

## Isolation and Purification of *Treponema pallidum* from Syphilitic Lesions in Rabbits

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*Treponema pallidum* were extracted from testicular syphilomas of corticosteroid-treated rabbits and purified by differential centrifugation. The steroid therapy allowed a longer holding time for infected rabbits, which produced greater treponeme yields, averaging  $1.58 \times 10^{10}$  treponemes per rabbit. The treatment, which also diminished cellular infiltration and increased the extracellular mucoid material in lesions, produced much cleaner suspensions than preparations from nontreated animals. Most of the treponemes in the purified suspensions were still motile, and none carried demonstrable host immunoglobulin. The preparations were free of recognizable host tissue debris and they contained, on the average,  $1.9 \times 10^{-7}$   $\mu\text{g}$  of protein per treponeme.

A preparation of any microorganism to be used for antigenic, chemical, or metabolic studies should be free of interfering contaminants from the growth environment. To obtain such a "clean" suspension of *Treponema pallidum* presents a problem because these organisms can be propagated only in experimental animals. The limited range of susceptible hosts (only rabbits develop lesions with an abundance of treponemes) and the unusual physical and biological characteristics of treponemes have made it very difficult to recover quantities of these organisms from syphilitic lesions and to separate them from contaminating host material. A number of investigators have made attempts to overcome these difficulties.

Chandler and Clark (2) filtered testicular extracts through membranes of graded porosity, but this failed to separate treponemes from tissue components. Schmale et al. (9) recovered much cleaner preparations by continuous particle curtain electrophoresis. However, this procedure damaged the organisms in that it converted one-third to one-half of the spirochetes in the cleaner fractions to spherical forms (i.e., ballooning of the outer envelope) and diminished the immunofluorescent staining of the rest, suggesting that they had lost some cellular constituent.

Rathlev and Pfau (8) were the first to describe treponeme purification attempts by density gradient centrifugation. They separated *T. pallidum* from both soluble and particulate components by sedimentation through a potas-

sium tartrate gradient, but the organisms were killed by the high salt concentration. Moreover, the authors gave no indication as to the efficiency of their procedure, or whether the organisms were still intact though dead. Very clean preparations were obtained by Thomas et al. (10) with continuous-flow zonal centrifugation in a cesium chloride gradient, and in a very recent report Baseman et al. (1) described impressive results using a simple discontinuous gradient of sodium and meglumine diatrizoates. Viable treponemes were not obtained by these methods, and the total number of recovered organisms, based on calculations of data presented, did not exceed  $10^9$  to  $2 \times 10^9$  treponemes per infected rabbit.

We have recently reevaluated, and slightly modified, an isolation procedure devised some years ago for preparing *T. pallidum* suspensions for immune agglutination (5). The findings presented here show that treponeme suspensions with high yields of apparently clean organisms can be recovered regularly. Moreover, 90% or more of the treponemes in these suspensions are motile.

### MATERIALS AND METHODS

**Syphilitic lesions in rabbits.** Male New Zealand white rabbits, weighing 3 to 4 kg, were housed in individual cages, in quarters maintained below 17 C. They were offered antibiotic-free pelleted feed and water ad lib. All animals were examined clinically and serologically to assure absence of *T. cuniculi* infection and acclimated to laboratory conditions for at least 1 week before inoculation.

*T. pallidum* inocula were frozen pooled extracts of rabbit testicular syphilomas. Infected tissues were ground with sand in a mortar, diluted with serum-saline (10% normal rabbit serum in 0.85% NaCl solution), centrifuged to remove gross debris, and then further diluted to a *T. pallidum* concentration of  $10^8$  to  $2 \times 10^8$  organisms/ml. Dimethyl sulfoxide, 10% final concentration, was added just before dispensing aliquants in plastic vials, which were frozen immediately and stored in a liquid nitrogen refrigerator. Regardless of storage time, almost 100% of the treponemes were motile when thawed at 37 C, and rabbits inoculated intradermally with 0.1 ml developed visible chancres after 1 to 2 days.

Rabbits were inoculated intratesticularly, 0.5 ml of suspension per testis, and placed on corticosteroid therapy from the 3rd post-inoculation day until sacrificed. Cortisone acetate, 6 mg per kg of body weight, was given intramuscularly once a day. This treatment markedly depressed cellular infiltration in lesions, and orchitis, usually palpable after 6 days in the absence of corticosteroid treatment, was not apparent until about the 10th day of infection. Thereafter, testicular enlargement and induration increased rapidly. Animals were sacrificed on the 12th or 13th day when the testes were two to three times normal size and had acquired a firm rubbery texture. Each animal was anesthetized with intravenous sodium barbital and exsanguinated by cardiac puncture. The testes were removed aseptically, trimmed of all extraneous tissue, rinsed free of blood, and minced with scissors.

It has been our experience that testes harvested at the optimal stage of infection are large, pale, almost bloodless, and filled with stringy, mucoid material. Crude extracts of such tissue contain, in addition to treponemes, some large, round nucleated cells but relatively few erythrocytes and almost no spermatozoa. Just before this time the testes are somewhat softer, with milky and less mucoid extracellular fluid; relatively clean suspensions of treponemes can be recovered from these tissues, but the yield is not large. Shortly after the infection has reached the mucoid stage, hemorrhage into the testes occurs. Once hemorrhaging has become generalized, the tissue must be discarded because treponeme numbers decrease abruptly and clean suspensions cannot be obtained.

Extractions of minced testes from each rabbit were routinely carried out in 250-ml Erlenmeyer flasks containing 60 ml of 0.075 M sodium citrate. The flasks were placed on a rotary shaker (60 to 70 rpm) at room temperature for 3 h. Under these conditions almost all treponemes were motile at the end of the extraction period, with very little decrease in motility after subsequent sedimentation and resuspension. After extraction, supernatant fluids were decanted from the testicular tissue and centrifuged at  $800 \times g$  for 10 min at room temperature in a swinging bucket head. The supernatant fluids were transferred to 30-ml screw-cap polycarbonate tubes, and the treponemes were sedimented by centrifugation at  $17,500 \times g$  for 30 min in a Beckman Spinco preparative ultracentrifuge, rotor model 42.1. The organisms were resuspended in 1 to 2 ml of appropriate medium with the aid of a

Vortex mixer and again centrifuged,  $500 \times g$  for 10 min. The supernatant suspensions from individual rabbits were examined by dark-field microscopy, and those free of visible contaminating material were pooled, diluted as desired, and stored in a liquid nitrogen refrigerator. Only an occasional suspension was discarded as unsatisfactory.

**Protein determinations.** Assays were performed by the Folin-Ciocalteu procedure on trichloroacetic acid precipitates, as described by Chase and Williams (3). Crystalline bovine serum albumin, standardized by Kjeldahl nitrogen determination, was used as the reference.

**Immunofluorescence.** Direct immunofluorescent tests to determine the presence or absence of host-derived immunoglobulins on treponemes were performed with fluorescein-labeled globulin from goat anti-rabbit immunoglobulin serum. Indirect immunofluorescent reactions were performed in a quantitative fashion with both human and rabbit syphilitic sera and with the corresponding fluorescein-labeled antiglobulins. The latter were high-titer commercial preparations and each was used at a dilution just below its "plateau titer" (6). The general fluorescent treponemal antibody (FTA) procedure has been previously described (7).

**Electron microscopy.** Specimens were mounted on grids with carbon-coated Formvar films and stained with either 1% potassium phosphotungstate or 1% neutral ammonium molybdate. The preparations were examined with an AEI EM6B microscope at 60 kV accelerating voltage.

## RESULTS

Some of the characteristics of *T. pallidum* suspensions prepared from eight groups of rabbits are recorded in Table 1. It will be noted that in seven of the groups the treponeme harvest exceeded  $10^{10}$  organisms per rabbit, with an overall average of  $1.58 \times 10^{10}$  treponemes per animal. In these same suspensions, protein assays ranged  $1.4 \times 10^{-7}$  to  $2.9 \times 10^{-7}$   $\mu\text{g}$  of protein per treponeme (average,  $1.9 \times 10^{-7}$   $\mu\text{g}$ ). The eighth group (preparation 4) was one in which three of six animals were discarded because of mild, atypical infections and the remaining animals were harvested despite recognition that testicular infections were suboptimal. This entire group might be considered a failure in relation to the results obtained with the others. We have no explanation for these aberrant results.

All suspensions were monitored by dark-field microscopy during their preparation to assess the removal of host cells. The final products appeared to be free of such elements. The preparations described here were not made for purposes that required maintenance of viability, and therefore specific motility counts were not done. However, it was noted that almost all

treponemes remained motile. Negatively stained preparations of the treponeme suspensions were examined by electron microscopy. No particulate background material was observed and the treponemal cells appeared intact.

Portions of various suspensions were used as antigens for FTA tests after appropriate dilution and addition of thimerosal (0.01%) as a preservative. Direct FTA tests with labeled antiglobulin to rabbit immunoglobulins were negative, indicating the absence of detectable host-derived antibody on the treponemes. When these same preparations were used for indirect FTA tests with syphilitic sera, the spirochetes stained brilliantly against a dark, totally non-fluorescing background. Moreover, such preparations, when stored at 4 C, maintained their staining characteristics for at least a year.

### DISCUSSION

One of the major difficulties in syphilis research is the isolation of *T. pallidum* from infected tissues and the purification of these organisms in sufficient numbers for investigative studies. This report describes a procedure for recovering treponemes from rabbit testicular syphilomas that, in our hands, has repeatedly yielded a rich harvest, averaging  $1.58 \times 10^{10}$  clean treponemes per animal, most of which were still motile.

Although several groups of investigators have previously described the isolation and purification of *T. pallidum* from tissues (1, 8-10), very little data pertaining to the recovered organisms have been presented heretofore. Only two reports have given figures from which treponemal yields could be ascertained. Thomas et al. (10), using continuous zonal centrifugation in cesium

chloride, purified treponemes from pooled testicular extracts of five rabbits. They recovered  $7.9 \times 10^9$  organisms ( $1.6 \times 10^9$  per rabbit) in three peak gradient fractions, one of which contained the bulk of the treponemes,  $1.1 \times 10^9$  per rabbit. Baseman et al. (1) sedimented testicular extracts through discontinuous gradients of sodium and meglumine diatrizoates and recovered  $10^9$  treponemes per rabbit. In neither preparation were the recovered organisms motile, and the yields were only about one-tenth those obtained by the method described here.

In addition to the quantitative aspect of our procedure, another important feature was the purity of the final treponeme suspensions. Microscopically, these were free of recognizable host tissue components, and immunofluorescence studies showed no attached immunoglobulins. In addition, protein concentrations averaged  $1.9 \times 10^{-7}$   $\mu\text{g}$  per treponeme, a figure one-third that of the cleanest fraction obtained by Thomas et al. (10), the only other group to report such assays. For figures such as these to be meaningful, it was essential to establish the integrity of the recovered organisms. The best evidence for this was the observation that most of the treponemes in our suspensions were still motile. In addition, negatively stained preparations examined by electron microscopy established that the cells were indeed intact. Despite these findings, no conclusions could be made as to the relative purity of our preparations in terms of complete freedom from host component contamination because of the lack of criteria by which to judge. Nevertheless, our figure of  $1.9 \times 10^{-7}$   $\mu\text{g}$  of protein per *T. pallidum* compared favorably with that of  $2.3 \times 10^{-7}$   $\mu\text{g}$  per organism for the cultivable Reiter treponeme, a spirochete of comparable size (Hardy and Nell, unpublished data).

TABLE 1. Quantitative values of *T. pallidum* suspensions prepared from testicular syphilomas of corticosteroid-treated rabbits

Prepn	Rabbits		Final <i>T. pallidum</i> suspension				
	No. inoculated	No. harvested	Vol (ml)	Treponeme concn ( $\times 10^9/\text{ml}$ )	Total protein ( $\mu\text{g}/\text{ml}$ )	Protein/treponeme ( $\times 10^{-7}$ $\mu\text{g}$ )	Treponeme yield/rabbit ( $\times 10^{10}$ )
1	3	3 <sup>a</sup>	30	2	300	1.5	2
2	1	1	10	1.35	270	2.0	1.35
3	4	4	25	2.14	460	2.2	1.34
4	6	3	32	0.6	230	3.8	0.64
5	6	6	32	2.0	580	2.9	1.06
6	4	4	25	2.64	375	1.4	1.65
7	6	5	45	2.0	320	1.6	1.8
8	6	5	40	2.3	370	1.6	1.84

<sup>a</sup> An occasional animal was discarded because of inadequate testicular infection or massive hemorrhage into the lesions.

The large treponeme harvests reported here could be directly attributed to the corticosteroid therapy given during the infection. The immunosuppressive action of this drug in rabbits permitted animals to be held longer than usual after inoculation, and this, in turn, allowed further multiplication of these slowly growing microorganisms. The cleanliness of the final suspensions was primarily due to other effects of steroid therapy: the depression of cellular infiltration in lesions, and the tremendous increase in the extracellular mucoid substance that is usually present only in a very small amount (11). However, the time at which animals were sacrificed was also important because the cleanest suspensions were obtained only at one stage of lesion development. For this reason it was very advantageous to initiate infections with a uniform inoculum from a frozen *T. pallidum* pool of predetermined infectiousness. The course of infection in different groups of animals was quite predictable and harvest times could be scheduled in advance.

Treponeme suspensions prepared in the manner described here were originally used for specific agglutination tests (5), and in the present study we have found them also completely satisfactory for immunofluorescence reactions. Cox and Barber (4) used suspensions prepared similarly for O<sub>2</sub> consumption determinations. Currently we are investigating their value for other studies.

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