Influence of a Bacillus on the Reversion of *Hemophilus influenzae* L Forms to the Bacterial Form (35059)

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Clive and Landman (1) reported that the reversion of L forms of a strain of *Bacillus subtilis* to the bacterial form was greatly enhanced by the addition to the media of killed bacilli or of extracts of the cell wall of this organism. During the past few years, I have used a large, spore-bearing bacillus (designated as Bacillus Y) for the same purpose. Its effect is most conspicuous on the L form of *Hemophilus influenzae*, of certain strains of streptococci and of *Bacillus sp.*, and on some of the so-called "stable" L forms of *Proteus* and *Salmonella*. This report describes the reversion to bacteria of the L forms of *H. influenzae* under the influence of Bacillus Y.

Methods. The strain of *H. influenzae* used was a Type B strain freshly isolated from the spinal fluid of a patient with meningitis. The development and properties of the L forms of *H. influenzae* have been described previously (2). The Bacillus Y is a large gram-positive spore-bearing bacillus which was found as a contaminant. The culture of Bacillus Y spreads slowly on the surface of agar as a thick dry film. To avoid contamination of the tested culture, the following technic was used: The Bacillus Y was inoculated on blood agar in a short streak (about 5 by 10 mm). A section of the agar extending 10 mm or more beyond the inculum was cut out and transferred to a petri dish with the inoculate side downward. On top of this agar section on the area corresponding to the Bacillus Y inoculum on the underside, agar blacks containing the L culture to be tested were placed face downward. This procedure allowed for the diffusion of products of the bacillus to the L cultures. To examine the development of the bacteria from the L cultures, thin slices of agar bearing the test culture were placed face upwards on these agar sections. The petri dish was sealed to prevent drying. The cultures were incubated at 32°. Blocks of agar with L colonies of *H. influenzae* were also submerged in a filtrate of 1–2-day-old culture of Bacillus Y. The appearance of L forms and bacterial colonies was different and reversion to bacteria was apparent by the inspection of the culture and could be checked by simple microscopic examination. Contamination with the fast-growing Bacillus Y would be immediately apparent.

The photographs illustrating the reversion to bacteria in the large bodies and L type colonies were made from stained agar preparations in which the growth was visible in the original position, both on the surface or embedded in the media (3), Figs. 1–5.

Results and Discussion. When a young culture of *H. influenzae* was transferred to the appropriate agar medium containing penicillin, almost all of the bacterial cells converted into large bodies. From a few of the large bodies, L colonies developed. The large bodies at the early phase of development from bacteria, that is, up to about 12-hr exposure to penicillin, returned to the bacterial form when they were transferred to a medium without penicillin. This occurred very rarely at the later phase of their development. Under the influence of Bacillus Y, many large bodies retained the ability to revert to the bacterial form even though they had been exposed to penicillin for as much as 24 hr. After longer exposure of the large bodies to penicillin, the Bacillus Y was not effective in promoting bacterial development. Exposure of the large bodies to the influence of staphylococcus or destroying the penicillin by penicillinase did not noticeably influence

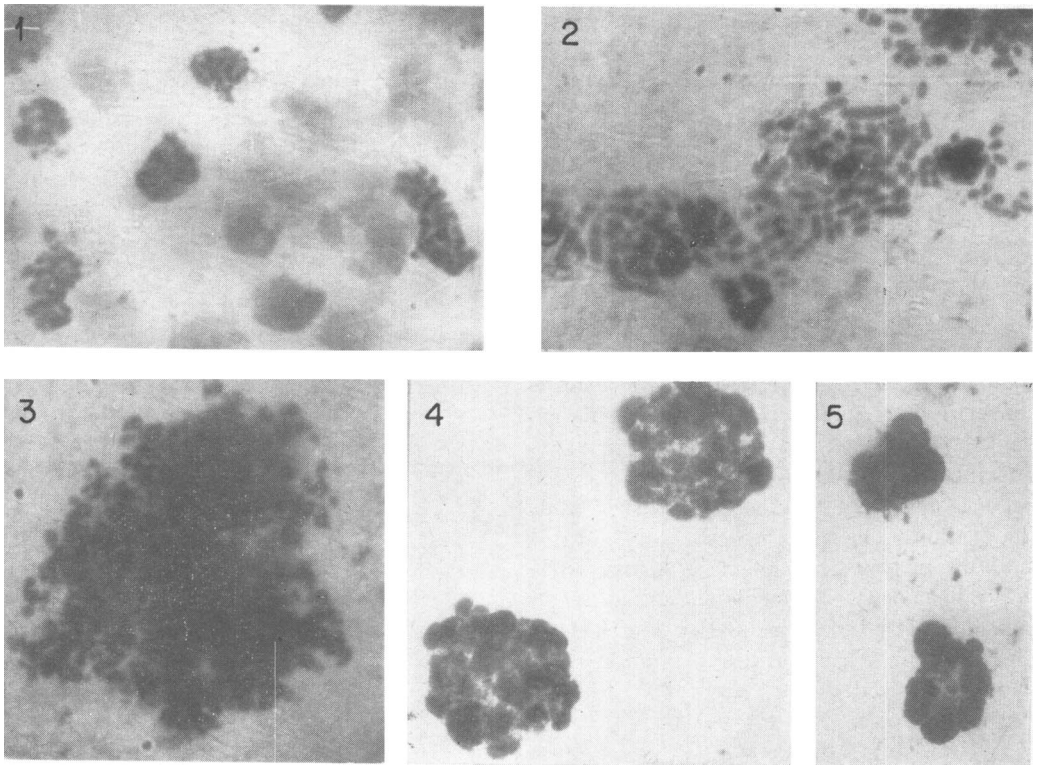

FIG. 1. Large bodies of *H. influenzae* on the surface of blood agar after 3-hr exposure to the influence of Bacillus Y. Some of the large bodies are deformed, darkly stained, and disintegrating into small forms; ×2250.

FIG. 2. Pleomorphic rods in an L colony of *H. influenzae*. These rods developed after exposure to Bacillus Y diffusible products for 24 hr; ×2250.

FIG. 3. Well developed L type colony of *H. influenzae* within blood agar; ×2250.

FIGS. 4 and 5. L colonies within blood agar after exposure to Bacillus Y for 24 hr; × 250.

the reversion of large bodies into bacteria. Moreover, other strains of bacillus found as contaminants did not promote reversion of L forms to the bacterial from.

The colonies of L forms produced from bacteria continued to enlarge in appropriate media for several weeks but after transplant to fresh medium, no growth or only a very few colonies developed. Without penicillin these were bacteria; with penicillin, L forms. Bacteria were not reproduced in the L colonies when the penicillin was destroyed by penicillinase, and rarely in L colonies growing in the immediate neighborhood of a staphylococcus culture. If a culture of L forms was submerged in a culture filtrate of Bacillus Y, reversion to bacteria occurred in every L colony. This occurred in various experiments as long as the L colonies were growing and contained well stained organisms. The different influence of Bacillus Y on the large bodies, and on the L colonies after long exposure to penicillin was probably caused by the continuous development of fresh growth in the L colonies.

The reversion of large bodies developed from bacteria under the influence of penicillin was observed microscopically (Fig. 1 and 2). After 3-hr exposure to the influence of Bacillus Y, most large bodies remained unchanged and faintly stained; some were disintegrating into darkly stained granules which later developed into bacterial forms. After 18- to 24-hr exposure to the influence of Bacillus Y, pleomorphic large rod-shaped bacteria grew out on the surface of the medium surrounding

darkly stained disintegrating large bodies (Fig. 2). The bacteria developed in the part of the L colonies embedded in the medium and produced sharply circumscribed spherical colonies. In general, L colonies exposed to Bacillus Y diffusible products contained less than a dozen to a few dozen small bacterial colonies inside their boundaries (Figs. 4 and 5). This indicates that reversion to bacteria did not occur *en masse,* but that very few of the L organisms reverted to bacteria. For comparison, an L colony within the agar is shown in Fig. 3. The L form organisms extended as groups or as single organisms growing into the medium, while the reverted bacteria remained together and pushed aside the medium. The bacteria recovered from the L forms in this strain of *H. influenzae* were very pleomorphic immediately after reversion, but after longer incubation regained the characteristic small size, gram-negative staining and rod shape.

Summary. Bacterial forms reappeared in L form colonies of various species of bacteria in the neighborhood of colonies of a large gram-positive spore-bearing bacillus, or immersed in the filtrate of a broth culture of this *Bacillus.* This effect was most conspicuous in L cultures of *Hemophilus influenzae.* The organisms in the L culture did not resume their bacterial form *en masse*; rather bacterial forms started to grow at a few sites within the L colonies. Several other strains of similar spore-bearing bacilli tested did not have a similar effect on the L forms.

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