L-FORMS OF MECHNIKOV'S VIBRIO AND NAG VIBRIO OBTAINED WITH TETRACYCLINE AND THEIR BIOLOGICAL PROPERTIES

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Stable L-forms were obtained by the action of tetracycline on Mechnikov's vibrio and the NAG vibrio, for the first time. Conversion of the L-forms of the vibrios by tetracycline is similar to that obtained by the use of penicillins. By passage of the L-forms three types of cultures were obtained: stable tetracycline-resistant L-forms, stable L-forms highly resistant to tetracycline, and tetracycline-dependent L-forms. Strains of vibrios having the typical complex of biological properties as well as atypical strains of vibrios were formed by reversion of the unstable L-forms.

KEY WORDS: vibrios; tetracycline; stable L-forms.

Preparations of the tetracycline series have been used with success for the treatment and prevention of several infectious diseases and, in particular, of cholera. The antibacterial action of tetracycline is linked primarily with a disturbance of protein synthesis on the ribosomes [3, 4]. According to one report [1], tetracycline disturbs the function of the mesosomes, and precursors of the cell wall accumulate in the cells through its action [5]. At the same time, when biosynthesis of the cell wall is disturbed, L-forms of bacteria can be produced (for example, under the influence of penicillin).

The object of this investigation was to study whether tetracycline has a L-converting action on vibrios.

EXPERIMENTAL METHOD

The following bacterial strains were used: Mechnikov's vibrio, NAG vibrios Nos. 69, 3350, and 116 (obtained from Professor Z. V. Ermol'eva) and No. 200 (obtained from the Saratov "Mikrob" Institute).

The nutrient media consisted of a 0.3% tryptic digest of bovine heart (pH 8.2) in agar with 20% normal horse or bovine serum (0.3% nutrient agar with 20% serum, pH 8.2).

Preparation of L-Forms. L-forms were obtained by Levashev's method [2]. Tetracycline hydrochloride was added to the nutrient medium in concentrations of 0.5 to 700 μg/ml. The concentration of the vibrios added to the medium was $2 \cdot 10^8$ cells/0.1 ml. The cultures were grown at 37°C. The seedings were checked every 24 h under the phase-contrast microscope.

Biological Properties of the L-Forms. Biochemical activity relative to glucose, lactose, galactose, rhamnose, sucrose, mannitol, xylose, and starch was studied on media described above with the addition of Andrade's indicator or bromthymol blue. The proteolytic activity was determined by the gelatin test.

The sensitivity of L-forms to trypsin, EDTA, lysozyme, and sodium dodecylsulfate was determined turbidimetrically at 630 mμ and also from the ability of the treated cells to grow on nutrient media.

The cytopathic effect of the L-forms of the vibrios on a tissue culture was investigated relative to a culture of human embryonic fibroblasts (HEF). The cytopathic effect also was observed in a parallel series

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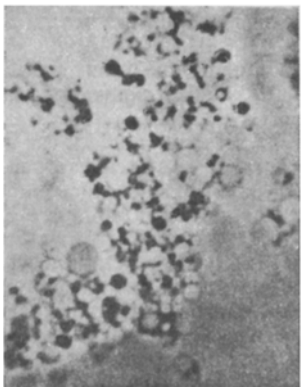

Fig. 1

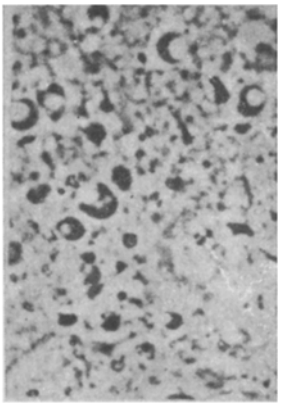

Fig. 2

Fig. 1. Morphological elements of L-forms of Mechnikov's vibrio 18 h after treatment with tetracycline. Phase-contrast microscopy, 1200 ×.

Fig. 2. Degeneration of morphological elements of L-forms of Mechnikov's vibrio 60 h after treatment with tetracycline. Phase-contrast microscopy, 1350 ×.

when tetracycline was present in the culture fluid containing L-forms and in the absence of tetracycline.

Reversion of L-Forms. Spontaneous reversion of the L-forms was investigated during their regular subculture for 1 year on nutrient media. To induce reversion, media containing various additives – diaminopimelic acid (DAP), glucosamine, and D-alanine – and also an extract of cell walls of vibrios were used.

EXPERIMENTAL RESULTS

During investigation of the morphological response to tetracycline, microstructural elements of L-forms were observed after 18 h within the concentration range of 2 to 12.5 μg/ml, but the processes of formation of the L-forms differed a little in the strains studied. For instance, Mechnikov's vibrio and NAG vibrio No. 200 were converted into spherical dark and light cells and vacuolated elements (Fig. 1). After 36-48 h, in a tetracycline concentration of 3-4 μm/ml, the whole population of seeded vibrios consisted of spherical and vacuolated bodies. In higher concentrations of tetracycline, mainly granular L-forms were observed. Microscopic observations showed that the spherical bodies were capable of reproduction and divided by transverse division, budding, and segmentation of large elements, i.e., the process of conversion and reproduction of L-forms of Mechnikov's vibrio and of NAG vibrio No. 200 under the influence of tetracycline was similar to that produced by penicillin. After culture for 60 h, processes of regeneration of the spherical elements were observed in the subcultures: they were filled with granular contents, they were vacuolated, and they disintegrated to liberate granular forms (Fig. 2).

The study of L-conversion of strains of NAG vibrio Nos. 69, 3350, and 116 showed that mainly granular L-forms were observed after the use of tetracycline within the dose range studied. Admittedly, after the first passages a few spherical forms appeared, but as a rule these were forms resulting from an incomplete cycle and they died rapidly (M-forms) [6].

Granular L-forms were subcultured on fresh media containing tetracycline in a concentration of 4 μg/ml. They grew as a rule in the depth of the agar column as brownish colonies with a flattened center, or else they grew diffusely and required high concentrations of agar (0.5-0.7%).

Subsequent passages of the subcultures were made in the thickness of serum-tetracycline agar with increasing concentrations of the antibiotic, and L-cultures also were seeded onto agar-serum media in order to establish stabilization. As a result of these experiments three types of L-cultures were obtained.

1. Stable tetracycline L-forms. These were obtained at the first passages and were grown in a tetracycline concentration of 4 μg/ml.

2. Stable L-forms highly resistant to tetracycline. These L-forms were able to grow on media containing 12-14 μg/ml of tetracycline.

3. Tetracycline-dependent L-forms. They could be subcultured only on media containing 4 μg/ml or more of tetracycline, and as a rule they died in the absence of the antibiotic.

The study of the biochemical properties of the L-cultures showed that they were unable to exhibit their biochemical activity with respect to the series of carbohycrates studied for a period of 7 days, unlike the original vibrios. Very weak splitting of sugars was observed on the 9th-11th day of cultivation. Stabcultures of the L-forms in gelatin as a rule did not cause liquefaction, or very slight liquefaction occurred on the 2nd-3rd day of cultivation, depending on the rate of growth of the L-forms tested.

A study of the ability of the tetracycline L-forms to give growth on penicillin media showed that all tolerated penicillin in a concentration of 5000 μg/ml well, without losing their ability to proliferate. Similar results were obtained with ristomycin and vancomycin.

The sensitivity of the L-forms of Mechnikov's vibrio and NAG vibrios Nos. 69 and 3350 to trypsin, EDTA, lysozyme, and sodium dodecylsulfate was investigated. Unlike the bacterial strains, the L-forms were sensitive to these agents, except lysozyme, from which it followed that they had no rigid cell walls.

When the cytopathic action of the L-forms on an HEF tissue culture was studied cytopathic changes appeared on the 3rd day in a series of tubes not containing tetracycline. The monolayer became less compact, its cells became partly vacuolated and rounded, and dark granules appeared. On the 5th day of cultivation only separate islands of cells remained on the coverslip.

In the series of tubes in which the nutrient medium contained tetracycline no such changes were found at these times. When culture fluid was seeded from the tubes onto nutrient media, characteristic growth of L-forms was observed, i.e., the L-forms retained their viability to tissue culture in the presence of tetracycline.

In the course of passage of the stable L-forms on nutrient media in the absence of tetracycline for 1 year reversion into vibrios was not observed. Only once was spontaneous reversion of a stable L-form of Mechnikov's vibrio observed at the 6th month of cultivation. In old subcultures, not transplanted for 3 months, no sign of reversion likewise was observed. During cultivation of L-forms of Mechnikov's vibrio and NAG vibrio No. 69 with DAP, D-alanine, glucosamine, and cell wall extract, reversion of these L-forms took place only in medium containing DAP and cell wall extract. A study of the biological properties of the reverted cultures showed that some of them had regained the properties of the original vibrios, but others were atypical (changes in morphology – swollen cells, disturbance of the rate of growth in broth, changes in biochemical properties, acquisition of resistance to tetracycline). Under these conditins the L-forms of NAG vibrios Nos. 200 and 3350 did not take place.

The study of the biological properties of L-forms thus showed that they have no cell wall and are analogous in their properties with penicillin L-forms of granular type. The ability of some strains of L-forms to revert on media with DAP suggests that they can be regarded as mutants for DAP. The other L-forms that did not revert on medium with DAP probably originated in a different manner.

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