Purification of *Treponema pallidum* from Infected Rabbit Tissue: Resolution into Two Treponemal Populations

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Virulent *Treponema pallidum* organisms, extracted from infected rabbit testes, were subjected to velocity sedimentation in discontinuous gradients of Hypaque, a high density, low viscosity material. After centrifugation of extracts at 20 C for 45 min at 100,000 X g, treponemes separated into two distinct bands based upon their relative velocities, although some variation was observed in the densities of the two bands and the number of treponemes per band. Rabbit tissue components sedimented more rapidly. Dark field and electron microscopy of preparations after velocity sedimentation indicated that treponemes retained general structural characteristics and no tissue contamination occurred in the treponemal fractions. Purification of treponemes in Hypaque resulted in their loss of motility and infectivity based upon animal inoculation. Antigenicity with respect to reactivity with antibody was preserved as shown by the high fluorescence intensity of treponemes in the fluorescent treponemal antibody adsorption test.

Successful in vitro cultivation of virulent *Treponema pallidum*, the causative agent of syphilis, would allow critical studies concerning the biology of the spirochete as well as the pathogenesis of the disease. Since no artificial medium exists in which Nichols strain of *T. pallidum* can grow and remain virulent, experimentally infected rabbits serve as the only readily available source of virulent treponemes. Unfortunately, attempts at purification of treponemes from rabbit tissue for purposes of chemical, enzymatic, and antigenic analysis have been of limited value. Rathlev and Pfau (4) reported the separation of *T. pallidum* from rabbit tissue by equilibrium density gradient centrifugation in potassium tartrate. However, the close proximity of treponemes to host tissue after isopycnic centrifugation of small amounts of material and the apparent high degree of aggregation made this method inadequate. Also, efforts to separate treponemes by membrane filtration (1) appear to be unsatisfactory. In the most detailed study to date, Thomas et al. (8) were unable to sharply band treponemes after zonal centrifugation in cesium chloride. Instead, treponemes sedimented over a wide range of buoyant densities which overlapped densities of testicular tissue.

It seemed feasible, however, to purify treponemes from host tissue by velocity sedimentation if a proper supporting medium for gradiens could be found. Gradients constructed of ficoll, sucrose, or dextran were not capable of separating treponemes cleanly from tissue contaminants. This study describes the use of Hypaque, a high density, low viscosity material for isolation of virulent *T. pallidum*. Not only were treponemes purified and concentrated to a high degree, but they were resolved into two distinct populations based upon sedimentation velocity.

**MATERIALS AND METHODS**

**Animals.** New Zealand White male rabbits weighing between 7 and 8 lbs (ca. 3.18 and 3.63 kg) were obtained from Pel-Freeze (Rogers, Ark.) and were housed in isolation cubicles set at 16 to 18 C prior to and during treponemal infection. Standards recommended by the U.S. Public Health Service Guide for the Care and Use of Laboratory Animals (9) were followed.

**Bacteria.** The virulent Nichols strain of *T. pallidum* and the avirulent Reiter strain were obtained through the courtesy of Paul H. Hardy, Jr., Johns Hopkins University. Virulent treponemes were passaged in rabbits, whereas the Reiter treponemes were cultivated in spiralate broth (BBL) supplemented with 10% normal rabbit serum and incubated under anaerobic conditions at 35 C.

**Extraction of virulent *T. pallidum*.** Rabbits were inoculated intratesticularly with approximately 5 x 10^7 virulent *T. pallidum* (Nichols strain) per testis. The animals were frequently examined for early signs of orchitis and were sacrificed 1 to 2 days after its...
detection. Animals were then injected intravenously with 100 mg of pentobarbital and bled out by cardiac puncture prior to removal of the testes. Treponemes were extracted from each testis by first mincing the infected tissue, then shaking the tissue (frequency of 74 cycles/min, amplitude of 3 in (7.62 cm/stroke) at room temperature in an atmosphere of 5% CO₂, 10% H₂, and 85% N₂ for 30 min in 5.0 ml of phosphate-buffered saline containing 3.0 mg of glutathione (PBS-G). The liquid extract was removed, and the tissue was reextracted as previously described. Each extract was centrifuged three successive times at 500 x g for 5 min to remove large tissue pieces and the majority of red blood cells and spermatozoa. The supernatant was then centrifuged at 18,000 x g for 15 min to pellet treponemes. The pellet, which contained treponemes and some host tissue components including blood cells, spermatozoa, and small-molecular-weight tissue fragments, was resuspended in 0.2 to 0.5 ml of PBS-G before layering onto gradients. At this point the treponemes were actively motile.

**Formation of gradients.** Discontinuous gradients of Hypaque-M, 75%, a brand of sodium and meglumine diatrizoate (Winthrop Laboratories), were prepared at room temperature by diluting stock Hypaque-M in PBS-G to the desired concentration. A layer of 60% Hypaque-M (1.5 ml) was formed in cellulose nitrate tubes (½ in diameter by 2 in [ca. 1.3 by 5.1 cm]; Beckman Instruments) followed by 1.0 ml each of 37.5, 25.0, and 19.0% Hypaque-M; these step gradients were prepared just before use. Renografin-76 (E. R. Squibb & Sons), a similar diatrizoate material, was also an adequate medium for gradients. A 0.1- to 0.2-ml sample of the crude treponemal suspension was applied to each gradient. The gradients were centrifuged at 20 C for 45 min at 30,000 rpm (100,000 x g) in either a Beckman SW50.1 or a SW65 swinging bucket rotor. Fractions (0.1 ml) were collected either by drop from the bottom of the tube or from the top by displacing the gradient with 75% Hypaque-M. Samples were also withdrawn from the top of the tube by using a Hamilton microsyringe.

**Density determinations.** After the gradients had been collected, a known volume from each fraction was drawn into a calibrated micropipette, and its weight was determined using a torsion balance.

**Enumeration of cells.** Because of inconsistencies in duplicate determinations of treponemal numbers by counting specific volumes under the dark field microscope, acryl reference particles (0.5-μm diameter, Colab Laboratories) were added to each fraction to yield approximately 50 particles per field at a magnification of 1,000 x. Numbers of treponemes, as well as latex particles, were counted per field and the ratio of treponemes to latex particles was determined. This value was multiplied by the number of latex particles per 0.1 ml of the original stock to determine the number of treponemes in each fraction.

**Electron microscopy.** Samples from gradients were prepared for microscopic analysis using the conventional pseudoreplica technique (5). One drop of sample was spread over a small section (1 cm² by 0.2 cm deep) of 2% agar containing 1% NaCl. After drying at room temperature, samples were covered with 0.75% collodion, and the excess collodion was blotted away with filter paper. Once dried, the collodion film was removed from the agar by water flotation and transferred to 200-mesh copper grids. Specimens were shadowcast with platinum-carbon pellets (Ladd) at a grazing angle of 10° and then observed in an AEI EM6B electron microscope with a 25-μm objective aperture at 60 kV.

**Fluorescence microscopy.** The standard fluorescent treponemal antibody absorption (FTA-ABS) test was performed on treponemes obtained from gradient bands to determine their general antigenic properties after velocity sedimentation.

**RESULTS**

**Purification of treponemes on gradients of Hypaque.** Numerous attempts to purify treponemes from host tissue on linear or discontinuous gradients of dextran, ficoll, or sucrose resulted in poor separation of treponemes from host tissue and a diffuse distribution of spirochetes over a wide range of gradient fractions. However, centrifugation of crude treponemal preparations on discontinuous gradients of Hypaque was much more satisfactory. Extractions of treponemes from individual or pooled rabbit tissues were combined, pelleted by centrifugation, and resuspended in 0.25 ml of PBS-G. One-tenth milliliter of the concentrated suspension was applied to each gradient (Fig. 1). As can be seen, three distinct bands were evident after centrifugation. The lower band (c) in gradient A contains numerous visible aggregates probably due to excess sample and resultant clumping. Gradients A and B were fractionated, and samples were counted as described in Materials and Methods. Large numbers of treponemes, apparently free of host tissue components, were detected in the two lower bands with the majority of treponemes located in band b (Fig. 2). The densities of bands b and c were 1.187 and 1.202, respectively. No treponemes were visible in the top band, which contained very small undetectable particles.

Although two bands of treponemes were observed regularly after velocity sedimentation in Hypaque, the distribution of treponemes in each band was variable (Fig. 3). Here treponemes were also equally distributed between the two bands. Since fractionation of gradients resulted in some mixing and distortion of the gradient, a calibrated microsyringe was carefully inserted into a similar gradient, and 10-μliter samples were removed from the regions around bands b and c. Under these conditions the trough between the two bands was much more pronounced, representing less than 20% of the value of either peak. It was also possible, on occasion, to observe a different sedimentation...
pattern (Fig. 4, tube A). Treponemal bands are again indicated by letters b and c; band d is comprised of blood cells and spermatozoa which sediment at a density of 1.235. In this gradient the two treponemal bands are close together but still remain distinct. On occasion, the lower treponemal band and the tissue components were so close that extreme care was necessary to prevent cross-contamination of the fractions.

For comparative purposes, Reiter treponemes grown in broth culture were pelleted and resuspended in PBS-G for application to gradient B (Fig. 4). Using identical conditions for velocity...
sedimentation in Hypaque, only one band appeared at a position similar to that of host tissue components (Fig. 4, tube B). If suspensions from uninfected testicular extracts were centrifuged in Hypaque gradients, a single band was observed at a density of 1.235. Modifications in the concentration of Hypaque at various levels of the step gradient did not substantially increase resolution and for the most part gave poorer separation. Also, longer centrifugation runs or higher rotor speeds resulted in less distinction between the two treponemal bands as the bands moved deeper into the gradient, possibly explained by a more rapid approach to isopycnic densities which might be identical in both treponemal populations.

It was of interest to see whether treponemes retained their banding properties after a second centrifugation in Hypaque. Several preparations were examined in which bands b and c were pooled, diluted to a volume of 30 ml in PBS-G, and centrifuged at 20,000 × g for 30 min at 4°C in a Sorvall RC-2B centrifuge to remove the Hypaque and pellet treponemes. The pellet was resuspended in PBS-G and subjected to a second velocity sedimentation in Hypaque. These treponemes were distributed in a single band at a density of 1.202 (data not shown). Unfortunately, considerable structural damage was incurred by the spirochetes as a result of these manipulations (see next section and Fig. 5c), and the significance of these findings in terms of the observed change in sedimentation velocity is difficult to assess.

**Electron microscopy of Nichols strain of T. pallidum after sedimentation in Hypaque.** Although banding of treponemes in Hypaque yielded satisfactory separation from tissue components as determined by dark field microscopy, further examination was performed with the electron microscope. Figure 5a is a micrograph from a section of band b withdrawn by microsyringe and processed as indicated in Materials and Methods. Treponemes are in very high concentration, and no significant clumping is apparent. Under higher magnification (Fig. 5b) treponemes appear to be intact, although some damage to the outer mucoid layer and envelope may have occurred. When specimens from band c were examined in a similar manner, no differences in the length of individual organisms or in the number and amplitude of coils could be observed when compared with organisms in band b. After a second centrifugation of treponemes in Hypaque, however, extensive damage was evidenced by released and ruptured axial filaments (Fig. 5c). Velocity sedimentation of crude treponemal extracts in Hypaque gradients routinely gave excellent separation with no identifiable host contamination, especially in the upper treponemal band (band b). However, on one occasion, a section of a spermatozoa tail was associated with a small aggregate of treponemes from band b (Fig. 5d).

**Fluorescence microscopy with purified T. pallidum.** After velocity sedimentation of crude treponemal extracts in Hypaque, bands b and c were removed, and loopfuls of the material were transferred to fluorescent-antibody slides (Clay Adams). All antigen preparations reacted with a high intensity of fluorescence in the FTA-ABS test.

**Biological activity of purified T. pallidum.** Treponemes lost motility after purification in Hypaque, as determined by dark field microscopy. In addition, injection of purified treponemes subcutaneously into rabbits resulted in no discernible skin reactions.

**DISCUSSION**

Velocity sedimentation of crude treponemal extracts in discontinuous gradients of Hypaque
proved to be highly successful for the separation and concentration of virulent *T. pallidum* from host tissue. After centrifugation of infected testicular suspensions, two bands of treponemes were evident. The number of treponemes in each band varied as did the density, although the most frequent sedimentation profiles indicated greater treponemal concentration in band b than in band c and densities of 1.187 and 1.202, respectively. The fact that treponemes extracted from infected testes were regularly resolved into two populations of cells based...
upon their relative sedimentation velocities was interesting, suggesting that two distinct classes of treponemes might exist during the infectious process. It might be argued that treponemes contain varying amounts of mucoid material, lipid, or other components as a result of cell growth and multiplication in rabbit testes and, therefore, would possess differing sedimentation velocities. However, since two obvious populations were detected rather than a broad spectrum, some interrelationship must exist, such as an early and late stage of a treponemal growth cycle which might be reflected in distinct variations in deoxyribonucleic acid content or in the amounts of outer mucoid or envelope layers. In addition, it is possible that two interacting types of treponemes are necessary for disease pathogenesis. It would be difficult to test the latter possibility at present since treponemes lose infectivity after sedimentation in Hypaque. *Bacillus megaterium*, however, can be separated into vegetative and sporulating populations (7), and *Bacillus subtilis* into competent and noncompetent cells (2), by sedimentation in diatrizoate without decreased viability.

It should also be pointed out that work by others (3, 6), including ourselves (unpublished data), indicates that treponemes are found both intracellularly in testicular cells and extracellularly during infection. What role intracellular organisms play in the etiology of disease, if they are indeed viable, remains uncertain, although differences between intra- and extracellular organisms may be reflected in banding variations.

In general, all treponemes applied to Hypaque gradients were recovered in the regions of bands b and c. Dark field and electron microscopy indicated that the organisms comprising each band remained intact, singular, and unassociated with host tissue components which banded at a higher density. Fluorescence microscopy demonstrated that treponemes retained antigenic determinants for the FTA-ABS test. It was impressive to observe the intensity of fluorescence using such a highly purified, concentrated preparation of virulent *T. pallidum* when compared with typical FTA-ABS treponemal antigen controls or crude treponemal preparations. Although the volume of the sample applied to each gradient was small, larger gradient tubes of Hypaque should accommodate increased sample sizes with no loss of resolution. It is also possible that zonal centrifugation of infected tissue extracts using Hypaque as the supporting medium would result in satisfactory separation of treponemes from host tissue.

Over the past years, one of the difficulties associated with research in syphilis has been the inability to efficiently isolate and concentrate virulent *T. pallidum* from tissue components. In this paper, a method for accomplishing this task is described. In addition, banding of treponemes into two classes is discussed, although its significance is not known at this time. Possibly more sophisticated techniques for examining the subpopulations of treponemes, including thin section and high resolution electron microscopy as well as radioimmunoassays, which were not attempted in the current study, will offer explanations for dual banding. The technique of velocity sedimentation in Hypaque offers a satisfactory means for separating virulent treponemes from host tissue for biochemical analysis which may clarify activities of the parasite, thereby suggesting means for interrupting the host-parasite interaction. Also, the use of Hypaque for purification may provide a technique which will allow development in a nontoxic medium of an effective vaccine for the control and prevention of syphilis.

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LITERATURE CITED


