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Mucopolysaccharide Material Resulting from the Interaction of *Treponema pallidum* (Nichols Strain) with Cultured Mammalian Cells

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During incubation of *Treponema pallidum* (Nichols strain) with cultured mammlian cells derived from normal rabbit testes (NRT), an amorphous material accumulated at the surface of the cultured cells. This material was randomly distributed on all tissue cells within the culture chambers. The amount of amorphous material was dependent on the treponemal inocula. With 3×10^8 organisms per ml, this material was readily apparent within 2 days; with 4×10^7 organisms per ml, this material was detectable within 4 to 5 days; with lower inocula, the accumulation of amorphous material was far less apparent. Deposition of this surface-associated material required attachment of treponemes to the cultured cells, and the amount deposited was related to the number of treponemes attached per cell. This amorphous material was not detected when NRT cells were incubated with preparations of *T. pallidum* that were heat or air inactivated. In addition, the accumulaton of amorphous material was not due to a soluble component from host testicular tissue or to a soluble component developing during treponemal infection. This was demonstrated by the inability of membrane filtered preparations of *T. pallidum* to induce the deposition of amorphous material at the surface of the cultured cells. The nature of this material appeared to be acidic mucopolysaccharide as indicated by its metachromatic staining properties, its stainability with ruthenium red, and its partial degradation by bovine and streptomyces hyaluronidase. This amorphous material that accumulated in vitro at the surface of cultured cells may be similar to the mucoid material that accumulates in vivo during syphilitic infection.

The occurrence of mucoid material within syphilitic lesions is characteristic of infection with *Treponema pallidum*. The earliest observations of degeneration of tissue with mucoid accumulation were made by Uhlenhuth and Mulzer (27) in 1912 and by Graetz and Delbanco (10, 11) in 1914; others have confirmed these findings (1, 12, 21). Scott and Dammin (18-20) expanded these earlier observations. At various times after inoculation with *T. pallidum*, infected tissues were excised and stained with toluidine blue. The metachromasia that was observed correlated with the infective process. As the number of treponemes increased, more pronounced metachromasia was recorded. The authors attributed this change to the occurrence of increased amounts of mucopolysaccharides. Prior treatment of infected tissue with bovine hyaluronidase abolished the metachromasia, suggesting that the predominant mucopolysaccharide was hyaluronic acid and/or chondroitin sulfate.

Turner and Hollander (24) reported greatly increased numbers of treponemes within lesions, and increased amounts of mucoid material in animals treated with cortisone. In a later publication, Turner and Hollander (25) confirmed the metachromatic observations of Scott and Dammin (18-20). In syphilis this mucoid material is most apparent in the early stages of rapidly progressing dermal and testicular lesions.

At present, opinion is divided concerning the derivation of this mucoid material. Turner and Hollander (26) suggested that this material was produced by the organisms, whereas Rice (17) suggested that the mucopolysaccharides were produced by the animal tissue in response to the stimuli of treponemal infection.

The determination of the origin of mucopolysaccharides in syphilitic infection is complicated by the relatively large amounts of mucopolysaccharides within uninfected testicular and dermal tissues. A partial solution to this problem is to utilize cultured mammalian cells instead of rabbits. Tissue culture is a biological tool that emulates the environmental conditions encoun-

tered by the organisms in vivo (6, 8, 9). The addition of *T. pallidum* to cultured cells results in the rapid attachment of the organisms to the cultured cells. With extended periods of incubation, a deposition of amorphous material was observed on the surface of the cultured cells. The purpose of this report is to examine both the derivation and the nature of this material.

MATERIALS AND METHODS

Animals. Healthy Dutch Belt rabbits weighing 4 to 5 pounds (ca. 1.8 to 2.2 kg) were used. They were housed at 19 to 22°C and given antibiotic-free food and water ad libitum.

Organism. *T. pallidum* (Nichols strain) was maintained by intratesticular passage. Approximately 1×10^7 to 3×10^7 treponemes were inoculated per testis. Daily intramuscular injections of cortisone acetate (Merck Sharp & Dohme), 6 mg/kg of body weight, were initiated 3 days postinoculation. After the development of a satisfactory orchitis, usually requiring 9 to 13 days, rabbits were sacrificed by intracardial injection of sodium pentobarbital. Testes were removed, rinsed in physiological saline, and placed in tissue culture medium containing 4 mM glutathione, 1 mM cysteine, and 1 mM dithiothreitol. The testes were sliced and extracted aerobically at room temperature for 20 to 30 min. The suspension was then centrifuged at $1,000 \times g$ for 7 min at 24°C to sediment particulate matter. A hemocytometer was used for counting of organisms. Dilutions were made with the filtrate of treponemal suspensions that were passed through a membrane filter (Millipore Corp.; pore size, 0.20 μm).

Cultured cells. The cells were derived from normal rabbit testes (NRT) as previously described (8). The tissue culture medium contained Eagle minimal essential medium with Hanks balanced salts and 4 mM $NaHCO_3$ buffered with 30 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid). This medium was supplemented with 10% fetal bovine serum (vol/vol) that had been heat inactivated at 56°C for 30 min. Fetal bovine serum was obtained from Reheis Chemicals (Kankakee, Ill.).

Cultured cells were grown to 20 to 100% confluency in Sykes-Moore chambers (22) that were incubated at 36°C. These chambers were then inoculated with suspensions of *T. pallidum*. The chambers were placed into vacuum desiccators that were evacuated and flushed five times with a gas mixture of 2.5% oxygen–92.5% nitrogen–5% carbon dioxide. This gaseous environment is referred to as 2.5% oxygen. Incubation was performed at 30°C.

Phase-contrast microscopy. The chambers containing treponemes and cultured cells were visualized daily by phase-contrast microscopy using 400× magnification with a Zeiss inverted microscope. Pictures were taken using 400× magnification with a Leitz microscope with an attached Leitz automatic exposure timing device. Due to optical problems with the Leitz microscope, the treponemes attached to the surface of the cultured cells were difficult to visualize in the photomicrographs. The film used was Kodak Tri-X pan for black and white prints.

Metachromatic staining. Crystalline toluidine blue was dissolved in physiological saline at a final concentration of 0.5% (wt/vol). The culture medium within Sykes-Moore chambers containing NRT cells was removed. Toluidine blue was added directly to the chambers for 2 min. The dye was then removed and the cultured cells were examined immediately.

Hyaluronidases. Crystalline type IV hyaluronidase derived from bovine testicular tissue (Sigma) was dissolved in tissue culture medium without fetal bovine serum to yield a final concentration of 3,000 National Formulary units/ml; the pH was approximately 6.8. A second type of crystalline hyaluronidase derived from *Streptomyces hyalurolyticus* (Miles Laboratories) was dissolved in tissue culture medium without fetal bovine serum to yield a final concentration of 20 turbidity reducing units/ml; the pH was approximately 7.0.

RESULTS

Studies have indicated that under the conditions previously described, maximum survival of *T. pallidum* occurred in the presence of cultured cells in an atmosphere of 2.5% oxygen (7) and at a temperature of 30°C (unpublished data). Experiments were performed to determine effects on treponemal survival with different inocula of organisms. Sykes-Moore chambers containing cells derived from NRT cells at 20% confluency were inoculated with 5×10^6, 4×10^7, and 9×10^7 treponemes per ml. After 3 days of incubation, a distinct amorphous material had accumulated at the surface of many of the cultured cells in the chamber inoculated with 9×10^7 treponemes per ml. In the other 2 chambers a similar surface-associated material was not observed at this time. After 5 days of incubation, all cultured cells within the chamber containing 9×10^7 treponemes per ml were almost completely covered with this amorphous material. In the chamber containing 4×10^7 treponemes per ml, some amorphous material was observed at the cultured cell surface; in the chamber containing 5×10^6 treponemes per ml, no amorphous surface-associated material was apparent. In these experiments, the majority of treponemes were actively motile for the 5 days of incubation.

The micrograph in Fig. 1 depicts control chambers that were incubated for 5 days without *T. pallidum*. Note the relatively smooth appearance of the surface of the cultured cells. The micrographs in Fig. 2 and 3 depict cultured cells after 5 days of incubation with 4×10^7 and 9×10^7 treponemes per ml. Note the amorphous material that has accumulated at the surface of the cultured cells. This material was randomly distributed on the cultured cell surface. It did not appear to be preferentially deposited in specific areas, such as the nuclear region or the outer periphery of the cells. In addition, all

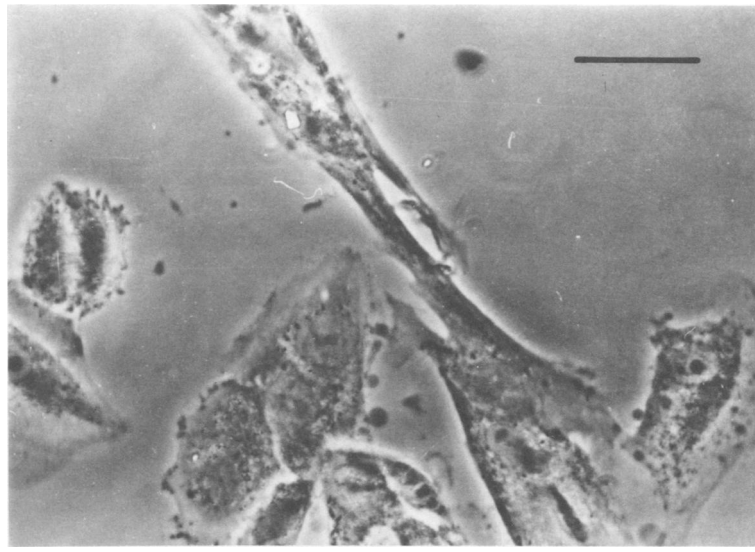

FIG. 1. *Cultured NRT cells at 20% confluency incubated for 5 days without treponemes. Bar, 20 μm.*

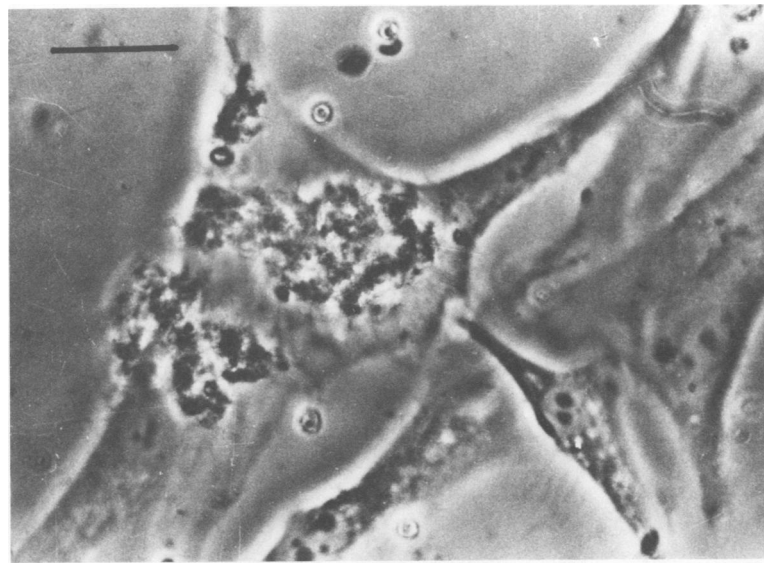

FIG. 2. *Cultured NRT cells at 20% confluency incubated for 5 days with 4×10^7 treponemes per ml. Bar, 20 μm.*

cultured cells exhibited amorphous material; some cells contained more than others.

Further experiments confirmed the observation that the accumulation of amorphous material on cultured cells was dependent on the size of the treponemal inoculum. Preparations of *T. pallidum* were concentrated by high-speed centrifugation (12,000 × *g* for 30 min) and adjusted to 3×10^8 treponemes per ml. Subsequent inoculation of NRT cells revealed an extremely rapid build-up of amorphous material within 2 days, as shown in Fig. 4. After 5 days, the NRT cells were heavily coated with amorphous material, as shown in Fig. 5.

The relationship of treponemal numbers to the deposition of amorphous material was demonstrated in a different way. Suspensions of *T. pallidum* were adjusted to 4×10^7 organisms per ml and added to Sykes-Moore chambers containing NRT cells at 20, 50, and 100% confluency. The availability of additional tissue cells in the latter two chambers resulted in fewer trepo-

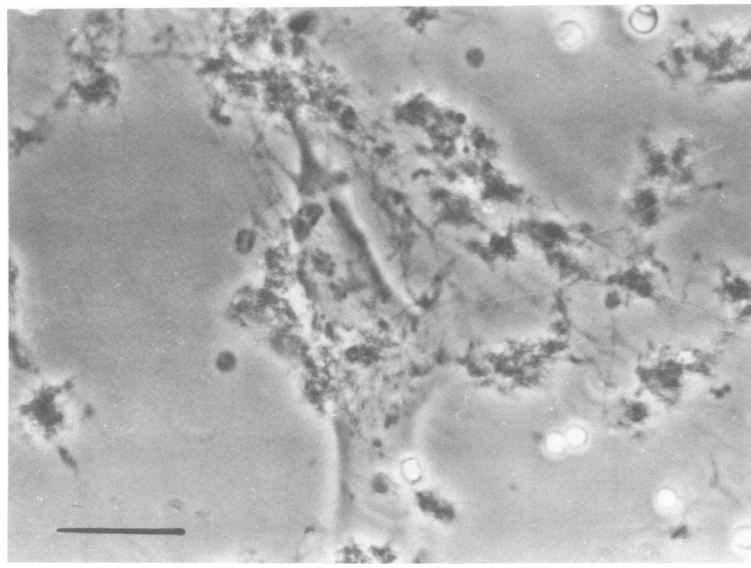

FIG. 3. *Cultured NRT cells at 20% confluency incubated for 5 days with 9×10^7 treponemes per ml. Bar, 20 μm.*

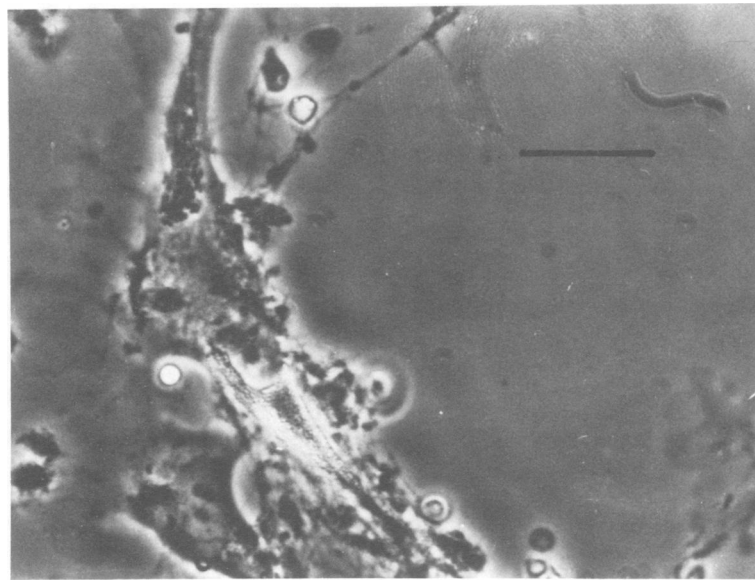

FIG. 4. *Cultured NRT cells at 20% confluency incubated for 2 days with 3×10^8 treponemes per ml. Bar, 20 μm.*

nemes attached per individual cultured cell. After 5 days of incubation, maximal amorphous material at the cultured cell surface was detected within the chamber containing the fewest cultured cells (largest number of treponemes per cell). The amount of amorphous material was similar to that in Fig. 2. Minimal amorphous material was detected in the chamber containing the most cultured cells, as shown in Fig. 6. The amount of surface-associated amorphous material in the remaining chamber was intermediate between the other two chambers. In all further experiments, cultured cells were used at approximately 20% confluency.

Experiments were performed to determine whether the deposition of amorphous material on the surface of the cultured cells was related to treponemal viability. Under these conditions of treponeme-cultured cell incubation, the majority of organisms were actively motile for 5 to 6 days. During this time, increases in surface-associated material occurred. If incubation were continued for another 6 days, increases in amounts of amorphous material were not observed. In fact, this material gradually dissipated from the cultured cell surfaces during this extended incubation period. To further examine the potential relationship of viable organisms to

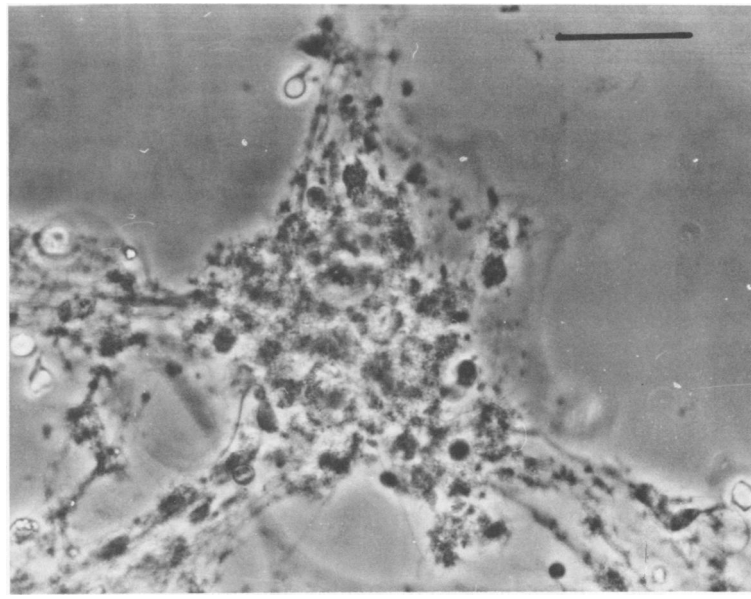

FIG. 5. *Cultured NRT cells at 20% confluency incubated for 5 days with 3×10^8 treponemes per ml. Bar, 20 μm.*

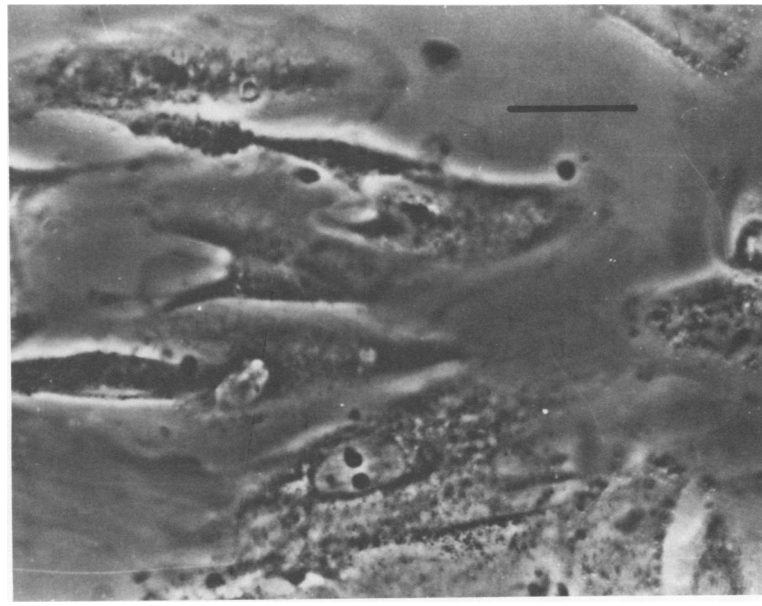

FIG. 6. *Cultured NRT cells at 100% confluency incubated for 5 days with 4×10^7 treponemes per ml. Bar, 20 μm.*

the deposition of amorphous material, the following experiments were performed. A freshly harvested treponemal suspension was divided in two. One portion was then heat inactivated (50°C for 5 min). Both preparations were added to NRT cells in Sykes-Moore chambers and incubated for 5 days. At this time, much amorphous material had accumulated at the surface of the cultured cells exposed to viable *T. pallidum*. In direct contrast, cell surface amorphous material was not detected in the chambers exposed to heat-inactivated *T. pallidum*.

Two other procedures were used to demonstrate the requirement of viable treponemes for deposition of amorphous material on the surface of cultured cells. In one, freshly harvested treponemes were incubated for 20 h in a test tube under aerobic conditions. At this time approximately 10% of the organisms were sluggishly motile and 90% were nonmotile. This preparation was then added to cultured cells and incubated in 2.5% oxygen. After incubation for 1 day, all treponemes were nonmotile. After incubation for 5 days, no amorphous material was apparent on the surface of the NRT cells. In a related experiment, a freshly-harvested preparation of *T. pallidum* was inoculated into chambers containing NRT cells. These chambers were then incubated in air instead of the usual atmosphere of 2.5% oxygen. Within 1 day, all organisms were nonmotile. After incubation for 5 days, no amorphous material was apparent at the surface of the cultured cells. In both of the above experiments, appropriate controls were performed that contained fresh preparations of treponemes incubated in 2.5% oxygen. Five days of incubation revealed the expected deposition of amorphous material on the surface of the cultured cells.

The amorphous material might result from host testicular components or treponemal by-products that were extracted in conjunction with the treponemes from the infected tissue. This component may have been heat labile or air labile. To explore this possibility, a freshly harvested suspension of *T. pallidum* was divided in two. One portion was then filtered through a membrane filter (Millipore Corp.) with a pore size of 0.20 μm. The filtrate was examined by dark-field microscopy and no treponemes were observed. This filtrate and the suspension of *T. pallidum* were added to separate chambers containing cultured cells. After incubation for 5 days, much amorphous surface material was detected in the treponeme-containing chamber. With the membrane-filtered preparation, no amorphous surface material was apparent.

One further variation in experimental protocol was tested to demonstrate the production of amorphous material. Suspensions of *T. pallidum* at 4×10^7 per ml were added to NRT cells, and the chambers were inverted so that the tissue cells were topside. During the subsequent 5 days of incubation, the treponemes remained actively motile and gradually settled to the bottom of the chamber. Examination of the cultured cells revealed relatively few attached organisms and, as shown in Fig. 7, no accumulation of amorphous material. In these chambers, some cul-

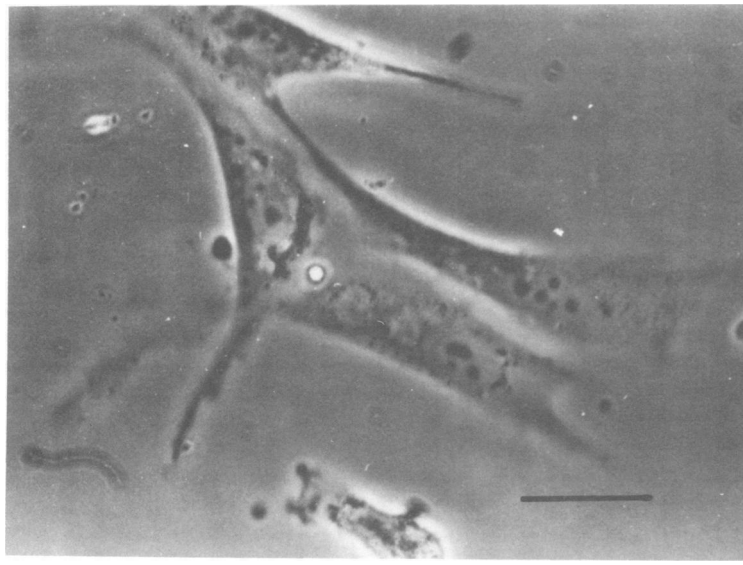

FIG. 7. *Cultured NRT cells at 20% confluency incubated inverted for 5 days with 4×10^7 treponemes per ml. Bar, 20 μm.*

tured cells had dislodged from the top, settled to the bottom, attached to the glass, and grown. These cells had numerous attached treponemes and, as shown in Fig. 8, were covered with amorphous material. Thus, within the same chamber, differences in the amount of amorphous surface-associated material were readily apparent. These differences appeared to be a direct reflection of the numbers of treponemes attached to the cultured cell surface.

Attempts were made to determine the nature of the amorphous material associated with the surface of the cultured cells. Sykes-Moore chambers were inoculated with *T. pallidum* and incubated for 5 days. The culture medium was then removed and replaced with toluidine blue. The amorphous material at the surface of the cultured cells was metachromatic, appearing as a deeply stained purple-violet color. This was in sharp contrast to the surface of the cultured cells that was stained a bluish color. The amorphous material was also stained with ruthenium red, appearing as a bright yellowish color contrasted with the slight reddish color of the cultured cells.

Previous reports of the in vivo accumulation of mucoid material during syphilitic infection had indicated that this material was partially degraded by bovine hyaluronidase (19, 20, 23, 25, 26). A suspension of *T. pallidum* at 9×10^7 treponemes/ml was added to NRT cells. After 5 days, a large amount of amorphous material was apparent. Bovine hyaluronidase and streptomyces hyaluronidase were then added to the chambers and incubated for 20 to 24 h. The presence of the enzymes greatly reduced the amount of amorphous material on the surface of the NRT cells. Figure 9 depicts cultured cells exposed to streptomyces hyaluronidase. These cultured cells, after incubation with hyaluronidase, were stained with toluidine blue. As expected, the occurrence of material that stained a purple-violet color was greatly diminished.

DISCUSSION

Four observations suggested that *T. pallidum* was responsible for the deposition of amorphous material at the surface of the NRT cells. First, the amount of amorphous material was dependent on the amount of treponemes. This was demonstrated in two ways. When various treponemal inocula were added to a constant number of cultured cells, differing amounts of amorphous material resulted. If the initial inoculum were below 4×10^7 treponemes per ml, little amorphous material was apparent. With increasingly larger inocula, the accumulation of surface-associated material on the NRT cells occurred at faster rates. The largest inoculum tested was 3×10^8 treponemes per ml; at this treponemal concentration, amorphous material was apparent 2 days postinoculation.

The relationship between treponemal concentration and the quantity of amorphous material was demonstrated in a second way. A constant inoculum of *T. pallidum* was added to varying amounts of cultured cells. When 4×10^7 treponemes/ml were incubated with NRT cells at 20,

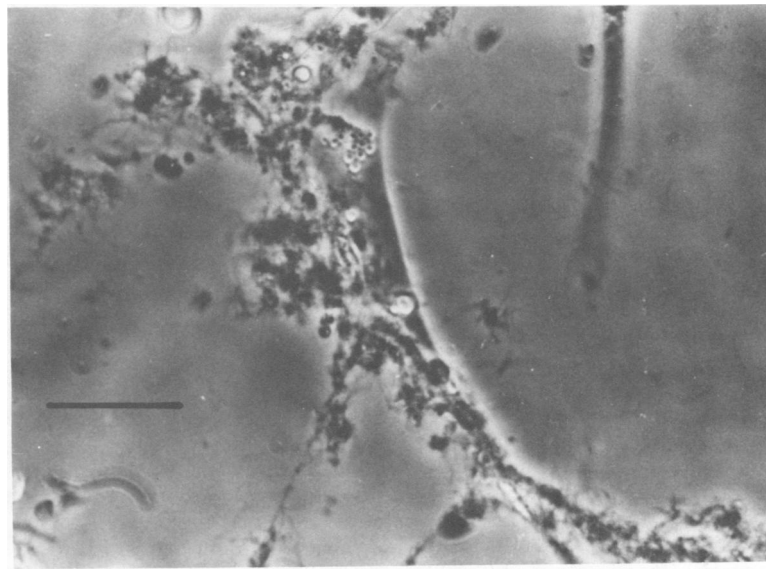

FIG. 8. *Cultured NRT cells at 20% confluency incubated inverted for 5 days with 4×10^7 treponemes per ml. This cell dislodged from the top surface and settled to the bottom of the chamber. Bar, 20 μm.*

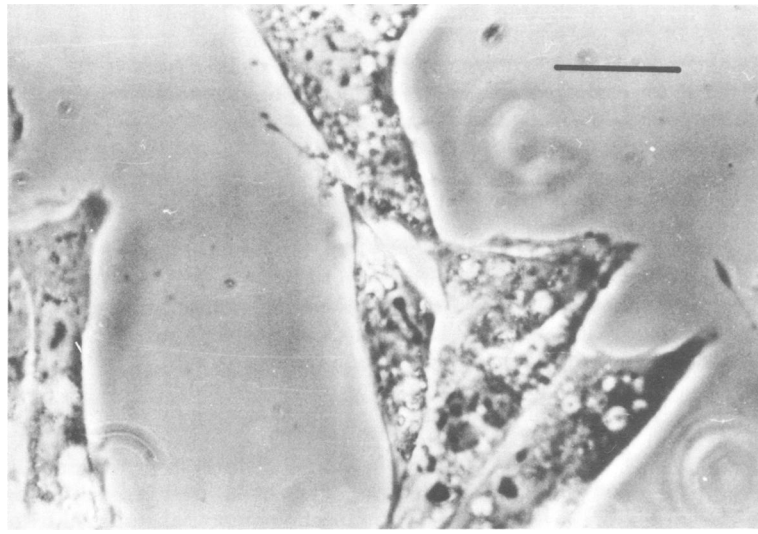

FIG. 9. *Cultured NRT cells at 20% confluency incubated for 5 days with 9 × 10^7 treponemes per ml. Streptomyces hyaluronidase was added for 20 to 24 h. Bar, 20 μm.*

50, and 100% confluency, far more amorphous material was detected in the chambers containing the fewest cultured cells. It is important to consider the ratio of treponemes per NRT cell. When cultured cells attain 100% confluency, they continue to grow. This results in tight packing and overlapping of individual cells. Thus, the chambers at 100% confluency probably contain at least 10 times as many cells as chambers at 20% confluency. With an inoculum of 4 × 10^7 treponemes per ml and NRT cells at 20% confluency, 50 to 80 treponemes were attached to each cell. With the same treponemal inoculum and NRT cells at 100% confluency, 5 to 10 treponemes were attached to each cell. Similar numbers of treponemes per cell occurred when NRT cells at 20% confluency were inoculated with 10^7 treponemes per ml. These low numbers produced little detectable amorphous material at the surface of the cultured cells.

The use of reduced temperatures for incubation was an important factor in these observations. The accumulation of amorphous material was most obvious with minimal numbers of cultured cells. NRT cells survived quite well at 30°C in 2.5% oxygen, but did not reproduce. Chambers containing 20% confluent cells after 5 days of incubation at 30°C remained at 20% confluency. Incubation at 37°C, however, resulted in cultured cell growth to 100% confluency during the 5-day period, and the accumulation of amorphous material was less apparent.

A second observation that implicated *T. pallidum* as the source of amorphous material was the requirement for viable organisms. In these experiments, the treponemes retained active motility for approximately 5 days. During this period there was a gradual build-up of amorphous material on the NRT cells. Incubation beyond 5 days did not result in further accumulation of amorphous material. In fact, this material dissipated with extended incubation. Furthermore, if treponemal preparations were inactivated by heating or by exposure to air, then added to cultured cells, no amorphous material was apparent after 5 days. In an alternative procedure, *T. pallidum* was incubated with the NRT cells and incubated in air instead of 2.5% oxygen. This resulted in loss of motility within 1 day. Amorphous material did not accumulate at the surface of these cultured cells.

A third observation suggested that *T. pallidum* produced the amorphous material. The procedure used for harvesting treponemes from infected testes results in extraction of host tissue constituents in conjunction with the treponemes. In addition, the treponemal preparations contain both soluble by-products of *T. pallidum* that occur during infection and inflammatory products from the host response to infection. Membrane filtrates of treponemal suspensions that were added to NRT cells and incubated for 5 days did not produce amorphous material. This indicated that the amorphous material does not emanate from the interaction of cultured cells with host tissue, treponemal by-products, or host-inflammatory products.

One final observation strongly implied that *T. pallidum* produced the amorphous material. Chambers were inoculated with treponemes and

incubated in an inverted position so that the cultured cells were topside. During the 5 days of incubation, the organisms remained actively motile and gradually settled to the bottom of the chambers. The NRT cells that were topside contained few attached organisms; amorphous material was not detected on these cells. A few NRT cells apparently dislodged from the top surface of the chambers and settled to the bottom. These cells had numerous attached treponemes and were heavily coated with amorphous material. Thus, within individual chambers, depending on the number of attached treponemes per cell, differing amounts of amorphous material were detected.

The amorphous material that accumulated at the surface of the NRT cells appears to be acidic mucopolysaccharide. It was partially degraded by bovine and streptomyces hyaluronidase; it exhibited metachromasia which is characteristic for mucopolysaccharides (2–4); and it reacted with ruthenium red, an inorganic dye that is specific for mucopolysaccharides (16). These mucopolysaccharides could be hyaluronic acid and/or chondroitin sulfate, or closely related polysaccharides that are susceptible to degradation by hyaluronidase.

T. pallidum (Nichols strain) has an outer surface layer that is apparently acidic mucopolysaccharide (5, 28). During treponemal attachment to cultured cells, this coat material may be extruded from the organisms to the surface of the cultured cells. In support of this possibility, Fitzgerald and Johnson (submitted for publication) have shown that *T. pallidum* (Nichols strain) under certain conditions of in vitro incubation has a tendency to form clumps. These clumped organisms contain acidic mucopolysaccharides. The authors postulated that these clumps resulted from treponemal synthesis of capsule-like material comprised of acidic mucopolysaccharides. This would explain the observations within this paper that the accumulation of amorphous material is of treponemal origin and is dependent on treponemal concentration, treponemal viability, and treponemal attachment to the cultured cells.

Four previous observations have suggested that the mucoid accumulation within syphilitic lesions is of treponemal origin. First, metachromatic tissue changes correspond to the progress of infection (19, 20, 23, 25, 26). Second, the number of organisms within infected tissue closely parallels the amount of mucoid material that accumulates (20, 23, 25, 26). Third, the more virulent strains of *T. pallidum* are specifically associated with increased mucoid accumulation (23, 26). Fourth, assays for mucopolysaccharide concentrations within the mucoid material obtained from testicular infections have shown a direct relationship between the numbers of treponemes present and the mucopolysaccharide concentration (Fitzgerald and Johnson, submitted for publication).

The observations reported within this paper provide further evidence that the mucoid material in syphilis is of treponemal, rather than host, origin. One qualification, however, is important in evaluating these observations. Cultured mammalian cells are capable of synthesizing acidic mucopolysaccharides (13–15). It is possible that the attachment of *T. pallidum* triggers this capability, resulting in excess mucopolysaccharide synthesis by the cultured cells.

It will be important to establish the specific derivation of this material. If it is primarily synthesized by *T. pallidum*, investigations can be undertaken to elucidate its potential role in the infective process. Turner and Hollander (26) and Turner (23) have suggested that mucopolysaccharide production is a virulence factor of *T. pallidum*. Fitzgerald and Johnson (submitted for publication) have demonstrated that the testicular fluid that accumulates during infection contains large quantities of mucopolysaccharides. The injection of this material resulted in an immunosuppressive phenomenon (Fitzgerald and Johnson, submitted for publication). Previously, in vivo infection was the only method available for observing this mucoid material. The use of cultured mammalian cells provides a feasible alternative. The interaction of cultured cells with *T. pallidum*, and the subsequent deposition of mucopolysaccharide, may prove to be a valuable biological tool for further characterizing the potential role of mucopolysaccharides in the pathogenesis of syphilis.

ACKNOWLEDGMENTS

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