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Prolonged Survival of Virulent *Treponema pallidum* (Nichols Strain) in Cell-Free and Tissue Culture Systems

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Survival of *Treponema pallidum* was found to be prolonged in the presence of tissue culture. Of the 12 cultures studied, cottontail rabbit epithelium (Sf1Ep) supported *T. pallidum* for the longest time. In horizontal Leighton tubes with reduced medium and an atmosphere of 5% CO_2 in N_2, the 50% survival time (ST_{50}) was 5 to 6 days for treponemes associated with monolayers of Sf1Ep cells. Comparable cell-free tubes had ST_{50} values of less than 4 days. In vertical Leighton tubes containing 6 ml of prereduced medium incubated aerobically, gradients of O_2 tension and redox potential were established. Attachment and survival of *T. pallidum* were greatest at a depth of about 10 to 20 mm. Motility was between 70 and 95% in this area throughout the first 14 days of incubation. Occasionally, greater than 50% motility was observed for as long as 21 days. The redox potential and O_2 tension in the optimal area of gradient cultures were reproduced by adjusting the medium depth in a shell vial culture system containing cells on a horizontal cover slip. Treponemes associated with the cell monolayer in both gradient and shell vial cultures were still virulent after 21 days in vitro. The dilution of testis extract and the concentration of *T. pallidum* were found to be important factors in survival of *T. pallidum*.

Many attempts have been made to cultivate *Treponema pallidum* in vitro during the past 70 years, and a number of investigators have reported the successful achievement of this elusive goal. Unfortunately, the relationship of these avirulent, cultivable treponemes to the causative agent of syphilis appears to be rather remote—if, indeed, any relationship exists. Failing to cultivate *T. pallidum*, many investigators attempted to develop survival media in which virulent organisms could be maintained for extended periods of time; they hoped that those studies would eventually lead to the development of a medium in which *T. pallidum* would replicate. Virtually all the studies concerning cultivation and maintenance of *T. pallidum* that were published up to 1966 have been ably reviewed by Willcox and Guthe (21). Most of those investigations emphasized the use of media containing reducing agents as well as the maintenance of strict anaerobic conditions because it was generally agreed that *Treponema* were anaerobic organisms and that O_2 severely limited their survival. Under these conditions, Weber (20), by using a modification of a medium devised by Nelson (16), found the ST_{50} (survival time, in days, during which 50% of the organisms remained motile) to be as long as 16 days. Unfortunately, as in studies by others (2, 9, 18), there was no correlation between the ST_{50} and virulence for rabbits, which was lost after 6 days in vitro.

Although all members of the genus *Treponema* that have thus far been cultured appear to be anaerobes, at least one is known to be oxygen tolerant (19). Furthermore, Kawata (12) demonstrated the presence of cytochromes in the Reiter treponeme, suggesting the possible use of oxygen. More importantly, Cox and Barber (4) showed that *T. pallidum* consumes O_2 at the same rate as a known aerobic *Leptospira*. Baseman et al. (1) demonstrated that optimal metabolic activity, as indicated by the use of glucose and pyruvate, and the rate and extent of protein synthesis by *T. pallidum* occurred in the presence of 10 to 20% O_2. Protein synthesis was inhibited after a few hours under anaerobic conditions. Although the experimental conditions demonstrating the use of O_2 by *T. pallidum* may not necessarily reflect the requirements for in vitro replication, this possibility cannot be eliminated. This is especially true in view of all previous attempts to cultivate *T. pallidum* under anaerobic conditions.

In recent years, tissue cultures from various mammalian species have been investigated as possible substrates for in vitro cultivation of *T. pallidum*. In 1975 Fitzgerald et al. (8) reported that in cultures of rabbit testis and human tumor cells under aerobic conditions, *T. pallidum* not

only attached to the cell surface but also entered the cells. Virulent treponemes could be detected up to 24 h later. Subsequently, it was reported by Sandok et al. that, under anaerobic conditions, *T. pallidum* remained motile and virulent for at least 5 days in cultures of human prepuce cells (18). Jones et al. (11) reported replication and subculturing of *T. pallidum* in cultures of baby hamster kidney cells under aerobic conditions. However, they could not consistently demonstrate rabbit infectivity of the tissue culture-passaged treponemes, nor did they demonstrate virulence of the treponemes in the terminal passages. The validity of these results must await independent confirmation.

The studies reported here were initiated to develop a medium that would be optimum for maximal survival of virulent *T. pallidum* and which, at the same time, could serve as a maintenance medium for mammalian tissue cultures. Our goal was to develop a tissue culture system in which we could eventually demonstrate replication of *T. pallidum*.

MATERIALS AND METHODS

Rabbits. The rabbits used in these experiments were 6- to 8-month-old New Zealand white males weighing 3.0 to 4.0 kg. Before rabbits were used, their sera were tested by the venereal disease research laboratory test and found to be nonreactive. Animals were housed individually at a temperature of 16 to 18°C.

T. pallidum. The virulent Nichols strain of *T. pallidum* used throughout this work was obtained from J. N. Miller of the University of California, Los Angeles. Large pools were maintained frozen and were used to inoculate rabbits as a source of treponemes for experimental work.

The organisms were propagated by intratesticular inoculation of 2.5×10^7 *T. pallidum* suspended in 0.5 ml of basal reduced medium (BRM), the formula for which is given below. The use of uniform inocula that had been stored frozen resulted in predictable infections requiring 12 days (±1 day) to reach a peak orchitis.

The testes were removed aseptically, freed of extraneous tissue, and minced with iris scissors. Extraction of the treponemes in each testis was carried out in 5 ml of BRM in a 50-ml Fernbach flask. Air was displaced from the flask with 5% CO_2 in N_2. The flask was sealed with a silicone rubber stopper and placed on a shaking machine (180 oscillations per min) at 33°C for 30 min. After extraction, the suspension was centrifuged at $500 \times g$ for 5 min, the pellet was discarded, and the treponemes in the suspension were counted.

Typically, this method of propagation and extraction yielded between 1.0×10^8 and 4.0×10^8 *T. pallidum* per ml, of which approximately 95% were motile. For survival studies in cell-free cultures, the *T. pallidum* suspension was diluted 1:20 in BRM. For tissue culture studies, to facilitate accurate enumeration of *T. pallidum* on cell monolayers, the concentration of *T. pallidum* was held constant at 5×10^6 per ml of culture fluid. In some of the cell-free cultures and in most tissue cultures, both the treponeme and the testis extract concentrations were held constant by making a preliminary dilution of the *T. pallidum*-containing extract in testis extract that had been cleared of *T. pallidum* by centrifugation at $12{,}000 \times g$ for 10 min.

The pools of frozen *T. pallidum* were prepared by adjusting the suspension with BRM and glycerol (final concentration 15%) to a final treponeme concentration of 2.0×10^8 per ml. Portions (1.2 ml) of the adjusted suspension were distributed in 2-ml plastic ampules, which were then frozen at a rate of 2°C per min and stored in liquid N_2. Pools have been kept in this state for at least 8 months with no perceptible loss in potency.

Recently, testes from rabbits treated with cortisone acetate as described by Hardy and Nell (10) have been used to prepare large frozen pools of *T. pallidum*. The use of cortisone acetate has resulted not only in greater yields of treponemes, but also in much cleaner preparations.

BRM. This medium was gradually developed by using Eagle minimum essential medium (MEM) (5) combined with reducing agents and other supplements. The BRM stock contains pyruvate, glutathione, and cysteine at the concentrations used by Kimm et al. (13), and dithiothreitol (DTT), which is known to act as a protective agent for SH groups and which presumably would maintain glutathione and cysteine in the reduced state (3, 15). The following components were combined in distilled water to a final volume of 100 ml to make up the BRM stock: 10 ml of 10× MEM in Earle balanced salt solution without L-glutamine and $NaHCO_3$, 1 ml of 200 mM L-glutamine, 3.75 ml of 7.5% $NaHCO_3$, 1 ml of 100× MEM nonessential amino acids, 1 ml of sodium heparin solution containing 100 U, 10 mg of sodium pyruvate, 40 mg of reduced glutathione, 20 mg of DL-cysteine hydrochloride, and 10 mg of DTT. In later studies, as indicated, the formulation of the stock medium was changed in that the concentrations of cysteine, glutathione, and DTT were decreased by 50%, and the volume of 7.5% $NaHCO_3$ was decreased to 3.38 ml per 100 ml of stock. This was referred to as BRM-50 stock. These stock media were membrane filter-sterilized (Millipore Corp.) and stored under 5% CO_2 in N_2 at 4°C for up to 1 month before use.

The complete medium (BRM or BRM-50) was prepared by mixing 77.5 parts of the stock medium, 2.5 parts of 1 M HEPES buffer (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; pH 7.3), and 20 parts of fetal bovine serum (FBS). The medium was preequilibrated with 5% CO_2 in N_2 at 33°C for 4 to 8 h before use. The final pH of the medium was 7.3 to 7.4.

In some experiments, medium lacking phenol red but containing resazurin was used. In these instances, the stock medium was prepared from 10× Earle balanced salt solution without phenol red plus a 100× vitamin solution and a 50× essential amino acid solution. Resazurin was added at a final concentration of 0.0001% at the time the complete medium was prepared.

Pre-equilibration of media. An analyzed gas mixture containing 5% CO_2 in N_2 and contaminated with

less than 0.3% O_2 (obtained from Matheson Gas Products, Newark, Calif.) was used for media pre-equilibration and displacement of air from culture tubes. Media pre-equilibration was carried out in sidearm flasks fitted with sterile packed cotton filters and sealed with silicone rubber stoppers. Flasks of media were subjected to three cycles of evacuation to 1 to 3 mm of Hg and refilling with 5% CO_2 in N_2. To ensure a large surface area for gas exchange, the volume of media was limited to one-third the volume of the flask. Displacement of air in culture tubes was performed by allowing a stream of 5% CO_2 in N_2 to flow into the tubes through a cotton-plugged Pasteur pipette at a rate of 2 liters/min for 1 min.

Survival studies in cell-free cultures. *T. pallidum* survival in BRM without tissue culture under an atmosphere of 5% CO_2 in N_2 was examined by the following procedure. A freshly harvested suspension of *T. pallidum* was diluted 1:20 in BRM and placed in tubes (16 by 125 mm), 2 ml per tube. The tubes were flushed with 5% CO_2 in N_2, sealed with a silicone rubber stopper, and incubated at 33°C. Samples of fluid were removed for counting at 24-h intervals, and the tubes were reflushed with 5% CO_2 in N_2.

Survival studies in tissue culture. All cell cultures, with the exception of the mouse sarcoma cell line, were cultivated in 8- or 16-ounce (ca. 0.236 to 0.473 liter) prescription bottles with Eagle MEM plus 10% FBS as the culture medium. For passage, confluent monolayers were dispersed with 0.1% trypsin and 0.02% Versene in calcium- and magnesium-free phosphate-buffered saline. The mouse sarcoma cell line was cultivated in RPMI medium 1630 plus 10% FBS and normally grew as a suspension culture (6). No antibiotics were added to any of the cultures.

For use in experiments, cells in 1 ml of Eagle MEM plus 10% FBS were planted either in a Leighton tube (16 by 125 mm) containing a cover slip (10.5 by 35 mm) or in a flat-bottomed shell vial (25 by 95 mm) containing a 22-mm round cover slip. The cultures were sealed with silicone rubber stoppers and incubated aerobically at 33°C for 2 to 3 days before use in the experiments. The cells were approximately 25% confluent at the time *T. pallidum* was added.

All experiments were performed at 33°C with freshly harvested *T. pallidum* suspensions. For experiments using Leighton tubes in the standard horizontal position, the culture medium was replaced with 1 ml of BRM containing approximately 5×10^6 treponemes per ml, and air was displaced with 5% CO_2 in N_2. Gradient cultures also contained cells grown out on Leighton tubes in the usual manner; however, after removing the culture medium, the tubes were placed in a vertical position, and *T. pallidum* diluted in BRM-50 was added in sufficient volume to cover the cover slip (ca. 6 ml). The cover slip assumed an angle of 10° from the vertical, the side with the tissue culture cells facing upward.

For studies in shell vials, the culture medium was replaced with *T. pallidum* diluted in BRM-50, the volume being varied to examine survival on the cell monolayer under different medium depths. The vials were briefly flushed with 5% CO_2 in air to stabilize the pH.

***T. pallidum* enumeration.** Enumeration of treponemes in fluids was performed by counting organisms in a 10-μl sample that had been placed under a 22-mm^2 cover slip. Care was taken to avoid bubbles or excess fluid along the edge of the cover slip. Random fields were counted until 100 treponemes were observed. Enumeration of treponemes on cover slip cultures of tissue cells was performed by inverting the cover slip onto a slide, gently swabbing the reverse of the cover slip to remove the excess culture fluid, and counting 5 random fields per cover slip. On gradient culture cover slips, one field was counted at each of the indicated distances from the top of the cover slip. Treponemes attached to both the cells and the cover slips were counted.

All observations were made at a magnification of ×800 with a Zeiss universal microscope equipped for dark-field illumination. The field diameter was measured with a stage micrometer, and the field area was calculated to be 4.26×10^{-4} cm^2.

The multiplication factors used to determine the number of treponemes per milliliter or per cover slip were derived by the following formulas.

For 10-μl fluid samples,

$$\text{multiplication factor} = \left(\frac{\text{cover slip area}}{\text{field area}}\right) \times \left(\frac{1{,}000\ \mu l}{10\ \mu l}\right) = \frac{4.84\ cm^2 \times 100}{4.26 \times 10^{-4}\ cm^2} = 1.14 \times 10^6$$

$$\text{number of } T.\ pallidum/\text{ml} = \left(\frac{T.\ pallidum \text{ counted}}{\text{fields counted}}\right) \times 1.14 \times 10^6$$

For cover slips containing tissue culture, the factor was calculated by dividing the cover slip area by the field area. The factor for a 10.5- by 35-mm cover slip was 8.62×10^3, and, for a 22-mm round cover slip, it was 8.91×10^3.

The use of a magnification of ×800 permitted observation of variations in treponemal motility and morphology as well as discrimination of motile and nonmotile treponemes. Subjective impressions of the morphology and motility were recorded for each sample counted.

Virulence of *T. pallidum*. The virulence of *T. pallidum* after various periods of in vitro incubation was determined by intradermal inoculation of 0.1-ml volumes of 10-fold dilutions of infected fluids or cells into the clipped backs of rabbits. Each site was examined daily for the appearance of typical lesions. Rabbits were reclipped as required.

All dilutions were made in fresh BRM, with care being taken to minimize exposure of the samples to air. Fluid samples were diluted directly. For tissue culture samples, the incubation medium was removed, and the cells and attached treponemes were scraped into 1 ml of fresh BRM and diluted.

Direct counts of each undiluted sample were performed and the number of motile *T. pallidum* was calculated. The data are expressed as the minimum numbers of motile *T. pallidum* required to produce a lesion. All lesions were found to contain typical, motile *T. pallidum*.

Medium components, chemicals, and storage. Tissue culture medium components and FBS were

obtained from Flow Laboratories. HEPES, reduced glutathione, DL-cysteine HCl, and DTT were supplied by Sigma Chemical Co. Resazurin and trypsin (1:250) were obtained from Difco Laboratories; heparin-sodium salt (100 U/mg) was obtained from Nutritional Biochemicals. All other chemicals were reagent grade.

Several lots of FBS were screened, and considerable variation in *T. pallidum* survival was observed among the different lots. The lot in which maximum survival occurred was used throughout this study. FBS was inactivated at 56°C for 30 min and stored in small portions at −20°C until use. HEPES was prepared as a 1 M stock solution (pH 7.3), heparin as a 1-mg/ml stock (100 U/mg), and resazurin as a 20-mg/ml stock. All were sterilized by membrane filtration (Millipore Corp.) and stored at 4°C.

RESULTS

Survival of *T. pallidum* in cell-free cultures. Figure 1 presents the combined results of 41 tubes in 13 experiments in which we examined *T. pallidum* survival in BRM under an atmosphere of 5% CO_2 in N_2. There was an apparent 38% drop in the number of treponemes during the first 24 h, with a return to the original number by the third day of incubation. Thereafter, the total number of treponemes steadily decreased. The ST_{50} based on the initial number of treponemes was between 4 and 5 days; the ST_{50} of the treponemes observed was between 6 and 7 days.

Survival of *T. pallidum* in Leighton tube tissue cultures. Table 1 summarizes the results of 43 experiments on the survival of *T. pallidum* carried out with 12 different cell cultures derived from rabbits, humans, rats, mice, and dogs. Most of the early experiments were conducted with adult rabbit testes and adult human foreskins because these were the cultures that we expected might most closely approximate tissues infected in vivo. In all of the experiments in which survival of the treponemes was 2 days or less, various media other than BRM were used. It was not until BRM was developed as a maintenance medium that survival was significantly extended. The Sf1Ep cells were selected for further study because they had a slow growth rate and could be maintained for relatively long periods without fluid changes. Also, they were more transparent than cells of other cultures, thus facilitating observation of the treponemes. As Fitzgerald et al. (8) described previously, many of the treponemes attached to the cells and appeared to be more vigorously motile than those that were free in the medium. However, contrary to their findings, we did not observe any organisms that appeared to be intracellular.

Table 2 summarizes the results of one representative experiment in which we compared survival of *T. pallidum* in Leighton tubes with Sf1Ep cells to that in Leighton tubes without tissue culture. As shown in the table, survival in BRM with Sf1Ep cells was at least 50% both on the cover slip and in the medium after 6 days in vitro. By comparison, survival under identical conditions, but without tissue culture,

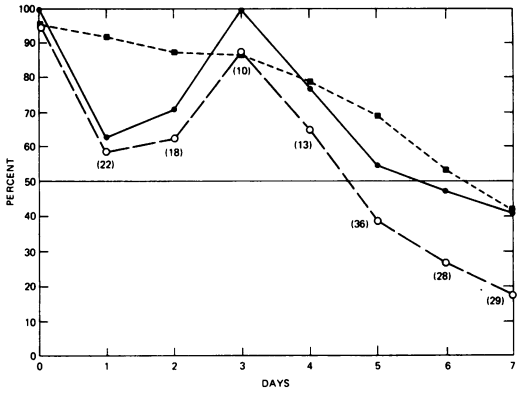

FIG. 1. *Survival of T. pallidum in BRM containing a 1:20 dilution of testis extract: 2 ml of suspension under 5% CO_2 in N_2 in a 16- by 125-mm tube. Symbols: ●, Percentage of initial number of treponemes; ○, percentage of initial number of treponemes motile; ■, percent motility of observed treponemes. Numbers in parentheses represent number of tubes counted on a given day. Data represent 41 tubes in 13 separate experiments. Initial number of treponemes ranged from 2.9 × 10^6 per ml to 2.7 × 10^7 per ml.*

TABLE 1. *Survival of T. pallidum in Leighton tube cultures of various mammalian tissues in an atmosphere of 5% CO_2 in nitrogen*

Cell strains or lines	Passage levels tested	No. of expts carried out	Maximum 50% survival in vitro (days)	
			Cell sheet	In tissue culture fluid
Rabbit testis (adult)[a]	2-5	9	5	5
Human foreskin (adult)[a]	14-23	8	>3	5
Human fetal lung[b]	6	1	<1	1
Human fetal kidney[a]	41-43	3	2	2
Rat peritoneal macrophages[a]	1	2	1	1
Rat nose[a]	26	2	<1	<1
Rat footpad[a]	23	2	<1	<1
Mouse sarcoma[a]	54	2	<1	<1
Dog kidney[a]	142-143	3	2	2
Cottontail rabbit epithelium (Sf1Ep)[b]	69-85	9	>8	>6
Rabbit embryonic skin[a]	4	1	>6	>6
Rabbit cornea[b]	447[c]	1	5	>4

[a] These cultures were initiated in our laboratory.
[b] These cultures were obtained from the Naval Biomedical Research Laboratory, Oakland, Calif.
[c] Approximate only; number of passages after 400 has not been recorded accurately.

TABLE 2. *Comparison of survival of T. pallidum in Leighton tube cultures of Sf1Ep cells with that in the absence of tissue culture*[a]

Tissue culture	% Motile treponemes at indicated time of incubation							
	Day 1		Day 4		Day 5		Day 6	
	CS[b]	Med[c]	CS	Med	CS	Med	CS	Med
Sf1Ep	94	94	76	89	79	70	50	86
None[d]	78	90	19	16	NC[e]	NC	NC	NC

[a] Incubation was carried out in BRM under an atmosphere of 5% CO_2 in N_2.
[b] CS, Treponemes on cover slip.
[c] Med, Treponemes in medium.
[d] Controls consisting of Leighton tubes containing cover slips but no tissue culture.
[e] NC, Not counted.

was less than 20% after 4 days. Although there was unquestionably a tendency for the treponemes to attach to the cell sheet, it has been our experience that the treponemes will also adhere to some degree to cover slips containing no tissue culture, especially when cellular or other debris is present. For example, in the experiment presented here, on day 1, 82 treponemes were counted in five fields on the cover slip without tissue culture, whereas 735 treponemes were counted in the same number of fields on the cover slip containing Sf1Ep cells.

Influence of redox potential on *T. pallidum* survival. Cell-free cultures were used to examine the effects of redox potential on survival. Redox potentials lower than those in tubes containing 2 ml of BRM were obtained by increasing the volume of medium in vertical test tubes (16 by 125 mm), and a higher redox potential was obtained by using 1 ml of medium in a horizontal Leighton tube. The inclusion of resazurin in the medium confirmed that more reduced conditions were obtained as the volume was increased. Resazurin was completedly oxidized in horizontal Leighton tubes. A progressively smaller portion of the medium was oxidized in 2-, 5-, and 10-ml tubes, with only the upper 20% being oxidized in 10-ml tubes. The ST_{50} in tubes containing 2 or 5 ml of BRM was 6 to 7 days; it was 4 to 5 days in tubes containing 10 ml of BRM and 2 to 4 days in cell-free Leighton tube cultures. These results suggested that extremes of redox potential resulted in shorter survival of *T. pallidum* in cell-free systems. The results in cell-free Leighton tube cultures were in direct contrast to the repeated findings of a 5- to 6-day ST_{50} in Leighton tube cultures containing tissue culture. It therefore seemed possible that one function of tissue culture was an alteration in redox potential and, hence, an increase in survival time over that in cell-free Leighton tube cultures.

Survival of *T. pallidum* in gradient tissue cultures. Gradient tissue cultures have provided a convenient method for observation of cell-treponeme interactions in a gradient of O_2 tension. Similar cultures have been described by Osgood and Krippaehne (17) and Leighton and Katsuta (14) in studies of cell proliferation and differentiation. Modification of their techniques has shown that proper conditions of O_2 tension and redox potential result in striking survival of *T. pallidum* in gradient cultures of Sf1Ep cells. Furthermore, in the absence of tissue culture, survival in gradient tubes was significantly shorter.

T. pallidum survival in these gradient cultures was examined both with and without tissue culture, under 5% CO_2 in N_2 and under air, and in modified BRM (i.e., lower concentrations of reducing agents). In the absence of tissue culture, relatively few *T. pallidum* adhered to the cover slip, and in no instance was survival as long in a gradient without tissue culture as it was in gradients when tissue culture was present. In the presence of Sf1Ep cells and in all combinations of media and atmosphere, an area containing numerous vigorously motile *T. pallidum*, many of which exhibited a serpentine type of motility, was observed. This area shifted downward if the culture was incubated under air or if the concentration of reducing agents was lowered. BRM-50 incubated under air appeared to allow maximum survival of *T. pallidum* in gradient cultures and was utilized in all subsequent experiments.

When the gradient was initiated, the cells were equally distributed on the cover slip. After several days of incubation, the cell density began to decrease with increasing depth. By day 7 after initiation of the gradient, cells on the upper 15 to 18 mm of the cover slip had reached confluency. The cell density rapidly decreased below that level, and, below 25 mm, the cells usually rounded up and detached from the glass. With continued incubation, the area of confluency extended slightly deeper. The cells on the upper portion of the cover slip began to die

about day 14, and the entire cell culture usually died between days 14 and 21.

Figure 2 is a bar graph representing the combined results of 11 experiments on *T. pallidum* survival in gradient cultures. Each bar represents the average percent motility of *T. pallidum* at the given depth in the medium on the given day of observation. The average number of treponemes per field is given above the bars. The percentage of treponemes motile in the medium is given in parentheses below the day of observation. It is readily apparent that in the deeper areas of the gradient, 23 and 29 mm below the surface, there was a rapid decline in motility to below 50% by day 7. At 5 mm below the surface, the average motility ranged from 42 to 72% during the 14-day incubation period.

Of greater interest are the results at the 11- and 17-mm levels. At these levels, the motility percentage remained between 83 and 92% through day 12, with the standard deviation ranging from 4 to 12%. On day 14 the average motility dropped to 70 to 75%. By day 12 there was a decline in the number of treponemes on the cell sheet. This phenomenon may have been due in part to the increasing density of the cell monolayer, with a concomitant tendency to mask the treponemes, as well as to an actual decrease in the number of organisms. This was accompanied by a decrease in number and percent motility of the treponemes in the medium. Therefore, the maintenance of a high percentage of motility over a long period of time may be somewhat misleading. However, the treponemes in the optimal survival areas of these gradient cultures remained regularly coiled and were vigorously motile for the life of the cell cultures. They appeared to be quite similar to freshly harvested treponemes, unlike those at the top of the gradient, those at the bottom, and those maintained in the absence of tissue culture. Furthermore, when the Sf1Ep cells were maintained for prolonged periods of time, vigorous motility was observed for up to 21 days.

Measurements of the redox potential and O_2 tension along these gradients have not been attempted because of the technical difficulties involved in such measurements. However, it seemed apparent that since O_2 had not been excluded from the gas mixture over the medium, a gradient had been established that permitted observation of *T. pallidum* survival under varying conditions of oxygen tension and tissue culture growth. Resazurin was used in these cultures and was found to be oxidized in the upper half of the gradient. The depth of the oxidized region corresponded roughly with the depth of the confluent cell growth and always included

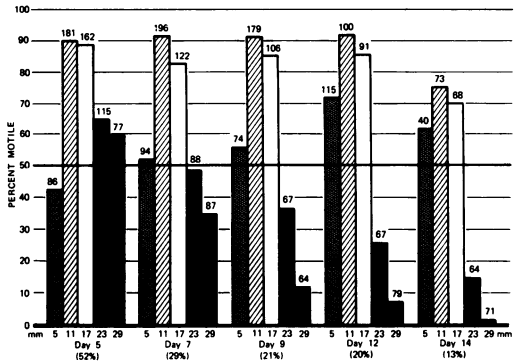

FIG. 2. *T. pallidum survival in gradient cultures of Sf1Ep cells in BRM-50 incubated under air. Each bar represents the average percent motility at the given depth. Numbers above the bars represent numbers of treponemes per field. Numbers below bars represent the depth (mm) at which the count was made. The numbers in parentheses are the percent motile treponemes in the medium on the indicated day of incubation. The data represent an average of 11 experiments in which the testis extract dilution ranged from 1:20 to 1:36 and the number of T. pallidum per ml ranged from* 4.8×10^6 *to* 5.9×10^6.

the area where *T. pallidum* survival was optimal.

In one experiment, *T. pallidum* survival in gradient cultures of dog kidney, adult human foreskin, and human fetal kidney cells was compared with that in gradient cultures of Sf1Ep cells. Survival was markedly reduced in all three cultures, with none having greater than 50% motility in any portion of the gradient by day 12, whereas in Sf1Ep gradient culture, motility was 82, 79, and 84% at the 5-, 11- and 17-mm levels, respectively, after 14 days in vitro. In addition, 2- to 10-fold fewer treponemes were counted on the cover slips of these alternate cell cultures as compared with the Sf1Ep culture cover slips.

Survival studies on *T. pallidum* in shell vial cultures. In an effort to achieve an optimum O_2 tension for all treponemes on the cover slip, we investigated a culture system in which cells were grown on 22-mm round cover slips placed in the flat bottom of shell vials (25 by 95 mm). Since these cover slips were horizontal, there was an equal distribution and a greater number of cells and viable treponemes on the cover slips. The medium depth was varied to examine *T. pallidum* survival under various O_2 tensions.

Transition to the shell vial culture system required some procedural modifications. Although small numbers of treponemes actively attached to a vertical (gradient culture) cover

slip, far greater numbers of treponemes settled onto the horizontal (shell vial) cover slip, making enumeration of *T. pallidum* virtually impossible. Thus, to avoid overloading the cell monolayer, the number of *T. pallidum* was initially standardized at 5×10^6 per vial regardless of the volume of the medium. This necessitated a dilution of the initial *T. pallidum* suspension 2- to 10-fold greater than in gradient cultures.

The depth of the medium in shell vials was varied from 5 to 25 mm. *T. pallidum* survival under 5 to 10 mm of medium was poor in every instance (less than 10% motile by day 4). With a depth of 15 or 20 mm, the ST_{50} on the cover slip was as long as 13 days in several experiments. However, in several other experiments the motility dropped below 50% within 3 or 4 days. The ST_{50} ranged from 12 to 21 days in the optimal area of the gradient culture tubes used as simultaneous controls and using the same treponeme suspension and media.

Effect of testis extract dilution and *T. pallidum* concentration on survival. Greater dilution of testis extract and lower concentration of *T. pallidum* were two of the most important modifications in shell vials as compared with gradient cultures. Other investigators previously reported that the dilution of testis extract was an important factor in the in vitro survival of *T. pallidum* in cell-free systems (13, 16). To determine whether the extract dilution and *T. pallidum* concentration used in the shell vial cultures affected *T. pallidum* survival, we carried out four experiments in gradient cultures designed to examine these factors. The results of one experiment, typical of the others, are shown in Table 3.

In the first group, *T. pallidum* was diluted directly to 8.8×10^5 per ml, the concentration used in shell vials with a 15-mm depth. In this instance the testis extract was diluted 1:203. The second group also contained 8.8×10^5 *T. pallidum* per ml; the third group contained 5×10^6 treponemes per ml. However, in the latter two groups the testis extract dilution was held constant at 1:30 by preliminary dilutions in treponeme-free testis extract.

It is readily apparent that *T. pallidum* survival was significantly greater in the second group than in the first. After 12 days in culture, *T. pallidum* survival in the center three fields in the second group was 71, 86, and 91%, respectively. By comparison, survival in the first group was 27, 25, and 18% in the corresponding fields. In addition, three to five times as many treponemes were found in the second group as in the first. These results suggested that the testis extract provided some factors essential for the survival of *T. pallidum* even in the presence of tissue culture.

TABLE 3. *Effect of testis extract dilution and T. pallidum concentration on survival in gradient cultures of Sf1Ep cells*

No. of *T. pallidum* per ml	Extract dilution	Depth of measurement	% Motile treponemes				
			Day 5	Day 9	Day 12	Day 14	Day 19
8.8×10^5	1:203[a]	5	0 (0/17)[b]	33 (1/3)	0 (0/2)	(0)	
		11	0 (0/25)	0 (0/18)	27 (4/15)	25 (1/4)	
		17	29 (4/21)	40 (2/5)	25 (2/8)	88 (7/8)	
		23	49 (17/35)	86 (6/7)	18 (2/11)	50 (1/2)	
		29	76 (13/17)	100 (6/6)	70 (7/10)	0 (0/10)	
8.8×10^5	1:30[c]	5	23 (9/39)	43 (3/7)	0 (0/3)	(0)	(0)
		11	60 (26/43)	88 (29/33)	71 (25/35)	84 (21/25)	17 (1/6)
		17	82 (95/116)	97 (63/65)	86 (24/28)	88 (7/8)	17 (2/12)
		23	92 (98/106)	92 (82/89)	91 (21/23)	59 (13/22)	7 (2/30)
		29	67 (31/46)	85 (17/20)	4 (2/51)	57 (4/7)	0 (0/45)
5×10^6	1:30[d]	5	22 (31/142)	0 (0/32)	95 (39/41)	84 (6/19)	90 (18/20)
		11	87 (193/222)	88 (101/115)	85 (71/84)	88 (84/96)	79 (27/37)
		17	87 (246/284)	70 (35/50)	93 (42/45)	77 (44/57)	73 (23/30)
		23	69 (120/173)	7 (4/61)	0 (0/39)	32 (8/25)	10 (3/31)
		29	63 (121/191)	3 (1/33)	0 (0/70)	0 (0/16)	1 (2/141)

[a] Testis extract containing 1.79×10^8 *T. pallidum* per ml was diluted 1:203 in BRM-50.

[b] Number of treponemes motile per number of treponemes counted in one ×800 field.

[c] Testis extract containing 1.79×10^8 *T. pallidum* per ml was diluted 1:6.76 in treponeme-free testis extract. This suspension was then diluted 1:30 in BRM-50. Treponeme-free testis extract was obtained by centrifugation of the testis extract at 12,000 × *g* for 10 min.

[d] Testis extract containing 1.79×10^8 *T. pallidum* per ml was diluted 1:1.19 in treponeme-free testis extract. This suspension was then diluted 1:30 in BRM-50. Treponeme-free testis extract was obtained as above in *c*.

T. pallidum survival was greater in the third group than in the second. A comparison of the results of the last two groups suggested that the optimal area for *T. pallidum* survival was shifted downwards with decreasing *T. pallidum* concentrations. Furthermore, in tubes containing resazurin, the resazurin was oxidized virtually to the bottom of the gradient in the second group, whereas in the third group it was oxidized only in the upper half of the gradient.

In another experiment, 10 μg of penicillin G per ml, which immobilized at least 90% of the treponemes within 24 h, was added to similar cultures containing 5×10^6 treponemes per ml. The resazurin in this group was oxidized throughout the gradient. This suggested that the treponemes themselves were contributing to the reduction of the medium. The concentration of viable *T. pallidum* therefore appeared to be an important factor in the establishment of conditions permissive for long-term survival of *T. pallidum*.

In view of the results of these experiments in gradient cultures, *T. pallidum* survival was again tested in shell vials. The procedure was modified by adding treponeme-free extract to the medium. The final dilution of extract was 1:30, approximately the same as that used in the gradient cultures. Preliminary results with 1×10^6 treponemes per ml and a medium depth of 25 mm showed that at day 16, 58% of the treponemes on the cover slip were still motile. In another group incubated with 5×10^6 treponemes per ml and at a medium depth of 25 mm, the treponemes on the cover slip were too numerous to count, but vigorously motile treponemes were still present after 21 days in culture.

Virulence of *T. pallidum* after maintenance in vitro. Table 4 summarizes the results of virulence tests performed after maintaining *T. pallidum* in the in vitro survival systems described above. In every instance, where tested, *T. pallidum* was found to be virulent for rabbits after various periods in vitro. Virulence could be maintained for at least 21 days in both gradient and shell vial cultures, with as few as 2.7×10^2 motile organisms producing a lesion, despite the fact that only 8 to 11% of the treponemes inoculated were motile. It is apparent that after 14 days in gradient culture, not only were the treponemes virulent, but a dark-field positive lesion could be produced by no more than 13 motile organisms (Table 5).

TABLE 4. *Virulence of T. pallidum after in vitro incubation*

In vitro system	Days in vitro	No. of motile *T. pallidum*/site[a]
Cell-free culture	6	$<5.5 \times 10^3$[b]
Leighton tube[c]	4	$<3.4 \times 10^5$[b]
Gradient culture[d]	14	$<1.3 \times 10^1$[b]
	21	2.7×10^2
Shell vial[d]	21	3.6×10^2

[a] Minimum number of motile *T. pallidum* producing a dark-field positive lesion.
[b] No end point was reached.
[c] Culture media only; *T. pallidum* on cell monolayer not tested.
[c] Cell monolayer only; *T. pallidum* in culture media not tested.

TABLE 5. *Virulence of T. pallidum after 14 days in gradient tissue culture*

Inoculum	Dilution	No. of motile *T. pallidum*/site	Day of lesion appearance[a]
Cover slip scrapings[b]	10^0	1.3×10^5	10
	10^{-1}	1.3×10^4	12
	10^{-2}	1.3×10^3	13
	10^{-3}	1.3×10^2	14
	10^{-4}	1.3×10^1	24
Positive control[c]		5×10^3	11

[a] All lesions were dark-field positive.
[b] Treponemes and cells scraped from cover slip suspended in 1 ml of BRM.
[c] *T. pallidum* freshly harvested from rabbit testis.

DISCUSSION

In the foregoing we have addressed ourselves primarily to the development of conditions for extended survival of virulent *T. pallidum* in tissue culture. Although we were unable to demonstrate overt multiplication of *T. pallidum* in any of the systems we used, it would be unreasonable to assume or expect that survival could be extended beyond the time that we have demonstrated. Only rarely can tissue cultures be maintained in the same vessel beyond 3 to 4 weeks, especially in the absence of a change of medium. We would expect that the supply of O_2, as well as essential nutrients for both treponemes and tissue culture cells, would be depleted. Even if the medium were changed several times during that period, the continued growth of the tissue culture cells would present problems. Furthermore, it must be kept in mind that, with changes of medium, rabbit testis extract is either highly diluted or removed. The extract may play an important—but as yet undetermined—role in the survival and possible replication of *T. pallidum* in vitro. Although we could demonstrate that addition of rabbit testis extract to dilute suspensions of treponemes

added considerably to the survival of the treponemes, we have not attempted to passage *T. pallidum* in the presence of added extract. It is worthwhile to note, however, that in several instances in which we replaced the maintenance medium with fresh BRM to prolong survival of *T. pallidum,* we noticed both qualitative and quantitative changes. That is, we not only found a higher percentage of motile treponemes, but they were more vigorously motile than before the fluid change. Unfortunately, more frequently than not, when fluids were changed we observed either a loss of motility or no effect. It is possible that had rabbit testis extract been included in the medium, the effect might have been observed more consistently. It is therefore of paramount importance to determine whether rabbit testis extract is vital to continued survival of subcultured organisms and, if so, whether a satisfactory substitute can be developed.

The role of tissue culture in *T. pallidum* survival appears to be very complex. It is generally acknowledged that oxygen is toxic to *T. pallidum.* Yet our results, as well as those of Cox and Barber (4) and Baseman et al. (1), suggest an O_2 requirement for some metabolic activities and possibly for replication. One possible function of tissue culture is to counteract oxygen toxicity. Any protective or nutritive functions of tissue culture seem to be effective only within a narrow range of conditions. In our experiments in gradient cultures incubated under air, vigorously motile treponemes were generally most numerous between depths of 11 and 17 mm in areas with heavy cell growth. However, organisms found attached to areas of equally heavy cell growth located near the top of the gradient, where O_2 tension was presumably highest, were sluggish. In addition, although treponemes in close proximity to cells in optimal areas of the gradient culture remained vigorously motile for the life of the culture (14 to 21 days), treponemes free in the medium of these cultures survived only slightly longer than cell-free controls, which had a 50% survival for no longer than 4 days. It is interesting to note that *T. pallidum* attached to gradient culture cover slips with Sf1Ep cells even when the tissue culture cells were on the underside of the cover slip. The attachment of treponemes to tissue culture cells, therefore, appeared to be an active rather than a passive phenomenon.

With respect to virulence of *T. pallidum* maintained in vitro, other investigators have reported that these organisms lose virulence for rabbits before the loss of motility (2, 9, 18, 20). This did not occur in our experiments. Even when less than 10% of the treponemes were motile after 21 days in culture, they still produced darkfield positive lesions in rabbits. It is probable that true loss of virulence does not occur in vitro any more than it does in vivo. It seems unreasonable to expect that an organism that has maintained virulence through 65 years of passage in rabbits should lose virulence after a short period in vitro. Rather, it seems likely that under some conditions in vitro, especially under strict anaerobic conditions, irreversible changes occur, and the organisms are no longer capable of replication, even though they may be motile; hence, the so-called loss of virulence before loss of motility is simply an inability of *T. pallidum* to multiply.

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ADDENDUM

After completion of this manuscript it was brought to our attention that Fitzgerald et al. (7) reported limited (7-day) virulence of *T. pallidum* in cultures of Sf1Ep cells in a medium containing DTT. This confirmed earlier observations reported by us (A. H. Fieldsteel. 1975. Studies on cultivation of *Treponema pallidum.* Progress Report IDB-VDP-07-128 for April-December 1975, Public Health Service contract N01 AI 42536 from the National Institute of Allergy and Infectious Diseases).

LITERATURE CITED

1. **Baseman, J. B., J. C. Nichols, and N. S. Hayes.** 1976. Virulent *Treponema pallidum*: aerobe or anaerobe. Infect. Immun. **13**:704-711.
2. **Clark, J. W., Jr.** 1962. Loss of virulence *in vitro* of motile *Treponema pallidum.* Br. J. Vener. Dis. **38**:78-81.
3. **Cleland, W. W.** 1964. Dithiothreitol, a new protective reagent for SH groups. Biochemistry **3**:480-482.
4. **Cox, C. D., and M. K. Barber.** 1974. Oxygen uptake by *Treponema pallidum.* Infect. Immun. **10**:123-127.
5. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432-437.
6. **Fieldsteel, A. H., P. J. Dawson, and J. Scholler.** 1966. Virus-free Friend virus-induced tumor: *in vitro* and *in vivo* characteristics. J. Natl. Cancer Inst. **36**:71-80.
7. **Fitzgerald, T. J., R. C. Johnson, J. A. Sykes, and J. N. Miller.** 1977. Interaction of *Treponema pallidum* (Nichols strain) with cultured mammalian cells: effects of oxygen, reducing agents, serum supplements, and different cell types. Infect. Immun. **15**:444-452.
8. **Fitzgerald, T. J., J. N. Miller, and J. A. Sykes.** 1975. *Treponema pallidum* (Nichols strain) in tissue cultures: cellular attachment, entry, and survival. Infect. Immun. **11**:1133-1140.
9. **Graves, S. R., P. L. Sandok, H. M. Jenkin, and R. C. Johnson.** 1975. Retention of motility and virulence of *Treponema pallidum* (Nichols strain) in vitro. Infect. Immun. **12**:1116-1120.
10. **Hardy, P. H., and E. E. Nell.** 1955. Specific agglutination of *Treponema pallidum* by sera from rabbits and human beings with treponemal infections. J. Exp. Med. **101**:367-382.
11. **Jones, R. H., M. A. Finn, J. I. Thomas, and C. Folger.**

1976. Growth and subculture of pathogenic *T. pallidum* (Nichols strain) in BHK-21 cultured tissue cells. Br. J. Vener. Dis. **52**:18–23.

12. **Kawata, T.** 1967. Presence of cytochromes in the Reiter treponemes. J. Gen. Appl. Microbiol. **13**:405–406.
13. **Kimm, G. E., R. H. Allen, H. J. Morton, and J. F. Morgan.** 1962. Studies on the *in vitro* survival of virulent *Treponema pallidum*. Am. J. Hyg. **75**:339–346.
14. **Leighton, J., and H. Katsuta.** 1973. Meniscus-gradient cultures of carcinomatous epithelial cell lines of bladder and kidney origin, p. 31–43. *In* S. Garattini and G. Franchi (ed.), Chemotherapy of cancer dissemination and metastasis. Raven Press, New York.
15. **Moore, W. B.** 1968. Solidified media suitable for the cultivation of *Clostridium novyi* type B. J. Gen. Microbiol. **53**:415–423.
16. **Nelson, R. A.** 1948. Factors affecting the survival of *Treponema pallidum in vitro*. Am. J. Hyg. **48**:120–132.
17. **Osgood, E. E., and M. C. Krippaehne.** 1955. The gradient tissue culture method. Exp. Cell Res. **9**:116–127.
18. **Sandok, P. L., S. T. Knight, and H. M. Jenkin.** 1976. Examination of various cell culture techniques for coincubation of virulent *Treponema pallidum* (Nichols strain) under anaerobic conditions. J. Clin. Microbiol. **4**:360–371.
19. **Smibert, R. M.** 1973. Spirochaetales, a review. CRC Crit. Rev. Microbiol. **2**:491–552.
20. **Weber, M. M.** 1960. Factors influencing the *in vitro* survival of *Treponema pallidum*. Am. J. Hyg. **71**:401–417.
21. **Willcox, R. R., and T. Guthe.** 1966. *Treponema pallidum*. A bibliographical review of the morphology, culture and survival of *T. pallidum* and associated organisms. Bull. WHO **35**(Suppl.):1–139.