Capacity of Virulent Treponema pallidum (Nichols) for Deoxyribonucleic Acid Synthesis

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Received for publication 15 November 1978

Treponema pallidum (Nichols) was extracted from infected rabbit tissue, and cell lysates were prepared for monitoring thymidine kinase and deoxyribonucleic acid polymerase activities. No thymidine kinase could be demonstrated in preparations of T. pallidum or the cultivable T. phagedenis biotype Reiter. Significant levels of deoxyribonucleic acid polymerase were detected in both treponemal samples. Interestingly, comparisons of polymerase activity among a spectrum of bacterial genera revealed a direct correlation between enzyme concentrations and estimated generation time. Incorporation of [3H]uridine and [3H]thymidine into macromolecules by intact T. pallidum and the Reiter treponeme was examined. Selective ribonuclease-deoxyribonuclease digestion and cesium chloride gradient banding demonstrated that T. pallidum, independent of the host, and T. phagedenis were capable of synthesizing deoxyribonucleic acid only from the [3H]-uridine precursor.

The inability to grow Treponema pallidum in culture remains a major experimental limitation. While we and others were attempting to develop appropriate techniques for T. pallidum cultivation in vitro, we were also paralleling these efforts with studies to clarify anabolic and catabolic activities of virulent treponemes. We have shown that T. pallidum selectively oxidizes specific substrates to CO₂ and identifiable end products (12), that it readily synthesizes a range of proteins (2, 3) and ribonucleic acid macromolecules (13), and that these activities are dependent upon several environmental variables including O₂ (4). Other investigators have described specific metabolic pathways (13) and a functioning electron transport system (10) coupled to oxidative phosphorylation (11). Deoxyribonucleic acid (DNA) synthesis, an important determinant of T. pallidum growth potential, has not been previously detected. The lack of this indicator as direct support for possible increases in treponemal cell numbers or improved culture conditions has been a considerable technical deficiency (1). In this paper, we examine thymidine kinase and DNA polymerase activities in T. pallidum extracts and describe methodology for measuring DNA synthesis in the intact microorganism.

MATERIALS AND METHODS

Bacteria. Virulent T. pallidum (Nichols) was grown intratesticularly in New Zealand white rabbits as previously described (8). Infected animals were treated 3 days postinfection with cortisone acetate (25 mg intramuscularly), and injections were continued daily for approximately 6 to 9 days, at which time maximal orchitis occurred. Rabbits were sacrificed, and testes were removed aseptically, minced, and shaken in an air atmosphere in 10 ml of modified Dulbecco minimal essential medium (MEM) containing 10% fetal calf serum (8). Tissue was reextracted twice more, and the combined extractions containing treponemes were centrifuged twice at 500 × g for 5 min to sediment the majority of tissue debris and contaminating animal cells. The supernatant was further clarified by centrifugation at 650 × g for 20 min over a Methocel-Hyapaque gradient (8). Nontreponemal components banded at the gradient interphase or were dispersed throughout the lower part of the gradient. The supernatant which contained only treponemes and soluble tissue products was used as the source of T. pallidum. Although these preparations were essentially devoid of tissue cells as determined microscopically, animal cell controls (low-speed tissue pellet resuspended in high-speed supernatant as previously described [12]) were established for certain assays. The avirulent treponeme, T. phagedenis biotype Reiter, was grown in Spirolate broth (Baltimore Biological Laboratories [BBL], Cockeysville, Md.) with 10% heat-inactivated normal rabbit serum and incubated anaerobically at 34°C. Leptospira interrogans var. canicola and a water leptoapire designated B5 were kindly provided by C. D. Cox (University of Massachusetts, Amherst). Both were cultured aerobically at 30°C in 0.2% tryptose–phosphate broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% heat-inactivated normal rabbit serum. Escherichia coli serotype O111:B4, Staphylococcus albus, Micrococcus lysodeikticus, Bacillus subtilis, and Salmonella typhi 642 were grown in "Trypticase soy broth (BBL) at 37°C with agitation.

Radioisotopes and chemicals. [5,6-3H]uridine...
Bacterial lysates. Except for T. pallidum, bacterial species were grown in 30-ml volumes of broth medium to mid-log phase as determined by optical density (650 nm) or Petroff-Hauser cell counts. All cultures, including T. pallidum preparations described above, were centrifuged at 12,000 × g for 15 min, washed once in phosphate-buffered saline (pH 7.4), and suspended in 0.75 to 1.0 ml of appropriate cold buffer. For thymidine kinase assays, the buffer (pH 8.0) consisted of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.15 M KCl, 0.0014 M β-mercaptoethanol, and 0.0002 M thymidine. For DNA polymerase measurements, buffer (pH 7.8) contained 0.05 M Tris-hydrochloride, 0.001 M ethylene-diaminetetraacetic acid (EDTA), 0.001 M dithiothreitol, and 5% glycerol. Test samples were iccd and subjected to sonic oscillation at 45 Hz at a setting of 3 (Sonifier cell disruptor model W 140; Heat-Systems Ultrasound Inc., Plainview, N.Y.) one to two times for spirochetes and four to six times for other bacteria, with a 45-second interval between each period of sonic oscillation. Samples were then centrifuged at 150,000 × g for 1 h, and the clear supernatant was assayed for enzymatic activity. For comparative purposes selected bacterial lysates were prepared by passing bacteria through a miniature French pressure cell (Aminoac; 5 ml maximal capacity) at 15,000 to 20,000 lb/in². Protein determinations (Bio-Rad protein assay, Bio-Rad Laboratories, Richmond, Calif.) were performed on bacterial lysates which were then either diluted to a concentration of 80 μg of protein per ml of buffer or, in the case of T. pallidum, used undiluted.

Thymidine kinase assay. Cleared bacterial lysate (10 μl) was added to 100 μl of reaction buffer (pH 8.0) (0.01 M Tris-hydrochloride; 0.025 M KCl; 0.01 M MgCl₂; 0.014 M β-mercaptoethanol; 0.015 M adenosine 5′-triphosphate [ATP]; 0.004 M NaF; 2.5 μCi [methyl-3H]thymidine). The reaction mixture was incubated at 37°C for 5, 15, and 30 min. Samples were collected on diethylaminoethyl (DEAE)-cellulose disks (DE-81, Whatman) previously soaked in 0.001 M ammonium formate buffer (pH 3.8). Each filter was washed with formate buffer and dried, and radioactivity was determined (6).

DNA polymerase assay. A 25-μl amount of bacterial lysate was added to 100 μl of reaction buffer (0.1 M Tris-hydrochloride; 0.025 M MgCl₂; 0.001 M dithiothreitol; 0.25 mM guanosine 5′-triphosphate, cytidine 5′-triphosphate, and ATP; 0.125 mg of bovine serum albumin; and 1 to 3 μCi of [3H]TTP [thymidine triphosphate]) plus 100 μl of DNA template. Calf thymus DNA was prepared by dissolving 0.25 mg of DNA per ml in 0.05 M Tris-hydrochloride containing 0.005 M MgCl₂ and 0.5 mg of bovine serum albumin per ml. After DNase I digestion for 20 min at 37°C and heat inactivation of the enzyme at 80°C for 15 min, the DNA template was either used immediately or stored at −20°C. Incubation of the test system was performed at 37°C for 30 min, and samples were precipitated with cold 10% trichloroacetic acid and collected onto 0.22-μm membrane filters (Millipore Corp., Bedford, Mass.) presoaked in 0.1 M sodium pyrophosphate to minimize background. Filters were washed with cold 5% trichloroacetic acid, dried, and counted.

Determination of DNA synthesis. To measure DNA synthesis in T. pallidum and the Reiter treponeme, spirochetes were exposed to [3H]uridine or [3H]thymidine at a final concentration of 10 μCi/ml of medium. T. pallidum was incubated in modified Dubbecco MEM as previously described (8). Under these conditions, treponemes remained actively motile throughout the 48-h period. The Reiter treponeme was grown in Spirfolate broth supplemented with 10% heat-inactivated normal rabbit serum. Incubation was performed at 34 or 4°C as indicated. Two separate procedures were employed to detect radiolabeled DNA in these cultures.

Procedures: (i) sensitivity of radioactive nucleic acid to enzyme digestion. Cultures of T. pallidum and T. phagedenis biotype Reiter were incubated aerobically and anaerobically, respectively. At the termination of the experiment, treponemes were pelleted, washed in phosphate-buffered saline and suspended in 600 μl of buffer (0.05 M NaCl, 0.01 M EDTA, and 0.03 M Tris-hydrochloride, pH 7.8), which was then divided into three 200-μl portions. To each sample, 10 μl of lysozyme (10 mg/ml stock) was added for 30 min at 37°C, followed by 25 μl of 10% sodium dodecyl sulfate. Samples were shaken gently to lyse the cells and obtain a clear solution (15). One sample was precipitated with cold 10% trichloroacetic acid and collected onto 0.22-μm Millipore filters. This sample represents total [3H]-associated counts. To the second sample, 10 μl of RNase A (5 mg/ml stock, heat-treated for 20 min at 80°C to inactivate contaminating DNase) was added for 60 min at 37°C. This sample was then trichloroacetic acid-precipitated and processed as described for sample 1. In this case, loss of radioactivity represented RNase-sensitive macromolecules, and counts retained were potential radioactive DNA. The third sample was processed identically to the second through the RNAse step. Then cold 95% ethanol was added to twice the volume, and the sample was stored overnight at 4°C. This step allowed the precipitation of remaining macromolecules which were then pelleted at 12,000 × g for 10 min at 4°C to free the material from sodium dodecyl sulfate-containing supernatant which would interfere with DNA activity. Ethanol was decanted, and the pellet was dried and dissolved in 200 μl of phosphate-buffered saline containing 0.002 M calcium chloride and 0.002 M magnesium sulfate (pH 7.8). These manipulations did not result in significant losses of radioactivity when compared to the RNAse-resistant counts in the second sample. Then 10 μl of DNAse I (10 mg/ml stock) was introduced, and incubation was continued for 2 h at 37°C. This sample was then precipitated with cold trichloroacetic acid, collected on filters, dried, and counted. Decreased radioactivity would represent...
DNase-sensitive macromolecules.

(ii) Isolation of radioactive DNA by CaCl gradients. Treponemal cultures were incubated with [H]uridine or [H]thymidine for 48 h. Treponemes were centrifuged and washed as described in the first procedure, except that pellets were suspended in 200 µl of buffer and then transferred to polyallomer centrifuge tubes. Each sample then received 12 µl of 10% sodium dodecyl sulfate and 10 µl of proteinase K (1 mg/ml stock). The latter enzyme addition was necessary to obtain consistent results (13). Samples were then incubated for 15 min at 37°C with periodic agitation. To these samples were added 0.35 ml of ethidium bromide (200 µg/ml stock) and 4.58 g of CaCl in 3 ml of the NaCl-EDTA-Tris buffer (pH 8.5). Gradients were overlaid with light mineral oil to prevent evaporation and centrifuged at 100,000 x g at 20°C for 65 h. The ethidium bromide-positive band in each sample was identified by ultraviolet transillumination and collected via syringe. A 0.1-ml sample was diluted in 15 ml of scintillation fluid (ACS; Amersham Corp., Arlington Heights, Ill.) for radioactive determination.

RESULTS

Absence of thymidine kinase activity in treponemes. We had attempted unsuccessfully to measure the uptake of radioactive thymidine by T. pallidum under a variety of experimental conditions. Because no significant increases in treponemal cell densities could be observed during these studies, the inability to measure accumulation of radioactive thymidine into macromolecules was not surprising. However, our observation that growing cultures of the Reiter treponeme did not incorporate [H]thymidine into macromolecules suggested potential enzyme deficiencies among the treponemes and prompted a search for thymidine kinase activity. E. coli lysates readily phosphorylate thymidine, which results in its adsorption to DEAE-cellulose disks due to increased negative charge (Fig. 1). Enzyme activity was linear during the 30-min incubation. When ATP was excluded from the reaction mixture, activity reached background levels. Bacterial lysates of T. pallidum and T. phagedenis biopsy Reiter exhibited no thymidine kinase activity during this or longer incubations. Increases in the amount of treponemal lysates or [H]thymidine to the treponemal reaction mixture did not increase enzyme activity above background. Background values were based upon the amount of radioactivity associated with DEAE-cellulose disks from test samples lacking ATP or in complete reaction mixtures minus bacterial lysate. Addition of treponemal sample to E. coli test systems did not significantly alter the expected level of kinase activity associated with E. coli preparations.

DNA polymerase in bacterial lysates. Because virulent T. pallidum cannot yet be grown in vitro, these microorganisms may be unable to synthesize DNA independent of the host environment. The absence of thymidine kinase activity in treponemal preparations reinforced the need to establish an alternative assay for measuring T. pallidum competence regarding DNA synthesis. We initially employed a standard DNA polymerase assay containing unlabeled carrier TTP. Under these conditions, significant polymerase activity was observed in E. coli and Reiter preparations but not with T. pallidum (Table 1). However, by omitting unlabeled thymidine triphosphate from the basic reaction mixture, measurable levels of polymerase activity could be observed in T. pallidum (Table 1). Also, under these conditions, polymerase activity was directly related to the amount of bacterial lysate added as well as to the presence of ATP (data not shown). It is apparent from these results that a considerable range in the specific activity (in counts per minute per microgram of lysate protein) of DNA polymerase exists in the three bacterial samples. To further clarify variations in polymerase activity among other bacterial genera and lend support to the credibility of the low values for T. pallidum, other bacteria were screened (Table 2). Except in the case of Bacillus, a direct correlation exists between enzyme activity and the estimated generation time of the microorganisms. Mixing experiments, designed to establish whether lysates with lower polymerase activity might be antagonistic to high activity lysate, resulted in additive effects.

Detection of radioactive DNA in treponemes by selective enzyme digestion. It was
apparent from the previous data that both *T. pallidum* and *T. phagedenis* lacked thymidine kinase but possessed measurable DNA polymerase activity. Since the avirulent Reiter treponeme multiplies in vitro, it seemed likely that uridine would be phosphorylated and converted to deoxythymidylate monophosphate. Thus, incorporation of radioactive uridine could be used as an indicator of DNA synthesis if properly analyzed. More importantly, *T. pallidum* may have similar metabolic capabilities. To substantiate this hypothesis, it was essential to demonstrate the existence of specific radioactive macromolecules by selective enzyme digestion. Our attempts to use differential alkali sensitivity of nucleic acids proved unsatisfactory. Of the total \([^3]H\)uridine-labeled macromolecules synthesized by *T. pallidum*, approximately 46% are RNase sensitive, and the remainder are DNase digestible. For *T. phagedenis*, about 62% of the radioactivity was digested by RNase, and the remaining counts were DNase sensitive. Note that thymidine is not incorporated into macromolecules by treponemes and that contributions by animal cell controls from the tissue extract are negligible.

The kinetics of DNA synthesis in cultures of *T. pallidum* was also monitored. Radioactive uridine was added to treponemal samples for 6, 24, and 48 h, and radioactivity was processed by enzyme digestion as in Table 3. Incorporation of \([^3]H\)uridine into DNA by *T. pallidum* continued for 24 h, after which no further increases were observed (Fig. 2). RNA synthesis also ceased after 24 h under these experimental conditions (13).

**Isolation of radioactive DNA from \([^3]H\)uridine-labeled treponemes.** To obtain further proof that *T. pallidum* was capable of synthesizing DNA during in vitro incubation, treponemal samples were prepared as outlined in Materials and Methods and examined on cesium chloride gradients. Ethidium bromide-positive bands formed in all preparations at densities of 1.70 g/cm\(^2\) for *T. pallidum* and 1.68 g/cm\(^2\) for *T. phagedenis*. However, only in those treponemal cultures previously incubated at 34°C with \([^3]H\)uridine was radiolabeled DNA recovered (Fig. 3). No radioactivity was detected in bands

**Table 1. DNA polymerase activity in lysates of *T. pallidum* and *T. phagedenis***

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>[methyl-(^3)H]TTP/assay:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with TTP carrier*</td>
</tr>
<tr>
<td></td>
<td>2 (\mu)Ci 3 (\mu)Ci 1 (\mu)Ci 3 (\mu)Ci</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.7c 2.7 31.0 NDd</td>
</tr>
<tr>
<td><em>T. phagedenis</em></td>
<td>0.15 0.3 4.7 9.6</td>
</tr>
<tr>
<td><em>T. pallidum</em></td>
<td>0 0.05 0.5 1.1</td>
</tr>
</tbody>
</table>

* Bacterial lysates were prepared by sonic oscillation as described in the text. *E. coli* samples lysed by the French pressure cell gave similar values.

* Concentration of unlabeled exogenous TTP was 0.25 mM.

* Radioactivity represents incorporation of \([^3]H\)TTP into acid-insoluble material and is expressed as counts per minute \((\times 10^{12})\) per microgram of lyase protein. These data are the average of triplicate determinations from three separate experiments. In all cases, no enzyme activity was detected in animal cell controls (see text).

* ND, Not done.

**Table 2. Comparison of polymerase activity among selected bacterial genera***

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Activity (cpm/µg of protein)*</th>
<th>Approx. generation time</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>31,010</td>
<td>30–40 min</td>
</tr>
<tr>
<td><em>M. lysodeikticus</em></td>
<td>27,215</td>
<td>30–40 min</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>25,180</td>
<td>30–40 min</td>
</tr>
<tr>
<td><em>L. biflexa</em> (B16)</td>
<td>5,205</td>
<td>4–h h</td>
</tr>
<tr>
<td><em>T. phagedenis</em></td>
<td>4,719</td>
<td>4–6 h</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>4,650</td>
<td>30–40 min</td>
</tr>
<tr>
<td><em>L. interrogans</em></td>
<td>1,340</td>
<td>10–12 h</td>
</tr>
<tr>
<td><em>T. pallidum</em></td>
<td>504</td>
<td>30 h</td>
</tr>
</tbody>
</table>

* The level of [methyl-\(^3\)H]thymidine triphosphate was 1 µCi per test sample. Values are the average of triplicate samples of sonically treated preparations from three separate experiments. Similar results were obtained with extracts from *E. coli*, *M. lysodeikticus*, and *B. subtilis* prepared by the French pressure cell.

* Estimate based upon in vivo growth in rabbits (16).

**Table 3. RNase and DNase sensitivity of radiolabeled macromolecules in *T. pallidum* and *T. phagedenis***

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>([^3]H)uridine (cpm)</th>
<th>([^3]H)thymidine (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total radioactivity</td>
<td>RNase sensitive</td>
</tr>
<tr>
<td><em>T. pallidum</em></td>
<td>13,270</td>
<td>6,084</td>
</tr>
<tr>
<td><em>T. phagedenis</em></td>
<td>47,400</td>
<td>29,367</td>
</tr>
<tr>
<td>Tissue cell control</td>
<td>120</td>
<td>87</td>
</tr>
</tbody>
</table>

* Results are the average of triplicate samples from three experiments. Radioactive uridine or thymidine of similar specific activities was added to bacterial cultures for 48 h before harvest. Samples were processed by the sequential steps outlined in the text.
from treponemes exposed to \(^{3}H\)uridine at 4°C or \(^{3}H\)thymidine.

**DISCUSSION**

Our purpose in the current investigation was twofold: (i) to demonstrate that virulent treponemes had the biosynthetic capability to synthesize DNA in vitro, and (ii) to establish assays that could be used as in vitro monitors of DNA synthesis and growth. Data on thymidine kinase activity in treponemal lysates indicated that the enzyme was either lacking, inactivated, or at a very low concentration. The absence of thymidine kinase in *Treponema* should not be viewed as novel or biologically restraining. Other groups of microorganisms also lack thymidine kinase, including *Neisseria* (9) and some fungi (7). How this property might have evolved to provide a selective advantage or to influence pathogenicity is unclear.

Detection of measurable amounts of DNA polymerase activity and utilization of \(^{3}H\)uridine for DNA synthesis based upon DNase sensitivity and CsCl banding reinforce the metabolic competence of *T. pallidum*. Although the low level of DNA polymerase activity in *T. pallidum* extracts was initially viewed as restrictive in terms of growth potential, comparisons with other bacterial species indicated that this enzyme level might be consistent with the estimated generation time of *T. pallidum*. It is interesting that such a correlation may exist between growth rate and DNA polymerase activity among procaryotes under the experimental conditions described here.

The DNA assays employed in this study are readily applicable to current and proposed investigations monitoring in vitro growth of *T. pallidum*. Increases in radiolabeled DNA occurred during the first 24 h after removal from infected tissue, possibly reflecting new or prior, in vivo committal to DNA synthesis (Fig. 2). After that period, DNA synthesis apparently ceased in motile treponemes, suggesting inherent metabolic deficiencies and/or suboptimal culture conditions. Certainly, actively growing cultures of *T. pallidum* should synthesize DNA at appropriate intervals before cell division, a criterion which should be used now that DNA assays are available. The unreliability and inadequacy of cell density determinations and other currently employed techniques for monitoring potential increases in *T. pallidum* numbers have been discussed (1).

It appears that *T. pallidum*, once extracted from infected tissues, is anabolically competent and capable of growth. Although the appropriate sequence and combination of biological variables to elicit this response remain elusive, identification of the DNA-synthetic capabilities in *T. pallidum* reinforces research efforts to develop satisfactory in vitro growth conditions.

**ACKNOWLEDGMENTS**

This work was supported by Public Health Service grant AI-18308 and Research Career Development Award 1-K04-AI-00178 from the National Institute of Allergy and Infectious Diseases to J.B.B.
LITERATURE CITED


