Vertical Transmission of *Treponema pallidum* to Various Litters and Generations of Guinea Pigs

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The transmission of congenital syphilis was studied in a 4-generation guinea pig family with 10 litters and 38 offspring. By use of one or all of the following tests (ELISA-IgM, polymerase chain reaction, and rabbit infectivity), transplacental infection was demonstrated through 5 litters and up to 4 generations. Twenty-eight (93%) of 30 animals were positive by ≥1 test, and 2 (7%) were negative by 1 or 3 tests. While transmission of the pathogen appeared to be unaffected by the maternal acquisition of immunity, signs of smoldering infection in the young was suggested by the decline in humoral responses in successive progeny and by unusual rabbit infectivity test results. With each pregnancy there was a remarkable booster in the maternal humoral response, which dropped significantly prior to term. These findings shed new light on the understanding and interpretation of serologic testing during pregnancy and the perinatal period.

The possibility that an untreated female congenitally infected with syphilis, when reaching the childbearing age, will be able to transmit the disease to her fetus (third-generation syphilis) has been the subject of much discussion by syphilologists [1-4]. The controversy started before the discovery of the causative agent of syphilis in 1905 and the elaboration of the Wassermann test in 1906. The latter events not only helped to dispel the concept of hereditary transmission, which included either parent, but also firmly demonstrated that generational transmission occurs only from mother to fetus. Despite the precise clinical descriptions of third- and even fourth-generation syphilis [1, 2, 4], the controversy continued for >50 years, as reinfection of a congenitally infected woman could not be totally excluded. With the introduction of penicillin in 1943, the essential tool for control of the disease became available. Consequently, third-generation syphilis was no longer of interest, and it is seldom mentioned or discussed in recent scientific literature and medical text books [5, 6]. Another important question which, to our knowledge, has not been addressed, is why an untreated syphilitic woman in the latent stage of the disease who is immune to reinfection and is not contagious to her partner can still transmit the disease to her progeny for a number of years [4, 7, 8].

We have elaborated the guinea pig model of congenital [9] and neonatal [10] syphilis. More recently, we applied highly sensitive and specific serologic, molecular, and biologic methods for detection of *Treponema pallidum* infection in guinea pigs with acquired and congenital infection [11]. By use of one or another of these procedures, we have seen third-generation syphilis in three families of guinea pigs (unpublished data). To further verify and consolidate these observations under more strict experimental conditions, we examined infected female progeny consisting of 10 litters and 4 generations. We also analyzed the pregnancy-associated changes in the humoral response of multiparous females and how it relates to maternal transmission of the disease.

Material and Methods

**Microorganisms.** *T. pallidum* subspecies *pallidum* (Nichols) obtained from rabbit testes at the peak of orchitis (10-12 days) was extracted into sterile RPMI 1640. The treponemal suspension was adjusted to a concentration of 5 × 10^9/mL in the same medium.

**Animals and infection.** Normal (3- to 5-month-old) C4D guinea pigs were used in this study. The animals were obtained from the Wadsworth Center’s animal facilities [12]. The C4D strain, genetically related to inbred strain 13, is susceptible to *T. pallidum* infection (ID<sub>50</sub> = 10<sup>7</sup> organisms [12]). The C4D animals have a genetically controlled total deficiency of the fourth component of complement [13]; however, their immunologic competence at the cellular and humoral levels is similar to that of complement-sufficient strains [14, 15].

Pregnant females were infected by intradermal (id) injection of 0.1 mL of fresh treponemal suspension at each clipped hind limb. Strict measures were taken to prevent cross-infection between fe-
males and breeders and between parents and offspring. To this end, id infection of pregnant females was done between 25 and 30 days of gestation. The gestation period in guinea pigs is ~68 days. A separate, serologically negative healthy male breeder was used for each mating. Since female receptivity is increased during postpartum estrous [16], in several instances a new healthy breeder was placed with the asymptomatic female immediately after parturition (~48 h). Male breeders were maintained for 2–3 months under observation for clinical symptoms and serologic signs of infection. Lymph nodes from 5 randomly selected breeders were also examined by polymerase chain reaction (PCR). Neonates were housed together with their mothers until they reached age ~3–4 weeks, when they were weaned and housed separately by sex, first in groups of 2 per cage, and then individually. Similar precautions were applied when dams suspected of being congenitally infected were selected for breeding.

**Specimen collection.** Mothers and offspring were periodically bled for serology from 1 week to 4 months after infection or birth. Organs such as inguinal lymph nodes (ILN), brain, and heart were collected from liveborn or recently delivered stillborn guinea pigs and examined by PCR or by rabbit infectivity test (RIT). When a pregnant female had to be sacrificed because of maternal dystocia, immediately after death, the abdomen was cut open and the entire uterus removed. The fetuses with the umbilical cord and placenta attached were then removed from the uterus. The umbilical cord was immediately cut to separate the placenta, and the fetuses were freed from any traces of maternal blood by rinsing with abundant sterile saline solution prior to the removal of the fetal membranes.

**SeroLOGY.** The specificity of guinea pig antitreponemal antibodies and immunoglobulin isotypes IgM and IgG were examined by ELISA as reported [9] using Percoll-purified 10% alcohol-fixed *T. pallidum* as described by Zeltzer et al. [17]. Optical densities ≥0.107 for IgM and ≥0.066 for IgG were considered positive. These values were ≥2 SD above values for noninfected animals determined in sera from normal young (n = 10) and adult (n = 10) guinea pigs. Positive and negative controls consisted of serum pools previously tested by ELISA and immunoblot. Seroconversion of rabbits used in the RIT was determined by VDRL test and in some animals also by the fluorescent treponemal antibody absorption test.

**PCR.** Organs from guinea pigs and rabbits were examined by nested PCR as described [18]. In brief, ~1 g of each guinea pig organ were carefully collected with individual sets of instruments to avoid DNA cross-contamination. Tissues were placed in 2× TE buffer, homogenized, and kept at ~70°C until testing. DNA extraction was done by absorption of the lysate with diatoms in the presence of guanidine thiocyanate. In vitro amplification of DNA was done by use of the *bmp* gene of *T. pallidum* [19] with primers Tp7 and Tp8 for the first PCR and primers Tp3 and Tp4 for nested PCR.

In each experiment, *T. pallidum* at concentrations of 10^2 and 10^3 organisms/mL in TE buffer containing tRNA as a carrier was included as an extraction control. *T. pallidum* chromosomal DNA at concentrations of 2.5, 25, and 250 fg were used as positive sensitivity controls. Admixtures of PCR reagents without DNA and tissues from normal rabbits or guinea pigs were used as negative controls. The PCR products were analyzed in 2% agarose-containing ethidium bromide. The specificity of the PCR product was further confirmed by Southern blot analysis using biotinylated probes as reported [11]. The target-probe hybrid was detected by a nonradioactive system of nucleic acid detection. Four laboratories located in three different floors of the building were used for (1) preparation of reagents, (2) preparation of the PCR mixture and DNA extraction, (3) amplification and dilution of samples for nested PCR, and (4) analysis of the PCR products.

**RIT.** This test was used as further confirmation of an animal infection per se and not of any individual organ. To this end, ILN and portions of brain and heart as a pool were teased under sterile conditions through 100-mesh wire with 2 mL of RPMI 1640 medium, and the suspension was injected intratesticularly (1 mL/testis) into VDRL-negative rabbits. The recipients were observed for 3 months for signs of orchitis and were bled every 2 weeks for serologic testing. At the end of the experimental period, multiple specimens from both testes were examined by PCR. The RIT was considered positive when the rabbits developed orchitis or seroconverted, the testes were positive by darkfield microscopy, or testicular specimens were positive by PCR.

**Results**

**Course of infection.** The onset and nature of the cutaneous reaction to *T. pallidum* infection in the original pregnant dam was similar to that previously described [9]. Between days 9–12, a darkfield-positive lesion developed at the sites of inoculation, which dissolved within 4–6 weeks after infection. No reactivation of skin lesion was observed during her subsequent pregnancy. In agreement with our previous studies [9], no obvious clinical signs of infection were noticed in offspring born to mothers with acquired or congenital syphilis even though the fetus with the umbilical cord and placenta was immediately cut to separate the placenta, and the fetuses were freed from any traces of maternal blood by rinsing with abundant sterile saline solution prior to the removal of the fetal membranes.

Female 564 was the source of 4 generations that included 10 litters with 38 offspring: 24 liveborn, 4 stillborn, and 10 (L2-GII and L1-GIV) sacrificed because of maternal dystocia. Organisms from 5 of the 10 fetuses and organs from 1 of 4 stillborn (L2-GIII) delivered were serologically examined by PCR and/or RIT. The original experimentally infected mother, her progeny, and a summary of the tests used are shown in figure 1. Offspring B (L1-GII) underwent 5 pregnancies in 1 year, and 3 offspring from this GIII provided the members of GIV.

**Nested PCR.** Three organs (ILN, heart, and brain) from 30 offspring were individually analyzed by PCR at sacrifice (age 30 min to 3 months; figure 1 and figure 2). In 23 of 30 animals, ≥1 tissue contained treponemal DNA by PCR, and the specificity of the reaction was verified by hybridization. Amplification products from heart, brain, and ILN by nested PCR and hybridization are shown in figure 2A and 2B, respectively. These organs were collected from 2 surgically removed offspring (GIV) sacrificed on 7 May 1997 (figure 1). Although ILN from both animals (lanes 4 and 7) were apparently negative by nested PCR, they were weakly positive after hybridization. Lymph
### Vertical Transmission of T. pallidum to Various Litters and Generations of Guinea Pigs

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**Figure 1.** Vertical transmission of syphilis in guinea pigs through various litters (Ls) and generations (Gs). Family 564, consisting of 10 Ls and 4 Gs, was examined by 2 or 3 methods: presence of specific IgM antibodies, polymerase chain reaction (PCR), and rabbit infectivity test (RIT). Of 10 Ls, 2 (*) were surgically removed before parturition and organs from 2 or 3 members and of 1 stillborn (L2-GIII) were analyzed by PCR or PCR and RIT. Except for babies D (L2-GII) and C (GIV, 5/8/97), remaining offspring were positive for *T. pallidum* by >1 test.

Node specimens from male breeders were all negative by PCR (data not shown).

**RIT.** Fourteen normal rabbits were inoculated intratesticularly with individual pool of tissues prepared from 14 offspring from GIII and GIV (figure 1). Except for the young born on 29 June 1997 (GIII), which were 5 days old, and those surgically removed from their mother on 7 May 1997 (GIV), the remaining guinea pigs were sacrificed and tested by RIT at age 30–60 days. None of the inoculated rabbits developed typical orchitis or seroconverted for a 3-month period. However, in 10 of 14 inoculated rabbits, 1–3 of 4–6 testicular specimens examined from each animal were positive for *T. pallidum* DNA by nested PCR (figure 3). Not all testicular specimens from a single rabbit were positive; however, all normal testes and control reagents were negative.

**ELISA-IgM.** The serologic analysis of representative animals of litters from G1 to GIV is shown in figure 4. Generation I is represented by id infected mother 564. GII–GIV are represented by animals potentially infected in utero. Except for offspring surgically removed, which were not tested serologically, most animals were examined at age 1–3 months, except members of L5-GIII, which were sacrificed at age 5 days. Most pups responded with rising levels of IgM antitreponemal antibodies, considered genuinely produced by the infected host.
Fourth-Generation Syphilis

Figure 2. Detection of T. pallidum DNA in organs of 2 surgically removed offspring (litter 1, generation IV) by nested polymerase chain reaction (A) and hybridization (B). Lanes 1 and 14, molecular weight controls. Amplification products from heart, brain, and inguinal lymph nodes of baby A are shown in lanes 2–4 and corresponding organs from baby B in lanes 5–7. Lanes 8 and 9, diatom-extracted treponemal DNA from 10^3 and 10^2 suspensions; lane 10, reagent control; lanes 11–13, T. pallidum DNA at concentrations of 250, 25, and 2.5 fg, respectively.

Figure 3. Detection of T. pallidum DNA by nested polymerase chain reaction (PCR) in rabbit testes inoculated with tissue extracts prepared from 2 guinea pig pups. Lanes 1 and 18, molecular weight controls. Amplification products from heart, brain, and inguinal lymph nodes of baby A are shown in lanes 2–4 and corresponding organs from baby B in lanes 5–7. Lanes 8 and 9, diatom-extracted treponemal DNA from 10^3 and 10^2 suspensions; lane 10, reagent control; lanes 11–13, T. pallidum DNA at concentrations of 250, 25, and 2.5 fg, respectively.

Discussion

These data provide strong evidence of third- and fourth-generation syphilis in the experimental host. The application of three specific and sensitive methods for detection of T. pallidum enabled us to detect congenital infection through various guinea pig generations. A reasonably good correlation was found between serology and PCR if L5-GIII is excluded; these animals were sacrificed 5 days after birth. The animals had an insufficient time to mount a measurable humoral response, although they were positive by PCR. Of the remaining 21 animals examined by the two methods, 14 (67%) were positive by both, 5 (24%) were positive only for IgM, 1 (4%) was only positive by PCR, and 1 (4%) was negative by both. The latter animal, baby C born on 8 May 1997, was the only animal negative by the three tests. It should be stressed that our ELISA, which uses alcohol-fixed T. pallidum, reacts with specific but not cross-reacting antibodies [9]. Thus, in the newborn, only IgM genuinely produced by the offspring and relatively low levels of maternally transmitted IgG antibodies specific for T. pallidum are detected. The kinetics of the humoral response in congenitally infected pups revealed similarities with human congenital infection. Indeed, the strategy recommended for distinguishing passively transferred from host antibodies in the asymptomatic infant is to follow the baby’s titer. The levels of maternal antibodies should fall within 2 months and disappear by age 6 months, whereas stable or increasing levels indicate active infection [20]. Our data showed an increasing level of IgM and IgG responses in most offspring examined ≥1 month (figure 4).

The sensitivity and specificity of PCR and RIT are similar when the same tissue is examined by both tests [21]. In this study, however, there was not such a close correlation. Several possibilities may account for this apparent discrepancy includ-
ing the following: A dilution factor was introduced in the RIT, as a pool of tissue specimens removed from each guinea pig, rather than single organs, was examined by RIT, while an extensive examination of multiple organs or multiple samples from the same organ in a single animal was examined by PCR. The fact that normal tissues and reagent controls were negative and that neither all organs nor specimens from a single organ were positive by PCR (figure 3) rule out the possibility of a false-positive reaction due to contamination during sample collection or processing. Moreover, as opposed to serology and PCR, the RIT was applied to offspring of late litters and generations that showed a decline in immune responses.

On the basis of empirical observations in humans (Kassowitz’s law [4]), it has long been suggested that reduction in transplacental infection is related to progress of the maternal disease and evolution of immunity. However, more recent studies in humans [7, 8] have demonstrated that although transmission of the infection by an untreated mother declines with progression of the disease, the possibility of fetal infection is not eliminated [7]. Thus, an untreated female can transmit the disease
to her progeny regardless of her immunologic status. Indeed, in our studies, what seems to decline in successive progeny is not the maternal transmission but the number or pathogenicity of the microorganism. This postulate is in conformity with the expression of a milder humoral response in successive progeny and the unusual results of the RIT. In the latter test, absence of orchitis and seroconversion was the rule, although the organisms were detected in 10 of 14 rabbits by examination of multiple testicular samples by PCR.

It may be argued that in the experimental model, it is always possible for the infection of the offspring to take place postnatally rather than in utero. This could occur when passing through the birth canal or by contact with infected maternal blood parturition. In the present study, this was highly unlikely; in 9 of 10 pregnancies, the females were congenitally infected and therefore, asymptomatic, and there was not 1 case of infection or seroconversion of male breeders.

Since parturition occurs at any time of the day and the grooming of the young and consumption of placenta and fetal membranes by the dam takes a variable period of time, the contact of the newborn with maternal blood cannot be totally excluded. However, postnatal infection could not account for the presence of T. pallidum (PCR- and RIT-positive) in organs obtained from members of L2-GII and L1-GIV (figure 1), which were surgically removed before delivery or from L5-GIII sacrificed on postpartum day 5. Neither could explain levels of IgM antitreponemal antibodies ≥2 SD within the first week of life in a number of offspring (A and C, LI-GIII; A and B, L2-GIII; and C, LI-GIV).

In our studies, analysis of the kinetics of the humoral response in multiparous females showed a consistent pattern of fluctuation in the maternal humoral response. As each pregnancy progressed, levels of IgM and IgG antitreponemal antibodies increased remarkably and then the titers substantially dropped before and immediately after delivery. This pattern appears to be related to a mobilization and replication of T. pallidum as shown by the obvious transmissibility to the fetuses. The fact that mobilization of the pathogen occurs after fetal implantation may be taken as an indication that T cell–mediated mechanisms (Th1) are down-regulated and that the humoral response, while unprotective, is not harmful either to the mother or the fetus. Th1 and Th2 immune responses are mutually exclusive [22, 23]. Immunoregulatory substances, including cytokines, are produced by both lymphoid cells and nonimmune components of the fetoplacental unit, such as uterine epithelium and trophoblast tissue (reviewed in [24]). The enhancing effect of pregnancy on specific and nonspecific humoral response has been reported by several investigators (reviewed in [25]), and there is increasing evidence suggesting a pregnancy-associated depression of cell-mediated immune responses [22, 26, 27]. In the field of syphilis, as early as 1920 Brown and Pearce [28] showed that syphilitic lesions in preg-

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**Figure 5.** Kinetics of humoral response to T. pallidum in congenitally infected mother B (generation II) during 5 pregnancies (A). For comparison, kinetics of humoral response of nulliparous congenitally infected female (B).

**Figure 6.** Kinetics of humoral response in multiparous female guinea pig with acquired syphilis resembles that of multiparous congenitally infected sow.
nant rabbits appeared to be suppressed, and Moore [29] in 1922 suggested that pregnancy itself has a suppressive effect on the clinical manifestations of the disease in humans.

In summary, this experimental model is the first to consistently demonstrate in third- and fourth-generation syphilis an association between congenital transmission and pregnancy-related changes in the immune response, the transmissibility of the disease beyond the stage of maternal chancre immunity, and a decline in immune response in successive congenitally infected progeny. These should provide aid in the current understanding of the immunopathology and clinical management of the disease in humans and in its epidemiologic surveillance.

Acknowledgments

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References