

# Biological Basis for Syphilis

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INTRODUCTION .....	29
OVERVIEW OF THE NATURAL HISTORY OF SYPHILIS.....	30
Primary Syphilis .....	30
Secondary Syphilis.....	31
CNS Involvement .....	31
Latent Syphilis .....	31
Tertiary Syphilis.....	32
Gumma.....	32
Cardiovascular syphilis.....	32
Late neurological complications .....	32
Congenital Syphilis.....	32
BIOLOGY OF <i>T. PALLIDUM</i> .....	33
Limited Metabolic Capacity .....	33
In Vivo and In Vitro Growth of <i>T. pallidum</i> .....	33
Paucity of Recognized Virulence Factors .....	34
INVASION .....	34
Attachment.....	35
Motility.....	35
Chemotaxis .....	36
INFLAMMATION AND IMMUNE RESPONSE .....	36
Inflammation .....	36
Dendritic Cells .....	37
Cytokine Production .....	37
Immune Clearance.....	37
Antibodies in Syphilis Immunity .....	38
Antibodies in Diagnostic Testing.....	38
EVASION OF THE IMMUNE RESPONSE AND CHRONIC INFECTION .....	39
Surface of <i>T. pallidum</i> .....	39
Tpr Proteins.....	40
GENETIC VARIATION AMONG SUBSPECIES AND STRAINS .....	42
<i>tpr</i> Genes .....	42
Other <i>T. pallidum</i> Genes .....	42
IMPLICATIONS FOR SYPHILIS CONTROL .....	42
Treatment of Infection .....	42
Macrolide Resistance .....	42
Vaccine Development.....	42
REFERENCES .....	43

## INTRODUCTION

Since its recognition in 15th-century Europe as a new disease, syphilis has been the subject of great mystery and legend. Speculation abounds, often with scant evidence, about famous persons who may have had syphilis—the implicated include political figures (165, 172, 210), musicians (87, 114, 254), and literary greats (113, 187, 258, 262). Many believe that syphilis was brought to Europe by Columbus and his sailors, although there is no objective proof of this theory (176). John Hunter, the eminent mid-18th-century Scottish physician and venereologist, in order to test whether syphilis and gonorrhea were

the same or different diseases, is said to have inoculated himself with pus from an individual infected with a sexually transmitted disease (46, 84, 159). Unfortunately for Hunter, the patient was infected with the etiologic agents of both diseases. This was also unfortunate for the medical community, as it caused decades of medical and scientific confusion. Finally Philippe Ricord's studies, published in 1838, clarified that syphilis and gonorrhea are indeed distinct infections (253). The greatest mystery of syphilis, however, is how the spirochete *Treponema pallidum* subsp. *pallidum* causes the many clinical features of disease. Despite the inherent difficulties in investigating this organism, researchers have been successful in uncovering some of the secrets of *T. pallidum*'s biology and the pathogenesis of syphilis, but much remains to be discovered. In previous issues of this journal, a 1999 review focused on the epidemiological and clinical aspects of syphilis (290) and a

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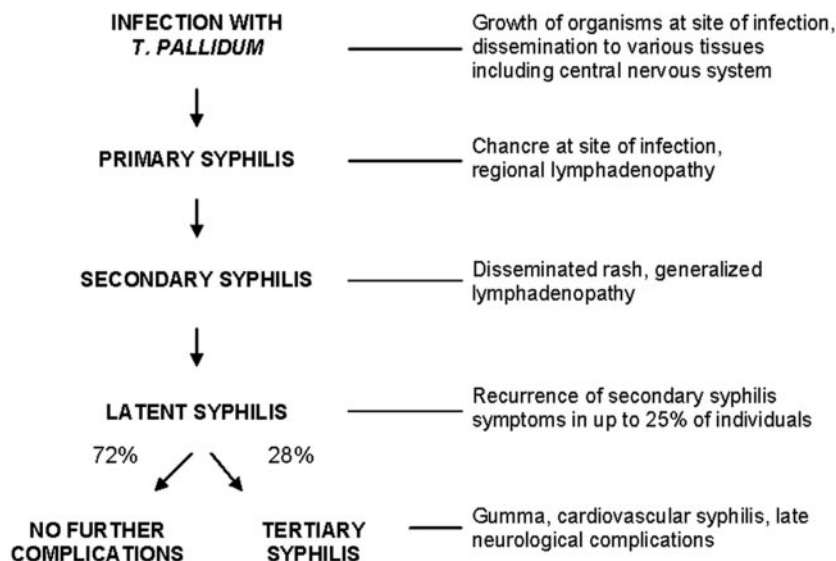


FIG. 1. Natural history of untreated syphilis, according to Gjestland (112).

2005 review provided a detailed description of secondary syphilis (20). Here, we address research on the biology of *T. pallidum* and syphilis pathogenesis, highlighting investigative efforts over the last five years.

Syphilis is a chronic disease, and *T. pallidum*'s only known natural host is the human. Syphilis is acquired by direct contact, usually sexual, with active primary or secondary lesions. Studies have shown that 16 to 30% of individuals who have had sexual contact with a syphilis-infected person in the preceding 30 days become infected (205, 274); actual transmission rates may be much higher (6, 106, 271). Infection also occurs when organisms cross the placenta to infect the fetus in a pregnant woman. In the United States, the incidence of syphilis during the Second World War was over 500,000 infections per year. Between the years 1945 and 2000, syphilis declined to 31,575 reported infections, with alternating peaks and troughs of infectious cases. Since 2000, there has been an increase in the number of syphilis cases in the United States, mainly among men who have sex with men (MSM) (47–50, 52); these outbreaks have been reported along the west coast of the United States and in New York. Similar increases in syphilis in MSM have been reported in western Europe and the United Kingdom (111, 145, 166, 288, 321). Outbreaks among MSM are associated with a rise in unsafe sexual behavior, perhaps a consequence of improved antiretroviral treatment for human immunodeficiency virus (HIV); in recent surveys, 37% to 52% of MSM reported multiple risk behaviors (69, 145, 146).

Compared to syphilis rates in developed countries, the worldwide burden of syphilis is formidable. The World Health Organization estimates that 12 million new cases of syphilis occur each year (107). The vast majority of these are seen in developing nations, but an increase in new cases has also been noted in eastern Europe since the dissolution of the Soviet Union (264, 336). Congenital syphilis is of particular concern in developing nations, where the lack of prenatal testing and antibiotic treatment of infected pregnant women results in congenital infection of the fetus. Congenital syphilis causes

spontaneous abortion, stillbirth, death of the neonate, or disease in the infant; a recent report from Tanzania estimates that up to 50% of stillbirths are caused by syphilis (330). Of particular importance to worldwide health is the recognition that syphilis infection greatly increases the transmission and acquisition of HIV (115, 300). These factors, along with the highly destructive nature of late disease, make syphilis an important public health concern.

## OVERVIEW OF THE NATURAL HISTORY OF SYPHILIS

Syphilis is a multistage disease with diverse and wide-ranging manifestations; the distinct stages of syphilis were first described in detail by Philippe Ricord in the mid-1800s (253). A schematic diagram of untreated syphilis is shown in Fig. 1.

### Primary Syphilis

Infection is initiated when *T. pallidum* penetrates dermal microabrasions or intact mucous membranes, typically resulting in a single chancre at the site of inoculation. Moderate regional lymphadenopathy is associated with the primary stage. The chancre usually becomes indurated and will progress to ulceration but typically is not purulent. In heterosexual men, primary chancres most commonly occur on the penis, but 32 to 36% of homosexual men have primary chancres in other sites, including the rectum, anal canal, and oral cavity (146, 204). In women, the primary chancre usually occurs on the labia or cervix. Because the chancre is painless and may be located in an inconspicuous anatomical site, diagnosis of syphilis in women and homosexual men is sometimes delayed until later disease manifestations become apparent. Clinical evaluation can also be complicated by the fact that the appearance of primary chancres in some individuals does not fit the classic description. Nonindurated lesions with irregular borders have been observed (67, 85), as have multiple and/or painful lesions,

especially in the anal area and in individuals who are infected with HIV (146, 259).

The primary chancre develops an average of 3 weeks after exposure; the incubation period ranges between 10 and 90 days (320). Based on intradermal inoculation of human volunteers with graded doses of *T. pallidum*, the 50% infectious dose is estimated to be 57 organisms (188); lesions develop more rapidly when the inoculum size is larger (189). The primary chancre heals spontaneously within 4 to 6 weeks, but may still be discernible in about 15% of patients at the onset of secondary syphilis (150, 204, 259). HIV-infected individuals are reported to be two to three times more likely to have concurrent primary and secondary disease than HIV-uninfected individuals (150, 259).

### Secondary Syphilis

Within hours of inoculation, and during the evolution of the primary stage, *T. pallidum* disseminates widely and organisms are deposited in a variety of tissues. Manifestations of secondary syphilis usually occur within 3 months of initial infection and are sometimes quite subtle. A detailed description of secondary syphilis can be found in the review by Baughn and Musher (20). Sore throat, muscle aches, malaise, and weight loss are examples of variable systemic symptoms of secondary syphilis. Generalized nontender lymphadenopathy occurs in up to 85% of cases (66, 141). The most common manifestation of secondary syphilis is a disseminated mucocutaneous rash. Pale and discrete macular lesions appear initially on the trunk and proximal extremities; these are followed by lesions of various morphologies among patients. At diagnosis, the most common types of secondary syphilis lesions are maculopapular (in 50% to 70% of patients), papular (12%), macular (10%), and annular papular (6% to 14%) (66, 141). In rare individuals, lesions may become necrotic, a condition called lues maligna (86); more often, secondary lesions are inconspicuous and may go undetected (66). Rash is frequently found on the palms of the hands and soles of the feet and, in 4% to 11% of patients, *T. pallidum* infection of hair follicles results in alopecia of the scalp (141, 204). There have also been infrequent reports of facial and body hair loss (1, 239).

Concurrent with the appearance of secondary lesions, about 10% of patients develop condylomata lata (66, 204). These enlarged lesions, appearing in warm and moist areas including the perineum and anus, are highly infectious. Localized inflammation of the oral cavity, tongue, and genital mucous membranes can cause mucous patches (66, 204); infrequently, secondary syphilis can be accompanied by gastric and renal involvement and hepatitis (118, 213). *T. pallidum* has been found in liver biopsy samples taken from patients with secondary syphilis (92, 170), while glomerulonephritis resulting from immunoglobulin-treponeme antigen complexes deposited in the glomeruli appears to cause kidney damage (17, 224, 315). Nephrotic syndrome may also be present. Approximately 5% of individuals with secondary syphilis experience the early manifestations of neurosyphilis, including meningitis and ocular disease (66, 301).

### CNS Involvement

Classically, neurological complications of syphilis have been associated with tertiary disease, but studies have demonstrated that penetration of the central nervous system (CNS) by *T. pallidum* occurs during earlier stages of disease. Neurosyphilis is defined by increased protein and leukocyte levels in the cerebrospinal fluid (CSF) or a reactive CSF-VDRL (Venereal Disease Research Laboratory) test. *T. pallidum* can be detected in the CSF of many patients with syphilis, sometimes so early after CNS invasion that pleocytosis and other CSF abnormalities may have not yet appeared. About 40% of early syphilis patients and 25% of individuals with latent infection meet at least one of the diagnostic criteria for neurosyphilis (183, 193). The prognostic implications of CNS invasion are difficult to determine. The majority of persons with CNS invasion by *T. pallidum* appear to resolve or control CNS treponemes; however, there are no known indicators for subsequent development of symptomatic neurosyphilis. A recent large neurosyphilis study indicates that a serum rapid plasma reagin (RPR) titer greater than or equal to 1:32 is significantly associated with neurosyphilis, regardless of syphilis stage or concurrent HIV infection (193).

Early symptoms of neurosyphilis may occur concurrently with primary or secondary syphilis or may follow resolution of secondary syphilis. Typical symptoms of acute early meningitis include fever, headache, nausea, vomiting, and stiff neck. Cranial nerve involvement may result in visual disturbances, including blurred vision and photophobia, hearing loss, and facial weakness (143). Ocular inflammation (uveitis) and numbness and pain in the extremities can also occur. Rarely, individuals with early neurosyphilis report memory loss and mental confusion. In the decade after the recognition of HIV infection, some investigators claimed that neurosyphilis symptoms are exacerbated in HIV-infected individuals; however, that conclusion has not been supported by more-recent large studies (193, 259). Also, HIV infection was not more likely to result in increased CSF pleocytosis (193). Despite the lack of association between HIV status and severity of CNS disease, standard benzathine penicillin therapy, particularly in those with concurrent HIV infection, may not be sufficient to resolve infection. Thus, neurosyphilis therapy is warranted in all patients with CSF abnormalities consistent with neurosyphilis (194).

### Latent Syphilis

Disseminated lesions and other manifestations of secondary syphilis usually resolve spontaneously within 3 months of appearance, and symptoms are absent for a variable period of time in untreated individuals. Latent syphilis is divided into two stages, based upon an approximation of the time of infection. For the first year after infection, patients are considered to have early latent syphilis; up to 25% may have recurrent secondary manifestations (112). Late latent syphilis is defined as asymptomatic infection of longer than one year or unknown duration. Serologic testing during the late latent stage is positive, but sexual transmission is unlikely. Organisms may seed the bloodstream intermittently during latent syphilis and can infect the developing fetus during pregnancy. Latent syphilis

ends when curative antibiotic therapy is administered or when manifestations of tertiary disease develop.

### Tertiary Syphilis

Today, when coincidental antibiotic therapy is common, late manifestations of syphilis are rarely seen. In contrast, the historic Oslo (112) and Tuskegee (161) studies help to provide an understanding of the late manifestations of disease. In the retrospective Oslo study (112), conducted in the preantibiotic era, approximately one-third of individuals with latent syphilis infection developed clinical manifestations of tertiary syphilis. These syndromes often did not appear until 20 to 40 years after the onset of infection. Progressive inflammation caused gumma (late benign syphilis), a localized form of tissue and bone destruction, in 15% of patients with untreated syphilis. Cardiovascular syphilis, usually seen as aortic insufficiency or aneurysm, was observed in 10% of untreated patients. Symptomatic late neurosyphilis was recognized in 6.5% of untreated patients. Although the Tuskegee study (161), in which 408 African-American men with untreated latent syphilis in Macon County, Alabama, were observed prospectively between the 1932 and 1972, provided information about late disease manifestations, the motivations of the investigators have been questioned by ethicists and human interest groups. Informed consent was not obtained from subjects and, regrettably, penicillin treatment was not offered to the subjects after its advent as syphilis therapy in the 1950s, leading to preventable morbidity and mortality.

**Gumma.** Granulomatous, nodular lesions with variable central necrosis may develop as early as 2 years after initial infection, although they usually appear much later. These destructive lesions most commonly affect the skin and bones, although they may also occur in the liver, heart, brain, stomach, and upper respiratory tract. *T. pallidum* can occasionally be identified in gummatous lesions (122, 301); lesions rarely heal spontaneously but resolve rapidly with appropriate antibiotic therapy. Unless they affect a critical organ, gummas usually do not cause serious complications, thus the term "late benign syphilis."

**Cardiovascular syphilis.** Prior to the advent of penicillin therapy, the majority of deaths due to syphilis were thought to result from cardiovascular involvement (260). Typically, syphilitic aortitis involves the ascending aorta; in most cases, aortitis is uncomplicated and asymptomatic. Complications occur in approximately 10% of individuals with untreated syphilis (160), the most common being aortic regurgitation. Other complications are coronary ostial stenosis and saccular aneurysm (153). Recently, PCR methods have detected *T. pallidum* DNA in an aortic aneurysm (223), confirming that damage results from actual infection of the aorta.

**Late neurological complications.** Within 5 to 10 years of untreated initial infection, early invasion of the CNS may progress to meningovascular syphilis, with complications due to cerebrovascular accident. Prior to this event, individuals may experience vertigo, insomnia, and personality changes; these symptoms are followed by arterial involvement that can be widespread or focal (201). Loss of consciousness and seizures may also be present.

Late parenchymatous syphilis, presenting as general paresis

or tabes dorsalis, appears two to three decades after infection. Symptoms of general paresis include personality changes, emotional instability, memory impairment, hallucinations, and hyperactive reflexes. Involvement of spinal cord posterior columns and dorsal root ganglia causes tabes dorsalis, which presents as sensory ataxia in the lower extremities, paresthesia, and sudden-onset vomiting or abdominal pain. Wide-based gait often results from the loss of position and vibratory senses. Loss of temperature and deep pain sensations can also occur; the latter may result in Charcot's joints (trophic lesions in ankles, knees, and hips). Optic nerve damage occurs in about 20% of cases of tabes (289), and both paresis and tabes can present with papillary abnormalities.

### Congenital Syphilis

*T. pallidum* can be transmitted from the bloodstream of the infected woman to her developing fetus at any time during pregnancy, although risk of fetal infection is much higher during early maternal syphilis (the first year of infection) than during later stages (284). Antibiotic treatment of the mother during the first two trimesters is usually sufficient to prevent negative outcomes, but later treatment or lack of treatment may result in fetal death, fetal damage, or birth of an infected infant. Destructive effects are thought to depend upon the immune response of the fetus and include spontaneous abortion, stillbirth, and premature delivery. Affected infants typically have low weight at birth, and infants with congenital syphilis may be underweight even relative to other infants of the same gestational age (196). Pulmonary hemorrhage, secondary bacterial infection, and severe hepatitis cause death of approximately 4% of *T. pallidum*-infected neonates soon after delivery (65, 104).

Like adult disease, congenital syphilis is divided into stages: early manifestations appearing in the first 2 years of life, late manifestations appearing after 2 years, and residual stigmata. Early manifestations are infectious and resemble severe symptoms of adult secondary syphilis; they usually become apparent 2 to 10 weeks after delivery. The first symptom seen in up to 50% of newborns with congenital syphilis is "snuffles." Invasion of *T. pallidum* into the nasal mucosa causes persistent rhinitis with a whitish discharge that is sometimes tinged with blood. *T. pallidum* can further invade the bones and cartilage of the nose and palate, leading to gummatous destruction later in life. Infants with early congenital syphilis commonly have skin lesions resembling those of adult secondary disease, sometimes accompanied by desquamation of the skin of palms and soles (68), condylomata lata, and mucous patches. Other evidence of infection includes anemia, hepatosplenomegaly, renal involvement, and jaundice. Osteochondritis of the long bones can lead to pain and lack of movement of the upper and lower extremities (Parrot's pseudoparalysis); radiographs of the long bones can be helpful in establishing the diagnosis.

Late manifestations of congenital syphilis occur after two years of life. Between the ages of 5 and 25, interstitial keratitis may cause damage to the cornea and iris, and eighth-nerve deafness may be apparent. Asymptomatic and symptomatic neurosyphilis, arthropathy, bilateral effusions of knees and elbows (Clutton's joints), and gummatous periostitis of the palate and nasal septum may also occur. Many of these manifes-

tations progress despite treatment. Hutchinson's teeth, peg-shaped notched upper incisors, is another characteristic late stigma of congenital syphilis. Other tooth deformities, saddle nose, and saber shins may also occur.

### BIOLOGY OF *T. PALLIDUM*

*T. pallidum* subsp. *pallidum* belongs to a family of spiral-shaped bacteria, the *Spirochaetaceae* (spirochetes), and is related to other pathogenic treponemes that cause nonvenereal diseases. *T. pallidum* subsp. *endemicum* (bejel), *T. pallidum* subsp. *pertenue* (yaws), and *T. carateum* (pinta) can be differentiated from *T. pallidum* by the clinical manifestations of their respective diseases and, more recently, by genetic differences (55, 58). *T. pallidum* varies from 6 to 15  $\mu\text{m}$  in length and is 0.2  $\mu\text{m}$  in diameter. In both clinical and laboratory settings, dark-field microscopy is used for visualization of this small organism. The spiral-shaped body of *T. pallidum* is surrounded by a cytoplasmic membrane, which is enclosed by a loosely associated outer membrane. A thin layer of peptidoglycan between the membranes provides structural stability. Endoflagella, organelles that allow for the characteristic corkscrew motility of *T. pallidum*, are located in the periplasmic space (155).

#### Limited Metabolic Capacity

The *T. pallidum* genome, known to be small (327, 328), was confirmed by the Genome Sequencing Project to be 1.14 Mb and to encode 1,041 putative proteins (105). Few bacteria have genomes smaller than that of *T. pallidum*; indeed, the genomes of many conventional gram-negative (e.g., *Escherichia coli* K-12, 4.6 Mb) and gram-positive (e.g., *Bacillus subtilis*, 4.2 Mb) bacteria are several times larger. The *T. pallidum* Genome Sequencing Project also confirmed what had already been suggested by ongoing experimental efforts: that *T. pallidum* has a striking lack of metabolic capabilities. The organism is able to carry out glycolysis (105, 217, 270) but lacks tricarboxylic acid cycle enzymes and an electron transport chain (105). Analysis of the *T. pallidum* genome suggests an absence of pathways for the use of alternative carbon sources for energy and for de novo synthesis of enzyme cofactors and nucleotides (105). Amino acid and fatty acid synthesis pathways are also lacking (105), but *T. pallidum* does carry enzymes for the interconversion of amino acids and fatty acids. With this dearth of biosynthetic pathways, it is suspected that *T. pallidum* derives most essential macromolecules from the host, using interconversion pathways to generate others.

To take up macromolecules from the host environment, specific transporters may be utilized by *T. pallidum*. Homologs of transporters for a variety of amino acids are found in the *T. pallidum* genome (105). The lipoprotein TpN32 is homologous to a member of the newly identified methionine uptake transporter family in *E. coli* (337), and the methionine-binding properties of TpN32 were recently described (83), suggesting that these are part of a methionine transport system in *T. pallidum*. Six *T. pallidum* transporters have specificity for cations (105). The most extensively studied of these is the ATP-binding cassette (ABC) transporter encoded by the *tro* operon. TroA (alternatively termed TROMP1), the cation-binding protein of the Tro complex, binds zinc (82, 105, 171) and manga-

nese (137), trace metals that may be required for enzyme function in *T. pallidum*.

*T. pallidum* homologs to *dct* (*Rhodobacter*) and *y4o* (*Rhizobium*) encode proteins that are likely to transport multiple sugars across the cytoplasmic membrane. In classical gram-negative organisms, the ABC transporter MglABC has specificity for galactose (126, 212). The homologous Mgl system in *T. pallidum* (21, 236, 299) may also bind galactose, but it has been speculated that, because of its inability to utilize galactose as a carbon source, *T. pallidum* may utilize MglABC as a glucose transporter (81). Ribose may be taken into the cell via an ABC transporter with homology to the RbsAC transporter system in the related spirochete *Borrelia burgdorferi*; however, carbon utilization studies demonstrated that ribose is not degraded by *T. pallidum* (217). Thus, the RbsAC homolog may function to transport other sugars or may be nonfunctional in *T. pallidum*.

Because the *T. pallidum* genome encodes no known homologs to porin proteins, it is unclear how nutrients are moved across the outer membrane into the periplasmic space. A recent study (135) suggests that Tp0453, a putative outer membrane protein, may perturb the outer membrane by insertion into its inner leaflet, allowing nonselective diffusion of nutrients into the periplasm.

#### In Vivo and In Vitro Growth of *T. pallidum*

Although *T. pallidum* was one of the first infectious agents to be associated with a human syndrome (269), efforts to elucidate molecular mechanisms of *T. pallidum* virulence have been hampered by certain characteristics of the organism. *T. pallidum* does not survive outside the mammalian host; infectious capability is lost within a few hours or days of harvest. To obtain sufficient organisms for experimental manipulation, *T. pallidum* must be propagated in rabbits (317). As yet, researchers have been unable to propagate *T. pallidum* in tissue culture more than 100-fold, an equivalent of about seven generations (95, 96, 220). With the expectation that providing fresh media and removing toxins from the culture system would extend its metabolic capabilities, Norris and Edmondson attempted to subculture *T. pallidum* in vitro (221) but were unable to maintain viable organisms. This inability of *T. pallidum* to survive and multiply outside the mammalian host is perhaps the greatest impediment to syphilis research.

The generation time of *T. pallidum* is unusually slow. Inoculation studies determined that *T. pallidum* doubles every 30 to 33 h in vivo (77, 189); extrapolating generation time from the number of replication cycles in tissue culture yields an in vitro generation time of 30 to 50 h (95, 220). Several biological factors may contribute to *T. pallidum*'s sluggish replication rate. Because it lacks a tricarboxylic acid cycle and an electron transport chain (105), *T. pallidum* depends upon glycolysis as the sole pathway for the synthesis of ATP. In fact, the theoretical energy yield of an organism that undergoes aerobic respiration, such as *E. coli*, is 38 ATP, 19 times greater than the 2 ATP synthesized from glycolysis alone. *E. coli* doubles approximately every 20 min, at least 90 times faster than *T. pallidum*, suggesting that low energy production is not the only factor that inhibits *T. pallidum* replication. Because of a lack of enzymes such as catalase and oxidase that detoxify reactive

oxygen species (105), the in vitro survival of *T. pallidum* is prolonged by low oxygen concentrations. Genes for several other oxygen-protective enzymes have been identified in the *T. pallidum* genome. Neelaredoxin (Tp0823) is hypothesized to convert superoxide to peroxide, which is then reduced to water by hydroperoxide reductase C (Tp0509). Each of these enzymes is regenerated by other *T. pallidum* proteins (136).

In addition to its sensitivity to oxygen, *T. pallidum* may have a limited stress response. The typical heat shock response regulated by  $\sigma_{32}$  is lacking (105, 296), possibly reflecting the sensitivity of the organism to growth temperature (95). At least one *T. pallidum* enzyme is unstable at normal body temperature (22), suggesting that the heat lability of enzymes may also contribute to the slow growth of the organism. Heat therapy for late neurosyphilis was introduced in 1918 by the Viennese psychiatrist Julius Wagner von Jauregg (326), a discovery for which he later won the Nobel Prize in Medicine. The regimen consisted of inoculating patients with malaria-infected blood and, 10 to 12 febrile episodes later, treating them with quinine. The high temperatures induced by this regimen, along with other methods of raising body temperature that were later introduced, presumably killed *T. pallidum* in the CNS. Doctors reported high percentages of complete or partial remission of general paresis symptoms (although the "treatment" killed an estimated 10% of patients) (301), causing heat therapy to be in vogue for more than a quarter of a century. It is clear that *T. pallidum* has limited heat tolerance; this, along with oxygen sensitivity and possibly other as-yet-unrecognized factors, may hinder the replication of *T. pallidum* both in vivo and in vitro.

Genetic intractability, related to the inability to grow the organism, is another hindrance to *T. pallidum* molecular research. Unlike the related spirochetes *Treponema denticola* (110) and *B. burgdorferi* (89, 268, 291), no system for genetic manipulation of *T. pallidum* yet exists. Because of the fragility of its outer membrane, genetic manipulation of *T. pallidum* may prove impossible. Heterologous expression in related organisms such as *T. denticola* (70) may be the most practical way to study *T. pallidum* genes and advance our understanding of this enigmatic organism.

#### Paucity of Recognized Virulence Factors

Despite its fragility to environmental factors, *T. pallidum* readily causes chronic infection and varied disease manifestations in the host. The *T. pallidum* genome sequence does not reveal any obvious classical virulence factors that could account for syphilis signs and symptoms. *T. pallidum* lacks lipopolysaccharide (LPS) (105), the endotoxin found in the outer membranes of many gram-negative bacteria that causes fever and inflammation. However, *T. pallidum* does produce a number of lipoproteins which may induce expression of inflammatory mediators via toll-like receptor 2 (TLR2) recognition (38, 173). Many gram-negative pathogens utilize type III secretion systems to insert virulence-related proteins into the cytoplasm of host cells (335); *T. pallidum* lacks homologs for the recognized type III components (105). Cytolytic enzymes or other cytotoxins have not been shown to play a role in syphilis pathogenesis, and a cytopathic effect of treponemes on cells in culture requires extraordinarily high numbers of bacteria (103). Therefore, the identification of several putative hemolysins in

the annotated *T. pallidum* genome was unexpected. These predicted proteins, however, have only weak similarity to known cytolysins, and recombinant preparations of these proteins showed no hemolytic activity. Thus, the true function of these proteins is unknown. Nonetheless, experimental studies and exploration of the *T. pallidum* genome have revealed significant new information about many aspects of syphilis pathogenesis.

#### INVASION

Despite a striking lack of metabolic capabilities, sensitivity to oxygen, and decreased viability in an environment warmer than body temperature, *T. pallidum* is able to invade and survive in a wide variety of tissues and organs. The detection of *T. pallidum* in the CSF of a high proportion of individuals with early syphilis, as well as the disseminated clinical manifestations of secondary, tertiary, and congenital syphilis, provide evidence of the highly invasive capabilities of the organism. Typical of other pathogenic spirochetes, including *Borrelia* and *Leptospira* species, dissemination is not only widespread, it is rapid. In rabbit infection, *T. pallidum* enters the bloodstream within minutes of intratesticular or intradermal inoculation (77, 301), and organisms applied to mucosa are found in deeper tissues within hours (190).

Direct evidence for the ability of *T. pallidum* to invade many different tissue types is provided by numerous studies of experimental infection. Blood from mice, monkeys, and rabbits during early stages of infection is infectious to naïve animals, indicating the ability of the organism to gain access to the bloodstream. Infectivity tests have also been performed on various tissues from these animals. The liver and lymph nodes of intradermally infected macaques were shown to contain live *T. pallidum* bacteria (317) and, after 45 days of mouse infection, the skin, brain, spleen, and lymph nodes were infectious to rabbits (261).

The rabbit is the most extensively studied animal model for syphilis. Rabbits are less expensive and easier to work with than monkeys, and unlike mice, which do not develop any signs of infection, rabbits produce disease manifestations that are similar clinically and histologically to human primary and secondary disease (9, 276). After intratesticular infection in rabbits, early researchers noted skin and bone lesions (317), indications of the presence of virulent *T. pallidum*. *T. pallidum* was demonstrated by microscopy in rabbit skin, testes, spleen, and lymph nodes after intradermal infection (276). After testicular infection of rabbits, treponemes were visible in the lymph nodes, brain, and aqueous humor, and in the CSF as early as 18 h postinoculation (73). *T. pallidum* RNA has also been detected by reverse transcription-PCR in the CSF of intravenously infected animals (306), and Marra et al. found that 6% of rabbits inoculated by the intrathecal method developed ocular syphilis (192), demonstrating that organisms are able to travel from the CSF to the eye. Not only do treponemes disseminate to distant sites but they also can persist in distant tissues during chronic infection. Unapparent or latent infection in experimental animals is routinely demonstrated by the ability of lymph node extracts to infect naïve animals (63, 138, 317); infectivity testing has also demonstrated *T. pallidum* in the blood and liver of intratesticularly infected rabbits (73).

The diverse manifestations of human syphilis also demonstrate the invasiveness of *T. pallidum*. Humans are initially infected with syphilis at anogenital and, more rarely, oral and nongenital dermal sites, yet the rash of secondary syphilis is a clear indication that organisms disseminate widely from the primary site of contact. *T. pallidum* has been detected directly in tissues and fluids far from the initial site of infection. Using PCR and infectivity testing, *T. pallidum* is routinely found in the CSF of individuals with early and latent syphilis (183, 193). *T. pallidum* has been detected decades after initial infection in tertiary gummatous lesions of the skin by silver staining and immunofluorescence microscopy (122, 160, 301) and by PCR (338).

### Attachment

As with many bacterial pathogens, the first step in *T. pallidum* invasion is the attachment of organisms to host cells. *T. pallidum* has been shown to attach to a wide variety of cell types including epithelial, fibroblastlike, and endothelial cells of rabbits and humans (99, 134, 168, 311). A reduction in attachment occurs when either bacterial or eukaryotic cells in culture are not viable (99). Organisms are also able to adhere to isolated capillary (241), kidney (102), and abdominal wall tissues (257) *ex vivo*. Microscopic examination of *T. pallidum* associated with cell cultures (98, 99) reveals that large numbers of organisms attach to cells by one or the other end. Some researchers have speculated that specialized *T. pallidum* adhesins are located at the tips of the organisms, but treponemes have also been observed to be attached to host cells along their length. It has been speculated that initial attachment can occur at multiple sites along the length of the bacterium but that the active motility of the attached treponemes serves to cause the bacterial adhesin molecules to migrate through the somewhat fluid outer membrane toward the tips, in a "capping" phenomenon. The number of treponemes that can attach to each host cell appears to be limited: the addition of virulent *T. pallidum* bacteria to cells with organisms already attached does not increase the total number of adherent *T. pallidum* bacteria per cell (134). This limitation of binding may occur because all of the receptors on the host cell are occupied, implying a specific adhesin-ligand interaction; integrins have been implicated as host cell receptors for *T. pallidum* (168). The nonpathogenic treponemal species *T. phagedenis*, *T. refringens*, and *T. vincentii* do not adhere to cultured cells (98, 99, 134), indicating that attachment is specific to pathogenic treponemes. Heat-killed *T. pallidum* organisms do not attach to cells (100), nor do *T. pallidum* organisms that have been rendered nonmotile by 22 to 23 h of incubation at 37°C (99). Immune rabbit (99, 134) or human (322) serum prevents the adherence of viable *T. pallidum* organisms to cell cultures, suggesting that specific surface antigens of *T. pallidum* may mediate attachment to host cells.

Components of host serum, cell membranes, and the extracellular matrix (ECM) have been shown to bind to *T. pallidum*, and ECM components have been demonstrated to be involved in mediating attachment of other pathogenic bacteria to host cells. *T. pallidum* attaches to fibronectin-coated coverslips (41, 101, 102, 234, 309); this attachment is enhanced by pretreatment of organisms with fibronectin (308) and is inhibited by antifibronectin antibodies (102, 309) or immune rabbit serum

(102). Pretreatment of cell monolayers with antifibronectin antibodies also inhibits the attachment of *T. pallidum* to these cells (234, 309). Other ECM components, such as laminin, collagen I, and hyaluronic acid, have been reported to bind to *T. pallidum* (39, 102). Except for the latter, attachment of *T. pallidum* to coated coverslips is blocked by antibodies directed against each component (102).

Three treponemal proteins with fibronectin-binding properties were discovered and partially characterized over 20 years ago (18, 310), but until recently the identity of potential *T. pallidum* adhesins was unknown. Cameron et al. (41) used computer analysis to search the *T. pallidum* genome for adhesin candidates. Two genes, referred to by their genome sequence designations *tp0155* and *tp0483*, were expressed as recombinant proteins and assayed for the ability to bind to fibronectin. Tp0155 binds to matrix fibronectin, while Tp0483 binds to both soluble and matrix forms of fibronectin (41); binding occurs in a dose-dependent manner. This finding suggests that one molecule may mediate attachment via fibronectin in the bloodstream, while the other is functional in tissues. Another recombinant *T. pallidum* protein, Tp0751, binds specifically to laminin (39), and antibodies against Tp0751 have been shown to inhibit the binding of *T. pallidum* to laminin-coated coverslips (40).

Binding to ECM components is a virulence factor in many microorganisms (97). The major sheath protein (Msp) of the related oral spirochete *T. denticola* binds fibronectin and laminin (94, 120), as well as other ECM components (88, 319), implicating Msp as an important molecule in colonizing host tissues. *Streptococcus pyogenes* binds to host ligands using a wide variety of adhesins (227). Immunization with an *S. pyogenes* fibronectin-binding protein, Sfb1, protects mice against intranasal challenge (119) but does not protect against subcutaneous challenge (197), suggesting that the variety of *S. pyogenes* adhesin molecules may allow the organism to penetrate a wide variety of tissue types. In the same way, *T. pallidum* adhesins that bind to different ECM components may contribute to the ability of the organism to penetrate different tissues and disseminate widely during infection.

### Motility

Motility is a virulence factor for many bacterial pathogens (157, 225). *T. pallidum* is a highly motile organism that propels itself by rotating around its longitudinal axis. This corkscrew-like motility is common to other spirochetes (45) and allows this group of organisms to swim easily through gel-like materials that hinder most other flagellated organisms (23). This ability allows some spirochetes to occupy unique ecological niches, such as sediments in pond and lake bottoms, the guts of certain arthropods, and the rumens of cows and sheep. The arrangement of spirochete flagella is also unique among the bacteria. Early electron microscopy studies of *T. pallidum* revealed filamentous structures wrapped around the cytoplasmic body (209, 303). These "axial filaments," now called endoflagella or periplasmic flagella, are located in the periplasmic space, between the cytoplasmic membrane and the outer membrane (155). Three to six fibrils insert at the ends of the organism and extend toward the center of the cell (155, 305). The fibrils have a typical flagellar structure with a long shaft and an

insertion apparatus made up of a hook, collar, and basal knob (142). Unlike most bacterial flagellar filaments, which are comprised of a single protein, the shaft of the *T. pallidum* flagella is made up of several major filament proteins (72, 229). Three proteins, FlaB1 (34.5 kDa), FlaB2 (33 kDa), and FlaB3 (31 kDa), make up the flagellar core (64, 226), and the core is covered with a sheath made up of subunits of the 37-kDa FlaA protein (152). Motility-related proteins that make up the flagellar motor/switch and assembly apparatuses have homology to those found in other bacteria (105, 127, 128, 174). Cellular (7, 10) and humoral (31, 125, 178) immune responses to endoflagella are induced during syphilis infection, and the major flagellar proteins are recognized by antibodies from infected humans (11, 31, 124, 125, 243) and rabbits (123, 178, 181). *T. pallidum* endoflagellar core subunits share antigenic epitopes with endoflagella of the nonpathogenic treponeme *T. phagedenis* biotype Reiter (28, 31, 125, 178, 243); there are also pathogen-specific flagellar sheath epitopes in *T. pallidum* (28, 178, 186). Despite their strong antigenicity, immunization with flagellar subunits from *T. phagedenis* and *T. pallidum* failed to induce complete protection against *T. pallidum* infection (63, 140), probably because the flagella are not exposed on the surface of the organism.

### Chemotaxis

Motile bacteria depend upon chemotactic responses to move toward favorable environments and away from hostile environments. The dissemination of motile bacterial pathogens in tissues may be facilitated by chemotaxis. Methyl-accepting chemotaxis transmembrane proteins (MCPs) and cytoplasmic chemotaxis proteins (Che) comprise the chemotaxis system of gram-negative bacteria, and *T. pallidum* has homologs for proteins of each of these systems. Four genes for MCPs are found in the *T. pallidum* genome (105, 116, 121). MCPs sense attractants and repellents in the environment; glucose and histidine are molecules that may have affinity for *T. pallidum* MCPs. Two operons in the *T. pallidum* genome contain genes for the Che response regulators (105, 117). Pathogenic processes such as crossing the endothelial barrier to reach the bloodstream are likely to depend upon mechanisms that allow *T. pallidum* to sense and respond to nutrient gradients.

### INFLAMMATION AND IMMUNE RESPONSE

Compared to the wealth of information about the disease-causing mechanisms of many bacterial pathogens, little is known about how *T. pallidum* causes the protean manifestations of syphilis. In the absence of cytotoxins and other known virulence factors, it is probable that inflammation and the ensuing adaptive immune response to *T. pallidum* cause the tissue destruction characteristic of syphilis infection.

### Inflammation

*T. pallidum* quickly gains access to deeper tissues and the bloodstream (190, 301), probably by traversing the junctions between endothelial cells (255, 311). *T. pallidum* has been shown to induce the production of matrix metalloproteinase-1 (MMP-1) in dermal cells (71). MMP-1 is involved in breaking

down collagen, which may help *T. pallidum* to penetrate tissues. The presence of a pathogen signals inflammatory and immune cells to migrate from the bloodstream to the site of infection. An early step in this homing mechanism is the expression of cell adhesion molecules on capillary endothelial cells, promoting the leakage of serous fluids and the migration of leukocytes out of blood vessels into infected tissues. Virulent *T. pallidum* induces cultured endothelial cells to express the adhesion molecules ICAM-1, VCAM-1, and E-selectin (169, 255). These are also activated by the 47-kDa *T. pallidum* lipoprotein Tpn47 but not by heat-killed *T. pallidum* or the nonpathogenic treponeme *T. phagedenis* (169, 255), suggesting that endothelial cell activation is a pathogen-specific, active process mediated by specific *T. pallidum* molecules. Preincubation with virulent *T. pallidum* organisms increases the ability of endothelial cells to bind to lymphocytes (256), and this binding is blocked by antibodies against the cell adhesion molecules (169), supporting a functional role for ICAM-1, VCAM-1, and E-selectin expression in the inflammation caused by *T. pallidum* infection.

During acute bacterial infection, polymorphonuclear lymphocytes (PMNs) are often the first cells to infiltrate the site of infection. PMNs are seen in very early syphilis lesions that are experimentally induced (214, 277, 312, 317) and naturally acquired (36), although infiltration is transient (33, 312) and the number of PMNs is low relative to that seen in other acute bacterial infections (228, 275). Dermal injection of synthetic analogs of Tpn17 and Tpn47 lipoproteins also induces transient infiltration by PMNs of the injection site (219, 280).

To kill ingested pathogens, phagocytic vacuoles of PMNs fuse with cytoplasmic granules that contain enzymes, superoxide radicals, and antimicrobial peptides. The antimicrobial rabbit neutrophil defensins NP-1, NP-2, and NP-5 have been detected at the site of syphilis infection within 24 h of inoculation with *T. pallidum* (33), and some defensins have been shown to neutralize the infectivity of *T. pallidum* in vitro (35). Cathelicidins are another class of antimicrobial peptides found in PMN granules, and six different cathelicidins have shown various levels of activity in *T. pallidum*-killing assays in vitro (76, 266). While these studies suggest that PMNs may be involved in early infection, the inability of PMNs to adequately control *T. pallidum* is demonstrated by the progression of infection following this mild localized early inflammatory response.

During bacterial infection, endothelial cells, dendritic cells, and macrophages recognize shared microbial patterns such as LPS, peptidoglycan, and the acylated moieties of lipoproteins. This recognition is mediated by receptors, the TLRs, found on the cell surface. The human embryonic kidney cell line HEK293, which lacks TLRs, is unresponsive to stimulation with the lipoprotein Tpn47 but is activated by Tpn47 when stably transfected with TLR2 (38). Stimulation is enhanced by the expression of CD14 (38, 279), a membrane protein that acts as a coreceptor to mediate lipoprotein signaling via TLR2 and LPS signaling via TLR4. The role of TLR2 in signaling the presence of *T. pallidum* lipoproteins was confirmed by studies with Chinese hamster ovary (CHO) cells that have a normal TLR4 receptor but whose TLR2 receptor is inactivated by a point mutation. As with HEK293 cells, it was found that stimulation by Tpn47 occurred only in CHO cells that had been



stably transfected with a gene that expresses a functional TLR2 receptor (173).

### Dendritic Cells

Dendritic cells (DCs) are also stimulated by synthetic microbial lipopeptides through the TLR2 pathway (139, 287). Specialized DCs called Langerhans cells are found in skin, the site of the majority of primary and secondary lesions; DCs are also found in the mucosa, the intestinal wall, and the heart, all potential sites of *T. pallidum* infection. In many bacterial infections, the bacteria are taken up by immature DCs at the site of infection, and the DCs then migrate to lymph nodes where they activate T cells (251). It has been shown that *T. pallidum* interacts with and is phagocytized by immature DCs in cell culture (37, 286). As they mature, DCs produce inflammatory cytokines in response to pathogenic stimulation. Expression of the inflammatory cytokines interleukin 1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-12, and tumor necrosis factor alpha (TNF- $\alpha$ ) in DCs is stimulated by exposure to whole *T. pallidum* organisms or to a synthetic lipopeptide representing the lipid portion of TpN47 (37). The TpN47 synthetic lipopeptide also stimulates immature DCs grown in cell culture to express the maturation markers CD54, CD83, major histocompatibility complex class II (the molecule on which antigen is presented to CD4<sup>+</sup> T cells), as well as other markers (37, 139, 286, 287). In an assessment of their functional activity, DCs that had been stimulated with whole *T. pallidum* organisms were more capable of stimulating T cells in vitro than DCs that had not been exposed to *T. pallidum* (286).

In a model developed to simulate human infection, the inner forearm of several volunteers was injected with TpN17 and TpN47 synthetic lipopeptides and suction was used to raise blisters at the sites (280). DCs present in blister fluids expressed the maturation marker CD83, as well as molecules involved in T-cell stimulation (280). CD83-positive DCs have also been found in disseminated skin lesions of human secondary syphilis (162).

Specific *T. pallidum* molecules that have been shown to stimulate DCs, the lipoproteins TpN17 and TpN47, are not surface localized. The initiation of lipoprotein signaling of DCs is not likely to occur until the organisms are being degraded, exposing the lipoproteins to the TLR2 receptors. This theory is supported by observations that longer times than usual are required for *T. pallidum* stimulation of DCs (37). A delay in DC maturation, resulting in a slower inflammatory response, could allow the early dissemination of *T. pallidum*, giving organisms the opportunity to penetrate organs and tissues before an active inflammatory response has been mounted by the host.

### Cytokine Production

Endothelial cell activation and migration of inflammatory cells are further augmented by the secretion of soluble immune factors called cytokines. In an in vitro system, exposure to intact and sonicated *T. pallidum* induces macrophages to express TNF- $\alpha$  (278), a cytokine that activates a number of components of the immune response. Stimulation of macrophages by purified *T. pallidum* lipopeptides, or with representative synthetic lipopeptides, also induces expression of proinflam-

matory cytokines. TpN47 activates the expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IL-12 (38, 242, 279). TNF- $\alpha$  production is also induced by TpN15, TpN17, and TpN38 (2, 248), and stimulation of macrophage cell lines with TpN17 activates IL-1 $\beta$  production (218). Additionally, TpN47 induces expression of the T-cell chemoattractant cytokines MIP-1 $\alpha$  and MIP-1 $\beta$  (281). Liver macrophages called Kupffer cells also produce TNF- $\alpha$  in response to stimulation with whole *T. pallidum* organisms or with the lipoproteins TpN47, TpN17, TpN15, and TmpA (191). Taken together, these findings implicate *T. pallidum* lipoproteins as potent inducers of inflammation during early syphilis infection.

### Immune Clearance

DCs act as a bridge between innate and adaptive immunity by presenting specific antigens to T cells in the lymph nodes (251), stimulating them to differentiate and migrate to the site of infection where they perform their specialized functions. After intratesticular infection of rabbits with an inoculum of 10<sup>8</sup> *T. pallidum* organisms, splenic T cells sensitized to *T. pallidum* antigens could be demonstrated in these rabbits as early as 3 days following infection (180). T cells induced in response to syphilis infection are highly reactive to several of the major *T. pallidum* proteins, including TpN47, TpN17, and TpN15—lipoproteins embedded in the outer leaflet of the cytoplasmic membrane—and TpN37, TpN35, TpN33, and TpN30—proteins that make up the core and sheath of *T. pallidum* flagella (7, 10). T cells were detectable at the infection site within 3 days postinfection (179) and reached peak concentrations at days 10 to 13, roughly the same time that the number of *T. pallidum* organisms in testis tissue reached its maximum. Macrophages also infiltrate the site of infection after 6 to 10 days and reach maximal numbers at approximately day 13. Between days 13 and 17 postinfection, the number of detectable *T. pallidum* organisms sharply declines (179). These studies suggest a mechanism similar to delayed-type hypersensitivity as the mode of immune clearance in *T. pallidum* infection.

Similarly, T cells and macrophages are found in rabbit dermal lesions (276), as well as in human primary chancres (90, 314, 325) and secondary lesions (90, 198, 325, 333). Helper (CD4<sup>+</sup>) T cells and cytolytic (CD8<sup>+</sup>) T cells are present in primary and secondary lesions (90, 314, 325). McBroom et al. suggest that many CD4<sup>+</sup> cells in secondary lesions are in fact macrophages expressing the CD4 receptor (198). mRNA for the cytokines gamma interferon (IFN- $\gamma$ ) and IL-2, which function to activate macrophages and stimulate proliferation of T cells, respectively, is also found in primary and secondary lesions (323). Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells produce IFN- $\gamma$ , and the lytic mediators granzyme B and perforin have been detected in syphilis lesions, suggesting that infiltrating CD8<sup>+</sup> T cells are activated (325). As *T. pallidum* is found almost exclusively extracellularly, the role of the CD8<sup>+</sup> lytic compounds in clearance of *T. pallidum* from the site of infection is unclear; however, granzyme B and perforin may be partially responsible for the tissue destruction characteristic of syphilis lesions.

A functional role for macrophages in immune clearance was first suggested by early reports that found intact and degraded *T. pallidum* organisms inside phagocytic vacuoles of macro-

phages (155, 275) and by the demonstration of phagocytosis of opsonized *T. pallidum* organisms in vitro by rabbit peritoneal macrophages (184). In response to cytokine signals from infiltrating T cells, macrophages migrate to the site of infection, are activated by interferon IFN- $\gamma$ , and ingest and kill organisms. In vitro phagocytosis and killing of *T. pallidum* by macrophages has been demonstrated, and phagocytosis is increased when organisms are opsonized by preincubation with serum from rabbits infected with *T. pallidum* (13, 15). Opsonizing agents are serum components, antibodies, or the complement protein C3b, which make the pathogen recognizable to macrophages via specific cell surface receptors. In macrophage recognition of *T. pallidum*, opsonization is accomplished by antibodies, both immunoglobulin G (IgG) (283) and IgM (16). *T. pallidum* antigens, including Tp92 and TprK, have been shown to induce production of opsonic antibodies (44, 54, 283). Antibodies against the VDRL (Venereal Disease Research Laboratory) antigen, a complex of cardiolipin, cholesterol, and lecithin, also increase the phagocytosis of *T. pallidum* by macrophages (16). Interestingly, it has been shown that those few treponemes remaining at the site of intratesticular infection after most have been cleared by the immune response are able to resist ingestion by macrophages (185), suggesting that this subpopulation of organisms may be able to avoid binding by opsonic antibody and therefore persist in the face of active immune clearance. The mechanism of this resistance has not been explored.

#### Antibodies in Syphilis Immunity

IgM antibodies are usually the first to develop after establishment of bacterial infection, followed shortly by IgG. In the intratesticular model of syphilis infection in rabbits, anti-*T. pallidum* IgM and IgG are detectable as early as 6 days postinfection (123, 181, 211). Specific IgM continues to be produced in infected rabbits and humans, even after disease symptoms have subsided (11, 14, 181), suggesting that exposure to *T. pallidum* antigens continually stimulates B cells. IgG persists throughout late latent syphilis in humans (11, 124), and strong IgG reactivity has been demonstrated in rabbit serum up to 17 months after infection (123). The antibody response elicited during infection is specific for a broad range of *T. pallidum* molecules (200, 222), including lipids found on the surface of *T. pallidum* (26), flagellar proteins (31, 243), lipoproteins (61, 148, 272, 273, 285), and various other proteins, including the Tprs (167). Some of these proteins are cross-reactive with antigens of the cultivable *Treponema* species (12, 125, 178), but a number are specific for *T. pallidum* subspecies, indicating that there are both shared and unique antigenic determinants in pathogenic and nonpathogenic treponemes.

Besides opsonization, there are other functions of antibodies produced during *T. pallidum* infection. Antiserum from *T. pallidum*-infected rabbits, presumably the IgG component, has been shown to block organisms from binding to cells in vitro, suggesting that attachment to host cells is mediated by treponemal adhesin molecules. In the presence of complement, anti-*T. pallidum* antibodies immobilize organisms (30, 216) and neutralize the ability of the organisms to produce typical dermal lesions (25, 316). Passive administration of whole serum and fractionated IgG from long-term-infected rabbits delays

lesion development in challenged rabbits during serum administration (24, 30, 232, 282, 313, 318, 332), but lesions develop at the inoculation site within days of discontinuing antibody administration (24, 332). This demonstrates that specific antibody alone, while inhibitory to the establishment of lesions, is not sufficient to kill *T. pallidum* and prevent infection.

#### Antibodies in Diagnostic Testing

The antibodies produced in response to syphilis infection are exploited by clinicians for diagnostic purposes. The first serologic test for syphilis, developed in the early 20th century, was the Wasserman test. This test is classified as nontreponemal because the antigen, comprised of lecithin, cholesterol, and cardiolipin, is not unique to *T. pallidum*. These lipids are thought to be derived from the host and incorporated into the membrane of the metabolically limited *T. pallidum* (105), producing a configuration that is antigenic. Decades later, the antilipoidal antibody VDRL and rapid plasma reagin (RPR) tests were developed (132, 237). Automation, antigen stability, the ability to use plasma rather than serum, and macroscopic observation (199) make the RPR test more conducive for use in clinical laboratories. The VDRL test continues to be used in some settings, although it has no advantages over the RPR for diagnosis of syphilis.

The sensitivities of the RPR and VDRL syphilis diagnostic tests depend upon the stage of disease. In disease of short duration, i.e., when the primary chancre has just appeared, antilipoidal antibody tests are often negative; after several weeks of infection, however, the tests are usually positive. Accordingly, the mean sensitivities during primary syphilis of the RPR and VDRL tests are 86% and 78%, respectively, while the sensitivities of both tests during secondary syphilis are 100% (164). Antilipoidal antibody reactivity can arise as a result of tissue damage from recent or concurrent infectious diseases such as hepatitis or underlying autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus; these antibodies can cause a false-positive RPR or VDRL test. Because autoantibodies increase as a result of aging, elderly people are also at risk for a false-positive result.

The *T. pallidum* immobilization (TPI) test, developed as a result of the discovery that serum from syphilis-infected patients inhibits treponemal mobility in the presence of active complement (216), was the first test to be specific for antitreponemal antibodies. Within a decade of its discovery, the TPI test was replaced by the more sensitive fluorescent treponemal antibody (FTA) test (80), later refined by an absorption step to the FTA-ABS test (149). These tests use anti-human Ig labeled with fluorescein to detect antibodies bound to *T. pallidum* organisms on slides. Hemagglutination, a technically simpler test developed in the same era as the FTA-ABS test, detects reactive antibody that agglutinates red blood cells sensitized with *T. pallidum* antigen (263). The *T. pallidum* particle agglutination assay (235) uses biologically inert gel particles in place of red blood cells and has fewer equivocal reactions than the hemagglutination test.

To diagnose neurosyphilis, the CSF can also be tested for antibodies induced in response to infection with *T. pallidum*. While insensitive, the CSF-VDRL test is very specific for neurosyphilis (133). The FTA-ABS CSF test has also been exam-

ined in neurosyphilis diagnosis (164) and, while more sensitive than the CSF-VDRL test, may not be specific for neurosyphilis (91), possibly because of the passive transfer of serum-derived antibodies to the CSF. Thus, some investigators suggest that the use of the FTA-ABS CSF test should be limited to ruling out neurosyphilis in cases with a negative test result (154, 195).

In an attempt to develop a test with excellent sensitivity, especially during the earliest syphilis stages, several laboratories have explored the use of recombinant *T. pallidum* antigens in either enzyme immunoassay (108, 151, 245, 267, 324) or immunoblot (8, 233) format. Several antigens that elicit high antibody titers during syphilis infection and are not cross-reactive with serum from patients with other common spirochetal diseases have been identified (108, 324). One antigen in particular, Tp0453, was shown to be highly sensitive in detecting infection during primary syphilis (324); additionally, Tp0453 did not react with serum from uninfected individuals or from individuals with leptospirosis, Lyme disease, or relapsing fever. Testing with recombinant *T. pallidum* antigens may improve syphilis diagnosis, especially the detection of early infection.

#### EVASION OF THE IMMUNE RESPONSE AND CHRONIC INFECTION

In the face of a hostile host environment and a strong specific immune response, how does *T. pallidum* survive? There are several mechanisms the organism might use to ensure its continued existence in the host. As discussed above, *T. pallidum* penetrates a broad variety of anatomical sites, including the central nervous system, eye, and placenta, tissues that may be "immune privileged" in that less surveillance by the innate immune system may occur in those sites. Organisms may survive in these tissues, slowly replicating and possibly reseeding other tissues. *T. pallidum* may also exploit its slow metabolism to survive in tissues, even those that are not immune privileged. It has been speculated that a minimum number of organisms, or "critical antigenic mass," is required to trigger a host response (177, 317). By maintaining infection with very few organisms in anatomical sites distant from one another, *T. pallidum* may prevent its clearance by failing to alert the immune response to its presence. In this scenario, *T. pallidum* may spend months to years in a quiescent environment, with organisms dividing very slowly. Indeed, it is quite likely that *T. pallidum* undergoes an even lower rate of division during latent disease, as late latent syphilis must be treated by a prolonged course of penicillin to prevent treatment failure (51). Unknown factors cause *T. pallidum* to begin dividing at a higher rate again in certain anatomical areas in a small percentage of individuals, leading to symptomatic late syphilis.

An important defense mechanism utilized by the host is iron sequestration. The host iron-binding proteins transferrin and lactoferrin cause free iron to be unavailable to bacteria, impairing their growth. *T. pallidum* has been reported to interact with both transferrin and lactoferrin (4, 5, 292), and the organism may be able to acquire iron from these host proteins (5). *T. pallidum* may also overcome host iron sequestration by utilizing enzymes that bind metals other than iron. Unlike many bacterial pathogens, *T. pallidum* lacks an electron transport chain, which is made up of enzymes that use iron as a

cofactor, and *T. pallidum* appears to have very few other enzymes or components that require iron (105). A regulated system for the uptake of metals such as zinc and manganese has been described (171, 238), suggesting that these metals may act as iron alternatives. As mentioned above, neelaredoxin is thought to be important for protection against damage by superoxide radicals (158, 175). The neelaredoxin enzyme is found mainly among primitive species of anaerobic bacteria and appears to bind iron or zinc. Studies of neelaredoxin were conducted with recombinant protein; the metal-binding properties of the native enzyme have not been examined.

#### Surface of *T. pallidum*

Some organisms survive in infected hosts despite the presence of strongly reactive antibodies directed against a number of *T. pallidum* proteins (11, 222) and activated T cells and macrophages in lesions (90, 314, 325). Outer surfaces are the first bacterial component to encounter the host and are often the targets of host adaptive immunity. Early researchers noted that antibodies in serum from infected animals did not readily bind to intact treponemes (80, 202). This observation was later supported by Penn and Rhodes (231), who found that only those treponemes that had been physically disrupted reacted with anti-*T. pallidum* antiserum, leading them to propose that the surface of *T. pallidum* is nonantigenic. As a follow-up to these observations, it was noted that treponemes were able to be labeled with radioactive iodine (230) or antibodies raised against *T. pallidum* (298) only when the outer layer had been disrupted by detergents or by incubation that effectively "aged" the organisms. Furthermore, in experiments involving the interaction of *T. pallidum*-specific antibodies with viable organisms—agglutination (131), neutralization (25), and opsonization (13, 184)—organisms must be incubated with antibodies for unusually long periods before effects are observed. Taken together, these observations suggest that there are few antigenic targets on the surface of the organisms, causing the binding and aggregation of antibodies to proceed slowly. The relative antigenic inertness of the *T. pallidum* outer membrane was first explored in two independent studies (249, 329) in which *T. pallidum*, visualized by freeze fracture electron microscopy (EM), was shown to have only rare integral proteins in its outer membrane, approximately 1% of the number found in the outer membrane of *E. coli*. In later experiments, outer membrane vesicles isolated from *T. pallidum* were analyzed for integral proteins by freeze fracture EM and tested for antigens by immunoblot analysis (32, 250). These studies confirmed the paucity of integral outer membrane proteins in *T. pallidum*, a characteristic that may help the organism escape immune detection and that has inspired researchers to call *T. pallidum* "the stealth pathogen" (265). The rare *T. pallidum* outer membrane proteins are implicated as likely to be very important in interactions with the host; for this reason, their identity has been the subject of intense research over the last two decades.

Many techniques have been used to explore the identity of *T. pallidum* outer membrane proteins. Earlier studies used phase partitioning with various detergents (79, 230, 244, 298), separation of membranes with acid (293), or density gradient ultracentrifugation of organisms lysed in a hypotonic solution (3). These methods revealed a subset of proteins that had been

previously discovered by immunoblotting of whole organism lysates (178). Several of these, including TpN47, were initially identified as surface-exposed proteins (3, 156), and much effort was devoted to confirming their location. However, further studies indicated that these proteins are not surface exposed but are more likely to be anchored in the inner membrane with portions extending into the periplasm (27, 147, 240, 304). By examining organisms microscopically after physical manipulations such as centrifugation and washing, or after treatment with detergents, Cox et al. substantiated the growing suspicion that the *T. pallidum* outer membrane is easily damaged by these procedures (75). Most bacteria with a double membrane have a peptidoglycan layer that is linked to the outer membrane by lipoprotein molecules, but in *T. pallidum* peptidoglycan is thought to associate with the more abundant inner membrane proteins (246). Additionally, *T. pallidum* lacks LPS (105, 130, 230, 247), a molecule that lends structural stability to bacterial outer membranes. The combination of these ultrastructural features is a likely explanation for the fragility of the *T. pallidum* outer membrane.

With conventional techniques being unable to positively identify *T. pallidum* outer membrane proteins, researchers turned to novel molecular (29, 129) and physical (74) methods. Two research groups used *E. coli* fusion vectors to screen *T. pallidum* genomic DNA for genes with export signals or transmembrane domains (29, 129). This method provides only indirect evidence for surface exposure and, because of their inherent differences, *E. coli* expression data do not always translate to biological meaning in *T. pallidum*. Accordingly, the TpN47 and TpN38 (MgIB) lipoproteins, originally identified as localized to the outer membrane by cloning *T. pallidum* DNA into *E. coli* (62, 93), were later shown to be inner membrane localized.

In an attempt to preserve the delicate structure of *T. pallidum*, researchers protected treponemes by suspending them within agarose beads during immunofluorescence labeling. These studies showed that serum from syphilis-infected individuals reacts with organisms only after they are treated with detergent, increasing the permeability of the outer membrane (74). Only detergent-treated organisms were stained by specific antisera to the major membrane lipoproteins TpN47, TpN17, and TpN15 (74), confirming that they are not surface exposed. This technique has been used to identify a phosphorylcholine lipid on the surface of *T. pallidum* (26) but may not be sensitive enough to detect rare outer membrane-spanning proteins. For the last 25 years, techniques used to identify surface molecules have focused on proteins that are well anchored in the membrane with membrane-spanning domains. A recent report identifies a protein that, rather than spanning the outer membrane, appears to loosely associate with its inner leaflet (135). The report of small proteins secreted into the medium surrounding in vitro-cultured *T. pallidum* bacteria (293) also raises the possibility that some surface-exposed proteins are loosely anchored in the outer leaflet of the outer membrane by the C or N terminus or by a lipid tail. Further research may continue to reveal previously unidentified surface molecules.

Recent research efforts to identify proteins that may be exposed on the surface of the organism have utilized computer analysis of the *T. pallidum* genome. Several proteins that are predicted to have a cleavable signal sequence, transmembrane

domains, and other characteristics of proteins that span the outer membrane have been identified (39, 54). As discussed above, three of these, Tp0155, Tp0483, and Tp0751, bind to ECM components and are candidate host-binding molecules (39, 41). Their potential involvement in *T. pallidum* binding to host cells strongly implicates these proteins as being outer surface localized.

### Tpr Proteins

Several other genes that encode candidate outer membrane proteins belong to the *tpr* gene family. The twelve *tpr* genes are divided into three subfamilies. The *tprC*, *tprD*, *tprF*, and *tprI* genes belong to subfamily I; *tprE*, *tprG*, and *tprJ* belong to subfamily II; and *tprA*, *tprB*, *tprH*, *tprK*, and *tprL* belong to subfamily III. The proteins encoded by *tprF*, *tprI*, and *tprK* are predicted to be located in the outer membrane (39, 54); the subfamily I proteins and TprK are predicted to have cleavable signal sequences. Most of the proteins encoded by the *tpr* genes (the Tprs) elicit an immune response in experimental syphilis (167, 208, 302). Antibody responses arise at different times after infection: anti-TprK antibodies are seen as soon as 17 days postinfection and are robustly reactive at day 30 (167, 208), while antibodies against the members of subfamilies I and II often are not detectable until 45 days after infection and reach peak titers at day 60 (167, 302). The time of development of antibodies to specific Tprs may reveal the timing of expression of the proteins that induced those antibodies. Regulation of expression of related proteins is referred to as phase variation and may be utilized by *T. pallidum* to down-regulate the expression of those Tprs against which an immune response has been mounted, while simultaneously up-regulating the expression of new Tprs. The expression of new proteins that are not recognized by the existing immune response may help *T. pallidum* maintain chronic infection. Additionally, immunological studies suggest that different strains of *T. pallidum* express different repertoires of Tpr proteins (167).

Besides its potential location in the outer membrane, other intriguing features have singled out the TprK protein as important to syphilis pathogenesis. A translational start site downstream of the site predicted in the *T. pallidum* genome sequence has been proposed (54) and confirmed experimentally by Hazlett et al. (138). This alternative TprK translational start introduces an N-terminal cleavable signal sequence, supporting the prediction that TprK is a membrane-localized protein (54). The *tprK* gene and predicted amino acid sequences are characterized by seven discrete variable (V) regions that are separated by stretches of conserved sequence; this is shown in Fig. 2. To date, diverse *tprK* sequences have been demonstrated in every *T. pallidum* strain (56, 163, 295). Syphilitic hosts are infected with multiple subpopulations of organisms, differentiated by their *tprK* sequences (57); Fig. 2 shows the diverse TprK sequences encoded by *T. pallidum* during a single infection. Diversity in *tprK* sequence accumulates over the course of infection (57, 163), and some V regions are more diverse than others. DNA sequence cassettes that correspond to diverse V-region sequences were discovered in an area of the *T. pallidum* chromosome separate from the *tprK* gene (57). These sequence cassettes are potential sequence donor sites and are presumed to replace small or large portions of V-

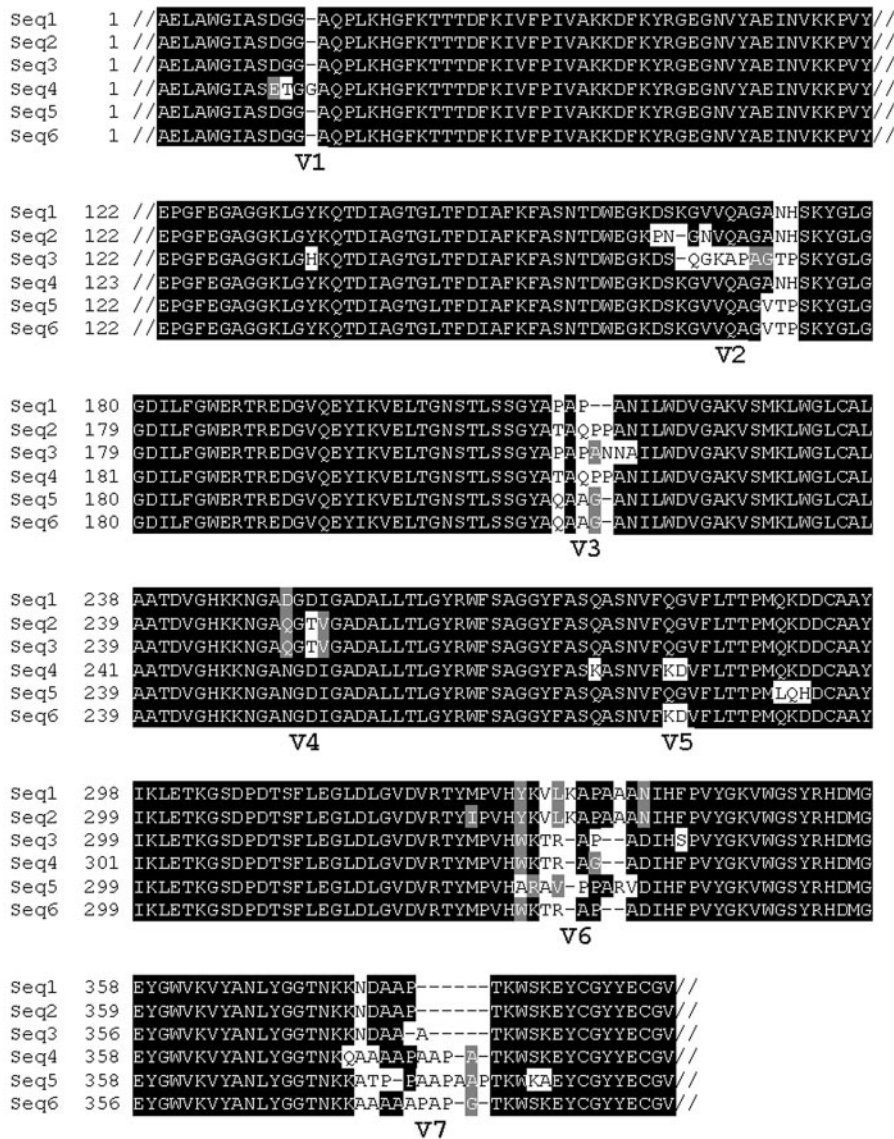


FIG. 2. Amino acid alignment of TprK showing six different sequences in the *T. pallidum* Sea81-4 strain harvested from a single infection. Conserved regions are indicated by black shading; V regions have white and gray shading. The V region that exhibits the greatest diversity is V6, with six different sequences; in comparison, only two different sequences are found in V1. For ease of visualization, the conserved C- and N-terminal portions of the protein and 64 amino acids of conserved sequence between V1 and V2 are truncated (//). (The GenBank accession numbers of the sequences shown are AY346063 to AY346068.)

region sequences in the *tprK* gene, thus creating new, diverse sequences by gene conversion (57). Together, the *tprK* expression site and the donor regions comprise approximately 4,000 bp in the minimal *T. pallidum* genome, suggesting that variation in *tprK* must play an important role in the survival of *T. pallidum*.

The TprK protein elicits both cellular and humoral immunity in infected animals. Antibodies to TprK that arise in response to *T. pallidum* infection are specifically targeted to the V regions (207, 208), while T cells recognize conserved TprK epitopes (208). Very slight changes in the amino acid sequence of a V region can abrogate the ability of antibodies to bind a certain V region (208), suggesting that antibody binding to V regions is highly specific. Multiple studies show that immuni-

zation with recombinant TprK protein attenuates lesion development upon challenge with virulent *T. pallidum* (54, 206, 207), and animals that are challenged with a *T. pallidum* strain expressing a TprK protein that is homologous to the immunizing protein are better protected than those challenged with a strain expressing TprK proteins with different V regions (207). These findings suggest that V-region-specific immunity is important in protection against progressive infection. Taken together, the immunologic characteristics and sequence variation of the TprK protein indicate that TprK undergoes antigenic variation, the first such system identified in *T. pallidum*. The host immune response may eliminate organisms that express TprK sequences against which a specific antibody response has been mounted. Generating new variation in TprK may help

variant *T. pallidum* organisms escape immune recognition, serving as another mechanism by which *T. pallidum* may be able to survive the host immune response and cause chronic infection.

## GENETIC VARIATION AMONG SUBSPECIES AND STRAINS

### *tpr* Genes

As discussed in the previous section, diversity in *tprK* has been observed in all syphilis strains examined thus far. The heterogeneity observed in the seven variable regions of *tprK* arises during rabbit passage (57, 163); diversity is also seen in *tprK* sequenced directly from patients with primary and secondary syphilis (163, 215). Some of the other *tpr* genes have also displayed variation, although the diversity is of a different type. Variation of *tprC* and *tprD* has been noted, but diversity exists only among strains, not within strains as with *tprK*. The *tprC* and *tprD* genes are located in different loci in the *T. pallidum* genome but share an identical sequence in the *T. pallidum* Nichols type strain (54, 105). Some of the non-Nichols *T. pallidum* strains also have identical *tprC* and *tprD* sequences; however, some strains carry an alternative gene in the *tprD* locus, called *tprD2*, that varies in sequence from *tprD* (59). Strains that carry *tprD2* have a slightly different gene in their *tprC* locus, and, unlike the identical *tprC* and *tprD* sequences, alternative *tprC* sequences are not identical to *tprD2* (302).

The *tprD2* sequence is also found in other pathogenic *T. pallidum* subspecies, including strains of the *T. pallidum* subspecies *pertenue* and *endemicum*, which cause the nonvenereal treponematoses yaws and bejel, respectively. The Gauthier strain of *T. pallidum* subsp. *pertenue* carries in its *tprJ* locus a gene that is a hybrid of *tprG* and *tprJ* sequences (297), as does one identified *T. pallidum* subsp. *pallidum* strain. As described above, regulation of the expression of different *Tprs* may help *T. pallidum* avoid the immune response, and sequence differences may also confer different functions to the proteins.

### Other *T. pallidum* Genes

Other genes have been examined for differences among strains of *T. pallidum* subsp. *pallidum*, as well as among the *T. pallidum* subspecies. The gene that encodes the TpN15 lipoprotein is identical among *T. pallidum* subsp. *pallidum* strains and the subspecies *endemicum* and *pertenue* (53). However, a sequence difference in a region upstream of the gene introduces a site for cleavage by the restriction enzyme Eco47III (55) in *T. pallidum* subsp. *pallidum* strains but not in strains of the other subspecies. A sequence change in the *gpd* gene also confers a restriction enzyme cleavage site that differentiates *T. pallidum* subsp. *pallidum* from *T. pallidum* subsp. *pertenue* and *endemicum* (43). The pathogenic *T. pallidum* subspecies are morphologically identical by microscopy, and the syphilis, yaws, and bejel infections induce many cross-reactive antibodies, precluding serological differentiation. Thus, unique restriction sites such as those in the genes that encode TpN15 and Gpd provide a genetic method for differentiating the syphilis spirochete from the other pathogenic treponemes.

## IMPLICATIONS FOR SYPHILIS CONTROL

### Treatment of Infection

After 60 years of use, penicillin still remains the drug of choice in syphilis treatment. Several *T. pallidum* proteins have been shown to bind penicillin (78, 246, 331). Penicillin-binding proteins often function in cell wall synthesis pathways; thus, penicillin kills susceptible bacteria by interfering with production of cell walls. Recently, one penicillin-binding protein, TpN47, was shown to have  $\beta$ -lactamase activity (60); paradoxically, this activity is inhibited by the products of the reaction and the organism remains penicillin sensitive. Guidelines published by the Centers for Disease Control and Prevention (51) specify oral doxycycline or tetracycline as alternative treatments in the case of penicillin allergy (except for pregnant women).

### Macrolide Resistance

In the 1990s, azithromycin emerged as a particularly attractive alternative to penicillin therapy for syphilis (144, 252). Azithromycin is given orally, in contrast to benzathine penicillin, which must be delivered intramuscularly in a large volume. Early syphilis has been shown to be successfully treated with a single dose of azithromycin (144), and large azithromycin treatment trials are ongoing. Recently, however, a study of *T. pallidum* samples from patients in San Francisco, Calif., Baltimore, Md., Seattle, Wash., and Dublin, Ireland, identified macrolide-resistant strains among these populations (182); this resistance was shown to be associated with a single base mutation in the 23S rRNA gene (182, 294). Macrolide resistance was particularly high in San Francisco (37% of samples collected in 2003) and Dublin (88% of samples collected in 2002) and lower, but present, in other cities (182). In cities with high rates of macrolide-resistant strains, penicillin should remain the drug of choice. In other cities, certain situations may warrant the use of azithromycin to treat syphilis; however, to ensure efficacy of treatment, it is essential that patients be monitored carefully with clinical reevaluation and serological testing.

### Vaccine Development

Syphilis infection increases the risk of transmitting and acquiring HIV infection. Not only are syphilis lesions a portal of entry for HIV but the immune cells that carry and are susceptible to the virus, macrophages and T lymphocytes, are found in abundance in syphilis lesions. There is also some evidence for the direct involvement of *T. pallidum* in facilitating HIV infection and progression. Virulent *T. pallidum* and the TpN47 lipoprotein up-regulate HIV replication in monocyte precursors that are infected with HIV (307). Additionally, TpN47 stimulation of monocytes up-regulates expression of the HIV receptor CCR5 (281). Prevention of syphilis infections would help to stem the rising numbers of new HIV infections in the world and would certainly prevent congenital syphilis in developing nations. These issues, in addition to the possible serious late sequelae of untreated syphilis, provide a clear rationale for active syphilis vaccine development efforts.

Thus far, only one animal study has demonstrated complete

protection against infection with *T. pallidum* (203). The immunization regimen, 60 intravenous inoculations with  $\gamma$ -irradiated *T. pallidum* over 37 weeks, was far too cumbersome and expensive to repeat in rabbits and too impractical to test in humans. In many other studies, immunization with *T. pallidum* molecules (endoflagella [63], 4D [34], Gpd [42], TmpB [334], Tp92 [44], TpN15 [53], TpN47 [19], TprF [302], TprI [109], and TprK [54, 206, 207]) was conducted and protection against infectious challenge was determined. Immunization stimulated the production of strongly reactive antibodies, some of which were shown to be opsonic (44, 54). These immunization regimens, however, induced only partial protection, shown by attenuated development and rapid healing of lesions. This lack of complete protection in the face of high antibody titers suggests that the cellular immune response is critical to early clearance of *T. pallidum*. The requirement for cellular immunity in syphilis protection is supported by the fact that passive transfer of antibodies against *T. pallidum* (24, 232, 316), or against one of its lipid components (26), does not completely protect against infection. Additionally, the recombinant molecules used for immunization in many of these studies are likely not to be folded in native conformation; thus, antibodies that bind to conformational epitopes of native antigen may not be produced by immunization. Finally, the first targets of the immune response, outer membrane proteins, are very rare in *T. pallidum*. The identification of molecules found on *T. pallidum*'s surface is likely to be essential in the development of an effective syphilis vaccine. It is hoped that their discovery and use in a multivalent vaccine will lead to the production of an effective vaccine for syphilis.

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