VIRULENCE IN LÉPTOSPIRAE

III. COMPARISON OF SENSITIVITIES OF VIRULENT AND OF AVIRULENT Leptospira icterohaemorrhagiae TO CULTURAL CONDITIONS

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The mechanism of virulence in leptospiroa is obscure. It has been shown that virulent, but not avirulent, leptospiroa survive and multiply in host tissues (Faine, 1957a, b). Conditions in vivo, optimum for growth and survival of virulent leptospiroa, differ obviously in osmotic pressure, salinity, temperature, and oxygen tension from those usually used in cultures, which may selectively favor avirulent leptospiroa. In these experiments, the fates of virulent and of avirulent strains of Leptospira icterohaemorrhagiae were compared under conditions partially resembling those in vivo, in culture media made approximately isotonic to body fluids, at 30, 37, and 40 C.

MATERIAL AND METHODS

Sources, maintenance, and counting of leptospiroa. L. icterohaemorrhagiae strains Jackson (avirulent) and Field (virulent) were obtained from Dr. J. C. Broom, London, in 1953. They were used in previous studies, where the methods of counting and of maintenance of all cultures were described (Faine, 1957a). An avirulent Field strain was also used. Its last passage through a guinea pig was in 1953. Strains Wijnberg (avirulent) and 21 (virulent) were obtained from Dr. L. Kirschner, Dunedin, New Zealand. Virulence was verified with the use of guinea pigs weighing 200 g. Virulent means LD<sub>50</sub> < 100 organisms; avirulent means LD<sub>50</sub> > 10<sup>6</sup>. Cultures were incubated in water baths controlled to ±0.5 C of the temperature stated.

Criteria for death of leptospiroa. Leptospiroa observed microscopically were considered to be dead when they were nonmotile and showed characteristic, classical degenerative changes, including the appearance of highly refractile granules throughout the length of the organism.

Culture media. Stock cultures were maintained in a modified Korthof medium containing, essentially, 10 per cent hemolyzed rabbit serum, 0.08 per cent Neopeptone (Difco), and 0.14 per cent NaCl buffered to pH 7.2 with 0.006 M phosphate, sterilized finally by filtration through a Seitz filter pad (Alston and Broom, 1958). This medium, containing a total, approximately, of 0.045 M concentration of salts, is hypotonic to body fluids. Media were made isotonic by adding 0.1 M concentrations of the required solute before filtration. Korthof-saline medium contained a total of 0.85 per cent NaCl in Korthof medium. Serum-saline medium contained 10 per cent pooled hemolyzed rabbit serum in 0.85 per cent NaCl buffered with 0.006 M phosphate to pH 7.2. This is very similar to the medium of Reiter and Ramme (1916).

RESULTS

Growth in saline media. Small inocula of young active cultures of virulent (Field) or of avirulent (Jackson) leptospiroa were transferred to saline or to Korthof media, at 30 or 37 C. After 19 hr in saline medium at 37 C, 60 per cent of avirulent leptospiroa were dead, 95 per cent were dead in 43 hr, and 100 per cent in 67 hr. After 67 hr incubation, 90 to 98 per cent of the leptospiroa were alive in cultures of the avirulent strain in Korthof medium at 37 C, and in saline or in Korthof media at 30 C, and in cultures of the virulent strain in saline or in Korthof media at 30 or 37 C.

In a similar experiment, cultures surviving after 5 days were subcultured in duplicate into saline and into Korthof media. One of each pair of subcultures was incubated at 30 C, the other at 37 C. Growth curves are shown in figure 1. Figures 2A and 3B show growth curves from typical, similar experiments. Avirulent leptospiroa did not survive in saline media at 37 C (figure 1E). Virulent leptospiroa survived in...
saline media at 37°C but would not grow in subculture (figure 1B, C, and F). Similar results were obtained when the experiments were repeated using Korthof-saline medium in place of saline medium.

The upper limit of NaCl concentration permitting growth of virulent leptospirae at 37°C was 0.9 per cent. Avirulent leptospirae were not inhibited at 37°C by 0.4 per cent NaCl added to Korthof medium.

*Figure 1.* Growth curves of virulent and of avirulent leptospirae in Korthof medium and in saline media at 30 or 37°C. A, C, D, and F were transferred from cultures grown in the media and at the temperatures shown. B was previously grown in Korthof medium at 30°C, and E in Korthof medium at 37°C. A. Virulent strain in Korthof medium; —— at 30°C, X——X at 37°C. B. Virulent strain in saline media at 37°C (2 experiments). C. Virulent strain in saline media at 30°C. D. Avirulent strain in Korthof media; —— at 30°C; X——X at 37°C. E. Avirulent strain in saline media at 37°C (2 experiments). F. Virulent strain in saline media at 37°C (2 experiments).

*Figure 2.* Growth curves of saline-adapted avirulent leptospirae. The parent culture A was grown in saline medium at 30°C. After 12 days, A was divided into two parts, one of which (B) was transferred to 37°C. A was also subcultured into saline medium at 30°C (C) and 37°C (D), and into Korthof medium at 30°C (E) and at 37°C (F).
The serum contained by ml; NaCl 37 was incubated approximately 30 X KCl 37 Na^2SO_4 at 37 C. The serum was also cultured in Korthof saline medium (B), or in Korthof medium (C). ○——○, growth at 30 C; ×——×, growth at 37 C.

**TABLE 1**

Growth of *Leptospira icterohaemorrhagiae* after 5 days in Korthof medium containing 0.1 % added salts, at 30 or 37 C

<table>
<thead>
<tr>
<th>Conditions of Growth</th>
<th>Strain</th>
<th>Avirulent Jackson</th>
<th>Avirulent Wijnberg</th>
<th>Virulent Field</th>
<th>Virulent 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt</td>
<td>Temp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>37</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NaCl</td>
<td>37</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Na^2SO_4</td>
<td>37</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>KCl</td>
<td>37</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

0 = fewer than 10^3 leptospiroae per ml; 1 = approximately 5 \times 10^4 per ml; 2 = approximately 5 \times 10^4 per ml; 3 = approximately 5 \times 10^4 per ml; 4 = approximately 10^6 per ml.

Culture was incubated at 30 or 37 C and all were compared after 5 days. All cultures in sucrose, glucose, or glycerol grew satisfactorily from approximately 10^4 leptospiroae per ml to approximately 10^6 leptospiroae per ml. All except the virulent Field strain were inhibited by Na^2SO_4 more at 37 than at 30 C. The Jackson strain was inhibited by KCl and the NaCl at 37 C, and Wijnberg was inhibited approximately 100-fold by NaCl at 37 C. The attenuated Field strain reacted more like the other avirulent strains than like its parent virulent strain (table 1).

**TABLE 2**

Growth of *Leptospira icterohaemorrhagiae* at 37 and 40 C

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Days incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37 C</td>
<td>40 C</td>
</tr>
<tr>
<td>Virulent (Field)</td>
<td>0</td>
<td>7.4*</td>
</tr>
<tr>
<td>Avirulent (Jackson)</td>
<td>0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Millions of leptospiroae per ml; 0 = less than approximately 10^4 leptospiroae per ml.

**Figure 3.** Growth of virulent leptospiroae. Serum containing *Leptospira icterohaemorrhagiae* was obtained by heart puncture from a moribund, infected guinea pig and incubated at 30 C or at 37 C (A). The serum was also cultured in Korthof saline medium (B), or in Korthof medium (C). ○——○, growth at 30 C; ×——×, growth at 37 C.

**Growth of freshly isolated virulent leptospiroae in serum.** Serum was obtained by heart puncture from a moribund guinea pig infected with the field strain of *L. icterohaemorrhagiae*. Portions of the serum were incubated at 30 and at 37 C, and the leptospiroae counted. Subcultures of the serum into Korthof or saline media were each incubated at 30 or at 37 C. The leptospiroae grew satisfactorily in the serum, or in Korthof or saline medium (figure 3). Washed suspensions of virulent and of avirulent cultured leptospiroae were resuspended in normal guinea pig serum and incubated at 30 or 37 C. In all suspensions, 90 to 100 per cent of leptospiroae were dead in 18 to 42 hr.

**Growth at 40 C.** Temperatures of approximately 40 C are reached in febrile animals infected with leptospiroae. Leptospiroae obviously survive these
temperatures in vivo. The fates of virulent and of avirulent leptospirae at 37 or 40 C in Korthof or saline medium are shown in table 2. Virulent leptospirae grew slowly in Korthof media at 40 C; avirulent leptospirae did not. Neither strain survived in saline media at 40 C.

DISCUSSION

Physical and chemical factors affecting the survival of microorganisms in vivo and possibly responsible for virulence have been reviewed by Dubos (1954), Faine (1957b) suggested that virulence in leptospirae might be a result of the ability of virulent organisms to survive and multiply in the host environment. Infection of young guinea pigs with avirulent L. icterohaemorrhagiae led to the same immediate host response as infection with virulent leptospirae, but avirulent leptospirae failed to survive and multiply or to cause pathogenic effects (Faine, 1957a). In mammals, concentrations of NaCl up to approximately 0.9 per cent may be found in tissues and in renal tubules (Walker et al., 1942) of carriers where leptospirae survive at 37 to 40 C. The rates of avirulent leptospirae at 37 C in either whole serum or in saline medium, or at 40 C in Korthof or in saline media were similar to those in vivo, although the cultural conditions are different nutritionally. However, the relative susceptibility of avirulent (Jackson strain) leptospirae to NaCl at 37 C in saline medium is unlikely to be due to nutritional deficiency in that medium. The same results were obtained in saline and in Korthof-saline media. Except for salt content, Korthof-saline medium is nutritionally the same as Korthof medium, in which avirulent leptospirae grew normally at 37 C.

It is interesting that the first isolation of L. icterohaemorrhagiae from infected guinea pigs in Europe was made in whole serum (Ungermann, 1916). In the experiments reported here, virulent leptospirae from infected guinea pigs grew rapidly in whole serum or in Korthof or saline medium at 37 C. Kligler and Ashner (1928) found that primary cultures of virulent L. icterohaemorrhagiae from the blood of infected guinea pigs grew better at 28 C in Noguchi's medium (Noguchi, 1918a) made with saline than in the same medium made with distilled water. Subcultures grew equally well in either medium. Anjow (1928) found that virulent L. ictero-

haemorrhagiae and nonpathogenic Leptospira biflexa grew in 10 per cent rabbit serum diluted in Ringer's solution, at 37 C. He did not test avirulent L. icterohaemorrhagiae. L. biflexa was generally more resistant than L. icterohaemorrhagiae to harmful surroundings. The ability of virulent leptospirae to survive in saline media or in serum at 37C could explain how they are selected in vivo. The failure of virulent leptospirae to survive subculture at 37 C or to grow at 40 C in saline medium does not necessarily invalidate this suggestion, because survival and growth under these cultural conditions may be separate problems. Although the ability to survive and multiply under the conditions described does not explain virulence in leptospirae it does provide evidence of an important difference between the virulent and avirulent leptosporal strains tested, which could be the basis of the ability of virulent organisms to survive in vivo.

Uhlenhuth and Zuelzer (1921) observed that unphysiological concentrations of 3.5 per cent NaCl damaged virulent and cultured strains of leptospirae. However, they also found that at room temperature, 3 per cent rabbit serum supported growth when diluted with water but not when diluted with 0.95 per cent NaCl. Media made with physiological saline or Ringer's solution as diluents would not support subculture. L. icterohaemorrhagiae grew after adaptation in 1 to 1.2 per cent NaCl (Shiga, 1924; Anjow, 1928). On the other hand, Noguchi (1918a) found that cultures of L. icterohaemorrhagiae incubated for 30 days at 26 C in 20 per cent rabbit serum, diluted either in water or in 8 per cent NaCl, grew to the same density. The discrepancies between these observations could be explained by assuming that higher serum concentrations might protect leptospirae from lysis (Noguchi, 1918b). Tsyss (1950) failed to find lysis in 48 hr in 2 per cent NaCl in an albumin medium.

The effects of NaCl in culture media could be due specifically to NaCl or generally to osmotic pressure. Avirulent leptospirae grew normally in media made isotonic with sucrose, glucose, or glycerol. The results in table 1 suggest a specific inhibitory effect of Na and Cl ions on some strains rather than an osmotic effect. These ions are not immediately lethal but merely prevent growth or survival of avirulent organisms above 37 C and of virulent organisms above 40 C.
Thus it is possible that the action of NaCl at the concentrations tested is different from lysis by high concentrations (Uhlenhuth and Zuelzer, 1921).

ACKNOWLEDGMENT

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SUMMARY

Avirulent strains of Leptospira icterohaemorrhagiae were susceptible to physiological concentrations of NaCl in culture media at 37 C but not at 30 C. Virulent strains grew normally at 37 C in media with NaCl or in serum from infected guinea pigs. At 40 C, virulent strains grew slowly in Korthof medium but not in media with NaCl. Avirulent strains failed to survive in Korthof medium at 40 C. The relative resistance of virulent leptospirae to conditions resembling those in vivo could explain their survival in infected animals.

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