Rate of Clearance of *Vibrio fetus* var. *venerealis* in the Rabbit

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The rate of clearance of *Vibrio fetus* var. *venerealis* was measured by using in vivo and in vitro methods. The rate of clearance from the circulating blood increased in immune rabbits and reached a plateau 4 days after immunization. Thereafter in vivo clearance did not increase further despite rising agglutinin titers. Bactericidal activity of freshly drawn blood paralleled the rate of in vivo clearance although it was less effective at 4 days. Bactericidal activity of immune serum was not demonstrated at the same intervals of time as the freshly drawn blood. Bactericidal activity of immune sera plus complement was demonstrated after 1 hr of incubation at 37°C in a candle jar. An effective systemic immune response to *V. fetus* var. *venerealis* can be elicited in the rabbit. These results suggest that humoral antibodies play an important role in effecting acquired immunity against *V. fetus* var. *venerealis*.

Bactericidal activity of serum may sometimes reveal information about host immunity although we cannot readily evaluate its relative effectiveness in circulating blood (4). Serum bactericidal systems have been extensively studied with gram-negative bacteria and have been found to depend on complement and complement-fixing antibodies (9, 10). On the other hand, the bactericidal effect demonstrable with freshly drawn blood (before clotting) may be expected to approximate the antibacterial activity expressed in vivo (4, 5) because it contains leukocytes and more closely simulates the in vivo environment.

In this work, *Vibrio fetus* var. *venerealis*, which causes infections limited to the genital system in the cow, was used. Bovine vibriosis is characterized by an early embryonic death and subsequent infertility (7). The serology of the disease has been extensively studied (6, 8).

Firehammer et al. (2) have studied the clearance from the blood of ewes and rabbits of *V. fetus* var. *intestinalis*, responsible for the disease in ewes, and concluded that the rate of clearance is increased in vaccinated animals as compared to nonvaccinated controls.

We have studied some aspects of immunity against *V. fetus* var. *intestinalis* by using rabbits as test animals. The kinetics of the bactericidal activity of freshly drawn blood and serum were investigated and compared with in vivo clearance rates.

**MATERIALS AND METHODS**

**Bacteriology.** *V. fetus* var *venerealis* was isolated from the genital tract of a heifer. After two subsequent subcultures on solid thioglycollate (Difco) in Roux flasks, the virulent strain was seeded in small samples and was flash-frozen in liquid nitrogen. The bacteria were subcultured after inoculation and recovery from rabbit blood. A selective medium described previously (3) was used. It is made of cysteine heart agar (Difco) containing 5% defibrinated sheep blood, 1:80,000 Brilliant Green, and 300 units of nystatin per ml. The inoculated plates were placed in a desiccator jar, and the air was replaced with 10% CO2, 85% nitrogen, and 5% oxygen. Incubation was carried out at 37°C for 3 to 5 days.

**Agglutination test.** The antigen was prepared by using a technique described previously (7). The opacity of the antigen was standardized at 50% transmittance (650 nm) with a spectrophotometer (Spectronic 20, Bausch & Lomb, Inc., Rochester, N.Y.). The titration for antibacterial activity was done by using twofold dilutions of serum starting at 1:20. The antibacterial end point was chosen as that tube whose transmittance was 75% less than the original after 24 hr of incubation at 37°C.

**Rabbits.** Female New Zealand albino rabbits weighing 5 to 7 lb (ca. 2 to 3 kg) were used in this study. No agglutinin titers against *V. fetus* var *venerealis* antigen were detected in the serum of these rabbits.

**Clearance assay (in vivo).** *V. fetus* was washed
from a 2-day-old culture on a plate and standardized at an optical density of 0.20 (650 nm). This reading gave consistently a suspension of 10^4 to 3 × 10^4 bacteria per ml. One milliliter of this suspension was injected into the marginal ear vein of the rabbit. The blood was taken from small arteries in the median part of the same ear, at regular time intervals. One heparinized microhematocrit tube (Pre-Cal, Clay-Adams Inc., N.Y.), 60 mm length, 0.55 mm inner diameter, was filled. The blood was dispensed on a culture plate and spread out with glass spreader. After a suitable incubation period, the colonies on the plates were counted. The clearance assay was performed after the initial injection and after reinjections of V. fetus at 2, 4, 7, 14, 21, and 35 days. Nonimmune rabbits injected with opsonized V. fetus were also used. For this test, standardized bacterial suspensions were opsonized with pools of immune or nonimmune rabbit sera for 30 minutes at 37 C. Then, 1 ml of this mixture was injected into the marginal vein of rabbits not previously injected with V. fetus, and the assays were performed as described above.

Bactericidal effect of freshly drawn blood (in vitro). V. fetus was standardized as described. Approximately 5 ml of blood was withdrawn from the median artery of the ear. Two milliliters of this blood was added to 0.1 ml of the suspension of bacteria in plastic tubes. The rest of the blood was used for serum agglutination and bactericidal tests. The blood and bacteria were immediately mixed by repeated inversion of the tube. Then, 0.1 ml of the blood was transferred to 0.9 ml of saline at 2, 5, 7, and 10 min and subsequently diluted and plated by using a calibrated microdropper (Linbro Chemical Co., Inc., New Haven, Conn.). The dropwise plating method of suitable dilutions of bacteria was used. The number of colonies growing from six replicate drops, respectively, were counted in those dilutions where the number of colonies ranged between 10 and 100. The mean colony count was calculated and expressed as colony-forming units per ml of original suspension. The percentage survival of bacteria was calculated by using 2.1 × 10^8 per ml as 100% survival. Variations in the plating method were within the normal percentage of error as determined in preliminary trials.

Bactericidal activity of the serum. Pooled sera of known agglutination titers were used. To 1 ml of different dilutions of the pooled sera, 0.3 ml of freshly reconstituted commercial guinea pig complement (Colorado Serum Co., Denver, Colo.), 0.1 ml of standardized suspension of bacteria, and 0.6 ml of diluent (10) were added. The tubes were mixed thoroughly and incubated at room temperature or at 37 C for various specified intervals of time. They were then diluted and plated by using the dropwise technique. Some tests were done at the same intervals of incubation as that used for testing whole blood. When using an incubation at 37 C for 1 hr, the tubes with loose caps were placed in a candle jar filled with the previously described gas mixture.

RESULTS

Clearance assay (in vivo). The clearance at 0 day (i.e., first experience of the rabbits with V. fetus) revealed the persistence of V. fetus at 180 min after injection (Table 1) Subsequently, V. fetus was cleared faster from the circulating blood, reaching a plateau at 4 days, at which time specific antibodies were first detected. The agglutinin titer increased considerably with subsequent immunization. Increases in agglutinin titer were not followed by corresponding increases in the clearance rate.

For opsonization of the bacteria, immune and nonimmune pooled sera were used with the inoculum of V. fetus. The clearance (Table 2) was increased after 30 min when using specific immune serum as compared to nonimmune serum where V. fetus cells were not detectably cleared from the circulating blood after 120 min. The

<table>
<thead>
<tr>
<th>Time after 1st injection (day)</th>
<th>Colony counts at various times</th>
<th>Serum agglutination titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>0 (6 rabbits)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 (2 rabbits)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4 (4 rabbits)</td>
<td>250</td>
<td>18</td>
</tr>
<tr>
<td>7 (6 rabbits)</td>
<td>315</td>
<td>22</td>
</tr>
<tr>
<td>14 (6 rabbits)</td>
<td>420</td>
<td>38</td>
</tr>
<tr>
<td>21 (6 rabbits)</td>
<td>410</td>
<td>53</td>
</tr>
<tr>
<td>35 (6 rabbits)</td>
<td>192</td>
<td>37</td>
</tr>
<tr>
<td>a</td>
<td></td>
<td>+, 500 or more colonies; ND, not done.</td>
</tr>
</tbody>
</table>
Table 2. Mean rate of clearance from the blood of live Vibrio fetus var. venerealis opsonized with immune and nonimmune pooled sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Colony count at various times</th>
<th>Serum agglutination titerb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Immune (2 rabbits)</td>
<td>255</td>
<td>77</td>
</tr>
<tr>
<td>Nonimmune (2 rabbits)</td>
<td>+c</td>
<td>+</td>
</tr>
</tbody>
</table>

a Serum and V. fetus cells were incubated at 37°C for 30 min.
b Two weeks after injection of opsonized V. fetus.
c +, 500 or more colonies.

4th day after the first injection (Fig. 1). The in vivo clearance rate and the blood bactericidal activity remained constant from the 7th to the 35th day after initial exposure. However, at 4 days the in vitro blood bactericidal activity was not as efficient as the in vivo clearance.

Bactericidal activity of serum. In these experiments pooled normal, immune and hyperimmune sera were tested. Complement-dependent bactericidal activity of serum was not apparent after 2, 5, and 10 min (Table 3). However, a bactericidal effect was demonstrated after incubation for 1 hr at 37°C in a candle jar employing either immune or hyperimmune serum plus complement. The control tubes with or without complement gave the same readings, so there was no need to adsorb the guinea pig serum.

DISCUSSION

The kinetics of the bactericidal activity of freshly drawn blood is a potentially useful approach to the study of bactericidal factors in vivo and their role in host defenses (4). We suggest that this method, coupled with the study of the rate of clearance of live bacteria from the circulating blood, can provide information about factors involved in immune clearance.

The vibriocidal effect of fresh blood was as effective 7 days (third injection) after injection as the in vivo clearance of V. fetus and remained so for the duration of our observations. This suggests that antibodies, immune leukocytes, or both, are effective in the immune mechanism to this organism. Our observations also indicate that the immune clearance of V. fetus persists and that there is no re-emergence of the organism as occurs with facultative or obligate intracellular bacteria (11).

The serum bactericidal effect did not develop as rapidly as that for Brucella (4). However, after 1 hr of incubation at 37°C, a bactericidal effect was observed with immune serum, which was more pronounced with hyperimmune serum. Berg (per-
sonal communication) also observed that immune serum from ewes inhibits the growth of *V. fetus* var. *intestinalis* in broth cultures. *V. fetus* opsonized with immune serum was cleared at an enhanced rate when added to the blood of normal rabbits (Table 2). Since exogenous complement was not added to the opsonizing serum, based on the kinetics and degree of bacterial reduction, clearance was attributed mainly to the opsonocytophagic effect. The opsonizing immune serum suppressed the antibody response of the injected rabbit, whereas rabbits injected with *V. fetus* opsonized with nonimmune serum were capable of mounting an agglutinin response (Table 2).

Subsequent challenge with *V. fetus* resulted in a gradual increase in serum agglutinin titers. This was not paralleled by an increase in the rate of in vivo clearance of *V. fetus*. However, hyperimmune serum appeared to be more bactericidal than immune serum (Table 3). It is possible that in vivo clearance can not be achieved before 90 to 120 min after injection of 10⁸ *V. fetus* cells, regardless of the immune status of the host.

Joos et al. (4) postulated the antagonistic effect of blocking antibodies in *Brucella abortus*. Our results, and similar data (1), showed no evidence of such an antagonistic effect since the bactericidal effect of immune serum decreased with the dilution of all antisera used.

The results suggest that the bactericidal activity of freshly drawn blood paralleled the rate of in vivo clearance. Accordingly, tests with freshly drawn blood may be useful in measuring the immune status of an animal in systems where this relationship has been established.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**