Treponema pallidum (Nichols Strain) in Tissue Cultures: Cellular Attachment, Entry, and Survival

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The interaction of Treponema pallidum (Nichols strain) with cultured cells was investigated under aerobic conditions. Cell monolayers derived from rabbit testicular tissue extended the survival of treponemes as indicated by active motility. Large numbers of organisms rapidly attached to cultured cells. Within 3 h, one to twelve actively motile treponemes were attached to 25 to 50% of the cells. In addition, T. pallidum attained intracellularity as early as 30 min after inoculation of the cell monolayers. In sharp contrast, T. phagedenis biotype Reiter and T. denticola did not attach and did not enter cultured cells. Most importantly, intracellular and/or attached T. pallidum retained virulence for at least 24 h. Similar observations of attachment and retention of virulence were detected with ME-180, a cell line derived from a human cervical carcinoma. Preliminary studies with superoxide dismutase indicated that this enzyme prolonged treponemal motility and retention of virulence in the presence of cultured cells. These data provide guidelines for further investigations of in vitro cultivation of T. pallidum.

Previous attempts to culture Treponema pallidum in vitro have been uniformly unsuccessful. The few reported successes have been either nonreproducible or have involved related nonpathogenic treponemes. Further, elaboration of factors or parameters that prolong in vitro survival has not led to subsequent cultivation.

Reports based on maintenance of motility have indicated that viable tissue significantly prolonged the survival of T. pallidum in vitro. Steinhardt observed motile treponemes for 25 days in a hanging drop preparation of rabbit testicular tissue (13). Similar extensions of survival were reported by Shaffer, using normal rabbit tissue, and by Gammel and Ecker, using syphilitic rabbit tissue (3, 11).

In addition to the use of tissue fragments, in vitro growth of T. pallidum has been attempted in the presence of cultured tissue cells. Perry maintained the motility of T. pallidum for 10 days by the addition of testicular epithelium from new-born rabbits in a microaerophilic environment (10). Bessemans and de Geest (2) were unable to grow T. pallidum in the presence of cultures of fibroblastic testicular cells; Kast and Kolmer (5) were unsuccessful using primary cultures of syphilitic and normal testes.

Generally, attempts to culture the organism

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sions (16). The presence of *T. pallidum* within such diverse tissue cells suggests that intracellularity might be of importance in growth and multiplication.

The purpose of this study was to determine whether the in vitro cultivation of *T. pallidum* could be accomplished by using cultured cells derived from rabbit testes under aerobic conditions. Attempts were made to relate survival of the organisms to intracellular location; survival of organisms was based on motility, as determined by dark-field microscopy, and virulence, as determined by the development of pathogenic lesions in the rabbit.

**MATERIALS AND METHODS**

**Rabbits.** Adult male New Zealand white rabbits with nonreactive venereal disease research laboratory tests were used in all experiments. The animals were maintained at 18 to 20 °C.

**Source of treponemes.** Rabbits were inoculated intratesticularly with the Nichols strain of *T. pallidum.* Organisms were harvested after the development of a satisfactory orchiitis, usually within 9 to 11 days. Testes were removed aseptically, placed in a suitable medium, and sliced. After extraction for 5 to 30 min, the suspension was centrifuged at 1,000 × g for 7 min to sediment gross tissue debris. The supernatant containing the organisms was removed and the number of treponemes was determined according to the method described by Miller et al. (8).

*Treponema phagedenis* biotype Reiter and *Treponema denticola* were grown and maintained as previously described (15).

**Reagents.** All chemicals used in this study were reagent grade. Superoxide dismutase derived from bovine erythrocytes was obtained through the courtesy of Miles Laboratories (Research Division) and from Truett Laboratories.

**Source and maintenance of cultured cells.** Normal rabbit testes were removed aseptically, placed in 0.14 M saline, equilibrated with 95% nitrogen-5% carbon dioxide, and transported within 30 min to the Research Department of the Southern California Cancer Center. The testes were rinsed in 0.14 M saline, cut and minced into small fragments, and then placed in 0.25% trypsin-0.01% ethylenediaminetetraacetic acid in phosphate-buffered saline at pH 7.8. The tissue fragments were continuously agitated on a magnetic stirrer for 25 min at 22 °C. The resulting cell suspension was centrifuged at 1,000 × g for 10 min to sediment the cells; the supernatant was discarded. The cells were resuspended in a basal tissue culture medium (BM) with heat-inactivated fetal calf serum (FCS) at a final concentration of 20%. The BM consisted of Eagle minimal essential medium with Hanks balanced salt solution supplemented with 2× amino acids and vitamins with 20 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid as a buffering agent. The cells were grown at 35 °C and the medium was changed as necessary. Primary cultures which had attained confluency were used to establish secondary cultures. An epithelial cell line (ME-180) derived from a human cervical carcinoma (18) was also tested under the same conditions as the cells derived from rabbit testes.

**Cell harvesting.** After inoculation of treponemes and the appropriate incubation of the monolayers at 35 °C, the culture medium was discarded. The monolayers were washed three times with 8 ml of BM. After the third washing, 3 ml of BM containing FCS or normal rabbit serum (NRS) was added to the monolayers and the cells were removed from the glass by gentle scraping with a rubber policeman. To break up cell clumps, the suspensions were rapidly pipetted five times with a 5-ml pipette. Cells harvested in this fashion retained their viability as measured by vital staining with trypan blue.

**Treponemal virulence and concentration.** To determine the number of virulent treponemes within or attached to the cultured cells, control suspensions of *T. pallidum* containing 10^3, 10^4, and 10^5 organisms per ml were prepared in BM with NRS and inoculated intradermally into the shaved backs of the same rabbits. Animals were observed daily for the initial appearance of characteristic lesions. The graph shown in Fig. 1 relates the number of treponemes injected to

![Fig. 1. Time of appearance of erythema and induration as a function of the number of inoculated *T. pallidum* (Nichols). Inocula were made intradermally in a volume of 0.1 ml into the shaved backs of white New Zealand rabbits housed at 20 °C. These data represent mean values for five separate experiments.](http://iai.asm.org/)
the day of appearance of pathognomonic erythema and induration.

**Preparation of cells for electron microscopy.** The cells were fixed in 3.6% glutaraldehyde (cacodylate buffered) either as suspended cells or in situ as a cell monolayer. After exposure to glutaraldehyde for 15 min, the suspended cells were pelleted by centrifugation, the supernatant was discarded, and fresh 3.6% glutaraldehyde was added for 1 h. Cells fixed in situ were exposed to 3.6% glutaraldehyde for 15 min. The cells were removed from the culture flasks by scraping with a rubber policeman; fixation was allowed to continue for 4 h. In both cases, the cells were then pelleted, fixed with 1% osmic acid, and processed as described for syphilitic rabbit testicular tissue (14).

**RESULTS**

The initial studies were designed to determine the influence of testicular tissue components on the motility of *T. pallidum* in a normal atmosphere. Infected rabbit testes were extracted in heat-inactivated NRS either undiluted or diluted 50% (vol/vol) with 0.14 M saline. After extraction, the tissue suspension was centrifuged at 1,000 × g for 7 min to sediment blood cells, small fragments of tissue, and spermatozoa. After centrifugation, half of the supernatant was removed and placed in a sterile tube, and the remaining portion was mixed with the pelleted tissue components. In this way, the identical preparations of *T. pallidum* were suspended in medium with and without tissue components.

The presence of tissue constituents extended the duration of motility of *T. pallidum* (Fig. 2). The time required for a 50% reduction in the motility of organisms (t50) in the presence of tissue constituents was 22 h as opposed to 4 h for organisms suspended in the cell-free medium. Loss of all detectable motility (t95) was observed after 30 h in the presence of tissue components, and after 5 h in the absence of tissue components. Other viable cell systems also prolonged treponemal survival. Motility was extended by the addition of leucocytes, erythrocytes, large (2 cm³) fragments of testes, and by contaminating bacteria.

In similar experiments in which an atmosphere of 95% nitrogen-5% carbon dioxide was used to provide an anaerobic milieu, no enhancement of treponemal motility in the presence of tissue constituents was observed.

Monolayers of cells derived from normal rabbit testes, in T-30 flasks, were inoculated with 3 ml of *T. pallidum* suspended in BM containing 10 to 50% heat-inactivated NRS. At the same time, flasks not containing cultured cells were inoculated with treponemes. The data from six experiments indicate that the presence of cell monolayers extended the persistence of treponemal motility (Fig. 3). The t50 for organisms incubated with cultured cells was 5.0 h in contrast to a t50 of 3.0 h for treponemes incubated in the absence of cells; the t95 was 10.4 h with cells and 5.5 h without cells.

To determine whether products released by the cultured cells were responsible for extending motility, medium was obtained from uninoculated cell cultures following contact with the cells for 0, 1, 5, 10, and 24 h. After centrifugation at 2,000 × g for 10 min to remove any detached tissue cells, all samples were adjusted to pH 7.2, (with 0.1 N NaOH) and inoculated with *T. pallidum*. No differences in motility were detected, indicating that the cells were responsible for the observed enhancement of motility, rather than released cellular products.

In observing treponemal motility in the presence of cultured cells, a distinct drop in the number of organisms was detected. In a series of six experiments, utilizing an inoculum containing an average of 6.4 × 10³ treponemes per ml, an immediate drop in the number of organisms was observed (Fig. 4). Within 10 min the count decreased 31% to 4.4 × 10³ treponemes per ml. After this rapid initial decline, a more gradual
decreased occurred. Six hours after inoculation, the count had dropped to $2.7 \times 10^7$ treponemes per ml, representing a decrease of 58%. In identical flasks not containing cell monolayers, no decrease in numbers was recorded during the same time period, suggesting that the drop in count was not due to lysis of the organisms, but rather to an attachment and/or entry of the treponemes into the cultured cells.

To explore this possibility, ME-180 cell cultures in Sykes-Moore chambers (17) were inoculated with *T. pallidum* and observed by phase contrast microscopy. Three hours after inoculation, actively motile treponemes (two to six per cell) were attached to 70% of the cells.

Similar observations were made with cultured cells derived from normal rabbit testes. Three hours after inoculation, actively motile treponemes (one to twelve per cell) were attached to the cells. A wide range of cell involvement was detected. The numbers of cells with attached organisms varied from 25 to 50%. This variation was not as pronounced with ME-180 cells. This may be a reflection of the heterogeneity of primary cultures relative to the homogeneity of cells of a cell line. With both types of cells, it appeared as if some of the treponemes were intracellular. This apparent observation is limited by the technical difficulties of documenting intracellularity by phase contrast microscopy.

To evaluate the specificity of this treponemal-cellular interaction, chambers containing cultured cells derived from normal rabbit testes were inoculated with approximate $10^7$ per ml suspensions of *T. pallidum*, heat-killed *T. pallidum*, *T. denticola*, and *T. phagedenis* biotype Reiter. In contrast to the attachment of virulent treponemes, heat-killed *T. pallidum* and the two nonpathogens did not attach to the cells. These findings suggest that attachment is neither a phagocytic nor a pinocytotic function of the cultured cells, but rather an active treponemal process associated with the pathogenic quality of organisms.

Viability of cultured cells, as assessed by vital staining with trypan blue, was unaffected by the presence of virulent *T. pallidum*. After 24 h, 85 to 90% of cells exposed to treponemes were viable. Identical percentages of viable cells were detected in cultures not exposed to treponemes.

Sykes and Miller (14) and Sykes et al. (16) reported the intracellular location of *T. pallidum* in tissue from infected rabbit testes and in tissue from human primary syphilitic lesions. To examine the possibility of intracellularity in tissue cultures, monolayers of cells derived from rabbit testes were inoculated with *T. pallidum* in BM with FCS or NRS containing 6 x $10^7$ organisms. After various periods of incubation, cells, after washing with BM to

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**FIG. 3.** Decrease in motility of *T. pallidum* (Nichols) with time. Organisms suspended in BM containing 10 to 50% heat inactivated normal rabbit serum were inoculated into T-30 flasks of cell cultures derived from rabbit testes. Symbols: (---) with cultured cells; (-----) without cultured cells. These data represent mean values for six separate experiments.

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**FIG. 4.** Numbers of *T. pallidum* (Nichols) observed by dark-field microscopy (x600) in the presence and absence of cultured cells. T-30 flasks were inoculated with an average of 6.4 x $10^7$ organisms. Symbols: (---) with cultured cells; (-----) without cultured cells. These data represent mean values for six separate experiments.
remove unattached organisms, were fixed for electron microscopy. Intracellular treponemes were observed 30 min after inoculation (Fig. 5). This finding of rapid entry correlates with the data represented in Fig. 4, in which a rapid decrease in treponemal numbers (31%) was demonstrable immediately after inoculation of cultured cells. Intracellular, morphologically intact organisms were observed at 1, 2, 4, 6, 11, and 18 h. Additionally, intact organisms were demonstrated both intra- and extracellularly 4 (Fig. 6) and 7 days after inoculation.

In related experiments using T. denticola and T. phagedenis biotype Reiter, no intracellular organisms were detected.

An important consideration in these studies was the assessment of virulence of intracellular or attached organisms. At various times after inoculation of monolayers, the cells were washed and 0.1 ml of washed cell suspension was inoculated intradermally into the shaved backs of rabbits.

Table 1 summarizes the data obtained from 10 experiments of this type. The estimated number of virulent treponemes based on the time of appearance of erythema and induration (Fig. 1) is presented. These studies demonstrate unequivocally that the intracellular and/or attached treponemes retained their infectivity. Cell monolayers that had been inoculated with $2 \times 10^7$ to $3 \times 10^7$ organisms per ml contained $10^8$ organisms per ml within 30 min. These findings correlate with data of Fig. 4 which indicated a rapid decline in numbers of extracellular treponemes after inoculation of cultured cells. After incubation for 24 h, $10^4$ treponemes per ml were detected.

In parallel experiments, no virulent organisms were detected in the absence of cultured cells after 9 h of incubation.

Nonspecific reactions to uninfected cultured cells derived from rabbit testes were not detected.

Related studies using cultures of the human cell line ME-180 revealed a similar interaction of treponemes with cultured cells. Cell attachment and/or penetration of $3 \times 10^4$ virulent T. pallidum per 0.1 ml of inoculum was demonstrable 16 h after cell-treponeme interaction. As expected, nonspecific side reactions did occur with ME-180 cells; control cell suspensions without T. pallidum produced areas of erythema 5 to 9 mm in diameter with slight induration between days 6 and 15. After day 15, these reactions regressed.

Preliminary experiments were undertaken to determine the effect of superoxide dismutase on the cell-treponeme interaction. Six thousand units of the enzyme in BM with NRS were added to cell monolayers. Identical cultures received BM with NRS without the enzyme. These cell monolayers were then inoculated with $5 \times 10^4$ treponemes and incubated for 24 h at 35 C. This inoculum is lower than that used in previous experiments listed in Table 1. When washed cell suspensions from the enzyme-containing cultures were inoculated intradermally, treponemal skin lesions became apparent in 21 days. In contrast, no skin lesions were produced after inoculation of cells not exposed to the enzyme. Besides extending retention of virulence of T. pallidum, superoxide dismutase also prolonged active motility of the organisms.

**DISCUSSION**

The use of aerobiosis, although representing a departure from previous efforts to culture T. pallidum, was considered appropriate since it more closely approximates environmental conditions during growth and multiplication of the organisms in vivo. Justification for this approach appeared to be confirmed by the finding that large numbers of treponemes attached to, and entered, cultured cells; these organisms retained their virulence for at least 24 h.

The reports of in vivo intracellularity of T. pallidum by Sykes and Miller (14) and by Sykes et al. (16) support further research utilizing cell culture systems. The presence of organisms within such a diversity of cell types suggests that an intracellular location was more than chance occurrence, and might play an integral role in the pathogenesis of both human and experimental syphilis. T. pallidum may enter cells to escape from an unfavorable environment; or perhaps some stage of treponemal growth may require an intracellular residence. Whatever the reason(s), cell cultures should provide a system that will permit correlation between in vitro studies and observed in vivo phenomenon.

In contrast to T. pallidum, T. denticola and T. phagedenis biotype Reiter, when inoculated intratesticularly into rabbits, were not seen within the cells of the intact organ; furthermore, no evidence of an intracellular location was detected within cell cultures derived from rabbit testes. This major difference between these two nonpathogenic treponemes and T. pallidum focused attention on the possible importance of intracellularity in the pathogenesis of T. pallidum infection.

The failure of killed T. pallidum to attach to cultured cells indicated that attachment was an active treponemal function unassociated with phagocytosis or pinocytosis. T. pallidum appeared to be specifically attracted to cultured cells. Within 3 h, numerous attached trepo-
Fig. 5. Electron micrograph of part of a cell fixed 30 min after infection with T. pallidum (Nichols). The cells were from an aerobic secondary culture derived from rabbit testes. Part of a treponeme (T) lying close to the cell nucleus (N) has been cut across a bend. Three axial filaments link the two oblique cross sections. The cytoplasm of the cell contains mitochondria (M) with some degeneration of cristae. There is some swelling of the endoplasmic reticulum and vacuolation of the golgi complex. The bar in both electron micrographs represents 1 μm. Magnification ×30,000.

Fig. 6. Electron micrograph of parts of two cells from a culture fixed 4 days after infection with T. pallidum (Nichols). The cells were from a secondary culture of rabbit testes that had been maintained under an atmosphere of N₂ 95%–CO₂ 5%. The nucleus (N) indicates a maturing cell and the cytoplasm of the lower cell shows some minor swelling of the rough endoplasmic reticulum. Parts of a morphologically intact treponeme (T) are lying between the cells. Magnification ×30,000.
TABLE 1. Virulence of T. pallidum (Nichols) in the presence of cultured cells of normal rabbit testes

<table>
<thead>
<tr>
<th>Time (h) in culture</th>
<th>No. of rabbits inoculated</th>
<th>Development of intradermal lesions</th>
<th>Day of EI* appearance</th>
<th>Estimated no. of virulent treponemes in 0.1 ml of inoculum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>2</td>
<td>4/4</td>
<td>5.0</td>
<td>5</td>
</tr>
<tr>
<td>1.0</td>
<td>4</td>
<td>8/8</td>
<td>6.2</td>
<td>5-7</td>
</tr>
<tr>
<td>3.0</td>
<td>2</td>
<td>2/2</td>
<td>7.0</td>
<td>7</td>
</tr>
<tr>
<td>6.0</td>
<td>8</td>
<td>12/13</td>
<td>8.0</td>
<td>5-11</td>
</tr>
<tr>
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<td>4</td>
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<td>2</td>
<td>4/4</td>
<td>8.0</td>
<td>7-9</td>
</tr>
<tr>
<td>14.0</td>
<td>2</td>
<td>4/4</td>
<td>10.0</td>
<td>10</td>
</tr>
<tr>
<td>24.0</td>
<td>2</td>
<td>6/6</td>
<td>12.0</td>
<td>7-21</td>
</tr>
</tbody>
</table>

* Erythema and induration.
* See Fig. 1. Cultures were inoculated with 2 x 10^7 to 3 x 10^7 treponemes per ml.

Treponemes were demonstrable by phase contrast microscopy at the surface of 70% of ME-180 cells and 25 to 50% of rabbit testicular cells. This multiple cell involvement was also evident in the virulence studies. Cell monolayers derived from rabbit testes, in T-30 flasks, contained approximately 3 x 10^4 cells. Thirty minutes after incubation, 3 x 10^7 treponemes were attached to the washed cells. Furthermore, this treponemal interaction occurred without significant killing of cultured cells.

Of considerable interest was the susceptibility of ME-180, a cell line derived from a human tumor, to infection with T. pallidum. A human cell line could be used to prepare clean preparations of treponemes that would facilitate immunological studies directed toward antigenic structure, immune response mechanisms, and vaccine efficacy. After attachment of treponemes and subsequent washing, the organisms could be forced to detach in a controlled fashion, yielding a relatively clean preparation free of contaminating rabbit constituents.

In some of the tissue culture studies, it appeared that a "shedding" of attached or intracellular T. pallidum, with subsequent reattachment or reentry, was occurring. After replacement of the culture medium with fresh medium, and further incubation, there was a significant decrease in virulent treponemes. The fluctuating extracellular treponemal count in the presence of cell monolayers also suggested a "shedding" phenomenon. The initial 6-h incubation revealed a gradual decline in the number of extracellular organisms (Fig. 4). Thereafter, there was a slight but reproducible fluctuation in numbers up to 24 h.

The BM used was relatively toxic in that T. pallidum did not survive beyond 5 h in the absence of cultured cells (Fig. 3). In view of this "shedding" of treponemes, this toxicity assumes major importance. Oxygen, which is detrimental to the organisms in vitro, may be the primary toxic factor in this cell culture system. The extension of treponemal survival in the presence of viable cells indicated that these cells provided a degree of detoxification. Inasmuch as oxygen at high concentrations is toxic to all cells, the cells must possess an effective mechanism(s) for neutralization of atmospheric oxygen toxicity.

Superoxide dismutase may be one of the major mechanisms for neutralization of this toxicity. This enzyme prevents the accumulation of highly destructive superoxide anions. The presence of superoxide dismutase within erythrocytes, brain, lung, liver, heart, and testes tissue, and within yeasts and bacteria, demonstrates its ubiquitous occurrence in nature. Recently, McCord et al. (7) proposed an enzyme-based theory of anaerobiosis related to the presence of superoxide dismutase within microorganisms. They found that aerobes contained large amounts of the enzyme, strict anaerobes contained none, and microaerophilic organisms contained low levels of the enzyme.

They proposed that superoxide dismutase was the primary factor that enabled organisms to survive in the presence of oxygen. In an extension of this work, Gregory et al. (4) successfully protected Escherichia coli from the lethality of superoxide anions by addition of superoxide dismutase.

This theory, which postulates the necessity of superoxide dismutase for survival of T. pallidum in an aerobic environment, raises some interesting possibilities. Within infected testes, superoxide dismutase could conceivably prevent oxygen toxicity and allow treponemes to grow and multiply in a favorable environment. Removal of T. pallidum from infected tissue might result in an accumulation of lethal superoxide anions. Addition of a viable cell system, such as testicular tissue, could provide a transient source of superoxide dismutase that would prolong survival of the treponemes until death of the tissues and loss of enzyme activity.

Preliminary studies using superoxide dismutase have been encouraging; they have shown that addition of the enzyme prolonged the motility and virulence of T. pallidum within this culture medium in the presence of cultured cells. These cells probably contain an endogenous source of superoxide dismutase, but the preliminary data have indicated that this level was inadequate to maintain extracellular treponemal survival. An exogenous source of super-

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oxide dismutase appears, therefore, to be essential.

In view of the possible "shedding" of organisms, any reduction in culture medium toxicity should enhance the possibility of successful in vitro cultivation of *T. pallidum*. If the toxicity shown in these studies was primarily due to oxygen, the advantages of adding superoxide dismutase are readily apparent.

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