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Electron Microscopy of *Treponema pallidum* (Nichols) Cultivated in Tissue Cultures of SflEp Cells

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The in vitro interaction between *Treponema pallidum* and SflEp cells during treponemal replication was investigated by using transmission electron microscopy. The SflEp cells grown on Teflon-treated cover slips after 12 days of cocultivation were fixed in situ, overlaid with agar, embedded, and vertically sectioned. Large numbers of treponemes were found extracellularly not only at the upper cell surfaces but also in the narrow spaces between the cells and between the cells and the cover slips. These narrow spaces supported treponemal growth and survival, as did those at the upper cell surfaces. Although few in number, organisms were also seen in cell vacuoles either surrounded by a membrane or free in the cytoplasm. Some extracellular treponemes attached to host cells by body spirals or the terminal end and formed electron-dense layers at attachment sites. Some treponemes were often surrounded with amorphous, extracellular material which appeared to "connect" them to host cell surface. After 12 days of cocultivation, host cells showed excessive vacuolation and appeared to be damaged. This did not seem to be due to treponemal infection alone, because cells from uninfected cultures also showed similar vacuolation.

Since *Treponema pallidum* was determined to be the causative agent of syphilis, many investigators have tried to cultivate it in vitro. Previous attempts were uniformly unsuccessful. Either the few reported successes have been nonreproducible, or the treponemes have been subsequently shown to be nonpathogenic (25). However, recently, Fieldsteel et al. reported the replication of the virulent Nichols strain of *T. pallidum* in tissue cultures of cottontail rabbit epithelium (SflEp) cells (5, 6) and, by scanning electron microscopy (SEM), showed that *T. pallidum* attaches to and replicates on the surface of SflEp cells growing in conventional monolayer cultures under an atmosphere of 1.5 to 5% oxygen at 33°C (5).

Numerous reports (7, 8, 14–16, 18, 19, 21) have described the interaction of *T. pallidum* and tissue cells and the nature of attachment as determined by electron microscopy. Fitzgerald et al. (8) and Repesh et al. (21) reported that *T. pallidum* caused no morphological changes of tissue culture cells. However, in further studies, Fitzgerald et al. (15) and Oakes et al. (18) described various degrees of the cultured cell disruption caused by coincubation with *T. pallidum*. This disruption may explain some of the histopathology of syphilitic disease. All of these observations were made with SEM and were done only after short incubation periods, in which little or no treponemal replication occurred. Hayes et al. (16) showed that the terminal structures on virulent *T. pallidum* represent specialized functional organelles that permit the initial surface colonization of host cells. However, in their studies, cultured cells were scraped from the cover slips before postfixation, although they were prefixed in situ. Zeigler et al. (27) and Fitzgerald et al. (8) demonstrated that material on *T. pallidum* reacted with ruthenium red by both transmission electron microscopy (TEM) and SEM. In further studies, Fitzgerald et al. examined putative glycosaminoglycan present on the treponemal surface layer, in vivo within ground substance and in vitro on the surface of cultured cells (10, 12, 13). They also suggested the presence of a treponemal mucopolysaccharidase (9, 11, 27) and related its potential role in syphilitic histopathology (7).

The present study has assessed by TEM the in vitro interaction of *T. pallidum* and SflEp cultured cells during treponemal replication. It was our aim to determine (i) the morphological changes of SflEp cells that occur during cocultivation and when they occur, (ii) the location of the treponemes in the cultures, (iii) the nature of the attachment of *T. pallidum* to the cells, (iv) whether treponemes enter the cells and, if so, the frequency of intracellular inhabitance in the cells in vitro, and (v) whether glycosaminoglycan material was present on the surface of the treponemes or the cells during in vitro replication.

MATERIALS AND METHODS

Animals. New Zealand White male rabbits (6 to 8 months old) were used for testicular passage of *T. pallidum* and as a source of inoculum into tissue cultures of SflEp cells.

***T. pallidum*.** The virulent Nichols strain was used throughout these studies and was passaged as described previously (5).

Tissue culture. An established cell line of cottontail rabbit epithelium (SflEp) was used throughout this study. Passage levels utilized in these experiments ranged from 73 to 85. The tissue cultures were infected with *T. pallidum* as previously described (6).

Electron microscopy. SflEp cells were cultivated with and without *T. pallidum* for 12 days as described above. Both infected and uninfected cells were grown on glass cover slips treated with Teflon by the methods of Chang (3). They were fixed in situ in cacodylate-buffered (pH 7.4) 1.2% glutaraldehyde–0.05% ruthenium red or in phosphate-buffered (pH 7.4) 1.2% glutaraldehyde for 3 h at room temperature, rinsed once with cacodylate or phosphate buffer, and overlaid with 1.5% agar to prevent the removal of treponemes during the following procedures. They were then thoroughly washed

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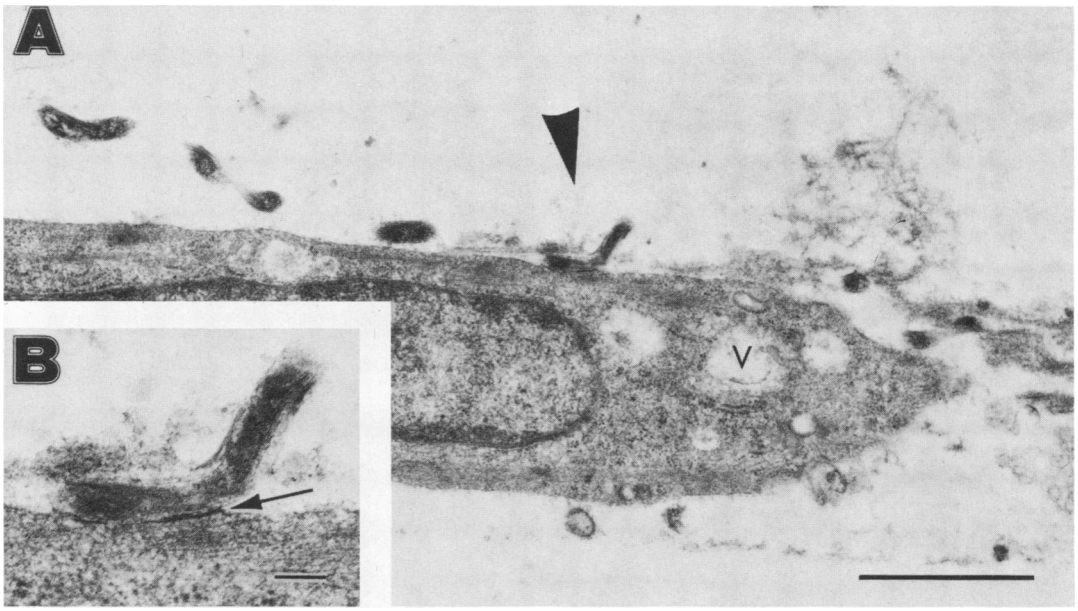

FIG. 1. Vertical sections of Sf1Ep cell cocultivated with *T. pallidum*. (A) Treponemes are seen above and on the cell showing vacuolation (V). Bar, 1 μm. (B) Higher magnification from (A) (arrowhead). Bar, 0.1 μm.

with buffers before postfixation (overnight at 4°C) in either cacodylate-buffered 1.3% osmium tetraoxide (OsO_4)–0.05% ruthenium red or in phosphate-buffered 1.3% OsO_4. The fixed specimens were thoroughly washed with distilled water and dehydrated in a graded ethanol series with a final dehydration in propylene oxide. They were then infiltrated with Polarbed 812 (Polaron Instruments Inc., Hatfield, Pa.) and placed upside down upon gelatin capsules filled with fresh resin. After polymerization, the blocks were separated from the cover slips by cooling with dry ice, cut into small cubes, and re-embedded in flat-surface silicone rubber embedding mold for vertical ultrathin sectioning.

The blocks were trimmed, vertically sectioned with a Sorval MT-2B ultramicrotome (with glass knives), and mounted on 200- or 400-mesh copper grids without support films. The sections were stained with uranyl acetate and lead citrate and viewed on a Philips EM-200, JEOL 100B, or JEOL 200CX electron microscope.

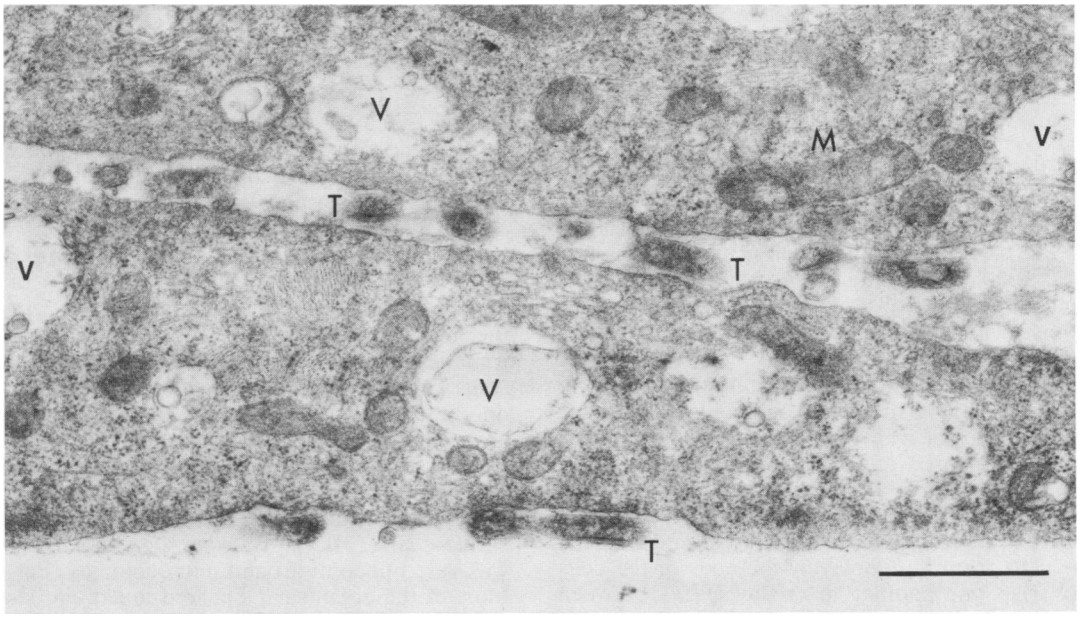

FIG. 2. Vertical section of Sf1Ep cells cocultivated with *T. pallidum*. Two cells showing vacuolation (V) are seen one above the other. Several treponemes (T) are located between the cells and under the lower cell, and demonstrate the characteristic morphology of *T. pallidum*. M, Mitochondrion. Bar, 1 μm.

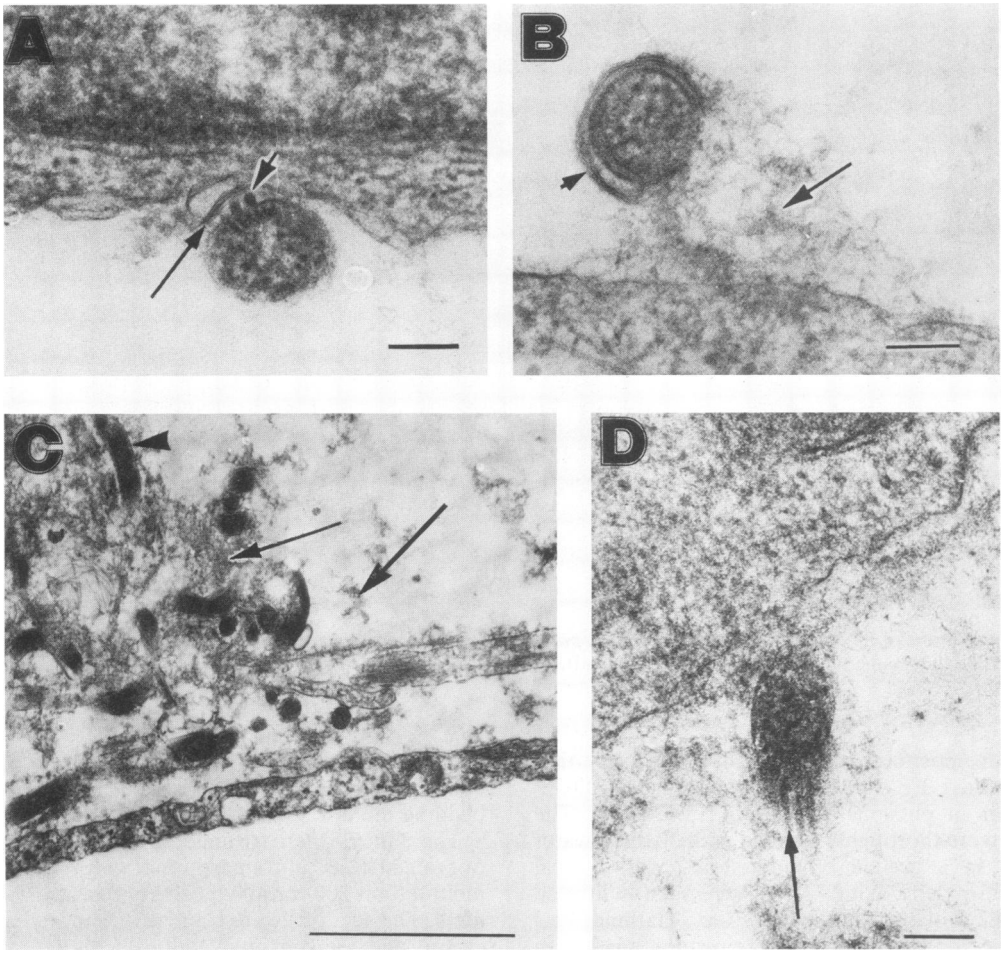

FIG. 3. Vertical sections of Sf1Ep cells cocultivated with *T. pallidum*. (A) A treponeme attaches to the lower surface of Sf1Ep cell by body spiral and forms electron-dense layers with the host cell membrane (arrow). Three periplasmic flagella can be clearly seen (arrowhead). (B) The cross sections of *T. pallidum* above Sf1Ep cells show amorphous material around the treponemes and the host cells (arrows). Outer envelope can be seen (arrowhead). (C) Microcolony of treponemes surrounded by amorphous material (arrows). (D) A treponeme appears to attach to the lower surface of the host cell by a terminal end. The periplasmic flagella are visible (arrow). Bars, 0.1 μm.

RESULTS

To assess the in vitro interaction of *T. pallidum* during treponemal replication, Sf1Ep cells grown on Teflon-treated glass cover slips were fixed in situ and overlaid with agar. Agar overlaying prevented the removal of treponemes and Sf1Ep cells during dehydration, embedding, and vertical sectioning and allowed the observation of more treponemes attached to host cells. In situ fixation and vertical ultrathin sectioning also gave more precise information about the location of the treponemes in the tissue culture.

All electron micrographs are orthogonally placed. Figures 1, 2, and 3 are typical of what was observed in specimens exposed to ruthenium red after 12 days of cocultivation. Sf1Ep cells appear as long thin cells. Internal structures such as nuclei, nuclear membrane, mitochondria, rough endoplasmic reticulum, and ribosomes are visible (Fig. 1 and 2). Even though these cells form a monolayer, the ends of the cells frequently overlap one another to create spaces between cells (Fig. 2). Frequently, spaces between the lower surface of the Sf1Ep cell and the cover slip were observed (Fig. 2 and 3A). Large numbers of individual treponemes were usually found extracellularly, not only on the upper Sf1Ep cell surface (Fig. 1) but also in the spaces between the cells and between the cells and the cover slips (Fig. 2). Microcolonies on the surface of the cells (Fig. 3C) as well as individual treponemes (Fig. 1A) were seen. Some of the extracellular treponemes, attached to the upper surface (Fig. 1) or to the lower surface (Fig. 2 and 3A) of Sf1Ep cells, made electron-dense layers (Fig. 1B and 3A, arrows) with the membrane of the cells at the site of attachment. Most treponemes observed attached to the cells by the body spiral (Fig. 1A and 3A). Few treponemes appeared to attach by only the terminal end (Fig. 3D). The treponemal outer envelope could be observed in many sections (Fig. 3B and C). Some extracellular *T. pallidum* organisms were surrounded with amorphous material that stained weakly with ruthenium red and appeared to connect them to the cell surfaces (Fig. 3B and C, arrows). This amorphous material was very apparent around microcolonies (Fig. 3C, arrows). Most of the organisms appeared intact and showed characteristic treponemal morphology. The spiral shape of the treponemal cell body was well preserved, and periplasmic flagella were usually visible. However, the outer envelope

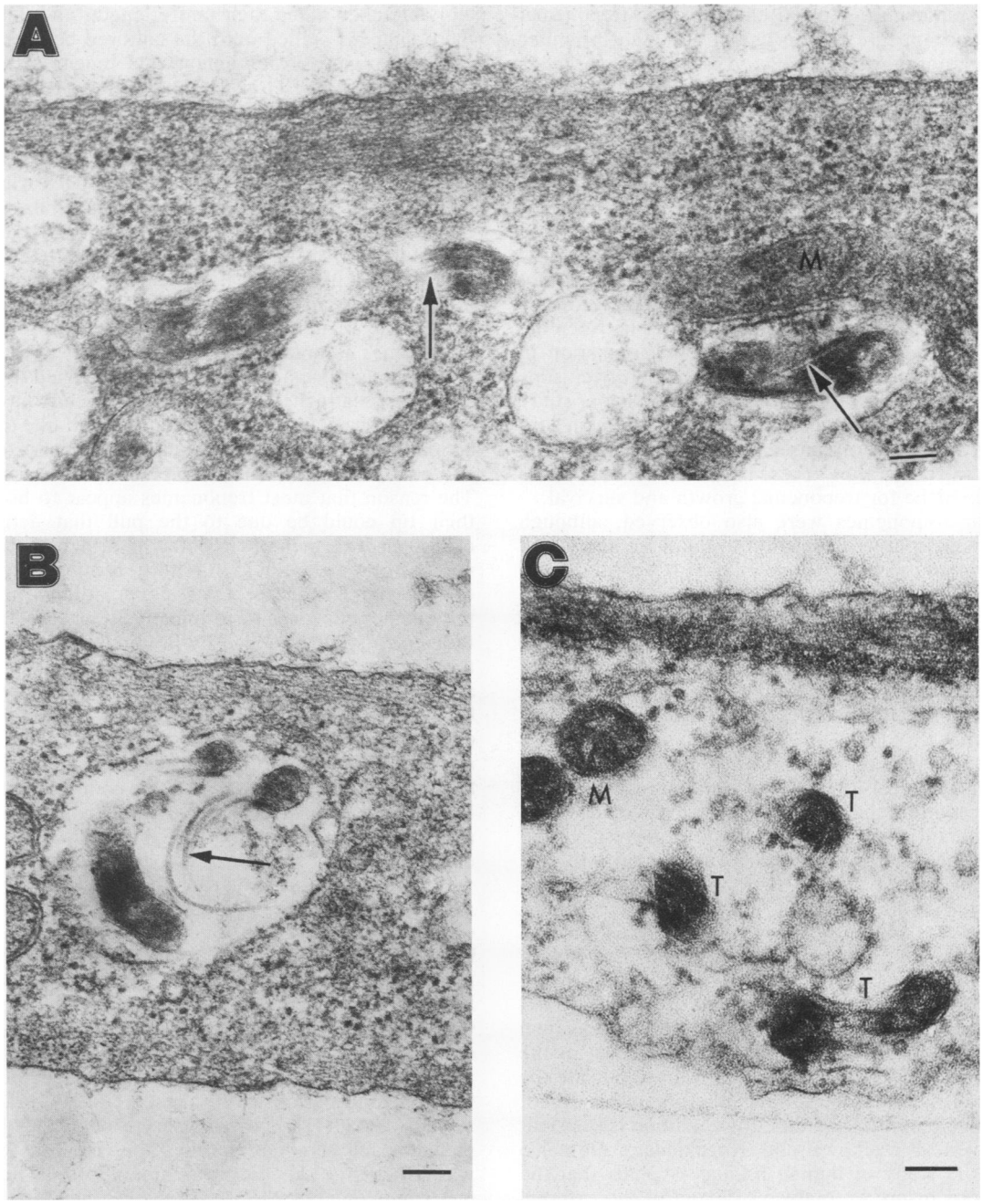

FIG. 4. Vertical sections of Sf1Ep cells cocultivated with *T. pallidum*. (A and B) Treponemes are located in vacuoles within the cytoplasm of Sf1Ep cells. The periplasmic flagella are visible (arrows). M, Mitochondrion. (C) *T. pallidum* (T) can be seen free in the cytoplasm. M, Mitochondrion. Bars, 0.1 μm.

was not always distinguishable. Figures 3B and C are examples of the sections which show the triple-layered structure of the outer envelope (arrowheads).

A few treponemes appeared to be intracellular (Fig. 4B), either surrounded by a membrane (Fig. 4A) or free (Fig. 4C) in the cytoplasm of the cultured cells. These intracellular organisms appeared not to be severely damaged morphologically. The periplasmic flagella were usually visible (Fig. 4A and B, arrows), although the outer envelope was not always distinguishable. In addition, some organisms were folded upon themselves and lost their spiral configuration.

The cultured cells infected with *T. pallidum* showed excessive vacuolation and appeared to be degenerate (Fig. 1, 2, and 4A) after 12 days of incubation. Uninfected cells cultured by the same methods also showed similar excessive vacuolation (data not shown).

DISCUSSION

In this TEM study, we observed the in vitro interaction of *T. pallidum* with Sf1Ep cells during treponemal replication. Large numbers of individual treponemes were usually found extracellularly above and on the upper surface of Sf1Ep cells as described in other reports (5, 8, 15, 16, 18, 21). The

extracellular treponemes showed characteristic treponemal morphology and appeared to be intact, although the outer envelope was not always visible. Treponemal colonies, described by Fieldsteel et al. (5), were also observed. In the original cultivation study by SEM, most of the treponemes were observed to be in microcolonies of five organisms or more. Few single or paired organisms were observed on the host cell surface. This may have been an artifact, because methods to prevent the removal of single organisms were not employed. In addition, many treponemes were also found extracellularly in the narrow spaces, between the cells and between the cells and the cover slips. These sites could not be observed by Fieldsteel et al. (5) with SEM. Fitzgerald et al. (14) also described extracellular treponemes between the cultured cells, using TEM. However, after prefixation in situ, the cultured cells were scraped from the cover slips before postfixation. The treponemal localization in our study should be more precise, since the cultures were fixed in situ, overlaid with agar, and prepared for TEM without being scraped from the cover slips. These narrow spaces appear to be a favorable niche for treponemal growth and survival.

Intracellular treponemes were also observed, although they were few in number in relation to the large number of extracellular organisms. Fitzgerald et al. (14) reported intracellular, morphologically intact *T. pallidum* organisms from 30 min after inoculation of the cell monolayers in vitro, suggesting that the presence of organisms within the cultured cells might play an integral role in the pathogenesis of experimental syphilis, and that some stage of treponemal growth may require an intracellular residence. Other reports support this view (2, 17, 20, 22, 23, 26). Sykes and Miller (23) reported in the study of experimental rabbit orchitis that morphologically typical *T. pallidum* organisms were found intracellularly within the cytoplasmic substance of fibroblasts and interstitial and Ledig cells, and they suggested that intracellular organisms might be associated with the phenomenon of latency. They also demonstrated intracellular *T. pallidum* within cells of a primary chancre from a human female (24) and suggested that difficulties could be associated with the observed intracellular location of treponemes which would render them inaccessible to humoral antibodies and to therapeutic substances. In contrast, Lauderdale et al. (17) reported in the study of experimental rabbit orchitis that the morphology of treponemes in the intracellular location differed from that of those outside the cells; they suggested that the intracellular organisms do not have pathogenic potential and that this condition is transient and short lived. Wrzolkowa and Kozakiewicz (26) also observed in the study of human primary syphilis that the intracellular organisms were always devoid of the external envelope, fragments of which were visible in the intracellular spaces; this suggested that treponemes enter the cytoplasm by way of endocytosis. Intracellular organisms in the present study appeared not to be severely damaged morphologically. However, treponemes in vacuoles frequently folded upon themselves and lost their spiral configuration. Since intracellular treponemes were relatively rare, the intracellular environment does not appear to favor multiplication of *T. pallidum* under the conditions studied. However, intracellular organisms might be associated with the latency of syphilis, or some stage of treponemal survival in the host might require an intracellular residence.

Fitzgerald et al. (8) and Repesh et al. (21), studying treponemal attachment to cultured mammalian cells by using SEM, reported that *T. pallidum* attached only at its very tip; however, in some of their micrographs the organisms apparently attached along their entire length, and during fixation the organisms settled onto the cultured cell surface, giving the appearance of full-length attachment rather than tip attachment. Hayes et al. (16) also demonstrated attachment to rabbit testicular cell membrane by a terminal end, suggesting that the extreme tip of *T. pallidum* serves as a specific organelle of attachment, and that it might seem likely that a specialized organelle mediates treponemal attachment. In the present study, extracellular treponemes attached to Sf1Ep cells primarily by body spiral as well as by a terminal end. Although we have considered that this may be an artifact of fixation, it remains possible that treponemes attaching at their tip could be observed in vertical sections. However, numerous dark-field observations of viable treponemes colonizing tissue culture cells have been made. Even though most appear to be attached by their tip, treponemes attached to tissue culture cells have been observed to oscillate from side to side much like a bow across a violin. The small point of attachment appears to be "fluid" and able to traverse the complete length of the treponeme. The reason that most treponemes appear to be attached at their tip could be due to the pull that actively motile organisms exert on their point of attachment. Since this point appears to be fluid in nature, it may slide toward one end of the treponeme. Therefore, at any instant, most treponemes appear to be terminally attached, when in reality they can also be attached by the body spiral. By reversing the spiraling motion, the treponeme can reverse its direction and the point of attachment will then slide down the spiral body to the opposite end. This fluid characteristic of the outer envelope has also been observed in *Leptospira interrogans*; Charon et al. (4) observed that antibody-coated beads attached to its outer envelope could traverse the entire length of the spirochete depending upon the direction of motion. We are uncertain of the percentage of organisms displaying this behavior; however, numerous treponemes within the same sample can be observed moving in this manner. With TEM, the exact point of attachment was observed in several tissue sections as an electron-dense layer between the Sf1Ep cell membrane and the outer membrane of the treponeme. To our knowledge, this electron-dense layer has not been reported by other investigators. Many serial sections were observed to find these election-dense points. Furthermore, agar overlaying to physically freeze the integrity of the samples appeared to improve the probability of observing these points of attachments.

Aikawa et al. (1) reported on erythrocyte entry by malarial parasites and demonstrated that the erythrocyte membrane was thickened at the attachment site when malarial merozoite entered the erythrocyte. The formation of an electron-dense layer at the attachment site of the treponeme appears to be different from that in malarial infection, because thickening of host cell membrane was not observed.

Extracellular treponemes and Sf1Ep cells were usually surrounded with amorphous material, which sometimes appeared to connect the organisms to the cell surface. Treponemal colonies formed on the cell surface were also surrounded with this material. This amorphous material was not strongly stained with ruthenium red as demonstrated by Zeigler et al. (27). The agar overlaying used in the present study may have prevented a strong ruthenium red staining. The putative glycosaminoglycan material was presumed to be from the Sf1Ep cells, and its function in the attachment of treponemes to the cell surface is questionable. The effect of this material upon treponemal growth and survival also is still unclear.

Fitzgerald et al. (15) and Oakes et al. (18) described various degrees of cultured cell disruption caused by coincubation with *T. pallidum*. In the present study, we observed excessive vacuolation in the cultured cells infected with *T. pallidum*; this gave the appearance of morphologically damaged cells. However, it does not seem that the morphological damage was caused by treponemal infection alone, because uninfected cultures also showed similar vacuolation under the conditions studied. Cell vacuolation may have been due to low oxygen tension and reducing agents in the culture medium.

The deterioration of the Sf1Ep cells usually does not appear for 9 to 11 days. Sf1Ep cells can maintain their normal integrity for several days under low (5%) oxygen tension, because they are slow-growing cells with a generation time of 25 to 30 h. They can adapt to a low oxygen environment, because their metabolic rate of oxygen consumption is slower than that of most tissue culture cells.

For this reason, serial passage of *T. pallidum* should be attempted no later than 7 days from initial infection. The abnormal appearance of the Sf1Ep cells under oxygen stress probably contributes to the termination of treponemal growth after 10 to 12 days in vitro.

Agar overlaying prevented the removal of treponemes and Sf1Ep cells during the preparation for electron microscopy and allowed us to successfully observe more treponemes attached to the host cells, even though it appeared to prevent strong ruthenium red staining of putative cell surface glycosaminoglycan. However, this problem may be resolved by agar overlaying after postfixation. In addition, in situ fixation and the vertical ultrathin sectioning also gave more precise information about the treponemal location in tissue culture.

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