Use of Antibiotics in the Preparation of Canine Kidney Tissue Culture Vaccines to Eliminate Leptospiral Infection Hazards

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ABSTRACT

ELLISON, D. (Walter Reed Army Medical Center, Washington, D.C.), J. RIGG, S. J. McCONNELL, A. D. ALEXANDER, AND R. H. YAGER. Use of antibiotics in the preparation of canine kidney tissue culture vaccines to eliminate leptospiral infection hazards. Appl. Microbiol. 13:595–599. 1965.—The potential leptospiral infection hazard in the use of vaccines prepared from canine kidney monolayer cultures was studied. Cell cultures were prepared from kidneys of dogs experimentally infected with *Leptospira* serotype *canicola*. Viable leptospires were found in kidney cell suspensions at the time of seeding, surviving trypsinization either at room temperature for approximately 2 hr or overnight at 4°C, even in the presence of antibiotics. In tissue cultures maintained without antibiotics, leptospires were cultured up to the time of involution of cells at 25 to 34 days of incubation. Cytopathogenic effects of leptospires on cultured kidney cells were not noted; neither was growth of leptospires remarkable. Generally, the leptospirosis culture titer decreased to $10^{-4} \text{ or } 10^{-2} \text{ at the 4th hr or 1st day of incubation to } 10^{-1} \text{ or negative by the 3rd or 4th day of incubation. The addition of either a combination of penicillin (100 units per ml) plus streptomycin (100 } \mu g/\text{ ml}) \text{ or polymyxin B (50 units per ml) plus dihydrostreptomycin (100 } \mu g/\text{ ml}) \text{ to seeding cell suspensions resulted in the elimination of viable leptospires by the 4th hr of incubation. From cell cultures treated with neomycin (100 } \mu g/\text{ ml}) \text{ or chloramphenicol (100 } \mu g/\text{ ml}) \text{, leptospires were recovered, respectively, after 24 and 48 hr, but not thereafter. It was apparent that antibiotics, particularly the combination of polymyxin B and dihydrostreptomycin, could be effectively used to eliminate leptospires in tissue culture. Other antibiotics with known antileptospiral activities probably would be effective also. If antibiotics are not used in canine kidney tissue culture employed for viral vaccine preparations, rigid testing for the presence of leptospires in donor dogs and tissue-culture vaccine is indicated."

The increasing use of animal kidney tissue cultures for vaccine production posed questions on the potential leptospiral infection hazards of such preparations. These questions were particularly pertinent in view of the recently proposed utilization of an attenuated measles vaccine prepared from canine kidney tissue cultures. The high incidence of renal leptospiral carriers has been demonstrated repeatedly in dogs (Alexander et al., 1957). The survival and viability of leptospires in monolayers of kidney cells derived from known infected animals and the effect of various antibiotics on leptospires in tissue cultures are reported.

MATERIALS AND METHODS

Experimental infections. Two leptospiral strains of *Leptospira* serotype *canicola* were employed. Hamsters were infected with strain Moulton, known to be pathogenic for this host. The strain was isolated originally in 1952 from the urine of a sick dog and had been maintained in culture with intermittent hamster passages. A recent isolate, strain Swafford, recovered from a sick dog in 1961 was used to infect puppies. Both strains were serially passed in hamsters 1 to 2 months prior to use in test animals. Hamsters weighing 20 to 30 g were employed as a source of kidney for tissue culture. They were infected by the intraperitoneal (ip) inoculation of a 0.5-ml dose of a 10% suspension (in physiological salt solution) of pooled...
livers and kidneys from two or three infected hamsters. These tissues were obtained 3 to 4 days postinfection, just prior to expected time of death. Hamster kidneys for tissue culture were removed 3 to 5 days postinfection. A 9-day-old Fletcher's culture of strain Swafford grown from the heart's blood of infected hamster was administered ip to 4- to 6-week-old puppies in a 1.5- or 4.0-ml dose. By the 4th or 5th day postinoculation, puppies were moribund. At this time, the puppies were killed, and the kidneys were removed aseptically for preparation of tissue cultures.

Preparation of kidney tissue culture. Techniques of either Melnick (1956) or Bodian (1956) with minor modifications were followed in the preparation of monolayer kidney cell cultures in three different series of experiments.

The former technique was used in initial studies conducted on infected kidneys from four hamsters and a dog, by use of basal medium containing a combination of 100 units of penicillin, 100 μg of streptomycin, and 100 μg of amphotericin B per ml. Cortex of kidneys was minced, washed several times, and trypsinized in 4 to 6 volumes of 0.25% trypsin (Difeo, 1:250 solution) for approximately 1 hr at room temperature. The preparation was centrifuged, and the tissue fragments were collected and subjected to a second trypsinization for 1 to 2 hr. The supernatant fluid was filtered through a layer of gauze. Cells were collected and washed several times and suspended in growth medium to provide a 1:500 dilution of cells. Tissue cultures were prepared by adding 2 ml of cell suspension to test tubes (16 by 150 mm). Tubes were tightly stoppered and incubated in a horizontal stationary position at 37 C. The basal medium used for wash fluids, trypsinization, and for growth medium was Hank's balanced salt solution containing the aforementioned antibiotics and 0.5% lactalbumin hydrolysate. Calf serum free from leptospiral agglutinins was added to the final cell suspension to provide a 10% concentration. Growth media were changed on the 3rd day of incubation. The calf serum concentration in sustaining fluids was 3 to 5%.

The same technique was used in a second series of tests conducted on different cultural preparations of an infected kidney from a dog, with the following exceptions: no antibiotics were used in the preparation of the final suspension used for planting cells; the concentration of bovine serum in sustaining fluids was reduced to 2%. The cell suspension was divided into four samples. To three were added (per ml): 100 μg of neomycin sulfate, 100 μg of chloramphenicol, or 100 units of penicillin plus 100 μg of streptomycin. The fourth portion was untreated. The different suspensions were planted on approximately 25 tubes. Tissue-culture tubes were incubated at 30 C.

Bodian's technique of preparing kidney tissue cultures by overnight trypsinization at 4 C was used to study the viability of leptospires in monolayer dog kidney tissue culture containing a combination of polymyxin B sulfate (50 units per ml) and dihydrostreptomycin (100 μg/ml). Renal cortex from an infected dog was divided into two portions; one was trypsinized in the presence of polymyxin B and dihydrostreptomycin, whereas the second was processed with no antibiotics. Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate was similarly used in these tests to wash minced kidneys. Approximately 15 to 20 volumes of 0.25% trypsin solution per gram of kidney tissue were used. After overnight trypsinization, suspensions were filtered through gauze and centrifuged; sedimented cells were washed and then suspended in growth media in a 1:600 dilution. The antibiotic-free final cell suspension was divided into three portions, one untreated and two treated with combinations of penicillin (100 units per ml) plus streptomycin (10 μg/ml) and polymyxin B sulfate (50 units per ml) plus dihydrostreptomycin (100 μg/ml). These three preparations and the suspension processed with antibiotics were separately planted, each in approximately 60 tubes. Cultures were incubated at 30 C. Sustaining fluids contained 5% bovine serum. The fluids were not changed in this experiment. The pH of tissue cultures (7.4 to 7.6) was maintained by periodic addition of a NaHCO3 buffer solution.

Examination for leptospires. In initial series of tests conducted with kidney tissue cultures derived from hamsters and one dog, and containing penicillin and streptomycin, leptospiral examinations were made on samples taken prior to planting and after 1, 2, 3, and 6 days of incubation. All samples were examined microscopically and culturally. Hamster inoculation procedures for isolation of leptospires were used for samples obtained on the 1st or 3rd, and on the 6th days of incubation. In two later experiments, microscopic and cultural observations on tissue cultures were extended through the 30th to 34th days of incubation, with periodic biweekly or weekly examinations. Generally, hamster inoculation procedures for isolation of leptospires were also included for alternate samples. The pH of all samples was measured.

Specimens for culture were diluted 10-fold in 10⁻² or 10⁻⁴, and 1-drop portions of the dilutions were inoculated into each of three tubes containing approximately 5 ml of Fletcher's leptosporal medium. Three hamsters each were inoculated with 0.5 ml of the sample to be tested. Methods for the examination of hamsters and cultures for the presence of leptospires have been described elsewhere (Yager et al., 1963).

Results
Attention was initially directed to the survival of leptospires in tissue cultures of infected kidneys prepared according to commonly employed techniques utilizing penicillin and streptomycin in concentrations of 100 units and 100 μg/ml, respectively. Five different groups of kidney cell cultures were established successfully; four were derived from hamsters, the fifth from a dog. The
Table 1. Effect of antibiotics on survival of leptospires in Leptospira serotype canicola-infected canine kidney tissue cultures

<table>
<thead>
<tr>
<th>Tissue culture technique</th>
<th>Antibiotic (conc per ml)</th>
<th>Dilution of tissue culture positive for leptospires on day of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate trypsinization at room temp, 2% bovine serum in sustaining media</td>
<td>Control, no antibiotic 100 units of penicillin + 100 ( \mu )g of streptomycin 100 ( \mu )g of neomycin 100 ( \mu )g of chloramphenicol</td>
<td>10(^{-4}) 10(^{-3}) 10(^{-2}) 10(^{-3}) 10(^{-2}) 10(^{-3}) 10(^{-2}) 10(^{-3}) 10(^{-2})</td>
</tr>
<tr>
<td>Overnight trypsinization at 4 C, 5% bovine serum in sustaining media</td>
<td>Control, no antibiotics 50 units of polymyxin B + 100 ( \mu )g of dihydrostreptomycin ( \dagger )</td>
<td>10(^{-2}) 10(^{-4}) 10(^{-2}) 10(^{-3}) 10(^{-3}) 10(^{-2}) 10(^{-2}) 10(^{-2}) 10(^{-3}) 10(^{-2}) 10(^{-4}) 10(^{-2})</td>
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</table>

* Positive by microscopic examination as well as by culture and animal inoculation technique.  
† Same findings on portion of kidney processed in presence of antibiotics.

kidneys employed for the preparation of tissue cultures in these and subsequent experiments contained profuse numbers of leptospires. Approximately 50 to 100 leptospires were revealed by microscopic examination (450 times magnification) of a drop (ca. 0.01 ml) of 10% suspension of a portion of the kidney, distributed under a cover slip (22 by 22 mm) on a slide. Findings in five groups of tissue cultures were essentially the same. Approximately two to five motile leptospires were seen in the final cell suspension used for establishing the tissue cultures. No leptospires were recovered from any of the tissue cultures after 24 hr of incubation. Microscopically, nonmotile organisms were still evident after the first day of incubation. The pH of the media during the 6-day period of observation ranged from 6.8 to 8.3 (Ellison et al., 1964).

By the time of completion of the initial studies, new federal specifications (Federal Register, 1963) were issued, prohibiting the use of penicillin in tissue cultures employed for the preparation of viral vaccines for human use. It was, therefore, deemed advisable to extend observations on the effects of other antibiotics on the viability of leptospires in tissue cultures and to determine viability of leptospires in tissue cultures devoid of antibiotics. Previous efforts to establish cell cultures without the use of antibiotics were unsuccessful because of microbial contamination. Preparation of tissue cultures by the technique of Bodian (1956) was included when it was learned that this procedure is frequently used in commercial vaccine production.

Findings in the two additional experiments are summarized in Table 1. For comparative purposes, portions of the kidney suspension were cultured in the presence of penicillin and streptomycin. Results affirmed previous observations. Addition of penicillin and streptomycin to leptospire-infected tissue-culture suspensions just prior to planting served to eliminate leptospires after 24 hr of incubation; moreover, leptospires were no longer viable by the 4th hr of incubation. The same results were obtained in tissue-culture samples treated with a combination of 50 units of polymyxin B and 100 \( \mu \)g of dihydrostreptomycin. In the preparation derived from tissues trypsinized overnight in the presence of polymyxin B and dihydrostreptomycin, viable leptospires were detected at time of planting but not after 4 hr of incubation. Leptospires were recovered from cell cultures treated with neomycin (100 \( \mu \)g/ml) after 24 hr of incubation, and from those treated with chloramphenicol (100 \( \mu \)g/ml) after 48 hr of incubation, but not thereafter. The antibiotics tested did not affect the establishment or maintenance of cell monolayer tissue cultures in concentrations used. The pH of the cell cultures ranged from 6.8 to 7.8.

Leptospires were isolated in control tissue cultures maintained with no antibiotics up to time.
of cell culture disintegration, on the 25th to 34th days of incubation. The leptospire culture titers in the cell monolayers prepared by the method of Melnick (1956) decreased from $10^{-4}$ on the 4th hr to $10^{-1}$ by the 11th day of incubation. On the 14th and 25th days of observation, titers increased to $10^{-2}$, providing ancillary evidence of the multiplication of leptospires in tissue culture. Microscopically, leptospires were seen only on specimens taken after 4 hr and 1 day of incubation. In the control series of tissue cultures prepared by overnight trypsinization, leptospires were isolated from a $10^{-2}$ dilution of tissue culture after 4 hr of incubation. Approximately 1 day later the titer increased to $10^{-4}$, thereafter gradually receding to $10^{-1}$ by the 34th day of incubation. Microscopically viable leptospires were seen in tissue-culture samples examined after 4 hr of incubation, but not in samples taken 1 to 7 days thereafter. On the 17th and subsequent days of incubation, leptospires again were seen microscopically. There was no evidence that leptospires had a cytopathogenic effect on kidney cells.

**DISCUSSION**

It was demonstrated consistently in this study that leptospires in infected kidneys can survive after subjection to trypsin treatments commonly used for the preparation of monolayer tissue cultures. In kidney cell cultures devoid of antibiotics, leptospires were recovered up to the time of involution of cell cultures, 25 to 34 days after planting. No cytopathogenic effects were noted in the leptospire-infected kidney cell cultures. The degeneration of tissue cultures occurred within the time interval usually seen with normal canine kidney tissue cultures.

Although the tissue-culture medium had essential ingredients and pH suitable for the propagation of leptospires, there was no evidence of remarkable growth of leptospires. The viabiliy of leptospires in infected cell suspensions processed by overnight trypsinization gradually decreased from the 1st to 34th days of incubation. In cultures established from cells trypsinized at room temperature, findings were similar up to the 12th day of incubation; from the 14th to 25th days of incubation, there was an apparent 100-fold increase in number of leptospires from approximately 200 to 20,000 organisms per milliliter. In conventional leptospiral media, the concentration of this strain would be in the order of $2 \times 10^8$ cells per milliliter after 5 to 7 days of incubation. The different findings in the two cell cultures may have reflected variable factors in the preparation and maintenance of the tissue cultures, e.g., trypsinization, bovine serum concentration, and pH regulation methods. The possibility that substances inhibitory for leptospires may have been contained or produced by kidney cells was recognized. The presence of such inhibitors in infected kidneys or urine had been noted by Kesy et al. (1958), Rudge (1958), and Stuart (1956). Yamanaka, Hiramune, and Fujita (1963) found that inhibitors were also present in kidneys from four noninfected animals, and could not be attributed solely to antibody. Generally, their presence was manifest by the more frequent isolation of leptospires from higher than from lower dilutions of kidney suspensions or urine of renal-carrier animals. Cultures for leptospires of serial dilutions of antibiotic-free kidney culture suspensions yielded no evidence of the presence of growth-inhibitory substances, although renal tissue inhibitors of leptospires may have been present. With the exception of chloramphenicol, the known growth-inhibitory substances for leptospires in the medium were the tested antibiotics, penicillin-streptomycin, neomycin, and polymyxin B-dihydrostreptomycin. These antibiotics were present in concentrations well above the known in vitro bacteriostatic or bactericidal levels (Felsenfeld et al., 1950; Kochoine and Mailloux, 1962; Spadlbrow, 1963; Van Thiel, 1957), and were responsible for the elimination of leptospires from tissue cultures. The antileptospiral effects of the penicillin-streptomycin and polymyxin B-dihydrostreptomycin treatments were relatively rapid when compared with those of neomycin and chloramphenicol. The observed inhibitory effect of chloramphenicol on leptospires in tissue cultures was surprising in view of the reported refractiveness of leptospires to the action of this antibiotic at comparable and higher concentrations (Dunn and Thompson, 1953; Spadlbrow, 1963).

Under the test conditions, polymyxin B sulfate (50 units per ml) in combination with dihydrostreptomycin (100 µg/ml) were antibiotics of choice for the treatment of tissue cultures to ensure absence of pathogenic leptospires. It was anticipated that other antibiotics with known antileptospiral activity, e.g., tetracyclines, erythromycin, oleandomycin, etc., could also be used effectively to rule out leptospiral infection hazards in preparation of vaccines from canine kidney tissue cultures. Alternatively, barring the use of antibiotics in canine tissue cultures, more rigid test criteria for the presence of leptospires would be necessary for donor dogs and tissue-culture vaccines (Yager et al., 1963).

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LITERATURE CITED


