MAJOR ARTICLE

Treponema pallidum Strain-Specific Differences in Neuroinvasion and Clinical Phenotype in a Rabbit Model

Lauren C. Tantalo,[1] Sheila A. Lukehart,[2] and Christina M. Marra[1,2]

Departments of [1]Neurology and [2]Medicine (Infectious Diseases), University of Washington School of Medicine, Seattle

Background. The relationship between neuroinvasion and other manifestations of syphilis and the infecting strain of *Treponema pallidum* is not known.

Methods. Six groups of 8 rabbits were intravenously infected with 1×10^8 organisms from 1 of 6 strains of *T. pallidum*. Rabbits were examined 2–3 times/week; blood and cerebrospinal fluid (CSF) were collected weekly and every 2 weeks, respectively, for 10–12 weeks. Degree of CSF pleocytosis and skin-lesion severity were estimated by the area under the white blood cell–versus-time and lesion-versus-time curves.

Results. Maximum serum Venereal Disease Research Laboratory test titers, time to maximum titer, degree of CSF pleocytosis, and severity of skin lesions differed significantly among infecting strains. Overall, *T. pallidum* was identified, by reverse-transcriptase polymerase chain reaction, in CSF from 13 (27.7%) of 47 rabbits and was never identified in CSF from rabbits infected with 1 of the strains. The time course of detection varied by infecting strain. Severity of skin lesions and of CSF pleocytosis were inversely correlated ($P = .005$).

Conclusions. There are particularly neuroinvasive *T. pallidum* strains, and the clinical phenotype of infection varies with infecting strain. This information could ultimately be used to identify patients at increased risk for neuroinvasion and, thus, at risk for neurosyphilis.

Studies from the first half of the 1900s demonstrated that *Treponema pallidum,* the bacterium that causes syphilis, invades the central nervous system (CNS) in as many as 70% of individuals by the end of the secondary stage of disease [1]. This invasion was demonstrated by identification of the organism in cerebrospinal fluid (CSF) or by identification of surrogate markers such as CSF pleocytosis, elevated protein concentration, or reactivity of the CSF Wassermann test (the predecessor of today's CSF-VDRL test). However, unlike the situation with other bacteria, CNS invasion did not necessarily mean that the CNS became persistently infected. Invasion might regress spontaneously without eliciting conventional CSF abnormalities, or it might elicit such CSF abnormalities, which could then spontaneously resolve or could persist as asymptomatic neurosyphilis [2]. Invasion was thought to precede all cases of symptomatic neurosyphilis; those individuals who were unable to clear invading CSF organisms were those who had "asymptomatic neurosyphilis," and many went on to develop the symptomatic forms of the disease. Investigators believed that the course of events after neuroinvasion was, at least in part, determined by the host response to infection. For example, several investigators noted a correlation between the severity of the secondary syphilis skin rash and identification of CSF abnormalities in early syphilis [1, 3]. On the other hand, Merritt noted that symptomatic neurosyphilis was rarely seen in individuals who had experienced severe skin manifestations [2]. Partial or ineffective treatment for early syphilis was also thought to increase the likelihood of symptomatic meningitis and ocular disease, perhaps because it downregulated the immune response and thus hampered the ability of the host to clear organisms that had invaded the CNS [2].

Unifying these observations regarding the host re-

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Reprints or correspondence: Dr. Christina M. Marra, Harborview Medical Center, Box 359775, 325 9th Ave., Seattle, WA 98104-2499 (cmarra@u.washington.edu).

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Table. 1. Characteristics of the 6 *Treponema pallidum* strains.

Strain name	Strain source	Disease stage of source patient	Year isolated
Sea 81-4	Chancre	Primary	1981
Bal 7	CSF	Posttreatment	1976
Bal 73-1	Aqueous humor	Congenital	1973
UW085B	Blood	Secondary	2001
UW099B	Blood	Secondary with asymptomatic meningitis	2001
Nichols	CSF	Secondary	1912

NOTE. CSF, cerebrospinal fluid

sponse to infection to explain the course of CNS syphilis is difficult. Specifically, if neuroinvasion precedes symptomatic neurosyphilis, how could neuroinvasion be more likely with more severe skin lesions but neurosyphilis be less likely with more severe skin lesions? One explanation for these apparently contradictory observations is that those patients with many skin lesions may have a greater concentration of *T. pallidum* in blood and are thus more likely to experience neuroinvasion. Such an argument has been used to explain the association between higher serum rapid plasma reagin (RPR) titers and identification of *T. pallidum* in CSF [4]. These individuals might also be more likely to clear CSF organisms, because of a robust immune response induced by the high organism load. On the other hand, patients with fewer skin lesions may have a lower concentration of organisms in blood. Exposure to lower concentrations of organisms could attenuate the host immune response, leading to impaired ability to clear those organisms that managed to invade the CNS and, thus, to greater likelihood of developing subsequent symptomatic neurosyphilis. Such a scenario is comparable to that postulated for patients who receive partial or ineffective therapy.

Some investigators also believed that there were strains of *T. pallidum* that were particularly neuroinvasive. In their 1944 text on syphilis, Stokes et al. cited experiments that showed that passage of *T. pallidum* in mouse brain rendered the organism more neurotropic in rabbits. In addition, they described several case reports of clinically identical forms of neurosyphilis developing in multiple sex partners of a single individual, presumably because of infection with a highly neurotropic strain [5]. However, in their 1946 text on neurosyphilis, Merritt et al. identified flaws in the available data and concluded, "…few syphililogists subscribe to the idea of neurotropism" [2] (p. 11).

Nonetheless, modern studies of related spirochetes, *Borrelia burgdorferi* and *B. turicatae,* provide biological plausibility for the hypothesis that there are particularly neuroinvasive strains of spirochetes. For example, Wilske et al. showed that, of the 3 *B. burgdorferi* genospecies that cause Lyme disease in Europe, *B. garinii* is the most neuroinvasive [6, 7]. Moreover, of the *B. garinii* serotypes, OspA serotype 4 is most frequently identified in *B. garinii* organisms isolated from CSF [8]. Similarly, serotype A of *B. turicatae,* an organism that causes relapsing fever, is more neuroinvasive than serotype B in a mouse model [9, 10]. In an unpublished preliminary study, we showed that *T. pallidum* could be amplified, by reverse-transcriptase (RT) polymerase chain reaction (PCR), from CSF of 33%–50% of rabbits infected intravenously with the Nichols strain of *T. pallidum.* Because of uncertainties regarding the impact of clinical manifestations of syphilis on CSF abnormalities and regarding the existence of neuroinvasive strains of *T. pallidum* in humans, we undertook a study using the rabbit intravenous infection model to examine these issues.

METHODS

Animals. Adult male New Zealand White rabbits (3.0 kg; R & R Rabbitry) free of serological evidence of *Treponema paraluiscuniculi* infection were housed individually at 18°C and were fed antibiotic-free food and water. Animal experimentation guidelines were followed in the conduct of this research.

Source of organisms. Six strains of *T. pallidum* (table 1) were propagated from frozen stocks by rabbit testicular passage, as described elsewhere [11]. Treponemes were extracted by mincing testes and then gently rotating the tissue in a solution of 50% normal rabbit serum (NRS) and 50% 0.14 mol/L saline. The extract was centrifuged at 400 *g* for 10 min at room temperature to pellet gross debris. Motile organisms in the supernatant were counted by darkfield microscopy and were adjusted to obtain a concentration of 1.0×10^8 organisms/mL.

Experimental design. Eight rabbits per strain were anesthetized with intramuscular injections of 20.0 mg/kg ketamine and 3.3 mg/kg xylazine and were inoculated intravenously with 1×10^8 *T. pallidum* in a volume of 1 mL. The back was kept clipped free of fur, to facilitate development of disseminated skin lesions. Rabbits were examined 2–3 times/week for skin lesions; the number of erythematous and indurated lesions was determined weekly. Blood (10 mL) was collected weekly for serological tests for syphilis. Every 2 weeks after inoculation—for 10 weeks in the group infected with Sea 81-4 and for 12 weeks in the other 5 groups—rabbits were anesthetized with intramuscular injections of 40.0 mg/kg ketamine and 6.7 mg/kg xylazine, and 0.5–1.0 mL CSF was collected by cisternal puncture using a 22-gauge, 1.5-inch Quinke needle. CSF white

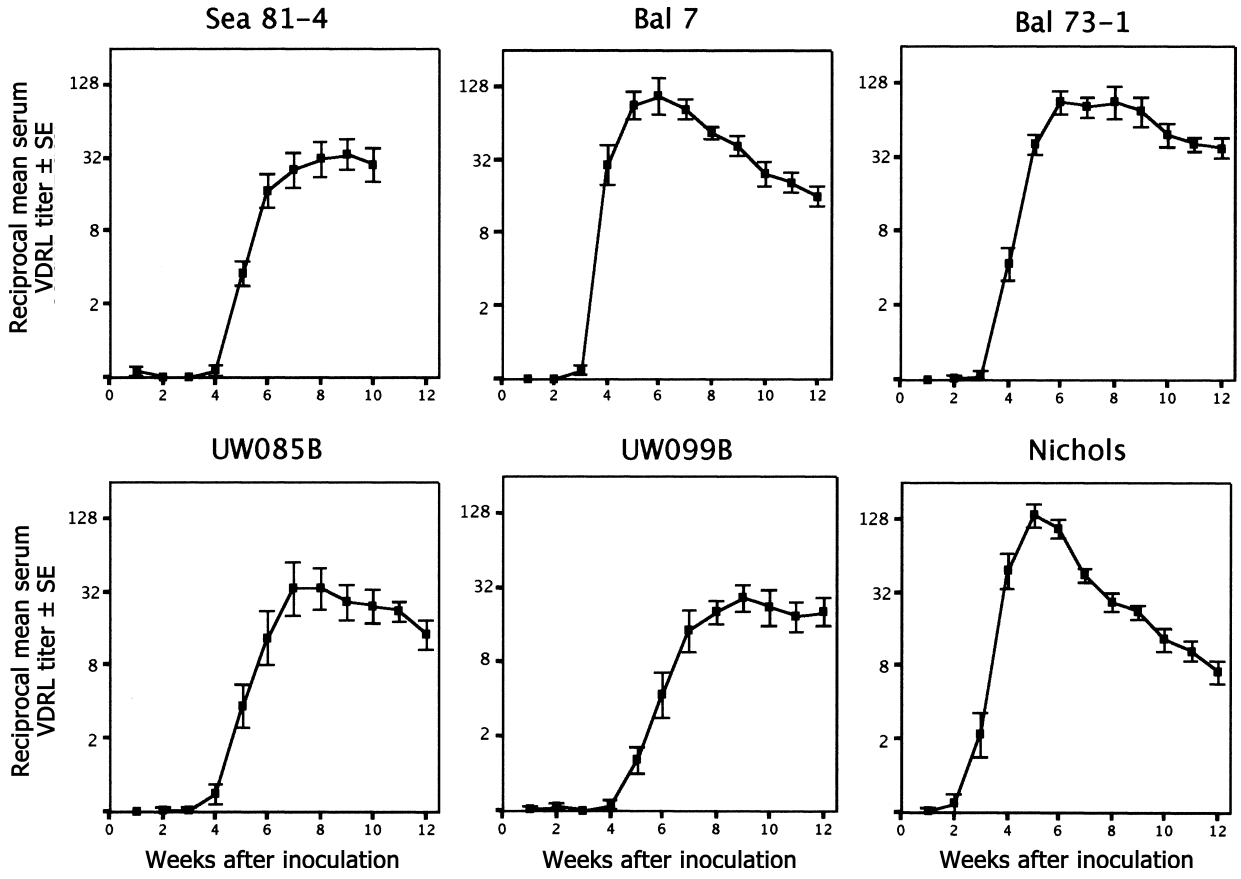

Figure 1. Geometric reciprocal mean ± SE serum VDRL titers after intravenous inoculation with 6 strains of *Treponema pallidum*

blood cell (WBC) and red blood cell (RBC) counts were performed immediately after collection using a Fuchs-Rosenthal bright-line counting chamber (Hausser Scientific). CSF (0.3–0.5 mL) was centrifuged at 12,000 *g* for 30 min at 4°C to pellet treponemes. The supernatant was removed and stored at −80°C for CSF-VDRL. The pellet was resuspended in Ultraspec RNA reagent (BiotecX) and stored at −80°C for RT-PCR. One of 8 rabbits in the group infected with Sea 81-4 died between weeks 1 and 2, and 1 of 8 rabbits in the group infected with the Nichols strain died between weeks 10 and 12; data from the remaining groups reflect all 8 rabbits.

Serologic analysis and CSF RT-PCR. Serum VDRL and fluorescent treponemal antibody-absorbed (FTA-ABS) tests were performed as described elsewhere [12]. CSF-VDRL was performed using standard methods [13]. RT-PCR detection of *T. pallidum* 16S rRNA in CSF was performed as described elsewhere [4].

Analyses. To avoid erroneous results due to blood contamination of CSF, only samples that contained <2000 RBCs/μL were considered in the analyses of CSF-VDRL [14]. Associations between categorical and continuous variables were assessed by Mann-Whitney *U* test or Kruskal-Wallis test. Associations between continuous variables were determined by Spearman rank correlation. Severity of skin lesions for each rabbit was estimated by calculating the area under the lesion number–versus-time curve. This approach accounts for both the number and duration of lesions. Similarly, the degree of CSF pleocytosis for each rabbit was estimated by calculating the area under the CSF WBC–versus-time curve, which, again, accounts for amount and duration of pleocytosis. $P < .05$ was considered to be significant.

RESULTS

Serologic evidence of systemic infection. All rabbits had serologic evidence of systemic *T. pallidum* infection. Specifically, by 6 weeks after inoculation, all rabbits had reactive serum FTA-ABS tests. Serum VDRL test reactivity is shown in figure 1. The maximum titer and time to maximum titer differed significantly among the 6 groups ($P < .005$ for both).

CSF abnormalities. The median CSF RBC count in all samples was 15.5 cells/μL. Eighteen (6.9%) of 262 CSF samples contained ≥2000 RBCs/μL. The number of samples with CSF RBC counts ≥2000 cells/μL was not greater for any inoculum strain ($P = .96$). In addition, there was no relationship between maximum CSF RBC and maximum CSF WBC counts ($P = .88$) or between maximum CSF RBC and degree of CSF pleo-

cytosis ($P = .38$). Degree of CSF pleocytosis was significantly associated with the infecting strain ($P = .005$). As shown in figure 2, rabbits infected with strain Sea 81-4—and, to a lesser degree, rabbits infected with Bal 7 and Bal 73-1—showed CSF pleocytosis (CSF WBC counts $\geq$10 cells/μL). Specifically, 43.8% of all CSF WBC values in rabbits infected with Sea 81-4 were above this cutoff, compared with 6.7% in those rabbits infected with Bal 7, 2.3% in rabbits infected with Bal 73-1, and 0% in rabbits infected with the remaining strains. Although infection with Bal 7 led to transient CSF pleocytosis that resolved after 8 weeks, infection with Sea 81-4 led to CSF pleocytosis that persisted throughout 10 weeks of observation.

Overall, *T. pallidum* was detected in CSF from 13 (27.7%) of 47 rabbits. Five (62.5%) of 8 rabbits infected with Bal 73-1 and 3 (37.5%) of 8 rabbits infected with UW085B had $\geq$1 positive samples. These 2 strains accounted for 13 (72.2%) of the 18 positive samples. *T. pallidum* was isolated from CSF of 2 rabbits infected with Bal 7 and 2 rabbits infected with the Nichols strain, at single time points, and was never isolated from CSF of rabbits infected with UW099B (figure 2).

The time course of detection of *T. pallidum* by RT-PCR varied among strains. Whereas *T. pallidum* was identified throughout the 12 weeks of observation in the CSF of rabbits infected with Bal 73-1, *T. pallidum* was not identified in CSF of rabbits infected with UW085B until 8 weeks after infection. Moreover, *T. pallidum* was identified at 2 and 4 weeks after inoculation in CSF from rabbits infected with the Nichols strain, but not thereafter, suggesting that they had cleared their CSF infection. Detection of *T. pallidum* in CSF by RT-PCR did not occur more often in rabbits with higher peak serum VDRL titers ($P = 1.00$), in those with higher maximum CSF WBC counts ($P = .33$), or in those with greater degrees of CSF pleocytosis ($P = .42$). Detection of *T. pallidum* in CSF was not merely a consequence of blood contamination of CSF. Specifically, detection of *T. pallidum* by PCR was not more common in the samples with the highest CSF RBC counts ($P = .86$).

Only 3 rabbits had reactive CSF-VDRL test results: 1 rabbit infected with Bal 73-1, at 10 weeks (titer 1:1); and 2 different rabbits infected with Nichols strain, at 4 weeks (titer 1:2) and 6 weeks (titer 1:1). One of the 2 rabbits infected with the Nichols strain that had a reactive CSF-VDRL result also had a positive CSF RT-PCR result at the same week.

Development of skin lesions and correlation with CSF abnormalities. We kept the skin of rabbits' backs clipped free

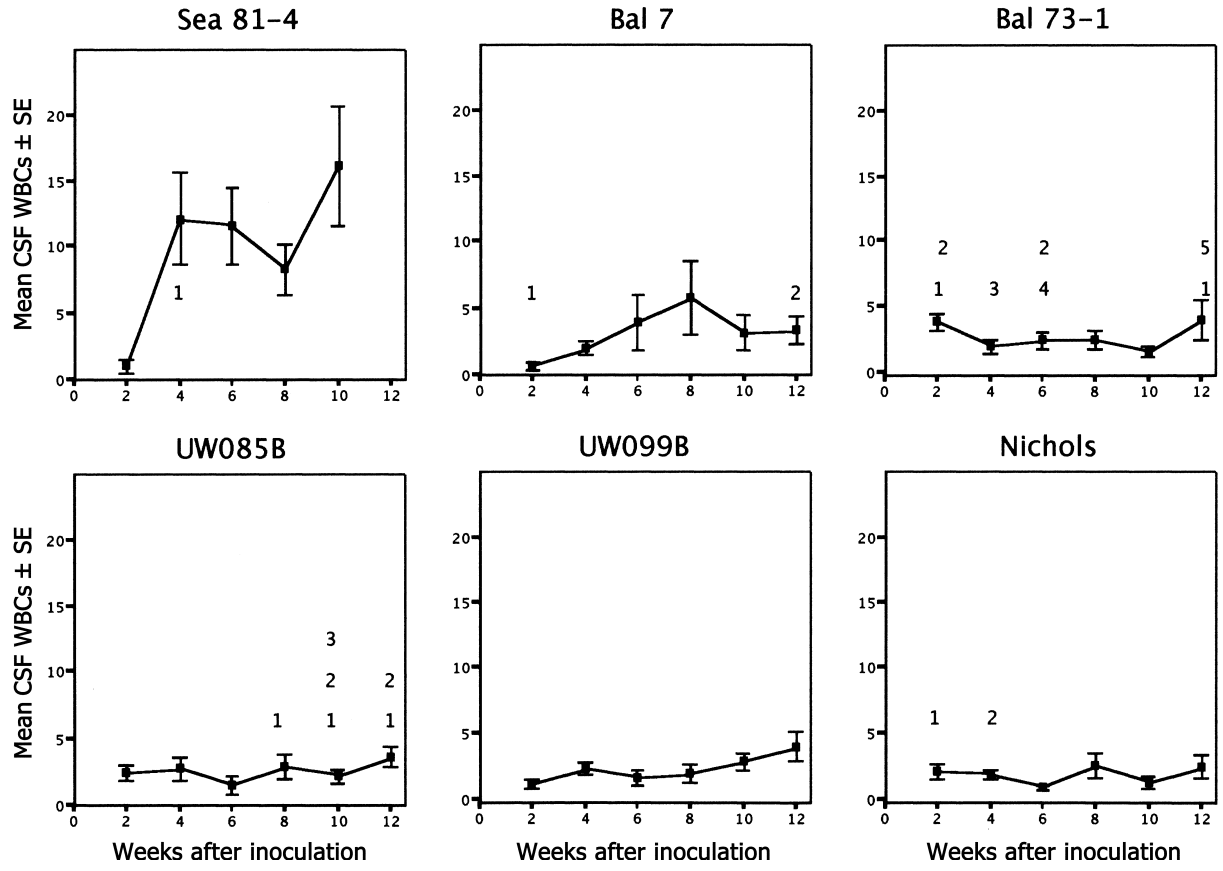

Figure 2. Mean ± SE cerebrospinal fluid (CSF) white blood cell (WBC) counts after intravenous inoculation with 6 strains of *Treponema pallidum*. The nos. (1–5) denote identification of *T. pallidum* by reverse-transcriptase polymerase chain reaction. Each no. is specific to a given rabbit.

of fur, which facilitated development of skin lesions, because we reasoned that the development of skin lesions might increase the overall bacterial load and the likelihood of CNS infection. The severity of skin lesions differed by infecting strain ($P = .001$) and was most severe for the Bal 7 and Nichols strains and least severe for Sea 81-4 (figure 3). Identification of *T. pallidum* in CSF by RT-PCR was not more common in those rabbits with more-severe skin lesions ($P = .30$). However, there was a significant inverse relationship between severity of skin lesions and degree of CSF pleocytosis ($r = -0.4$; $P = .005$).

DISCUSSION

In humans, invasion of the CSF by *T. pallidum* may be followed by spontaneous clearance without development of an inflammatory response, by development of a transient inflammatory response, or by development of a persistent inflammatory response. The third outcome defines asymptomatic neurosyphilis, which is the precursor of symptomatic neurosyphilis [2]. Our model recapitulates these events and suggests that clinical course may be determined by the infecting strain. For example, infection with the Bal 73-1, UW085B, and Nichols strains led to neuroinvasion by *T. pallidum* without CSF pleocytosis, and rabbits infected with the Nichols strain cleared their CSF organisms. It is of note that CSF-VDRL reactivity was also seen in rabbits infected with the Nichols strain. Because VDRL antibody opsonizes *T. pallidum* [15], it may have contributed to clearance of CSF organisms. Whereas *T. pallidum* was identified throughout the 12 weeks of observation in the CSF of rabbits inoculated with Bal 73-1, *T. pallidum* was not identified until 8 weeks after infection in CSF of rabbits infected with UW085B, was identified in CSF only very early in infection in rabbits infected with Sea 81-4, and was never identified in CSF from rabbits infected with UW099B. Conversely, infection with Bal 7 led to transient CSF pleocytosis that resolved after 8 weeks, whereas infection with Sea 81-4 led to CSF pleocytosis that persisted throughout 10 weeks of observation. Longer follow-up of rabbits infected with Bal 73-1 and UW085B might have allowed us to identify clearance of CSF organisms or development of CSF inflammation after neuroinvasion. Reactivity of the CSF-VDRL results at 10 weeks in 1 rabbit infected with Bal 73-1 supports the contention that clearance might have been demonstrated with longer observation. Similarly, sampling CSF at very early time points in rabbits infected with Sea 81-4 might have allowed us to demonstrate neuroinvasion preceding CSF pleocytosis. The differences that we observed in neuroinvasive capacity of the 6 *T. pallidum* strains suggest that,

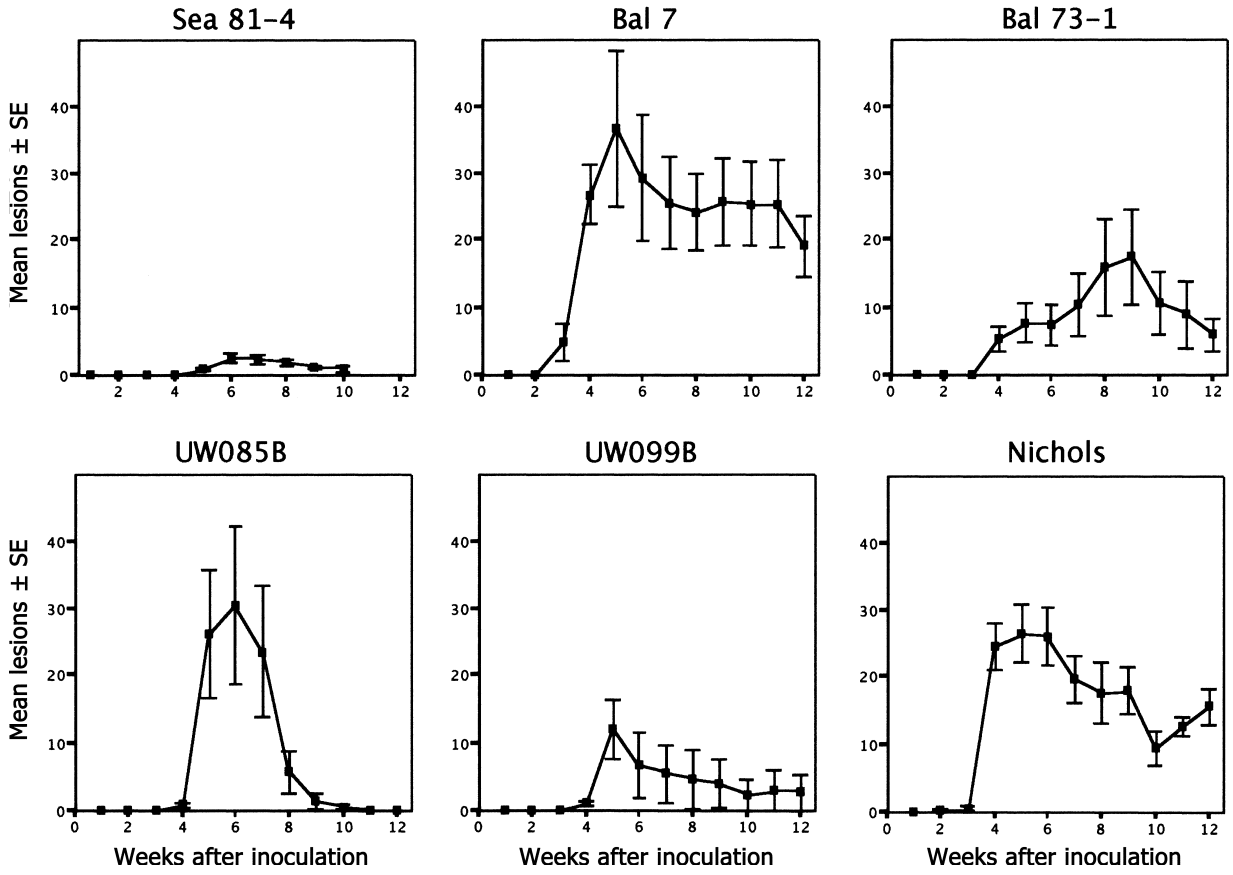

Figure 3. Mean ± SE number of indurated lesions after intravenous inoculation with 6 strains of *Treponema pallidum*

as with *Borrelia* species, there are strains of *T. pallidum* that are particularly neuroinvasive.

Studies from the preantibiotic era in humans suggested that there was a relationship between the skin manifestations of early syphilis and involvement of the CNS. Individuals with "florid" skin lesions were more likely to have CSF abnormalities in early syphilis, and lack of skin lesions was associated with a greater likelihood of developing symptomatic neurosyphilis [1–3]. We expected to see a positive correlation between severity of skin lesions and CSF abnormalities. Instead, we show that CSF pleocytosis, but not the identification of *T. pallidum* in CSF, was inversely related to the severity of skin lesions. This association was most striking in rabbits infected with Sea 81-4, the strain that induced persistent CSF pleocytosis. The explanation for this unexpected finding may be that the persistent CSF pleocytosis that was seen in rabbits infected with Sea 81-4 and not in rabbits infected with other strains reflects true asymptomatic neurosyphilis. Thus, our data may support the association between neurosyphilis and poor skin-lesion development observed in humans in the preantibiotic era.

Use of a rabbit model enabled us to perform a study that is not possible in humans. However, its use poses certain challenges. Because each rabbit underwent repeat cisternal punctures, it is possible that their CSF was contaminated by blood organisms at the time of the procedure. It is also possible that contamination of CSF with RBCs at 1 time point could elicit CSF pleocytosis at a subsequent time point because of chemical meningitis. We do not think that these potential limitations apply to our work. Detection of *T. pallidum* in CSF was not more common in rabbits with higher CSF RBC counts, was not uniform across strains, and clustered in time in those strains that demonstrated clear neuroinvasion. These findings argue strongly that contamination of CSF with blood organisms did not occur. Similarly, CSF pleocytosis was not uniform among rabbits, and there was no correlation between CSF WBC count and CSF RBC count, supporting the contention that the CSF pleocytosis that we observed was not due to CSF RBC contamination. We did not see a relationship between CSF WBC count or serum VDRL titer and detection of *T. pallidum* in CSF, as has been noted in humans [4]. The reason for this difference may simply be that the relatively small number of positive RT-PCR test results limited the power of the analysis, or it may relate to more-severe disease in the infected humans who had wider ranges of serum RPR titers and CSF WBC counts than were seen in the rabbit model.

Our data have important implications for studies of the pathogenesis, diagnosis, and treatment of neurosyphilis in humans. They support the validity and utility of the intravenous infection model, which, in future studies, could be used to dissect the immune response that is responsible for clearance of organisms from the CNS. In addition, the model may allow for phenotypic or genotypic identification of *T. pallidum* strains that are particularly neuroinvasive, thus providing clues regarding the virulence factors involved in CNS infection. This information could ultimately be used to identify patients who are at particular risk for neuroinvasion and, thus, at risk for neurosyphilis. These individuals could be targeted for more-extensive evaluation, such as CSF examination, or even for empiric therapy to prevent or treat CSF infection. Further study is required to identify the characteristics of neuroinvasive *T. pallidum* strains.

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