Biology of *Borrelia hermsii* in Kelly Medium

HERBERT G. STOENNER

National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana 59840

Received for publication 29 April 1974

More than 800 *Borrelia hermsii* in mouse plasma were required for establishment of growth in an artificial medium (Kelly), but only a single organism of a fully adapted strain (25th subculture) was required for a successful subculture. As judged by generation time, maximal concentration in culture, and length and motility of the organism, the process of adaptation extended through at least 11 subcultures. Because the organisms regularly died shortly after the logarithmic growth phase, transfers at 7- to 10-day intervals were required to maintain continuous cultures.

The first satisfactory medium for growing *Borrelia hermsii* was described recently by Kelly (3). He maintained this organism in continuous cultivation for 8 months, during which time it retained its infectivity for mice. The maximal concentration of organisms was obtained after 7 days of incubation at 35°C, when numbers varied from $3 \times 10^7$ to $5 \times 10^7$ borreliae per ml. This report deals primarily with the behavior of the organism in culture medium, changes in morphology and motility associated with adaptation to the medium, and other parameters of growth and survival of *B. hermsii* in this medium.

**MATERIALS AND METHODS**

The method of preparing the medium was slightly modified from that described by Kelly. The basal medium, bovine albumin solution, 7% gelatin solution, and serum were prepared and stored separately, as prescribed. However, instead of dispensing them individually into 9-ml culture tubes, all components except 7% gelatin were pooled, mixed, and filtered before dispensing. Also, these components were filtered under air pressure through an ultrafine, sintered-glass filter instead of a 0.22-μm membrane filter. The 7% gelatin was added last, and the complete medium was then dispensed into 9-ml culture tubes.

The strain of *B. hermsii* used was obtained from *Ornithodoros hermsi* collected by W. Burgdorfer near Spokane, Washington (4). Serial passages were made in Swiss mice of the Rocky Mountain Laboratory stock every 48 h, or less, until the spirochtemia was adequate for successful subculture in medium. Transfers to fresh medium were made at 7- to 10-day intervals to assure continuous cultivation of the organism. Portions (2 ml) of some of the passages were preserved by adding sterile glycerine to a concentration of 10% before freezing them at −70°C in an alcohol bath.

Spirochetes in the medium and citrated mouse plasma were counted under a cover slip with a dark-field microscope. As delivered from a 0.005-ml calibrated capillary pipette (Kimble microcapillary pipette), 0.0025 ml of medium was deposited on a slide, surrounded by a ring of a mixture containing petrolatum and plasticine (2:1, wt/wt), and covered with a cover slip, and sufficient pressure was applied to extend the drop to a diameter of about 8 mm. Counts were made of dilutions containing 3 to 12 organisms per 0.0025 ml; the entire area was searched for borreliae at a magnification of ×125. All counts were made in duplicate and averaged. It should be emphasized that cover slips, slides, and capillary tubes must be cleaned thoroughly.

A method was devised for culturing a single spirochete in sterile capillary tubes (90 mm long, 0.4 mm inner diameter). In this method, the number of spirochetes in vigorously growing cultures (5 to 7 days old) or mouse plasma was first established by direct count. Decimal dilutions in sterile medium were then made until the highest dilution contained less than one organism per 0.01 ml of medium. When necessary, intermediate dilutions also were made to obtain the desired concentration of organisms. Tubes were then filled two-thirds full and inverted to form an air space at both ends, and the clean end was mounted vertically in plasticine. After all tubes had been filled with medium of a given dilution, the free ends of the capillary tubes were passed briefly through the edge of a Bunsen burner flame. Tubes were again inverted, and the seared end was mounted vertically in plasticine. After tubes were incubated at 35°C for 10 to 13 days, they were examined for growth, which was restricted to the lower two-thirds of the tube.

The process of adaptation was studied by comparing growth characteristics, generation times, maximal yield, and survival of 4th, 11th, and 25th subcultures. Frozen portions of these subcultures were thawed simultaneously and used as inocula when the preceding parameters were compared in the same batch of medium.

**RESULTS**

Kelly established cultures by inoculating the medium with 0.05 ml of blood from infected
mice. However, he did not indicate the number of organisms required to regularly establish a culture. During the early course of these investigations, it became apparent that relatively large numbers were required to obtain a culture from infected mouse blood. Attempts to establish cultures with citrated blood from mice infected with mild spirochetemias rarely were successful.

In initial trials to determine the minimal number of *B. hermsii* necessary for initiating growth, tubes of medium were inoculated with conventionally prepared samples from decimal dilutions of blood. Results were erratic, and sometimes growth did not occur in tubes inoculated with as many as $1.7 \times 10^4$ organisms. Exposure of the organism to oxygen incidental to thorough mixing of dilutions was considered injurious and possibly responsible for the erratic results. Titrations were then made by inoculating tubes of medium with varying quantities of infectious blood of mice that had different levels of spirochetemia. Tubes were examined for growth at varying intervals for 40 days after inoculation. Under these conditions, borreliae grew in every tube inoculated with blood containing at least $2.38 \times 10^4$ organisms, but in only one of four tubes inoculated with $8 \times 10^3$ spirochetes.

Repeated attempts were made to obtain growth of *B. hermsii* in capillary tubes inoculated with citrated plasma from infected mice. In most experiments, the organism survived for 2 to 3 days but did not multiply. Occasionally, growth occurred in tubes inoculated with $10^3$ or more organisms, but results were too erratic for the method to be useful. Further investigations are being made of the variables that influence growth of unadapted strains in capillary tubes.

The 28th passage was considered fully adapted to the medium, as judged by generation time, maximal concentration in culture, and length and motility of the organism. The number of organisms required for a successful transfer was determined in capillary tubes and conventional 9-ml culture tubes. A 5-day-old culture determined to have $8 \times 10^2$ borreliae per ml was appropriately diluted so that the last two highest dilutions contained 0.8 and 0.4 organisms per 0.01 ml. If only a single organism was required for a successful transfer, growth should occur in 80 and 40% of the capillary tubes, respectively. The results obtained (Table 1) indicate that essentially all of the organisms in a fully adapted culture were capable of reproduction, whether cultivated in capillary tubes or 9-ml culture tubes.

| No. inoculated | Capillary tubes | 9-ml culture tubes |
|---------------|----------------|-------------------|
| 800           | Not done       | 4*/4              |
| 80            | 10/10          | 4/4               |
| 8             | 10/10          | 4/4               |
| 0.8           | 12/15          | 6/8               |
| 0.4           | 7/20           | Not done          |

* Numerator, Number of tubes with growth; denominator, number inoculated. Capillary tubes were incubated for 12 days, and 9-ml culture tubes were incubated for 40 days before final readings were made.

Several serotypes that were recovered at initial infection and after three relapses, from a single mouse have been carried in continuous culture through 35 passages. Changes in morphology and motility were noted as these isolates were cultivated. Borreliae in early passages were actively motile and, in addition to continuous rotation on their own axes, they propelled themselves forward and backward. For the first 4 to 6 days after inoculation of medium, many organisms alternately contracted their coils, reduced their length by one-half, and then extended themselves to their full length. This activity was seen only in early passages and in cultures containing less than $10^4$ organisms per ml. When the organism was adapted to growth in the medium, as indicated by reaching a maximal concentration of at least $10^5$ organisms per ml, motility of the organism was limited essentially to rotation on its own axis. Organisms were rarely seen propelling themselves through the medium.

The length of the organism was influenced by the number of passages, age of culture, and size of inoculum. In early passages of cultures containing less than $10^4$ organisms, the length as measured by dark-field microscopy varied from 10 to 22 $\mu$m, which is comparable to its in vivo size in the mouse. As maximal growth of early passages was reached, the length increased, but did not reach that of fully adapted strains. After full adaptation, the length varied from 21 to 52 $\mu$m when maximal concentration was reached in a culture. However, those in fully adapted cultures were shorter during the early growth phase in passages initiated with less than $10^4$ borreliae.

The influence of adaptation on the dynamics of growth and survival in medium is shown in Fig. 1. Only actively growing cultures were used as inocula. The generation time (1) of the fourth
subculture, when calculated for the first 6 days of growth, was 43 h, whereas that of the 11th subculture was 24 h and that of the 25th was 16 h. The maximal concentrations of borreliae in the 4th, 11th, and 25th subcultures were attained in 14, 12, and 10 days, respectively. On the basis of motility, organisms of the 4th subculture survived the longest after maximal concentration was attained.

This medium did not support growth for extended periods. Within 2 days after maximal growth was achieved, motility of organisms was noticeably reduced and declined gradually until death of the culture 10 to 16 days later. To maintain continuous cultivation of a culture, it was necessary to transfer organisms to fresh medium within 3 to 4 days after the maximal concentration of organisms was obtained. Sometimes, transfers made later did not result in growth, even though the organisms transferred were motile.

**DISCUSSION**

The medium described by Kelly was shown to be excellent for supporting growth of adapted strains of *B. hermsii*, but rather inefficient for isolating the organism directly from mouse blood. Dodge (2) was able to isolate *B. recurrentis* from blood of patients in Ethiopia with Kelly medium and three other media with a Trypticase soy yeast base, but was unable to subculture these isolates in any of the four media used.

Kelly medium supported growth of fully adapted strains so effectively that only a single organism was necessary to establish subcultures. However, its ability to support growth for extended periods was limited. Shortly after maximal growth was attained, reproduction essentially ceased and the motility of all organisms in a culture declined simultaneously. If a significant percentage of organisms continued to reproduce, one would expect to observe a declining number of borreliae with motility characteristics of a young culture, but these were never seen. The reason for death of organisms in culture has not been established, but conceivably it could be due to an accumulation of toxic by-products of metabolism or exhaustion of a critical nutrient. Acid is produced by the organism, and the life of a subculture can be prolonged by neutralizing the acid with sterile sodium hydroxide solution (R. Kelly, personal communication).

The modified method of preparing medium certainly has advantages over that described by Kelly in which quantities of basal medium, 7% gelatin, and serum were dispensed individually in 9-ml tubes. The final product by the modified method is more uniform in composition, and the chance of contaminating the medium is greatly reduced. Modification of the composition of the medium was not attempted, but serum from other animal sources was evaluated as a substitute for rabbit serum. That of guinea pigs and sheep was unsatisfactory, but commercially available foetal calf serum supported growth of adapted strains comparable to that of rabbit serum-supplemented medium. However, more "breed nests" or clumps of actively dividing organisms were seen during the early phase of growth in calf serum-supplemented medium.

The medium devised by Kelly and the use of capillary tubes for establishing a population...
from a single organism should facilitate studies on genetics of *B. hermsii* and the dynamics of antigenic changes associated with relapses of infection in animals. One serious problem, however, is the rather low efficiency of the medium in cultivating the organism as it exists naturally in animals. Some modification of the medium or culture procedure apparently is necessary to improve the efficiency of the medium for directly isolating borreliae from the blood of animals.

**LITERATURE CITED**

1. Buchanan, R. E., and E. I. Fulmer. 1929. Physiology and biochemistry of bacteria, p. 19. Williams and Wilkins Company, Baltimore.
2. Dodge, R. W. 1973. Culture of Ethiopian strains of *Borrelia recurrentia*. Appl. Microbiol. 25:935–939.
3. Kelly, R. 1971. Cultivation of *Borrelia hermsi*. Science 173:443–444.
4. Thompson, R. S., W. Burgdorfer, R. Russell, and B. J. Francis. 1969. Outbreak of tick-borne relapsing fever in Spokane County, Washington. J. Amer. Med. Ass. 210:1045–1050.