Appearance of Mouse-Lethal Toxin in Liquid Cultures of *Bordetella pertussis*

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The mouse-lethal toxin present in liquid cultures of most smooth strains of *Bordetella pertussis* is known to originate in the cytoplasm of the organism but to be most lethal for mice when released into the supernatant fluid. It is also recognized that cell degeneration and lysis occur in liquid cultures during the stationary and decline phases of growth. For these reasons, it is generally believed that most of the toxicity demonstrable in liquid cultures at the time of harvest is released during the later stages of cultivation, when high alkalinity and aging of cells favor lysis. However, the results reported here have indicated that high levels of mouse-lethal toxicity arise during very early log phase and that the peak of toxicity is reached before the end of the log phase. No further increase in toxicity was observed during stationary and decline phases. The very early appearance of toxicity could not be explained by the presence in the inoculum of a proportion of dead and degenerating cells, and it is concluded that the toxin is produced mainly by actively growing cells. This was confirmed by tests on organisms growing in continuous culture. Electron-microscopic examination of cells from a very early log-phase culture revealed the presence of large numbers of small vesicles on the cell walls of about 5% of the population. It is suggested that these vesicles may be associated with the releases of toxin from living cells. It is concluded that no useful reduction in the toxicity of cultures would result from harvesting before the end of the log phase of growth.

The heat-labile, mouse-lethal toxin present in liquid cultures of most smooth strains of *Bordetella pertussis* has been shown to originate in the cytoplasm of the organism but to be most lethal for mice when released into the supernatant fluid. If the organisms are removed from a culture by centrifugation, most of the toxic activity is found in the supernatant fluid. The cell suspension is initially of comparatively low toxicity, but the toxicity increases gradually during storage due to release of toxin from the organisms. Large quantities of toxin can be released by disrupting the cells (1, 2, 4, 7, 8, 10–12, 17; L. B. Holt, Round Table Conf. Pertussis Immunization, Prague, 1962, p. 170; J. Pekarek, Round Table Conf. Pertussis Immunization, Prague, 1962, p. 157).

For these reasons, the toxin has been called an "endotoxin" [the mouse-lethal, thermolabile toxin has been called "endotoxin" by Pennel and Thiele (12) and by Anderson (1), but some later workers have used the term "endotoxin" to describe the cell wall lipopolysaccharide (9, 14); because of this confusion the term "endotoxin" has been avoided in this paper], and it has been generally assumed that the toxicity of liquid cultures is largely due to leakage of toxin from lysed and dead cells, mainly towards the end of the growth cycle, when high alkalinity and aging of cells favor lysis.

The presence of mouse-lethal toxin in *B. pertussis* cultures causes problems in the preparation of innocuous vaccines. Therefore, it was of practical importance to investigate whether less-toxic cultures of *B. pertussis* could be obtained by harvesting cultures before the end of the usual cultivation period of 72 hr.

**MATERIALS AND METHODS**

*Broth and shake flasks.* Cultures were grown in a complex liquid medium containing casein hydrolysate, yeast dialysate, and salts as described by Cohen and Wheeler (6).

Erlenmeyer flasks (1-liter capacity) containing 500 ml of the broth were incubated at 36 C on an inclined rotary shake table turning at 144 rev/min.

*Strain.* A recently isolated strain, code number BR1, was used during the entire investigation. It was selected from a cough plate, purified on Cohen and Wheeler blood-agar plates and slopes, and was stored in lyophiles. A lyophile was opened to begin a fresh stock culture line on Cohen and Wheeler
blood-agar slopes, no culture line being continued beyond the 10th subculture. 

BR1 was a typical smooth strain. It agglutinated with smooth antiserum, contained hemagglutinin, histamine-sensitizing factor, and intracerebral mouse-protective antigen, and caused hemolysis on Cohen and Wheeler blood-agar plates. Shake-flask cultures were invariably extremely toxic for mice.

Inoculation and sampling. Several slopes were inoculated from a slope of the stock culture line and were incubated at 35°C for 36 to 48 hr. Each slope was harvested in 5 ml of saline, and the pooled harvest was used to inoculate shake flasks so that each flask received an inoculum equivalent to the harvest from one-half to one slope.

Samples were removed from the flasks at intervals during the cultivation, and total cell count, percentage viability, and pH at room temperature were measured. The pH of each of the samples was adjusted to 7.0 to 7.5 with 0.5 N HCl, Merthiolate was added to 0.01% (w/v), and the samples were stored at 2°C for 1 week before testing for toxicity.

Bacterial count. Total cell counts were measured in a calibrated E.E.L. colorimeter (Evans Electросelenium Ltd., Halstead, Essex, U.K.), the primary standard being the U.S. National Institutes of Health opacity reference equivalent to 10 × 10⁶ organisms/ml.

Viable counts were made by plating out in duplicate, on Cohen and Wheeler blood-agar, 0.1-ml quantities of serial 10-fold dilutions of culture made in 1% (w/v) Casamino Acids solution.

Toxicity test. Toxicity tests were carried out with

4- to 6-week-old mice of the Commonwealth Serum Laboratories white strain in groups of 10. Each group was weighed, and each mouse received 10 × 10⁶ organisms by intraperitoneal injection. Toxicity values were assigned on the third day according to the following scale: 8 to 10 dead, +++; 4 to 7 dead, ++++; 1 to 3 dead, ++; none dead but weight reduced, +; none dead and weight regained, -. Saline-injected controls were always included and invariably gave a negative toxicity value.

RESULTS

Three parallel shake-flask cultures were sampled at 22, 46, 72, 99, and 119 hr after inoculation. Measurement of total cell count, pH, percentage viability, and toxicity of each sample produced the results shown in Fig. 1. To observe clearly whether age and pH had any effect upon toxicity, the cultivation period was deliberately extended beyond the usual 72 hr to 119 hr.

A further set of three parallel shake flasks was sampled at 0, 11, 25, 49, and 54 hr after inoculation. The growth curves and toxicity of these flasks are shown in Fig. 2.

As very little growth had occurred by the 11th hr after inoculation, it seemed possible that the high levels of toxicity seen at that time might have resulted from disintegration of the dead cells contained in the inoculum. (Unpublished results have shown that harvests from slope cultures are about 10 to 40% viable.)

To determine whether the conditions existing in a freshly inoculated shake flask could cause release of toxin from the cells of the inoculum, it was necessary to treat a freshly inoculated shake flask in such a way as to prevent the normal growth of the culture. As any such treatment could itself have some unexpected effect upon toxicity, a variety of methods was used, since it is unlikely that all would have the same effect upon toxicity.

A freshly inoculated shake flask was divided into four samples, which were treated as follows.

Sample 1. Growth was prevented by anaerobio-
sis. The sample was placed in a screw-capped jar and was incubated stationary at 36 C. It was sampled at 24 hr.

Sample 2. Growth was prevented by the addition of antibiotic. The sample was placed in a 500-ml Erlenmeyer flask, and sterile chloramphenicol solution was added to about 0.5 µg/ml; the sample was incubated at 36 C on an inclined rotary shaketable in the usual way. It was sampled at 0 and 24 hr.

Sample 3. This was the untreated control. The sample was placed in a 500-ml Erlenmeyer flask and was incubated at 36 C on an inclined rotary shaketable in the usual way. It was sampled at 0 and 24 hr.

Sample 4. This sample was disrupted in a French press at 10 tons/square inch in the cold.

In addition, a 1-liter flask containing 500 ml of saline (instead of broth) was inoculated and incubated at 36 C on an inclined rotary shaketable in the usual way; it was sampled at 0 and 24 hr.

All eight samples were tested for toxicity as described in Materials and Methods, except that the test dose was 1.0 ml per mouse in each case. The results of these toxicity tests are shown in Table 1.

If the mouse-lethal toxin was released from actively growing cells, as the results indicated, it might be expected that cultures grown continuously at reasonably high dilution rates would also be highly toxic. Samples were taken from a continuous culture of strain BR1 operating at dilution rates between 0.066 and 0.081 hr⁻¹ (pH controlled at 7.50 ± 0.02 by automatic addition of 1 N HCl). Results of toxicity tests carried out on these samples are shown in Table 2. Examination of these samples with an electron microscope showed very uniform cultures with almost no cell debris and only an occasional cell with any sign of degeneration.

### Table 1. Toxicity of the inoculum in shake-flask cultures of B. pertussis

<table>
<thead>
<tr>
<th>Treatment of freshly inoculated culture</th>
<th>Time of sampling</th>
<th>Toxicity value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated, normal shake-flask culture</td>
<td>0 hr</td>
<td>++</td>
</tr>
<tr>
<td>Incubated in presence of chloramphenicol (0.5 µg/ml)</td>
<td>24 hr</td>
<td>++++</td>
</tr>
<tr>
<td>Incubated in absence of nutrients</td>
<td>24 hr</td>
<td>+</td>
</tr>
<tr>
<td>Disrupted anaerobically</td>
<td>24 hr</td>
<td></td>
</tr>
<tr>
<td>Disrupted in French press</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ In a group of 10 mice, 8 to 10 dead, ++++; none dead but weight reduced, +; none dead and weight regained, −.

### Table 2. Toxicity of samples from a continuous culture of B. pertussis

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>Toxicity value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>+++</td>
</tr>
<tr>
<td>0.066</td>
<td>++++</td>
</tr>
<tr>
<td>0.075</td>
<td>++++</td>
</tr>
<tr>
<td>0.081</td>
<td>++++</td>
</tr>
</tbody>
</table>

¹ In a group of 10 mice, 8 to 10 dead, ++++; 4 to 7 dead, +++. At higher magnification, some of the cells were seen to have a large number of small vesicles or "blebs," which appeared to be formed by evagination of the cell wall. A more detailed study of the cells from a batch culture in very early log phase showed that the vesicles were present in about 5% of the cell population. These vesicles are shown in Fig. 3.

**DISCUSSION**

Previous studies have shown that the cytoplasm of B. pertussis contains large quantities of mouse-lethal toxin which can be released by disrupting the cells. It is also well known that considerable degeneration and lysis of cells take place during the decline phase in a batch culture, possibly partly as a result of the high pH. Our own unpublished results support these observations.

These two separate observations have led to the generally held assumption that the toxicity of liquid cultures of B. pertussis at the time of harvest is released mainly towards the end of the cultivation period and is the result of degeneration and lysis of cells. Pittman has said that the "age of bacteria at the time of harvest, no doubt, has an effect upon the permeability of the cell walls, thereby influencing toxicity," and that "alkalinity favours lysis and thereby promotes toxicity" (13). If this were the explanation for the toxicity of liquid cultures at the time of harvest, toxicity of cultures would be expected to rise markedly during the stationary and decline phases of cultivation.

Toxicity tests carried out on samples taken during cultivation in shake flasks indicated that this is not the case. The toxicity values of these samples, in terms of toxicity per organism, reached their peaks during the active growth of the cultures and thereafter remained fairly steady or fell slowly (Fig. 1). There was no marked rise in toxicity during the decline phase, even when the cultivation period was extended to 5 days; by this time, the percentage viability had fallen to zero and the pH had risen to 8.92 to 8.97.

The very early appearance of toxicity suggested either that a large quantity of toxin had been
FIG. 3. Electron micrograph of B. pertussis showing vesicles (cells stained by phosphotungstic acid). × 100,000.
introduced with the inoculum, or that toxicity arose very early in the cultivation cycle. In a further shake-flask experiment, however, freshly inoculated flasks were virtually nontoxic, but high levels of toxicity appeared after only 11 hr of cultivation (Fig. 2). The very early appearance of toxicity could not be attributed to release of toxin from the dead cells in the inoculum, since a sample from a freshly inoculated shake flask which had been mechanically disrupted in the cold had a negative toxicity value. That the inoculum itself was of negligible toxicity was confirmed when normal growth in a series of freshly inoculated cultures was prevented by anaerobiosis, by absence of nutrients, or by the presence of chloramphenicol. After 24 hr of incubation, all of these cultures were nontoxic.

These results from shake-flask cultures suggested that the mouse-lethal toxin is released by the actively growing organism and that the main part of the toxicity of liquid cultures arises when the culture is in the active growth phase. This was supported by tests on samples from a continuous culture operated at dilution rates up to 0.081 hr⁻¹ with controlled pH. These samples were all highly toxic (Table 2), although almost no cell degeneration was evident under the electron microscope.

These results indicated that the release of toxin from growing cells is very rapid (Fig. 2, Table 2); possible mechanisms for release based upon diffusion of toxin from the cells or washing off of toxin from the cell walls do not seem adequate to explain such vigorous production. With a view to discovering a possible mechanism for the release of the toxin, a detailed electron-microscopic study of cells from a very early log-phase culture was undertaken.

About 95% of the cell population showed delicate surface convolutions, but no surface structure which could be interpreted as a possible mechanism for the release of toxin.

About 5% of the cells were evenly stained, smooth, and without surface convolutions. Many of these cells possessed large numbers of small vesicles or "blebs," which appeared to be formed by evagination of the cell wall. Various stages in the formation of a vesicle seem to be distinguishable (Fig. 3). Apparently, a bulge forms in the cell wall, which then elongates and pinches off leaving a completely detached sac.

It is possible that these vesicles are associated with the release of the mouse-lethal toxin from *B. pertussis* cells. It is also possible that these vesicles represent nothing more than the earliest stages in the degeneration of aging cells, but evidence from continuous culture suggests that this is not the case. The vesicles were seen in a proportion of the cells taken from a continuous culture operating at a dilution rate of 0.081 hr⁻¹, and there was almost no evidence of degeneration in the cells in this culture.

It has not yet been clarified why only a small proportion of the cells contained these vesicles.

It would be of interest to examine sections of cells to determine whether there is any organization of cell membranes at the site of a vesicle, such as has been demonstrated in penicillinase-producing *staphylococci* (3).

This study has shown that the mouse-lethal toxin of *B. pertussis* is produced by actively growing cells. It is clear that, in the production of liquid cultures for preparation of vaccine, less toxic cultures would not be obtained simply by harvesting before the end of the log phase.

Evidence that significant amounts of mouse-lethal toxin are actively produced by living cells further supports the theory that the toxin is important in the human virulence of the organism, as suggested by Bordet and Gengou (5), Sprunt, Martin, and McDearman (15), and Standfast (16).

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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