FURTHER STUDIES ON THE IMMUNIZATION OF RABBITS TO TOXIGENIC CORYNEBACTERIUM DIPHTHERIAE BY INJECTIONS OF NONTOXIGENIC DIPHTHERIA BACILLI

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Frobisher and Parsons (1943) reported that rabbits injected with broth cultures of living avirulent (nontoxigenic) Corynebacterium diphtheriae developed significant resistance to subsequent injections of living cultures of virulent (toxigenic) C. diphtheriae.

Their experiments involved 21 immunized and 11 control animals. These were tested with a challenge dose that was fatal to all of the 11 control rabbits, which died on an average of 3.6 days after the dose was administered. Of the immunized animals 4 survived, the other 17 dying after an average of 7 days. In all, 48 per cent manifested some degree of resistance, including the 4 survivors. Resistance was ascribed to mobilization of cutaneous defenses, which effected, not a neutralization of toxin by antitoxin, but toxin localization. Antitoxin was not present. Apparently resistance was related to a greatly enhanced tissue reactivity and was presumably engendered by somatic antigens of the bacilli against a heterologous antigen—the exotoxin.

The present investigation was undertaken to extend and verify these observations and to collect further information regarding the phenomena observed.

MATERIALS AND METHODS

Infusion broth and agar. These were prepared with veal or pork, according to the methods outlined in the Manual of Methods of the Society of American Bacteriologists, with the following modifications: (1) Neopeptone (Difco) was used in 1 per cent concentration, and (2) the meat infusion was heated to 80 C before pressing out the juice.

Synthetic medium. This was used in experiments to study the role of thiamine in the effectiveness of the antigens. The formula is given in the description of the experiments in which it was used.

 Cultures. The avirulent strains of corynebacteria (cd107b and My654a) used as antigens were the same as those used by Frobisher and Parsons (1943), and tests for avirulence and atoxigenicity were not repeated. The virulent strain (EHD70) used for challenge doses was also the strain used by these workers. Broth cultures, 48 hours old, were used for both immunizing and challenge doses.

Inoculations. Several immunization programs were conducted with variations

1 This study was aided by a grant from The Rockefeller Foundation.

2 In later experiments using numerous controls no control animal has survived the same challenge dose.
in the route of inoculation, the total amount of antigen administered, the intervals between the injections of antigen, and the numbers of antigenic stimuli. However, within the limits employed these variations appeared to have little or no effect on the degree of resistance of the rabbits to the subsequent challenge dose of virulent *C. diphtheriae*. The most commonly employed procedure for immunization consisted of ten 1-ml doses at intervals of 3 to 4 days. For the subcutaneous and intracutaneous inoculations the animals’ backs were prepared by shaving with electric clippers.

The challenge doses were the same throughout the study. They consisted of 0.2 ml of a 48-hour broth culture of virulent *C. diphtheriae*, a dose which, with the strain employed, is uniformly fatal to normal rabbits. In general, it was administered 8 to 10 days after the last antigenic inoculation.

**EXPERIMENTS**

1. Veal-grown Antigens

At the time the work herein described was instituted, veal was being used routinely in this laboratory for preparing meat infusion media. Accordingly, veal infusion broth was used for the cultivation of the avirulent diphtheria bacilli with which the animals in this series of experiments were immunized. In all respects the procedures were made as nearly as possible like those previously used.

*Experiment 1a.* Ten rabbits were immunized with veal-grown antigen, 8 for a period of 4 weeks and 2 for 8 weeks. Following the challenge dose of virulent *C. diphtheriae*, all the animals died—7 within 24 to 48 hours, 1 on the third day, 1 on the fourth day, and 1 on the sixth day. All controls died at about the same rate. Because of the complete absence of resistance in the test animals, the experiment was repeated, only the period of immunization varying.

*Experiment 1b.* Eleven rabbits were immunized with veal-grown antigen, the immunization period being 5 weeks. Following the challenge dose, all of the animals died—5 within 2 days, 3 on the third day, and 3 on the fourth day.

The results of these two experiments were in surprising contrast to those previously obtained, i.e., the animals developed no resistance to virulent *C. diphtheriae*. The average survival time of the total of 21 test rabbits was 2.6 days and that of all 12 control (nonimmunized) rabbits was 2.7 days (table 1).

These two failures led to a careful review of the original work and revealed that the avirulent organisms with which the first animals had been immunized had been cultured in pork rather than veal infusion broth. This was because at the time of the original experiments pork was more readily available than veal. Immunizations were therefore repeated (exp. 2, 3, 4, 5a, and 5b) using cultures in pork infusion broth.

*Experiment 2.* Immunization with pork-grown antigen was started in 6 rabbits, but 3 died of nonspecific causes during the immunization period of 5

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*Dosages varied from 7 ml of antigen given in 5 doses during 1 month to 46 ml of antigen given in 47 doses during 7 months.*
weeks. Therefore, only 3 animals received the challenge dose of virulent *C. diphtheriae*. Of these 3, 2 survived and 1 died, but death did not occur until the seventh day. All 10 control animals died within 2 to 5 days.

**Experiment 3.** Six rabbits were immunized with pork-grown antigen over a period of 5 weeks. Following the challenge dose 2 animals survived, 2 died on the fifth day, 1 on the fourth day, and 1 on the third day.

**TABLE 1**

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent, veal-grown antigen*

<table>
<thead>
<tr>
<th>EXPERIMENT NUMBER</th>
<th>PERIOD OF IMMUNIZATION</th>
<th>RABBITS</th>
<th>SURVIVAL TIME</th>
<th>AVERAGE SURVIVAL TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>weeks</td>
<td></td>
<td></td>
<td>days</td>
</tr>
<tr>
<td>1a</td>
<td>4</td>
<td>8</td>
<td>2-6</td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>5</td>
<td>11</td>
<td>2-4</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>21</td>
<td>2-6</td>
<td>2.6</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>12</td>
<td>2-5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

**TABLE 2**

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent, fresh pork-grown antigen*

<table>
<thead>
<tr>
<th>EXPERIMENT NUMBER</th>
<th>PERIOD OF IMMUNIZATION</th>
<th>RABBITS</th>
<th>SURVIVORS</th>
<th>LIFETIME OF NONSURVIVORS</th>
<th>AVERAGE LIFETIME OF NONSURVIVORS*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>weeks</td>
<td></td>
<td></td>
<td>days</td>
<td>days</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>7.0</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>3-5</td>
<td>4.2</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>5-8</td>
<td>6.0</td>
</tr>
<tr>
<td>5a</td>
<td>14</td>
<td>5</td>
<td>1</td>
<td>3-6</td>
<td>4.5</td>
</tr>
<tr>
<td>5b</td>
<td>29</td>
<td>3</td>
<td>1</td>
<td>5-11</td>
<td>8.0</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>22</td>
<td>8</td>
<td>3-11</td>
<td>5.4</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>10</td>
<td>0</td>
<td>2-5</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Blood taken just before inoculation of virulent organisms was tested for antitoxin. Tests for 0.01 unit per ml were negative.

**Experiment 4.** Five rabbits were immunized with pork-grown antigen over a period of 5 weeks. Following the challenge dose 2 animals survived, 1 died on the eighth day, and 2 on the fifth day.

**Experiment 5a and 5b.** Eight rabbits were immunized with pork-grown antigen, 5 for a period of 14 weeks and 3 for 29 weeks. Of the first group of 5, following the challenge dose, 1 survived, and 1 died on each of the sixth, fifth, fourth, and third days. Of the 3 subjected to the longer immunization period, 1 survived, 1 died on the eleventh day, and 1 on the fifth day.

As shown in table 2, it is evident that the use of antigens cultivated in pork media afforded definite protection against virulent diphtheria bacilli. Of 22 rabbits immunized, 8 (36 per cent) survived the challenge dose. Others gave
evidence of increased resistance as evidenced by the 5.4-day average survival time of the 14 remaining rabbits. All of the 10 control rabbits died within 5 days or less.

A probable confirmation of the importance of fresh pork in the preparation of these antigens was later obtained inadvertently. Because of severe wartime shortages of meat fresh pork became unavailable. A preparation called, commercially, "pork-sausage," and probably consisting largely of corn meal and other nonporcine material, was used in cultivating antigens for one immunization experiment involving 12 rabbits. The results (table 3) were like those obtained with veal-grown antigens. At most only slight resistance was produced in the test animals. The average survival time was only 3.4 days as compared with 2 days for the controls. If the sausage contained fresh pork, which seems very unlikely, it must have been present in very small amounts, and its properties must have been modified by the spices and other materials mixed with it and by the processing to which it had been subjected.

**TABLE 3**

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent antigen prepared with "pork-sausage" infusion broth*

<table>
<thead>
<tr>
<th>PERIOD OF IMMUNIZATION</th>
<th>RABBITS</th>
<th>SURVIVAL TIME</th>
<th>SURVIVAL TIME AVERAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>days</td>
<td>days</td>
</tr>
<tr>
<td>weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>2-6</td>
<td>3.4</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>2</td>
<td>2*</td>
</tr>
</tbody>
</table>

* Compare also controls in tables 1, 2, 4, and 5.

**II. Reactions to the Challenge Dose**

The local reaction to the challenge dose in most of the animals immunized with organisms grown in a fresh pork base (not "pork-sausage") medium was characteristic. An area of very marked edema, 6 to 12 cm, often more, in diameter, developed within 24 to 48 hours. Sometimes the whole flank of the animal was involved. This was gradually absorbed over a period of several days, and a corresponding but somewhat smaller area of necrosis developed. In contrast to these edematous reactions, the control animals, as well as most of the animals injected with organisms grown in media other than fresh pork infusion (including "pork-sausage"), developed much smaller lesions with little or no edema and much less extensive necrosis. Apparently resistance was closely related to the extent of the skin reaction.

As originally described, the resistant animals showed little or no evidence of general intoxication at any time, whether or not they survived, until a few hours before death if they died after several days. The controls and nonresistant (veal and "pork-sausage") animals were obviously ill within 24 to 36 hours after administration of the challenge dose. Evidently toxin was absorbed rapidly from the local lesion in the control and nonresistant animals but was held *in situ* in the resistant animals.
III. Tests for Antitoxin

In order to have some confirmation of the observation that the survival of animals in these experiments is not dependent on the development in them of antitoxin, some of the test animals in this series were bled before receiving the challenge dose. The serum of 6 of the 8 animals surviving the challenge dose was examined and in each instance was found to contain less than 0.01 unit per ml. The sera were not assayed at lower levels.

IV. Effect of Thiamine

From the results described above it was inferred that fresh pork contains some factor which is of critical significance in the antigenicity of avirulent diphtheria bacilli in regard to virulent diphtheria bacilli. Data on the amino acid and vitamin content of veal and pork were obtained from the American Meat Institute. According to these data an important difference between pork and veal is in the thiamine content, which is decidedly greater in pork.

Further experiments (exp. 6, 7, 8, 9, and 10) were conducted to verify the earlier results with veal-grown antigens and to determine whether or not thiamine had any influence on the phenomenon under study. The thiamine effect was studied with thiamine-enriched

\[\text{Sodium lactate}\] 6 ml
\[\text{Glucose}\] 1 g
\[\text{MgSO}_4\] 1 g
\[\text{K}_2\text{HPO}_4\] 4 g
\[\text{NaCl}\] 6 g
\[\text{Tryptophane}\] 200 mg
\[\text{Cysteine hydrochloride}\] 200 mg
\[\text{Glycine}\] 200 mg
\[\text{Glutamic acid}\] 2 g

Heat to dissolve.
Adjust with \(\frac{n}{10}\) NaOH to pH 7.8 or 8.0.
Boil vigorously for 5 min.
Add distilled water to restore volume.
Cool to room temperature.
Filter through a good grade of filter paper.
Dispense and sterilize in the autoclave (15 lb, 20 min).

To each 100 ml of this base add aseptically 0.2 ml of vitamin solution 1 or 2.

\begin{align*}
\text{Vitamin Solution 1} & \\
\text{Ascorbic acid} & 100 \text{ mg} \\
\text{Niacinamide} & 40 \text{ mg} \\
\text{Riboflavin} & 8 \text{ ml} \\
(100 \text{ mg} \% \text{ in } \text{H}_2\text{O}) & \\
\text{Pyridoxine} & 1 \text{ ml} \\
(100 \text{ mg} \% \text{ in } \text{H}_2\text{O}) & \\
\text{Calcium pantothenate} & 2 \text{ ml} \\
(100 \text{ mg} \% \text{ in } \text{H}_2\text{O}) & \\
\text{H}_2\text{O} & 89 \text{ ml} \\
\text{Sterilize by Seitz filtration.}
\end{align*}

\begin{align*}
\text{Vitamin Solution 2} & \\
\text{Thiamine chloride} & 350 \text{ mg} \\
\text{Sterilize by Seitz filtration.}
\end{align*}
(1907) and the formula of Pappenheimer, Mueller, and Cohen (1931). However, the strains of C. diphtheriae in use failed to grow in serial subcultures in any of these three media. Therefore, the synthetic medium described below was developed. This maintained the organisms in indefinite serial transfer.

Experiment 6. Six rabbits were immunized with veal-grown antigen for a period of 5 weeks. Following the challenge dose of virulent C. diphtheriae all the animals died, 1 within 2 days, 1 on the third day, 3 on the fourth day, and 1 on the fifth day.

Experiment 7. Ten rabbits were immunized with veal-grown antigen for a period of 5 weeks. Following the challenge dose all the animals died, 6 on the second day and 4 on the third day.

Experiment 8. Ten rabbits were immunized with thiamine-enriched veal-grown antigen for a period of 5 weeks. Following the challenge dose all the animals died within 2 days.

<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reaction of rabbits to a virulent challenge dose following immunization with avirulent antigen prepared with veal infusion media with and without added thiamine</strong></td>
</tr>
<tr>
<td><strong>EXPERIMENT</strong></td>
</tr>
<tr>
<td><strong>NUMBER</strong></td>
</tr>
<tr>
<td>6*</td>
</tr>
<tr>
<td>7*</td>
</tr>
<tr>
<td>8†</td>
</tr>
<tr>
<td>Controls</td>
</tr>
</tbody>
</table>

* Veal infusion medium without added thiamine.
† Veal infusion medium with added thiamine.

Experiment 9. Four rabbits were immunized with antigen grown in synthetic medium without thiamine for a period of 5 weeks. Following the challenge dose all the animals died, 1 on the fourth day, 2 on the fifth day, and 1 on the seventh day.

 Experiment 10. Three rabbits were immunized in the usual manner, using antigen cultivated in synthetic medium with thiamine. Following the challenge dose all the animals died, 1 on the third day and 2 on the fifth day.

The results of these experiments, summarized in tables 4 and 5, corroborated the earlier evidence of the relative inefficacy of veal-grown antigens, since the average survival time of 46 animals receiving veal-grown antigens (exp. 1a, 1b, 6, 7, and 8) was 2.6 days and that of 4 controls 2.6 days. These animals all died, whereas 36 per cent of the animals receiving pork-grown antigens survived and those dying lived longer (avg 5.4 days) than the controls (avg 2.6 days).

These experiments also served to demonstrate that thiamine is apparently not the factor in pork which determines the protective antigenicity of avirulent C. diphtheriae. The average survival time of 41 rabbits (exp. 1a, 1b, 6, 7, and 9) receiving low-thiamine antigen (veal and synthetic media) was about 3.5 days;
that of 13 rabbits (exp. 8, 10) receiving high-thiamine antigen (veal and synthetic media) was 2.5 days. All these rabbits died.

**DISCUSSION**

A review of all the experiments reveals that only those rabbits that were immunized with avirulent organisms cultivated in fresh pork infusion media developed any definite resistance to infection with virulent diphtheria bacilli.

**TABLE 5**

*Reaction of rabbits to a virulent challenge dose following immunization with avirulent antigen prepared with synthetic media with and without added thiamine*

<table>
<thead>
<tr>
<th>EXPERIMENT NUMBER</th>
<th>PERIOD OF IMMUNIZATION</th>
<th>RABBITS</th>
<th>SURVIVAL TIME</th>
<th>AVERAGE SURVIVAL TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>weeks</td>
<td>rabbits</td>
<td>days</td>
<td>days</td>
</tr>
<tr>
<td>9*</td>
<td>5</td>
<td>4</td>
<td>4-7</td>
<td>5.2</td>
</tr>
<tr>
<td>10†</td>
<td>5</td>
<td>3</td>
<td>3-5</td>
<td>4.3</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*Synthetic medium without thiamine.
†Synthetic medium with thiamine added.

**TABLE 6**

*Summary table: Reactions of rabbits to virulent challenge dose following immunization with avirulent antigen prepared in a variety of media*

<table>
<thead>
<tr>
<th>MEDIUM USED FOR CULTIVATING ORGANISMS</th>
<th>RABBITS</th>
<th>SURVIVORS</th>
<th>PERCENTAGE OF SURVIVALS</th>
<th>AVERAGE LIFETIME OF NONSURVIVORS</th>
<th>MAXIMUM LIFETIME OF NONSURVIVORS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>days</td>
<td>days</td>
</tr>
<tr>
<td>Medium containing fresh pork</td>
<td>22</td>
<td>8</td>
<td>36</td>
<td>5.3</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>4</td>
<td>19</td>
<td>7.0</td>
<td>13</td>
</tr>
<tr>
<td>Totals</td>
<td>43</td>
<td>12</td>
<td>28</td>
<td>6.0 (±0.5)</td>
<td>13</td>
</tr>
<tr>
<td>Medium not containing fresh pork</td>
<td>66</td>
<td>0</td>
<td>0</td>
<td>3.2</td>
<td>7</td>
</tr>
<tr>
<td>Controls</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>2.9</td>
<td>5</td>
</tr>
</tbody>
</table>

*Data from Frobisher and Parsons (1943).
†Data from present report.

A summary table (table 6), in which data on animals immunized with antigens grown in fresh pork media are contrasted with data on animals immunized with antigens grown in veal and synthetic media and with data on the control animals, brings into sharp relief the difference in the protection afforded. It seems obvious that fresh pork contains some factor which determined the efficacy of avirulent *C. diphtheriae* as an antigen inducing resistance in rabbits against virulent *C. diphtheriae*. 
An effort to learn the nature of this factor served merely to demonstrate that thiamine is not the responsible agent.

These studies have amply corroborated the earlier finding (Frobisher and Parsons, 1943) that under proper experimental conditions rabbits that receive repeated doses of cultures of avirulent diphtheria bacilli develop a resistance to, and in many cases survive, doses of virulent diphtheria bacilli that are invariably fatal to normal rabbits. Two important additional facts have also been established: (1) as between the media used here, a fresh pork base medium is essential to antigenic effect; (2) thiamine is not per se responsible for the antigenic effect.

The implications of these findings are fairly obvious with respect to media used in the preparation of antigens heretofore regarded as of little efficacy, such as dysentery and cholera vaccine, etc., and the improvement of bacterial antigens already in use, such as typhoid and pertussis. The antigens might be made more effective by the inclusion in their culture media of some essential factor such as the yet unknown "pork factor" described here.

The mechanism of the protection afforded by the avirulent diphtheria bacilli is noteworthy but not understood. Allergy apparently is not significant, for there is no enhanced skin reactivity to the homologous somatic antigen of the avirulent bacilli, but only to the heterologous antigen—the exotoxin of the virulent organisms. That resistance and survival are not due to the presence of antitoxin in the blood stream was pointed out by Frobisher and Parsons (1943) and was again demonstrated in these studies. Judging by the appearance of the local reaction and the relatively "bright" appearance of the test rabbits following the challenge dose, it would seem that there is some local tissue reaction which binds the toxin, delaying its general absorption or, in the case of the survivors, entirely preventing absorption by holding the toxin in situ until the animal has built up its own antitoxic (and possibly antibacterial) antibodies to combat the infection.

In a general sense this is reminiscent of the observations by Abernethy and Francis (1937) that "some factor or change occurring in the serum in response to bacterial pneumococcal infection is capable of being mobilized in tissues and thereby reacting locally with the C substance" and that "the state of reactivity of the tissue cells is also essential for cutaneous response to C."

Whatever the nature of the phenomenon, it is obvious that some protection is afforded. In view of this fact, as well as of the mounting evidence that what is generally considered an adequate program of toxoid immunization is not always sufficient to prevent diphtheria (Eller and Frobisher, 1945; Turner, 1942), it seems permissible to suggest again that consideration be given to the idea that the immunizing agents used to protect children against diphtheria should contain properly cultivated bacterial antigens as well as antigens to stimulate anti-exotoxin.

**SUMMARY AND CONCLUSIONS**

Eighty-eight rabbits were repeatedly inoculated with living cultures of avirulent *Corynebacterium diphtheriae*. Twenty-two of the 88 animals received organisms which had been cultivated in a pork infusion medium. Of these 22,
8 (36 per cent) survived a subsequent challenge dose of virulent C. diphtheriae which was uniformly fatal to nonimmunized animals. The other 14 animals in this group of 22 survived an average time of 5.3 days as contrasted to the 2.9-day average survival time of 31 control (nonimmunized) animals.

This is in contrast with 66 rabbits which received inoculations of avirulent C. diphtheriae cultivated on media not containing fresh pork. Of these 66 animals, none survived the challenge dose of virulent C. diphtheriae, and their average survival time of about 3 days was essentially the same as that (2.9 days) of the 31 control animals. Thiamine was shown not to be the essential antigen-adjuvant in the pork. The implications of these results have been discussed briefly with respect to immunization procedures in general, and especially those against diphtheria.

Partial or complete protection against virulent diphtheria bacilli was engendered in rabbits by injecting into them living cultures of avirulent diphtheria bacilli which had been cultivated in a fresh pork base medium.

Avirulent C. diphtheriae cultivated in certain media not containing fresh pork were incapable of engendering any significant resistance against the virulent organisms.

Fresh pork contains some factor which is critical for the antigenicity of the avirulent diphtheria bacilli under the conditions of these experiments. This factor is apparently not thiamine.

The resistance of the immunized animals was not due to the presence of demonstrable antitoxin in the blood stream, and the mechanism of the protective action is not antitoxic. It appears to depend rather on a local binding action in subcutaneous tissues, where the unneutralized toxin causes extensive necrosis.

REFERENCES


