

PRODUCTION OF BCG VACCINE IN A LIQUID MEDIUM
CONTAINING TWEEN 80 AND A SOLUBLE
FRACTION OF HEATED HUMAN SERUM

I. PRODUCTION AND VIABILITY OF THE CULTURE

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Adequate biological or immunological criteria are still lacking for the standardization of the vaccines made from living attenuated bovine tubercle bacilli (BCG). It is probable that, eventually, standardization will be based on quantitative measurements of the humoral or cellular response of the host to the specific bacterial antigen which induces protective immunity against tuberculous infection. As long as the mechanism of antituberculous immunity remains unknown, however, it may be necessary to standardize the vaccine in terms of less specific criteria: the level of attenuation of the BCG culture, the amount of bacterial material present in the vaccine preparation, the viability and physiological activity of the cells which it contains, etc. All these different characteristics are probably of importance in determining the extent to which BCG is capable of multiplying *in vivo*, and its effectiveness in establishing tuberculin allergy and resistance against infection. In the hope of minimizing both qualitative and quantitative variations in the activity of the vaccine, most BCG workers have advocated a rigid adherence to the preparative techniques worked out by Calmette and his associates (1, 2). It is almost certain, however, that, despite all precautions taken in the preparation of the culture and in the distribution of the vaccine, the batches of final product differ greatly in biological activity.

According to the classical technique, the BCG culture must be grown on the surface of some synthetic medium (Sauton for example) where it forms a pellicle of varying but not inconsiderable thickness (1, 3). This mode of growth results in marked heterogeneity of physiological environment since the ease of gaseous exchanges and of access to nutrients are naturally influenced by the position of the bacterial cells with reference to the atmosphere and to the fluid medium. Removal of the bacterial pellicle from the nutrient solution and the preparation by grinding of a fine bacterial suspension are procedures that

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introduce further elements of heterogeneity and that bring about the death of many of the microorganisms. Loss of viability probably occurs as a result of the physical trauma caused by grinding and of the physiological disturbances following upon the sudden changes of environment to which the bacterial cells are exposed. It is well known, furthermore, that the BCG cells which have survived the operations inherent in the preparation of the vaccine soon begin to die in the fluid used for distribution of the final product. This fact has been acknowledged in the recommendation that the vaccine be used within a very few days following its preparation. Some figures may be in order to give concrete meaning to these general observations. The standard BCG vaccine, as distributed, contains approximately 10^9 bacterial cells per cc. (as determined by direct microscopic count); quantitative determinations carried out in our laboratory indicate that, within 48 hours after it has been issued, the vaccine contains at the most only 10^4 to 10^5 viable units per cc. and that its viability continues to decrease rapidly during the following few days. Thus, the vaccine contains at best only a small fraction of 1 per cent of viable cells at the time of its use.¹ Under these conditions, it is entirely meaningless and indeed misleading to prescribe the dose of vaccine in milligrams or cubic centimeters, as these weight and volume units give no idea whatever of the number of living organisms in the product.

In order to facilitate standardization of the final product, attempts are being made in several places to stabilize the vaccine by desiccation *in vacuo*. It is well to realize that no method of desiccation—however effective and dependable—can correct the elements of heterogeneity resulting from the procedures presently in use for the preparation of the culture and of the bacterial suspension. The purpose of this and the following paper is to show that one can obtain BCG cultures consisting of a very large percentage of living bacterial cells; and that these cultures retain for several weeks at least their viability and the power to induce in guinea pigs both tuberculin allergy and increased resistance to infection with virulent bacilli.

EXPERIMENTAL

Cultures.—The experiments on cultivation of BCG were carried out with four cultures available in the United States, and with one obtained from Copenhagen (through the courtesy of Dr. J. Holm) in the form of a sample of standard vaccine. As the five cultures exhibited essentially the same behavior in preliminary experiments, only one of them was studied more extensively. This culture (labelled ((Phipps)) in our collection) was received on May 21, 1947, from Dr. Joseph Aronson of the Henry Phipps Institute in Philadelphia.² It has since been

¹ It must be realized that the number of viable units does not represent the number of viable cells as the vaccine contains many microscopic clumps, each one of which gives rise to only one colony while consisting of many living organisms.

² We have been informed by Dr. Aronson that he had received this culture from the Pasteur Institute of Paris under the code number BCG Number 793, series 2 (February 21, 1946).

maintained in our laboratory in the Tween-bovine albumin medium described previously (4) by transferring, at 1 to 3 week intervals, one drop of culture to 5 cc. of the same liquid medium and incubating at 37°C. for 8 days. At the end of the incubation period, the culture is placed in the refrigerator (at 4–5°C.) until needed.

As far as can be judged, the BCG culture Phipps has retained unaltered the morphological and biological characteristics that it possessed when first received from Dr. Aronson. Thus, it exhibits on oleic acid-bovine albumin agar a distinct colonial morphology intermediate between the spreading "serpentine" type of the fully virulent strains and the unoriented, heaped appearance of the completely avirulent variants (5, 6). Its virulence also appears to have remained constant; 0.2 cc. of a 7 to 10 day old culture in Tween-bovine albumin medium, injected into the tail vein of mice, gives rise to discrete pulmonary lesions but fails to cause progressive disease and death (7).

In the final stages of the present study, the Phipps culture described above was compared in a few experiments with a new subculture of the same strain received in the form of a sample of standard BCG vaccine again from Dr. Aronson (in March, 1949). As far as could be judged from the limited number of observations made (type of growth in liquid and on agar media, response of guinea pigs to the intracutaneous injection of serial dilutions of cultures grown in liquid Tween-serum filtrate medium (8)), the two subcultures could not be differentiated either *in vitro* or *in vivo*.

Methods Used for Measuring the Viability of Cultures.—The numbers of viable cells (or clumps of cells) were determined by enumeration of colonies on oleic acid-bovine albumin agar, using techniques which have been described in detail elsewhere (4, 9). All culture dilutions were made in 0.1 per cent bovine albumin in distilled water. In a few cases the dilutions used for plating were inoculated also into 5 cc. of liquid Tween-bovine albumin medium (4) distributed in 5 cc. amounts in tubes 25 mm. in diameter. Readings of the extent of growth (colonial counts or evaluation of turbidity) were made at weekly intervals.

Production of Dispersed Growth of BCG in Liquid Medium.—As pointed out in earlier publications, it is possible to obtain submerged and diffuse growth of tubercle bacilli by adding to ordinary synthetic media certain wetting agents and the albumin fraction of serum. An attempt was therefore made to adapt this cultural technique to the production of BCG vaccine.

The basal medium was the same as that previously described (4). It did not contain either glucose or glycerine. Tween 80 was added in a final concentration of 0.02 per cent before autoclaving.

In the past, we have used almost exclusively as source of albumin the fraction V separated from bovine plasma by alcohol precipitation.³ Unfortunately, this material is not readily available to workers outside the United States. Moreover, as BCG cultures may be used for vaccination of human beings, it appears undesirable to add to the culture media in which they are grown any protein of animal origin that might act as antigen. We have attempted therefore to work out an inexpensive and convenient method for the separation from human serum of a fraction that would have the same growth-promoting properties as the fraction V separated from plasma by the Cohn method.

The technique which has given the best results in our laboratory is based on the fact that, at acidic reactions, serum albumin can be heated to high temperatures without undergoing denaturation and without losing the associated properties responsible for the enhancement of

³ Bovine plasma fraction V is available commercially from Armour Laboratories, Chicago.

growth of tubercle bacilli. The serum globulins, on the contrary, become completely denatured under the same conditions. The technique is as follows:—

Serum, diluted with an equal volume of physiological saline, is acidified to pH 2.0–2.5 by the addition of normal HCl (approximately 0.5 to 0.8 cc. of normal acid per 10 cc. of serum). It is then heated at 65–70°C. for 20 minutes. The heated serum, which should remain fluid and transparent, is cooled, and brought back to pH 6.5 by the careful addition of 0.1 N sodium hydroxide. There separates an abundant precipitate consisting chiefly of denatured globulins, while a large part of the albumin remains in solution. The denatured insoluble proteins are then removed by filtration through filter paper or by centrifugation. In some cases, however (and particularly is this so in the case of human sera), the precipitate is of such a fine colloidal nature as to prevent separation. Separation may then be effected by adding to the heated serum brought back to pH 4.5–5.0 a small amount (approximately one-tenth the volume) of a water-immiscible organic solvent (chloroform or ether for example). Gentle agitation causes immediate agglutination of the precipitate which can then be readily separated from the soluble part by filtration.

Sterilization of the filtrate can be carried out by a second filtration through bacteriological porcelain candles. In our laboratory these are first cleaned with water, then baked in an electric oven at 560°C., and washed again with water before being autoclaved. Although it is possible, and convenient, to sterilize albumin solutions by filtration through asbestos pads of the Seitz type, care must be taken that the pads are thoroughly washed before use as they often release impurities which interfere with the growth of tubercle bacilli. It is advisable to heat the sterilized filtrate at 55°C. for 20 minutes in order to distill off the traces of organic solvent left after removal of the precipitate of denatured proteins.⁴

The concentration of albumin in the filtrate of heated serum can be determined by precipitation with 5 per cent trichloroacetic acid or 2.5 per cent sulfosalicylic acid.

The basal medium was distributed in 25 cc. amounts in Erlenmeyer flasks of 125 cc. capacity and autoclaved. To each of these flasks was also added 5 cc. of the filtrate of heated human serum prepared as described above.⁵

Each flask was inoculated with 3 cc. of BCG culture grown for 7 to 10 days in the same medium.

After incubation for 1 week at 37°C. the new growth could be seen on the bottom of the flask as an abundant fine sediment that could be readily resuspended by gentle manual shaking to give a macroscopically homogeneous suspension. Microscopic examination revealed the presence of clumps of various dimensions (some containing probably up to one thousand bacilli) in addition to many smaller clumps and isolated bacterial cells. The bacilli were uniformly acid-fast but the cells were somewhat shorter than those present in a sample of vaccine prepared from the same strain by the conventional technique at the Henry Phipps Institute.

⁴ There is no evidence that heating at acid reaction and filtration after neutralization will exclude the filterable viruses—the serum hepatitis virus for example—that may be present in the original sample of human serum. Should the method described in the present paper come to be used in the preparation of vaccine for injection into human beings, it would be advisable to obtain the heated serum filtrate from a serum sample proved to be free of serum hepatitis virus, or rendered free of it by irradiation or by treatment with nitrogen mustard.

⁵ Many of the experiments described in the present report were carried out with samples of filtrates of heated human serum received through the generous cooperation of Mr. C. E. Bender of Microbiological Associates, Coral Gables, Florida.

Viability of BCG Cultures in Tween-Serum Filtrate Medium

Flask of BCG cultures in the Tween-serum filtrate medium were incubated for 8 days at 37°C. and stored in the refrigerator (approximately 4°C.) for various periods of time (0, 3, or 6 weeks). The number of viable cells (or clumps of cells) in these cultures was determined by spreading 0.15 cc. of 10^{-6} culture dilutions (in 0.1 per cent bovine albumin) on the surface of oleic acid-albumin agar. The number of viable units, computed from the number of colonies detectable after 3 weeks' incubation at 37°C., is reported in Table I.

In a parallel experiment, tubes containing 5 cc. of oleic acid-bovine albumin liquid medium were inoculated with serial dilutions (in 0.1 per cent bovine albumin) of similar BCG cultures stored for 0, 3, or 6 weeks in the refrigerator, as well as of a standard BCG vaccine. This vaccine had been prepared at the Henry Phipps Institute in Philadelphia; it was shipped by Dr. Aronson to our laboratory at the Rockefeller Institute in New York, where it was received the following day and immediately tested. The turbidity of this standard vaccine was approx-

TABLE I
Viability of BCG (Phipps) Culture in Liquid Medium Containing Tween and the Filtrate of Heated Human Serum

Preparation	Incubation time 37°C.	Storage (ice box)	Colonial count* (per cc. of culture)
	<i>days</i>		
I	8	0	12×10^3
II	8	0	33×10^3
II	8	3 wks.	40×10^3
III	8	0	13×10^6
III	8	6 wks.	36×10^6

* Average of counts made on 5 plates of oleic acid-albumin agar. Colonial counts do not describe exactly the number of viable cells as the bacterial suspensions contained many microscopic clumps in addition to single cells.

The differences between the different counts are within the experimental error.

imately half that of the cultures in Tween-serum filtrate medium. On microscopic examination, it was found to consist of a mixture of single cells and of clumps of various sizes in proportion not unlike those present in the Tween cultures. The bacilli in the standard vaccine were uniformly acid-fast and appeared longer than those in the Tween cultures.

The tubes of oleic acid-bovine albumin medium inoculated with graded dilutions of these different preparations were incubated at 37°C. The extent of growth after 14 days' incubation is recorded in Table II.

As seen from the results presented in Tables I and II, growth could be initiated in liquid medium and colonies developed on oleic acid-albumin agar, following inoculation with extremely high dilutions of fresh and stored cultures in Tween-serum filtrate medium. It is clear therefore that a large percentage of the cells present in these cultures were living and physiologically active and that no detectable decrease in their viability occurred during the 6 weeks' storage at 4°C. It will be noted also that the standard BCG vaccine behaved as if it

contained far fewer viable cells than the Tween cultures (less than one hundredth as many). This finding, duplicated with samples of vaccine obtained from other sources, suggests that the preparations of standard vaccine contain a very large percentage of dead cells unless one assumes that these cells are viable but are unable to grow on the albumin media.

TABLE II
Growth in Oleic Acid-Bovine Albumin Liquid Medium of Graded Inocula of Different Preparations of BCG

Inoculum cc.	Standard BCG vaccine	BCG cultures in Tween-serum filtrate medium		
		Fresh culture	Culture stored 3 wks.	Culture stored 6 wks.
0.5×10^{-2}	+++	++++	++++	++++
0.5×10^{-3}	+++	++++	++++	++++
0.5×10^{-4}	++	+++	+++	++++
0.5×10^{-5}	—	++	+++	+++
0.5×10^{-6}	—	+	++	++

TABLE III
Effect of Glucose on the Growth and Viability of BCG in Tween-Serum Filtrate Medium

Glucose concentration per cent	No. of viable units* per cc. of culture after the following periods of incubation			
	1 wk.	2 wks.	3 wks.	4 wks.
0	17×10^6	36×10^6	31×10^6	19×10^6
0.1	28×10^6	54×10^6	43×10^6	33×10^6
0.5	39×10^6	49×10^6	83×10^6	60×10^6

* Calculated from number of colonies detectable after agar plates had been incubated for 3 weeks at 37°C.

As mentioned in the text, the Tween-serum filtrate cultures consisted in large part of microscopic clumps. The number of living cells was therefore much larger than the numbers of colonies growing on agar.

Effect of Glucose on the Abundance and Viability of Growth of BCG in Liquid Tween-Serum Filtrate Media.—Glucose or glycerine is not essential to secure rapid multiplication of tubercle bacilli in the Tween-albumin medium. It is known on the other hand that these substances can increase markedly the yield of growth in this medium—as measured by dry weight of the bacterial cells or turbidity of the cultures. The purpose of the following experiment was to determine the effect of glucose on the number of viable cells present in BCG cultures after various periods of incubation.

Glucose was added aseptically in 0.1 to 0.5 per cent final concentration to a number of flasks containing 30 cc. of Tween-serum filtrate medium prepared as described earlier in this report. The stock glucose solution (50 per cent) had been autoclaved with 0.01 *M* citric acid in order to prevent the development of the toxic substances which are formed during heating at neutral or alkaline reactions. The culture flasks were inoculated with 3 cc. of an 8 day old BCG culture grown in the same medium without glucose. Samples of cultures were taken aseptically at weekly intervals and 0.02 cc. of graded dilutions (in 0.1 per cent bovine albumin) was spread on the surface of oleic acid-albumin agar. The number of viable units per cubic centimeter of culture was computed from the number of colonies visible after 3 weeks' incubation at 37°C. (Table III).

The results presented in Table III suggest that addition of glucose to the medium can increase somewhat the yield of bacterial cells. It is true that the differences in numbers of viable units in cultures with and without glucose incubated for 1 and 2 weeks, fall probably within the limits of error of the enumeration technique. Nevertheless, these differences acquire some significance by virtue of the fact that the turbidity of the cultures increased more rapidly with glucose (both in the 0.5 and 0.1 per cent concentration) than without, reaching after 2 weeks in the former case a level approximately twice as high as in the latter.

In contrast with the findings indicating an enhancing effect of glucose on growth, is the observation that an excess of the sugar (0.5 per cent) brought about a marked decrease in the number of viable units on prolonged incubation (3 and 4 weeks). A similar and even more striking decrease in viability occurred when glycerine (0.5 per cent) was added to the medium instead of glucose. Microscopic study revealed that, after 2 to 3 weeks' incubation at 37°C., in the Tween-serum filtrate medium containing 0.5 per cent glucose or glycerine, there were present a large number of bacterial cells that exhibited abnormal morphology and that had lost their acid-fast staining property. These alterations continued to increase on further incubation and finally resulted in extensive disintegration of the cells. No similar change was observed over the same period of time in the flasks to which neither glucose nor glycerine had been added. No analysis has been made of the mechanism of this toxic effect. It appears possible that, under the conditions of submerged growth determined by the presence of Tween in the medium, the rate of oxygen diffusion was not rapid enough to allow complete oxidative utilization of the higher concentrations of glucose or glycerine; and that the tubercle bacilli suffered a biochemical injury resulting from semi-anaerobic metabolism.

As the goal of the present study was the preparation of BCG cultures to be used as experimental living vaccines, viability was deemed a more important criterion than yield of bacterial cells in the selection of the culture medium. For this reason glucose and glycerine were omitted from the Tween-serum filtrate media used in the investigation of the antigenic activity of BCG, to be reported in the following paper.

SUMMARY

Diffuse, submerged growth of BCG bacilli has been obtained in liquid media containing 0.02 per cent Tween 80 and the soluble fraction of human serum heated under acid conditions (pH 2.5) at 65°C.

In the absence of glucose or glycerine,—which had a detrimental effect on viability—these cultures consisted predominantly of cells that were living and that remained viable during prolonged storage at temperatures ranging from 4 to 37°C.

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