Adherence of *Treponema pallidum* subsp. *pallidum* in the Rabbit Placenta (43804)

ANDREW W. O. BURGESS,* LOIS J. PARADISE,*,[1] DON HILBELINK,† AND HERMAN FRIEDMAN*

Department of Medical Microbiology and Immunology, and Department of Anatomy,† University of South Florida College of Medicine, Tampa, Florida 33612*

Abstract. **Congenital syphilis is the consequence of transplacental passage of *Treponema pallidum*. A system was developed to deliver virulent *T. pallidum*, Nichols strain, through an isolated uterine horn of a pregnant rabbit in order to investigate the mechanism by which *T. pallidum* is able to cross the placenta. While the pregnant rabbit was anesthetized, the ovarian artery and the uterine vein were cannulated and attached to a peristaltic pump. *Treponema pallidum* ($2–5 \times 10^8$ in 10–15 ml RPMI-1640) were circulated via the peristaltic pump throughout the horn for 2 hr, after which the placentas were removed, fixed in formalin, and embedded in paraffin. This system was used to investigate treponemal binding to rabbit placenta at Day 20 and 26 during the gestation period of the rabbit (29–32 days). Examination of 5-μm Dieterle silver stained tissue sections revealed (i) a greater number of spirochetes in the later gestational stage placentas (Day 26) than in the earlier placentas (Day 20), (ii) organisms adhering to the trophoblastic tissue surrounding the maternal blood channels, and (iii) organisms appearing to be in the process of penetrating the trophoblastic tissue or that had completely penetrated from the channels into the trophoblastic elements. We suggest that *T. pallidum* may be adhering to placental components that are differentially expressed during gestation of the rabbit.** [P.S.E.B.M. 1994, Vol 207]

Congenital syphilis is an infectious disease caused by the spirochete, *Treponema pallidum*, that is acquired transplacentally (1). It was generally believed that *T. pallidum* are unable to cross the placenta and infect the fetus before 18 weeks gestation until spirochetes were demonstrated in two fetuses that were at 9 and 10 weeks gestation, indicating that *T. pallidum* can also cross the placenta in the first trimester (2).

T. pallidum readily attaches to a variety of different cultured mammalian cells (3, 4). Pathogenic treponemes, unlike the avirulent species of treponemes, attached to the cultured cells (4–6) via terminal end structures, suggesting that they are specialized organelles which permit the initial surface colonization of host cells (7). Cell membranes also appear refractory to specific challenge with *T. pallidum* shortly after the initial surface parasitism (7). Together these studies provide evidence of ligand-receptor interactions between *T. pallidum* and host cells.

Successful induction of fetal infection has been reported in pregnant rabbits given multiple intravenous inoculations of large numbers of *T. pallidum* subsp. *pallidum* (8) and in Syrian hamsters infected with *T. pallidum* subsp. *endemicum* (9, 10). Wicher *et al.* reported an experimental model in which asymptomatic congenital syphilis infection occurs in guinea pigs given a single dose of *T. pallidum* subsp. *pallidum* (11). Obviously, the initial *T. pallidum*-placenta interaction would be as impossible to study in these experimental congenital syphilis animal models as in placental tissue obtained from syphilitic mothers, since the infection has been progressing for some time.

Therefore, we designed a method to investigate the penetration of *T. pallidum* from maternal to fetal tissues by circulating *T. pallidum* through an isolated uterine horn at Day 20 and 26 during gestation of a pregnant rabbit (29–32 days). Dieterle silver stained

[1] To whom requests for reprints should be addressed at Department of Medical Microbiology and Immunology, College of Medicine, University of South Florida, 12901 Bruce B. Downs Boulevard, Tampa, FL 33612–4799.

Received March 8, 1994. [P.S.E.B.M. 1994, Vol 207]
Accepted June 12, 1994.

0037-9727/94/2072–0180$10.50/0

sections revealed a greater number of treponemes attaching to Day 26 than Day 20 placentas, suggesting that the organisms are adhering to one or more placental components that may be present in higher quantities on Day 26 than on Day 20 gestation.

Materials and Methods

Bacteria. Virulent *T. pallidum* subsp. *pallidum*, Nichols strain, (*T. pallidum*) (obtained from James N. Miller, UCLA) was maintained by intratesticular passage in New Zealand White rabbits (Cummings, Lutz, FL) (12). Each testis received 1–5 $\times$ 10^7 treponemes. Daily intramuscular injections of 20 mg cortisone acetate (Sigma Chemical Co., St. Louis, MO) were given beginning the following day (13). After orchitis developed, usually within 10–13 days, the animals were euthanized by intracardiac injection of sodium pentobarbitol. Organisms were extracted in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heated (56°C for 30 min) normal rabbit serum at room temperature on a model 361 orbital shaker (Fisher Scientific, Fair Lawn, NJ) then adjusted to the appropriate concentration for passaging. Numbers of organisms were determined by darkfield microscopy.

For experiments, testes were exhaustively extracted at room temperature (14). Extracted organisms were separated from host debris by centrifugation at 250*g* for 10 min at room temperature. The treponemal suspension was then adjusted to the appropriate concentration for use in the experiments.

Surgical Preparation of the Pregnant Rabbit. After injection of ketamine hydrochloride (40 mg/kg body wt, im) to a pregnant rabbit, sodium pentobarbital, 6.67 mg/ml 0.85% NaCl (saline), was administered via a 23-gauge pediatric scalp vein needle in the ear vein. The volume delivered was titrated by the animal's response to stimulus.

Using aseptic precautions, the abdomen of the animal was opened and the skin pulled back to allow full access to the internal organs. The uterus was placed on a layer of sterile gauze previously soaked in sterile saline. A polyethylene (PE) cannula (Clay Adams, Parsippany, NJ) (o.d. less than 0.024 in), attached to a multichannel peristaltic pump (Buchler Instruments, Fort Lee, NJ), was placed into the ovarian artery. A PE 60 cannula (0.048-in o.d.), also attached to the peristaltic pump, was inserted into the uterine vein. A vascular clamp was placed on the ovarian vein, and the uterine artery was closed with a suture. Blood vessels connecting the right and left uterine horns were clamped with a hemostat. At this point, the vasculature of the uterine horn was isolated with "inflow" through the ovarian artery and "outflow" through the uterine vein. The open end of the "inflow" cannula was placed in a reservoir containing 5 units heparin/ml saline and the vasculature was flushed with this solution for 1 hr. Then both cannulae were placed in another reservoir containing 2–5 $\times$ 10^8 freshly extracted or heat-killed (56°C for 1 hr) *T. pallidum* in 10–15 ml RPMI-1640 in order to produce a closed circulation system. Organisms were circulated throughout the placentas at a constant rate of 0.8 ml/min for 2 hr. Thereafter, unbound treponemes were washed out of the placentas with saline for 1 hr. The placentas were removed, their positions with respect to the ovary were noted, and they were fixed in 10% neutral buffered formalin for paraffin processing.

Dieterle Spirochete Stain. Paraffin sections were stained by the Dieterle spirochete stain (15, 16). Briefly, 5-μm sections were collected on slides coated with aminoalkylsilane (17). After tissues were cleared of paraffin and hydrated to distilled water, sections were placed in preheated (56°C for 30 min) uranyl nitrate in a 57°C oven for 1 hr. Sections were then dipped once in distilled water followed by another dip in 95% alcohol. After a 3-min incubation in 10% alcoholic gum mastic (Sigma Chemical Co., St. Louis, MO), sections were dipped once in 95% alcohol. Slides were immersed in distilled water for 1 min, then were allowed to drain for 15 min. Sections were transferred to a preheated (56°C for 30 min) solution of 1% silver nitrate solution in the oven for 4 hr. After two dips in distilled water, sections were placed in the developer solution until they were pale yellow to light tan, usually 6 min. Sections were sequentially dipped twice in distilled water, 95% alcohol, acetone, and two changes of xylene, then were mounted under coverslip with Permount (Fisher, Fair Lawn, NJ). Treponemes, appearing as tight even spirals, stain dark brown to black while background stains pale yellow to light tan. Dieterle stained testicular sections (Fig. 1) were used as controls in each experiment for staining and for demonstrating typical treponemal morphology.

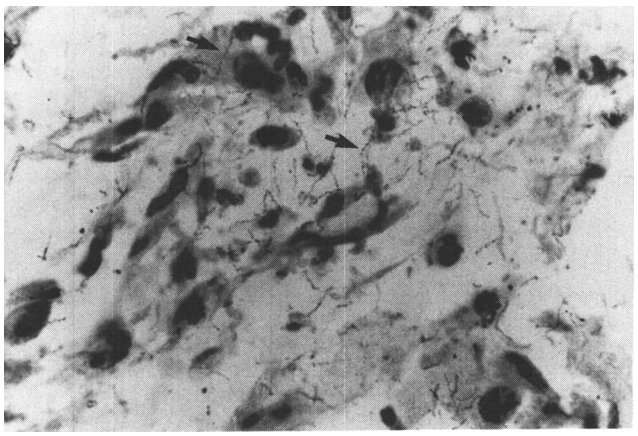

Figure 1. Dieterle-stained section (×1000) from rabbit testis control illustrating the morphology of classic appearing spirochetes (arrows).

Results

Surgical Preparation. A schematic diagram of the experimental design used to deliver virulent *T. pallidum* through an isolated uterine horn of a pregnant rabbit to investigate treponemal attachment to the rabbit placenta is shown in Figure 2. In order to verify that the medium was circulating through the placentas, hematoxylin and eosin stains were performed on 5-μm sections from control placentas (no surgical manipulation) and placentas that were exposed to *T. pallidum* and subsequently flushed for 1 hour. Examination of the labyrinth (Fig. 3) from these sections revealed that fewer than 1% maternal red blood cells remained in the flushed placentas (Fig. 4B) as compared with control placentas (Fig. 4A) indicating adequate circulation of the medium and flushing of the placentas.

***T. pallidum* Attachment.** In order to examine the adherence of *T. pallidum* to placentas at different stages in gestation of the rabbit, 20- and 26-day placentas were exposed to 2–5 × 10^8 *T. pallidum* for 2 hr. Dieterle stained sections from both 20- and 26-day placentas yielded classic appearing treponemes when compared with morphologically similar treponemes from rabbit testis control (Fig. 1). However, at Day 26 gestation, all three rabbits and all the placentas were positive for *T. pallidum,* whereas at Day 20, only one of three rabbits and 2 of 11 placentas were positive (Table I). In order to test for nonspecific binding of treponemes to host tissue, 4.7 × 10^8 heat-killed *T. pallidum* were circulated throughout the uterine horn of one rabbit at Day 26 gestation. All four placentas were negative for *T. pallidum*. These data demonstrate preferential binding of viable *T. pallidum* to 26-day placentas in comparison with 20-day placentas.

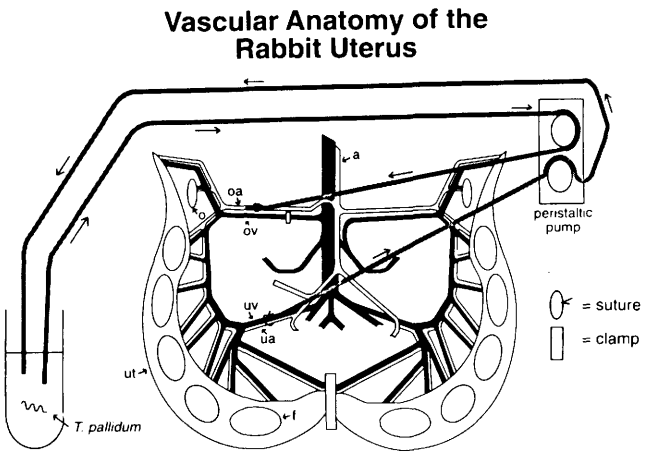

Figure 2. Schematic diagram of the vascular anatomy of the pregnant rabbit uterus. Aorta (a), fetus (f), ovary (o), ovarian artery (oa), ovarian vein (ov), uterine artery (ua), uterine vein (uv), uterine tube (ut). PE cannulae, attached to a multichannel peristaltic pump, were placed into the ovarian artery and into the uterine vein. A vascular clamp was placed on the ovarian vein and the uterine artery was closed with a suture. Blood vessels connecting the right and left uterine horns were clamped with a hemostat. 2–5 × 10^8 treponemes were circulated throughout the placentas for 2 hr. Arrows along the cannulae indicate the direction of treponemal flow.

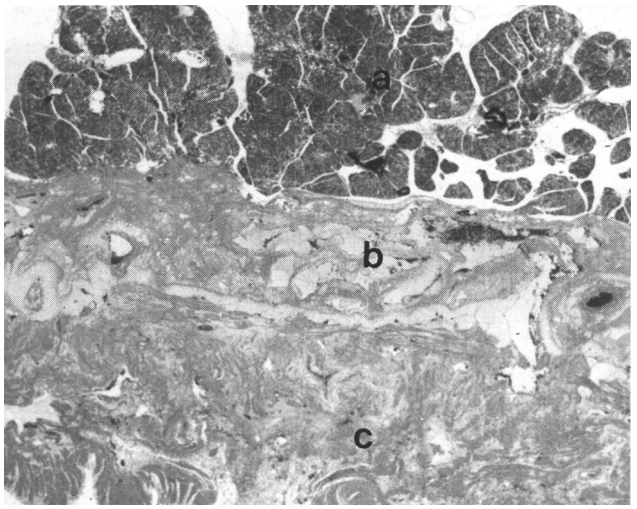

Figure 3. Hematoxylin- and eosin-stained cross-section of 26 day gestation section (×10). (a) Placental labyrinth, (b) decidua, (c) uterine wall.

Location of *T. pallidum*. Examination of sections revealed treponemes adhering to trophoblastic tissue surrounding maternal blood channels in the placental labyrinth (Fig. 5A, Arrow 1). Other treponemes appeared to have left the maternal blood channel and to have penetrated the trophoblastic tissue (Fig. 5A, Arrow 2, and 5B, Arrow 1 and 2). Some treponemes seemed to have been in the process of penetrating the trophoblastic tissue since they were still partially in the maternal blood channels (Fig. 5B, Arrow 3).

Discussion

The first major interaction between a pathogenic microorganism and its host is its attachment to a eucaryotic cell surface (18). *T. pallidum* is known to attach to a variety of cultured mammalian cell surfaces (4–6). Components of basement membranes and extracellular matrices are implicated as possible host cell molecules for mediation of treponemal adherence (19–22). It was shown *in vitro* that *T. pallidum* adherence involved fibronectin, laminin, collagen I, and collagen IV, all structural components of basement membranes and extracellular matrices (19). In addition, the treponemes attached to a 90-kDa protein located in the extracellular matrix extracted from rabbit epithelial cells, which suggest that this treponeme can react with multiple matrix proteins (21). However, other investigators maintain that fibronectin alone is involved in cytadherence of *T. pallidum* to host cell surfaces (22).

Models of experimental congenital syphilis have been developed in rabbits given multiple intravenous injections of high concentrations of *T. pallidum* throughout pregnancy (8), in hamsters infected with *T. pallidum* subsp. *endemicum* (9, 10) up to 2 months

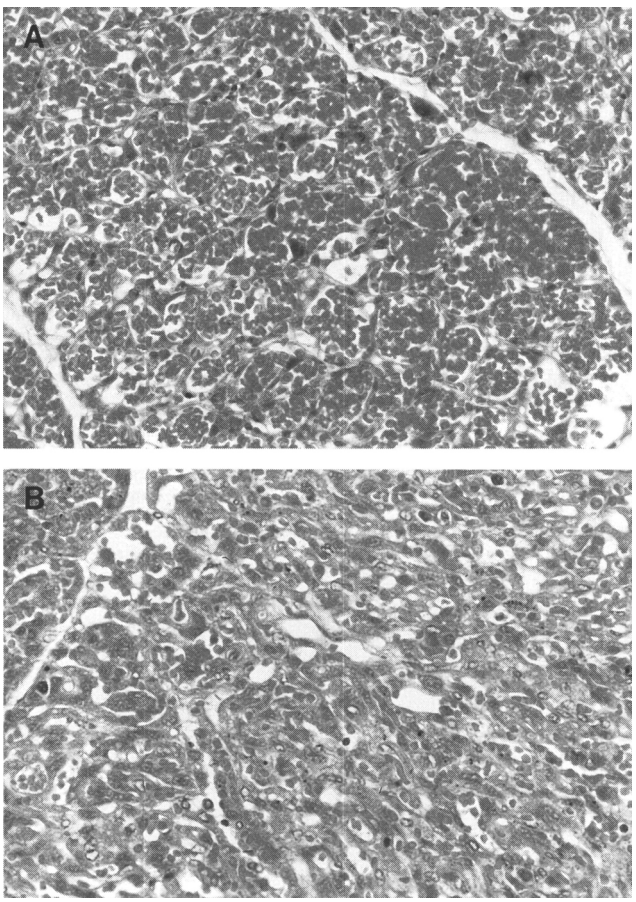

Figure 4. (A and B) Hematoxylin- and eosin-stained sections (×400) of the labyrinth from (A) control placenta (no surgical manipulation) and (B) placentas that were exposed to *T. pallidum* and flushed for 1 hr. An extremely low number of maternal red blood cells remained in the flushed placenta as compared with control placenta.

before or at early stages of pregnancy, and in inbred guinea pigs infected with a single dose of *T. pallidum* 1 to 3 months prior to parturition, or 30 to 40 days prepartum (11). These models, in addition to placental tissue obtained from syphilitic mothers, do not, however, permit the investigation of the initial interaction between the treponemes and placental tissue since it is impossible to determine when the organisms reach the placenta from the time of inoculation or infection. Furthermore, basic pathological changes are present in late syphilitic placentas which would obscure observation of the initial interaction with normal tissues. These changes include relative immaturity of the villi, obliteration of villous vessels due to endothelial and perivascular fibroblastic proliferation, and focal proliferative villitis, with or without necrosis, infiltrated by polymorphonuclear leukocytes, lymphocytes, or plasma cells (23–25).

Our rabbit model was developed to investigate the mechanism by which virulent *T. pallidum* is able to localize in the placenta. Each uterine horn of the rabbit possesses a dual blood supply consisting of the ovarian

Table I. Number of Rabbits and Placentas Positive for *T. pallidum*

Day gestation	# Positive rabbits[a]/ total # rabbits	# Positive placentas[b]/ total # placentas[c]
20	1/3	2/11
26[d]	3/3	10/10

[a] A positive rabbit contained at least one positive placenta.
[b] Three sections were examined from each placenta. The average number of treponemes found in 300–700 high power microscopic fields (×100 oil immersion objective lens fields) was determined for each section. At least one treponeme was observed in each section from a positive placenta.
[c] The number of placentas/rabbit varied from 2 to 5.
[d] 4.7×10^8 heat-killed *T. pallidum* (56°C for 1 hr) were circulated throughout the uterine horn of one rabbit used as a control for nonspecific binding. All four placentas were negative for *T. pallidum*.

blood vessels and the uterine blood vessels (26). In this model, the uterine horn was isolated, and organisms were introduced into the horn through the ovarian artery and exited through the uterine vein. Organisms could travel only one route through the placentas since

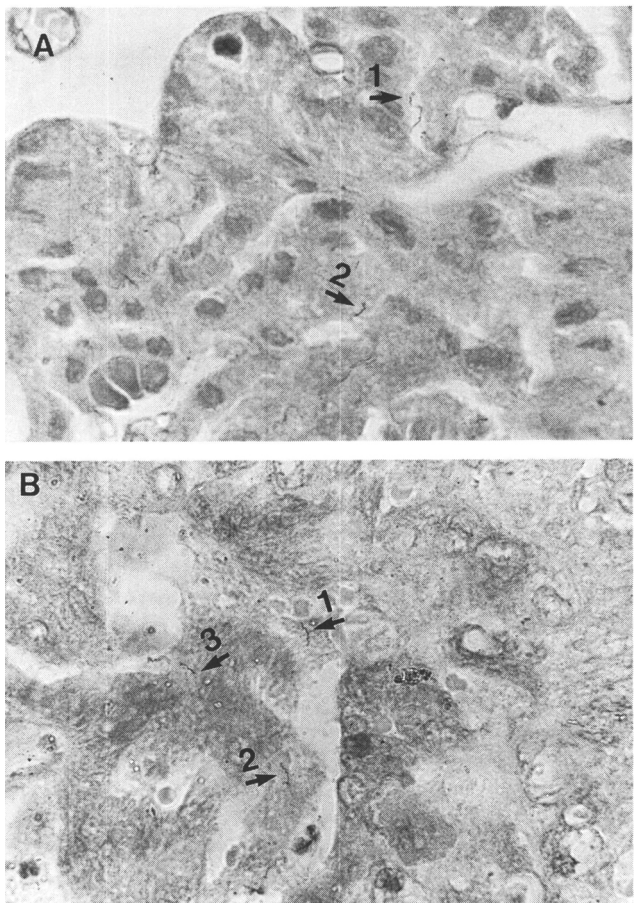

Figure 5. (A and B) Dieterle-stained sections (×1000) from Day 20 (A) and Day 26 (B) gestation placentas demonstrating *T. pallidum* (arrows) interaction with trophoblastic tissue.

the ovarian vein and the uterine artery were clamped. Adequate circulation of the medium and medium containing the organism was established since hematoxylin and eosin stains of sections showed extremely low numbers of maternal red blood cells from all placentas and Dieterle stained sections showed the presence of organisms without respect to the position of each placenta.

Treponemes were only detected in the placental labyrinth and not in the decidua or uterine wall of the placental sections. In the placental labyrinth, where the exchange of materials between the fetal blood and maternal blood takes place, organisms seemed either to be adhering to the trophoblastic tissue surrounding the maternal blood channels, or to be in the process of penetrating or to have penetrated already into the trophoblastic elements.

Although the rabbit placenta is described as labyrinth hemodichorial with two layers of trophoblast separating the maternal blood spaces from the fetal vessels and other extraembryonic connective tissue, and the human placenta as villous hemomonochorial with only one layer of trophoblast (27), similarities in the tissue organization between the two exists. In both cases, the layer of trophoblast in close association with the maternal blood is syncytial (27). Basement membranes are beneath the trophoblastic layer(s), and the fetal endothelium is beneath the basement membranes. Attachment to the trophoblastic layer in our model, particularly the syncytiotrophoblast surrounding the maternal blood spaces, is expected, since this is the first layer of tissue (28) that the organisms must traverse in order to reach the fetal blood system.

In human congenital syphilis, very few organisms are found in the placenta, even though there may be a large number in the fetus (1). In the first trimester, there is no association between congenital syphilis and fetal loss, and organisms are very rare in the fetus (2). Second-trimester fetal loss usually occurs late in the trimester and is associated with few organisms (1). In the third trimester, the severity of the disease is associated with large numbers of organisms (1, 29). In this report, *T. pallidum* bound to Day 26 placentas more consistently than to Day 20 placentas, since all 10 placentas were positive for *T. pallidum* at Day 26 while only 2 of 11 placentas were positive at Day 20. At Day 20 gestation, only one of three rabbits containing the two positive placentas was positive. Furthermore, binding is an active treponemal process since heat-killed *T. pallidum*, previously shown to be unable to attach to cultured cells (30), did not attach to the trophoblastic layer in the rabbit examined. Preferential binding of *T. pallidum* to Day 26 placentas may account for the larger number of organisms associated with third trimester congenital syphilitics. It may also suggest that *T. pallidum* is adhering to one or more placental components that may be present in larger quantities at Day 26 than at Day 20, since the placenta is a short-lived organ undergoing rapid development.

At this time, the placental differences contributing to *T. pallidum* adherence at Day 26 as opposed to Day 20 is not known. An increase in the amount of epidermal growth factor receptor has been observed when early, middle, and late isolated human trophoblast cells were compared (31). Furthermore, the greatest concentration of human placental pregnancy-specific β_1-glycoprotein occurs in the late placenta (32). These results indicate that there are at least two types of proteins that are present in higher quantities at Day 26 than at Day 20. Thus, there may well be a new molecule present that enables *T. pallidum* to bind preferentially to membranes of the Day 26 placenta.

In our system, there is no histological evidence indicating that *T. pallidum* has reached the amniotic layer, since no organisms were observed associated with this layer. This may be a function of time since the tissues were exposed to the organisms for only 2 hr. It is currently not known how *T. pallidum* enters the amniotic fluid. Potential routes of entry could be transamniotic, from an infected uterus, placenta, or umbilical cord, or from an infected fetus (33).

Congenital syphilis becomes a recognizable disease after the fetus begins to become immunocompetent (1). Also, in humans, none of the characteristic pathological changes of the placenta occur until the appearance of the fetal immune system at about the fourth month of pregnancy (34). Our model is relevant for the study of *T. pallidum* placental interaction because at any time during gestation, treponemes can be localized in the placenta in the absence of any immediate pathological changes.

This work was partially supported by the PHS Training Grant DA07245. The authors wish to thank Walter McAllister and Tim Ganey (Shriners Hospital, Tampa, FL) for their assistance in preparation of placental sections and other histological work.

1. Judge DM. Congenital syphilis. Prog Clin Biol Res **281**:87–106, 1988.
2. Harter CA, Benirschke K. Fetal syphilis in the first trimester. Am J Obstet Gynecol **124**:705–711, 1976.
3. Fitzgerald TJ. Pathogenesis and immunology of *Treponema pallidum*. Annu Rev Microbiol **35**:29–54, 1981.
4. Fitzgerald TJ, Miller JN, Sykes JA. *Treponema pallidum* (Nichols strain) in tissue culture: Cellular attachment, entry, and survival. Infect Immun **11**:1133–1140, 1975.
5. Fieldsteel AH, Stout JG, Becker FA. Comparative behavior of virulent strains of *Treponema pallidum* and *Treponema pertenue* in gradient cultures of various mammalian cells. Infect Immun **24**:337–345, 1979.
6. Fitzgerald TJ, Johnson RC, Miller JN, Sykes JA. Characterization of the attachment of *Treponema pallidum* (Nichols strain) to cultured mammalian cells and the potential relationship of attachment to pathogenicity. Infect Immun **18**:467–478, 1977.

7. Hayes NS, Muse KE, Collier AM, Baseman JB. Parasitism by virulent *Treponema pallidum* of host cell surfaces. Infect Immun **17**:174–186, 1977.
8. Fitzgerald TJ. Experimental congenital syphilis in rabbits. C J Microbiol **31**:757–762, 1985.
9. Kajdacsy-Balla A, Howeedy A, Bagasra O. Syphilis in the Syrian hamster: A model of human venereal and congenital syphilis. Am J Pathol **126**:599–601, 1987.
10. Kajdacsy-Balla A, Howeedy A, Bagasra O. Experimental model of congenital syphilis. Infect Immun **61**:3559–3561, 1993.
11. Wicher K, Baughn RE, Wicher V, Nakeeb S. Experimental congenital syphilis: Guinea pig model. Infect Immun **60**:271–277, 1992.
12. Miller JN. Spirochetes in Body Fluids and Tissues. Illinois: CC Thomas, p23, 1971.
13. Lukehart SA, Baker-Zander SA, Lloyd RMC, Sell S. Effect of cortisone administration on host-parasite relationships in early experimental syphilis. J Immunol **127**:1361–1368, 1981.
14. Blanco RB, Radolf JD, Lovett MA, Miller JN. Correlation of treponemicidal activity in normal human serum with the presence of IgG antibody directed against polypeptides of *Treponema phagedenis* biotype Reiter and *Treponema pallidum*, Nichols strain. J Immunol **137**:2031–2036, 1986.
15. Dieterle RR. Method for demonstration of *Spirochaeta pallida* in single microscopic sections. Arch Neurol Psychiat **18**:73–80, 1927.
16. Van Orden AE, Greer PW. Modification of the Dieterle spirochete stain. J Histotechnol **1**:51–53, 1977.
17. Rentrop M, Knapp B, Schweizer J. Aminoalkylsilane-treated glass slides as support for in situ hybridization of keratin cDNAs to frozen tissue sections under varying fixation and pretreatment conditions. Histochem J **18**:271–276, 1986.
18. Finlay BB, Falkow S. Common themes in microbial pathogenicity. Microbiol Rev **53**:210–230, 1989.
19. Baughn RE. Role of fibronectin in the pathogenesis of syphilis. Rev Infect Dis **9**:S372–S385, 1987.
20. Fitzgerald TJ, Repesh LA, Blanco DR, Miller JN. Attachment of *Treponema pallidum* to fibronectin, laminin, collagen IV, collagen I, and blockage of attachment by immune rabbit IgG. Br J Vener Dis **60**:357–363, 1984.
21. Steiner BM, Sell S, Schell R. *Treponema pallidum* attachment to surface and matrix proteins of cultured rabbit epithelial cells. J Infect Dis **155**:742–748, 1987.
22. Thomas DD, Baseman JB, Alderete J. Fibronectin mediates *Treponema pallidum* cytadherence through recognition of fibronectin cell binding domain. J Exp Med **161**:514–525, 1985.
23. Braunstein H. Congenital syphilis in aborted second trimester fetus: Diagnosis by histological study. J Clin Pathol **31**:265–267, 1978.
24. Ohyama M, Itani Y, Tanaka Y, Goto A, Sasaki Y. Syphilitic placentitis: Demonstration of *Treponema pallidum* by immunoperoxidase staining. Virchows Archiv A Pathol Anat **417**:343–345, 1990.
25. Russel P, Altshuler G. Placental abnormalities of congenital syphilis. Am J Dis Child **128**:160–163, 1974.
26. Del Campo CH, Ginther OJ. Vascular anatomy of the uterus and ovaries and the unilateral luteolytic effect of the uterus: Guinea pigs, rats, hamsters, and rabbits. Am J Vet Res **33**:2561–2577, 1972.
27. Enders AC. A comparative study of the fine structure of the trophoblast in several hemochorial placentas. Am J Anat **116**:29–68, 1965.
28. Samuel CA, Jack PMB, Nathanielsz PW. Ultrastructural studies of the rabbit placenta in the last third of gestation. J Reprod Fertil **45**:9–14, 1975.
29. Baughn RE. Congenital syphilis: Immunologic challenges. Pathol Immunopathol Res **8**:161–178, 1989.
30. Fitzgerald TJ, Miller JN, Sykes JA. *Treponema pallidum* (Nichols strain) in tissue cultures: Cellular attachment, entry, and survival. Infect Immun **11**:1133–1140, 1975.
31. Chen CF, Kurachi H, Fujita Y, Terakawa N, Miyake A, Tanizawa O. Changes in epidermal growth factor receptor and its messenger ribonucleic acid levels in human placenta and isolated trophoblast cells during pregnancy. J Clin Endocrinol Metab **67**:1171–1177, 1988.
32. Beck T, Schweikhart G, Stolz E. Immunohistochemical location of HPL, SP1 and beta-HCG in normal placentas of varying gestational age. Arch Gynecol **239**:63–74, 1986.
33. Wendel GD, Sanchez PJ, Peters MT, Harstad TW, Potter LL, Norgard MV. Identification of *Treponema pallidum* in amniotic fluid and fetal blood from pregnancies complicated by congenital syphilis. Obstet Gynecol **78**:890–895, 1991.
34. Walter P, Blot P, Ivanoff B. The placental lesions in congenital syphilis. Virchows Arch **397**:313–326, 1982.