

Separation of *Treponema pallidum* from Tissue Debris Through Continuous-Particle Electrophoresis

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Treponema pallidum can only be cultured in living animal tissue, such as rabbit testes. However, the extract of these organisms from the testicular material leaves the *T. pallidum* contaminated with tissue debris. This paper describes the separation of *T. pallidum* from the debris by continuous-particle electrophoresis. The importance of equilibration time before electrophoresis is discussed.

Treponema pallidum at present can be propagated only in living animal tissues. Large numbers of *T. pallidum* can be obtained from rabbit testes inoculated with a rabbit-adapted strain—the Nichols strain. Practical usage of such *T. pallidum* for test antigens, vaccine material, or antigen studies necessitates removal of the testicular debris as efficiently and thoroughly as possible. Previous investigators have sought to accomplish this by various techniques of centrifugation, including differential centrifugation (2), tartrate gradients (3), and cesium chloride gradients in zonal rotors (Thomas et al., *personal communication*). As yet, however, separation by these methods has not come into routine laboratory use. This paper describes the separation of *T. pallidum* from rabbit testicular debris by using a Continuous-Particle Electrophoresis apparatus (Beckman Instruments, Inc., Fullerton, Calif.). A method is outlined, and pictures of the "clean" material are shown.

MATERIALS AND METHODS

Preparation of *T. pallidum* suspensions. New Zealand male white rabbits, 6 to 8 lb, whose sera were nonreactive in serological tests for syphilis were inoculated intratesticularly with 0.5 ml of a treponemal suspension (40 to 50 motile *T. pallidum* per high dry field). After 8 to 12 days, rabbits with a definite orchitis were killed, and the testes were removed, trimmed of fat, and cut into small pieces approximately 0.25 inch (0.64 cm) thick. The pieces were put

into a flask, and 15 ml of 0.15 M NaCl-0.0055 M PO (potassium salt), pH 7.2, buffer was added for each testis to be extracted. The mixture was slowly stirred for 1 hr in the cold (4 C), and the supernatant fluid was decanted and centrifuged at 1,000 × *g* for 10 min. The resulting supernatant fluid was decanted and centrifuged at 30,000 × *g* for 10 min. The sediment from the 30,000 × *g* centrifugation was resuspended in 0.001 M barbital buffer, pH 8.6 (Fig. 1). Adequate suspension of the treponemes in the buffer was accomplished by repeated aspiration into and expulsion from a syringe equipped with a 14-gauge cannula. The suspended treponemes were allowed to stand for 18 hr at 4 C, during which time the suspending buffer was changed at least once.

Before electrophoresis, the treponemes were centrifuged at 30,000 × *g* for 10 min and resuspended in the curtain buffer (*see below*) at a concentration to give definite turbidity (1 to 2 McFarland units).

Continuous particle electrophoresis. The separation of *T. pallidum* from tissue debris was carried out with a "Continuous-Particle Electrophoresis" apparatus fitted with a cooling faceplate. The crude material was slowly pumped into the top of a chamber containing the "curtain buffer." As the material passed downward in the curtain, it passed through the field of two vertical electrodes where it was separated into the various fractions by electrophoresis. The bottom of the curtain contained 40 tubed outlets, where the individual samples were collected. The curtain buffer and electrode rinse buffer were 0.001 M barbital, pH 8.6. Unless otherwise indicated, electrophoresis was carried out at 50 v/cm, 11 ma, with an 18 ml/min curtain flow rate. The apparatus, including the voltage supply, was turned on and stabilized at least 0.5 hr before the start of any run.

The treponemal material was introduced by gravity feed to give the thinnest line compatible with unin-

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rupted flow. Separation was observed visually through the graduated viewing port. After the position of the bands had stabilized (approximately 5 min), the fractions were collected.

After completion of a run, the material in each collection tube was centrifuged at $30,000 \times g$ for 10 min, and the sediment was examined by phase and dark-field microscopy. Direct and indirect fluorescent-antibody staining was used to confirm the serological identity of the treponemes and treponemal structures. Fluorescein-labeled anti-*T. pallidum* globulins used for direct fluorescent-antibody staining had been sorbed with Reiter treponemes to provide maximal specificity. The indirect fluorescent antibody staining was performed in the same manner as the Fluorescent Treponemal Antibody-Absorption (FTA-ABS) test with the continuous-particle electrophoresis-harvested treponemes being used as antigen (1).

The percentage of treponemes recovered as "clean" was crudely estimated by resuspending the sediment in each tube in 0.5 ml of saline. A drop of each suspension was placed under a cover slip, and 10 high dry fields were examined. The average number of treponemes per high dry field was used to calculate the percentage of "clean" treponemes.

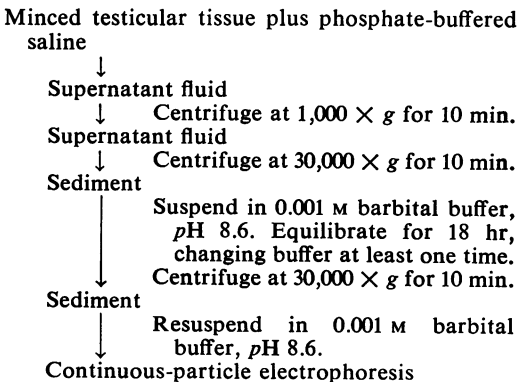
RESULTS

Examination by phase and dark-field microscopy of the sediment from each of the centrifuged collecting tubes showed the following pattern: tubes 25-28, debris; tubes 29-31, *T. pallidum* plus debris; tubes 32-34, *T. pallidum* in spiral and sphere form.

We found that equilibration in the barbital buffer for at least 18 hr was necessary to produce a maximal separation of the treponemes from the debris. Furthermore, this equilibration process increased the percentage of "clean" treponemes recovered. However, it resulted in conversion of 30 to 50% of the spiral forms to sphere forms.

Approximately 70% of the treponemes recovered were found in the "clean" fractions. Figure 2 shows the sphere forms resulting from

FIG. 1. Flow sheet of treponemal preparation for electrophoresis.



the equilibration process. The absence of debris in Fig. 2 is characteristic of "clean" treponemal preparations.

Serological responsiveness of the treponemes or spheres, or both, was assessed by direct and indirect fluorescent-antibody procedures. The spiral forms were found to fluoresce less brilliantly after passage through the continuous-particle electrophoresis apparatus than before passage (2+ versus 3+ subjective readings) by either direct or indirect fluorescent-antibody techniques. Subjectively, the sphere forms retained their brilliance after electrophoresis.

DISCUSSION

The continuous-particle electrophoresis apparatus allows the production of virtually clean *T. pallidum*. However, the term "clean" treponemes needs further definition. Although these treponemes may be free of particulate testicular debris, they nevertheless stain with goat anti-rabbit immunoglobulin G.

The separation of *T. pallidum* from rabbit testicular debris has many possible applications. Before production of a vaccine against syphilis infection, it will be necessary to purify the organism sufficiently not only to produce a clean



FIG. 2. High-power photograph of the "clean" treponeme preparation after electrophoresis. The sphere forms with their inner structures are clearly seen. Note the lack of background debris.

vaccine preparation, but also to study the antigens of the organism without the presence of foreign substances. Routine serological testing with the FTA-ABS test has been complicated by the presence of background fluorescence caused, in part, by the contaminating rabbit testicular debris and protein. It is now possible to prepare *T. pallidum* without this contaminating background particulate matter.

The decrease in fluorescent-antibody staining by the spiral treponemes after passage through an electric field needs further study. It is possible that treponemal antigens are removed by this passage. If so, the recovery of these antigens should give a product important in understanding the reactivity in indirect fluorescent-antibody tests using the treponeme. These removed antigens could be important in the further development of tests which detect specific antibodies against *T. pallidum*.

Indirect fluorescent-antibody techniques using the sphere forms do not show this striking loss of staining. However, this may be caused by exposure of more antigenic sites by ballooning of the envelope encompassing the sphere, or by a larger fluorescent area being observed. The production of the sphere form is not an irreversible phenomenon since the sphere can be reverted to a spiral form by equilibration in an isotonic buffer. The phenomena of sphere formation and reversion have been recently reviewed (4).

The phenomenon of "equilibration" of particles in various buffers was studied by us during the development of the present technique. The direction and rate of migration of treponemes, bacteria, cell walls, and cellular components were strongly influenced by the pH, ionic content, and ionic strength of the buffer in which the material was suspended before electrophoresis, and by the length of time the material was suspended in this buffer. Advantage can be taken of this fact in the

electrophoretic separations. For instance, we found that when the treponemes were allowed to stand in isotonic saline and resuspended in the barbital buffer just before electrophoresis, not only was separation unacceptable but, during the time of the electrophoretic run, there was shifting of the bands apparently caused by gradual equilibration with the suspending buffer. However, extraction into saline followed by equilibration in barbital buffer for 18 hr before electrophoresis resulted in a good per cent recovery of "clean" *T. pallidum*.

The rapidity of separation will be important if any large-scale separation is undertaken. Using the present continuous-particle electrophoresis apparatus, approximately 5 ml of processed testicular extract was passed through the machine per hour. Although this volume was small, the number of treponemes was large (200 per high dry field). Furthermore, the apparatus could run relatively unattended for long periods of time.

Limiting factors in determining how often the apparatus must be checked are the size of the collecting tube for each fraction and rare unaccountable shifts in band position.

Although we have described one workable technique, it is likely that further refinements in separation are possible.

LITERATURE CITED

1. Hunter, E. F., W. E. Deacon, and P. E. Meyer. 1964. An improved FTA test for syphilis, the absorption procedure (FTA-ABS). *Pub. Health Rep.* 79:410-412.
2. Portnoy, J., and H. J. Magnuson. 1956. *Treponema pallidum* complement-fixation (TPCF) test for syphilis. *Amer. J. Clin. Pathol.* 26:313-322.
3. Rathlev, T., and C. J. Pfau. 1965. Purification of the pathogenic *Treponema pallidum* by density gradient centrifugation. *Scand. J. Clin. Lab. Invest.* 17:130-134.
4. Willcox, R. R., and T. Guthe. 1966. *Treponema pallidum*. bibliographical review of the morphology, culture and survival of *T. pallidum* and associated organisms. *World Health Organ. Bull. (Suppl.)* 35:35-44.