THE SUSPENSION CULTIVATION OF, AND THE USE OF ALTERNATIVE CELL LINES FOR THE IN VITRO CULTIVATION OF, TREPONEMA PALLIDUM SUBSPECIES PALLIDUM

DISSERTATION

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By

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In vitro propagation of Treponema pallidum subsp. pallidum (Nichols strain) can be achieved by co-cultivation of the bacteria with Cottontail Rabbit Epidermal (Sf1Ep) cells. In vitro cultivated T. pallidum has several advantages over treponemes grown in rabbit testes. These advantages include less contamination by rabbit proteins and the absence of antibodies against T. pallidum antigens. This study had two objectives: (1) to achieve suspension cultivation of Sf1Ep cells and (2) to develop procedures for achieving the replication of T. pallidum in those cell cultures. The final procedure was as follows: fifty ml of Eagles MEM with 10% fetal bovine serum and 200 cm^2 of either polystyrene beads (Cytospheres) or collagen coated dextran beads (Cytodex-3) were inoculated with $6x10^6$ Sf1Ep cells. After two days, the cell cultures were inoculated with $2x10^7$ T. pallidum freshly harvested from rabbit testes. Cultures were placed in an atmosphere of 4% oxygen, 5% carbon dioxide and 91% nitrogen at 34°C. The treponemal suspension cultures were stirred for

1 minute every 45 minutes at 30 rpm. Seven cultures yielded an average of 7.2×10^8 T. pallidum per flask (36 fold increase) after 12 days of incubation with a maximum yield of 1×10^9 treponemes.

Sf1Ep cells have been the sole cell line used for the in vitro cultivation of T. pallidum. A study was undertaken to determine if other cell lines can support growth of T. pallidum. Rabbit skin fibroblasts (RAB-9), nude mouse ear (NME) cells, and normal rabbit testis fibroblasts (RT) were compared to Sf1Ep cells for their ability to support in vitro multiplication of T. pallidum. RAB-9 cells supported multiplication of treponemes equal to that of Sf1Ep cells. NME and RT cells also supported growth but to a lesser extent than Sf1Ep cells. Utilization of alternative cell lines may lead to improved in vitro growth of T. pallidum including possible serial passage.

ACKNOWLEDGMENT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The spirochete, Treponema pallidum subsp. pallidum, is the causative agent of venereal and congenital syphilis. T. pallidum is 5-15μm in length and 0.15μm in diameter. This spiral shaped organism is motile with either a rotational or flexing motion. Motility is the result of two sets of three flagella that originate at both ends of the organism and wrap around the length of the treponeme between the cell wall and the outer membrane. T. pallidum is a microaerophilic organism and utilizes glucose as its major carbon and energy source (1,2,3,24,36). The subsp. pallidum can be differentiated from subsp. endemicum and pertenue (causative agents of endemic syphilis and yaws respectively) by the ability to produce lesions in rabbits, hamsters, or guinea pigs (table 1). The three subspecies also differ in the nature of the diseases they cause in humans. Venereal syphilis is sexually transmitted and systemic. Endemic syphilis and yaws are manifested by cutaneous lesions and are transmitted via direct contact.

Venereal syphilis consists of three separate stages. Primary syphilis is manifested by a papule at the organism's initial site of entry. The surface of the papule hardens to

become what is known as a chancre, which contains many treponemes and is highly infectious. In the absence of treatment the lesion will heal in 4-6 weeks. Secondary syphilis develops 2-10 weeks after the primary lesion heals. This stage is characterized by a maculopapular rash that can occur anywhere on the body. The lesions contain large numbers of treponemes and are very infectious. Approximately 1/3 of untreated individuals will resolve the infection on their own. If left untreated, tertiary syphilis can occur anywhere from 2-20 years after the initial infection. Tertiary syphilis can manifest itself as cardiovascular syphilis, neurosyphilis, or granulomatous lesions affecting any part of the body. T. pallidum is present in very low numbers in these lesions and tertiary syphilis is usually not infectious.

Table 1: Laboratory differentiation of Treponema pallidum subspecies.

T. pallidum	Cutaneous lesion produced in		
subsp.	Rabbits	Hamsters	Guinea pigs
pallidum	+	-	-
pertenue	+	+	-
endemicum	+	+	+

Since its discovery in 1905 the study of T. pallidum has

been hampered by the lack of a satisfactory animal model for syphilis, and the inability to cultivate the organism in vitro. Virulent T. pallidum subsp. pallidum was first cultivated in vitro by Fieldsteel et al. (8,9) in 1981, and confirmed in 1982 by Norris (26). In vitro multiplication was achieved by co-cultivation of T. pallidum with cottontail rabbit epidermal (Sf1Ep) cells in chemically pre-reduced tissue culture media under low oxygen tension. Much of the early work on in vitro cultivation of T. pallidum dealt with defining the optimum dissolved oxygen concentration and the effects of the addition of reducing agents and scavengers of oxygen radicals to the media (5,16,25). A number of different cell lines have been used for the study of T. pallidum. These uses include the study of the attachment of T. pallidum to tissue culture cells and the use of this attachment to prolong the survival of T. pallidum in vitro (see table 2 for ref.). Despite the large number of cell lines studied, only cottontail rabbit epidermal (Sf1Ep) cells have been used for the in vitro cultivation of T. pallidum. Sf1Ep cells are used for cultivation because these cells were the most effective cell line for prolonging the survival of T. pallidum in the gradient cultures employed prior to succesful in vitro cultivation of this organism (5). At this time, it is not known what the tissue culture cells are providing to the treponemes, but all attempts at cell free culture have been unsuccessful. Fetal bovine serum was also found to be an

Table 2: literature survey of the studies concerned with the relationship between tissue culture cells and T. pallidum subsp. pallidum.

Use	Cell Line	ref
1. Study of attachment and the possible relationship to pathogenicity	rabbit capillary tissue	30
	rabbit testicular cells	12,13,14,17
	rabbit kidney	12
	rabbit epidermis	12
	rabbit spleen	12
	rabbit lung	12
	human cervical carcinoma	12
	human epidermoid carcinoma	12
	human urothelial carcinoma	12
	human testicular tumor	12
	human epithelial cells	12,13,14,17
	human foreskin cells	14
	rat glioma	12
	rat cardiac cells	14
	rat skeletal muscle cells	14
2. Study of the factors affecting attachment to mammalian cells	baby rabbit genital organ	42,43,44,45
	cottontail rabbit epithelium	40

Table 2: Continued.

3. Prolonging the survival of *T. pallidum* in vitro	rabbit testis cells	5,6,10,39
	rabbit kidney	11
	rabbit embryonic skin	5,6
	rabbit cornea	5
	cottontail rabbit epithelium	5,6,11
	mouse sarcoma	5
	nude mouse ear fibroblasts	5
	dog kidney	5
	rat peritoneal macrophages	5
	rat nose	5
	rat footpad	5
	rat glial cells	11,33,34
	human prepuce cells (newborn)	34
	human skin tumor	39
	human fetal kidney	5
	human skin epithelium	11
	human adult foreskin	5,6
	human fetal lung	5
	human cervical carcinoma	10
4. Cultivation of *T. pallidum*	cottontail rabbit epithelium	8,9,27

essential factor for cultivation of T. pallidum in tissue culture (7,28). Individual lots of serum vary greatly in their ability to support growth of T. pallidum. Although many refinements have been made on this system of cultivation, the basic method used today remains the same.

Difficulties in scaling up the present system of cultivation in static tissue culture monolayers, limit the growth of large numbers of T. pallidum. Suspension culture of anchorage dependent cells is one method currently used for the large scale cultivation of viruses and the production of cell-derived compounds (4,18,21,22,23,37). Research needs for large numbers of T. pallidum are currently met by cultivation of the treponemes in rabbit testes. One rabbit can supply between $5x10^9$ and $1x10^{10}$ T. pallidum. Use of rabbit propagated treponemes is hindered by the need to separate the treponemes from contaminating rabbit tissue. T. pallidum is a very fragile organism and it is difficult to manipulate without causing irreparable damage. In vitro cultivation allows collection of large numbers of T. pallidum with much less extraneous material than rabbit grown treponemes.

A problem which has limited progress in the improvement of this system was the number of experimental groups that could be set up in a single experiment. The amount of rabbit testis extract (TEx) usually limited the number of variables that could be included in a single cultivation experiment. An average pair of infected testis yielded about 15 ml of TEx

which was enough to set up 40-45 T-25 flasks. Six to eight T-25 flasks are required per experimental group. Therefore, about 5-7 groups could be set up per pair of infected rabbit testis.

Microtiter plates as cultivation vessels would require considerably less TEx than T-25 flasks. With microtiter plates, many variables and more detailed analysis of each variable could be tested in a single experiment. Furthermore, in the present system, considerable effort has been made to optimize treponemal growth with Sf1Ep cells. If other cell lines are to be examined for their ability to support the growth of T. pallidum, it is most probable that several of the culture conditions will have to be altered for optimum treponemal growth. Cultivation experiments in microtiter plates would expedite the optimization of those conditions.

Despite the ability to cultivate the organism in tissue culture, T. pallidum cannot be serial passaged in vitro. The greatest multiplication that can be achieved is slightly over 100 times the inoculum. This limited growth would suggest that some essential substance is not being supplied to the treponemes in the tissue culture system. One advantage that Sf1Ep cells have over other cell lines for cultivation of T. pallidum is that Sf1Ep cells grow relatively slowly. The cell growth rate is important because the treponemes and tissue culture cells are in competition for the nutrients in the medium. Removing the spent medium to replace it with fresh

medium is not advantageous since it also removes many of the treponemes that are floating free in the medium. It is possible that a different cell line may provide the missing factor or factors necessary for serial passage of T. pallidum. Nude mouse ear (NME) cells and normal rabbit testis fibroblast (RT) cells were shown to be almost as effective as Sf1Ep cells for prolonging the viabilty of T. pallidum in gradient tissue cultures (5).

This report describes a method for the suspension cultivation of T. pallidum. This study consisted of two separate phases. The goal of the first phase was to cultivate Sf1Ep cells on microcarrier beads. Although many cell types have been cultivated on microcarriers, little information regarding epidermal cells cultured in suspension has been published (20). After successful culture of Sf1Ep cells on microcarriers, infection of the suspension cultures with T. pallidum was attempted. Factors such as oxygen concentration, number of tissue culture cells and stirring rate were very critical parameters to define.

A system was developed for the cultivation of T. pallidum in microtiter plates. The conditions that were varied were the number of Sf1Ep cells, and the volume of medium per well.

To determine if other cell lines can be utilized for in vitro cultivation, NME, RT, and rabbit skin fibroblast (RAB-9) cells were compared to Sf1Ep cells in microtiter plates for their ability to support in vitro replication of T. pallidum.

The glucose levels of the cultures were monitored as a measure of the relative metabolic rates of the culture systems.

CHAPTER 2

MATERIALS AND METHODS

Media. The medium used for the propagation of Sf1Ep cells in suspension was Eagles Minimal Essential Media Atox (EMEM) (American Biorganics Inc., N. Tonawanda, NY) prepared with endotoxin free water. The Treponema pallidum culture medium (TPCM) used for treponemal cultivation is described in Table 3.

Fibronectin Purification. Rabbit plasma was collected, via heart puncture, using 0.4% sodium citrate as the anti-coagulant. Fibronectin was purified by affinity chromatography using 40 ml of Affi-Gel gelatin (BioRad Laboratories, Richmond, CA). After application of 50 ml of plasma (0.5 ml/minute at room temperature), the column was washed with PBS until the eluent was free of protein as determined by absorbance at 280 nm. The fibronectin was then eluted from the column with 1.0 M sodium bromide pH 5. The purified fibronectin was dialysed for 2 days against 2 liters of 0.05 M NaCl at room temperature with changes of dialysate three times a day. The fibronectin was sterilized by filtration through a 0.2μ, low protein binding, membrane filter (Millipore, Bedford, MA) and frozen at -20° C until

Table 3: Composition of media for T. pallidum cultivation.

Component[a]	Amount/100ml TPCM	
Earls balanced salt solution (10x)[b]	10.0	ml
Essential amino acids (50x)[c]	0.5	ml
Non-essential amino acids (100x)[c]	0.5	ml
Vitamins (100x)[c]	0.5	ml
Glucose	250.0	mg
l-Glutamine (200mM)	1.0	ml
$CoCl_2$ (0.5 mg/100 ml)	0.1	ml
Cocarboxylase (0.2 mg/100 ml)	0.1	ml
Mannitol (10 g/100 ml)	0.1	ml
Histidine (5 g/100 ml)	0.1	ml
Sodium Bicarbonate	253.5	mg
MOPS Buffer (1M pH 7.5)	2.5	ml
Sodium Pyruvate	10.0	mg
Dithiothreitol [DTT]	15.0	mg
Superoxide dismutase [SODase]	2500.0	units
Catalase	1000.0	units
Pyrogen-free water to 100 ml		

Table 3: Continued.

[a]All components from Sigma Chemical Co. St Louis, MO unless otherwise indicated.

[b] Concentrations of stock solutions in parentheses.

[c] Flow Laboratories Inc. McLean, VA

All ingredients except DTT, SODase, and catalase are combined, the pH is adjusted to 7.4, the TPCM is sterilized by filtration and stored under an atmosphere of 95% N_2/5% CO_2. The other three components are hydrated, filter sterilized separately and added to the TPCM just prior to use.

use. The purified fibronectin was run on an SDS-PAGE (8-25%) gradient gel on a Phastsystem (Pharmacia, Piscataway, NJ). The gel was silver stained for visualization of the protein.

Preparation of Beads. Before use, the collagen coated dextran beads (Cytodex-3, Pharmacia) were hydrated in 10 mM phosphate buffered saline pH 7.4 (PBS) for 4 hours at room temperature. The beads were then washed 3 times with PBS by allowing the beads to settle to the bottom of the bottle, carefully removing the liquid above the beads, and adding more PBS. The beads were sterilized by autoclaving. After cooling, the beads were washed twice with MEM and brought to a final concentration of 4mg/ml. The beads were stored at 4° C until needed. Before initiating cell cultures on dextran beads, 40 mg of beads were incubated with 3 mg of purified rabbit plasma fibronectin and 30 mls of MEM in a siliconized 125 ml Magna-flex stirring flask (Wheaton Instruments, Millville, NJ)(figure 1). Five ml of FBS (HyClone,Logan,UT) were added to the flask and the flask was stirred at 40 rpm for 1 hour on a variable speed stiring table (Wheaton Micro-stir).

Polystyrene beads, (Cytospheres, Lux, Newbury Park, CA) were supplied dried and presterilized. The polystyrene beads were aseptically suspended in MEM with 10% fetal bovine serum at a concentration of 120 mg/ml and stored at 4° C until use. The cell cultures using polystyrene beads contained 600 mg of

figure 1: A 125 ml Wheaton Magna-flex stirring flask.

beads, 30 ml of MEM and 4 ml of FBS.

Tissue Culture Cells. The cottontail rabbit epidermal (Sf1Ep) cells were supplied by the Naval Biomedical Research Laboratory (Oakland, CA). The Sf1Ep cells were subcultured every 2 weeks at a ratio of 1:4. The nude mouse ear (NME) cells were isolated in the laboratory of A. Howard Fieldsteel at the Stanford Research Institute (Palo Alto, CA). The NME cells were subcultured every 7 days at a ratio of 1:3. The adult female rabbit skin fibroblasts (RAB-9) were from the American Type Culture Colllection (Rockville ,MD) ATCC no. CRL 1414. The RAB-9 cells were subcultured every 7 days at a ratio of 1:5.

The rabbit testis cell cultures were initiated as follows. Mature New Zealand White rabbit testis were minced and rinsed twice in 10 ml of sterile phosphate buffered saline pH 7.2 (PBS). The testis were then suspended in 10 ml of 0.25% Trypsin in PBS and placed on a shaker for 30 minutes. The fluid was removed and combined with 10 ml of MEM with 10% FBS. The cell suspension was centrifuged at 500x g for 10 minutes. The cells were resuspended in MEM and inoculated into plastic tissue culture flasks with MEM and 10% FBS. The cells were incubated at 34°C in an atmosphere of 5% CO_2/95% air, and were refed every third day. After 7 days, the cells were subcultured at a ratio of 1:4. The majority of the cells

present were fibroblast-like after 3 passages and these are the cells that were used for these experiments.

Initiating the Suspension Cultures. The desired number of Cottontail rabbit epidermal cells were added to the flask and the atmosphere in the flask was purged with a mixture of 5% CO_2/95% Air. The flask was then placed on the stirring table in a 34° C incubator and stirred at 40 rpm for 1 minute out of every 45 minutes. After twenty-four hours, 10-15 ml of fresh MEM was added to the flask.

Observing the Cell Cultures. Cell attachment was observed by staining with methylene blue, after cold methanol fixation. Cells were counted by the released nuclei method of Sanford et al. (35). Briefly, the cell coated beads were incubated for 1 hour in 0.1M citric acid containing 0.1% crystal violet. After incubation, the beads were vigorously mixed and the released nuclei were counted with a hemocytometer.

Preparation of Treponemes for Inoculum. The virulent Treponema pallidum subsp. pallidum (Nichols) used throughout this study was maintained and passaged in rabbit testes as follows. Adult male new zealand white rabbits (7-9lbs) were inoculated intratesticularly with $1x10^8$ frozen T. pallidum per

testis. The testis were aseptically removed 10-11 days post infection. The testis were minced and placed in TPCM (80gm tissue/100 ml TPCM). The minced testes were shaken for 20 minutes at room temperature. The fluid was removed and centrifuged at 500x g for 5 minutes to remove gross tissue debris. The supernatant was removed, the treponemes counted, and the treponemes required for inoculum were removed. The remaining fluid was centrifuged at 12,000x g for 10 minutes to remove the remaining treponemes. The supernatant was heat inactivated for 30 minutes at 56° C and then centrifuged at 20,000x g for 10 minutes. The supernatant from this last spin was the testis extract (TEx). The treponemes used for inoculation were diluted in fresh TPCM.

Inoculation of Suspension Cultures. After two days, suspension cultures of Sf1Ep cells were prepared for inoculation with T. pallidum by replacing the MEM with TPCM. Half of the media from the suspension culture was removed and replaced with TPCM. This was done three times for each flask. The final addition of TPCM brought the total volume to 60 ml per flask. Two ml of TEx and $2x10^7$ vigorously motile T. pallidum were added to each flask. The flasks were placed in a controlled gas incubator (Forma Scientific, Marietta, OH) at 34° C on a variable speed magnetic stirrer (Techne) in an atmosphere of 5% CO_2, 4% O_2 and 91% N_2. The cultures were

stirred at 35 rpm for 1 minute out of every 45 minutes.

Cultivation of T. pallidum in Microtiter Plates. Cell cultures were harvested by incubating the cultures with a solution of trypsin-versene [Irvine Scientific, Irvine, CA]. Cells were counted with a haemo-cytometer and adjusted to the appropriate concentration for inoculation into microtiter wells. One-half ml of cell suspension was added to each of the wells [except the corner wells] of a Falcon 24-well microtiter plate. The plates were placed in a CO_2 incubator at 35°C for two days. To infect the cell monolayers with T. pallidum, the EMEM was removed by aspiration and replaced with TPCM with 10% fetal bovine serum and 27µl of rabbit testis extract. The plates were then placed in an incubator containing an atmosphere of 5% carbon dioxide, and 95% nitrogen. The plates were allowed to equilibrate in this atmosphere for 1 hour. The wells were inoculated by adding 50 µl of a suspension of treponemes containing 6.4×10^6 treponemes/ml. The plates were immediately returned to the incubator and the above equilibration procedure was repeated. Afterwards, the plates were transferred to a tri-Gas incubator (Forma Scientific, Marietta, OH) containing an atmosphere of 3.5% oxygen, 5% carbon dioxide and 91.5 nitrogen at 34° C. The mirotiter plate wells used for the examination of alternative cell lines contained 1.5 ml of medium.

Enumeration of Treponemes from Suspension Cultures. Three ml of a homogeneous suspension of beads were placed in a 15 ml conical polypropylene centrifuge tube. The beads were allowed to settle and approximately 2 ml of media was transferred to a second tube. One ml of Trypsin-EDTA (Irvine Scientific, Santa Ana, CA) was added to the culture beads. After gentle agitation, the beads were allowed to settle and the Trypsin-EDTA was transferred to the second tube. Two more ml of fresh Trypsin-EDTA was added to the culture beads, the tube was flushed briefly with 95% N_2 and 5% CO_2 and allowed to incubate with intermittent agitation for 15-20 minutes. The contents of the second tube were added to the first tube and the tube was flushed briefly with 95% N_2 and 5% CO_2. The tube was agitated vigorously and the detached treponemes were counted by darkfield microscopy, as previously described (8).

Enumeration of Treponemes from Microtiter Plates. The media was removed from a well using a pasteur pipet and placed in a conical polypropylene centrifuge tube. The well was rinsed with 250µl of PBS and the PBS was placed in the tube. The wells were filled with 500µl trypsin-versene and the plates were placed in an incubator at 34° C with an atmosphere of 5% carbon dioxide and 95% nitrogen for 10 minutes. The trypsin-versene was removed by pasteur pipet and placed in the

tube. The treponemes were then counted by dark-field microscopy.

Passage of T. pallidum from Tissue Culture Flasks T. pallidum cultures in T-25s were harvested with trypsin EDTA on day seven. The cells and treponemes were passaged into T-25s with 10 ml of TPCM, 10% FBS, and 0.34 ml of TEx. The passaged cultures contained from $3\text{-}6\text{x}10^6$ treponemes.

Passage of T. pallidum from Suspension Cultures Homogeneous suspensions of beads were removed from suspension cultures of treponemes. The beads were inoculated into T-25s containing $4\text{x}10^5$ Sf1Ep cells and 10 ml of TPCM. The T-25s contained from $3\text{-}5\text{x}10^6$ treponemes.

Glucose Assay. Glucose levels in the spent media were determined with the glucose (HK) reagent (Sigma Chemical Co.). This reagent uses a coupled enzyme reaction (hexokinase and Glucose-6-phosphate dehydrogenase) to determine glucose concentration. Briefly, 10µl of the spent media was added to 1ml of glucose reagent and then incubated at 37° C for 5 minutes. The absorbance of the mixture was then measured at 340 nm. The glucose concentration was calculated from the millimolar absorptivity of NADH at 340 nm.

Scanning Electron Microscopy. Treponema pallidum was cultivated in suspension for 10 to 12 days as described above. Beads were removed from the culture and washed twice in phosphate buffered saline (10mM pH7.3). The beads were placed on glass cover slips coated with poly-L-lysine and allowed to sit at room temperature for 20-30 minutes. The samples were immersed in glutaraldehyde (4%, 0.1M phosphate buffer pH 7.3) for 1 hour. After fixation, the samples were washed twice in phosphate buffer and postfixed in 2% osmium tetroxide for 1 hour. The samples were washed twice in distilled water and dehydrated in a graded ethanol series (10, 30, 50, 70, 95, 100, 100, 100%). After dehydration, the samples were critical point dried (Polaron) and mounted on aluminum stubs with silver conductive paint. The samples were sputter coated with Au/Pd in a Polaron sputter coating apparatus and viewed in an ETEC Autoscan U-1.

CHAPTER 3

RESULTS

Fibronectin Purification. Gradient (8-25%) SDS-PAGE of the purified fibronectin showed a single band with an M_r of approximately 220 kD. The average yield of fibronectin from 50 ml of plasma was 12.3±6.0 mg (n=14).

Suspension Cultivation of Sf1Ep Cells. A minimum of 16 µg of fibronectin per cm^2 of bead surface was necessary for attachment and growth of Sf1Ep cells on collagen coated dextran beads. At lower concentrations of fibronectin, attachment was inconsistent. By the second day, the cells would clump and detach from the beads. Purified fibronectin was not required for cell attachment and growth on polystyrene beads. The cells grew well on both types of microcarrier beads. Figure 2 is a growth curve of Sf1Ep cells on polystyrene beads. The cells reached confluence after 8 days in culture. Figure 3 shows methylene blue stained Sf1Ep cells grown on dextran beads. The cells appear to cover the surface of the beads. Several beads are clumped together with the cells bridging the space between the beads. This clumping was common using either dextran or polystyrene beads.

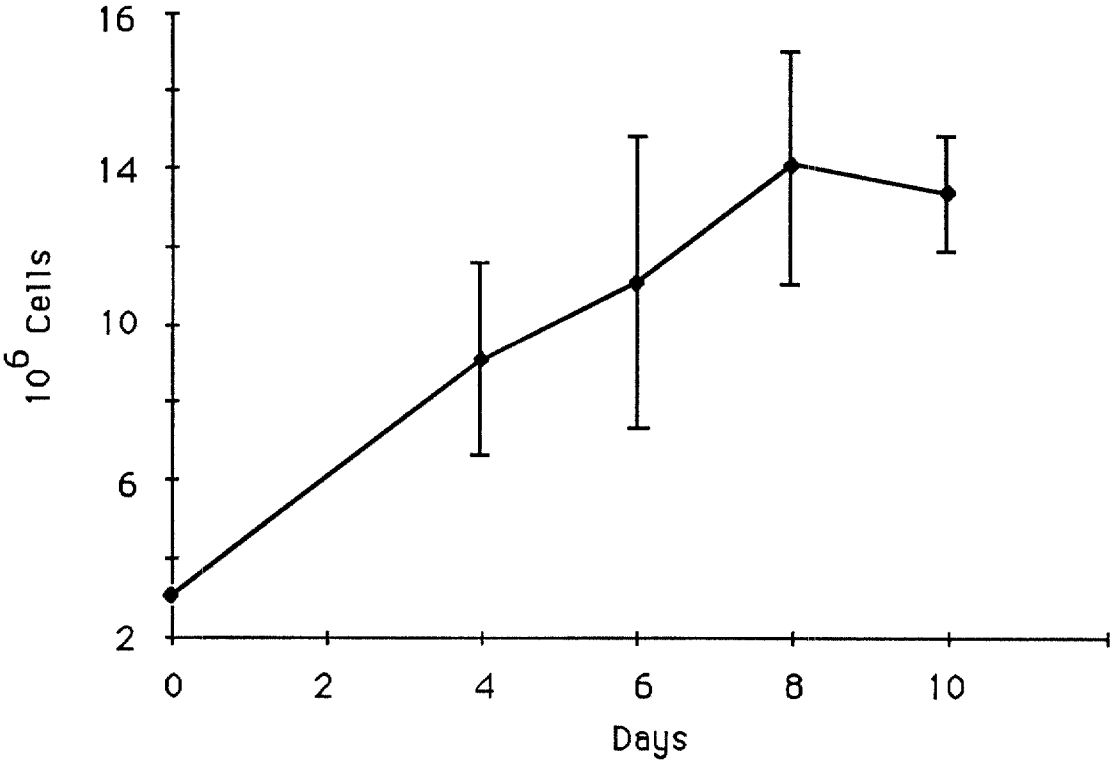

figure 2: Sf1Ep cells grown on polystyrene beads, $3x10^6$ cells were inoculated onto 600 mg of beads in 40 ml of MEM with 10% FBS. Cell counts via released nuclei method. Each point is the mean (± standard deviation) of 5 cultures.

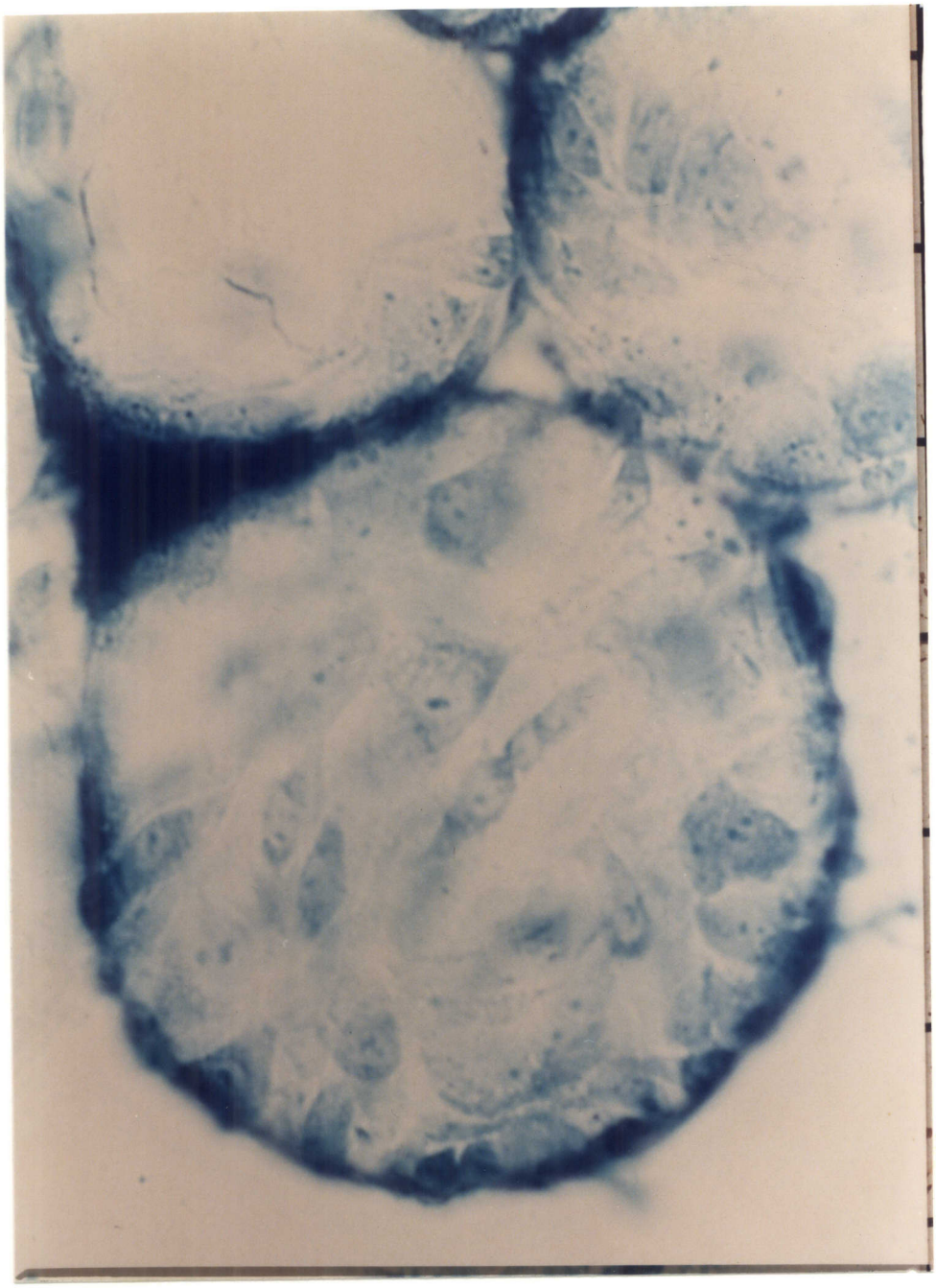

figure 3: Bright-field micrograph of methylene blue stained, Sf1Ep cells on dextran microcarrier beads. (600x).

Suspension Cultivation of Treponema pallidum. T. pallidum was successfully cultivated in vitro using either polystyrene or collagen-coated dextran microcarriers. Due to their ease of use, polystyrene microcarriers were used for these studies. The treponemal cultures were stirred for 1 minute out of every 45 minutes at 30 rpm. Constant stirring of the cultures caused the treponemes to detach from the cells and no measurable treponemal multiplication took place. Oxygen concentrations of 5%, 10% and 15% were used for the treponemal suspension cultures. The atmosphere in the flask was replaced with the desired oxygen concentration and the flask was sealed. An oxygen concentration of 5% resulted in significantly greater growth of T. pallidum than the other two oxygen concentrations. Further studies on oxygen concentration showed that a concentration of approximately 4% was best for suspension cultivation of T. pallidum (table 4). The treponemal cultures incubated at a constant oxygen concentration of 4% averaged a 36 fold-increase on day 12 with a maximum increase of 51-fold (figure 4).

Figures 5-9 are scanning electron micrographs of T. pallidum attached to Sf1Ep cells grown on microcarrier beads. These photographs were taken after 10 days in culture. Figure 5 demonstrates the attachment of the Sf1Ep cells to the polystyrene bead. The cells spread out and lie very flat on

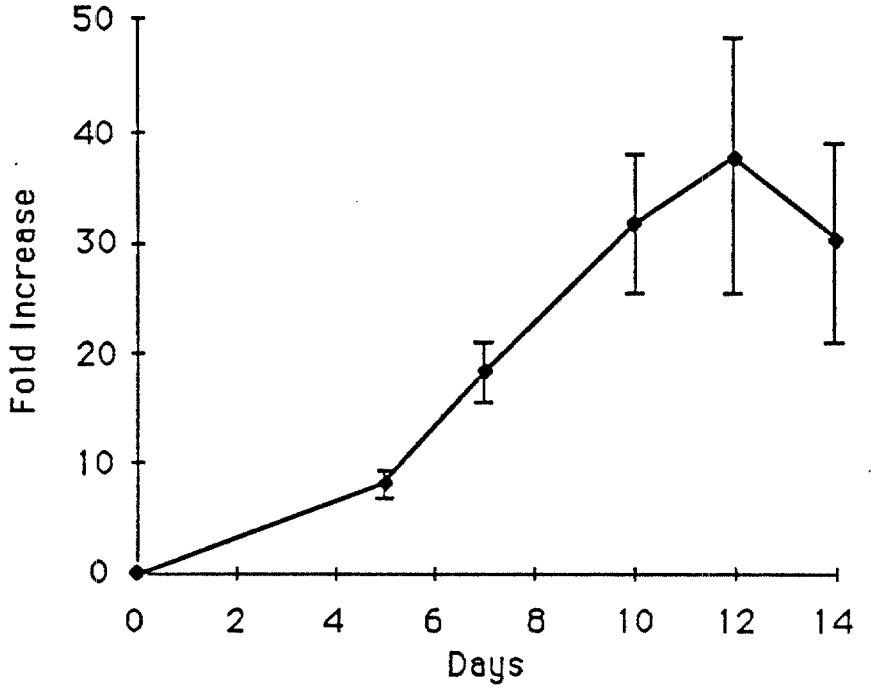

figure 4: Growth of T. pallidum in suspension on polystyrene beads. $2x10^7$ treponemes were added per flask on day 2 of the cell culture. Bacteria were counted via dark field microscopy on the indicated days. Each point is the mean (± standard deviation) of 5 cultures.

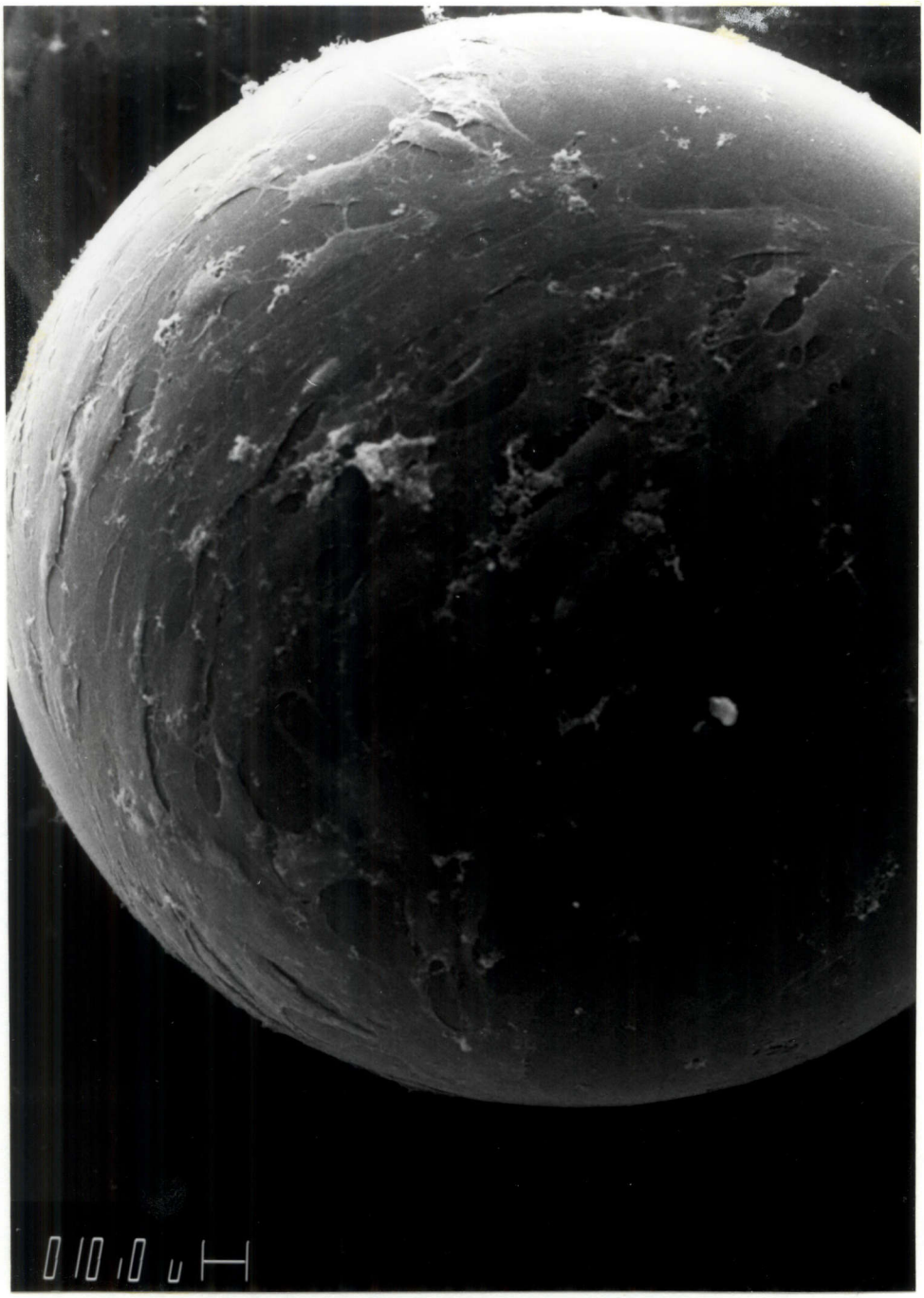

figure 5: Scanning electron micrograph of T. pallidum attached to Sf1Ep cells growing on polystyrene beads. Microcolony of treponemes (arrow). Bar, 10µm.

the surface of the bead. This is in contrast to the cells attached to the collagen-coated dextran beads where the cells are more rounded and project away from the bead surface (figure 6). Microcolonies of treponemes can be seen on the surface of some of the cells (figures 7 and 8). The microcolonies of treponemes were very common in the suspension cultures. However, not all treponemes were in these clumps. Many individual treponemes could also be seen on the cell surfaces.

Table 4: The oxygen concentration used for suspension cultures of T. pallidum.

Oxygen Concentration[a]	Maximum Fold Increase[b]	n
3.5	35.8±5.8	6
4.0	36.3±10.0	5
5.0	16.3±1.8	6
10.0	3.3±0.9	5
15.0	0.9±0.3	4

[a]± 0.2%

[b]Mean ± standard deviation

A density of $1x10^5$ Sf1Ep cells per ml of TPCM was optimal

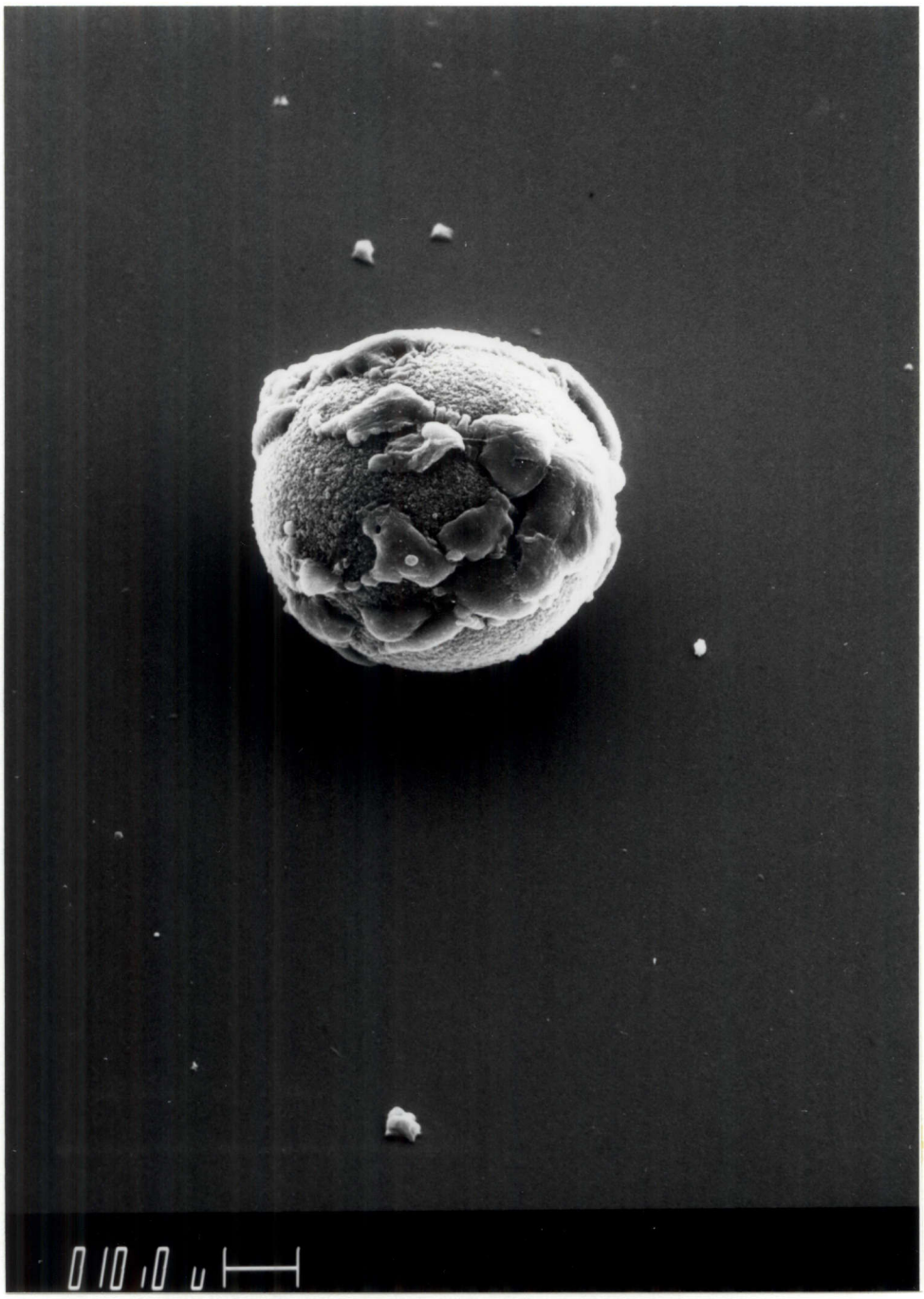

figure 6: Scanning electron micrograph of T. pallidum attached to Sf1Ep cells growing on dextran beads. Bar, 10µm.

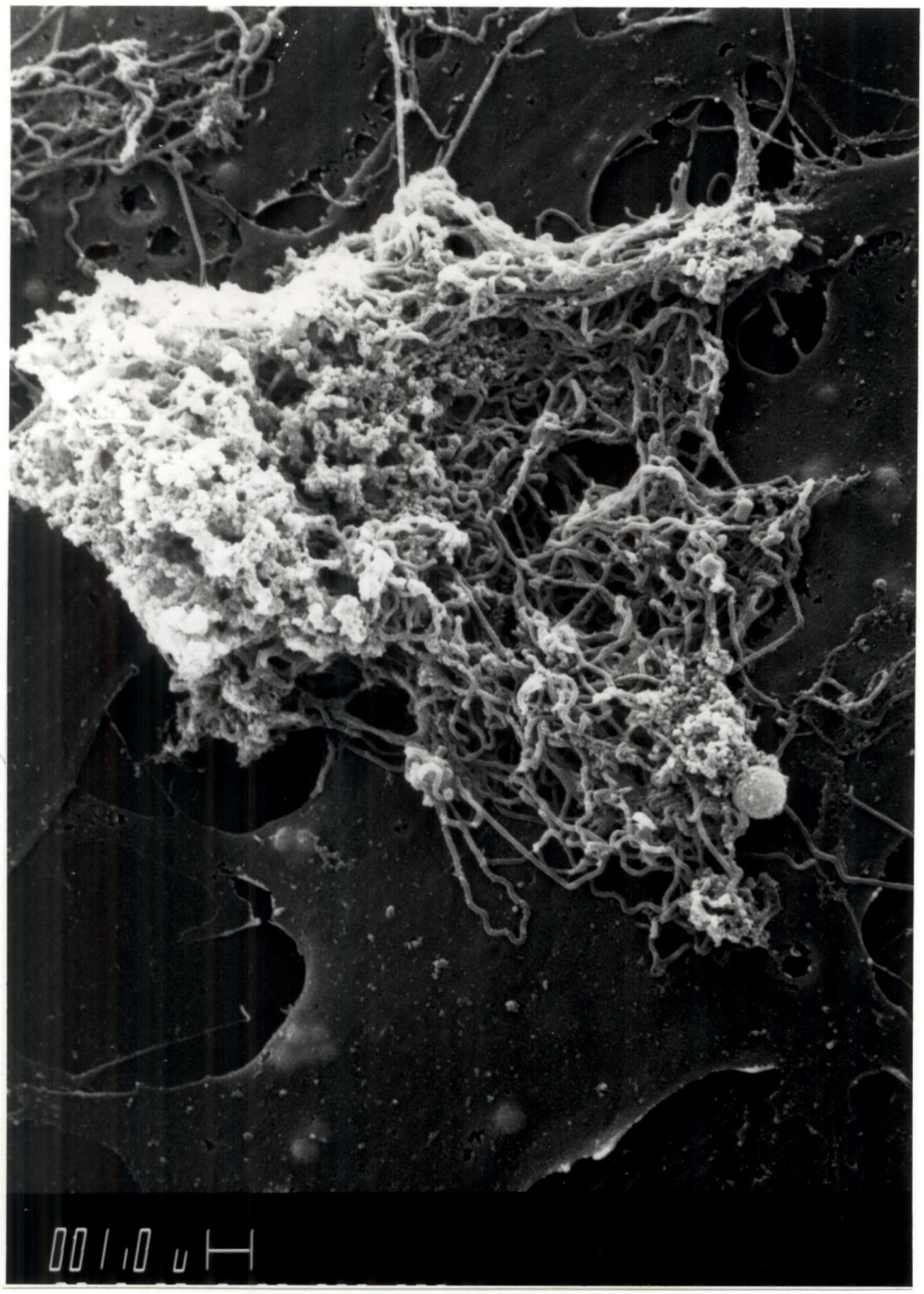

figure 7: A microcolony of treponemes indicated by the arrow in figure 5. Bar,1.0μm.

figure 8: A microcolony of treponemes. Bar, 1.0μm.

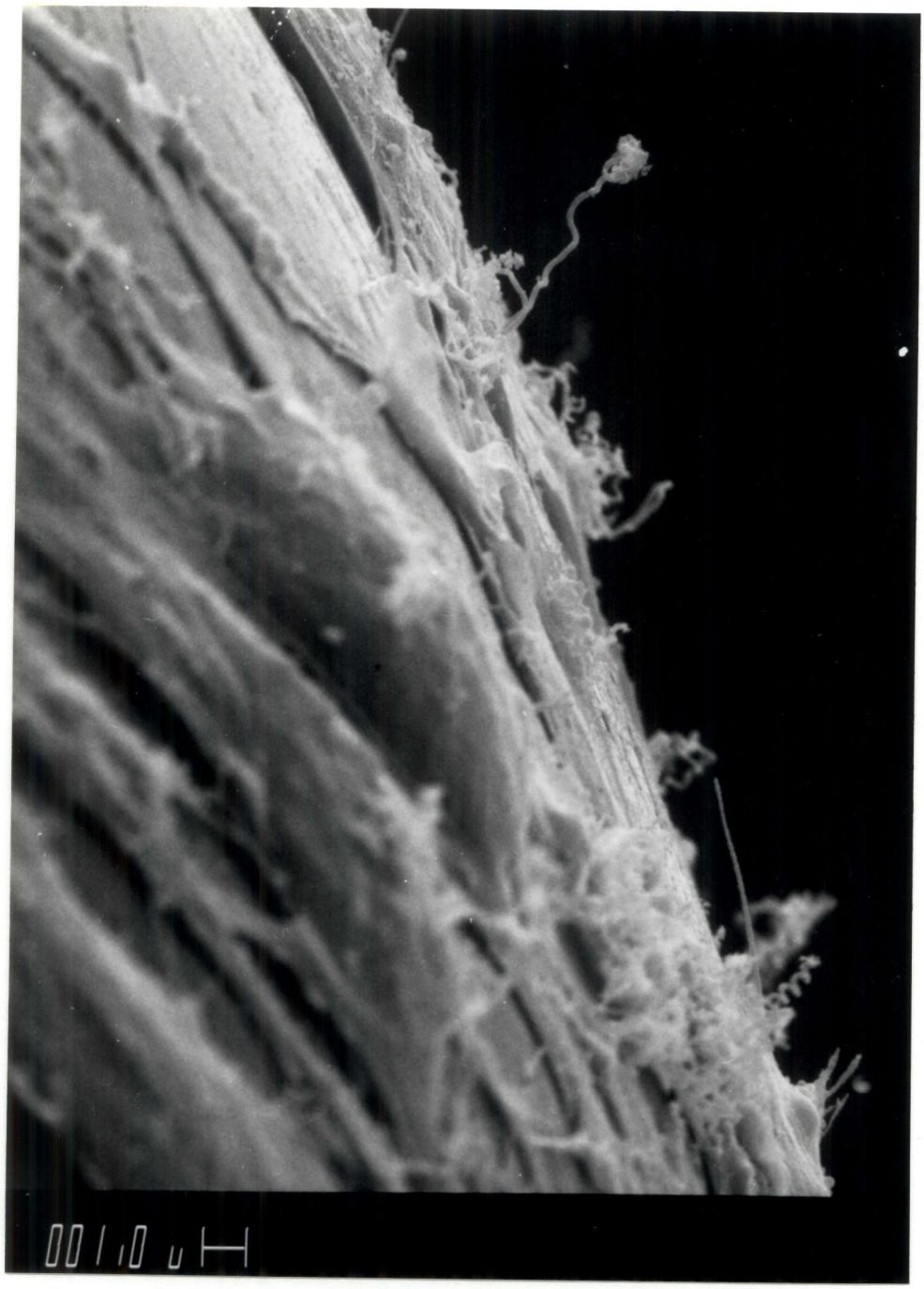

figure 9: Sideview of treponemes attached to Sf1Ep cells on polystyrene beads. Bar, 1.0µm.

for the growth of T. pallidum (table 5). Doubling the cell density decreased peak growth an average of 40%. Reducing cell density to $5x10^4$ Sf1Ep cells per ml of TPCM also reduced the maximum growth approximately 40%. Refeeding the treponemal cultures on day 7 with either 10 ml of TPCM or 25 mg of glucose did not significantly alter the maximum fold increase (table 6).

Cultivation of T. pallidum in Microtiter Plates

The wells seeded with $5x10^4$ Sf1Ep cells and either 1.25 or 1.5 ml of TPCM suported the greatest fold increase of T. pallidum (fig 10). The growth of T. pallidum in the wells compared favorably with the growth in T-25 plastic tissue culture flasks. In five separate experiments the maximum fold increase of T. pallidum in the wells averaged 74.5±28.6 and the T-25s averaged 65.3±15.1.

Comparison of Different Cell Lines. All three cell lines tested supported the replication of T. pallidum. Table 7 contains the fold increase and glucose assay results for the different cell lines at 2 different cell seeding densities. The maximum fold increase of T. pallidum obtained with the RAB-9 cells was equal to that of the Sf1Ep cells. The testis cells and the NME cells supported a lower maximum fold increase than the other two cell lines. The Sf1Ep cells

Table 5: The number of Sf1Ep cells used for suspension cultures of T. pallidum.

Cell Number/ml TPCM	Maximum Fold Increase[a]	n
$5x10^4$	26.9±5.7	4
$1x10^5$	41.6±6.7[b]	6
$2x10^5$	26.8±3.9	4

[a]Mean ± standard deviation

[b]The mean maximum fold increase of this group was significantly different from the other groups (SNK multiple range test, a=0.05).

Table 6: Refeeding suspension cultures of T. pallidum on day 7.

Group	Maximum Fold Increase[a]		n	p[b]
	Control	Experimental		
BRMM (10 ml)	33.9±5.4	34.9±8.5	6	0.616
Glucose (25 mg)	41.5±7.5	39.2±3.8	5	0.648

[a]Mean ± standard deviation

[b]Two-tailed paired-t test compared to control culture.

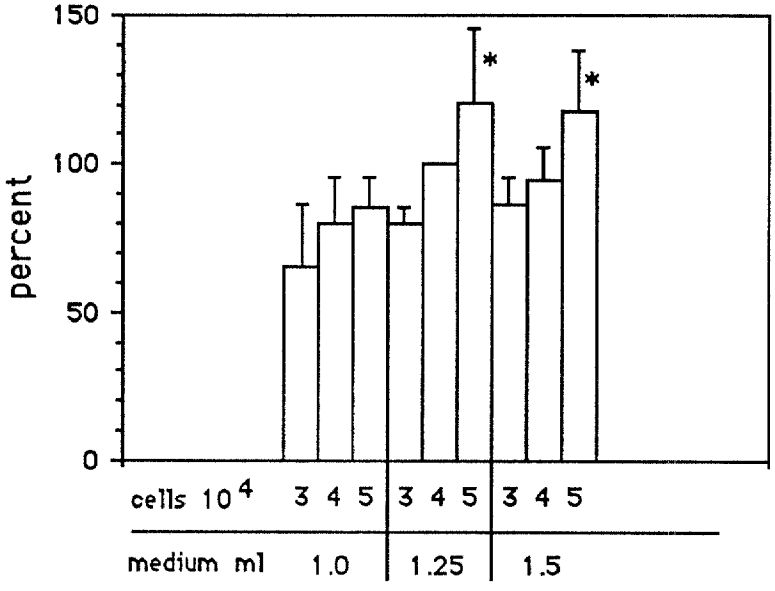

figure 10: Growth of T. pallidum in microtiter plates with Sf1Ep cells. The results are expressed as (fold increase x/fold increase with $4x10^4$ cells and 1.25 ml TPCM)x100. * groups are significantly greater then the other groups (SNK multiple range test, alpha=0.05).

Table 7: Fold increase[a] of T. pallidum and (glucose concentration[b] mg/ml) of experiments using four different cell lines for in vitro cultivation. Results are the mean ± standard deviation of 6 experiments.

Cell Type / Cell Number	day 6	day 8	day 10
Sf1Ep			
$2.5x10^4$	7.21±1.6 (164±13)	19.8±6.1 (169±20)	19.0±3.1 (150±18)
$5.0x10^4$	10.2±3.6 (144±22)	21.4±5.3 (158±15)	27.6±5.6 (132±17)
RAB-9			
$2.5x10^4$	11.9±1.9 (125±20)	28.3±5.1 (119±33)	36.2±4.0 (95±41)
$5.0x10^4$	12.4±1.6 (110±25)	22.2±2.5 (89±31)	23.7±9.3 (54±31)
NME			
$2.5x10^4$	7.3±0.4 (150±18)	12.8±1.7 (135±24)	18.8±6.6 (96±6)
$5.0x10^4$	8.9±2.2 (137±29)	14.7±5.4 (111±17)	18.1±10.4 (83±22)
RT			
$2.5x10^4$	9.2±1.3 (135±21)	16.0±3.3 (110±20)	20.5±3.6 (92±18)
$5.0x10^4$	8.3±1.7 (111±13)	13.1±2.9 (79±13)	18.2±3.6 (62±19)

[a]Fold increase = $\frac{\text{motile treponemes on day counted}}{\text{motile treponemes in inoculum}}$

[b]Glucose concentration on day 0 = 199.0±5.4mg/dl

Table 7: Continued.

Cell Type / Cell Number	day 12	day 15	day 17
Sf1Ep			
$2.5x10^4$	30.5±9.4 (143±9)	26.7±10.4 (128±21)	27.8±10.2 (124±36)
$5.0x10^4$	42.0±11.0 (135±36)	44.8±14.2 (111±34)	45.7±10.4 (97±31)
RAB-9			
$2.5x10^4$	51.4±9.8 (72±35)	37.5±4.2 (42±28)	25.3±9.2 (35±27)
$5.0x10^4$	20.7±17.0 (40±32)	11.5±13.4 (22±21)	1.1±2.1 (16±14)
NME			
$2.5x10^4$	21.0±5.0 (87±17)	15.5±4.2 (51±12)	10.8±9.6 (37±30)
$5.0x10^4$	19.2±10.5 (60±24)	16.3±7.5 (30±28)	9.0±10.4 (24±22)
RT			
$2.5x10^4$	21.2±7.8 (71±18)	17.4±6.0 (51±21)	8.5±4.2 (46±26)
$5.0x10^4$	13.5±4.7 (36±21)	7.6±4.4 (23±18)	4.3±3.4 (28±21)

supported greater fold increase of the treponemes when seeded at the higher cell density (figure 11). The other 3 cell lines supported the highest fold increase at the lower cell density (figures 12,13,14). Figure 15 consists of growth curves for all four cell lines at the initial cell density that resulted in the largest fold increase.

All microtiter plate cultures were observed by phase contrast microscopy before harvesting. The RAB-9, NME, and testis cells attained confluency by day 8. The Sf1Ep cells did not become confluent even after 17 days in culture, although the cell density did increase.

In Vitro Passage of T. pallidum Table 8 contains the results of five experiments passing T. pallidum from suspension culture into T-25 tissue culture flasks. In one of these experiments treponemes were passed from suspension culture into another suspension culture. The first experiment was very successful but subsequent experiments were not. In one experiment treponemes from the same suspension culture were passed into both suspension and T-25 cultures. There was no difference in the total fold increase between the cultures passed into static or suspension cultures.

The results of experiments passsing T. pallidum from T-25 to T-25 with several different cell lines are in Table 9. The first passage experiment using RAB-9 cells was very successful

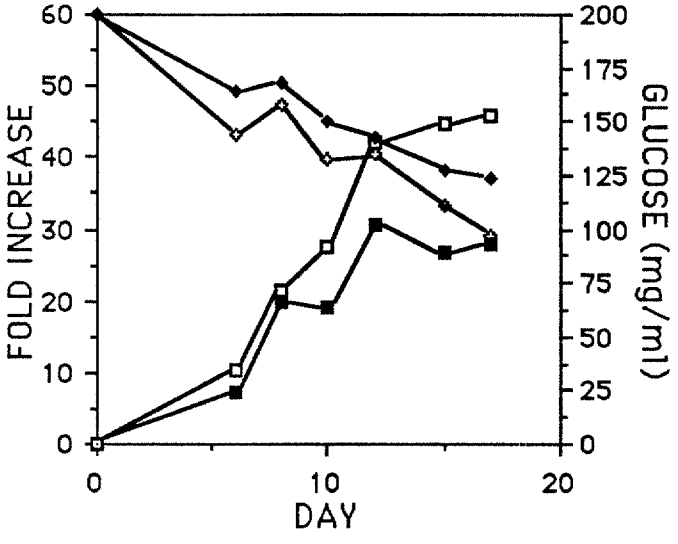

figure 11: Growth of T. pallidum with Sf1Ep cells. Fold increase of treponemes with ■ $2.5x10^4$ and □ $5x10^4$ Sf1Ep cells. Glucose concentration mg/dl with ◆ $2.5x10^4$ and ◇ $5x10^4$ Sf1Ep cells.

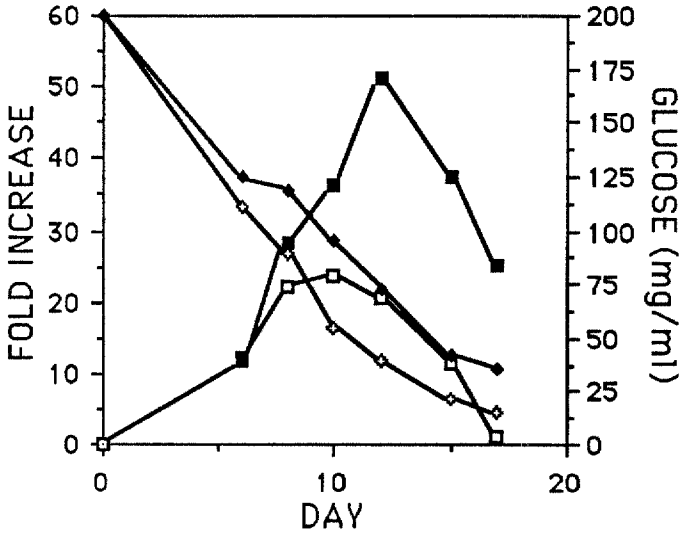

figure 12: Growth of T. pallidum with RAB-9 cells. Fold increase of treponemes with ■ $2.5x10^4$ and □ $5x10^4$ RAB-9 cells. Glucose concentration mg/dl with ◆ $2.5x10^4$ and ◇ $5x10^4$ RAB-9 cells.

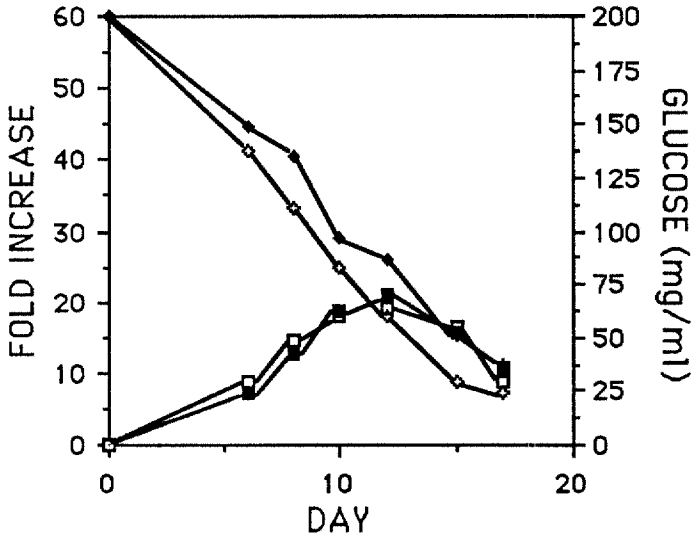

figure 13: Growth of T. pallidum with NME cells. Fold increase of treponemes with ■ $2.5x10^4$ and □ $5x10^4$ NME cells. Glucose concentration mg/dl with ◆ $2.5x10^4$ and ◇ $5x10^4$ NME cells.

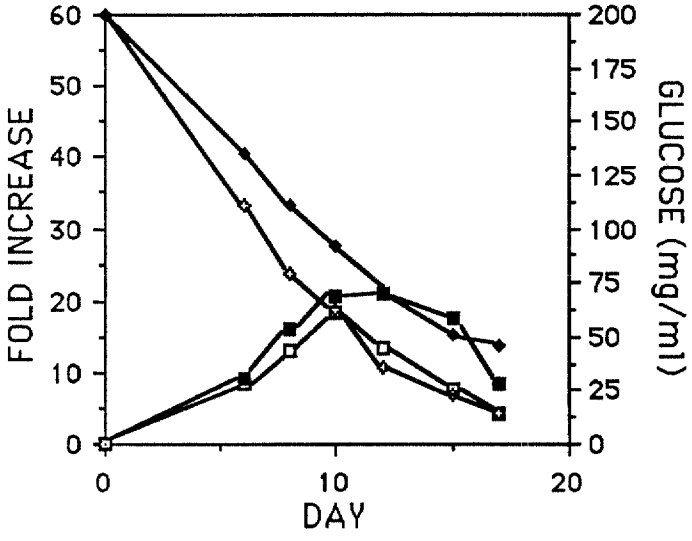

figure 14: Growth of T. pallidum with RT cells. Fold increase of treponemes with ■ $2.5x10^4$ and □ $5x10^4$ RT cells. Glucose concentration mg/dl with ◆ $2.5x10^4$ and ◇ $5x10^4$ RT cells.

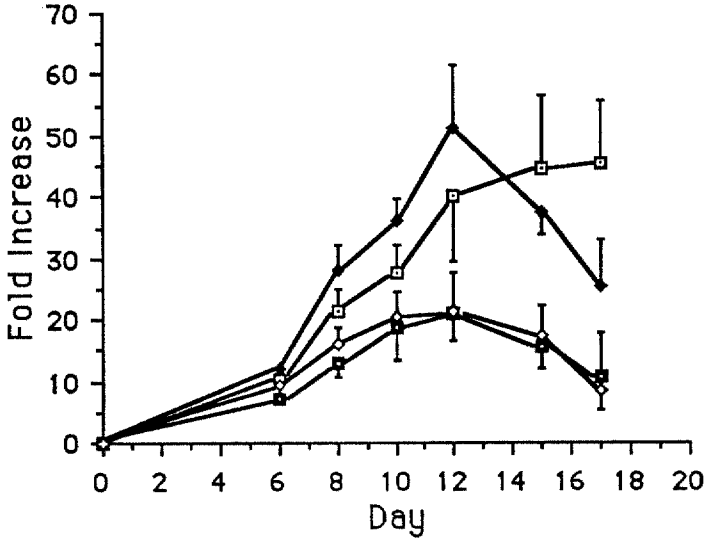

figure 15: Growth of T. pallidum with four different mammalian cell lines. All cells were seeded at $2.5x10^4/2cm^2$ well except for Sf1Ep cells which were seeded at $5.0x10^4/2cm^2$ well. □ Sf1Ep, ◆ RAB-9, ■ NME, ◇ RT. Each point is the mean (± standard deviation) of 6 cultures.

Table 8: In vitro passage of T. pallidum from suspension culture to T-25.

Experiment	Fold Increase When Passaged	Maximum Fold Increase[a] 1st Passage
1	21	80.5(1691)
2	18.6	3.5(65.1)
3a	22.7	1.8(40.9)
3b	22.7[b]	1.7(38.6)
4	12.5	3.5(43.8)
5	25	2.1(52.5)

[a]Total fold increase in parentheses

[b]Passaged into suspension culture containing $6x10^6$ Sf1Ep cells.

Table 9: In vitro passage of T. pallidum from T-25 to T-25.

Cell Type	Fold Increase When Passaged	Maximum Fold Increase[a] 1st Passage	Maximum Fold Increase 2nd Passage
RAB-9	23.1	17.2(397)	4.6(1826)
RAB-9	4.6	2.6(12)	ND[b]
RAB-9	15.3	3.2(49)	ND
RAB-9	8.9	4.8(42.7)	1.1(47)
RT	10	6.4(64)	ND
RT	5.6	5.5(30.8)	ND
RT	9.6	3.6(34.6)	ND
NME	10.8	3.3(35.6)	ND
NME	9.1	2.3(20.9)	ND
Sf1Ep	10.4[c]	4.1(42.6)	1.4(59.7)

[a]Total fold increase in parentheses

[b]Not done

[c]Passaged into flasks containing $4x10^5$ Sf1Ep cells

but none of the other experiments approached this level of total fold increase.

CHAPTER 4

DISCUSSION

These studies have examined the use of microcarrier beads for the growth of Sf1Ep cells in suspension. Although either type of microcarrier bead can be used for suspension cultivation of T. pallidum, the use of the polystyrene beads was preferred for two reasons. First, purified fibronectin does not have to be added to cell cultures with polystyrene beads. This saved both time and money spent purifying fibronectin from rabbit plasma. The second reason was that T. pallidum grown on polystyrene beads was easier to harvest. Ease of removal of treponemes from the beads was important both for enumeration of T. pallidum and for collecting treponemes for other uses. In order for suspension cultivation to be an alternative for large-scale cultivation of T. pallidum, the method used to harvest treponemes must be as gentle as possible. Removal of treponemes from dextran beads required prolonged incubation in trypsin which could damage the treponemes and release contaminating material from tissue culture cells. The difference in the strength of attachment of the cells to the beads was probably due to the use of purified fibronectin with the dextran microcarriers.

This does not mean that dextran beads are not useful for suspension cultivation of T. pallidum. Dextran beads were much better than polystyrene beads for studies where direct dark-field observation of treponemes attached to cells on microcarrier beads was desired. The polystyrene beads refract too much light for dark-field observation to be possible.

These methods of suspension cultivation of Sf1Ep cells resulted in clumping of the beads due to bridging of the cells between the beads. This was probably aggravated by intermittent stirring of the cultures rather than continuous agitation. Although clumping resulted in a decrease in the surface area available for cell growth, it was not detrimental for the suspension cultivation of T. pallidum. The maximum yield of Sf1Ep cells in suspension cultures was slightly less then that obtained in static cultures with comparable surface area. This was probably a result of the beads clumping in suspension culture. Clumping may also account for some of the variability seen in the numbers of both cells and treponemes. Large clumps of beads made it difficult to remove homogeneous samples from the suspension cultures. Pipets with large tip openings were necessary to obtain representative samples for counting.

A stirring rate of 30-40 rpm was used because it was strong enough to stir up the beads but not so strong that the treponemes would be sheared off the cells. Varying the rate

or frequency of the stirring was very difficult since this could change the optimum oxygen concentration. Therefore, the rate and frequency of stirring were held constant and then the optimum oxygen concentration was determined. The optimum concentration of oxygen found for the suspension cultures was very interesting. Although the media in the suspension cultures was much deeper than that of the static cultures (25 mm vs 3 mm), the optimum oxygen concentration was nearly the same (4% vs. 3.5%). Apparently the gentle, intermittent stirring used in the suspension cultures was enough to distribute the oxygen throughout the media.

The number of Sf1Ep cells used in T. pallidum suspension cultures was very important. Too few cells results in decreased growth of treponemes. Suspension cultures required more cells per volume of media than static cultures ($1x10^5$ vs. $4x10^4$ cells/ml). This may be due to the inaccessability of a portion of the total cell surface area while the beads were crowded together at the bottom of the flask. This problem should be minimized by the intermittent stirring of the beads. Increasing the number of cells resulted in a decrease in the maximum growth of T. pallidum. This was probably due to the more rapid depletion of the nutrients and the increased rate of acidification of the media as the result of the higher cell density. Optimum cell density was therefore a compromise between available surface area for

treponemal attachment and nutritional requirements of the treponemes and tissue culture cells.

Scanning electron micrographs show T. pallidum attached to Sf1Ep cells on the surface of polystyrene beads. The microcolonies of treponemes are very similar to those seen in static cultures of T. pallidum (8). This similarity indicates that the relationship between the treponemes and the cells is the same in static and suspension cultures.

Attempts were made to increase treponemal yield by refeeding the cultures. Adding 10 ml of fresh TPCM on day 7 to the static treponemal cultures grown in 150cm^2 tissue culture flasks resulted in a significant increase in maximum number of treponemes (data not shown). However, refeeding the suspension cultures of T. pallidum with either 10 ml of TPCM or 25 mg of glucose did not significantly increase treponemal growth.

The metabolism of the host cells is known to have an effect on T. pallidum. Wong et al (43) demonstrated that cycloheximide, an inhibitor of eucaryotic protein synthesis, inhibits the attachment of T. pallidum to baby rabbit genital organ cells. Cox observed that cycloheximide inhibited the growth of T. pallidum in the in vitro tissue culture system (unpublished observations). Stamm and Bassford (38) demonstrated that cycloheximide does not inhibit the incorporation of radiolabeled amino acids by T. pallidum.

Therefore the site of action of cycloheximide has to be the tissue culture cell protein synthesis. Because host cell metabolism is so important, any alteration of the media that effects the cells may also effect the treponemes. Production lots of fetal bovine serum were screened for the ability to support the in vitro growth of T. pallidum. These experiments demonstrated that the FBS lots with higher levels of some hormones supported increased multiplication of the treponemes. Changes in the composition of the media such as the addition of hormones and serum supplements are being worked out initially in the static cultures because they are easier to establish and use less material. Once these conditions are optimized in the static cultures, they will be utilized in the suspension cultures. These refinements may allow for suspension cultures to be maintained for longer periods of time with higher yields of treponemes.

With a maximum 51 fold increase (1.02×10^9) in the growth of T. pallidum, suspension cultures approach the yield seen in static cultures. For large-scale cultivation, it would be desirable to increase the volume of the cultures ten times or more while maintaining the same fold increase. Large-scale cultivation of T. pallidum would provide enough organisms to make practical many diverse studies of the biochemical and immunologic nature of T. pallidum. This would include characterization of the antigenic determinants of T. pallidum

as well as purification and characterization of enzymes and other proteins important to the physiology of this organism.

The ability to cultivate T. pallidum in microtiter plates greatly simplifies the use of the in vitro cultivation system. The use of microtiter plates also means that many more groups can be set up per experiment. The limitations imposed by the requirment for TEx is greatly reduced with microtiter plates. Approximately 550 wells can be set up with the TEx required for 45 T-25s. Microtiter plate cultures also require fewer tissue culture cells and less FBS than T-25s. Microtiter plate cultures can be used to examine such variables as the effects of potential growth factors on T. pallidum. Microtiter plates would also be an excellent method for determining antibiotic sensitivity.

It is not surprising that increasing the volume of media in the wells resulted in increased growth of the treponemes. However, a further increase in media volume is not possible since the wells can only hold 1.5 ml without the danger of overflow. The significant difference in the treponemal fold increase resulting from different cell density again points out the importance of the tissue culture cells for the treponemes.

It is interesting that cell lines other than Sf1Ep cells can support the in vitro growth of T. pallidum. Especially intriguing is the fact that a murine cell line can support in

vitro replication of T. pallidum even though this treponeme cannot grow in mice. It must be emphasized that the media used for in vitro cultivation of T. pallidum was optimized for use with Sf1Ep cells and changes in the media may lead to increased replication with other cell lines.

The glucose level in the media appears to be important for the replication of T. pallidum. The glucose level at the start of the cultures was determined by assay to be 199.0±5.4mg/dl. When the number of motile treponemes peaked in all the cell cultures, the glucose level was 68±12 mg/dl. The number of motile treponemes began to decrease as the glucose level continued to drop. After reaching its maximum, the number of motile treponemes did not decrease when cultivated with Sf1Ep cells, and the glucose level in these cultures did not drop below 97.0±31 mg/dl. It is to be expected that glucose would be very important for T. pallidum since glucose is a major source of both carbon and energy for this organism (1,2,3,24,36). This could also explain why attempts at refeeding suspension cultures with either TPCM or glucose were unsuccessful. The amount of glucose added to the cultures would only increase the concentration by 41 mg/dl. This is well below the levels at which the motility of the microtiter plate cultures began to decrease.

Just adding glucose alone would not be enough to enable the treponemes to multiply indefinitely. Other changes in the

media such as decreasing pH and the depletion of other nutrients also limit treponemal multiplication. The growth of T. pallidum is also influenced by the cells they are attached to. Even while the glucose levels are still high, the rate of treponemal growth is different for each of the four cell lines. The growth rate of T. pallidum was greatest with the RAB-9 cells and slightly lower with the Sf1Ep cells. The treponemes grew at an even slower rate with the NME and RT cells (Figure 10). Since the media is the same for all four cells lines, the only source of variance is the cells and whatever the cells are supplying to the treponemes.

Recent evidence regarding the structure of T. pallidum may explain why the multiplication ceases before the glucose is completely exhausted. Freeze-fracture studies have shown that T. pallidum has 10-15 fold fewer intramembranous particles (IMP's) in it's outer membrane than the nonpathogenic treponemes, Treponema phagedensis and Treponema denticola (31). If T. pallidum lacks outer membrane transport proteins for glucose, then it would have to rely on passive diffusion to obtain glucose. Passive diffusion would require a higher extracellular concentration of glucose than would active transport. Passive diffusion is also much slower than active transport and could explain T. pallidum's slow growth rate. T. pallidum has a generation time of 30-33 hours in vivo (41). T. phagedensis (7-9 hours,ref. 15) and T.

denticola (12-14 hours, ref. 32) by comparison have much shorter generation times than T. pallidum.

Available cell surface area also appears to be important for T. pallidum. The number of motile treponemes reaches a plateau when cultivated with Sf1Ep cells even though the glucose level remains fairly high. The maintainence of motile treponemes with Sf1Ep cells is in marked contrast to the cultures with the other three cell lines in which the number of motile treponemes decreased rapidly after peaking. This difference could be explained by the slow growth rate of the Sf1Ep cells and their inability to attain confluency in these cultures. Sf1Ep cultures initiated with $5x10^4$ cells supported a higher fold increase of T. pallidum than those with $2.5x10^4$ cells because the greater cell number provided more surface for the treponemes to attach. All the other three cell lines utilized were able to become confluent within 8 days of initiating the culture. Thus the surface area was adequate for treponemal attachment so glucose levels became the limiting factor. The lower initial cell number supported higher fold increase with RAB-9, NME, and Testis cells because they consumed less glucose while still supplying enough surface area for the treponemes. Similar results were observed with the optimum cell number in suspension culture. Too few cells result in less treponemal growth.

The observation that the Sf1Ep cells do not become

confluent in cultivation with T. pallidum is contradictory to previous reports (8,27). Our laboratory has observed however, that Sf1Ep cells in culture show a marked decrease in growth rate after 10-15 passages. Previously we had believed that these cells were not as suitable for supporting the in vitro cultivation of T. pallidum as earlier passages. These experiments show that these slower growing cells can be used if they are seeded at greater density than the faster growing earlier passage cells.

Suspension culture provides a very easy method for attempting passage of T. pallidum. Unfortunately the attempts to passage trepoenemes in suspension were not very successful, with the exception of a single experiment. One possible explanation for the single successful passage is the introduction of fresh treponemes with the TEx. This explanation however, is not very likely. The TEx is heat inactivated prior to use and T. pallidum is known to be killed by even less heat exposure than that given to the TEx. The heat inactivated TEx was also examined by dark-field microscopy prior to use in the passage experiments and no motile treponemes were observed. It is also possible that these treponemes were a nonvirulent cultivable species of treponemes. Since no confirmation of virulence was done after this experiment, that possibility cannot be completely ruled out. It should be pointed out however, that the cultivable

treponemes grow to a much greater density than did the treponemes in this passage experiment.

The treponemal passage experiments using RAB-9, NME and RT cells passaged from T-25s were also not very successful. Again the single good passage experiment using RAB-9 cells is difficult to explain. In this case however, the virulence of these treponemes was proven using rabbit intradermal injections of the treponemes after the second passage. These treponemes caused a chancre on the rabbits skin thus confirming their pathogenicity.

While somewhat discouraging, these negative results do not rule out the future possibility of in vitro serial passage of T. pallidum. Further work with either these or other cell lines may lead to successful passage. It must also be remembered that the in vivo environment does not consist of a single isolated cell type but instead is a blend of cell types working together in a complex interaction. Therefore serial passage of T. pallidum may require cocultivation of several cell types to more closely reproduce the in vivo environment that the treponemes require in order to multiply. Suspension cultivation could be a method to easily combine different cell types in one culture vessel. Different cell lines could be inoculated onto beads seperately and then combined in whatever ratio is desired.

CHAPTER 5

LITERATURE CITED

1. **Austin, F. E., and C. D. Cox.** 1986. Lactate oxidation by Treponema pallidum. Curr Microbiol. **13**:123-128.

2. **Barbieri, J. E., and C. D. Cox.** 1979. Pyruvate oxidation by Treponema pallidum. Infect Immun. **25**:157-163.

3. **Barbieri, J. T., F. E. Austin, and C. D. Cox.** 1981. Distribution of glucose incorporated into macromolecular material by Treponema pallidum. Infect Immun. **31**:1071-1077.

4. **Clark, J. M., and M. D. Hirtenstein.** 1981. Optimizing culture conditions for the production of animal cells in microcarrier culture. Annals NY Acad Sci. **369**:33-46

5. **Fieldsteel, A. H., F. A. Becker, and J. G. Stout.** 1977. Prolonged survival of virulent Treponema

pallidum (Nichols Strain) in cell-free and tissue culture systems. Infect Immun. **18**:173-182.

6. **Fieldsteel, A. H., J. G. Stout, and F. A. Becker.** 1979. Comparative behavior of virulent strains of Treponema pallidum and Treponema pertenue in gradient cultures of various mammalian cells. Infect Immun. **24**:337-345.

7. **Fieldsteel, A. H., J. G. Stout, and F. A. Becker.** 1980. Role of serum in survival of Treponema pallidum in tissue culture. In Vitro. **17**:28-32.

8. **Fieldsteel, A. H., D. L. Cox, and R. A. Moeckli.** 1981. Cultivation of virulent Treponema pallidum in tissue culture. Infect Immun. **32**:908-915.

9. **Fieldsteel, A. H., D. L. Cox, and R. A. Moeckli.** 1982. Further studies on replication of virulent Treponema pallidum in tissue cultures of Sf1Ep cells. Infect Immun. **35**:449-455.

10. **Fitzgerald, T. J., J. N. Miller, and J. A. Sykes.** 1975. Treponema pallidum (Nichols Strain) in tissue culture: Cellular attachment,entry and

survival. Infect Immun **11**:1133-1140.

11. **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.

12. **Fitzgerald, T. J., R. C. Johnson, J. N. Miller, and J. A. Sykes.** 1977. Characterization of the attachment of Treponema pallidum (Nichols Strain) to cultured mammalian cells and the potential relationship ofattachment to pathogenicity. Infect Immun. **18**:467-478.

13. **Fitzgerald, T. J., P. Cleveland, R. C. Johnson, J. N. Miller, and J. A. Sykes.** 1977.Scanning electron microscopy of Treponema pallidum (Nichols strain) attached to cultured mammalian cells. J Bacteriol. **130**:1333-1344.

14. **Fitzgerald, T. J., L. A. Repesh, and S. G. Oakes.** 1982. Morphological destruction of cultured cells by the attachment of Treponema pallidum.Br J

Vener Dis. **58**:1-11.

15. **Gelperin, A.** 1949. Morphology, cultural characteristics, and a method for mass cultivation of the reiter spirochete. Am J Syph. **33**:101-113.

16. **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.

17. **Hayes, N. S., K. E. Muse, A. M. Collier, and J. B. Baseman.** 1977. Parasitism by virulent Treponema pallidum of host cell surfaces. Infect Immun. **17**:174-186.

18. **Hayle, A. J.** 1986. Culture of respiratory syncytial virus infected diploid bovine nasal mucosa cells on cytodex-3 microcarriers. Arch Virol. **89**:81-88.

19. **Hespell, R. B., and E. Canale-Parola.** 1971. Amino acid and glucose fermentation by Treponema denticola. Arch. Microbiol. **78**:234-251.

20. **Katayama, H., S. Itama, H. Koizumi, and M.**

Tsutsui. 1987. Epidermal cell culture using sephadex beads coated with denatured collagen (Cytodex-3). J Inv Dermatol. **88**:33-36.

21. **Levine, D. W., D. I. C. Wang, and W. G. Thilly.** 1979. Optimization of growth surface parameters in microcarrier cell culture. Biotech & Bioeng. **21**:821-845.

22. **Mered, B., P. Albrecht, and H. E. Hopps.** 1980. Cell growth optimization in microcarrier culture. In Vitro. **101**:859-865.

23. **Mered, B., P. Albrecht, H. E. Hopps, J. C. Petricciani, and J. Salk.** 1981. Propagation of poliovirus in microcarrier cultures of three monkey kidney cell lines. J Biol Stand. **9**:137-145.

24. **Nichols, J. C., and J. B. Baseman.**1975. Carbon sources utilized by virulent Treponema pallidum. Infect Immun. **12**:1044-1050.

25. **Norris, S. J., J. N. Miller, J. A. Sykes, and T. J. Fitzgerald.** 1978. Influence of oxygen tension, sulfhydral compounds, and serum on the

motility and virulence of Treponema pallidum (Nichols strain) in a cell-free system.Infect Immun. **22**:689-697.

26. **Norris, S. J.** 1982. In vitro cultivation of Treponema pallidum : independent confirmation. Infect Immun. **36**:437-439.

27. **Norris, S. J., and D. G. Edmondson.** 1986. Factors affecting the multiplication and subculture of Treponema pallidum subsp. pallidum in a tissue culture system. Infect Immun. **53**:534-539.

28. **Norris, S.J., and D. G. Edmondson.** 1986. Serum requirement for the multiplication of Treponema pallidum in a tissue-culture system:association of growth-promoting activity with the protein fraction. Sex. Trans. Dis. **13**:207-213.

29. **Ovcinnikov, N. M., and V. V. Delektorskij.** 1975. Treponema pallidum in nerve fibers. Br J Vener Dis. **51**:10-18.

30. **Quist, E. E., L. A. Repesh, R. Zeleznikar, and T. J. Fitzgerald.**1983. Interaction of Treponema

pallidum with isolated rabbit capillary tissues. Br J Vener Dis. **59**:11-20.

31. **Radolf, J. D., M. V. Norgard, and W. W. Schulz.** 1989. Outer membrane ultrastructure explains the limited antigenicity of virulent Treponema pallidum. Proc Nat Acad Sci. (in press).

32. **Rose, N. R., and H. E. Morton.** 1952. Morphological variation of treponema. Am J Syph. **36**:17-37.

33. **Sandok, P. L., H. M. Jenkin, S. R. Graves, and S. T. Knight.** 1976. Retention of motility of Treponema pallidum (Nichols Virulent Strain) in an anaerobic cell culture system and in a cell-free system. J Clin Micro. **3**:72-74.

34. **Sandok, P. L., S. T. Knight, and H. M. Jenkin.** 1976. Examination of various cell culture techniques for coincubation of virulent Treponema pallidum (Nichols I Strain) under anaerobic conditions. J Clin Micro. **4**:360-371.

35. **Sanford, K. K., W. R. Earle, V. J. Evans, H. K.**

Waltz, and J. E. Shannon. 1951. The measurement of proliferation in tissue culture by enumeration of cell nuclei. J Nat Cancer Inst. **11**:773-775.

36. **Schiller, N. L., and C. D. Cox.** 1977. Catabolism of glucose and fatty acids by virulent Treponema pallidum. Infect Immun. **16**:60-68.

37. **Sinskey, A. J., R. J. Fleischaker, M. A. Tyo, D. J. Giard, and D. I. C. Wang.** 1981. Production of cell-derived products: virus and interferon. Annals NY Acad Sci. **369**:47-59.

38. **Stamm, L. V., and P. J. Bassford.** 1985. Cellular and extracellular protein antigens of Treponema pallidum synthesized during in vitro incubation of freshly extracted organisms. Infect Immun. **47**:799-807.

39. **Steiner, B., G. H. W. Wong, L. Drummond, and S. Graves.** 1983. The role of respiratory protection on increased survival of Treponema pallidum (Nichols) when cocultivated with mammalian cells in vitro. Can J Microbiol. **29**:1595-1600.

40. **Steiner, B. M., S. Sell, and R. F. Schell.** 1987.

Treponema pallidum attachment to surface and matrix proteins of cultured rabbit epithelial cells. J Infect Dis. **155**:742-748.

41. **Turner, T. B., and D. H. Hollander.** 1957. Biology of the treponematoses. WHO Monogr Ser no. 35.

42. **Wong, G. H. W., B. Steiner, S. Faine, and S. Graves.** 1983. Factors affecting the attachment of Treponema pallidum to mammalian cells in vitro. Br J Vener Dis. **59**:21-29.

43. **Wong, G. H. W., B. Steiner, S. Faine, and S. Graves.** 1983. Effect of serum concentration and metabolic inhibitors on the attachment of Treponema pallidum to rabbit cells. J Med Microbiol. **16**:281-293.

44. **Wong, G. H. W., B. Steiner, and S. Graves.** 1983. Effect of rabbit sera taken at different periods after infection on treponemal motility, treponemal attachment to mammalian cells in vitro, and treponemal infection in rabbits. Br J Vener Dis. **59**:220-224.

45. **Wong, G. H. W., B. Steiner, and S. Graves.** 1986. Effect of four serum componentson survival of

Treponema pallidum and its attachment to rabbit cells in vitro. Genitourin Med. **62**:1-3.