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# Retention of Motility of *Treponema pallidum* (Nichols Virulent Strain) in an Anaerobic Cell Culture System and in a Cell-Free System

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Optimum parameters for retention of motility of *Treponema pallidum* (Nichols virulent strain) were found by anaerobic co-incubation of the treponeme with rat glial cells and anaerobic incubation in spent medium obtained from glial cells originally grown aerobically.

The successful in vitro cultivation of virulent *Treponema pallidum* will probably follow the discovery of environmental parameters that extend its survival time during incubation. To date, the short-term survival of the virulent treponeme has been accomplished in various complex artificial media (3, 7, 8) and in attempts to grow it in cell cultures (2, 9, 10). However, since the microorganism has yet to be cultivated in vitro, the investigation of cell culture systems for this purpose remains a viable alternative to studies based solely on the use of artificial media. In addition, recent work by Sykes and Miller (5), Sykes et al. (6), Ovcinnikov and Delektorskij (4), and Fitzgerald et al. (2) on the detection of intracellular treponemes suggests that a utilizable cell culture system for treponemal co-incubation could provide valuable insights into the mechanism(s) of host-parasite interaction (10). To date there are no reports of a universally acceptable system for the co-incubation or cultivation of *T. pallidum* in cell cultures. Consequently, we have sought to develop such a system.

Modified Eagle medium (prereduced Eagle minimal essential medium [1] plus 10% newborn calf serum, $PRMEM_{10}$) was prepared as described in Table 1. This basal medium was diluted with unreduced Eagle medium plus 10% newborn calf serum ($MEM_{10}$) to obtain a gradual variation in redox potential from −408 to −232 mV $E_{cal}$ (Tables 2 and 3). Although rat glial cells (kindly supplied by W. Bondareff) exhibited no growth under anaerobic conditions after 48 h at 33.5 C (Table 2), cytopathological effects were minimal or undetectable. The cells could be repassed in aerobic medium after at least 96 h of anaerobic cultivation in various dilutions of $PRMEM_{10}$.

The glial cell cultures co-incubated with *T. pallidum* were maintained under deoxygenated $N_2$ in sealed (air-tight) Leighton tubes containing removable cover slips for subsequent dark-field microscopic examination. New Zealand rabbits (6 to 7 lb [2,721 to 3,175 g]) inoculated with $10^7$ to $3 \times 10^7$ microorganisms/testes were killed 8 to 9 days later after observing the initiation of an orchitis. When freshly harvested virulent treponemes were inoculated into anaerobic glial cell cultures (1 to 5 treponemes/glial cell) and incubated 24 h at 33.5 C, motile treponemes were detected, both in the supernatant fluid (at about 1/5 to 1/10 the initial concentration) and attached to the cells (Table 3). The treponemes could not be dislodged by agitation with a Vortex tube mixer, and they appeared to have become elongated, compared with unattached nonmotile microorganisms, without any evidence for division. In our earlier experience media having a redox potential of $-250 \pm 50$ mV $E_{cal}$ appeared to be optimal for this system (Tables 2 and 3). The longevity of motility of *T. pallidum* has been extended well beyond the time reported by Fitzgerald et al. (2).

In another series of experiments, unreduced $MEM_{10}$ was used to cultivate approximately 3.0 $\times 10^7$ rat glial cells in 32-oz (0.946-liter) bottles for 48 h at 33.5 C. The supernatant fluid was removed and diluted in $PRMEM_{10}$ (pH 7.4 to 7.6) under deoxygenated $N_2$ or 50% $N_2$ plus 50% $H_2$. The controls consisted of fresh $MEM_{10}$ diluted in $PRMEM_{10}$, as described above. Freshly harvested treponemes (0.3 ml or approximately $10^7$ to $3 \times 10^7$) were inoculated into each tube containing 10 ml of medium. Percentage of motility was estimated at 24-h intervals by counting 50 to 100 random fields or about 30 to 70 treponemes from triplicate tubes under dark-field microscopy using a 40× objective.



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TABLE 1. *Preparation and components of PRMEM*$_{10}$

| Procedure | Solution designation | Components | Final concn (per liter) |
|---|---|---|---|
| 1. Prereduce the salt solution[a] by heating in autoclave for 8 min at 121 C, at slow exhaust. Cool to room temperature under deoxygenated $N_2$. | Salt solution | NaCl | 5,440 mg |
| | | KCl | 320 mg |
| | | $MgSO_4 \cdot 7H_2O$ | 140 mg |
| | | $KH_2PO_4$ | 48 mg |
| | | $Na_2HPO_4$ | 48 mg |
| | | Glucose | 800 mg |
| | | $CaCl_2 \cdot 2H_2O$ | 112 mg |
| | | Resazurin | 1 mg |
| 2. Add the reducing agents in powder form under $N_2$. Adjust pH to 7.8 to 7.9 and sterilize in stoppered tubes or flask at 121 C for 25 min.[b] Cool to room temperature. | Reducing agents | Reduced glutathione | 1,200 mg |
| | | L-Cysteine | 800 mg |
| | | Sodium thioglycolate | 700 mg |
| 3. Mix appropriate volumes of the serum solution[c] with the reduced salts solution to obtain a final concentration of 10% serum plus 1× concentration of all the other nutrients listed. | Serum solution | 50× MEM essential amino acids | 20 ml |
| | | 100× MEM vitamin mixture | 10 ml |
| | | 200 mM glutamine | 10 ml |
| | | 1.4% $NaHCO_3$ | 20 ml |
| | | 0.2 N NaOH | 20 to 30 ml |
| | | Newborn calf serum | 100 ml |
| 4. Mix appropriate proportion of unreduced MEM$_{10}$ with reduced MEM$_{10}$ (Tables 2 and 3) under deoxygenated $N_2$ to obtain media at various redox potentials. | | | |

[a] The sodium, potassium, and magnesium salts, the sodium and potassium phosphate buffers containing glucose, the calcium chloride, and the resazurin solutions were prepared separately at 10× concentration prior to mixing the final solution.

[b] The pH was adjusted with about 0.5 ml of 10 N NaOH, and the stoppered tubes and Pyrex flasks were sterilized in an autoclave press (Bellco, Vineland, N.J.).

[c] The serum solution was adjusted with 0.2 N NaOH to pH 7.8 prior to mixing with the reduced salts to obtain a final pH of 7.4 to 7.6.

Results from our earlier preliminary work using the protocol above suggested that the optimal conditions for the long-term retention of treponemal motility in spent medium were: (i) a redox potential of $-250 \pm 50$ mV $E_{cal}$ (i.e., the medium mixture of one part of PRMEM$_{10}$ to nine parts of spent medium); (ii) a pH range of 7.3 to 7.6; (iii) a deoxygenated atmosphere of 50% $N_2$ plus 50% $H_2$; and (iv) an incubation temperature of 33 ± 1 C. The data in Table 4 are typical of results obtained from a minimum of six independent experiments performed under optimal conditions. The estimated time at which motility drops to 50% for PRMEM$_{10}$ diluted with the supernatant fluid from glial cells is about the same under both $N_2$ and $N_2$ plus $H_2$ (i.e., 50 to 60 h) (Table 4). However, the estimated time to reach 10% motility under $N_2$ plus $H_2$ is longer than that obtained under $N_2$ alone. The 10 and 50% motilities obtained for the controls (i.e., mixtures of fresh MEM$_{10}$ and PRMEM$_{10}$) are considerably shorter (i.e., 15 to 20 h). Thus, the supernatant fluid appears to contain some factor(s) not available in fresh MEM$_{10}$ that enhances the retention of treponemal motility.

In summary, we have presented evidence that the retention of motility of *T. pallidum* in cells and cell-free supernatant fluid is greatly enhanced under anaerobic conditions in appropriately reduced media. This evidence is in sharp contrast to previous reports using tissue culture systems for the co-incubation of *T. pallidum*, in which no attempt was made to control the redox potential (9, 10) and/or strict anaerobiosis was not maintained during incubation (2, 9). We are now in a position to perform a series of metabolic experiments which shall be less affected by rapid decreases in *T. pallidum* viability in cell-free and in cell cul-

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TABLE 2. *Rat glial cell cultivation under anaerobic conditions using various dilutions of PRMEM$_{10}$*

| Mixture of PRMEM$_{10}$/MEM$_{10}$ (%) | $E_{cal}$[a] (mV) at pH 7.6 | Cell count[b] (× $10^5$) |
|---|---|---|
| 100/0 | −408 | 1.61 ± 0.43 |
| 70/30 | −380 | 2.37 ± 0.32 |
| 30/70 | −334 | 3.40 ± 0.32 |
| 10/90 | −232 | 2.07 ± 0.28 |
| 0/100 | +48 | 3.87 ± 0.43 |
| Aerobic control | +48 | 5.23 ± 0.87 |

[a] Redox potentials were obtained at room temperature with a saturated KCl combination calomel-platinum electrode (Orion model 96-78, Orion Research, Cambridge, Mass.).

[b] Initial inoculum was 2.2 × $10^5$ rat glial cells per Leighton tube in triplicate samples. The cells were cultivated 48 h at 33.5 C under deoxygenated $N_2$ except for the aerobic control, which was cultivated in air. Counts were performed on a Coulter counter after the cells were removed from the glass surfaces by a solution containing 0.05% trypsin and 0.05% ethylenediaminetetraacetic acid.

TABLE 3. *Motility retention of virulent T. pallidum co-incubated with rat glial cells*[a]

| Mixture of PRMEM$_{10}$/MEM$_{10}$[b] (%) | Motility (%)[c] | |
|---|---|---|
| | Supernatant fluids | Attached treponemes |
| 100/0 | 0 | 0 |
| 70/30 | 45 ± 17 | 92.8 |
| 30/70 | 77 ± 8 | 95.0 |
| 10/90 | 81 ± 10 | 100.0 |
| 0/100 | 0 | 0 |

[a] The cultures were incubated for 24 h under deoxygenated $N_2$ at 33.5 C.

[b] The redox potentials for each mixture of PRMEM$_{10}$/MEM$_{10}$ are given in Table 2.

[c] Percentage of motility was determined by counting 50 to 100 treponemes from Leighton tubes with cover slips containing approximately 2.2 × $10^5$ rat glial cells in 1 ml of medium inoculated with 3 × $10^5$ to 10 × $10^5$ treponemes in 0.05 ml. Cover slips with attached glial cells were placed face down on glass slides and inspected under dark field microscopy using a 40× objective lens.

ture systems. In addition, a useful by-product of the present report is that it may be possible to ship fresh (unfrozen) human isolates in the cell-free medium from many parts of the world for immediate laboratory use.

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TABLE 4. *Motility retention of virulent T. pallidum in reduced fresh Eagle medium and in reduced supernatant fluids from rat glial cells*[a] *diluted with PRMEM$_{10}$*

| Culture medium[b] | $M_{50}$[c] | | $M_{10}$[c] | |
|---|---|---|---|---|
| | $N_2$ | $N_2 + H_2$ | $N_2$ | $N_2 + H_2$ |
| Fresh MEM$_{10}$ | 16.5 | 18.0 | 41.5 | 48.0 |
| Supernatant fluids | 51.5 | 57.8 | 71.5 | 119.0 |

[a] Rat glial cells ($10^7$ to 3 × $10^7$) were cultivated for 48 h at 33.5 C aerobically in MEM$_{10}$, and then the supernatant fluid was collected and mixed with PRMEM$_{10}$, as described in the text and in footnote *b* below.

[b] Nine parts of supernatant fluid and nine parts of fresh MEM$_{10}$ were each diluted with one part of PRMEM$_{10}$ by volume to obtain a final $E_{cal}$ of −250 mV at pH 7.6 at room temperature.

[c] The estimation of the time in which the motility of the treponemes dropped to 50% ($M_{50}$) and to 10% ($M_{10}$), respectively, was obtained from triplicate samples of a plot of percentage of motility versus time. The cultures were incubated at 33.5 C.

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