ON THE MODE OF RELEASE OF TETANUS TOXIN FROM THE BACTERIAL CELL

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By classical textbook definition an exotoxin, of which tetanus toxin is generally considered an example, is one that is found chiefly in the medium apart from the cells in contrast to the endotoxin which can be separated only by rupture of the cell itself (Jordan and Burrows, 1946; Wilson and Miles, 1946; Smith and Martin, 1948).

Recently Reynaud (1951) has shown that large amounts of tetanus toxin are found closely associated with the cells and could be obtained from the cells by extraction with hypertonic solutions or by the use of ultrasonic waves to rupture the cells. Stone (1952, 1953) used lysozyme to produce lysis of the tetanus bacillus and was able to obtain increased yields of tetanus toxin in the medium as measured by minimum lethal dose although the Lf value showed no rise.

Since these recent studies would tend to refute the classical views of the source of tetanus toxin, it was considered timely to investigate further the source of the toxin in relation to the cell. This report deals with such a study.

MATERIALS AND METHODS

Medium and culture. The 1951 Mueller toxin production medium (Mueller, 1951) generally was used for growth of the culture (Harvard strain) and for toxin production. Only one and two day growths were studied for toxin source. The lysozyme used in this study was a crystalline product obtained from Armour Laboratories.

Bacterial counts. Bacterial counts of the organism were performed by preparing serial dilutions in hundredths in peptone-saline solution and transferring one ml or one-tenth ml of the proper dilution to a tube containing approximately 50 ml of fluid thioglycolate medium. If the proper dilution was selected, colonies evidently representing individual clostridia were distributed throughout the medium 18 to 24 hours after inoculation, and these colonies could be counted readily. Since there was usually good agreement in adjacent tubes sometimes representing different dilution tubes of the same culture and since repeated counts of the same types of cultures were similar, this was considered a valid method for determining the number of organisms per ml.

Washing of cells. The cells were washed with a one per cent peptone solution in physiological saline. The cells of 5 ml of the one or two day culture were spun down at top speed in the International clinical centrifuge for 15 minutes. After the supernatant was decanted, 5 ml of the peptone solution were used to resuspend the sedimented cells. Repeated centrifugation and resuspension were carried on the desired number of times depending upon the conditions of the experiment.

Minimum lethal dose. The minimum lethal dose was determined in mice by calculation from the death time using a previously determined method (Ipsen, 1941).

EXPERIMENTAL RESULTS

Since previous studies appear to reveal that tetanus toxin is located within the cell or attached to the surface of the cell, an attempt was made first to wash all the toxin from the surface. Despite repeated washings of the culture, however, (in some cases up to 15 or 20 washings in a period of 5 to 7 hours) tetanus toxin could still be detected in the supernatant fluid, usually in fairly constant amounts for each wash fluid following the first or second washing. Table 1 shows the number of mouse minimum lethal doses per ml in particular supernatant fluids for a series of different trials.

It would be impossible for any soluble toxin that was present apart from the cells of the original culture to be detectable for more than one or two washings. It is assumed, therefore, that the toxin found in the supernatant fluids has arisen as a result of the presence of the cells, and this could be possible in any one of at least 5 different ways. Five possible mechanisms by which toxin could be released from the cells are listed as follows: (1) the presence of an enzyme

TABLE 1

Mouse minimum lethal dose (M.L.D.) per ml in supernatant fluids from washings of 1 and 2 day cultures

SUPERNATANT NUMBER	M.L.D. PER ML									
	1 day culture						2 day culture			
1	60	190	275	230	125	65	800	2,900	1,200	800
2	20	30	90	57	75	4	400	420	—	—
3	—	—	—	—	—	—	250	430	—	—
4	—	40	35	59	—	5	350	<300	325	—
5	15	—	—	—	40	4	130	—	—	125
6	—	—	—	—	—	—	200	—	—	—
7	—	—	—	—	—	—	175	—	—	—
8	—	30	30	29	—	—	250	—	175	—
10	5	—	—	—	30	—	—	—	—	80
12	—	—	—	—	—	—	—	—	325	—
15	—	—	—	—	—	—	—	—	500	50
20	5	—	—	—	—	—	—	—	—	—
Original whole culture.....	700	2,000	2,200	2,400	2,000	590	9,000	14,000	7,400	6,400

from the cells producing toxin in the supernatant; (2) the presence of toxicity in the supernatant due to the failure of some cells to sediment; (3) the release of toxin from the interior of the cell by autolysis; (4) the release of toxin from adsorption on the surface of the cell; and (5) the diffusion of toxin through the cell membrane. Since it was impossible to study these eventualities directly and positively, indirect methods and the process of elimination were used in order to determine which of these methods was the most feasible for allowing the presence of soluble toxin to become separated from the cells. The succeeding paragraphs deal with elimination of the possibilities that are unlikely.

Presence of an enzyme in the medium. Since a fairly complex medium is usually essential to produce appreciable quantities of toxin, it seemed unlikely even at first glance that this was the source of the toxin in a supernatant containing only peptones and sodium chloride. An enzyme might produce toxin in so simple a medium if it were released from the cells into the peptone-saline wash liquid. Evidence to rule out this possibility is shown by the following experiment. The supernatant fluid of a two day culture, containing 2,000 minimum lethal doses per ml and 230,000 organisms per ml, was filtered through a Berkfeld candle. This yielded a filtrate containing no organisms and 850 minimum lethal doses per ml. One ml of the filtrate was added then to each of two tubes containing 4 ml of the regular toxin production medium. One tube was placed in the incubator for one day, and the other tube was maintained in the cold room for the same length of time. The minimum lethal dose titers of these tubes after the one day period were 87 and 125 per ml, respectively. Since originally 170 minimum lethal doses per ml were present in each tube, there was an actual drop in toxin titer and not a rise as would be expected if a toxin producing enzyme were present in the culture fluid.

Presence of cells in the supernatant. Centrifugation for 15 minutes failed to sediment all the organisms, and therefore it was necessary to determine whether or not the cells in the supernatant were responsible for the toxicity in mice. A cell-free supernatant prepared by Berkfeld filtration still contained a considerable amount of toxin. This is evident in the experiment (concerned with the presence of an enzyme) in which a filtered supernatant was found still to have 850 minimum lethal dose units per ml despite the fact that the count per ml was zero. A repetition of this gave similar results.

As additional evidence that the number of organisms in the supernatant does not account for the minimum lethal dose, table 2 reveals that there is no uniformity in the count as compared with the minimum lethal dose. This table shows that the first supernatant has the greatest number of minimum lethal doses per ml whereas subsequent supernatant washings with lower minimum lethal dose values may have far greater numbers of organisms per ml.

TABLE 2

Comparison between minimum lethal dose (M.L.D.) values and bacterial counts in supernatant fluids

SUPERNATANT NO.	COUNT PER ML	M.L.D. PER ML
1	850,000	125
2	100,000	75
5	3,550,000	38
10	525,000	31
Repeat experiment		
1	300,000	65
2	470,000	4
3	10,000	5
4	240,000	4
Repeat experiment		
1	105,000	2,000
2	280,000	1,450
3	150,000	900

Release of toxin due to cell rupture. Since repeated centrifugations and washings continued to yield only small quantities of toxin in each supernatant, it seemed unlikely that repeated trauma on the cells for up to 15 or 20 washings, in some instances, would cause the breakage of only a few cells for each washing. Even less likely would be the repeated finding of approximately the same number of minimum lethal doses per ml for any washing taken at random (after the first or second washing) if cell rupture were the chief cause of the presence of toxin in the supernatant. It would be more plausible to expect small amounts of toxin at first as the more delicate cells broke, followed by large quantities of toxin as repeated damage caused the majority of the organisms to rupture. Since this presumption was not deemed sufficient to rule out autolysis as the mechanism, a series of experiments was performed to confirm or deny it.

In order to determine the effect of standing at cold room or incubator temperatures a group of experiments were run for this purpose. In most cases cell suspensions were allowed to stand (after 8 or 9 washings) in the cold room or incubator for one or more days. Cell counts were performed before and after standing in order to determine count drops (possibly due to autolysis), and toxin titers were done on the supernatants after standing in order to determine the minimum lethal dose rise during that period of time. Table 3 shows the results of this set of experiments. It is evident that there is a considerable amount of irregularity in count drop by comparison with minimum lethal dose rise. Detectable drops in count were not always present although there was always considerable rise in minimum lethal dose. In addition, there were instances in the same experiment, i.e., using the same culture, whereby an equal or lesser count drop was accompanied by a greater yield in toxin. There were also cases in which the incubated sample yielded less toxin than the cold room sample of the same culture although incubation almost invariably caused a much greater drop in count. A 50 per cent drop in minimum lethal dose titer was considered as the detoxification loss as a result of incubation for one day since other determinations have indicated that this would be the approximate extent of toxin loss. The figures in table 3 would indicate also that standing for 3 or 4 days would result

TABLE 3

Comparison between cell count drop and minimum lethal dose (M.L.D.) rise as a result of incubator or cold room storage

TREATMENT OF CULTURE	COUNT PER ML IN MILLIONS BEFORE STORAGE	COUNT PER ML IN MILLIONS AFTER STORAGE	COUNT DROP PER ML IN MILLIONS	PER CENT DROP IN COUNT	M.L.D. PER ML AFTER STORAGE
CR—1 day	100	100	0	0	1,400
CR—1 day	100	120	0	0	2,000
CR—1 day	165	110	55	33	500
*CR—1 day	140	90	50	35	3,800
*CR—1 day	165	100	65	40	900
CR—1 day	85	50	35	40	400
*CR—1 day	200	100	100	50	700
CR—1 day	180	80	100	55	900
*CR—1 day	255	115	140	55	1,100
CR—3 days	110	20	90	80	1,400
CR—4 days	80	10	70	88	2,000
*CR—3 days	100	2	98	98	2,300
*CR—3 days	90	2	88	98	3,800
*Inc—1 day	110	1	110	100	350
Inc—1 day	85	1	85	100	250
Inc—1 day	255	1	255	100	1,400
*Inc—4 days	140	1	139	99	7,000

CR = cold room; Inc = incubator.

* Culture was placed in cold room or incubator after one washing; all others received 8 or 9 washings.

TABLE 4

The effect of time on the amount of toxin released from tetanus organisms

DAY OF TEST	SUPERNATANT NO.	M.L.D. PER ML IN SUPERNATANT	COUNT PER ML OF SEDIMENT IN MILLIONS	SUPERNATANT NO.	M.L.D. PER ML IN SUPERNATANT	COUNT PER ML OF SEDIMENT IN MILLIONS
1	1	290	110	2	29	155
2	1	275	7	1	323	19
	2	47	2	2	83	12
	5	62	2	5	123	5
	10	35	<1	10	55	2
3	1	326	0.01	1	314	0.01

The horizontal line separating each day of test indicates that the suspended sediment was maintained in the cold room for 1 day at that point.

TABLE 5

Comparison of the effect of storage on viable counts and density readings

EXPERIMENT NUMBER	ORIGINAL CULTURE OR SEDIMENT			COLD ROOM OR INCUBATOR FOR 1 DAY		
	Count per ml in millions	M.L.D. per ml in supernatant	Density reading	Count per ml in millions	M.L.D. per ml in supernatant	Density reading
19	152	5	0.268	—	20	0.280
	153	600	0.327	160	1,750	0.283
	152	5	0.268	—	20	0.285
	153	600	0.330	107	1,700	0.331
22	215	0	0.485	175	7,000	0.498
	150	0	0.520	150	4,000	0.502
	250	0	0.485	170	—	0 486

frequently in far more toxin in the supernatant even though the decrease in bacterial count was not necessarily greater.

Since the length of time appeared to have some influence on the extent to which toxin rose, another experiment was performed in an attempt to confirm the possibility that length of time of standing had more effect on the amount of toxin in the supernatant fluid than the decrease in bacterial count. In this experiment two samples of a one day culture were centrifuged simultaneously. In one case the first supernatant was tested and the sediment was resuspended in peptone-saline. In the other instance the second supernatant was tested and the second sediment was resuspended in peptone-saline. Both tubes were kept in the cold room for one day and each culture then was washed 10 times. The final sediment of both tubes was suspended in the washing solution and once more maintained in the cold room for one day. The supernatants were tested again for toxin and the sediments for viable cells. The results of this experiment are summarized in table 4. It seems evident by a study of this table that maintenance of the culture for one day allows the release of approximately the same amount of toxin, which is in the vicinity of 300 minimum lethal doses per ml, whereas the 15 minute wash fluids contain much less toxin. The amount of toxin in the supernatant appears to have no relationship to the loss of viable bacteria. For example, the drop in count after the first day in the cold room was more than 100 million whereas the drop in count after the second day in the cold room was not more than two million, yet in each instance the minimum lethal dose was at approximately the same level. A possible explanation could be that a large proportion of the cells died after the first day in the cold room, but the amount of autolysis was sufficient for only 300 minimum lethal doses per ml and that more autolysis of these cells occurred later so that the second day in the cold room also released about 300 minimum lethal doses per ml by autolysis. This explanation, however, is not likely since so much uniformity of toxin release would not be expected to result from a haphazard breaking of dead cells. In addition, we believe it seems reasonable to assume that the majority of the dead cells would be more apt to rupture as a result of the repeated, though mild, agitation during the washing procedure than by merely standing in the cold room.

It is evident at this point that maintaining culture for periods of one or more days yields large drops in count due to the death of the organisms, but this is not necessarily followed by autolysis or rupture of the cells. A more direct approach to studying whether or not these dead cells have undergone lysis or whether they are chiefly intact could be through periodic density readings. It can be observed in table 5 that there was no significant change in density in most instances although in many of these cases large decreases in the number of viable bacteria were apparent. In addition large amounts of toxin were found in the supernatant so that the indi-

cations are that generally large amounts of toxin are being released, the bacteria are dying in varying numbers, but the densities of the suspensions are remaining fairly constant.

To show a possible relationship between the amount of toxin released and the number of cells lysed, lysozyme was used to break up virtually all the cells in a given culture or suspension, and the number of lysed organisms (determined by count drop) per minimum lethal dose rise in supernatant was calculated. The average number of organisms lysed to yield a one minimum lethal dose rise in toxin for one day cultures was about 10,000. For two day cultures, including whole cultures and suspended sediments of cultures, the results were a range of 180 to 1,700 lysed organisms per minimum lethal dose rise with a mean value of approximately 750. By comparison suspended sediments of two day cultures were stored for one or more days in the cold room or incubator and these sediments required from 10,000 to 440,000 cells to produce a one minimum lethal dose rise in titer. This was calculated by suspending the sediments in toxin-free peptone and determining the number of minimum lethal doses per ml in the supernatant as well as the drop in viable count per ml of the sediment at the end of the storage period. Thus, if the drop in cell count was 120,000,000 per ml and there were 5,000 minimum lethal doses per ml in the supernatant, it could be assumed that if the toxin rose as a result of the death and autolysis of the cells, for each minimum lethal dose present in the fluid, 24,000 cells were lysed. These results do not agree with the findings in artificial lysis of two day cultures or sediments which require only 750 cells to produce one minimum lethal dose or less than 4,000,000 organisms to produce the above mentioned 5,000 minimum lethal dose. It is possible but again not likely that 120,000,000 organisms died, but only about 3 per cent of these underwent autolysis to yield the 5,000 minimum lethal dose per ml of toxin found in the supernatant fluid.

A series of experiments was performed then on one and two day cultures in order to compare the effect of agitating for 15 minutes with centrifuging for 15 minutes or of centrifuging for 3 hours with centrifuging for 15 minutes. A control tube allowed to stand for 3 hours was compared with the tube centrifuged for 3 hours. In general the tube that was shaken fairly vigorously for 15 minutes revealed approximately the same minimum lethal dose value in the supernatant and same count in the sediment as the tube that was centrifuged for 15 minutes. In addition, 3 hour centrifugation as well as 3 hour storage at room temperature usually gave higher minimum lethal dose values than the samples treated for 15 minutes. Three hour room temperature storage frequently gave equal or higher toxin levels than 3 hour centrifugation. Sediment counts were about the same in all cases. Apparently the length of time rather than the type of treatment is the more important feature. If cell breakage were the cause of the release of toxin, it would be expected that a harsher form of treatment such as continued agitation would rupture the cells more readily than centrifugation or merely standing. Also 3 hour centrifugation should show consistently far higher minimum lethal dose values than 3 hour standing.

It appears logical to conclude by the above groups of experiments that autolysis is not the source or cause of all the toxin found in the supernatant washings.

Release of adsorbed toxin by washing. If the toxin was attached only weakly to the surface of the cell by adsorption, washing would rapidly remove most of the toxin in large quantities during the first few washings, and later washings would tend to drop in minimum lethal dose to negligible levels. Since this situation does not exist, it is probably true that the attachment is strong or else adsorption does not account for the toxin in each washing. If it were a strong attachment, the likelihood is remote that storage alone for one day could release sometimes 50 times more toxin than repeated washings. Repeated washings generally are considered to be the more efficient method of separation of materials.

In addition, adsorption alone still does not answer the question of the source of manufacture of the toxin. If the toxin is not produced outside the cell in the medium, it is produced probably inside and still must diffuse out to the surface in order to become adsorbed. The remote possibility that toxin was formed on the surface of the cell and then became attached to the surface was considered not likely in view of the previous facts.

Diffusion through the cell membrane. None of the evidence discovered seemed to oppose this possibility. Molecules of toxin size might be considered of too great magnitude to diffuse readily through the cell membrane, but some

form of selective secretion could occur. This phenomenon is probably true of enzymes that are produced within the cell and found in the fluid medium. The possibility also occurred that a small prosthetic toxin molecule diffused through the cell membrane and combined with a protein in the medium, but the fact that the toxin is still present in the absence of protein in the medium would tend to rule out this eventuality.

The facts that each 15 minute washing allows a fairly uniform amount of toxin to be released from the cells, that longer periods of time result in larger amounts of toxin, and that drops in count are variable or may be absent in the presence of the release of similar quantities of toxin are all compatible with a diffusion process.

DISCUSSION

The existing evidence appears to associate the toxin of *Clostridium tetani* with the interior of the cell. The present report indicates that the toxin is liberated somehow from the cell itself and can be found entirely independent of the cell despite the fact that it almost undoubtedly was within or attached to the cell only a short time previously. In view of the results of several experiments the cell-free toxin does not appear to be the product of enzymatic action on the medium, the result of washing adsorbed toxin from the surface of the cell, nor can its presence be explained entirely by autolysis of the organisms. A process of diffusion or some form of secretion or excretion through the cell membrane can explain the occurrence of tetanus toxin apart from the cells as a result of repeated washings. A longer period of storage, as one might expect by diffusion, results in the presence of greater amounts of toxin in the medium. This may, however, eventually reach an equilibrium which the following experiment will support. Repeated centrifugation in which the original washing solution was merely withdrawn and repipetted into the same tube to break up the sediment did not give minimum lethal dose rises equivalent to those found in the same number of repeated washings with fresh washing solution each time. If diffusion approaching equilibrium is the explanation, it can be considered that the presence of toxin in the medium inhibits the rate of continued diffusion. If cell rupture were the reason for the presence of toxin, the amount of toxin would increase similarly after each washing regardless of whether the wash solution were the same one or a fresh one.

Assuming that a form of diffusion releases small amounts of toxin into the supernatant fluid, calculations were made of the percentages of diffusion of one and two day cultures for the first supernatant (i.e., a long period of storage) and for later supernatants (i.e., a short period of storage). The calculation was accomplished by determining the amount of toxin per ml in the supernatant and the total amount of toxin per ml in the whole culture. The latter was determined by completely lysing the culture with lysozyme. The percentage of toxin in the supernatant compared to the total toxin was considered as the per cent of diffusion. The per cent diffusion in the first supernatant ranged from 2.5 to 14.0 and in the second or later supernatants from 0.3 to 2.5 for 9 determinations on one day cultures. For 8 determinations on two day cultures the per cent ranges for first supernatant and later supernatants were 0.3 to 5 and 0.09 to 0.8, respectively. The mean percentage values for first supernatants of the one and two day cultures were 7.6 and 1.6, respectively. Two independent samples of two day cultures were stored as suspended sediments for a day in the cold room and the percentage diffusion determined. The results were 1.5 and 1.8 per cent comparing favorably with the mean value for two day whole cultures of 1.6 per cent.

Although diffusion from the cells appears to explain the presence of tetanus toxin in the fluid, autolysis is not necessarily completely eliminated as a source. Small amounts of the total toxin in the medium of early cultures may be due to the rupture of weak cells either during the natural state of growth or during the various ways of handling the samples presented in this paper. An explanation of the source of toxin during natural toxin production could be summarized in the following statements. Toxin is produced entirely within the cell. Small amounts of toxin are being released into the medium by diffusion from the cells during the first two days with possibly very minute amounts released by autolysis. Autolysis becomes more and more manifest after 3 days of growth so that the greater proportion of toxin found in the medium at the time of harvest is actually due to autolysis of the older cells.

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SUMMARY

One and two day growths of *Clostridium tetani* were studied for the source of toxin in relation to the bacterial cell. Investigation was made of 5 different possible mechanisms by which toxin was being released constantly from the cells into the supernatant fluid even after 15 to 20 washings with a one per cent peptone solution in physiological saline. The 5 mechanisms studied were: (1) Release of an enzyme from the cells into the medium where toxin could be synthesized; (2) presence in the supernatant fluid of unsedimented cells to account for the toxicity of the supernatant from washed cells; (3) rupture of the cells resulting in release of toxin into the medium; (4) by dialysis of toxin through the cell membrane; and (5) by release from adsorption on the surface of the cells. The conclusion was drawn that most of the toxin that was found in each supernatant wash fluid was due to diffusion of the toxin from within the cell into the surrounding medium. The probable mechanisms involved in the release of toxin during the 6 days of incubation for toxin production were discussed.

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