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Retention of Motility and Virulence of *Treponema pallidum* (Nichols Strain) In Vitro

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A maintenance medium for *Treponema pallidum* was designed to hold its E_h at the optimum for that organism, −10 to −110 mV. After 100% motile (freshly harvested) *T. pallidum* was inoculated into the medium, the motility of the treponemes decreased to 80% after 2 days, 50% after 3.5 days, and 0% after 9 days during incubation at 34 C. Full virulence was retained for 2 days, but it dropped rapidly thereafter, and the treponemes became avirulent by day 5.

Despite numerous attempts since 1906 to culture virulent *Treponema pallidum*, the causative agent of syphilis, it is generally agreed that this has never been achieved (15). The best result obtained to date is the survival of *T. pallidum* in artificial medium, as indicated by its retention of motility and virulence for rabbits (6, 14, 15). However, with this medium it is apparently necessary to enrich it with unknown tissue components by shaking the minced, infected rabbit testis with the medium for several hours. The medium that we have formulated appears not to require this enrichment. By simply shaking the minced, infected rabbit testis with a few milliliters of medium in a petri dish for 2 to 3 min to obtain the inoculum, excellent motility of the *T. pallidum* (>80%) and potent virulence (>60%) can be retained for 2 days after inoculation. Therefore, this medium could be useful for the transportation of clinical specimens, as well as for a starting point in attempts to grow *T. pallidum* in vitro.

MATERIALS AND METHODS

Preparation of T. pallidum inoculum. *T. pallidum* was propagated in the testes of large, male New Zealand rabbits that had previously been shown to be free of reagenic antibody (rapid plasma reagin card test; Hynson, Wescott and Dunning, Inc., Baltimore, Md.). Approximately 5×10^7 viable *T. pallidum* were inoculated per testis, resulting in a well-developed orchitis within 8 to 11 days. Rabbits were housed at 16 to 19 C. Elution of *T. pallidum* from the minced orchitic testis involved two sequential 2- to 3-min washings with 5 ml of the medium described below. The 5 ml of medium was added to the minced testis in a petri dish and gently swirled for 2 min. This sample was discarded since it contained much particulate matter, erythrocytes and, sometimes, fibrin clots. The second 5-ml sample was collected under N_2 and immediately used as the inoculum without centrifugation or any attempt to remove or minimize particulate matter. A 3% inoculum (0.3 ml/10 ml of medium) was consistently used, and the final number of motile *T. pallidum* in the maintenance medium usually ranged from 10^5 to 10^6/ml.

Maintenance medium. The final composition of the maintenance medium is shown in Table 1. The medium was made in two parts as described below; the parts were combined to make the complete medium just prior to inoculation with *T. pallidum*. To make 1 liter of medium, 470 ml of part 1 and 530 ml of part 2 were combined, or pro rata for smaller volumes.

Maintenance medium A. The following components were dissolved in double-distilled water to a volume of 460 ml in a 500-ml conical flask with a magnetic spin bar: NaCl, 4.6 g; KCl, 230 mg; $(NH_4)_2SO_4$, 96 mg; $MgSO_4 \cdot 7H_2O$, 119 mg; $CaCl_2 \cdot 2H_2O$, 83 mg; glucose, 1.15 g; fructose, 1.92 g; sorbitol, 1.92 g; sodium pyruvate, 53 mg; cocarboxylase, 3 mg; adenine, 15 mg; yeast extract (Difco Laboratories, Detroit, Mich.), 1.92 g; and resazurin, 0.3 mg. The mixture was prereduced by autoclaving at 121 C for 8 min and cooling under slow exhaust. Immediately upon removal from the autoclave, the medium was continually flushed with oxygen-free nitrogen until cool. The latter was obtained by passing nitrogen gas, which was deoxygenated, through a hot copper column prior to use. After the medium had been cooled, 10 ml of buffer-bicarbonate solution was added, which contained (per 10 ml); $NaHCO_3$, 276 mg; KH_2PO_4, 116 mg; K_2HPO_4, 418 mg; and Na_2HPO_4, 34.55 mg. In addition, glutathione (reduced; 0.6 g), cysteine (0.4 g), and sodium thioglycolate (0.35 g) were added with gentle stirring. The pH of the solution was adjusted to 8.2 with several drops of 10 N NaOH. The medium was maintained under a stream of nitrogen until it was carefully added to nitrogen-flushed, anaerobic tubes (18 by 142 mm;

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TABLE 1. *T. pallidum maintenance medium*[a]

Component	Concn (mg/liter)	Molarity (mM)
NaCl	4,600.0	7.90×10^{-1}
$NaHCO_3$	276.0	3.29
KCl	230.0	3.08
$(NH_4)_2SO_4$	96.0	7.26×10^{-1}
KH_2PO_4	116.0	8.52×10^{-1}
K_2HPO_4	418.0	2.40
Na_2HPO_4	34.55	2.43×10^{-1}
$MgSO_4 \cdot 7H_2O$	119.0	4.83×10^{-1}
$CaCl_2 \cdot 2H_2O$	83.0	5.64×10^{-1}
Glucose	1,150.0	6.38
Fructose	1,920.0	1.07×10^{-1}
Sorbitol	1,920.0	1.05×10^{-1}
Sodium pyruvate	53.0	4.80×10^{-1}
Glutathione	600.0	1.95
Cysteine	400.0	3.30
Sodium thioglycolate	350.0	3.07
L-Glutamine	210.0	1.44
L-Arginine	75.58	4.34×10^{-1}
L-Cystine	17.27	7.18×10^{-2}
L-Histidine	22.31	1.44×10^{-1}
L-Isoleucine	37.79	2.88×10^{-1}
L-Leucine	37.72	2.88×10^{-1}
L-Lysine	41.75	2.86×10^{-1}
L-Methionine	10.80	7.23×10^{-2}
L-Phenylalanine	23.03	1.39×10^{-1}
L-Threonine	34.55	2.90×10^{-1}
L-Tryptophan	7.20	3.50×10^{-2}
L-Tyrosine	25.91	1.43×10^{-1}
L-Valine	33.11	2.83×10^{-1}
Cocarboxylase	3.00	6.20×10^{-3}
Adenine	15.00	1.10×10^{-1}
Isobutyric acid	2.85	3.23×10^{-2}
Coenzyme A	1.5×10^{-2}	2.00×10^{-3}
α-Lipoic acid	1.50	7.20×10^{-3}
p-Aminobenzoic acid	1.50	1.09×10^{-2}
Biotin	1.5×10^{-3}	6.00×10^{-3}
Choline chloride	8.22	5.88×10^{-2}
Folic acid	7.3×10^{-1}	1.66×10^{-3}
Inositol	8.94	4.96×10^{-2}
Nicotinic acid	0.15	1.22×10^{-3}
Nicotinamide	3.22	2.64×10^{-2}
Pyridoxal·HCl	1.47	7.22×10^{-3}
Pyridoxine·HCl	0.75	3.65×10^{-3}
Pyridoxamine·2HCl	1.00	4.15×10^{-3}
Putrescine·2HCl	2.50	1.55×10^{-2}
Riboflavin	0.22	5.80×10^{-4}
Thiamine·HCl	0.87	2.58×10^{-3}
Calcium pantothenate	0.87	1.83×10^{-3}
Cyanocobalamin	1.5×10^{-2}	1.00×10^{-5}
Yeast extract	1,920.00	
Bovine serum albumin (delipidified)	7,500.00	
Resazurin	0.30	1.30×10^{-3}

[a] In addition, bovine serum ultrafiltrate (15 ml/liter) and newborn calf serum (72 ml/liter) were added. The final pH was 7.25 to 7.45, and the final E_{cal} was −250 to −330 mV.

Bellco Glass, Inc., Vineland, N.J.) to minimize any oxidation of the medium. The medium (4.6 ml) was added to the tubes with a pipette preflushed with nitrogen a few times, taking the precaution not to blow through the pipette. The tubes were tightly sealed with no. 1 rubber stoppers and autoclaved in a press (Bellco Glass, Inc.) at 121 C for 15 min employing a fast exhaust. This part of the reduced medium was never made sooner than 1 day before use.

Maintenance medium B. The following components were made to a final volume of 530 ml: L-glutamine, 0.21 g; L-arginine, 75.58 mg; L-cystine, 17.27 mg; L-histidine, 22.31 mg; L-isoleucine, 37.79 mg; L-leucine, 37.72 mg; L-lysine, 41.75 mg; L-methionine, 10.80 mg; L-phenylalanine, 23.03 mg; L-threonine, 34.55 mg; L-tryptophan, 7.20 mg; L-tyrosine, 25.91 mg; L-valine, 33.11 mg; isobutyric acid, 2.85 mg; coenzyme A, 15 μg; α-lipoic acid, 1.5 mg; *p*-aminobenzoic acid, 1.5 mg; biotin, 155 μg; choline chloride, 8.22 mg; folic acid, 0.73 mg; inositol, 8.94 mg; nicotinic acid, 0.15 mg; nicotinamide, 3.22 mg; pyridoxal·HCl, 1.47 mg; pyridoxine·HCl, 0.75 mg; pyridoxamine·HCl, 1.0 mg; putrescine·2HCl, 2.5 mg; riboflavin, 0.22 mg; thiamine·HCl, 0.87 mg; calcium pantothenate, 0.87 mg; cyanocobalamin, 15 μg; bovine serum albumin (Pentex Biochemical, Kankakee, Ill.; fatty acid free), 7.5 g; bovine serum ultrafiltrate (Colorado Serum Co., Denver, Colo.), 15 ml; and newborn calf serum (International Scientific Industries, Cary, Ill.), 72 ml. The medium was adjusted to pH 7.25 to 7.45 and sterilized by filtration through a washed (0.45 μm) membrane filter (Millipore Corp., Bedford, Mass.). The stock solutions (amino acids, vitamins, etc.) of this mixture were prepared in advance, stored frozen, and then pooled just prior to use.

Final medium. The tubes containing 4.7 ml of maintenance medium A were opened under nitrogen, and 5.3 ml of maintenance medium B was added. The tubes were tightly stoppered again. The final pH of the complete medium was 7.25 to 7.45. It was isotonic, as measured by the stability of washed sheep erythrocytes in it. Its redox potential ranged from −250 to −350 mV E_{cal}, which is −10 to −110 mV E_h, the optimum range for the maintenance of motility of *T. pallidum*.

Determination of motility. The tubes of medium inoculated with *T. pallidum* (10^6 to 10^7 organisms/10 ml) were incubated at 34 C without shaking. At 24-h intervals, two freshly mixed samples per tube were removed aseptically and under nitrogen from each of three replicate tubes. A total of 400 treponemes was examined at each time interval, giving a theoretical standard error of ±5%. Each treponeme was scored as motile or nonmotile. Motile treponemes always showed either a flexing, snapping, or rapid spinning motion, as distinct from a slow, rolling motion or obvious Brownian movement.

Determination of virulence. As part of this study, the Dutch Belt breed of rabbits was standardized with respect to their formation of syphilitic lesions after intradermal inoculation of virulent *T. pallidum*. The observations of Turner and Hollander

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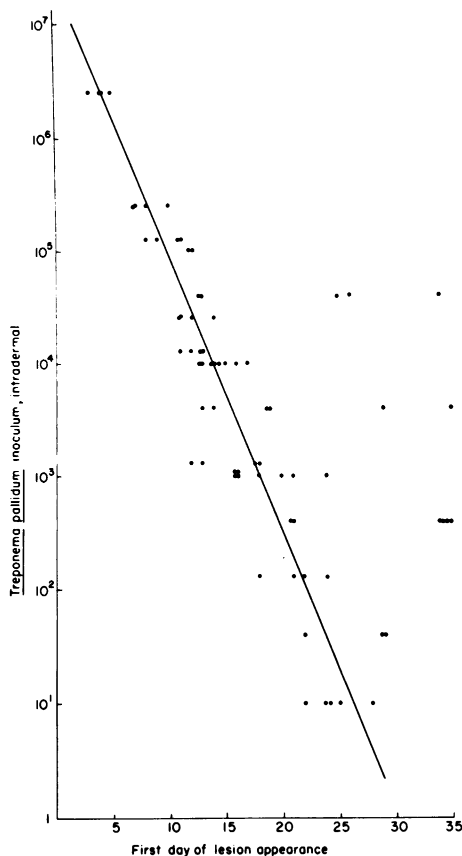

FIG. 1. *Standardization of male Dutch Belt rabbits with respect to graded intradermal inocula of virulent T. pallidum. Each point represents one dermal syphilitic lesion.*

(13) were substantiated in that a direct relationship was observed between the number of *T. pallidum* inoculated per site intradermally and the length of time elapsing before the first appearance of the dermal syphilitic lesion (Fig. 1). This relationship aided in establishing an estimation of the virulence of a *T. pallidum* sample when taken from the in vitro maintenance medium. To obtain these data we shaved the rabbits and marked their backs into grids. Each of four rabbits was injected intradermally with 0.1 ml of a *T. pallidum* sample which had been incubated at 34 C for varying intervals of time after inoculation of the maintenance medium. The rabbits were kept shaved and housed at 16 to 19 C for maximal lesion development. Two lesions that formed rapidly were excised and minced in saline. Motile *T. pallidum* were observed in both.

Nelson-Diesendruck medium as modified by Ajello et al. Nelson-Diesendruck medium (7), currently used in the *T. pallidum* immobilization test, was made as described by Ajello et al. (1).

Weber medium. Weber medium (14) was prepared as originally described, except it was modified by substituting acid-hydrolyzed gelatin with acid-hydrolyzed casein since the gelatin was unavailable. We compared this medium with our own for the maintenance of treponeme motility. In addition to utilizing the medium under nitrogen, we also used it under liquid paraffin containing 0.1% 2,6-di-*tert*-butyl-*para*-cresol, which is an antioxidant, as described in the original work of Weber.

Redox potential determination. Redox values (E_{cal}) were obtained with a combination platinum-calomel (saturated KCl) electrode (Orion model 96-78, Orion Research, Cambridge, Mass.) connected to a pH meter (Orion model 601 digital ionalyzer). The E_{cal} reading, in millivolts, was taken 10 min after inserting the electrode into the medium. Readings of E_h (redox potential based on the standard hydrogen electrode) were made by adding 241 mV to the E_{cal}, which is the potential of the saturated calomel electrode at 25 C (4).

RESULTS

As shown in Fig. 2, the *T. pallidum* motility remained above 80% for 48 h. Motility$_{50}$, the time required for 50% of the treponemes to become nonmotile, was 83 h, while the motility dropped to zero by day 9 after inoculation. The graph is a composite of nine experiments. The poorest motility$_{50}$ obtained was 64 h, the best was 94 h, and the average was 83 h. The variation seemed to be due to the difference in the inoculum of *T. pallidum*. An inoculum larger than 3% of the total volume resulted in a more rapid drop in the percentage of motility.

The drop in motility in the Nelson-Diesendruck medium and the Weber medium (under nitrogen and under oil), under the conditions we used, was considerably faster than in our new medium. Motility$_{50}$ ranged from 14 to 18 h. This was not due to an unsuitable redox potential since in both cases it was within the optimum range for *T. pallidum*, −250 mV to −350 mV E_{cal} (5; S. R. Graves and R. C. Johnson, unpublished data).

Retention of virulence of *T. pallidum* in our medium, as shown by the development of dermal lesions after intradermal inoculation of rabbits with samples taken at different intervals of time, was still 100% at day 1 and 60% at day 2, but it fell rapidly thereafter to become completely avirulent by day 5 (Fig. 1 and Table 2).

There was no increase in the number of *T. pallidum*, indicating that no multiplication occurred. However, by 4 days after inoculation, the remaining motile treponemes appeared to be much longer than those freshly isolated from infected rabbits.

During the course of the experiment, the redox potential of the medium tended to increase, from approximately −300 mV to about −250

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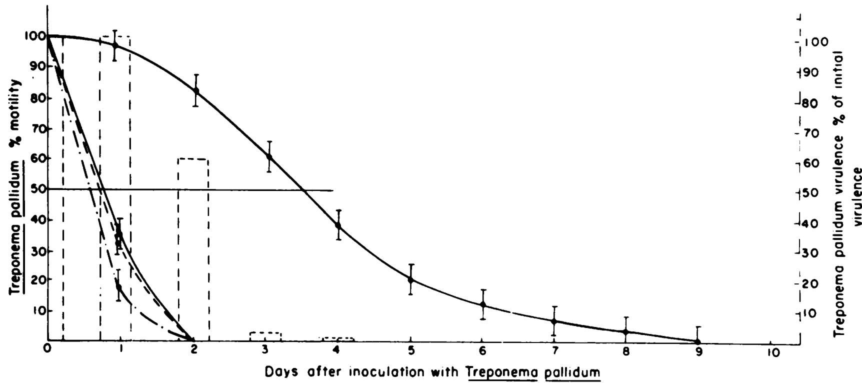

FIG. 2. *Loss of motility (⫲) and virulence (⌐¦) of T. pallidum in our maintenance medium compared with the loss of motility of T. pallidum in the Nelson-Diesendruck medium (1, 7) (——), Weber medium (14) under nitrogen (----), and Weber medium under liquid paraffin containing antioxidant (-·-·-).*

TABLE 2. *Drop in motility and virulence of T. pallidum in the maintenance medium at 34 C*

Days after inoculation with *T. pallidum*	Motility (%)	No. of rabbit with dermal lesions	Time of lesion development (days)	Original *T. pallidum* virulence remaining (%)
0	100	4/4	11	100
1	97	4/4	11	100
2	84	4/4	12	60
3	61	4/4	23	0.1
4	39	1/4	37	0.002
5	21	0/4		
6	13	0/4		
7	8	0/4		
8	4			
9	0	0/4		
10	0			

mV E_{cal} at day 9, probably due to oxygen leaking into the tubes.

DISCUSSION

The value of this work is not so much in the maintenance of motility and virulence of *T. pallidum* per se, but in the direction this work leads to research aimed at growing this organism in vitro. Culture of the organism in vitro will probably be necessary before a practical vaccine can be developed against syphilis.

The earliest work of Nelson (6) required a 12-h (technique A) or a 3-h (technique B) period of elution of testis for sufficient nutrients to accumulate in the medium. Without this procedure of elution or at least the factor present in beef serum or beef serum ultrafiltrate (11), the *T. pallidum* would survive for only 12 to 24 h. We confirmed the work of Nelson using a $motility_{50}$ of 18 h and the basic method of medium preparation as was modified and improved (7, 8, 10, 12) by Ajello et al. (1).

Weber (14), in an extensive study, reportedly improved the Nelson medium to give enhanced survival of the organism. He eluted the infected testis for 2 h. In our minor modification of his medium, we found it to be no better than the medium of Nelson although the inoculum we used was rapidly eluted within 2 to 3 min. This time sequence was used only as a control for our medium and was not an attempt to repeat the work of Nelson and Weber. It appears that the new medium we have developed contains at least some of the nutritional factors that were originally eluted from the rabbit testis.

With regard to known nutrients, it has recently been demonstrated that glucose and pyruvate are utilized by *T. pallidum* yielding CO_2 (J. C. Nichols and J. B. Baseman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, D3, p. 52), that various amino acids are incorporated into protein (2), that cocarboxylase and adenine prolong motility (9), and that K^+ is advantageous (3). These observations have been taken into account in the design of the new medium.

In general, as long as a tested compound did not reduce the retention of motility, it was added to the medium. The reason for this was that it was thought that a combination of compounds might enhance motility retention, even though each compound individually was without an enhancing effect.

Redox potential is important in maintaining *T. pallidum* motility. Metzger and Smogor (5) and, more recently, Graves and Johnson (unpublished data), have shown that E_{cal} values of -250 to -350 mV are optimal. Nelson and coworkers and later Weber did not measure the redox potential of their media. Their media, as we prepared them, were in fact within the optimal range.

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The loss of virulence in our maintenance medium was more rapid than the loss of percentage motility. Motility and, presumably, viability were still 20 to 40% at the point when the culture became avirulent. Weber (14) also observed this phenomenon. This suggests that the synthesis of an important bacterial substance essential for virulence, possibly an outer envelope, did not occur in vitro.

The consistent, albeit subjective, observation similar to that of Nelson (6) that the treponemes elongated over the course of several days in the maintenance medium gives some cause for cautious optimism about the possibility of cultivating this bacterium. This is especially true since it was the treponemes that retained their motility the longest that appeared to increase in length.

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