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Characterization of the Attachment of *Treponema pallidum* (Nichols Strain) to Cultured Mammalian Cells and the Potential Relationship of Attachment to Pathogenicity

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The interaction of *Treponema pallidum* (Nichols strain) with 19 different cultured mammalian cell types was examined. These types included cells derived from testis, kidney, spleen, lung, epidermis, cervix, urethra, and nerve tissue of human, rabbit, or rat origins. They represented normal and malignant cells, epithelial and fibroblastic morphology, cell lines, and cell strains. Large numbers of organisms attached to the cultured cells; this attachment prolonged the time of retention of active treponemal motility. Attachment was examined in terms of the number of treponemes inoculated, cultured cells present, and actively growing versus stationary cultured cells; the motility of the treponemes; the viability of the cultured cells; and the different cell passages. In sharp contrast to the attachment of *T. pallidum,* 11 nonpathogenic treponemes failed to attach to cultured cells. Immune syphilitic rabbit serum prevented the attachment of *T. pallidum* to cultured cells, as indicated by phase contrast microscopy and rabbit inoculations. This blockage of attachment by immune serum occurred without interfering with active motility of the organisms. Results are discussed in terms of the potential relationship of attachment to the pathogenicity of *T. pallidum.*

An important step in the initiation of the disease process for certain microbial pathogens is the attachment of the organisms to various host tissues (21). Attachment is mediated through specific surface components of the organisms that interact with specific surface components of the host cells. Microbial virulence is the result of many complex, interacting factors. Selective attachment to certain tissues or cells may be one of these factors. A number of reports have correlated attachment capabilities with virulence (1, 5, 10, 13, 15, 16).

Syphilis can be described as a generalized infection. Shortly after the entry and penetration of *Treponema pallidum,* organisms rapidly disseminate to several organs and tissues, probably via the blood stream and lymphatics. This occurs in both human and experimental rabbit syphilis. Within minutes after intratesticular inoculation of rabbits, organisms can be detected within the popliteal lymph nodes; within 2 h, only 7% of the injected treponemes remain within the testicular tissue (4). Little is known about the mechanisms that enable *T. pallidum* to localize within various organs and tissues after being transported from the site of initial entry. It remains to be determined whether this localization is related to the ability of *T. pallidum* to specifically attach to cells in vivo.

In assessing the feasibility of using tissue culture for in vitro cultivation of *T. pallidum,* we observed a rapid attachment of large numbers of organisms to the cultured cells (7). This confirmed the earlier preliminary observation by Wright (29) concerning attachment of *T. pallidum* to cultured cells. Further studies to explore the significance of this observation indicated that attachment extended the times of retention of treponemal motility and virulence (7, 8, 9). Other reports have confirmed these findings (19, 20). Of further interest is that two nonpathogenic treponemes, *T. denticola* and *T. phagedenis* biotype Reiter, and heat-killed *T. pallidum* failed to attach to cultured cells (7). The purpose of this report is to characterize the attachment phenomenon as it may relate to the pathogenicity of *T. pallidum.*

MATERIALS AND METHODS

Rabbits. Adult male New Zealand white rabbits weighing four to six pounds (ca. 1.81 to 2.72 kg) were used. Animals were screened for *Treponema paraluis*

cuniculi infection by physical examination and serological testing with the Rapid Plasma Reagin circle card test (Hynson, Westcott, and Dunning, Baltimore, Inc.). Rabbits were kept at 19 to 20°C and given antibiotic-free food and water ad libitum.

T. pallidum. The Nichols strain of *T. pallidum* was maintained by intratesticular passage in New Zealand white or Dutch Belt rabbits. Each testis received 1×10^7 to 3×10^7 treponemes. Daily intramuscular injections of cortisone acetate (Merck, Sharp, & Dohme, West Point, Pa.) at 6 mg/kg were given beginning on day 3 after inoculation of testes. After an orchitis had developed, usually within 10 to 14 days, the animals were sacrificed by intracardial injection of sodium pentabarbitol. Testes were removed, rinsed in physiological saline, and placed into tissue culture medium. The basal tissue culture medium contained Eagle minimal essential medium with Hanks balanced salt solution supplemented with 2× amino acids, 2× vitamins, 4 mM $NaHCO_3$, 1 mM dithiothreitol, 4 mM glutathione, and 1 mM cysteine buffered with 30 mM *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid. Heat-inactivated (56°C for 30 min) fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.) was added at 10 to 20% (vol/vol). The tissue was sliced and extracted 10 to 20 min under aerobic conditions without shaking. The suspension was centrifuged at room temperature at 1,000 × *g* for 7 min to sediment particulate matter, and the supernatant containing the organisms was decanted. Due to their poor chance of survival, treponemes were not used if the testicular tissue was hemorrhagic or if low yields of organisms ($<10^8$ treponemes per rabbit) were obtained.

Nonpathogenic treponemes.. In addition to *T. pallidum,* the following organisms were utilized: *T. phagedenis* biotype Kazan, *T. phagedenis* biotype phagedenis, *T. phagedenis* biotype Reiter, *T. refringens* biotype refringens, *T. refringens* biotype Noguchi, *T. denticola, T. denticola* biotype ambiguum, *T. denticola* biotype TD-2, *T. vincentii, T. scoliodontum,* and *T. denticola* biotype microdentium. The medium (14) and the methods for assessing growth of these organisms (11) have been described. After cultures had grown to mid-logarithmic stage, they were adjusted to about 1×10^7 to 3×10^7 organisms per ml and inoculated into Sykes-Moore chambers containing the cultured cells. The cultured cells survived well in the presence of this growth medium. These experiments were performed in an atmosphere of either air or 95% nitrogen–5% carbon dioxide. Microscopic observations were made periodically for 24 h.

Source and maintenance of cultured cells. The host species and tissue origin of the cultured cells are listed in Table 1. The following cell lines were obtained from the American Type Culture Collection: LLC-RK_1, SflEpNBL-11, TRK-1, RPMI 2650, and C6. Human skin epithelial cells were provided through the courtesy of Carl Seiter of Reheis Chemical Co.; HEp 2, KB, 751G, 833K, and 253J were provided through the courtesy of Arthur Elliott, Patrick Cleveland, and David Bronson of the University of Minnesota. ME 180 was initially propagated from a human cervical carcinoma (26).

Primary cultures from testes of New Zealand white or Dutch Belt rabbits and lung tissue of Dutch Belt rabbits were established as previously described (7). Similar procedures were used to establish two different types of primary cultures from New Zealand white rabbit kidneys. NRK_1 was predominantly fibroblastic-like; it was derived from whole kidney. NRK_2 was predominantly epithelial-like; it was derived from the medulla of the kidney. For studies on treponemal attachment, cell cultures that had been passaged between two and nine times were used.

Primary cultures were also established from Dutch Belt rabbit spleen tissue. The spleen was removed, minced, and passed through a fine-mesh wire screen. The cells were suspended in tissue culture medium and centrifuged. The cell pellet was suspended in distilled water for 30 s to lyse the erythrocytes, diluted with tissue culture medium, centrifuged, and resuspended in tissue culture medium. The cells were then incubated for 24 h at 37°C; the unattached cells were decanted and placed into another culture vessel and incubated at 37°C. Cells began attaching to the glass surface and within 1 week were confluent. These cells were predominantly epithelial-like. For studies on treponemal attachment, second-passage cultures were used.

Primary cultures were established from Dutch Belt rabbit dermal tissue. A portion of the epidermis was removed, cut into pieces of about 1 mm^3, and placed into culture medium. After 3 days of incubation at 36°C, the tissue pieces were discarded and fresh medium was added. The resulting cell growth was predominantly fibroblastic-like. For studies on treponemal attachment, second-passage cultures were used.

The basal tissue culture medium for growth of cells was identical to that used for extraction of *T. pallidum,* except that dithiothreitol, glutathione, and cysteine were omitted.

Phase contrast microscopy. All phase contrast observations were made with cultured cells grown in Sykes-Moore chambers (25) by using a magnification of ×400. Cultured cells at 20 to 50% confluency were inoculated at 36°C with freshly harvested organisms adjusted to 1×10^7 to 7×10^7 treponemes per ml. The number of attached organisms was estimated by observing a minimum of 20 fields that contained at least 100 cultured cells.

Rabbit serum. For experiments involving the interaction of *T. pallidum* with serum, freshly isolated normal rabbit serum (NRS) and immune rabbit serum (IRS) were used. Blood was removed by cardiac puncture and centrifuged immediately. All sera were heat inactivated at 56°C for 30 min. The history of exposure to *T. pallidum* for rabbits used as sources of immune sera follows: IRS^a was from a male rabbit 4 months after intratesticular inoculation with 3×10^7 treponemes; NRS^a was from a male rabbit; IRS^b was from a male rabbit 7 months after intradermal inoculation with 10^8 treponemes followed by two challenges 1 and 2 months before this experiment; NRS^b was from a male rabbit; IRS^c was from a female rabbit 2 years after intradermal inoculation with 10^8 treponemes followed by 20 challenges about 1 month apart; NRS^c was from a female rabbit; IRS^d was from three male rabbits 1 to 2 years after intradermal inoculation with 3×10^7 treponemes followed by eight challenges 1 month apart; and NRS^d was from 3 male rabbits. For

NRS sera, blood was removed from rabbits with a nonreactive Rapid Plasma Reagin circle card test. Both NRS and IRS were isolated and used the day of the experiment.

Challenge injections were performed intradermally with inocula of 0.1 ml at six to eight sites on the shaved backs of rabbits. Each site was injected with freshly harvested *T. pallidum* adjusted to 1×10^7 to 4×10^7 treponemes per ml. Solid resistance was evidenced by the failure of lesions to develop at any of the inoculation sites.

Serum treatment of *T. pallidum*. Treponemes were extracted in tissue culture medium for 10 to 15 min containing dithiothreitol (2 mM), glutathione (8 mM), and cysteine (2 mM), but without fetal bovine serum. After centrifugation at $1{,}000 \times g$ for 7 min, the organisms were decanted and diluted with medium to the appropriate concentration. The treponemal suspension was then added to serum to yield a final serum concentration of 50%. These preparations were placed in test tubes and flushed with 3% oxygen, 5% carbon dioxide, and 92% nitrogen as previously indicated (9). This step is referred to as preincubation. After 22 or 23 h of preincubation at 36°C, samples were removed, added to cultured cells, and incubated for 1.5 to 3 h at 36°C. This step is referred to as incubation with cultured cells.

Virulence determinations. Procedures for preincubation and incubation with cultured cells were followed as described above. For each sample to be tested for virulence, 0.1 ml was injected intradermally into the shaved backs of rabbits. These experiments were repeated twice with four rabbits; the data represent the average of eight separate sites per sample. After preincubation, each preparation containing either normal or immune serum was injected intradermally. At the same time, 2 ml of each preparation was incubated for 2 h with a confluent monolayer of cultured cells in T-15 flasks. After this incubation, a sample of each culture fluid was removed and injected intradermally; these samples represent unattached treponemes. The remaining fluid from each culture was discarded, and the monolayers were washed three times with tissue culture medium. Two milliliters of fresh medium was then added to each preparation, the cultured cells were disrupted as previously described (7), and samples were injected intradermally; these samples represent attached treponemes. The incubation period corresponding to the day of erythema and induration appearance was related to virulent numbers of treponemes as indicated previously (7).

RESULTS

Initial studies had indicated that *T. pallidum* attached to NRT (normal rabbit testes) cells and ME180 cells (7). In an attempt to find another cell type that would be superior, the organisms were incubated with a variety of cell types (Table 1). Cells were grown to 20 to 50% confluency in Sykes-Moore chambers, inoculated with 1×10^7 to 7×10^7 treponemes per ml, and incubated at 36°C in 3% oxygen, 5% carbon dioxide, and 92% nitrogen (9).

All 19 cell types interacted with the organisms. Attachment of treponemes began to occur within minutes after inoculation. Within the individual chambers, attached organisms retained motility about 24 h longer than unattached organisms. In addition, each of the 19 different cell types extended the time of retention of treponemal motility. In general, treponemal motility was observed for about 4 to 6 days in the presence of cultured cells compared to 2 to 4 days in the absence of cultured cells. Differences in the number of attached organisms per cultured cell were detected (Table 2). With some cell types, 1 to 5 treponemes were attached per

TABLE 1. *Cultured cells utilized in attachment experiments*

Designation	Origin	Source	Established cell line	Predominant morphology
NRT-DB	Rabbit	Testes (Dutch Belt)	No	Fibroblastic-like
NRT-NZ	Rabbit	Testes (New Zealand white)	No	Fibroblastic-like
NRK_1	Rabbit	Kidney (New Zealand white)	No	Fibroblastic-like
NRK_2	Rabbit	Kidney (New Zealand white)	No	Epithelial-like
SflEpNBL-11	Rabbit	Epidermis (cottontail)	Yes	Epithelial
LLC-RK_1	Rabbit	Kidney (New Zealand white)	Yes	Epithelial
TRK-1	Rabbit	Kidney	Yes	Epithelial
BTW-1	Rabbit	Spleen (Dutch Belt)	No	Epithelial-like
BTW-A	Rabbit	Epidermis (Dutch Belt)	No	Fibroblastic-like
BST-DB	Rabbit	Lung (Dutch Belt)	No	Epithelial-like
HSE	Human	Epidermis	Yes	Epithelial
ME180	Human	Cervical carcinoma	Yes	Epithelial
KB	Human	Epidermoid carcinoma	Yes	Epithelial
751G	Human	Urothelial carcinoma	No	Epithelial
253J	Human	Urothelial carcinoma	Yes	Epithelial
RPMI 2650	Human	Quasi-diploid tumor	Yes	Epithelial
833K	Human	Testicular tumor	No	Epithelial
HEp 2	Human	Epidermal carcinoma	Yes	Epithelial
C6	Rat	Glioma	Yes	Epithelial

TABLE 2. *Attachment of T. pallidum to various cultured cells*

Cultured cells	% Confluency of cultured cells	Treponemal inoculum	Incubation (h)	Range of treponemes per cell
NRT-DB	50	3×10^7	3	10–15
NRT-NZ	25–50	3×10^7	5	10–30
NRK_1	25–50	2×10^7	3	10–15
NRK_2	25–50	2×10^7	3	10–15
SflEpNBL-11	25	4×10^7	4	10–15
LLC-RK_1	25	4×10^7	4	10–15
TRK-1	50	3×10^7	4	10–15
BTW-1	20	7×10^7	3	40–50
BTW-A	20	7×10^7	3	40–50
BST-DB	20	6×10^7	5	10–30
HSE	25–50	3×10^7	3	1–15
ME180	50	1×10^7	3	2–6
KB	20	1×10^7	4	1–3
751G	50	7×10^7	3	10–30
253J	50	7×10^7	3	20–40
RPMI 2650	25–50	5×10^7	1	1–3
833K	20	1×10^7	3	1–5
HEp 2	20	1×10^7	4	1–3
C6	50	3×10^7	4	10–15

cell, whereas with other cell types 40 to 50 were attached per cell. Although there were differences in the number of attached treponemes per cell, it was noteworthy that with each of the 19 cell types virtually all cells within each individual chamber had attached treponemes. We did not find a cell type in which *T. pallidum* failed to attach.

In previous research, NRT cells were used up to the ninth passage. It was important to assess potential differences with different passages. *T. pallidum* was inoculated into chambers containing either primary or secondary, seventh-, or ninth-passage NRT cells. No major differences in treponemal attachment or prolongation of survival were detected. In further experiments, treponemes attached just as well to actively growing cells (20 to 50% confluent) as to stationary cells (100% confluent). The organisms, however, survived for longer periods in the presence of confluent cells. This may be related to the number of cultured cells present. When treponemes were inoculated into chambers containing different quantities of cells, motility was retained for proportionately longer periods with increasing cell numbers.

Previous work had indicated that heat-killed *T. pallidum* failed to attach to cultured cells (7). In close agreement, preparations of *T. pallidum* that were incubated aerobically for 24 h and were nonmotile ("aged") failed to attach. Attachment was also dependent upon the physiological status of the cultured cells. Treponemes were added to NRK_1 and NRT cells that were freeze-thawed and rendered nonviable, as indicated by trypan blue permeability, and to NRK_1 and NRT cells that were viable. After 3 h, with the viable NRK_1 cells, about 25 to 35 treponemes were attached to each cell. With the nonviable NRK_1 cells, attachment occurred but was reduced; only one to three treponemes were attached per cell. After 3 h of incubation of treponemes with NRT cells, every cell of the viable preparation had attached organisms, whereas only 3 of 25 cells of the nonviable preparation had attached treponemes. This reduction in treponemal attachment in the presence of nonviable cells was accompanied by a shorter period of retention of active motility.

The microscopic appearance of *T. pallidum* attached to NRT cells is shown in the phase contrast pictures of Fig. 1, 2, and 3. Treponemes above and below the field of focus are slightly blurred. As previously shown with scanning electron microscopy (6), the treponemes did not exhibit a preference for one specific area of the cell. They appeared to be randomly distributed on the cell surface. Similar numbers of treponemes were attached to each cell. The difference in the sizes of the individual cultured cells is readily apparent. All three pictures were taken using one culture chamber. Figure 1 shows a very wide cell in the process of division. Figure 2, which has the same magnification as Fig. 1, shows two cultured cells, one that is wide and another that is elongated with a thin cytoplasm. Figure 3 has a higher magnification of attached treponemes.

The number of treponemes attached per cell is dependent upon the initial inoculum. Correspondingly, more treponemes attached with larger inocula. However, as previously indicated (7, 8), the percentage attached per total inoculum remains at about 50 to 60%. When 10^6, 10^7,

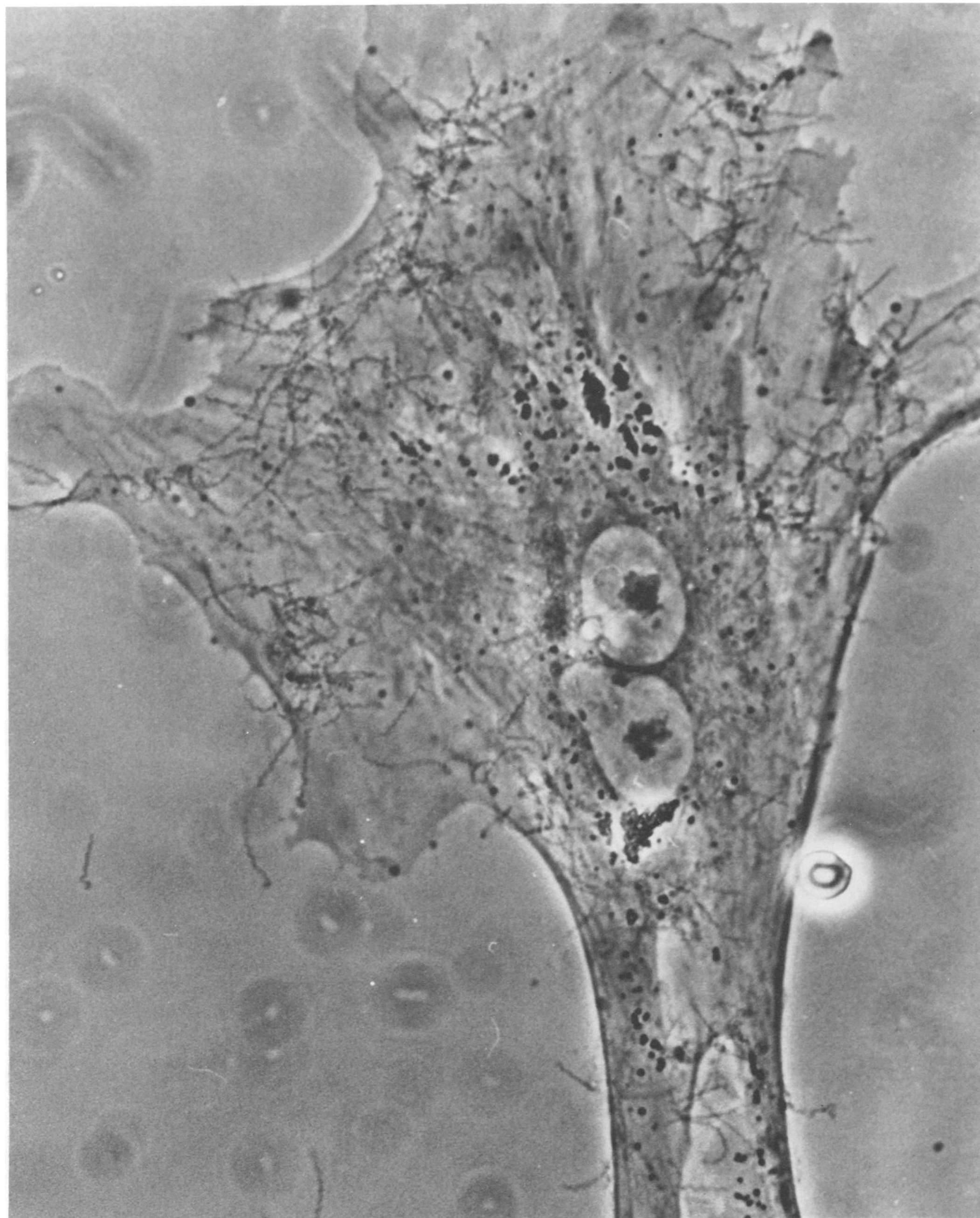

FIG. 1. *Cultured cells derived from normal rabbit testes showing the large number of attached T. pallidum. × 1,030.*

or 10^8 treponemes per ml were inoculated, about half of the total number of treponemes visualized per microscopic field had attached.

An important finding related to these studies was that *T. pallidum* appears to attach in vivo in a fashion similar to the in vitro attachment to cultured cells. During extraction of organisms from testicular tissue, large numbers of actively motile treponemes attached to small pieces of tissue were frequently observed. The same observations were made with samples removed from treponemal skin lesions. These organisms

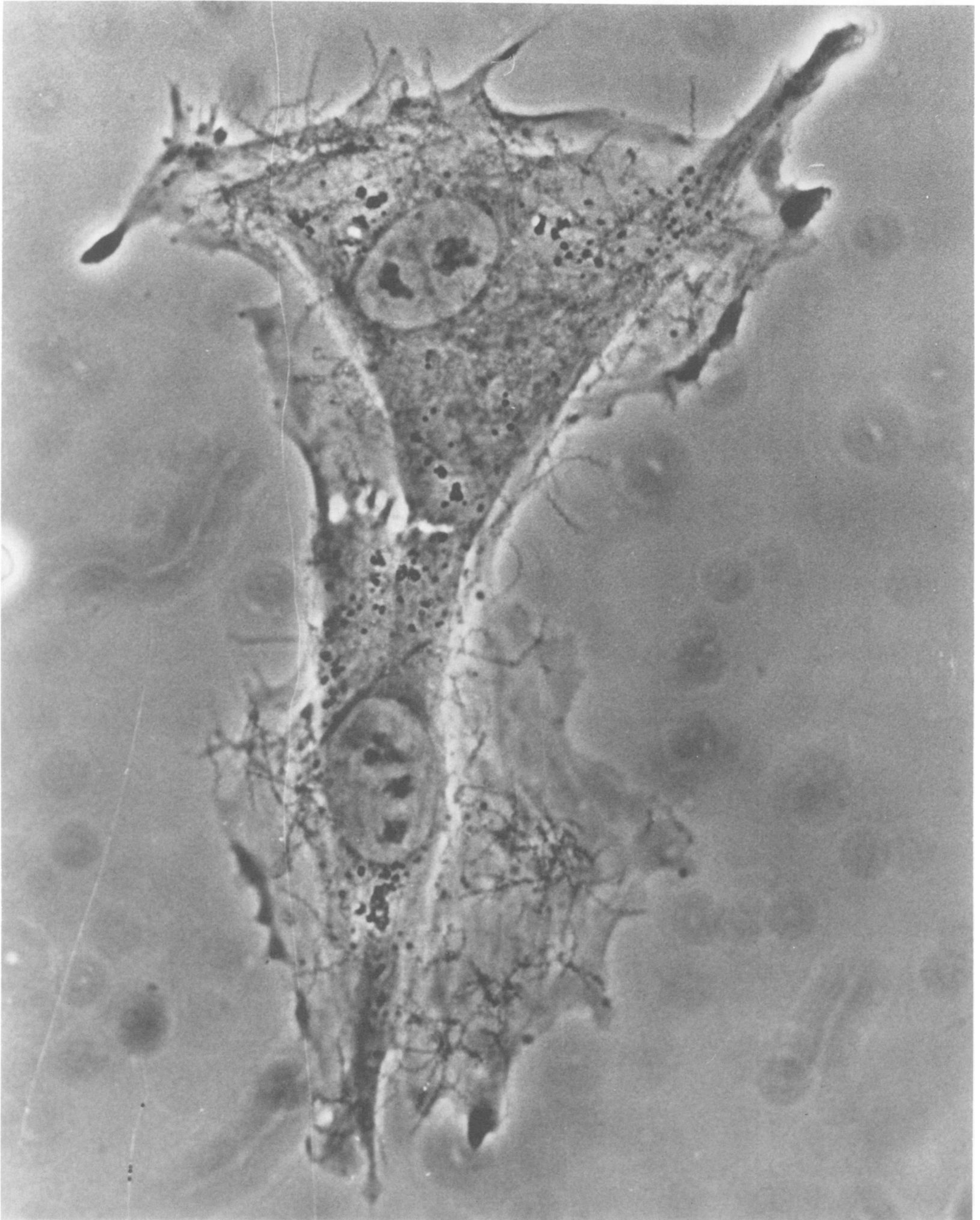

FIG. 2. *Two other cultured normal rabbit testes cells with numerous attached T. pallidum. Note the size variations of the cultured cells relative to Fig. 1.* × *1,030.*

were attached end on as previously described for cultured cells (6).

In previous work, two nonpathogenic treponemes failed to attach to cultured cells (7). These experiments were extended to other nonpathogenic treponemes (Table 3). Treponemal suspensions were inoculated into chambers containing NRT cells. Some of these anaerobic treponemes were rapidly inactivated in air. For this reason, all cultures were incubated both in air and in an atmosphere of 95% nitrogen–5% carbon dioxide. Observations were made periodically for 24 h.

None of the 11 nonpathogens attached to the

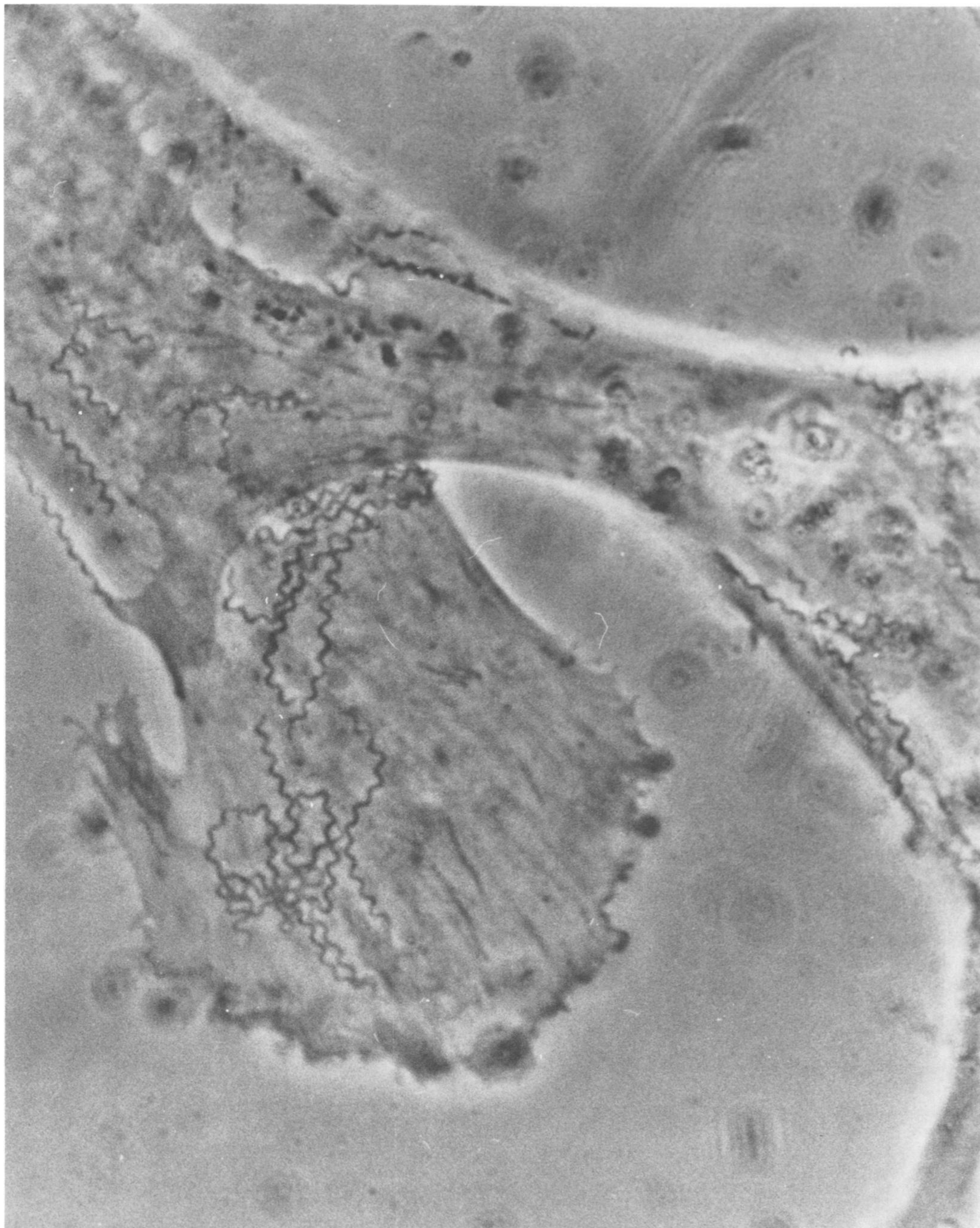

FIG. 3. *Higher magnification of T. pallidum attached to a portion of a cultured normal rabbit testes cell.* × *2,060.*

NRT cells during incubation in air or in nitrogen–carbon dioxide. After incubation for a few hours, some of the organisms lost motility and settled on top of the cultured cells. Lack of attachment by these organisms was demonstrated by inverting the chambers so that the cultured cells were topside. After 20 min, reexamination of the chambers indicated that virtually all of the cultured cells were free of organisms. When identical procedures were followed with *T. pallidum,* the organisms remained attached to the cells.

In related experiments, *T. phagedenis* biotype Kazan, *T. phagedenis* biotype Reiter, and *T.*

vincentii were incubated with other cell types (Table 4). Incubations were also performed in air and in nitrogen-carbon dioxide. After 24 h, these nonpathogens did not attach to these different cell types.

Additional experiments were designed to determine whether attachment of *T. pallidum* was influenced by IRS. When organisms were suspended in NRS or IRS at a concentration of 5 $\times 10^6$ to 4 $\times 10^7$ treponemes per ml and immediately incubated with cultured cells, no differences in numbers of attached treponemes were detected. However, if *T. pallidum* was preincubated with NRS or IRS before incubation with cultured cells, differences in the number of attached organisms became apparent. Preliminary experiments indicated that a preincubation of about 15 to 20 h was required for significant inhibition of attachment.

A series of experiments were performed by using freshly isolated NRS and IRS from various rabbits (Table 5). The history of *T. pallidum* exposure of each rabbit used as a source of IRS is described in Materials and Methods. The preparations of *T. pallidum* and rabbit sera were preincubated for 22 or 23 h. The percent motilities of organisms in the NRS and IRS suspensions were quite similar within each experiment after this preincubation step. Additionally, at this time, the number of treponemes was identical in both the NRS and IRS preparations; no clumping of treponemes was detected in either preparation.

After preincubation, the organisms were incubated with cultured cells for 1.5 to 3 h, and an approximate estimate of the number of attached treponemes per 100 cultured cells was made. In each of five separate experiments, far fewer treponemes were attached in the presence of IRS as compared to NRS. The percent blockage of attachment ranged from 73 to 100%.

These experimental procedures were then applied to virulence assessments. The data from two separate experiments with four rabbits per experiment involving 1 and 26 h of preincubation are presented in Table 6. The treponemal concentration in each experiment was 5 $\times 10^6$ to 7 $\times 10^6$ organisms per ml. In agreement with previous findings, after 26 h of preincubation, the percent motilities in both preparations were similar. In experiment 1, treponemal motility in NRS was 92%, and in IRS, 80%; in experiment 2, treponemal motility in NRS was 98%, and in IRS, 94%.

After 1 h of preincubation, no significant differences in time of lesion appearance were evident. Similar numbers of virulent treponemes in the NRS and IRS preparations were detected in the preincubation sample, in the sample representing unattached treponemes, and in the sample representing attached treponemes. After 26 h of preincubation, differences were apparent; IRS delayed lesion appearance 5 to 8 days with each sample. The data (Table 6) indicate the estimated virulent numbers based on the time of lesion appearance (7). In general, preincubation with IRS for 26 h resulted in an approximate two-log decrease in number of virulent treponemes with each sample. In addition, lesion development was affected by the longer period of IRS preincubation. Every site inoculated developed treponemal lesions (48/48 for NRS and 48/48 for IRS). After 1 h of preincubation, typical dark-field-positive lesions occurred at all sites for both NRS and IRS preparations. After 26 h of preincubation, 21 of 24 NRS sites were dark-field positive and one site developed an atypical lesion; 15 of 24 IRS sites were dark-field positive and 7 sites developed atypical lesions. These atypical lesions were erythematous,

TABLE 3. *Interaction of T. pallidum and nonpathogenic treponemes with NRT-NZ cells after 24 h of incubation*

Organism	Range of treponemes per cell
T. pallidum (Nichols strain)	30–70
T. phagedenis biotype Kazan	ND[a]
T. phagedenis biotype phagedenis	ND
T. phagedenis biotype Reiter	ND
T. refringens biotype refringens	ND
T. refringens biotype Noguchi	ND
T. denticola	ND
T. denticola biotype ambiguum	ND
T. denticola biotype TD-2	ND
T. denticola biotype microdentium	ND
T. scoliodontum	ND
T. vincentii	ND

[a] None detected.

TABLE 4. *Interaction of nonpathogenic treponemes with various cultured cells after 24 h of incubation*

Organism	Cultured cells	Range of treponemes per cell
T. phagedenis biotype Kazan	C6	ND[a]
	Sf1EpNBL-11	ND
	LLC-RK$_1$	ND
	TRK-1	ND
	HSE	ND
	NRT-NZ	ND
T. phagedenis biotype Reiter	LLC-RK$_1$	ND
	Sf1EpNBL-11	ND
	NRT-NZ	ND
T. vincentii	LLC-RK$_1$	ND
	Sf1EpNBL-11	ND
	NRT-NZ	ND

[a] None detected.

TABLE 5. *Blockage of attachment of T. pallidum to cultured cells by IRS, as indicated by phase contrast microscopy*

Expt	Treponemes per ml	Preincubation of treponemes for 22-23 h with serum		Attachment of serum-treated treponemes to cultured cells after 1.5-3 h	
		Serum[a]	% Motile	Approximate no. of treponemes per 100 cells	% Blockage
1	1.0×10^7	NRS[a]	76	600	73
		IRS[a]	76	165	
2	4.0×10^7	NRS[b]	68	100	80
		IRS[b]	60	20	
3	7.0×10^6	NRS[c]	92	400	100
		IRS[c]	76	0	
4	1.4×10^7	NRS[c]	52	350	100
		IRS[c]	64	0	
5	4.0×10^6	NRS[d]	98	750	83
		IRS[d]	98	125	

[a] Histories described in Materials and Methods.

TABLE 6. *Blockage of attachment of T. pallidum to cultured cells by IRS as indicated by intradermal inoculation of rabbits*

Treponemal[a] preincubation without cultured cells and incubation with cultured cells	NRS[b]			IRS[b]		
	Day EI[c] (range)	Day EI (avg)	Estimated treponemes per ml	Day EI (range)	Day EI (avg)	Estimated treponemes per ml
1 h of preincubation	5–8	6.2	3×10^6	7–8	7.4	8×10^5
1 h of preincubation followed by 2-h incubation with cultured cells						
Unattached treponemes	5–7	6.0	4×10^6	7–8	7.1	1×10^6
Attached treponemes	7–8	7.1	1×10^6	7	7.0	1×10^6
26 h of preincubation	6–7	6.2	3×10^6	9–18	13.5	8×10^3
26 h of preincubation followed by 2 h of incubation with cultured cells						
Unattached treponemes	6–8	6.6	2×10^6	8–16	11.8	2×10^4
Attached treponemes	7–17	8.6	2×10^5	11–18	14.0	6×10^3

[a] 5×10^6 to 7×10^6 treponemes per ml.
[b] History listed in Materials and Methods.
[c] EI, Initial appearance of erythema and induration.

slightly indurated, and did not increase in size (beyond 10 mm) or ulcerate.

DISCUSSION

A number of findings reported in this paper extend previous observations. Initial studies had indicated that *T. pallidum* readily attached to NRT and ME180 cells; this attachment was accompanied by a prolongation in treponemal survival (7, 8). This treponeme-tissue cell interaction has now been extended to 17 other cell types; these included cells derived from testes, kidney, spleen, lung, epidermis, cervix, urethra, and nerve tissue of human, rabbit or rat origins. They represented normal and malignant cells, epithelial and fibroblastic morphology, cell lines, and cell strains. Although slight variations were observed, one cell type did not emerge as clearly superior to others in extending the time of retention of treponemal motility. Differences were detected in numbers of treponemes attached per individual cell. The complexity of the various different cultured cell types makes specific comparisons between types difficult under these experimental conditions. Inherent limitations include: differing growth sensitivities of the cultured cells to pH, to serum concentration, to medium content, and to the 3% oxygen environment; difficulties in obtaining identical numbers of cultured cells within each Sykes-Moore chamber; and differences in the amount of surface area available for treponemal interaction between the smaller epithelial and larger fibroblastic cells.

Additionally there is a spectrum of cell size variations within an individual chamber. The photographs of NRT cells (identical magnifications) (Fig. 1 and 2) were taken from the same Sykes-Moore chamber. Figure 1 shows a very wide cell in the process of division; Figure 2 shows two cells, one that is wide and one that is elongated with a very thin cytoplasm. Because of these size variations, it is not feasible to make exact counts of the number of treponemes per cell. We feel that it is more appropriate to list an approximate range of attached treponemes per cell (Table 2). Due to the numerous inherent limitations, we are not emphasizing the differences in the number of attached treponemes per cell. At this time, it is important to emphasize only that *T. pallidum* interacts with a wide variety of cultured cells.

With any given cell type, *T. pallidum* did not exhibit a predilection for only certain cells within the individual Sykes-Moore chamber. Virtually every cell present was susceptible to treponemal attachment, and similar numbers of organisms were attached to each cell within the chamber. Heat-killed *T. pallidum* failed to attach to cultured cells (7). In agreement, "aged" preparations of *T. pallidum* that were nonmotile also failed to attach. This emphasizes that attachment is an active treponemal process (7, 8). In addition, the status of the cultured cells influenced the attachment of *T. pallidum*. With nonviable cultured cells, far fewer treponemes attached, with consequent poorer survival of the organisms.

Attachment of the treponemes began to occur immediately after inoculation of the cultured cells, and large numbers of organisms were attached within a few hours. The number of attached treponemes was related to the number initially inoculated. In general, about 50 to 60% of the added organisms attached as indicated in previous studies (7, 8). This percentage remained relatively constant despite variation in inoculum size. This observation suggests that only a certain population of the organisms were capable of attaching.

The data concerning the lack of attachment by the 11 nonpathogenic treponemes must be carefully evaluated. These organisms were grown in vitro in nutrient medium, whereas *T. pallidum* was grown in vivo within testicular tissue. It is certainly possible that the inability of nonpathogenic treponemes to attach to cultured cells results from the different growth conditions. However, it is also possible that the ability of *T. pallidum* to attach actually reflects a specific virulence determinant lacking in the nonpathogens. Other reports have correlated attachment of microorganisms in vitro with virulence. Ogawa et al. (15, 16) demonstrated that the ability of *Shigella* to induce disease was related to their ability to infect cultured cells. The initial step of cell infection was attachment of organisms. Virulent *Shigella, S. flexneri* and *S. dysenteriae*, readily attached and infected cultured cells; avirulent *Shigella* did not attach. Furthermore, *S. sonnei,* which causes relatively mild symptoms in human dysentery, infected cultured cells, but its infectivity rate was considerably less than that of the more virulent *Shigella*. Swanson (22) characterized the attachment of *Neisseria gonorrhoeae* to cultured mammalian amniotic cells. Virulent gonococci (colony type 2) markedly adhered to cultured cells; avirulent gonococci (colony type 4) adhered poorly. In further studies (23) using two additional cell types and iodine-labeled gonococci, type 4 attached less readily than type 2.

To strengthen the proposed relationship between attachment of *T. pallidum* and virulence, it will be very important to examine the capabilities of other pathogenic treponemes to attach to cultured cells.

An additional important observation in these studies was that the attachment of *T. pallidum* appears to occur in vivo. During extraction of *T. pallidum* from infected rabbit testes, organisms attached to small pieces of host tissue were routinely observed. Identical observations were made with samples of rabbit skin lesion material from an intradermal infection. These organisms were actively motile and attached end on as previously described for cultured cells (6).

The data obtained with immune rabbit sera were of interest in our attempt to understand the pathogenesis of experimental syphilis. Other reports indicate that immune serum is capable of blocking microbial attachment. Ogawa et al. (16) used antisera to prevent the attachment of *Shigella* to cultured cells. Punsalang and Sawyer (17) reported that gonococcal antibody inhibited the attachment of piliated gonococci to human epithelial cells. Jones and Rutter (12) observed that antisera prevented attachment of *Escherichia coli* to the small intestines of piglets. Williams and Gibbons (28) found that antibodies in secretions impaired the attachment of bacteria to epithelial surfaces. At this point, we can only state that IRS contains a factor(s) not found in NRS that prevents attachment of *T. pallidum* without interfering with treponemal motility. This factor was not immediately active; its demonstration required a period of pre-sensitization of the organisms. Blocking of treponemal attachment was not apparent when the preincubation step was omitted. If the organisms were added to the cultured cells at the same time as the IRS, similar numbers of treponemes

were attached in the NRS and IRS preparations after 26 h of incubation.

The recent work of Bishop and Miller (2) may have some application to the data presented in this paper. They demonstrated a neutralizing factor(s) within IRS that correlates with the development of immunity in experimental syphilis. The protocol of Bishop and Miller involved an incubation period of 16 h and a concentration of about 10^4 treponemes per ml. Heat-inactivated IRS slightly reduced the numbers of virulent organisms. With higher concentrations of about 10^6 treponemes per ml, the IRS did not reduce virulence. The heat-stable neutralizing factor could be similar to the factor that prevents attachment of *T. pallidum* to cultured cells. In our system, we used a higher concentration, about 5×10^6 to 7×10^6 treponemes per ml and observed blockage effects. This may have been due to the use of medium containing dithiothreitol (9), which permitted longer incubation periods (26 h).

In summary, the observations concerning treponemal attachment may explain some of the pathogenic mechanisms operative in syphilis. The data in Table 2 indicate that *T. pallidum* is capable of interacting with a variety of different cell types. This is particularly interesting in view of the ability of *T. pallidum* to localize within almost any tissue or organ of the body.

The observations concerning blockage of attachment by IRS may help to explain the immunity that develops in experimental syphilis. Humoral factors would have ready access to challenge treponemes and could prevent the organisms from disseminating and attaching to host tissue. Four other reports support this possibility (3, 4, 18, 27); when previously immune rabbits were challenged with *T. pallidum,* the organisms remained localized for a number of days at the site of injection and failed to disseminate to the draining lymph nodes. Unattached treponemes would then be susceptible to phagocytosis, lymphocyte inactivation, and other host defense mechanisms. On the other hand, some organisms might establish an intracellular residence; these organisms would be "protected" from the influence of the circulating humoral factors. If we can extend these findings to human syphilis, it would be interesting to speculate that humoral factors which prevent attachment, and consequent localization, form during the early stages of infection and neutralize extracellular organisms, resulting in the disappearance of the primary and secondary lesions. Organisms that have gained an intracellular residence may then be important in latency (24), in the recurrence of secondary manifestations, and in the occurrence of tertiary lesions.

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