Preservation of *Borrelia kansas* and *Plasmodium berghei*

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*Borrelia kansas* and *Plasmodium berghei* have been stored after slow freezing in thioglycollate-glycerol medium for a 6-month period. During this time, 75% or more of the *Borrelia* remained motile, many intracellular malarial parasites exhibited amoeboid movement, and the growth pattern of both organisms in mice remained unchanged.

There are several problems associated with the maintenance of obligate parasites in the laboratory: propagation may be expensive both in terms of labor and the cost of the animal host; in addition, a factor to be considered is the ease with which these organisms undergo variation within the vertebrate host. Storage procedures for borrelia and malaria have been described (2, 3, 4) which seem to require freezing the organisms in their arthropod host or freezing them in a high concentration of blood of the vertebrate host. The method described here involves dilute blood, allowing for the utilization of fewer organisms in the presence of less antibody, if it happens to be present. The method produces negligible damage to the erythrocyte or observable killing of the parasite. Therefore, the probability of the original strain being overgrown by a more resistant variant which may also be very different in virulence, antigenicity, or other properties is very small.

**MATERIALS AND METHODS**

Albany strain of white mice was infected with *Borrelia kansas* or *Plasmodium berghei* by inoculation of a suitable dilution of infected tail blood into the peritoneal cavity. On the second day after infection with borrelia and the seventh day after infection with malaria, blood was removed for storage. One part blood was added to 25 parts sterile Thioglycollate Medium (Difco) containing 10% glycerol. This material was then mixed and distributed into either 1- or 5-ml vials which were sealed in a flame.

The slow freezing process used consisted of 45 min at 5°C followed by 45 min at 0°C and then storage in either liquid nitrogen (−195°C) or in a dry ice chest (−55°C). When ampoules were removed from storage for use, they were thawed quickly at 34°C and inoculated without further dilution into mice.

**Counts.** Thin wet mounts of tail blood of borrelia-infected mice were examined under dark field, and the number of organisms was determined in each high dry field (40× objective). Ten fields on each of three different wet mounts were counted, and the average number of organisms per field was determined.

The parasitemia in malaria-infected mice was determined from Wright's stain of thin cover slip smears of tail blood. In such preparations, even distribution of all cellular elements was obtained; parasitemias determined by different individuals, and from all areas of the smears, were in good agreement. No less than 2,000 cells were used for each determination.

**RESULTS**

At all time intervals, direct microscopic examinations of thawed borrelia preparations showed most erythrocytes and leukocytes to be in good condition: red cells retained their shape and granules of leukocytes were in motion within the cells. Seventy-five to 80% of the borrelia organisms remained actively motile, suggesting minimal damage during freezing and storage. Erythrocytes in malaria-infected blood seemed to be more fragile, resulting in some hemolysis; however, most red cells remained intact, and the motile intracellular parasites were seen.

In Table 1, the change in numbers of borrelia with the day of infection is given for three intervals of storage in the dry ice chest. The picture of infection for each interval was the same; i.e., 3 to 13 organisms per field on the first day of infection, an increase to between 77 and 200 organisms per field on the second day, and further increase to more than 200 organisms per field followed often by a decrease in numbers and death of the animal in 5 to 7 days. One can also see individual variation in host resistance to infection in three mice which did not differ in counts from the others during the first 2 days of infection but which did recover. However, there did not seem to be any change in virulence of
TABLE 1. Pattern of growth of *Borreria kansas* in mice at various times after freezing

| Interval of storage | Mouse no. | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 14 | 15 |
|---------------------|-----------|----|----|----|----|----|----|----|----|----|----|----|----|
| 1 week              | 1         | 3.0a| 77 | >200|200 |    |    |    | 200|    |    |    |    |
|                     | 2         | 4.0 | 93 | >200|200 |    |    |    | 100|    |    |    |    |
|                     | 3         | 5.0 | 133| >200|200 |    |    | 0.01|0.06|1.4 |0.02|0   |0   |
|                     | 4         | 6.6 | 150| 200 |    | 0.05|0   | 0.02|0.06|0   |0   |    |    |
|                     | 5         | 6.2 | 131| >200|    |    |    |    |    |    |    |    |    |
| 3 weeks             | 1         | 11.3|149 | >200|    |    |    |    |    |    |    |    |    |
|                     | 2         | 10.7|130 | >200|    |    |    |    |    |    |    |    |    |
|                     | 3         | 10.0|120 | 7.9 |    | 0.06|0.03|0.04|0   |0   |0   |0   |0   |
|                     | 4         | 12.3|200 | 200 |    |    |    |    |    |    |    |    |    |
|                     | 5         | 11.4|200 | 200 |    |    |    |    |    |    |    |    |    |
| 6 months            | 1         | 5.1 | 141| 200 |200 |    |    |    |    |    |    |    |    |
|                     | 2         | 12.7|150 | 200 |200 |    |    |    |    |    |    |    |    |
|                     | 3         | 12.5|174 | 200 |200 |    |    |    |    |    |    |    |    |
|                     | 4         | 9.2 | 167| 200 |200 |    |    |    |    |    |    |    |    |
|                     | 5         | 10.6|167| 200 |200 |    |    |    |    |    |    |    |    |

* Number of organisms per high dry field. Average of 75 to 100 fields.

a Per cent parasitemia calculated from no less than 2,000 cells.

b Death.

Table 2 shows the change in parasitemia in *P. berghei* with the day of infection. Here, again, it appears that the rate of appearance of the parasites, the height of parasitemia, and the death rate of the animals did not change over this period of storage in dry ice.

**DISCUSSION**

These experiments are the result of a search for a suitable medium in which to maintain cells and parasites over a period of hours for student laboratory work and over a period of months during an investigation concerned with physiological changes of the host produced by infection. For the latter, it was of great importance to insure against any change in the virulence of the parasite.
The method used does present certain advantages: diluted blood can be used, a very large majority of the organisms remain motile, and no vial opened over a 2-year period has proven to be either noninfectious or reduced in virulence.

No studies have been undertaken to determine the role of each component of the medium on the stability of the blood components. However, it would appear that the tonicity is excellent for both blood cells and parasites; in addition, the low oxidation-reduction potential of the medium may enhance the survival of the anaerobic borrelia because they remain viable in this medium without glycerol for about 24 hr. The addition of glycerol (or other nonelectrolytes) apparently reduces the gross deviations in salt concentration from normal during the freezing process; a great increase in salt concentration within cells during freezing seems to be responsible for a major part of the damage to living cells rather than the formation of ice (1).

The slow freezing process allows time for cellular permeation to take place, and, in this case, the low temperature of the nitrogen tank did not seem to have any advantage over that of the CO₂ box.

Virulence, as measured by the pattern of appearance of the organisms in the blood and the time of death of the animals, remains essentially the same. It is felt that this is related to the fact that most of the parasites appear viable at all time intervals when examined directly with the microscope.

It would also be desirable to examine the organisms further for changes in antigenicity and drug sensitivity after preservation by this method.

ACKNOWLEDGMENT

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ERRATUM

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Volume 20, no. 2, pages 224 to 226: The organism referred to as “Borrelia kansas” is correctly termed “Borrelia turicatae, Kansas strain.”