THE EFFECT OF LYSOZYME ON THE PRODUCTION OF TETANUS TOXIN. II. MOUSE M.L.D.*

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It has previously been reported[3] that during the production of tetanus toxin in the Mueller Medium, the medium becomes dark and turbid in about 24 hours, followed by clearing in four or five days. When clearing occurs, maximum toxin production or release is obtained as measured by Lf units. The simultaneous occurrence of clearing and presence of maximum Lf value has been attributed to "autolysis" of the organisms with simultaneous release of toxin.[3] An attempt to demonstrate an increased yield in toxin production by artificial lysis of the organisms with lysozyme revealed no rise in Lf titer, although there was a marked decrease in time of flocculation due to the presence of lysozyme during the early stages of growth.[4]

The present report deals with the effect of lysozyme on tetanus cultures as measured by their toxicity in mice.

Materials and Methods

The medium used for the production of tetanus toxin was prepared according to the most recent Mueller formula, consisting chiefly of "N-Z case," veal infusion, glucose, and several salts, amino acids, and vitamins.[2] The culture of *Clostridium tetani* (Harvard strain) was planted from the frozen state into a fluid thioglycollate medium and grown for 24 hours prior to inoculation into the toxin production medium proposed by Mueller. The lysozyme was a crystalline product prepared from egg white. Dilutions of the enzyme were made in Sørensen's phosphate-buffer at pH 6.29.

M.L.D. determination. A sample of the culture for lysozyme treatment was removed from a regular production lot and divided into three portions of 1.9, 1.9, and 2.0 ml. To the two 1.9 ml. samples were added 0.1 ml. of a lysozyme solution (100 mg. per ml.) and 0.1 ml. of the phosphate buffer respectively. The three tubes now containing 2.0 ml. each were incubated at 35° C. for six hours. Dilutions of these samples were then made in peptone-saline solution for mouse injection. Three separate dilutions were prepared from each of the three samples on each day of growth for injection into mice. Two mice weighing 16-22 grams were each injected intramuscularly into the left thigh with 0.5 ml. of each dilution. The minimal lethal dose was calculated on the basis of the result in each mouse injected according to the method of Ipsen.[1]

Gram stains were prepared from specimens of lysozyme-culture mixture, buffer-culture mixture, and plain culture after the tubes had been incubated for four hours.

Results

When samples from early phases of growth (24, 48, and 72 hours) were treated with lysozyme (5 mg. per ml. final concentration), there was an initial flocculation, occurring in 10 to 15 minutes, followed by a marked

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clearing which was complete in about two hours. This phenomenon did not occur with the two control samples containing either buffer solution and culture or culture only. In addition, clearing was not as evident in the lysozyme tube with the four-, five-, and six-day growths when natural autolysis was occurring most rapidly or was in its final stages.

Stained preparations made during various stages of growth confirmed the clearing effect of lysozyme on the culture. Stains from one-, two-, and three-day samples that were incubated with lysozyme showed rare Gram-negative cells, many ghost cells, and much débris, whereas corresponding control

TABLE 1

COMPARATIVE MEAN LOGARITHMIC M.L.D. VALUES ON LYSOZYME-TREATED AND CONTROL CULTURES OF ONE- TO SIX-DAY GROWTH DURING THE PRODUCTION OF TETANUS TOXIN

Days of growth of culture	*Lysozyme plus culture** *Experiment no.*				*Buffer plus culture** *Experiment no.*				*Culture only** *Experiment no.*			
	III	IV	VI	VII	III	IV	VI	VII	III	IV	VI	VII
1	3.40	4.15	...	<3.30	3.01	3.66	...	3.06	3.08	3.76	...	3.22
2	4.99	5.34	...	5.56	3.87	4.51	...	3.80	3.89	4.37	...	3.94
3	5.59	5.77	5.86	5.78	5.14	5.29	5.43	5.19	5.35	5.30	5.21	5.12
4	...	...	6.31	6.43	...	...	6.27	6.28	...	...	6.09	6.25
5	...	...	...	5.99	...	...	...	5.98	...	...	...	6.02
6	...	...	6.05	5.80	...	...	6.06	5.89	...	...	6.03	5.85

* Placed in incubator for six hours prior to dilution for M.L.D. determination.

samples contained large numbers of short and long rods typical of the Harvard strain of *Clostridium tetani.* In this respect, also, four-, five-, and six-day cultures were less visibly affected by lysozyme, since the enzyme-treated and control cultures were alike in revealing very few whole organisms. In the older cultures the organisms had apparently already undergone lysis prior to the addition of lysozyme.

The effect on toxicity of the treatment of tetanus cultures with lysozyme is shown in Table 1. The figures in this table represent the geometric mean of the M.L.D. values on six mice in most instances. It is evident from this table that only small amounts of toxin were present in the medium after 24 hours of growth, regardless of whether or not the culture was treated with lysozyme. There was, however, an increase in the amount of toxin present in the 24-hour culture as a result of lysozyme treatment in Experiments III and IV. In Experiment III, the logarithmic mean of the M.L.D. value of the lysozyme culture was 3.40, whereas the mean M.L.D. values of the buffer plus culture control, and the culture only were 3.01 and 3.08, respectively. These figures indicate a per cent increase in M.L.D. for

lysozyme-treated over the controls of 145 and 110 whereas there was only a 17% difference between the controls. For Experiment IV the mean differences were approximately the same with per cent rises in M.L.D. value of lysozyme-treated cultures over the controls of 210 and 144. The difference between the controls in Experiment IV was 27 per cent. Table 1 also reveals that the maximal effects of lysozyme in increasing the M.L.D. in mice was on two-day cultures. On the three-day culture there was a sharp drop in effectiveness of lysozyme. Four-, five-, and six-day growths revealed little or no effect due to lysozyme.

TABLE 2

DIFFERENCES BETWEEN MEAN M.L.D. VALUES OF LYSOZYME-TREATED TETANUS CULTURES AND THE CONTROL CULTURES

Days of growth	*Mean M.L.D. of:*		*Factor of difference in value between*	
	Lysozyme plus culture L	*Buffer-culture controls* B-C	*L and average of* B-C	B and C
1	8,320	2,560	3.31	1.0
2	226,000	14,200	15.9	1.2
3	575,700	184,350	3.1	1.1
4	2,355,000	1,703,000	1.4	1.2
5	980,000	999,500	1.0	1.1
6	874,000	939,000	1.1	1.1

The increase in M.L.D. value as a result of lysozyme treatment for the six days of growth in comparison with the difference in M.L.D. value of the controls (buffer plus culture and culture only) is summarized in Table 2. This table reveals that there is some rise in M.L.D. on the first three days of growth as a result of treatment with lysozyme, the most marked rise in M.L.D. being in the two-day culture with a maximal rise of 15.9 times, while the difference between the two controls was only 1.2. No significant difference can be noted in Table 2 in mouse M.L.D. between the lysozyme-treated and the control samples for the four-, five-, and six-day growths.

The results of lysozyme treatment on the four- to six-day growth as compared with the untreated cultures can also be seen in Table 1. The M.L.D. values in this table reveal no increases as a result of lysozyme activity on the culture at five and six days. There appears to be a slightly higher mean M.L.D. value for the lysozyme-treated culture in both Experiment VI and VII on the fourth day, but the significance of this difference might be considered doubtful.

Table 2 and Experiments VI and VII of Table 1 appear to indicate also that the maximal mouse M.L.D. value is reached after four days of incuba-

tion by this method of toxin production. This peak is followed by a slight decrease in M.L.D. on the five- and six-day-old cultures. Evidently when the peak M.L.D. value is reached, lysozyme treatment thereafter does not increase the toxicity of cultures to mice.

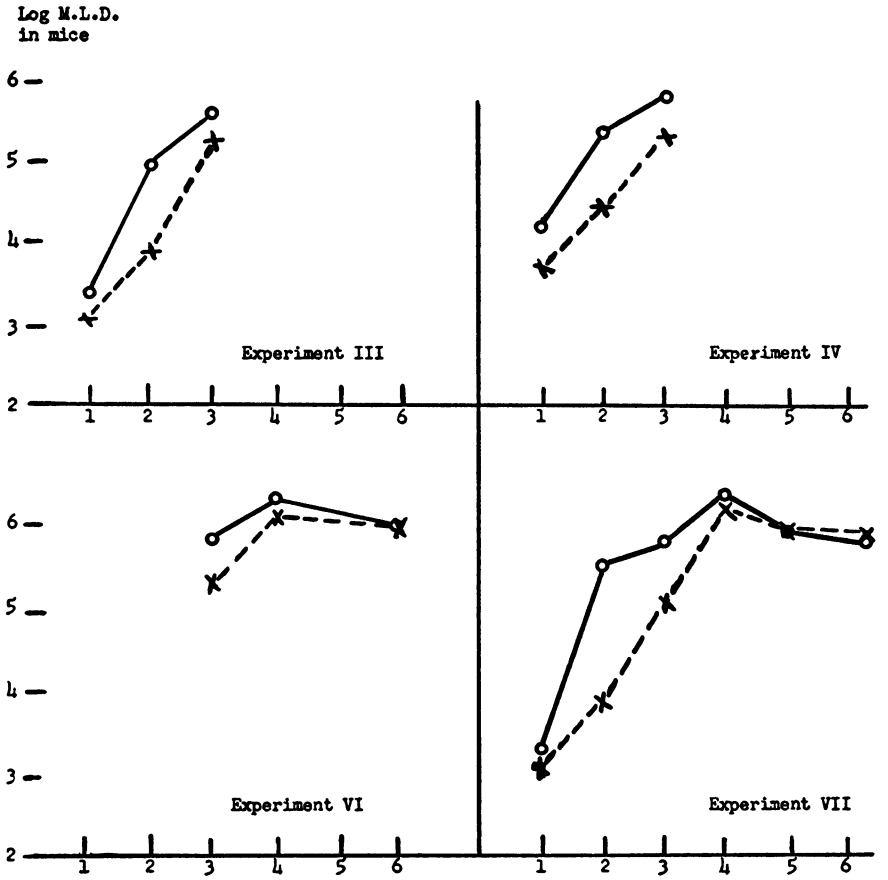

FIG. 1. The log of the M.L.D. in mice for each day of incubation comparing samples of cultures treated with lysozyme (circles and solid lines) and the average results of the controls (x's and dotted lines) for each of four experiments.

Figure 1 illustrates graphically the results of Tables 1 and 2. For each day of growth the M.L.D. value rises in each experiment to a peak at four days, and following the three-day growth, the lysozyme curve and the curve representing the average of the controls approach each other, indicating decreasing difference in M.L.D. value for lysozyme versus controls.

In order to make more certain that the lysozyme activity was directed on the cells and not on some material in solution, supernatants of cultures centrifuged for 30 minutes were treated in the same manner as whole cultures after each of the first three days of growth. On none of these three

days was there any evidence of toxin present in either lysozyme-treated or untreated supernatant fluids at the lowest dilutions tested which were 1-100, 1-2000, and 1-50,000 for one, two, and three days' growth, respectively. Untreated, whole culture controls for these three days showed mean M.L.D. values of 2,200 for the one-day growth, 12,000 for the two-day growth, and 288,000 for the three-day growth.

In order to rule out the possible toxicity of lysozyme itself as an influential factor in causing the increased M.L.D., lysozyme alone in 0.2 mg. quantities was injected into each of two mice. This represents nearly ten times the greatest amount of lysozyme introduced into mice in the lysozyme-toxin experiments. The mice were observed for seven days and showed no signs of toxicity at any time during this period.

Discussion

As a method of further evaluating the Mueller hypothesis that tetanus toxin was liberated into the medium at the time the organisms underwent autolysis on about the fifth day of growth, lysozyme was used to lyse these cultures artificially in the early (first three days) stages of growth. As a result of induced lysis of the tetanus bacilli, M.L.D. values in mice were increased as much as 16 times on two-day cultures, at which time the maximum effect of lysozyme occurred.

Although there was a marked enhancement of the toxicity to mice of tetanus broth cultures as a result of treatment with lysozyme on the first three days of growth, there were no instances in which maximal yields of toxin were obtained during these three days despite apparently complete lysis. This can be explained by the possibility that the organisms had not as yet manufactured maximal amounts of toxin even after three days of growth and that the only effect of lysozyme is to liberate all the existing toxin from the cells.

It seems to be evident that the greatest yield of toxin as measured by mouse M.L.D. can be obtained after four days of incubation. At this time the culture shows its earliest gross and microscopic evidence of being autolyzed and is similar in appearance to younger cultures that have been incubated with lysozyme for four hours. Also at this time, lysozyme begins to show a lack of activity on the culture in respect to increasing the M.L.D. value in mice. After four days of growth, the mouse M.L.D. begins to drop so that it is lower after five days and still lower after six days. This decreasing toxicity undoubtedly represents natural self-detoxification of the tetanus toxin, a phenomenon which is commonly known and accepted. During the last two days of growth lysozyme has no effect on toxicity.

Supernatants from centrifuged early cultures (one, two, and three days) that were treated with lysozyme resembled the untreated supernatants in showing no signs of toxicity for mice. Corresponding whole cultures showed normal M.L.D. values during these days. This once more indicates that the

toxin is associated with the cell and is not liberated until the cell ruptures. It does not, however, necessarily follow that the toxin is present or remains within the cell membrane, since it may possibly be adsorbed to the surface of the cell, remaining there until the cell is lysed.

Summary

1. Samples from tetanus cultures were treated with lysozyme, to produce lysis, during each day of six days of growth.

2. Lysozyme produced lysis of the organisms during the first three days of growth, but had no effect during the last three days since autolysis had already occurred.

3. The toxicity of the cultures, measured as mouse M.L.D., was markedly enhanced by lysozyme treatment of the first three days' growth, but there was no effect on cultures four to six days old.

4. The maximal M.L.D. value in mice was obtained after four days of growth regardless of whether or not the cultures were treated with lysozyme.

5. Supernatant fluids, both untreated and lysozyme-treated, from centrifuged cultures, one to three days old, showed little or no toxicity for mice.

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References

1 Ipsen, J.: Die Auswertung des direkten Giftwerte des Tetanusgiftes als Beispiel der biomathematischen Ausnutzung der Absterbedauer. Arch. exp. Path., Lpz., 1941, *197,* 536.

2 Mueller, J. H.: Unpublished data, 1951.

3 Mueller, J. H. and Miller, P. A.: Unidentified nutrients in tetanus toxin production. J. Bact., Balt., 1948, *56,* 219.

4 Stone, J. L.: The effect of lysozyme on the production of tetanus toxin. I. Studies with flocculation. J. Bact., Balt., 1952, *64,* 299.