THE VIRULENCE AND IMMUNOGENICITY OF SALMONELLA TYPHOSA
GROWN IN CONTINUOUS CULTURE

SAMUEL B. FORMAL, L. S. BARON, AND WALTER SPILMAN

Division of Immunology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center,
Washington 16, D. C.

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In recent years the technique for the continuous cultivation of microorganisms has been adapted for a variety of uses. Gerhart (1946) and Barnes and Dewey (1947) have reported on the advantage of continuous cultivation over conventional methods for obtaining increased yields of bacterial cells, and von Hofsten et al. (1953) have extended the method to the cultivation of fungi.

Continuous cultivation techniques offer several advantages. In some instances, the formation of a given enzyme may be inhibited by a constituent of the growth medium. By employing continuous culture, it is possible to grow large amounts of bacterial cells in a medium containing such low concentrations of the inhibiting constituent that enzyme formation is unaffected (Cohn and Torriani, 1953). In addition, metabolic products which may block the formation of some enzymes do not accumulate under conditions of continuous growth (Monod, 1950).

Workers in the field of microbial genetics have also employed continuous culture as a tool in the study of mutation (Novick and Szilard, 1950, 1951), while others (Bryson and Szybalski, 1952) have used a modification of the technique as a means of selecting bacterial mutants which are highly resistant to such substances as antibiotics.

Continuous cultivation techniques may also be applicable to the production of bacterial vaccines where the advantage of obtaining large quantities of cells in a relatively short period is obvious. On the other hand, a shift in the bacterial population during continuous growth from one of high protective potency to one of reduced immunogenicity would restrict the usefulness of this method in the large-scale production of organisms for vaccines. It cannot be denied that the opportunity exists for mutation and selection during continuous growth. Therefore a study of the effects of growth in continuous culture on the stability of the virulence and immunogenicity of Salmonella typhosa was undertaken.

MATERIALS AND METHODS

Continuous culture apparatus. A diagram of the continuous culture apparatus used in this work is given in figure 1. Several methods of injecting fresh medium into the growth tube have been used by other workers. In the present study, a Brewer automatic pipetting machine proved to be satisfactory, since the amount of medium introduced into the growth tube and the frequency of injection can be easily and accurately controlled with this apparatus. When less than 10 injections per min were required, it was found necessary to activate the pipetting machine by means of an automatic timer.

In pilot experiments, the culture in the growth tube was agitated by bubbling air through it; however this invariably resulted in excessive foaming. This difficulty was eliminated by employing a magnetic stirrer. Clumps of cells tended to accumulate on the sides of the growth tube after several days, but this difficulty was obviated by coating the vessel with silicone.

The component parts of the apparatus were sterilized separately and reassembled under aseptic conditions. This assembly process was greatly simplified by employing male and female "luer-lok" adapters to join the rubber tubing of the components.

Cultures. Two strains of S. typhosa were employed: a mutant of strain Ty2 which did not require tryptophan, and strain V58. Strain Ty2 has been utilized extensively by workers for experimental typhoid studies and for vaccine production. It exhibits maximum virulence for mice, and is agglutinated by anti-Vi but not by anti-O serum. The Panama Carrier strain (V58) is employed by most investigators in the U. S. for vaccine production. It is less virulent for mice than strain Ty2 when administered as an 18-hr
EFFECT OF CONTINUOUS CULTIVATION ON S. TYPHOSA

Figure 1. Diagram of continuous culture apparatus

A = Source of sterile medium
B = Brewer automatic pipetting machine
C = Automatic timer
D = Magnetic stirrer
E = 37°C water bath
F = Growth tube
G = Siphon
H = 3-way stopcock
I = Outlet to collection tank
J = Outlet to sampling tube

Culture media. The basic ingredients for all culture media used in these experiments were: dibasic potassium phosphate, 7 g per L; monobasic potassium phosphate, 3 g per L; and magnesium sulfate, 0.1 g per L. Glucose and ammonium sulfate in various concentrations were added to this basal medium, and in some experiments, cystine and tryptophan were also incorporated.

Mouse virulence tests. Suspensions of cells for challenge experiments were prepared from the Ty2 strain of S. typhosa collected directly from the continuous culture apparatus in a flask maintained at 0 to 3°C. When a sufficient volume had been obtained, the bacteria were centrifuged and resuspended in saline to a density of approximately $5 \times 10^8$ cells per ml. White mice (Bagg strain) weighing 14 to 16 g were then injected intraperitoneally with 0.5-ml quantities of dilutions of the cell suspensions. Groups of 20 mice containing equal numbers of males and females were used to test each dilution. Deaths were recorded after 72 hr and the LD$_{50}$ and Standard Error (S.E.) were estimated by the method of Miller and Tainter (1944).

Whenever the mouse virulence titrations were performed on samples from the continuous culture apparatus, parallel determinations on the mouse virulence of strain Ty2 grown on agar also were conducted. For this portion of the work, lyophilized ampules of Ty2 prepared 10 years previously were employed. A new ampule was opened for each virulence determination, and the mouse virulence of these cells grown on nutrient agar was assumed to be relatively stable. Thus this additional test was included to provide some indication of the variation in susceptibility of the mice to experimental typhoid infection over the course of the experiment.

Preparation of vaccines. The organisms used for the preparation of vaccines were collected from the continuous culture apparatus in a flask maintained at 0 to 3°C. As soon as a sufficient volume had been obtained, the cells were harvested by centrifugation.
Dehydrated vaccines were prepared from cells of strain Ty2 by washing the harvest with acetone, collecting the precipitated bacterial cells on a Buchner-type fritted glass filter, and drying the organisms by air suction through the bacterial mass. Final traces of acetone and moisture were removed by desiccation in vacuo. When the cells were to be assayed for their mouse protective potency, they were suspended in saline to make a concentration of 24 mg cells per 100 ml. The total nitrogen content of each vaccine was determined on 5-ml aliquots by the micro-Kjeldahl method.

Heat-killed vaccines were prepared from cells of strain V58 by suspending the harvest in saline to make a concentration of 10⁶ cells per ml, and then heating the suspension for 1 hr at 56 C. Phenol was then added to give a final concentration of 0.5 per cent. Finally the total nitrogen content of the vaccine was determined on 5-ml aliquots by the micro-Kjeldahl method.

**Mouse protective potency tests.** Vaccines prepared from cells grown in the continuous culture apparatus were compared with a standard vaccine consisting of cells cultivated by conventional methods. Groups of mice containing equal numbers of males and females were immunized by single intraperitoneal injections of 0.5 ml-volumes of 5- to 10-fold dilutions of the vaccine. Six days later the mice were challenged. Mice immunized with the acetone-killed and dried organisms were challenged with 100 million cells of an 18-hr culture of strain Ty2 suspended in saline, while mice receiving the heat-killed phenol-preserved organisms were challenged with one to two thousand cells of an 18-hr culture of strain V58 suspended in 5 per cent hog gastric mucin. These challenge suspensions sufficed to fatally infect at least 85 per cent of the control unvaccinated mice.

The ED₅₀ and Standard Error (S.E.) of these protection tests were estimated by the method of Miller and Tainter (1944).

**Bacterial counts.** Viable bacterial counts were determined by the usual pour plate technique, averaging the number of colonies on 4 to 6 plates. Total bacterial counts were determined in a Petroff-Hauser counting chamber with each recorded count representing the average of four separate determinations.

**Serological tests.** The phenomenon of O inagglutinability exhibited by *S. typhosa* is taken by most workers to be indicative of a high Vi antigen content. Strains not agglutinated by the O antiserum are considered to have more Vi antigen than cultures which are agglutinated by O antibody.

The ability of strain Ty2 grown in continuous culture to resist agglutination by typhoid O antiserum was examined daily. Cells were collected from the apparatus, centrifuged, and resuspended in saline. One-half ml of the suspension was added to 0.5-ml volumes of dilutions of a typhoid O antiserum (titer 1:2560). The tests were incubated for 2 hr at 37 C and then overnight at 4 C before reading.

The same bacterial suspension was also tested for its ability to react with Vi antibody. The identical procedure was employed as described above except that a serum prepared against a Vi-containing strain *Paracolobactrum ballerup* (Vi titer 1:1280) was used.

**RESULTS**

*S. typhosa* strain Ty2 was grown in continuous culture for 24 days in the basal medium which had been supplemented with an excess of glucose (2 g per L) and a suboptimal amount of ammonium sulfate (52 mg per L). Fresh culture medium was added to the growth tube (volume = 320 ml) at a rate of 2 ml per min without any change in the density of the growing culture. With the culture in the "steady state," this is equivalent to 0.53 divisions per hour (Monod, 1950).

The density in terms of per cent light transmission, the viable count, and the total count from the third to the eighth day of the experiment is shown in figure 2. Although no further counts were done after the eighth day, the turbidity of the culture during the remaining 15 days of the experiment remained constant.

Virulence tests were conducted on the effluent culture of strain Ty2 taken from the growth tube on the third, eighth, and twenty-third day and also on 16-hr agar-grown suspensions of the same strain. The results of these tests are given in table 1. The data show that over the course of the experiment the mice were equally susceptible to typhoid challenge, and that bacteria taken from the apparatus after 23 days of continuous growth were as virulent for mice as they were when continuously cultured for only 3 days.

The immunogenicity of acetone-killed and dried vaccines prepared from Ty2 cells collected after 6 and 24 days of continuous culture was
The continuously cultured cells were examined at intervals during the experiment for their resistance to agglutination by typhoid O antiserum titer (1:2560). Cells collected during the first 15 days were not agglutinated by the antiserum in a dilution of 1:80, but cells collected subsequently were agglutinated by progressively higher serum dilutions and finally reached the titer of the serum on the nineteenth day of continuous culture (table 3). These results were obtained in several experiments and varied only in the time the cells became O-agglutinable. However, it is interesting to note that although these organisms became O-agglutinable, no concomitant decrease in mouse virulence or immunogenicity was observed.

Experiments were also conducted in which S. typhosa strain Ty2 was cultured continuously in the basal medium supplemented with ammonium sulfate, 2 g per L and glucose, 390 mg per L. Here the nitrogen was in excess while the glucose was in suboptimal concentration. Fresh culture medium was added to the growth tube (volume = 320 ml) at a rate of 2 ml per min.

The results insofar as the virulence and im-

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**TABLE 1**

The mouse virulence of Salmonella typhosa strain Ty2 grown in continuous culture*

<table>
<thead>
<tr>
<th>Days of Continuous Culture</th>
<th>Strain Ty2 Grown in Continuous Culture</th>
<th>Strain Ty2 Grown on Nutrient Agar (16-hr Suspension)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Cells x 10⁶ Cell suspension</td>
<td>Vi-titer</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vi-titer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17/20</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>6.9 x 10⁶</td>
</tr>
<tr>
<td>6.9 x 10⁶</td>
<td></td>
<td>1:1280</td>
</tr>
<tr>
<td>1.7 x 10⁶</td>
<td></td>
<td>9/20</td>
</tr>
<tr>
<td>32.7 x 10⁶</td>
<td></td>
<td>8.2 x 10⁶</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>8.2 x 10⁶</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>19/20</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1/20</td>
</tr>
<tr>
<td>43.2 x 10⁶</td>
<td></td>
<td>18/20</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>8.8 x 10⁶</td>
</tr>
<tr>
<td>8.8 x 10⁶</td>
<td></td>
<td>1:1280</td>
</tr>
<tr>
<td>2.2 x 10⁶</td>
<td></td>
<td>1:1280</td>
</tr>
<tr>
<td>0.5 x 10⁶</td>
<td></td>
<td>1:1280</td>
</tr>
</tbody>
</table>

* Medium deficient in nitrogen.
† The mouse virulence of strain Ty2 grown on nutrient agar is included to give an indication of variation in mouse susceptibility to experimental typhoid infection over the course of experiment.
‡ Titer to which challenge suspension reacted in an antiserum prepared against Paracolobactrum ballerup.
§ Titer to which the challenge suspension reacted in an antiserum prepared against Salmonella typhosa strain 0-901.
TABLE 2

Comparison of the immunogenicity for mice of acetone-killed and dried suspensions of Salmonella typhosa strain Ty2 grown in continuous culture with a standard acetone-killed and dried preparation

<table>
<thead>
<tr>
<th>Description of Vaccine</th>
<th>Nitrogen Content</th>
<th>Vaccine* Dose in MI</th>
<th>ED50†</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/5 ml</td>
<td>0.025 0.005 0.001 0.0002 0.00004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella typhosa Ty2 grown in continuous culture for 6 days</td>
<td>0.10</td>
<td>36/40 30/40 17/40 15/40 5/40</td>
<td>0.0008</td>
<td>0.0005</td>
</tr>
<tr>
<td>Salmonella typhosa Ty2 grown in continuous culture for 24 days</td>
<td>0.10</td>
<td>36/40 33/40 26/40 13/40 11/40</td>
<td>0.0005</td>
<td>0.0004</td>
</tr>
<tr>
<td>Standard acetone-killed and dried vaccine</td>
<td>0.15</td>
<td>33/40 30/40 19/40 13/40 8/40</td>
<td>0.0007</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

* 12 mg of dried bacilli suspended in 50 ml saline.
† Challenge dose: 100,000,000 S. typhosa Ty2 suspended in saline and injected intraperitoneally.
‡ Numerator denotes number of survivors; denominator denotes total number vaccinated.

TABLE 3

Reactions with Vi* and O† antibodies of Salmonella typhosa strain Ty2 grown in continuous culture (medium deficient in nitrogen)

<table>
<thead>
<tr>
<th>Days of Continuous Cultivation</th>
<th>Vi-Titer†</th>
<th>O-Titer‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-15</td>
<td>1:1280</td>
<td>&lt;1:80</td>
</tr>
<tr>
<td>16</td>
<td>1:1280</td>
<td>1:320</td>
</tr>
<tr>
<td>17</td>
<td>1:1280</td>
<td>1:160</td>
</tr>
<tr>
<td>18</td>
<td>1:1280</td>
<td>1:640</td>
</tr>
<tr>
<td>19-24</td>
<td>1:1280</td>
<td>1:2560</td>
</tr>
</tbody>
</table>

* Antiserum prepared against a Vi-containing strain of Paracolobactrum baliereup.
† Anti-serum prepared against S. typhosa strain 0-901.
‡ Tests incubated for 2 hr at 37 C and overnight at 4 C.

immunogenic tests are concerned were the same as those described previously. The cells grown continuously for 8 days were as virulent and immunogenic for mice as conventionally cultured preparations. However, in contrast to the previous experiment, the cells became O-agglutinable by the eighth day. The total nitrogen content of the experimental vaccine in this experiment was slightly greater than that of the previous experiment (0.12 mg per 5 ml as compared with 0.10 mg per 5 ml). While this might have resulted from errors in experimental technique, it could also have been due to the difference in nitrogen content of the two media in which the bacteria were grown. The latter explanation would agree with the findings of Virtanen and De Ley (1948) who found that cells of Escherichia coli contained less nitrogen when cultured on a low nitrogen medium than on a medium with larger concentrations of nitrogen.

The results so far described were obtained with strain Ty2 as the test culture. This strain had been selected for this study, since on the basis of laboratory criteria, acetone-killed suspensions of these cells have been demonstrated to be superior vaccines (Henderson et al., 1951, Landy, 1953). Nevertheless, heat-killed, phenol-preserved vaccines prepared from S. typhosa strain V58 are widely used for immunization. It was of interest therefore to determine the effect of continuous cultivation on the immunogenicity of strain V58. In these experiments, heat-killed, phenol-preserved preparations of the cell harvest were used as immunizing agents. The bacteria

Figure 3. Density, viable count, and total count of Salmonella typhosa strain 58 grown in a medium deficient in cystine and tryptophan.
TABLE 4
Comparison of the immunogenicity for mice of heat-killed, phenol-preserved suspensions of Salmonella typhosa strain V58 grown in continuous culture with a standard heat-killed, phenol-preserved preparation

<table>
<thead>
<tr>
<th>Description of Vaccine</th>
<th>Nitrogen Content</th>
<th>Vaccine* Dose in ML</th>
<th>ED50†</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>*mg/5 ml</td>
<td></td>
<td>0.15 0.015 0.0015 0.00015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella typhosa strain V58 grown in continuous culture for 7 days</td>
<td>0.12</td>
<td>38/40 29/40 12/40 7/40</td>
<td>0.0042</td>
<td>0.0018</td>
</tr>
<tr>
<td>Standard heat-killed phenol-preserved vaccine</td>
<td>0.15</td>
<td>25/40 6/40 3/40</td>
<td>0.085</td>
<td>0.088</td>
</tr>
</tbody>
</table>

* One billion cells per ml.
† Challenge dose: 1-2 thousand S. typhosa strain V58 suspended in 5 per cent hog gastric mucin.
‡ Numerator denotes number of survivors; denominator denotes total number vaccinated.

were cultured for 7 days in the basal medium containing ammonium sulfate, 1.8 g per L; glucose, 0.65 g per L; tryptophan, 0.2 mg per L; and cystine, 0.2 mg per L. In this medium both amino acids were in suboptimal concentrations. Fresh medium was added to the growth tube (volume = 360 ml) at a rate of 1.4 ml per minute with no change in the density of the culture. The density, viable count, and total count of the culture effluent from the growth tube over the course of the experiment are given in figure 3, while the results comparing the immunogenicity of the vaccine prepared from the effluent cells collected on the seventh day with a standard heat-killed, phenol-preserved vaccine are given in table 4. It is apparent that the vaccine prepared from cells grown in continuous culture equalled and possibly exceeded the standard in mouse protective potency.

**DISCUSSION**

The studies outlined were designed to determine the effect of continuous cultivation on the virulence and antigenic properties of two widely used and studied strains of *S. typhosa*. Although directed primarily at determining the suitability of continuous cultivation techniques for the production of acceptable vaccines, the experiments described led to certain findings of more general interest.

In the present instance, the organisms were first grown in a medium deficient in nitrogen, but with an adequate source of glucose. Cells of *E. coli* grown continuously in a nitrogen-deficient medium are said to contain an increased carbohydrate fraction. Since the Vi antigen, established as being of major importance in the mouse virulence of typhoid strains, is known to be a polysaccharide, it appeared likely that a nitrogen deficient medium would favor the growth of Vi cells. Data have been presented which show that strain Ty2 can be continuously cultured for at least 3 weeks in this medium without loss of mouse virulence or immunogenicity. However, after 2 weeks, a measurable change occurred in the culture, i.e., cells previously able to resist agglutination by typhoid O antiserum were no longer able to do so.

When the same strain was grown continuously in a glucose deficient medium, a more rapid development of O-agglutinable cells was noted. However, as in the previous experiment, this was not reflected in a detectable loss in mouse virulence or immunogenicity. This experiment lasted only 8 days, and it is possible that if the cells were cultured continuously for a longer period of time under similar conditions, a reduction of mouse virulence and immunogenicity might have been observed. The relatively rapid loss in ability to resist agglutination by O antiserum can probably be attributed to the limited glucose concentration in the growth medium, an observation which is in agreement with the findings of Gladstone (1937).

These results would appear to differ from the observations of Felix and Pitt (1934), who found that an O-inagglutinable strain of *S. typhosa* was more virulent and immunogenic for mice than another O-agglutinable culture. However, in the case reported here the same strain was studied both before and after it became O-agglutinable. Thus, several explanations may be offered. The change which an O-inagglutinable culture undergoes when it becomes O-agglutinable

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may not be accompanied by a decreased content of Vi antigen, or perhaps some factor in the cell other than the Vi antigen is also of importance in determining mouse immunogenicity. Although these may be logical hypotheses, we know of no data to support them. A more likely explanation in our opinion is that in some cases when an O-inagglutinable culture becomes O-agglutinable, the accompanying decrease in Vi antigen content may be too small to measure by mouse virulence or protection tests.

It is also interesting to note that the total nitrogen content of the vaccines prepared from organisms grown in continuous culture was lower than those made from cells cultured by conventional methods. Since it was necessary to concentrate the bacteria grown in continuous culture in order to obtain the required vaccine density, the bacteria were centrifuged, and the sediment either acetone-killed and dried or resuspended in saline and heat-killed. Thus a total nitrogen content lower than that of the unwashed standard heat-killed vaccine could have resulted from this "washing" procedure. However, in the case of the acetone-killed and dried vaccines, both the standard and the experimental agents were washed. Thus in this instance at least, washing cannot account for the difference in nitrogen values which were noted. It does seem likely that cultivating the bacteria which made up all the experimental vaccines in simple synthetic media could have resulted in immunizing agents of lowered nitrogen content (Virtanen and De Ley, 1943). However, this point remains to be elucidated.

From the results reported, cells of S. typhosa grown by continuous cultivation techniques would meet the presently accepted criteria for use in a vaccine for immunization of humans. Organisms cultivated by this method are as immunogenic as conventionally grown preparations when tested by the mouse protective potency tests presently employed as the standard method of assay.

Some investigators have been critical of this test and are of the opinion that it is not justifiable to correlate mouse protective potency with human immunogenicity since the typhoid infection produced in the mouse does not resemble the disease in man. While this point is well taken, no substitute for this test has yet been widely considered or officially accepted. Recently, carefully controlled field and laboratory studies on pertussis vaccine have indicated that, in this case at least, a positive correlation between mouse protective potency (as determined by intracerebral challenge) and human immunogenicity may occur (Evans and Cockburn, 1951). It is hoped that work now in progress in this laboratory (Edsall et al., 1954) and in a controlled field trial will determine whether a similar correlation exists with typhoid vaccine. Until further results along these lines are available, however, it would appear that there is no alternative but to continue to employ the mouse protective potency test for assaying typhoid vaccines, and to be guided by the results of such tests in selecting procedures for vaccine preparation.

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SUMMARY

The effect of continuous cultivation on the mouse virulence and immunogenicity of strains of Salmonella typhosa was studied. Two strains were employed.

Strain Ty2 was continuously cultured for 24 days in a medium deficient in nitrogen with no discernible loss in mouse virulence. Dehydrated vaccines prepared from the cultures taken early in the experiment and on the twenty-fourth day were of comparable immunogenicity for mice, and both experimental vaccines were of the same order of potency as a standard preparation. However, a measurable change in the culture was observed after the fifteenth day of continuous growth since organisms previously able to resist agglutination by typhoid O antisemum became O-agglutinable. When strain Ty2 was cultured continuously in a medium deficient in glucose, the cells became O-agglutinable by the eighth day. At this time no loss in mouse virulence or immunogenicity was noted.

The second culture, strain V58, was grown continuously for 7 days. A heat-killed phenol-preserved vaccine was prepared from organisms on the seventh day. This preparation proved to be equal or possibly superior to a standard heat-killed vaccine in mouse protective potency. The significance of these results is discussed.
REFERENCES


