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Bioluminescence Methods for the Rapid Assay of the Specific Activity of Lyophilized BCG Vaccine

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Abstract

The specific activity of a lyophilized BCG vaccine (CFU/mg biomass) is one of the most important characteristics of this product. The classical microbiological method of the BCG vaccine activity assay is very time-consuming and gives results only in 4-5 weeks. The bioluminescent determination of specific activity based on the use of the firefly luciferase-luciferin system makes it possible to greatly shorten the duration of the assay. Since the 1970's, the ATP method has been successively applied for studying the specific activity of different BCG strains. In 2008, the bioluminescent ATP method was proposed and evaluated for analyzing lyophilized BCG vaccine preparations. The duration of analysis decreased from several weeks to 2 days. This method was used in the International collaborative study to evaluate and establish WHO Reference Reagents for BCG vaccine. In 2016, a new protocol was published describing the use of the ATP method to assay the specific activity of the lyophilized BCG vaccines. The duration of the assay decreased to 2 h. A good correlation between the CFU value and the intracellular ATP content was shown.

Keywords: ATP assay; BCG viability; Colony forming units; Lyophilized BCG vaccine; Tuberculosis

Introduction

The firefly luciferin-luciferase system is widely applied in so called "rapid microbiology" because it can be used to determine an intracellular ATP concentration in several minutes. This concentration is proportional to the content of viable cells in the sample [1-4]. When specific activity needs to be determined for slowly growing cells, the ATP method can conveniently replace plate counting. The lyophilized preparations of attenuated strains of viable bacteria *Mycobacterium bovis* (BCG vaccine) are largely used for vaccination against tuberculosis [5]. Specific activity (CFU/mg) is one of the most important characteristics of these medicinal preparations. The microbiological determination of the specific activity of BCG vaccines is a very time-consuming procedure that is hard to reproduce because of the ability of mycobacteria to aggregate [6].

An alternative method for assaying the specific activity of BCG vaccines is the bioluminescent method, which is based on the quantitative determination of intracellular adenosine-5'-triphosphate (ATP) with the use of the firefly luciferase [7-9]. This enzyme catalyzes the oxidation of D-luciferin by oxygen in the presence of ATP. The reaction is accompanied by the emission of visible light. The intensity of the emitted light is proportional to the ATP content in the analyzed sample [1-4]. Since the registration of bioluminescence takes just several seconds, the assay time depends only on the duration of the sample pretreatment stage. Thus, the use of the ATP method enables the implementation of a rapid assay for the specific activity of BCG vaccines.

Rapid bioluminescent estimation of cell viability in the BCG vaccine

Since the 1970's, the bioluminescence method has been used to determine the activity of BCG cells [10-13]. A good correlation has been established between the intracellular ATP content and the number of viable cells in liquid and lyophilized preparations of BCG cells [14-17]. The bioluminescent method for the rapid assay of the specific activity of lyophilized BCG vaccines was described in details and validated in 2008 [8]. According to this method, the lyophilized BCG vaccine is reconstituted in a Dubo growth medium and incubated for 24 h at 37°C. This procedure is required to restore the metabolic activity of the cells and, consequently, to increase the concentration of intracellular ATP. The intracellular ATP is extracted by treating mycobacterial cells with a boiling EDTA-containing Tris-acetate [8,16,18,19] and then assayed by the bioluminescent method. A good correlation was demonstrated between the ATP content and the CFU values. The use of this method reduced the total duration of analysis from 30 days to 36 h. The developed method was tested in six laboratories of different countries and accepted by WHO as a reference method for the assay of specific activity of the lyophilized BCG vaccine [7].

The initially proposed protocol was later modified by Kolibab et al. [19]. The authors revised the step that involved a 24 h incubation of a reconstituted BCG vaccine in Dubo growth medium with the following procedure: instead, they reconstituted lyophilized BCG vaccines in Sauton's SSI media that contained apyrase and incubated them for 30 min at room temperature. The addition of apyrase enabled the authors to degrade extracellular ATP and enhance the sensitivity of the analysis.

A new modification of the protocol was proposed [20,21]. A special kit of lyophilized reagents was developed to make the analysis more simple and reproducible. The kit includes an ATP-reagent (a mixture of

a thermostable luciferase, luciferin, buffer components and stabilizers [22]), ATP-standard (an aqueous solution with a precisely known content of ATP was prepared and then lyophilized) and apyrase. All the reagents can be readily used after the addition of pure water. According to Ugarova et al. [20], the assay of intracellular ATP in BCG vaccine includes the following steps:

1. Reconstitution of lyophilized BCG vaccine: A 1 ml aliquot of saline is added to an ampule with a lyophilized BCG vaccine. The ampule is incubated for 15 min at room temperature and then placed in a temperature controlled shaker and stirred for 60 min (250 rpm, 37°C). The ampule is then cooled in air to room temperature for 3-5 min. This stage is most important for obtaining correct and reproducible results [23].

2. Degradation of extracellular ATP: In a test tube, 100 µl of a vaccine suspension is gently mixed with 20 µl of apyrase solution and incubated at room temperature for 10 min without stirring.

3. Extraction of intracellular ATP: Then, the vaccine suspension is supplied with 1 ml dimethyl sulfoxide (DMSO), mixed, and incubated (1 min, room temperature). The use of DMSO reduces the duration and labor intensity of the ATP extraction and improves the reproducibility of the method because this highly efficient organic reagent is able to extract intracellular ATP from the BCG cells at room temperature [3,4,24].

4. ATP assay: 100 µl of the ATP-reagent is added to 20 µl of the ATP extract in DMSO, after which the bioluminescence signal is recorded (Ivial, RLU). A high operational stability of the ATP-reagent [25] provides good reproducibility of the measurements.

5. Determination of the ATP content in BCG samples and the use of the ATP-standard: A lyophilized ATP-standard is reconstituted with saline and then treated with DMSO under the same conditions as the BCG vaccine. The bioluminescence signal is measured (Istandard, RLU) as described above for the vaccine. The ATP content in the lyophilized BCG vaccine is calculated by the formula

(ATP), pmol/mg=(ATP)standard · Ivial/Istandard, pmol/mg

Where (ATP)standard is the content of the ATP-control in the initial saline solution, pmol.

The linear relationship between the bioluminescence signal (I) and the concentration of ATP eliminate the need to obtain a calibration curve.

6. The specific activity (10^6 CFU/mg) of lyophilized BCG sample is calculated from the measured ATP content in BCG samples using the formula:

10^6 CFU/mg=K · ATP, pmol/mg

K is the coefficient of proportionality between intracellular ATP content (ATP, pmol/mg) and the specific activity (10^6 CFU/mg) measured by plate counting. To determine the *K* coefficient, several dozen samples of different batches of the studied BCG vaccine were assayed in parallel by microbiological and bioluminescent methods. It was found that the coefficient K equals to 0.36 ± 0.02. K is the slope of the linear correlation between the intracellular ATP content and the CFU. This simplified the calculation and increased its accuracy.

Conclusion

The proposed ATP method for the determination of the specific activity of lyophilized BCG vaccine involves a rapid rehydration and reactivation of the lyophilized BCG cells and modified conditions for their lysis and the bioluminescence measurement. The designed reagent kit does not require a time-consuming preparation before the assay. These modifications reduced the duration of the analysis from 36 h [8] to 2 h [20] and significantly simplified the individual stages of the analysis. The measured intracellular ATP content (ATP, pmol/mg) was directly proportional to the specific activity (10^6 CFU/mg) (with the proportionality coefficient K=0.36 ± 0.02) in the concentration range of the manufactured BCG vaccine preparations. The method is reproducible and stable, and it is characterized by an ease of processing measurement results, which is especially important when the method is implemented in the production environment.

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