Cytotoxic Factor in the Blood and Plasma of Animals During Leptospirosis

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Whole blood and plasma from animals in the acute stage of leptospirosis contained a toxic factor which produced a cytopathic effect on fibroblastic L cell monolayers. Firm adsorption of cytotoxic factor (CTF) to L cells occurred within 1 h. The highest titer of CTF in plasma was reached at 24 h and declined after 48 h after the inoculation of leptospires. Toxic effects were also obtained with intracerebral inoculation of mice with plasma containing CTF. Mice showed signs of motor instability and muscular spasms shortly after inoculation with CTF. Death usually occurred within 1 h.

The involvement of toxins in the pathogenesis of leptospirosis has been suspected for some time (17, 18, 22). Miller and Wilson (13) noted that damage to the endothelium of capillaries in the interstitial tissue of the kidneys of hamsters occurred in the absence of leptospires in these damaged areas, suggesting the possibility of circulating toxin. Another indication of the possible role of toxins in the pathogenesis of leptospirosis was the repeated observation that antibiotic therapy is ineffective unless treatment is initiated early in the disease (11, 22, 23).

Toxicity of leptospiral cultures was demonstrated as early as 1926 when Fukushima and Hosoya (7) showed that materials toxic for guinea pigs were present in lysed cultures of Leptospira interrogans serotype icterohemorrhagiae. More recently, Yam et al. (24) produced a skin reaction in rabbits consisting of erythema and swelling after intradermal injection of a protein fraction prepared from the supernatant fluid of cultures of the serotype pomona.

Arean et al. (2) described a toxic substance in liver and kidney extracts from guinea pigs infected with icterohemorrhagiae. Clinical and pathological manifestations of leptospirosis in guinea pigs followed intraperitoneal inoculation with extracts from liver and kidney tissue from infected animals, indicating that these tissues were the optimal site for the production of toxin.

This study was done to determine whether toxin was also present in the blood after the inoculation of different hosts with a variety of virulent leptospiral serotypes.

MATERIALS AND METHODS

Test organisms. The following organisms were used in this study: L. interrogans serotype pomona strains 3341 and MLS (13), serotype pomona strain Pomona (24), serotype canicola strain Wood (obtained from Dellen Laboratories, Omaha, Neb.), serotype icterohemorrhagiae strain SC 2165 (obtained from Lyle E. Hanson, University of Illinois, Urbana, Ill.), L. biflexa strain CDC (obtained from Mildred Galton, Center for Disease Control [CDC] Atlanta, Ga.). Pasteurella multocida was obtained from the departmental culture collection. All organisms were virulent for hamsters with the exception of serotype pomona strain Pomona and L. biflexa CDC. Death of the hamsters occurred in 4 to 7 days after inoculation with virulent leptospires.

All leptospiral cultures were maintained in Fletcher semisolid medium (Difco) with 10% rabbit serum at room temperature, and were grown in Stuart liquid medium (Difco) with 10% rabbit serum at 30 C for inoculation into hamsters or other animals. P. multocida was maintained at 4 C in cystine tryptic agar (Difco) deeps and grown at 37 C in Trypticase soy broth (BBL) for inoculation into hamsters.

Experimental animals. Hamsters and mice were obtained from colonies bred in the departmental animal facilities. Guinea pigs were obtained locally (Eari Daubert, Omaha, Neb.), and dogs were obtained from Dellen, Inc., Omaha, Neb.

Inoculation procedures. Three-week-old hamsters were inoculated intraperitoneally with 0.5 ml of 7-day cultures of serotype pomona strains 3341, MLS, and Pomona, and serotype icterohemorrhagiae SC 2165 and L. biflexa CDC. Guinea pigs (250 g) were inoculated intraperitoneally with 1.0 ml of 7-day cultures of serotype icterohemorrhagiae SC 2165. Dogs were inoculated intravenously with 2.0 ml of 7-day cultures...
of serotype canicola strain Wood. All cultures contained approximately 10⁸ leptospires per ml.

**Bleeding procedures.** The animals were bled from the heart at various intervals beginning as early as 4 h and ending at 120 h after inoculation. Whole blood was collected from the animals with and without anticoagulant. Either sodium citrate or heparin was used as anticoagulants. A 7% solution of sodium citrate was drawn into a plastic syringe before bleeding. An amount of blood was then taken from the animals so as to achieve a concentration of 1 part of sodium citrate in 10 parts of blood. Heparinized plasma was obtained by rinsing a plastic syringe with sodium heparin (100 USP U/ml) just before bleeding.

**Tissue culture.** The L 293 mouse (L) cell line (5) and the HeLa cell line (8) were used in this study. Both cell lines were grown in Eagle minimal essential medium (MEM) with 5% calf serum. Penicillin (100 U/ml) and streptomycin (200 mg/ml) were added routinely to the medium. The cells were also treated periodically with 0.03 mg of kanamycin (Kantrex, Bristol Laboratories, Syracuse, N.Y.) per ml and examined by the method of Chanock et al. (4) for *Mycoplasma*. Stock cultures of both cell lines were grown in 32-oz (ca. 907 g) prescription bottles. Cells were transferred to Leighton tubes with cover glasses and grown to a complete monolayer in Eagle MEM with 5% calf serum at 37 C.

**Treatment of L cells and HeLa cells with blood, serum, or plasma.** Tissue culture monolayers were inoculated immediately with freshly drawn, whole blood from infected animals. Plasma and serum were either inoculated immediately or stored at -20 C until used. As controls, blood, plasma, and serum from noninfected animals were treated the same as for infected animals. Monolayers grown on cover slips in Leighton tubes were examined for a cytopathic effect (CPE). A detailed microscope examination of the cells was made after staining of the cover slips with the May-Grunwald Giemsa stain (10).

**RESULTS**

The effect of L cells of blood, serum, and plasma from hamsters infected with virulent leptospires. After 18 h of incubation at 37 C, L cells inoculated with 0.2 ml of blood or citrated plasma from hamsters infected with serotype pomona MLS began to detach from the glass surface at the periphery of the monolayer. Later, holes throughout the monolayer representing areas where cells had detached gave it a moth-eaten appearance. Individual cells undergoing degeneration appeared rounded, and their nuclei were pyknotic. Other cells were elongated and became separated from one another. Complete detachment of the monolayer occurred in approximately 24 h. Serum and heparinized plasma from infected animals produced no CPE. Cells treated with blood, serum, or plasma from normal animals showed no CPE. The same results were obtained with different combinations of animal hosts and leptospiral serotypes (Table 1).

The effect of plasma from infected hamsters on HeLa cells. Earlier studies using whole cultures of virulent leptospires (12, 14) as well as CPE factor prepared from culture filtrates (24) showed that a CPE occurred only on fibroblastic but not epithelial cells; consequently it was of interest to determine whether this was also true for cytotoxic factor (CTF).

HeLa cell monolayers, grown and maintained under the same conditions as L cells, were inoculated with 0.2 ml of citrated plasma containing CTF prepared from hamsters infected with 3341 or MLS. As a control, L cells were treated in the same manner as HeLa cells. Plasma from hamsters infected with serotype pomona strains 3341 and MLS had no effect on HeLa cells but showed a CPE on L cells as described for viable leptospires and culture filtrate.

Adsortion of CTF to L cells. Hamster and guinea pig plasma containing CTF was inoculated in 0.2 ml amounts onto L cell monolayers. After incubation at 37 C for 1 h, the medium covering the cells was removed and placed on fresh monolayers from which the growth medium had been removed. The plasma-treated monolayers were washed with 2 ml of Eagle MEM which was decanted and placed on fresh L cell monolayers. A 2-ml amount of Eagle MEM was added to the washed monolayers and incubated at 37 C to see whether CPE would occur. Controls consisted of L cells treated with CTF which was not removed from the monolayer.

The L cell monolayers inoculated with medium containing CTF removed from treated monolayers or with subsequent washings from the treated monolayers showed no CPE, suggesting that adsorption to, and possible penetration of, L cells by CTF occurred within 1 h. Additional evidence of adsorption was the appearance of CPE in the washed L cell monolayers after fresh medium was added.

**Table 1. Combination of animal hosts and leptospiral serotypes producing the cytotoxic factor**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Serotypes</th>
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<tbody>
<tr>
<td>Guinea pig</td>
<td>Icterohemorrhagiae strain SC 2165</td>
</tr>
<tr>
<td>Dog</td>
<td>Canicola strain Wood</td>
</tr>
<tr>
<td>Hamster</td>
<td>Pomona strain 3341 and MLS, icterohemorrhagiae strain SC 2165</td>
</tr>
</tbody>
</table>
Quantitation of CTF in plasma of infected hamsters collected over a 120-h period. It was of interest to determine the earliest time after inoculation of hamsters that CTF could be detected in plasma and the maximal titer of CTF obtainable during the acute stage of the infection.

Thirty hamsters were inoculated intraperitoneally with 10⁴ leptospires of the serotype pomona strain MLS. The plasma of five hamsters was tested separately for CTF activity at 4-h intervals through 24 h. No CTF was demonstrated in plasma at 8 h, but four out of five hamsters showed CTF at 12 h, and all hamsters were positive for CTF through 24 h (Table 2).

Another 29 hamsters were inoculated with pomona MLS, and plasma was pooled from four hamsters at 12 h and from five hamsters each at intervals of 24 h through 120 h. Each pool was titrated on L cells by preparing twofold dilutions of plasma in Eagle MEM from 1:2 through 1:64. A 0.2-ml amount of each dilution was inoculated onto L cells containing 2.0 ml of Eagle MEM with 2% calf serum. The cells were incubated at 37 C and observed for 24 h. The end point was read as the highest dilution of plasma clearly showing sloughing of the cell monolayer. The CTF titer rose abruptly between 12 and 24 h, reaching a titer of 1:16 which was maintained through 48 h. Thereafter, the titer declined gradually and leveled off at 96 h after inoculation (Fig. 1).

**Intracerebral titration of CTF in mice.**

Mice were inoculated intracerebrally with 0.03 ml of undiluted and twofold dilutions of pooled plasma from groups of five hamsters bled at 24, 72, and 120 h. No viable leptospires were present in plasma after treatment of the plasma with penicillin (100 U/ml). One group of mice was inoculated with normal plasma treated in the same manner as plasma from infected animals. Another group was inoculated with 0.03 ml of 10 mg/100 ml bilirubin which served as a control for the possible toxicity of the high concentration of bilirubin (10 mg/100 ml) usually found in the plasma of hamsters during acute leptospirosis.

The titration of plasma from infected hamsters showed decreasing activity in mice after 24 h (Table 3), which correlated with the results obtained in the tissue culture system (Fig. 1). However, the titers were never as high as those obtained with the tissue culture system. Only 13% of the mice inoculated with undiluted normal plasma died, whereas 67% died that were inoculated with undiluted plasma taken from infected hamsters at the three time intervals (Table 3). Bilirubin did not appear to be a contributing factor in the death of mice inoculated with plasma from infected hamsters since none of the 18 mice inoculated died.

Mice inoculated with infected plasma showed signs of instability with muscular spasms particularly in the head and neck region. Extreme respiratory distress was evident just prior to death which usually occurred within 1 h. Sections of the brain stained with hematoxylin and eosin showed no significant pathological changes.

**Effect on L cells of plasma from hamsters inoculated with avirulent and saprophytic leptospires and P. multocida.** To determine whether CTF was related to virulence and whether it was produced by bacteria other than *Leptospira*, five hamsters were inoculated with the avirulent Pomona strain of the serotype

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**Table 2. CTF activity in plasma of hamsters at 4-h intervals after inoculation of serotype pomona strain MLS**

<table>
<thead>
<tr>
<th>Time of bleeding (h)</th>
<th>No. of CTF positive hamsters*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0/5</td>
</tr>
<tr>
<td>8</td>
<td>0/5</td>
</tr>
<tr>
<td>12</td>
<td>4/5</td>
</tr>
<tr>
<td>16</td>
<td>5/5</td>
</tr>
<tr>
<td>20</td>
<td>5/5</td>
</tr>
<tr>
<td>24</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* Numerator indicates number of CTF positive hamsters. Denominator indicates number of hamsters inoculated.
pomona, and another five hamsters were inoculated with a saprophytic L. biflexa strain CDC which is also avirulent. Five hamsters were also inoculated with a virulent strain of P. multocida. This species was selected since it also produces bacteremia and hemorrhagic lesions in hamsters. Hamsters inoculated with the avirulent pomona and biflexa strains were bled at 48 h, and those inoculated with P. multocida were bled during the acute stage of infection (24-48 h). L cells were inoculated with plasma from each hamster. All L cell monolayers were incubated at 37 C and observed for 48 h.

None of the plasma samples produced a CPE. These results indicated that an infection with virulent leptospires must occur to produce CTF and that not all bacteria causing bacteremia and hemorrhagic disease in hamsters produce CTF.

**DISCUSSION**

It was shown in this study that CTF was present in the blood and plasma but not in the serum of three animal species infected with three leptospiral serotypes. The CPE on L cells in 24 h was similar to that described by Solotorovsky and Johnson (20) for Shigella neurotoxin on cell monolayers. Although CTF was adsorbed by L cells within 1 h, total destruction of the monolayer took 24 h, suggesting that either CTF does not destroy the cytoplasmic membrane or its activity on the membrane is delayed because of low concentrations in the plasma. Toxins acting on the cell membranes such as purified staphylococcal alpha toxin cause disintegration of cell monolayers in 30 min (3).

The selective action of CTF on L cells (fibroblastic) and not HeLa cells (epithelial) correlates with the activity of whole viable leptospires (14) and their products (24) on these cell types. This may be attributed to the affinity of CTF to the mucopolysaccharide on the surface of fibroblastic cells (15). Another indication of the affinity of CTF for mucopolysaccharides was the inactivation of CTF when heparin, a mucopolysaccharide, was used as an anticoagulant. The loss of CTF activity in the serum after clot formation could be associated with the adsorption of CTF to sialic acid, which is a mucopolysaccharide component of the fibrinogen molecule.

A significant difference between the activity of whole leptospires, supernatant fluid from cultures, and CTF on cell monolayers was that CTF produced extensive CPE in 24 h, whereas the supernatant fluid and whole cultures required 6 (24) and 9 (12, 14) days, respectively, for comparable CPE to occur. This may be because host tissues provide a more complete medium for the production of toxin than the culture media currently used for growing leptospires. Anthrax toxin was also initially demonstrated in plasma and tissues of infected guinea pigs (19).

Cytotoxicity of plasma was related to the virulence of leptospiral serotypes since plasma from animals inoculated with avirulent and saprophytic leptospires failed to produce a CPE on L cells. A leptospiral infection had to occur for CTF to appear in the blood. It was also shown that no CTF could be demonstrated in the blood or plasma of hamsters infected with P. multocida. The production of CTF in the blood and plasma may be characteristic of leptospiral infections; however, many other infections involving a variety of pathogenic microorganisms must be examined before this can be regarded as a certainty.

Titration in tissue culture of CTF in the plasma of infected hamsters over 120 h showed that toxicity appeared early in the infection (12 h postinoculation) before any overt symptoms were noted. These results agreed with those of Arean et al. (2) who also showed that material extracted from guinea pig tissues was toxic only when obtained from animals killed early during the infection. Since it is generally agreed that treatment with antibiotics is not effective in leptospirosis unless it is initiated early, it is possible that a toxin such as CTF is an important factor in the pathogenesis of this disease. It is difficult, however, to explain the decline in the CTF titer 48 h after the infection and before death of the hamsters.

In vivo studies supported the findings with tissue culture monolayers that plasma from

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**Table 3. Intracerebral titration in mice with plasma from hamsters infected with serotype pomona strain MLS**

<table>
<thead>
<tr>
<th>Time (h) bled after inoculation</th>
<th>Dilution of plasma*</th>
<th>No. of mice killed</th>
<th>No. of mice inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Undiluted</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>72</td>
<td>Undiluted</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>120</td>
<td>Undiluted</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Normal plasma control</td>
<td>Undiluted</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>Bilirubin control</td>
<td>10 mg/100 ml</td>
<td>0</td>
<td>18</td>
</tr>
</tbody>
</table>

* All mice were inoculated with 0.03 ml.

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*INFECT. IMMUNITY*
animals during acute leptospirosis contained a toxic factor. However, until purification of the toxic factor is achieved, it can only be assumed that the in vivo effects produced in experimental animals are due to the same factor which is toxic for L cells. Titration of plasma from infected hamsters by intracerebral inoculation in mice suggested that the same factor may be operative in both systems since a similar trend of decreasing toxicity of plasma at 48 to 72 h was seen in tissue culture and mouse titrations. The symptoms of instability and muscular and respiratory distress after intracerebral inoculation of plasma were similar to those seen in hamsters with leptospirosis just before death. Leptospires in the brain (16) and antigen-antibody interaction in brain tissue (6, 9) have been advocated as the probable causes of neurological symptoms in man and animals with leptospirosis. However, these studies support the belief of others (1, 21) that toxins might also be the cause of neurological manifestations in man and animals.

It is evident that a toxic substance(s) is circulating in the blood of animals during acute leptospirosis as demonstrated in tissue culture and mice. This preliminary study supports the suspicions of many investigators regarding the role of toxins in the pathogenesis of leptospirosis. Purification and characterization of CTF is necessary to determine whether one or more toxic substances are involved. Furthermore, it is not known at this time whether the origin of CTF is leptosporal or a product of the interaction of leptospires with host tissue.

ACKNOWLEDGMENTS
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LITERATURE CITED