Endotoxic Activity of L-Forms Derived from *Salmonella paratyphi* B.* (27804)

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L-forms exhibit many of the biosynthetic capabilities of the bacterial form from which they are derived. For example, L-forms of group A streptococci continue to secrete hemolysin, streptokinase, and deoxyribonuclease. The type-specific M protein, which is normally a constituent of the cell wall, is released into the medium(1). Carey and Baron(2) have shown that penicillin induced spheroplasts of *Salmonella typhosa* possess endotoxic activity for mice. The quantity decreased to one-tenth of that found in intact bacteria. We have followed the endotoxic activity of *S. paratyphi* B L-forms during successive transfers to determine whether endotoxin is a normal constituent of these forms or not. It was of special interest to determine whether stabilization of L-forms would result in a loss of synthetic capacities in regard to endotoxin.

Materials and methods. L-forms were derived from *S. paratyphi* B 777 obtained from O. E. Landman, Fort Detrick, Md. The medium used was essentially the same as described by Landman and Ginoza(3) with some modifications (suggested by them). It contained 2.8% Albimi brucella broth, 0.2M NaCl, 0.9% agar, 3×10^{-3}M $CaCl_2$, 10% unheated horse serum (Colorado Serum, Denver, Colorado), 1% rabbit red cell lysate (cells lysed in 3 vol. distilled water; stroma removed by centrifugation at 12,000 *g*), and 300 units penicillin/ml. The brucella broth, NaCl, and agar were autoclaved together in 88% of the final volume. After cooling to 45°C, the other constituents were added and plates were poured. Plates inoculated with 10^7 to 10^8 bacterial forms by the spread method showed confluent L-form growth in 10 to 14 days at 30°C. L-forms were collected from the surface of 10 to 20 plates by suspension in a diluent composed of 0.5M NaCl, 3×10^{-3}M $CaCl_2$, and 10% serum(3) with the aid of a bent glass rod. Much of the growth remained embedded in the agar after this procedure, but the L-form suspensions were relatively free of agar contamination. The preparations were examined by phase contrast microscopy for the presence of bacterial forms. For transfers, 0.1 ml of this suspension was spread on the surface of new plates. The preparations were washed twice by centrifugation at 5,000 *g* and were finally resuspended in the same diluent. Some of the preparations were suspended in saline and dialyzed overnight against distilled water. L-form membranes were produced by resuspending L-forms in distilled water with rapid pipetting. Membranes were removed by centrifugation at 25,000 *g*. Bacterial forms of *S. paratyphi* B were grown in brucella broth overnight on a shaker and washed twice in distilled water. All preparations were lyophilized and stored at room temperature in a vacuum desiccator.

Endotoxic activity was measured in mice made hyperreactive to endotoxin by intravenous injection of BCG 10 days prior to challenge(4). LD_{50} values were determined by injecting intravenously 2-fold dilutions of material into groups of 5 mice. LD_{50} values were calculated by the method of Reed and Muench(5). To obviate differences in susceptibility with different batches of mice, an LD_{50} with a standard preparation of *S. paratyphi* B was determined in each experiment. Results were expressed as a ratio to this standard.

Results. The morphology of the L-forms and bacteria used in these experiments is seen in Fig. 1. These L-forms were stable in that they did not revert to bacterial forms when medium without penicillin was used. Results on endotoxic activity are given in Table I. The LD_{50} values of the bacteria

* This investigation was supported by grant from NIAID of USPHS.

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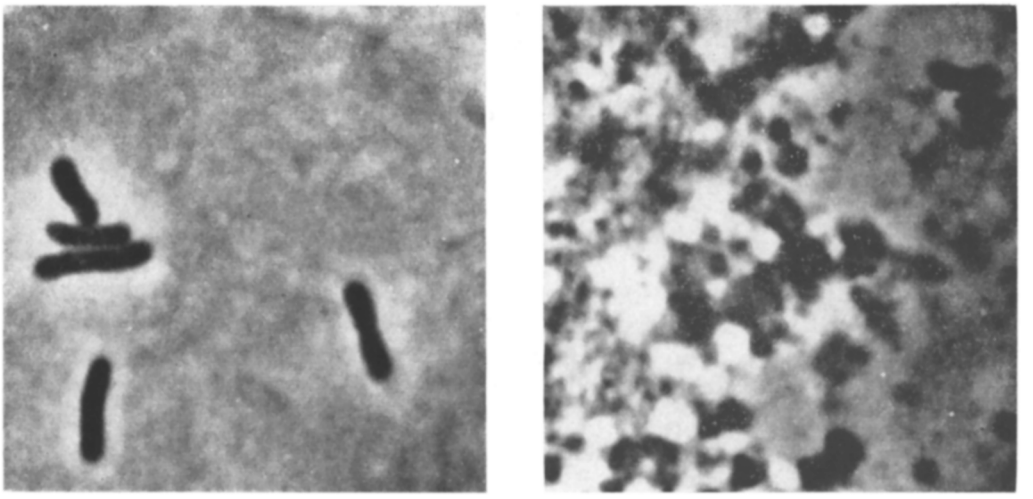

a b

FIG. 1. Phase contrast photomicrographs of (a) bacterial forms and (b) L-forms of *S. paratyphi* B. The suspending medium was agar in both cases. × 3700.

ranged from 1.2 μg to 1.4 μg in 5 determinations with a mean of 1.32 μg. The toxicity of the L-form was 5.5 to 9 times less than that of the bacterial form and remained relatively constant after 8 transfers. L-form membranes were slightly less toxic than intact L-forms in the one experiment tried. Dialysis of L-forms gave a decrease in toxicity in one instance and no significant change in another.

Discussion. Serologic evidence(6) indicates that cell wall constituents are found in L-forms of salmonellae. Since endotoxin of gram-negative bacilli is located for the most part in the cell wall(7), it is not surprising that toxicity is also found in L-forms. Our results indicate that after conversion to the L-form, bacteria continue to produce endotoxin. If this were not the case, the endotoxin present in the original bacterium should be diluted out during successive transfers.

TABLE I. Relative Toxicity of L-Form Preparations for BCG Vaccinated Mice.

No. of transfers	Relative toxicity* Intact	Membrane	Dialyzed
1 with penicillin	8.9	—	—
3 with penicillin	5.5	—	—
6 with penicillin	6.2	9.0	9.1
6 with penicillin followed by 2 without penicillin	7.2	—	7.5

* $\frac{LD_{50}\ L\text{-form}}{LD_{50}\ bacteria}$

Summary. L-forms derived from *S. paratyphi* B are 5.5 to 9.0 times less toxic than their parent bacterial form. Their toxicity remains relatively constant during successive transfers in the L-state.

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Received September 17, 1962. P.S.E.B.M., 1962, v111.