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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Now Method of Evaluating the Rate of Growth of TB Bacilli.

Adam Bekierkunst and Teofil Szulga
Polski Tygodnik Lekarski 7, 817-820(1952)

When TB bacilli were cultivated on the surface of liquid media, the amount of their growth was determined by weighing the dessicated membrane which had grown on the surface of the medium and was then removed from time to time. It was an inaccurate method, because it was not possible to use a strictly defined inoculum, and the degree of growth in different tubes was not always the same. This question became technically simpler, when it was established that a culture of the bacilli could be obtained under the surface of the liquid media, by using as inoculum the hematogenic emulsion of the bacilli. Youmans (1) availed himself of this discovery in order to determine the regular cycle of development of the culture, the degree of growth and the lifetime of one generation, by marking the amount of nitrogen in the bacilli. This system, however, was too complicated in defining the influence of different physical and chemical factors on the bacilli.

The evaluation of the rate of growth alone has lately gained greater importance, seeing that tuberculostatic combinations are being sought, and it therefore became necessary to find a method which could, with sufficient objectivity, define not only the action checking the growth of the bacilli, but also the action reducing the rate of their multiplication. Accordingly, Youmans (2) gave up his first method and suggested in 1949 a new method of calculating the rate of growth of the bacilli. He assumes that the rate of growth does not depend on the amount (strength) of inoculum (substance used in inoculation), and that in liquid cultures the growth is observed for the first time, when a certain definite number of cells of the bacillus appear. The greater the inoculum, the sooner one can observe the growth. This correlation between the time at the lapse of which the growth is noticed and the amount of inoculum, has been used by the author to evaluate the speed of the growth. This method, much simpler than the former ones, has been recognized by the American Trudeau Society(3). Apart from this method, there also exists another one: it is based on the examination of the degree of turbidity (muddiness) of the liquid medium in the course of the growth of the bacilli, by means of a photoelectric nephelometer (4). In following this method it is necessary to use the medium of Dubos, in order to obtain a steady and even turbidity of the medium. The disseminating agent "Tween 80", which is a component part of the medium, is not neutral to the bacilli, especially in view of its other constituents, such as the antibiotics (5). Besides, in this method the use of a very expensive implement is required.

We have tried to apply the second Youmans method in our laboratory and we have established that it is difficult to determine the time at which the growth of the bacilli is observed for the first time. Youmans, in describing his method, also stresses this difficulty. Our experiments have shown that this is an obstacle, hindering the reproduction of individual experiments.

The object of our work was to devise a method which would permit to evaluate the speed of growth of the bacilli, by growing cultures on glass slide. We record the results of this preliminary research.

Experiment 1.

Some sputum containing the bacilli was rubbed onto the glass, dried, (or desiccated), treated with 6% sulphuric acid for 3 minutes, then rinsed twice in distilled water. The glasses then were placed in 15 test tubes (2 glasses in each), containing a medium with 6 units/ml of penicillin. It is the medium of Sauton, changed by us (i.e. Bekierkunst and collaborators). Here is the composition of the medium: hydrolysate of casein 2.0 g, asparagin 5.0 g, monopotassium phosphate 5.0 g, ferro-ammoniacal citrate 0.05 g, citric acid 1.5 g, magnesium sulfate 0.5 g, potassium sulfate 0.5 g, glycerol 15 g, distilled water 1000 g. pH is raised to 7 by means of sodium hydroxide. The medium is poured into the test tubes, 6 ml into each; after sterilization the yolk of hen's egg (0.1 ml) is added to it, as well as an appropriate amount of penicillin.

The tubes with glasses in them were placed in the incubator, and the glasses were taken out every 8 hours. After the staining of the preparation by the Ziehl-Neelsen method, the bacilli were counted in order to establish their average number in a "unit". We call unit every group of the bacilli; it may consist of 1, 2, 3, 4, and more cells. We also noted the average number of the bacilli in the sputum, before preparing the cultures. The records of this experiment are shown in table 1. The results of the first experiments are given in table 2.

On the following day we repeated the experiment with the sputum of the same patient, which was kept in the icebox. The results are shown in table 3.

A similar experiment has been performed with the fresh sputum of the same patient, obtained 3 days later. The result of the experiment is shown in table 4. Considering that the glasses were taken out every 8 hours, we could fairly accurately reproduce the process of development of the culture, in proportion to the multiplication of the bacilli. We have ascertained that the bacilli do not multiply for about 32 hours, and that after that period a slow multiplication takes place, before the growth enters its logarithmic phase. The interdependence between the duration of the growth and the number of the bacilli is shown in figure 1. The latter shows the results of three experiments, made at different times with the sputum of the same patient.

We see from table 5, that the generation time after 72 hours of cultivation is, on the whole, longer than the time counted after 4 or 5 days. This may be due to the fact that we then still have to deal with the preliminary stage of logarithmic growth, when the growth rate has not yet reached its peak. We estimate the time of duration of one generation,

by checking the average number of the bacilli before the preparation of the culture or after 24 hours, and the average number of the bacilli in a "unit" after 4 or 5 days of culture. We use here the following formulas:

$$n = \frac{1 gb - ga}{2}$$

$$g = \frac{t^2}{n}$$

- b - average number of bacilli in a "unit" after a fixed time of culture
 a - average number of bacilli in a "unit" before the preparation of culture or after 24 hours
 n - number of generations, grown within time t
 t - time of incubation
 g - duration of one generation

We have established that it is better to count the average number of the bacilli in a "unit" after 24 hours, because during that period the morphotic elements of the sputum undergo dissolution (lysis) under the action of the enzymes contained therein. It is also easier to count the bacilli after 4 days, because then they are fewer in single groups. To verify this result as well as to check whether the generation time of the bacilli of the same person was the same, his sputum was taken a second time (9.X), and simultaneously five series of the same experiment were set up. In order to obtain the average, the glasses were taken out after 24 hours and after 96 hours. The result of this experiment is shown in table 6.

We have thus ascertained that it is possible to evaluate the length of time of one generation and that this time, in case of one patient, does not change, at least not within the period of our investigation (i.e. from 7.1 X to 9.X). That period amounts to 24.5 hours with a probable error of ± 0.22 .

There is a certain difficulty in counting the bacilli. They sometimes cling together closely and sometimes are scattered disorderly, so that it is difficult to count them. In order to see in how far it affects the reckoning, observed by us, we have set side by side the results of counting of the same preparation by three members of our staff.

As we have already mentioned, this research is preliminary. Many other methodical questions suggest themselves, and the object of our work is to find their solution. Our further investigation aims at the application of our system in bacteriology and the antibiotics of the bacillus of TB.

Trans. fr. the Polish
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