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BACTERIOLOGY

THE L-FORM OF *B. PROTEUS VULGARIS*

I. A STUDY ON THE DYNAMICS OF THE FORMATION OF *B. PROTEUS VULGARIS* WITH SLIDE CULTURE METHOD AND OBSERVATIONS MADE UNDER PHASE-CONTRAST MICROSCOPE*

LI HUI (李 輝), LI TIEN-LIN (李天玲),
(*Department of Microbiology, Shangtung Medical College*)
AND LEI AI-TE (雷愛德)
(*Division of Medical Illustration, Tientsin Medical College*)

INTRODUCTION

The L-form of bacteria, first observed by Klieneberger (1935) while she was studying *Streptobacillus moniliformis*, is a special morphological form of bacteria. Dienes (1949), Левашев (1957) and others proved that under the effect of penicillin, *B. Proteus* can produce the L-form. Stempen and Hutchinson (1951), Pulvertaft (1953), and Лешков (1955) studied the process of its formation. Pease (1957) first observed the L-form of *B. Proteus* under electron microscope and proved that under the influence of penicillin, the vegetative form loses its cell wall, becoming a protoplasmic body with further alterations of its external form and finally resulted in a group of irregular protoplasmic masses. With the aim of observing the whole process of L-form formation in *B. Proteus*, we carried out vital studies on slide cultures, observed the organisms under phase-contrast microscope and took time-lapse photographs.

The result of 15 repeated observations with this method is reported in this paper.

MATERIAL AND METHOD

(1) *Type of bacteria.* We used a freshly isolated, identified Proteus strain from a patient with diarrhea, cultured it in agar slant, and designated it as "P 2" strain.

(2) *Culture media.*

Beef extract	3.5 g
Proteos (Difco)	10.0 g
NaCl	5.0 g
Sucrose	100.0 g
Agar (Difco)	10.0 g
Distilled water	1000.0 g

(pH = 8.0)

* Received June 1, 1964.

All components of the culture media without sucrose were autoclaved at 15 lbs for 15—20 minutes. After autoclaving, the required amount of sucrose, 10 ml 20% $MgSO_4 \cdot 7H_2O$, 15—20% sheep serum, and 10—1 000 units of penicillin were added, making up the total volume of 1 000 ml.

(3) *Slide culture.* We designed a simple culture method based on Foubrune's oil chamber method. On a microscopic slide, two small strips of glass, measuring approximately 20 × 5 mm, were first placed at a distance of 10—15 mm apart. Then we dropped on the slide the above mentioned culture media in its liquid form, filling the space between the two strips of glass. After solidification, the four hour culture was transferred to this media with a platinum loop, covered with a piece of coverslip and sealed with paraffin. It was then placed on the warm stage of the microscope and observations were made at a constant temperature of 37°C.

(4) *Photomicrography.* With the slide culture in place, using phase-contrast optical equipment, we observed the changes and made time-lapse photomicrographs at intervals varying from 5—10 min for 3—15 days.

Results

(1) *Preliminary preparations.* We put the typical *B. Proteus* "P 2" strain on the above described penicillin sugar plate and incubated it at 37°C. During the period of 3—4 days, typical "3 A" and "3 B" colonies with a diameter of 50—100 μ were seen (Fig. 1 a, b, and c). The centre of these colonies grew into the culture media, becoming black in colour with transparent edges.

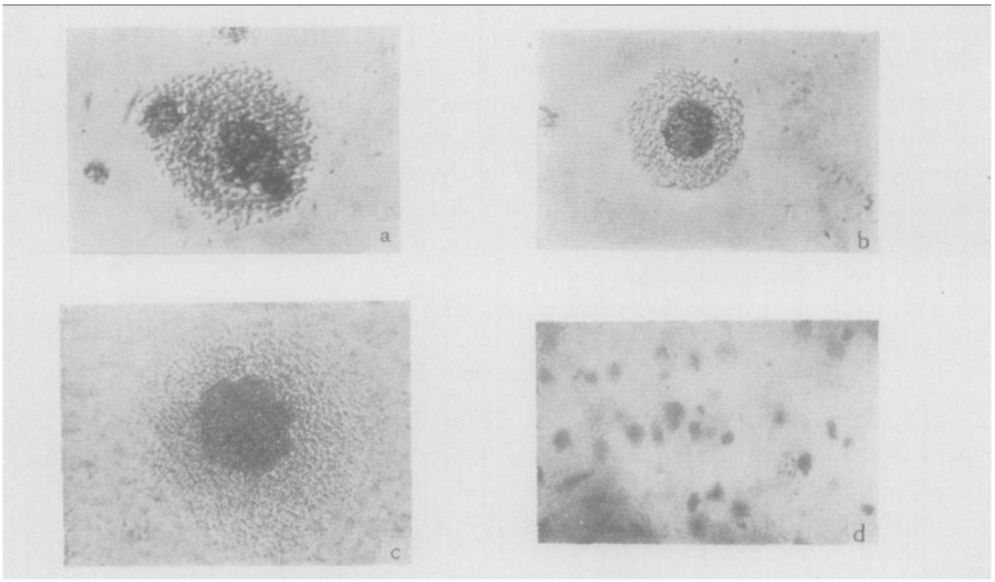

Fig. 1. Colonies of L-form on agar plate.

a, b: "3A" colonies (×50); c: "3B" colony (×40);
d: "3C" colony (×10).

The "3 A" colonies were then transferred to a microscopic slide and covered with a coverslip. One could observe a large amount of round, ball-like spherical bodies with

granules. The size of these spherical bodies varied a great deal, the smallest being approximately 2 μ and the largest 20 μ. Inside these spherical bodies could be seen many granules and vacuole-like structures of different sizes. There were also a large number of granules of approximately 0.3—1 μ in size outside these bodies, similar to those seen within (Fig. 2).

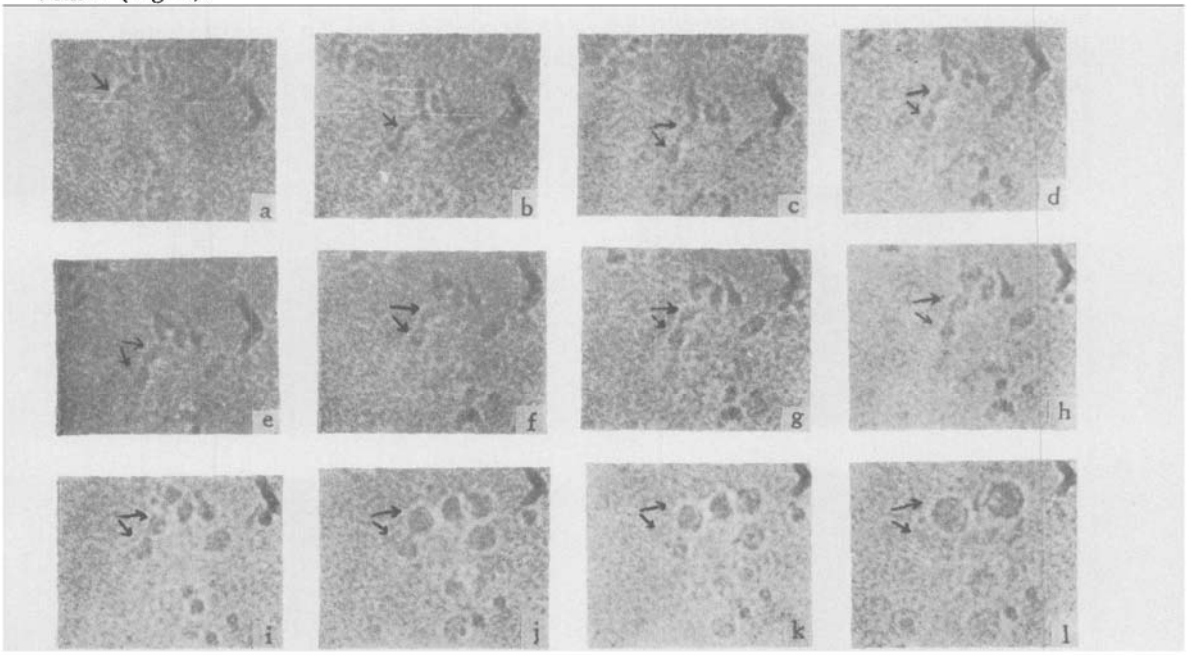

Fig. 2. Process of the formation of the L-form of *B. Proteus*. (Observations made on slide culture under phase-contrast microscope. ×900, oil immersion magnification.)

a: 1 hr; e: 3¾ hr; i: 6 hr;
b: 1¼ hr; f: 4 hr; j: 7½ hr;
c: 1½ hr; g: 4½ hr; k: 8 hr;
d: 3½ hr; h: 5 hr; l: 10 hr.

The colonies formed on the penicillin-sucrose nutrient agar plate could be subcultured. To simplify the subculturing procedure and to facilitate observations on the growth of the L-form, we tried the test-tube method with media mentioned above, as used in the semi-solid culture method for facultative anaerobic bacilli. The growth of the L-form of *B. Proteus* by this test-tube method was more abundant than cultures made by the plate method. In areas near the surface of the culture media were many colonies of approximately 0.5 μ in diameter. In about a week's time, there appeared many string-like and reticular structures between colonies and we temporarily called these "3 C" colonies (Fig. 1d).

(2) *Observations on the process of the formation of the L-form of B. Proteus.* Observations made on these prepared slides described above under phase-contrast microscope at a constant temperature of 37°C revealed slight swelling of the bacilli in about one hour's time. Binary fission could be seen at 1½ hour. After 3½ hours, some bacilli formed extrusions and the bacterial body became curved. In about 4 hours, the swelling became more obvious, some were round or spherical, while others "deer-horn" shaped. At this stage, one could notice the protoplasmic fluid flowing towards the extruded portion of

the body and the bacterial bodies becoming transparent. After 4½—10 hours the bacterial bodies vanished entirely, and, in the field, one could only find spherical bodies. These eventually grew in size, and attained approximately 5 μ in diameter (Fig. 2). After 24 hours, some of these spherical bodies appeared to be full of granular structures (Fig. 3 a, b), diffuse or aggregated in arrangement, giving a reticular appearance (Fig. 3 b). In others, only vacuole-like structures (Fig. 3 c, d, e, f) existed and many of these were packed together, giving a foamy look (Fig. 3 g). Vacuoles grew in size and some could attain almost half the size of the whole spherical body (Fig. 3 e). Some of the vacuoles broke and gave off granules.

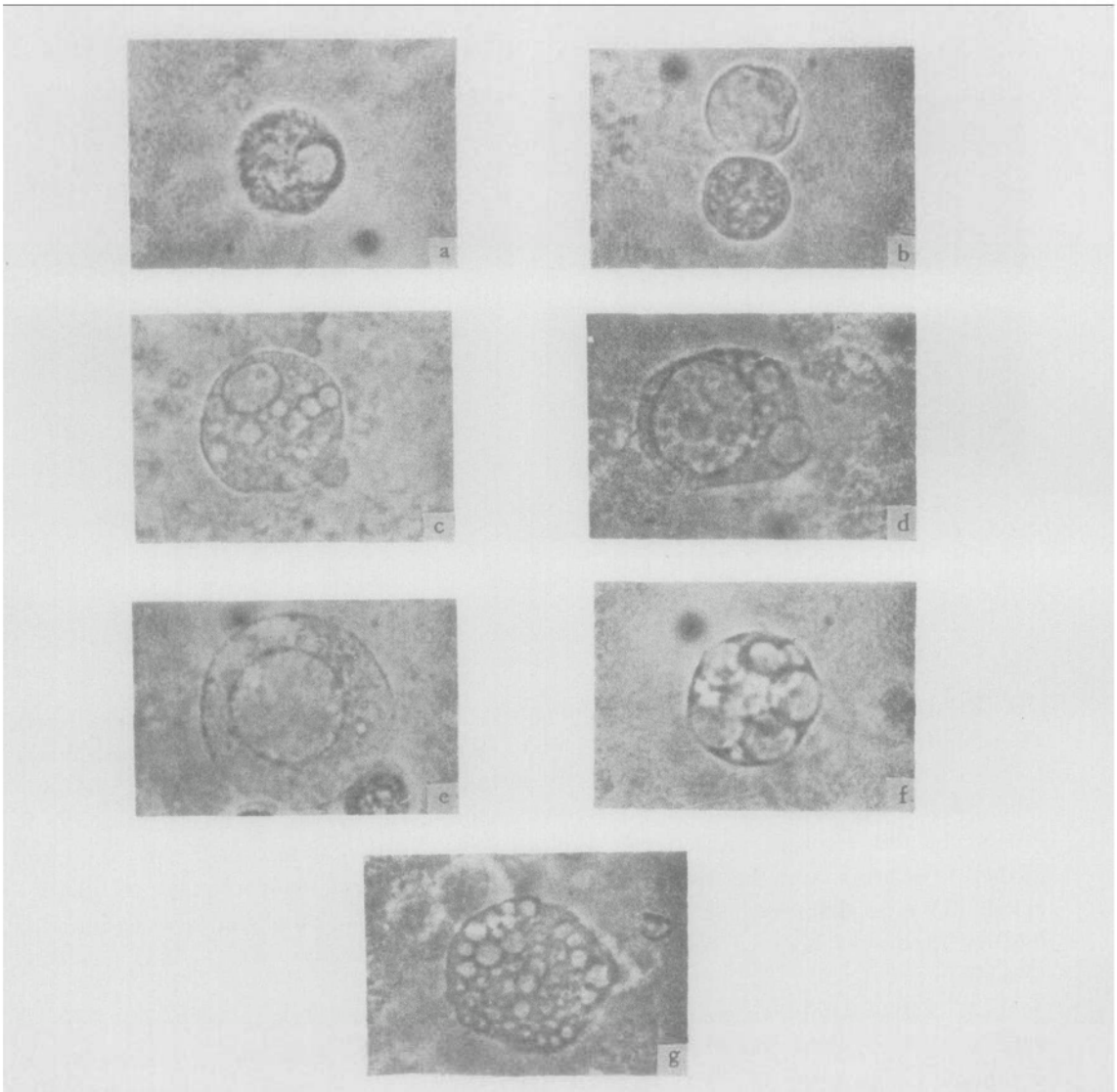

Fig. 3. Morphology of structure of spherical bodies of L-form of *B. Proteus*. (Observations made on slide culture under phase-contrast microscope. ×900, oil immersion magnification.)

a, b: granular structures; c, d, e, f: vacuole-like structures;
g: foamy look of spherical bodies.

After 7 days, colonies appeared on the slide culture. In the central portion of the colonies were many granules which in turn grew into the culture media. In the edge of the colonies, spherical bodies were most abundant.

Discussion

The L-form of the bacteria is the variant (Тимаков and Каган 1961) of bacteria which can be induced by the influence of many factors. To produce the L-form of bacteria with penicillin, the concentration of the penicillin in the culture media is important, because various strains of bacteria vary in their susceptibility to penicillin. The amount of penicillin to be used should be determined through experiments. We tested 21 strains of *B. Proteus* and found that only the "P 2" strain, under the influence of 200 μ penicillin per ml culture media, could produce the L-form regularly. Some strains could not be transformed to its L-form even with 1 000 μ of penicillin per ml culture media.

To induce the transformation of L-form of bacteria, many workers have emphasized the use of horse serum in the culture media (Weibull and Lundin 1961, Dienes 1948), because it can eliminate the toxic effects of some of the components which made up the media. Instead of the horse serum, we employed sheep serum with excellent results not only with *B. Proteus,* but have also induced successfully the L-form of *S. typhosa, Sh. flexner, E. coli, Staphylococcus aureus* and *C. diphtheria* etc. (To be presented in a later report.)

All workers used the nutrient agar plate method for passage of L-form, but this method is not economical and the plate may very easily be contaminated. We, therefore, suggested the use of the above described test-tube method. Generally speaking, the growth of the L-form is more favourable under slightly anaerobic condition. Because the L-form of bacteria is very fragile, agar-agar is required for support. The test tube method provides both these requirements.

We have used the test tube method for the passage of 47 generations of the L-form of *B. Proteus* "P 2" strain with very satisfactory results.

Using the slide culture method, under the phase-contrast microscope, we studied the dynamic of the transformation of *B. Proteus* "P 2" strain to its L-form. Experiments suggested that under the influence of penicillin at 37°C, the first noticeable change was the swelling of the bacterial bodies. (If the culture were placed at room temperature for 12 hours, besides the swelling of the bodies, one could also notice the elongation of these bacterial bodies—forming filament-like structures.) During this stage, binary fission might still be seen in some bacterial bodies. On further incubation at the same temperature, one could see many extrusions of various sizes and shapes from the swollen spherical bodies, and, as time went on, these extrusions grew in size and eventually broke off to form new spherical bodies. Binary fission could no longer be seen at this stage. If the culture was maintained at this condition for about 7 hours, spherical bodies continued to grow in size and the bacterial bodies would disappear entirely, leaving a clump of spherical bodies of various sizes and arrangements, inside which were found many granules and vacuole-like bodies of different sizes intermixed or grouped together.

Park and Strominger (1957) suggested that the mechanism of the action of penicillin on bacteria was to influence the proper utilization of the "cell wall precursor" in the cytoplasm which later would form the cell wall, and therefore hindered the synthesis of the latter. According to Bisset (1956), during the development and fission of the bac-

teria, there should exist a synchronization between the division of chromatin, synthesis and growth of the protoplasmic material and the synthesis and growth of the bacterial cell wall. Under normal conditions, the fission of the chromatin and the fission of the bacteria are in harmony; the fission of the chromatin and the growth of the protoplasm, together with the synthesis and fission of the bacterial cell wall, should all synchronize. The synthesis of bacterial cell wall proceeds continuously, growing inwards and finally resulting in two bacterial bodies (Conti and Gettner 1960; Chapman 1960).

From our own observations, the transformation of *B. Proteus* "P 2" strain to its L-form under the influence of penicillin can be divided into two successive stages.

In the first stage, the destructive action of the penicillin on the mechanism of the cell wall formation influenced the synchronizing effect required at the time of growth and fission of the bacteria. At this time, the formation and division of the chromatin as well as the growth of the protoplasm proceeded normally, but the synthesis of the bacterial wall was hindered, thus resulting in bacterial cell wall defect. The continuous growth of the bacterial protoplasmic material bulged through the defected wall and led to the formation of extrusions of various sizes and shapes, hence hindered the binary fission process. Finally, the bacterial cell wall was entirely destroyed and the extrusions continued to grow. As a result, spherical bodies of various sizes were formed. Since the synchronization between the division of the chromatin and of the bacterial body was hindered, the cell stopped its binary fission. However, the synthesis and fission of the chromatin proceeded as usual, therefore in the protoplasm of the spherical bodies there accumulated more and more granular and vacuole-like structures. With Feulgen's staining method and fluorescence microscopic examination with acridine orange, we observed that these granular and vacuole-like structures contained chromatin substance. The finding and discussion will be made in a separate report (Li Hui *et al.* 1964). The spherical bodies and granules gradually accumulated, and under favourable agar content in the culture media, in a period of 3—7 days, typical colonies of the so-called "fried-egg" type, specifically associated with the L-form type of bacteria, appeared (Razin 1961).

The L-form thus formed was very unstable. On the withdrawal of the penicillin, they very easily resumed their original bacterial form. In the second stage of the L-form formation, they became stable through repeated passage. This L-form would not return to its original bacterial form even if the penicillin were entirely withdrawn. The number of passage required was different for different strains of bacteria.

Conclusion

A discussion on the formation of L-form of the *B. Proteus vulgaris* under the influence of penicillin is given in this paper. Using slide culture method and phase-contrast microscopic observations, the formation of the L-form of the *B. Proteus* could be divided into two stages. The first stage was a destruction of the synthetic mechanism of the bacterial cell wall and the loss of the synchronous effect of its growth and division, resulting in the formation of spherical bodies and granules; the second stage was the process of stabilization of the L-form.

Some of the cultural requirements in inducing the L-form formation were also discussed.

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