NUCLEAR CHANGES IN LIVING CELLS OF A VARIANT OF BACILLUS ANTHRACIS

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Chromatinic bodies or nuclear structures have been demonstrated in bacteria by numerous workers using a variety of technics. Many of the methods employed involve the use of killed cells and give relatively little information concerning the actual changes occurring during multiplication of the bacteria. In recent studies by Eisenstark, McMahon, and Eisenstark (1950), Tulanse (1949), and Stempen (1950) nuclear changes have been followed in living cells with the aid of the phase contrast microscope.

The purpose of the present investigation is to observe chromatinic or nuclear changes in young, multiplying bacteria with the aid of the dark phase contrast microscope.

METHODS

Bacillus anthracis, variant RS63, from Dr. C. F. Robinow’s collection was employed in this study. The organism was maintained on slants of nutrient agar and for observation under the phase microscope was cultivated directly on a no. 1 microslide coverslip. A small drop of 0.7 per cent nutrient agar cooled to 40°C was placed in the center of the slide. The agar surface was very lightly inoculated with cells from a one to two-hour old agar slant culture which had been subcultured from an 18 to 24-hour agar slant culture. One-half of a no. 1 cover glass was then gently lowered until supported by the agar mound, thus flattening the inoculated surface. Melted vaspar was used to seal the coverslip to the slide. Sufficient air was present in the chamber thus formed to supply the needs of the organisms for some time. The slide cultures were held at room temperature. Impression film preparations were also made at various intervals of time from agar cultures and fixed in osmic acid vapors. The cells were hydrolyzed in N HCl at 60°C for 3 to 7 minutes, rinsed in distilled water, mordanted in 1 per cent formaldehyde for 3 minutes, rinsed in distilled water, stained for 30 seconds in 0.3 per cent aqueous basic fuchsin, rinsed in distilled water, and transferred to tap water (Smith, 1950). Aqueous mounts were made and examined under the microscope.

Photomicrographs of the living cells at a magnification of 1,240 diameters were taken with Eastman Kodak contrast process panchromatic film using a 97× dark phase medium oil immersion objective, N.A. 1.25 and a 10× compensating ocular. Contrast was enhanced by the use of a Wratten E filter. The stained organisms were photographed at a magnification of 700 diameters using a Zeiss condenser of N.A. 1.4, a Leitz 63× apochromatic oil immersion objective, N.A. 1.4, and a 10× compensating ocular. Wratten G and H filters were employed, and oil was used on both the condenser and the slide.
Figures 1–11, 15–16. Dark phase contrast photomicrographs at different stages of multiplication of *Bacillus anthracis*, variant RS63. Age of the culture in minutes is indicated at the right of the figure numbers. Negative images, magnification 2,700X.

Figure 12. Photomicrograph of a nuclear stained impression preparation of *Bacillus anthracis*, variant RS63, from a 240 minute culture. Magnification 1,240X.
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Figures 13-16
RESULTS

Young living cells of *B. anthracis*, variant RS63, show differentiation into dark and light areas when examined with the dark contrast phase microscope. Comparison with fixed, hydrolyzed, and stained cells, as for example the stained preparation from a 4-hour culture shown in figure 12, reveals that the light areas in the living cells correspond in position and configuration to the darkly staining chromatinic bodies. The dark areas correspond to the lightly stained cytoplasmic regions in the fixed cells. In the light of this correspondence of position and configuration it is deemed permissible to describe the light areas in the living cells as chromatinic material. For greater ease of comparison with stained preparations the picture as presented by the phase microscope was reversed by photographic means and negative rather than positive images of the living cells are presented in the dark contrast phase photomicrographs.

The best results from the standpoint of differentiation of the cells were obtained when the slide culture was prepared from a 1 to 2-hour agar slant culture. Immediate examination of such a slide culture disclosed a few cells showing internal differentiation. Further incubation at room temperature yielded a much greater number of differentiated cells. Undifferentiated cells had a uniform purplish appearance. On standing, generally not longer than 30 to 60 minutes, a gradual differentiation of viable cells into the previously mentioned light and dark areas could be seen. Usually slight elongation of the cell occurred during this period. In those cells which at first present a homogenous purplish appearance but apparently die, the change is a gradual clearing of the cell with the final appearance being that of a transparent cell containing scattered small, purplish granules. These cells neither grew nor showed further change in appearance over a period of hours.

The chromatinic body divisions in the living cells were quite easily followed. Division of the bodies occurred on lines perpendicular to the long axis of the cell. It was initiated by the appearance of a break in the continuity of the chromatinic body on one side next to the cell wall, the break progressing across the body, generally yielding a U-shaped structure or less frequently a V-shaped structure before complete separation occurred. Division commonly was initiated on the same side of the cell, that is, all of the U or V-shapes opened toward the same side of the column of cells. As division progressed, the bodies being formed moved apart until complete separation left them lying side by side.

The number of chromatinic bodies per cell appears to be two but cytokinesis and karyokinesis did not progress at the same rate in the actively multiplying cells. The cell elongated as chromatinic body division progressed, but not until two divisions were nearly completed did a new transverse cell wall become apparent. All of the steps in the process described before for living cells can be found duplicated in the photomicrograph of stained cells from the 4-hour culture.

Many instances in which a chromatinic body appears to be made up of a series of parallel strands were noted during observations with the phase microscope and several are evident in the photographs. The strands lie crosswise of the cell and increase in number as the chromatinic body increases in size and begins to divide.
It is difficult to determine the exact number of strands present at the time of chromatinic body division, but two are quite plainly indicated (particularly on projection of the originals) in figure 6, four or more are suggested in figures 8 and 10, and at least six, or possibly eight, strands show in figure 11.

As cell division progressed the initial sharp contrast diminished somewhat and, although the divisions of the chromatinic bodies could be followed, the same clarity of detail did not exist in all of the cells. At 24 hours the cells under the phase contrast microscope showed much less differentiation than they did at 6 hours. In a few cells, however, chromatinic bodies were still discernible. The cells were also somewhat shorter and less regular in form.

Endospores were numerous in the 1 to 2-hour cultures. They were found both free and lying within the spore mother cell. The resting spore appeared as a darkly outlined oval, its interior being light as it also appears under the light field microscope. The germinating spore had the uniform purplish appearance of the undifferentiated cells in the phase microscope and of the cytoplasmic areas in the differentiated cells. The development of the homogenous purplish appearance and a slight enlargement were the first visible indications of spore germination. The emergence of the bacillary body from the spore is becoming evident in figures 10 and 11. Chromatinic body differentiation is faintly evident in figure 11 and plainly evident in figure 13. The body presents the U-shape observed in the actively multiplying cell. Whether this represents the first division of a single chromatinic body within the young cell or the second division, the second chromatinic body being hidden by the spore case in this event, is difficult to determine. The young bacillus apparently emerges through the end of the oval-shaped spore. Whether the spore case is ruptured or dissolved at the point of emergence was not discernible. The spore case was discarded, apparently intact except for the open end, at about the time the first cell division was completed.

**Discussion**

The existence of structures which have been described as nuclei or chromatinic bodies in various bacteria is well verified by studies with the phase microscope. The excellence of the agreement in the shape and position of these bodies within the cell at various times and their behavior during cellular multiplication as demonstrated by both ordinary and phase microscopy leave no doubt that they are the same structure. It is of interest to note that none of the chromatinic bodies in the living cells of *B. anthracis*, variant RS63, presented the dumbbell shape often described in stained preparations. The absence of dumbbell forms in stained preparations of this bacillus is also noteworthy. Such forms were observed by Stempen (1950) in preparations of *Escherichia coli* stained with aqueous basic fuchsin but not mordanted with formaldehyde. He found, however, that the dumbbell shape was not present in the living organisms and suggested that the typical stained picture might be related to the effect of either fixation or acid hydrolysis.

The chromatinic bodies in stained preparations of young, actively multiplying cells are usually described as being rod shaped with the spherical nucleus oc-
curring only in the resting stages. Generally, they appear to be very nearly as long as the cell is wide and to have a quite well-defined edge. This has led to the idea of their being chromatinic structures rather than the complete nucleus, for no limiting nuclear membrane could be demonstrated, the latter in all probability being beyond the resolving power of the light microscope. In the living cells investigated here the light bands seen in the phase microscope present this rod appearance upon first examination although they appear to be broader than in the stained preparations. Closer examination reveals that many of the structures are very definitely round and that they do not have the sharp outline of the chromatinic bodies in stained preparations. The central portion of the body is quite dense, and this “core” is surrounded by a cortex of less dense material. No limiting membrane was observed, but the whole structure occupies a well-defined area within the cell. The diameter of the whole structure appears to be very nearly equal to the inside width of the cell.

This appearance of the bodies was not limited to any one period during the growth of the cells but was evident throughout the period of observation. The cells were actively multiplying during the 225-minute period of close observation. The bodies in the very young cells derived from the germinating spore also presented this appearance. These observations indicate that this is the normal shape of these structures throughout at least the early period of active multiplication.

The general appearance of roundness in the optical plane strongly suggests that the complete structure is a sphere. Such a structure would give the appearance of greater density in its center with a decrease in density toward its perimeter. The part played by the resolving power of the optical system in the appearance of these structures must be kept in mind. The inability of the light microscope to resolve the fine structures of these spherical bodies would tend to add to the appearance of increased density at their centers. This type of structure would indicate that these bodies are nuclei rather than merely clumps of chromatinic material.

The repeated occurrence of these round structures during the period of active multiplication suggests that there may be a resumption of the resting state of the nucleus between its divisions. During division there was often a loss of clarity but in one instance (figure 4) a mitotic type of figure was observed. In relation to this the instances (figures 6, 8, 10, 11) in which the parallel strands increased in number as the nuclear division progressed are of great interest. The mitotic type of figure was not in evidence in these instances but there was apparently an even distribution of the strands to the daughter nuclei. The possibility exists that these strands are chromosomes, but it is questionable whether the size of these structures will permit resolution within the limits of the optical system. Further study is required before a definite conclusion as to their nature can be drawn. If they are chromosomes, however, it appears that each nucleus may contain at least four.

The resemblance of these figures to those reported for various strains of *B. anthracis* by Flewett (1948) is noteworthy.
SUMMARY

Actively multiplying cells of *Bacillus anthracis*, variant RS63, were studied with the aid of the dark phase contrast microscope. Evidence is presented for the existence in a spherical form of nuclear structures in young, actively multiplying cells of this bacillus and for the possibility that the nucleus assumes a resting state between all divisions.

REFERENCES

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