QUANTITATIVE ASSESSMENT OF PHAGOCYTOSIS BY MACROPHACES IN VITRO BACILLI OF DIFFERENT VIRULENCE

Report I

Principles of the Method and Its Reliability

Translation No 1674

April 1966

U. S. ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK, FREDERICK, MARYLAND

QUANTITATIVE ASSESSMENT OF PHAGOCYTOSIS BY MACROPHAGES IN VITRO OF ANTHRAX BACILLI OF DIFFERENT VIRULENCE

Report I

Principles of the Method and Its Reliability

[Following is the translation of an article by T. N. Maslova, Gamaleya Institute of Epidemiology and Microbiology, AMN, USSR, published in the Russian-language periodical Zhurnal Mikrobiologii Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology), No 11, 1965, pp 124-126. It was submitted on 17 Mar 1965. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

It was demonstrated in previous works (Maslova, 1963; Ginsburg and Maslova, 1963) that the cellular elements of a culture of macrophages (obtained from aseptic exudates from the abdominal cavity of guinea pigs) possess an expressed capability to phagocytize anthrax bacilli. There was great interest in assessing quantitatively the capability of the cellular elements of such cultures, obtained from normal and immune guinea pigs, *to phagocytize bacilli of a different degree of virulence.

Up until now the problem concerning the best quantitative expression of the results of the phagocytic reaction has remained the subject of extensive discussion and has attracted the attention of a number of authors (Hanks, 1940; 1958; Suponitskaya, 1952; Mutsiniek, 1953, 1955; Latysheva, 1955; Gurvich, 1956; Linz and Mandelbaum, 1959, 1960).

The generally accepted method for recording the results of the reaction of phagocytosis is the determination of the number of particles, captured by the phagocytizing cell in a unit of time. The calculation amounts to the determination of the average number of bacteria, absorbed by one phagoctye in a unit of time (Wright index), and the determination of the percentage of phagocytes which are absorbing particles (Hamburger index) or recording the number of extracellular bacteria found. Often the final selection of the parameters for the quantitative assessment of phagocytosis is determined by the characteristic peculiarities in the flow of the process and the overall mission of the investigations.

Attempts to quantitatively assess phagocytosis of anthrax bacilli were undertaken by Smith and Gallop (1956) and Keppie et al. (1963). In comparing results the authors determined the number of viable bacteria, which had not perished as a result of phagocytosis, and the phagocytic index. In their tests the authors used the spore form of the anthrax causative agent, which were not multiplying during the period of observation.

*The culture of macrophages which was obtained from normal or immune guinea pigs will subsequently be called the normal or immune culture of macrophages correspondingly.

This considerably eased the carrying out of the calculations.

The mission of the present work was to develop a method, with the help of which it would be possible to evaluate the process of phagocytosis of anthrax bacilli in the vegetative form by cells of a culture of macrophages, and to use it in the future for comparative investigations in tests with anthrax causative agents with a various degree of virulence.

The utilization of methods, which had been developed for assessing the results of phagocytosis of other species of bacteria, for the quantitative characteristics of phagocytosis of anthrax bacilli, is made difficult by the following circumstances: 1) the process of phagocytosis of anthrax causative agents in vitro is accompanied by their multiplication, and here the bacilli form long interweaving chains, which practically excludes the possibility of measuring their length directly under the microscope; 2) the dimensions of the chains somewhat exceed the dimensions of the phagocytizing cells, and the part of the chain, which was initially entrapped by the cell, is usually insignificant in comparison with its remaining length; 3) the destruction of the chain in the process of phagocytosis takes place both within and outside of the macrophage as a result of the liberation of a bacteriolytic substance by the latter in the direction of the chain.

Taking this into consideration, we attempted to develop a method for determination, under which the stated peculiarities of phagocytosis of anthrax bacilli would not influence its result.

As materials for the calculations we used preparations which reflected the processes of in vitro phagocytosis by cell-macrophages (obtained from normal and anthrax immune guinea pigs) of virulent (variant 71/12 II Tsenkovskiy vaccine) and avirulent (strain STI-1) strains of anthrax bacilli after 45 and 90 minutes, and 3 and 5 hours following infection.

With the help of a MBI-1 microscope, equipped with a MNF-2 device and a drawing device, the sector of the preparation which is found in the field of vision of the objective was projected with a magnification of 182 times [1] onto a standard sheet of paper (203 x 288 mm) and sketched with colored pencils. Then the preparation was shifted, and into the field of vision came a section of new cells and microbes, which were sketched anew. These operations were repeated until the entire sheet of paper was filled.

1. This magnification was selected based on considerations for the optimum illumination of the image and the ease for performing the subsequent measurements and calculations.

In this manner 32 preparations were processed and 70 sketches of their separate sectors were obtained. At the selected scale of magnification each filled sheet of paper corresponded to a sector of preparation which in area equaled 1.77 mm^2.

For each sector which was transferred onto paper, the following data were determined by means of calculations and measurements: 1) the overall number of cell-macrophages (Ko), 2) the number of phagocytized

cells (Kph) and 3) the summary length of the chains of bacilli [2] which are found in a given sector of the preparation, taking into consideration the segments of the chains which are encompassed by the cells (l). On the basis of the enumerated absolute indices we determined a series of relative parameters, which also served subsequently as the main criteria for comparing the results of the process of phagocytosis. These parameters included the index of the intensity of phagocytosis (a), the percentage of phagocytosis (f), and the index of the density of the preparation (p).

2. The length of the chains was measured by a curvometer with divisions of 1 cm.

The index of the intensity of phagocytosis (a), characterizing the activity of the "attack" of the macrophages on the bacilli, was determined by the ratio of the number of phagocytized cells to the summary length of the chains captured by the phagocytes, and in actuality meant the number of phagocytized cells present in a unit of length of the chain.

$$a = \frac{\text{Kph (cells)}}{\text{l (mm)}} .$$

The percentage of phagocytosis showed what number of macrophages from the total number which were found in the preparation took part in the phagocytic reaction:

$$f = \frac{\text{Kph}}{\text{Ko}} .$$

The index of density of the preparation specified the number of macrophages belonging to a unit of space of the preparation:

$$p = \frac{\text{Ko (cell)}}{\text{S (mm)}^2} .$$

The enumerated parameters were determined for each sketched sector of the preparation, and then averaged out for the preparation as a whole.

On the basis of the average values of the parameters, f, a, and p for each type of strain and interval of time we determined the fourth parameter L according to the following formula:

$$L = \frac{p \cdot f}{100 \cdot a} \quad \frac{\text{mm of length of bacteria}}{\text{mm}^2 \text{ of preparation}} .$$

This parameter characterized the summary length of the chains of bacilli (considering the segments trapped by the cells), belonging to a unit of space in the preparation at a given moment of time. In accordance with this the parameter L was called the density of infection of the preparation and was the indirect index of growth and multiplication of the bacilli.[3]

3. The density of infection may also be determined by means of calculating its values for each sketched sector of the preparation and the

subsequent averaging of the data. However, this method is more laborious, especially with a large number of sketches.

Thus, the method adopted makes it possible to quantitatively describe the process of phagocytosis for each of the preparations with the help of three parameters: a, f and L. These data are then averaged for several preparations. The results obtained and an analysis of them will be presented in report II.

For the purpose of determining the reliability of the data which was obtained with the help of the proposed method for the quantitative evaluation of the reaction of phagocytosis, an analysis was carried out on accuracy, taking into consideration the following factors which are capable of influencing the results of the calculations: 1) accuracy of the observance of the initial doses of the cellular and bacterial population, 2) degeneration of macrophages during cultivation, 3) unavoidable deviations in the time for taking the preparations from the test tubes, 4) errors committed when sketching the enlarged sector of the preparation and when conducting measurements.

As a result of the analysis and on the basis of morphological observations, it was established that degeneration of macrophages in the period of cultivation was insignificant (less than 0.5%) and did not influence the results of the calculations. The remaining factors led to the manifestation of errors. The results of determining the reliability of data, obtained with the help of our method for calculations, and which are presented in the table, showed that the maximum error, committed when performing the quantitative evaluation of phagocytosis with a consideration for the main sources of errors, did not exceed $\pm$ 14% from the value of the determined parameter and was found within permissible limits.

Conclusions

The method developed makes it possible to quantitatively assess the phagocytosis of anthrax bacilli with a various degree of virulence in a culture of macrophages. The results obtained were reliable.

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Relative errors in the quantitative method of assessing experimental data

Source of error	Frequency of errors (in %) which influence the parameters characterizing the process of phagocytosis			
	a	f	p	L
Inaccuracy in observing the initial dose of cellular population	-	-	2	-
Inaccuracy in observing the initial dose of bacterial population	-	-	-	1.5
Errors in the time of withdrawal of the preparations	2	2	2	2
Errors committed when sketching the magnified sector of the preparation and when making calculations	9.55	8.9	3	9
Total error	11.55	10.9	7	12.5