/2/2005