SYPHILIS
AND
THE ENDEMIC TREPONEMATOSES

CLINICAL, (HISTO-) PATHOLOGICAL AND LABORATORY STUDIES

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CONTENTS

CHAPTER 1. GENERAL INTRODUCTION

1.1.

TREPONEMATOSES, IMPORTANT TO MANKIND

Herman Jan H. Engelkens M.D., and Ernst Stolz M.D.

Submitted for publication

CHAPTER 1. GENERAL INTRODUCTION

1.1. TREPONEMATOSES, IMPORTANT TO MANKIND

Treponemal diseases important to mankind are sexually transmitted syphilis and the endemic nonvenereally transmitted treponematoses (yaws, pinta, and endemic syphilis). Historically, the most important disease caused by treponemes is syphilis. During the last few decades, yaws, endemic syphilis and pinta have been largely ignored. These diseases, however, have not been eradicated (1-10). The causative agents of these chronic bacterial diseases belong to the order *Spirochaetales*, the family *Treponemataceae* and the genus *Treponema*. The order *Spirochaetales* comprises the families *Treponemataceae* and *Spirochaetaceae*. The *Treponemataceae* can be subdivided in: the genus *Treponema* (pathogenic, saprophytic, and special strains, for example the Nichols strain), the genus *Borrelia* and the genus *Leptospira*, respectively (11).

Species pathogenic for humans are enumerated in Table 1. Hitherto, it has not been possible to cultivate these species in artificial media or in tissue culture, which greatly hampers research (12,13). Moreover, it has not been possible to distinguish the causative agents of venereal syphilis and the endemic treponematoses routinely by morphological or immunological criteria or by DNA hybridisation studies. DNA homology studies (14) appear to support the view that syphilis and yaws are possibly caused by the same organism ("unitarian" theory) (15). Shared antigens give rise to cross-reactive antibodies common to all treponemal diseases, which so far precludes a differential diagnosis on the basis of serological tests. However, clinical and geographical differences between the treponematoses do exist.

T.pallidum, a Gram-negative bacterium, is a microaerophile, which requires low concentrations of oxygen for optimal *in vitro* survival. This slender, close-coiled, regular spiral-shaped organism, 6-15 micrometer in length, with an average breadth of 0.15 micrometer, with a variable number of regular coils, has a wavelength of approximately 1 micrometer and an amplitude of 0.2 to 0.3 micrometer (13). At each end of the protoplasmic cylinder, periplasmic flagella (axial filaments, axial fibrils, endoflagella) are present within the outer membrane, which are assumed to play a part in locomotion. Cytoplasmic fibrils (microtubules) extend along the inner layer of the cytoplasmic membrane, below the

periplasmic flagella. The outer membrane resembles the outer membrane of Gram-negative bacteria. A rotary motion with flexion and back-and-forth motion is considered characteristic. The best method to visualise the microorganism is with darkground illumination or phase contrast microscopy (13,16-18).

In the following paragraphs some aspects of the clinical manifestations and research of venereal syphilis are described. Separately some of the current problems with venereal syphilis due to the AIDS epidemic are mentioned. After this paragraph the endemic treponematoses are reviewed, with special emphasis on the current resurgence of yaws.

Table 1 Causative organisms and names of treponematoses important to mankind.

Treponema pallidum subspecies *pallidum* syphilis, sifilis, lues, lues venerea, Great pox, Morbus Gallicus
Treponema pallidum subspecies *pertenue* yaws, framboesia tropica, pian, bouba, buba, paru, parangi
Treponema pallidum subspecies *endemicum* endemic syphilis, nonvenereal syphilis, bejel, firjal, loath, radesyge, button scurvy, sibbens, dichuchwa, njovera, skerljevo, rewan, frenjak
Treponema carateum pinta, carate, azul, mal del pinto, cute, cativa

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1.2.

VENEREAL SYPHILIS

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Genital ulcer disease (review).
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1.2. VENEREAL SYPHILIS

Introduction

Syphilis is caused by *Treponema pallidum* subspecies *pallidum* (*T.pallidum*) (Latin: pallidum = pale or pallid). In 1905 Schaudinn (1871-1906) and Hoffman (1868-1959) were the first to describe this Gram-negative bacterium (1).

Transmission of syphilis occurs primarily by sexual contact. Mucous membranes and epithelial defects are presumably the portals of entry. Infection in utero with *T.pallidum* may occur by passage through the placenta and may result in early spontaneous abortion, stillbirth, or congenital syphilis in the newborn (2,3). Rare transmission is also possible by direct inoculation, for example by a needle accident, tattooing, blood transfusion or transplantation.

During several centuries syphilis has been present as a pandemic. The pandemic character has been greatly restricted in developed countries, due to the availability of arsenicals and later penicillins and other antibiotics, the introduction of large serological screening programmes and health education. However, syphilis is still far from eradicated (4-7). In many developing countries syphilis is still highly prevalent. As a consequence, congenital syphilis is prevalent in these countries (2,8).

Clinical features

In syphilis an early (primary, secondary, early latent) and a late (late latent, tertiary) stage are recognised. Considerable overlap exists between the different stages.

Primary stage

After an incubation period of usually two to four weeks (full range 9 to 90 days), the first lesion of syphilis may be observed at the site of exposure, most commonly the genitals (Figs.1,2) or the anorectal area. In 2-10% of cases the primary chancre may also be found on extragenital locations, for instance the lips, tongue, fingers (Fig.3), hands, arm or nipple (9,10). The primary lesion consists of a small solitary papule, which develops into a moist erosion or ulcerating painless lesion, with a sharply demarcated border, both indurated and infiltrated. However, multiple lesions may occur. Atypical forms are not rare. In case of

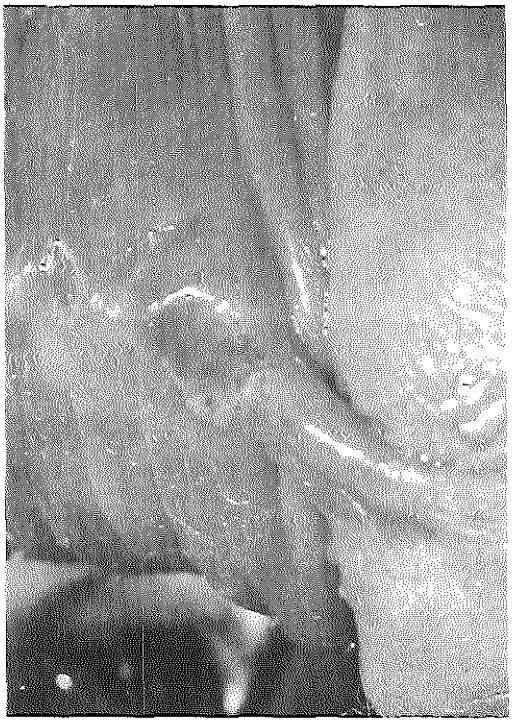

Figure 1. Penile chancre in primary syphilis.

Figure 2. Primary lesion near the posterior commissure on the right labium majus.

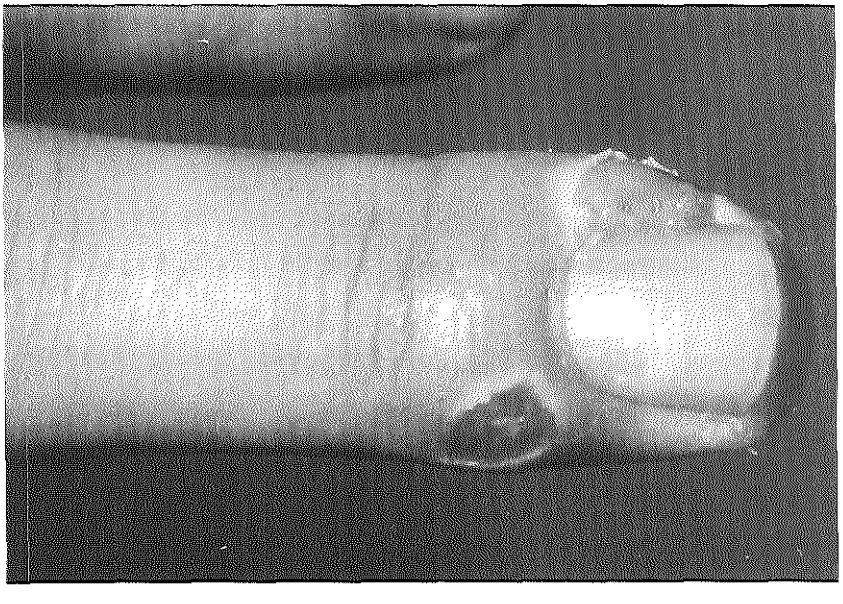

Figure 3. Primary syphilitic chancre on the fingers.

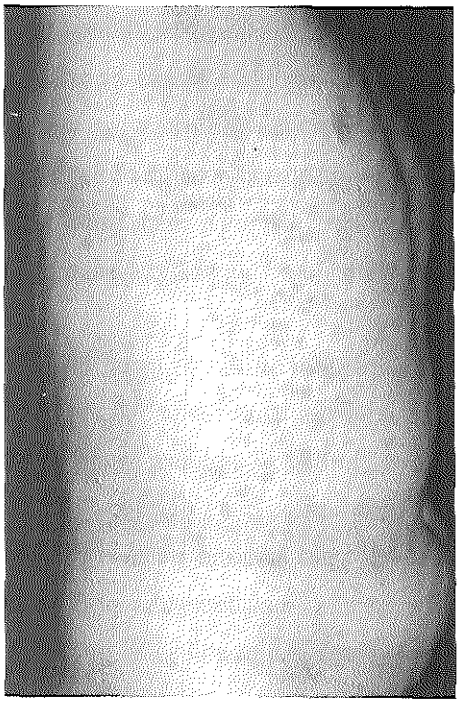

Figure 4. Skin rash in secondary syphilis.

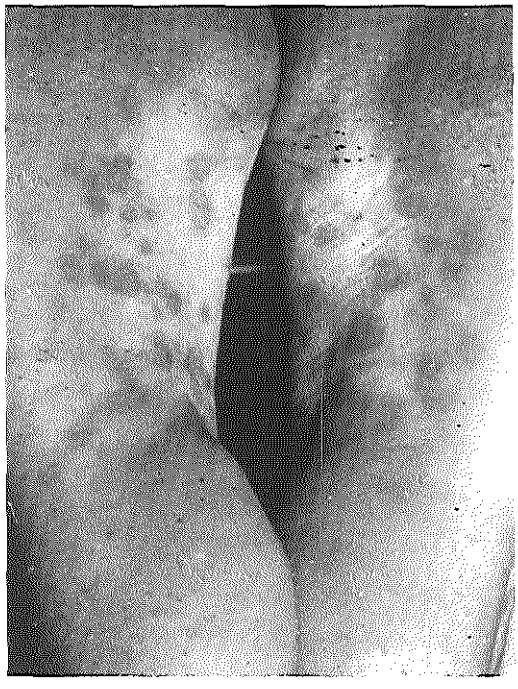

Figure 5. Macular syphilides on the soles of the feet.

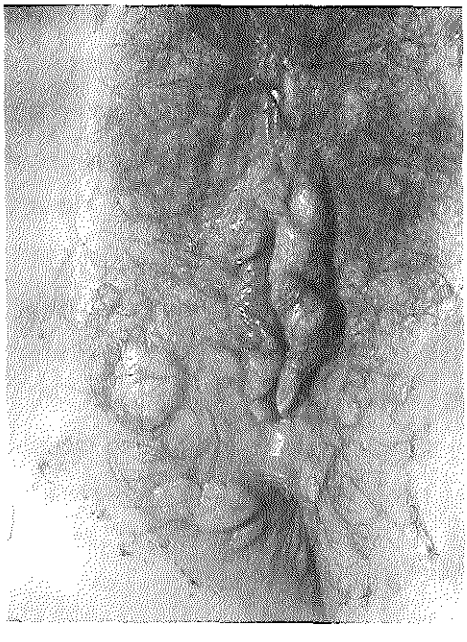

Figure 6. Condylomata lata.

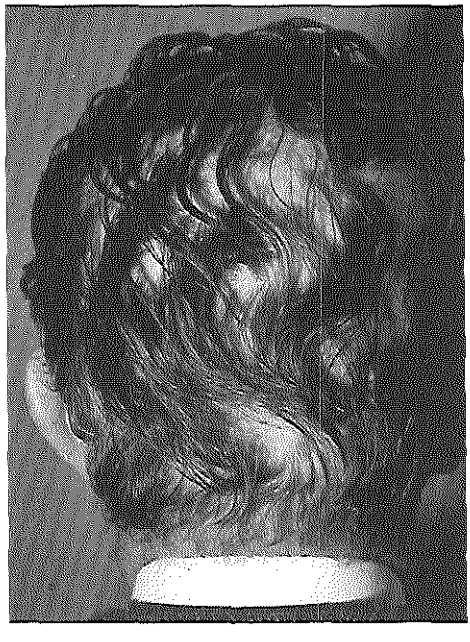

Figure 7. "Moth-eaten" alopecia.

secondary infection lesions may be painful. In most cases regional unilateral or (more often) bilateral painless lymphadenopathy is present. In the natural course of the disease the primary lesion(s) will disappear after approximately three to eight weeks, seldom leaving a scar. Complications include phimosis, paraphimosis, secondary infection and oedema of the labia. Based on observations in experimental animals, it is assumed that in humans soon after infection dissemination of *T.pallidum* occurs via the bloodstream or lymphatic system.

Secondary stage

After complete spontaneous resolution or during regression of the primary chancre secondary lesions appear usually within ten weeks after infection (full range 1-6 months), often preceded by a prodromal phase, with malaise, flu-like symptoms, generalised lymphadenopathy and/or arthralgia. A wide variety of skin lesions may develop (11,12). Classical signs are a skin rash, lymphadenopathy, condylomata lata and mucous patches (plaques muqueuses). The classical rash is a temporary macular, maculopapular or papular rash, affecting the whole body including the mucous membranes, palms and soles (Fig.4,5). Skin lesions in secondary syphilis may not be as rich in treponemes as the primary stage skin lesion. However, condylomata lata, another cutaneous feature of disseminated secondary syphilis (Fig.6), frequently are teeming with treponemes. Condylomata lata and mucous patches bear treponemes superficially and are considered to be very infectious. Condylomata lata develop specifically on warm and moist regions of the body, for example around the anus, on the vulva, on the scrotum or in the axillae. Mucous patches may be found on any mucous membrane, particularly in the oral cavity, the genitals, or the anal mucosa. Small areas of hypopigmentation on the neck and back may give rise to a "collar of Venus", syphilitic leukoderma. A temporary loss of hair, diffuse alopecia or the characteristic "moth-eaten" alopecia (Fig.7) may be noted (13,14). Virtually every organ may be affected in secondary syphilis, for instance the eyes, liver, kidneys, or the musculoskeletal system. Even in the early stage of syphilis the central nervous system may already be invaded (15,16).

In the natural course of the disease lesions will disappear in about one to three months. Approximately one-fifth of patients suffer from new symptoms of the secondary stage, in 90% of cases within one year.

Latency

Latency is characterised by a positive serology and absence of clinical signs or symptoms, without abnormalities in the cerebrospinal fluid. Neurosyphilis and cardiovascular syphilis

must be excluded. The latent stage is divided into an early latent stage and a late latent stage, with the intention to reflect the differential infectivity of the two stages and to guide therapy. In some nations early latency is classified as syphilis of less than one year's duration (following initial infection), in others of less than two years' duration. Late latent syphilis is defined as infection of longer than one or two years' duration, depending on the country in which the diagnosis is made. Patients with early latent syphilis are considered to be potentially more infectious for their sexual partner(s) than patients with late latent syphilis. Even after adequate antibiotic therapy is given, some serological test results may remain positive for many years or even lifelong. In untreated latent cases, early lesions may reappear, or the late tertiary stage sequelae of syphilis may occur, which may have life-threatening consequences.

Tertiary stage

Approximately one-third of patients with untreated syphilis will develop late manifestations, that is, late benign syphilis (17%), cardiovascular syphilis (10%), or neurosyphilis (8%) (17). The time of appearance of the tertiary stage is highly variable; usually the development of late manifestations takes many years. Virtually every organ may be affected in the late stage of syphilis.

If nonvital structures are involved, late benign syphilis or gummatous syphilis is diagnosed. Late benign syphilis most frequently involves the skin and the bones (18). Late skin syphilis may be of a superficial, nodular or nodulo-ulcerative, or tubercular type, or assume a deeper gummatous form. Gummas are granulomatous lesions ranging from millimetres to several centimetres in size. Gummas may be found in any organ, with a predilection for the skin, liver, spleen, bone, nervous system. They may have an indolent or an aggressive destructive character.

In the late stage of syphilis, cardiovascular involvement may occur. If involvement of the vasa vasorum of the aorta results in medial necrosis of the aorta, syphilitic aortitis is observed. Life-threatening complications may develop, for instance aortic insufficiency, formation of aneurysms in the ascending aorta, coronary stenosis and, rarely, myocarditis. In patients who have contracted syphilis before the onset of adolescence, cardiovascular sequelae are extremely rare (19).

Another late manifestation of tertiary syphilis is neurosyphilis. In the Netherlands, of all

late sequelae asymptomatic neurosyphilis is the most common form of neurosyphilis. In this stage without clinical illness, abnormalities in the cerebrospinal fluid are present (20). The central nervous system involvement classically has been divided into two types, namely parenchymatous (for example general paresis, dementia paralytica, tabes dorsalis) and meningovascular involvement (for example meningitis, meningo-myelitis, infarction). However, the actual clinical manifestations can be quite variable, mixed or incomplete. Especially due to penicillin therapy, late (tertiary) stage disease has become rare in the United States of America and in Europe. In large parts of the world, however, the late manifestations of syphilis still form a hazard to public health. For further information on the late stage of syphilis, the reader is referred to other publications (18,19,21,22).

Histopathology

In syphilis two types of pathological change are of major importance: a perivascular infiltration by lymphoid cells and often plasma cells is part of a vasculitis, the socalled obliterative endarteritis, with swelling and proliferation of small blood vessels in the involved area. Furthermore a gumma, which can cause severe local destruction in any organ of the body, is a focal nonsuppurative inflammatory lesion. It has a zone of obliterative endarteritis with central avascular necrosis and is surrounded by a mixture of mononuclear leucocytes and epithelioid cells, enclosed by a fibroblastic wall. The histopathological changes in syphilis may mimic those found in a wide variety of other skin conditions. An extensive histopathological study of early syphilis and yaws will be described in chapter 4.

Differential diagnosis

Syphilis is considered the 'great imitator'. The differential diagnosis is broad. Primary syphilis should be differentiated from genital herpes, chancroid, donovanosis, lymphogranuloma venereum, Behçet's syndrome, cancer, scabies, trauma, balanitis or balanoposthitis, tuberculosis, endemic treponematoses, many causes of oral ulcers, anal fissures or warts. The secondary stage of syphilis may resemble a broad array of other diseases presenting with generalised skin eruptions. Most common are pityriasis rosea, pityriasis versicolor, viral exanthems, drug eruptions, psoriasis, seborrhoeic dermatitis, lichen planus, scabies, endemic treponematoses, granuloma annulare, erythema multiforme and Stevens-Johnson syndrome. Mucous patches must be differentiated from herpes, Behçet's

syndrome, or Stevens-Johnson syndrome. Condylomata lata may mimic condylomata acuminata.

In particular in latency, positive findings in serological tests must be differentiated from, for example, positive findings in the endemic treponematoses, or latent congenital syphilis, and from biological false positive laboratory results. Some titres in serological tests may remain positive for life, even after antibiotic treatment. The differential diagnosis of gummatous syphilis includes many diseases with formation of granulomatous or ulcerative processes, for example leprosy, tuberculosis, sarcoidosis, chronic pyoderma, iododerma, deep fungal infections or malignancy.

Laboratory tests

The most widely used laboratory methods to diagnose treponemal infection are: the demonstration of the pathogen by dark-field microscopy, fluorescent methods, silver staining methods of biopsy specimens, and the demonstration of specific antibodies in body fluids by means of serological tests (23).

Serological tests can be used in a population as a screening device to assess the prevalence of treponemal infection, to demarcate the area of infection or in the individual patient to confirm the diagnosis and for follow-up after treatment.

Two types of serological tests are of major importance, detecting specific antibodies to treponemal antigens in socalled anti-treponemal tests, (demonstrable approximately two weeks after infection) and non-specific antibodies to cardiolipin antigen in the anti-lipoidal tests (present after approximately four weeks). The most widely used tests are treponemal tests such as the *T.pallidum* haemagglutination assay (TPHA), the fluorescent treponemal antibody - absorbed (FTA-ABS) test and nontreponemal tests such as the Rapid Plasma Reagin (RPR) card test or the Venereal Disease Research Laboratory (VDRL) test (24,25). Measuring serial titres of nontreponemal tests is useful in assessing serological response to therapy of early syphilis. Tests using recombinant DNA-derived antigens for the diagnosis of syphilis have recently been developed (25-28). The newly developed *Treponema pallidum* membrane protein TmpA ELISA has a sensitivity and specificity comparable to the TPHA. This may possibly be a treponemal test with a potential for monitoring the effect of antibiotic treatment (25). The use of serology in the diagnosis of congenital syphilis was recently thoroughly reviewed by Boot *et al.* In the serodiagnosis of congenital syphilis a special test, the socalled

19S(IgM)-FTA-ABS test is used (29).
Until now, no serological test can be used to differentiate between syphilis and the endemic treponematoses.

Therapy for early syphilis

Throughout the world differences in the treatment of syphilis exist. For early syphilis (primary, secondary, or latent syphilis of not more than two years' duration) large doses of penicillin are the treatment of choice: benzathine penicillin G, 2.4 million units intramuscularly in one dose (30,31) or aqueous procaine penicillin G, intramuscularly, 1.2 million units each day for 10 consecutive days (30). In several centres, for example at the University Hospital Rotterdam-Dijkzigt, higher doses are administered, for instance benzathine penicillin G, 2.4 million units, three times at weekly intervals. Recent problems with the treatment of syphilis in some HIV-seropositive patients are discussed in chapter 1.3.
In case of penicillin allergy, tetracycline or doxycycline (orally) are the alternatives. In penicillin-allergic pregnant women the alternative is erythromycin. However, erythromycin may fail to cross the placental barrier, so very strict follow-up of mother and child is prerequisite.
With penicillin treatment, (apart from possible allergic reactions) the socalled Jarisch-Herxheimer reaction may occur: an acute febrile condition often with transient reactions such as headache, hypertension, hyperventilation and myalgia. The incidence of this reaction is highest during treatment of late primary or early secondary syphilis. The aetiology of the Jarisch-Herxheimer reaction remains unknown (32).

Therapy for late syphilis

In 1989, for the treatment of late syphilis (late latent syphilis of more than two years' duration or of indeterminate duration, late benign or cardiovascular syphilis), the World Health Organization recommended intramuscular injection of aqueous procaine penicillin G, 1.2 million units daily, for 20 consecutive days. Another regimen consists of benzathine penicillin G, 2.4 million units weekly for three consecutive weeks. For cardiovascular syphilis the former treatment regimen is preferred (30). For neurosyphilis, aqueous crystalline penicillin G, 12-24 million units administered as 2-4 million units every four hours, intravenously, is advised, for 14 days. For patients allergic to penicillin, doxycycline

or tetracycline are recommended, for 30 days. For penicillin-allergic pregnant women erythromycin is the treatment of choice (30).

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1.3.

SYPHILIS IN THE AIDS ERA

Herman Jan H. Engelkens, M.D., Jaap J. van der Sluis, Ph.D.,
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Syphilis in the AIDS Era

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For several centuries syphilis has been present as a pandemic. In developed countries the pandemic character has been restricted due to the availability of arsenicals and later penicillins, but syphilis is still far from being eradicated. In the United States the incidence of syphilis in 1987 was the highest since 1950.[1] In developing countries the burden of sexually transmitted diseases (STD), including syphilis, is huge. As a consequence, congenital syphilis still is highly prevalent in these countries.

In its natural course syphilis evolves to a state of latency. During latency the number of treponemes present in the host is the result of a dynamic equilibrium between the growth of the treponemes and their "eradication" by host defenses. In this stage the disease can be recognized only by positive results of serologic treponemal tests. Even after adequate antibiotic therapy these tests may remain positive for years or lifelong. In untreated cases the tertiary stage of syphilis may develop, usually after several decades, with possible life-threatening consequences.

Immune Concepts

The humoral and cellular branches of the immune system play an important role in the defense against treponemes. The presence of anti-treponemal antibodies and sensitized T lymphocytes during early syphilis does not always prevent chronicity of the disease.

Eight decades after the discovery of *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) as the causative organism of venereal syphilis, the human immunodeficiency virus (HIV) was identified as the causative agent of the acquired immunodeficiency syndrome (AIDS). AIDS itself is a pandemic with disastrous consequences. The selective tropism of HIV for immunocompetent cells is responsible for the immunodeficiency of HIV disease. It is not difficult to imagine that the immunodeficiency state in HIV disease will have consequences for the natural course of co-infections such as syphilis.

Sexual contact is the principal mode of transmission of both diseases. Genital ulcer disease (syphilis, chancroid, genital herpes) has been associated with an increased risk of HIV-1 infection.[2,3] Thus, rapid spread of HIV infection may occur in areas of the world where genital ulcerations are prevalent. Disturbance of the integrity of mucosal epithelia is a major route for the spread of HIV infection. Genital ulcers provide a possible portal of entry for HIV; stimulated T lymphocytes and activated macrophages may be present as possible target cells for HIV. It has been documented that as many as 70% of AIDS patients have a positive history and/or positive serology for syphilis.[4] The long incubation time of AIDS often obscures whether the HIV infection was followed by or preceded by infection with *T. pallidum*.

Variations

Alteration in the natural history of syphilis has been described in HIV-1-seropositive persons. This has been noted previously in patients who were immunosuppressed by other causes. For example, in a patient receiving immunosuppressive therapy a remarkable course of syphilitic infection (hepatitis) recently has been documented.[5] It is well known that corticosteroids induce an alteration in the evolution of syphilitic infection in laboratory animals.[6]

In HIV-1-positive individuals, atypical and aggressive clinical manifestations of syphilis have been described. Syphilitic skin manifestations, similar to those in early reports of "malignant" syphilis, may be seen.[7] Several reports have described atypical skin lesions of syphilis in HIV-positive individuals. In HIV-

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seropositive individuals, syphilis involving the eye has received special notice by several ophthalmologists. These ocular manifestations often are difficult to discern from cytomegalovirus- or HIV-induced eye disease. The occurrence of accelerated progression to neurosyphilis with more aggressive characteristics has been reported recently[8,9]; this was explained by a potentiating effect of HIV on syphilitic infection. Invasion by *T. pallidum* of the central nervous system can already occur in the early stage of syphilis.[10] A problem is whether neurologic impairment is due to HIV infection, syphilis, or another entity. Care must be taken with the use of terms like "neurologic manifestations in syphilis" and "neurosyphilis." Cerebrospinal fluid examination before treatment of patients presenting with syphilis recently has been advised.[10] This examination may also be important after treatment has been given, especially for those at high risk for HIV infection.

Although early syphilis in HIV-infected individuals usually is accompanied by reactive serologic treponemal test results, false-negative results in HIV-positive patients have been reported.[11] Occasionally, very high titers have been reported.[12] Dark-field examination and special stains for detection of treponemes in patient materials (silver staining, direct fluorescent antibody staining) remain of utmost importance in the diagnosis of syphilis.

Therapeutic regimens for syphilis have been questioned, especially in HIV-positive/AIDS patients.[8,9] Despite treatment of patients suffering from early syphilis, progression to neurosyphilis within a short period of time has been reported. Earlier, for different complicating infections in AIDS like toxoplasmosis, pneumocystis, and cryptococcal infection, changes in treatment regimens have been proposed. Several investigators suggest adaptation of therapy for syphilis, proposing, for example, the treatment regimen for neurosyphilis for all immunocompromised individuals who have contracted syphilis[13] and "maintenance therapy."[8] Moreover, it has been suggested that all HIV-infected individuals with neurologic impairment be treated as if they have neurosyphilis.[13] There is as yet no agreement about this important issue. No changes in therapy for early syphilis have been recommended by the Centers for Disease Control (CDC)[14] and the World Health Organization (WHO).[15] According to the CDC and WHO it is essential that careful follow-up and evaluation of therapy be performed by means of repeated serologic testing in controlled studies.

No reports have been published on simultaneous infection with HIV and the other, still important, non-sexually transmitted treponematoses like yaws. This is not expected in the near future, since these infections have a varied geographic distribution. It would be of great importance to continue the study of the course of these diseases, which are supposed not to affect the cardiovascular and the central nervous system, in contrast to sexually transmitted syphilis. Furthermore, the precise role of simultaneous STD in HIV-2 infection is not yet clear.[3]

Conclusions

Since the introduction of AIDS in the modern world, problems in diagnostic and therapeutic methods have been encountered. The diagnosis, treatment, and follow-up of a classic disease like syphilis in patients with HIV infection remain difficult. Nearly forgotten manifestations of sexually transmitted syphilis from the prepenicillin era (e.g., "malignant" syphilis) have appeared in the AIDS era. Whether the current problems with syphilitic infection were already present in the pre-AIDS era remains to be seen.[16]

Careful monitoring of HIV-infected syphilitic patients is essential. Adaptation of treatment regimens for syphilis may be necessary. We also propose that all patients diagnosed with syphilis should be tested for HIV and vice versa. HIV testing has caused and is still causing debates in many countries, like ours, where informed consent for testing is required.

Mechanisms of a possible interaction between *T. pallidum* and HIV in the pathogenesis of syphilis require further study. More research is needed to evaluate existing serologic tests and new methods for screening syphilis and other STDs in the AIDS era.

In the face of global attention focused on HIV disease, it remains of utmost importance to continue careful study and health education about the other STDs.

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ENDEMIC TREPONEMATOSES
PART I. YAWS

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Endemic Treponematoses
Part I. Yaws

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The causal organism of yaws (framboesia tropica, pian, parangi, paru, buba, bouba), a chronic relapsing tropical disease, is *Treponema pallidum* subspecies *pertenue* (*T. pertenue*), belonging to the order *Spirochaetales*, family of the *Treponemataceae*, genus *Treponema*. Other pathogenic treponemes are *Treponema pallidum* subspecies *pallidum* (*T. pallidum*), causative organism of venereal syphilis, *Treponema pallidum* subspecies *endemicum*, causative organism of endemic syphilis (bejel), and *Treponema carateum*, causative organism of pinta. For *Treponema carateum* propagation an animal model has not yet been developed and it is still considered to be a separate species.[1] *T. pertenue* has been passaged successfully in rabbits and hamsters, thus providing both a source of microorganisms for study and an experimental model.[2] Currently, *T. pertenue* cannot be distinguished from the other pathogenic treponemes morphologically[3,4] or by laboratory tests.[5]

Recently, an alarming resurgence of yaws has been observed in several countries in the tropics.[6] Millions of children are at risk of contracting this disease, which can destroy bone and tissue. In most endemic areas the current extent of yaws is not fully known; presumably there is considerable underreporting. The current resurgence of yaws in the world is due partly to a false sense of security caused by the decline in number of patients after the mass campaigns.[7] Yaws is still a serious health problem in different countries of the endemic areas in the world, from which people might emigrate to the United States and Europe.

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History

In 1905, Castellani discovered the presence of spirochetes in ulcers from patients suffering from parangi in Ceylon.[8] The microorganisms resembled those observed by Schaudinn and Hoffmann, who were the first to describe the causative agent of venereal syphilis earlier that year. Because cutaneous lesions in yaws frequently resemble raspberries, the disease was called "framboesia tropica" and the skin lesions "frambesiomas" (Dutch: *framboos* = raspberry).

Yaws is an ancient disease, which in theory arose around 10,000 BCE in the Afro-Asian landmass from a mutant form of pinta, more adapted to tropical circumstances. According to Hackett, four different diseases exist, but the different treponemes share a common origin, probably a pathogenic ancestor in animals.[9] It has been suggested that the four clinical entities known today are in fact one disease caused by *T. pallidum* (unitary theory). Changing environmental conditions are held responsible for the different epidemiological patterns of the treponematoses.[10]

Before 1950, exposure to *T. pertenue* was likely for about half of the 400 million people living in the belt between the Tropics of Cancer and Capricorn.[11] The World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) supported mass treatment campaigns, executed with great success in the 1950s and 1960s.[5,6,12] The world's largest yaws campaign was held in Indonesia, directed by Dr. Kodijat.[13] Due to historical ties between Indonesia and The Netherlands, yaws has always been of interest to Dutch investigators.[14,15] Today, yaws still is one of the main topics of investigation of our department in Rotterdam.

Epidemiology

Distribution

Yaws is prevalent in rural, very warm tropical regions with high humidity and heavy rainfall. In many en-

demic areas the prevalence of yaws has been greatly reduced.[6] Today the main reservoir can be found throughout Africa.[6,16] Yaws has never been eradicated in any of the nations of the African continent. A low-level transmission has persisted over the years after the mass campaigns. In parts of West and Central Africa the incidence is again increasing. For example, in Ghana, Benin, Togo, and the Central African Republic the situation resembles the pre-campaign era.[17] Recently, increasing seroreactivity also has been reported in Nigeria, the Ivory Coast, and Mali (Meheus, VIth African Regional Conference on sexually transmitted diseases[18]). In Southeast Asia and the Pacific Islands residual foci of infection persist in several countries,[19,20] for example in Indonesia and Papua New Guinea, where widely dispersed foci of infection exist.[20,21]

Only a few countries in the Americas continue to report sporadic cases, specifically Suriname, Guyana, Colombia, Venezuela, Brazil, and Haiti.[6,22]

Age and Sex

Yaws is contracted mostly in childhood. Nearly all new cases are found in children under 15 years of age, with a peak between 6 and 10 years of age.[23] A sex difference has been described by some authors.[15,23] Others assume that boys and girls are equally affected.[24]

Transmission

Direct, personal skin-to-skin contact is the major route of transmission in yaws. Breaks in the skin (excoriations, scratches, bites, traumata, etc.) provide an entry for the treponemes. Transmission is facilitated under overcrowding conditions where open lesions on the skin are common and where no protective clothing is worn. Infectivity is present particularly under humid tropical circumstances.

Theoretically, indirect transmission by fomites and insects is probable, but no evidence exists. In 1931 and 1942 remarkable outbreaks of yaws were observed among workers in South African gold mines, pointing to the importance of close bodily contact while at work under very warm, moist conditions[25,26]; it was impossible for insects to survive under comparable circumstances.[27]

In West African baboons (*Papio cynocephalus*) a reservoir of a yaws-like treponeme was described by Fribourg-Blanc et al. on serological grounds in the early 1960s.[27,28] There is no certainty about the significance of this reservoir for humans. In general it is assumed that transmission of the infection to the fetus does not occur in yaws. Román and Román recently have summarized literature suggesting that children can be infected congenitally.[29] Since positive treponemal test results in the newborn can be explained by transplacental passage of IgG, and since a serologic test to differentiate yaws from venereal syphilis is not available, further study in this direction is needed to draw conclusions.

As a rule, sexual transmission does not play a role in yaws. The presence of genital lesions, although a rarity, can cause diagnostic problems.[30]

Clinical Features

Classification

Several classification systems in yaws were used in the past.[5,23] Most often four stages (primary, secondary, tertiary, and latent) have been discerned in yaws, as in venereal syphilis. In the primary stage initial skin lesions develop at the site of infection; secondary stage skin lesions, resulting from the widespread dissemination of treponemes, resemble initial skin lesions except in number and size; deformities can occur in the tertiary stage. However, an overlap between the stages can occur. According to the International Nomenclature of Yaws Lesions,[31] it seems more practical to distinguish between an early stage in which contagious skin lesions occur, and a late stage, in which lesions are not considered contagious. Early yaws comprises the primary and secondary stage; late yaws comprises the tertiary stage.

Early Yaws

Initial lesions, often pruritic, appear after an incubation period of 9 to 90 days (average 3 weeks).[5] Classically, the initial manifestation of yaws is the "mother yaw," one or more nontender papules that later become crusted (Fig. 1) and ulcerated. The lower extrem-

Figure 1. Early yaws: a crustopapillomatous lesion on the arm (West Sumatra, Indonesia, 1988). Serology: VDRL +, TPHA 2+, FTA-ABS 3+ (courtesy of J. van der Stek).

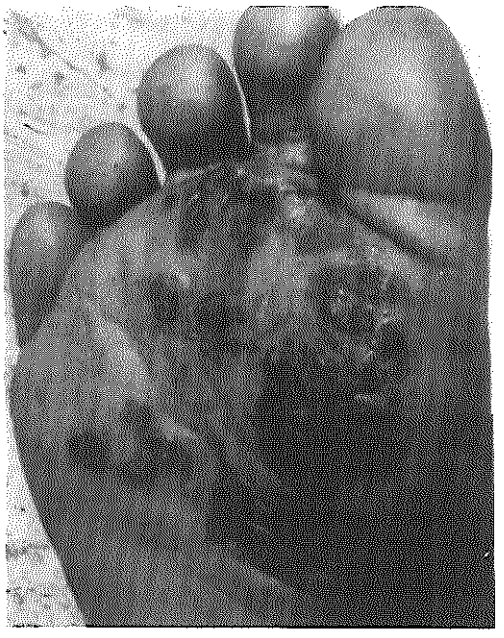

Figure 2. Multiple ulcerations on the footsole as the first manifestation of yaws. Dark-field examination revealed the presence of many treponemes. Positive treponemal test results.

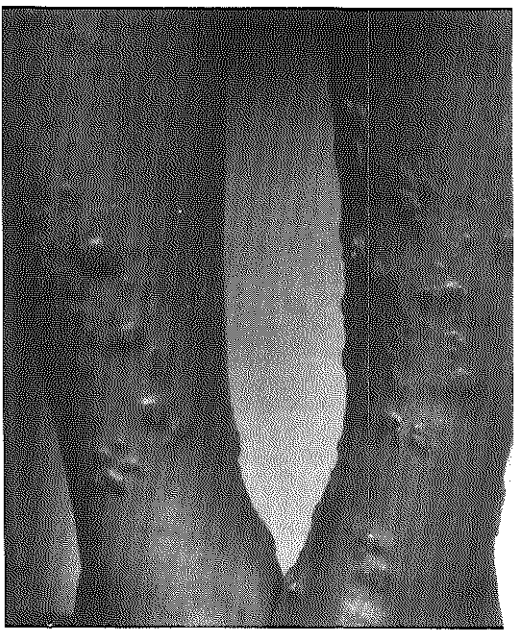

Figure 4. Multiple lesions on the lower legs. Dark-field examination was positive (Indonesia). Positive treponemal test results.

ities is the site of predilection (Fig. 2). Initial lesions can develop into friable ulcerated proliferative papillomatous lesions. These lesions contain numerous treponemes. After or during spontaneous disappearance of initial lesions, relapses of more disseminated lesions ("daughter yaws") can occur (Figs. 3, 4), which may be preceded or accompanied by malaise, fever, and generalized lymphadenopathy. Early stage skin lesions often resemble initial lesions. Macules, papules, and nodules also can be seen. Palms and soles regularly show hyperkeratosis (crab yaws). Even in the early stage bone and joint manifestations can occur[32,33]; most important are osteitis and periostitis. Lesions in the early stage of yaws usually disappear spontaneously, sometimes leaving slight pigmentary changes. However, severe secondary infection can be a life-threatening complication and scarring is no exception.

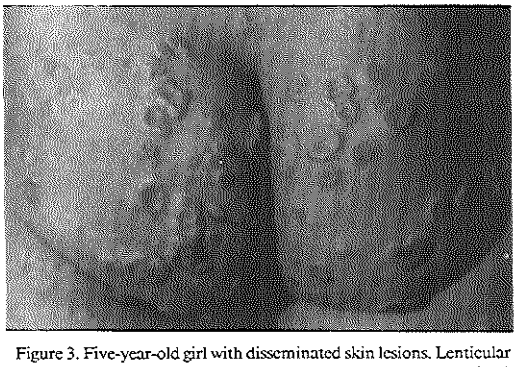

Figure 3. Five-year-old girl with disseminated skin lesions. Lenticular and nummular crustopapillomatous lesions on the buttocks for 3 months. Serology: VDRL ++, TPHA 2+, FTA-ABS 3+.

The skin lesions show climatological and seasonal influences: in warm, rainy seasons these are more florid and abundant than during drier periods of the year. During the dry season patients can present with atypical cutaneous lesions, often macular in character and more scanty.

Some authors have described an "attenuated" clinical picture of yaws after mass campaigns,[34,35] with less florid skin lesions. In areas with a low prevalence, solitary, small, flat, dry, and hidden lesions of short duration were seen in contrast to the classical skin lesions. Vorst argued that endemic treponematoses show extremely variable syndromes depending on the frequency of the infection in a certain community.[36] During our clinical study (Stolz, Jubianto Judanarso,

1988) in Sumatra, Indonesia, we saw that many patients still were suffering from classical early yaws (see Figs. 3, 4).[37]

Latency

After the early skin manifestations have subsided, a latent period of variable duration follows, which can be recognized only by positive serological test results. This period can be interrupted by one or more relapses of skin lesions.

Late Yaws

In the majority of patients latency lasts a lifetime. An estimated 10% of the patients enters a destructive late stage after 5 to 10 years or even longer.[5] Irreversible lesions of bone,[32] cartilage, soft tissue, and the skin are notorious. For example, gangosa (destructive ulcerative rhinopharyngitis), gummata, juxta-articular nodes, contractures, and saber tibiae can be the late consequences. Gondou (Fig. 5), rare exostoses of nasal and adjacent bones, even can occur early in the course of the disease. Hyperkeratotic lesions can occur in both late and early yaws.

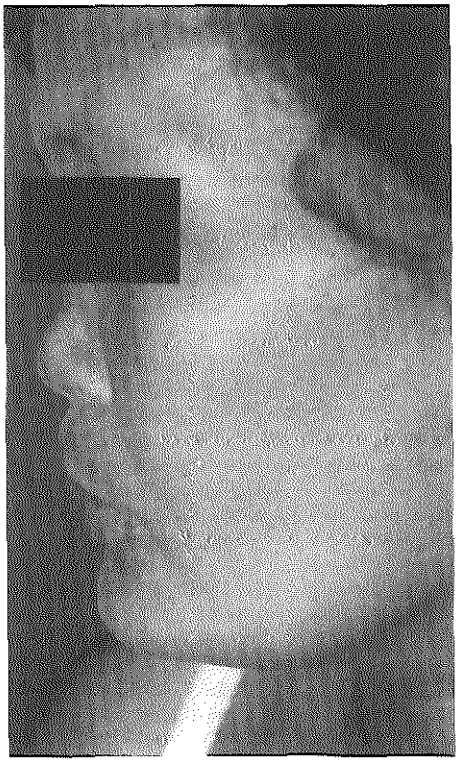

Figure 5. Gondou in Suriname.

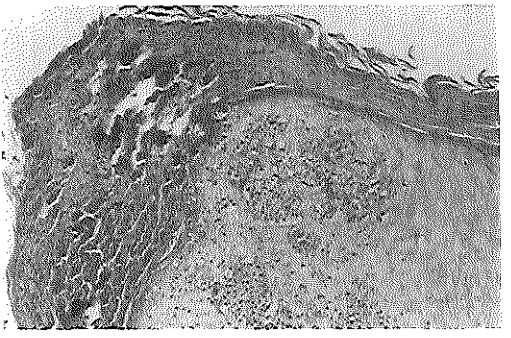

Figure 6. Skin showing multiple microabscesses in a hyperplastic epidermis (hematoxilin-azophloxin, original magnification ×150).

In general it is assumed that no neurological and cardiovascular abnormalities occur in yaws. In a recent review it has been suggested that cardiovascular and neurological involvement can indeed occur in yaws, comparable to syphilis.[29] In a neuro-ophthalmological study, ocular and neurological abnormalities in late yaws patients were described by Lawton Smith et al. in 1971.[38] Cerebrospinal fluid abnormalities, optic atrophy, abnormal pupils, and perivascular sheathing were noted. Since confusion between yaws and other treponematoses is possible, and since a test to differentiate yaws from the other treponematoses is not available, the initiation of new studies is recommended to draw definite conclusions.[29]

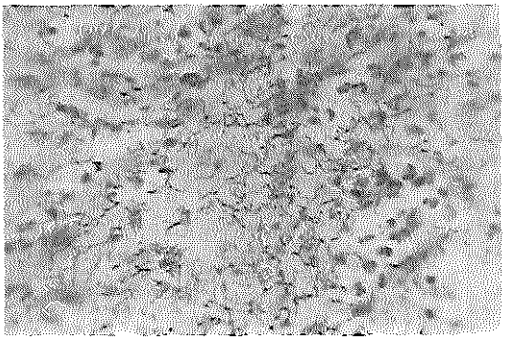

Figure 7. Numerous treponemes in the epidermis (Steiner method, original magnification ×600).

Histopathology

In early yaws papillomatous epidermal hyperplasia is the main feature. In addition, there is focal spongiosis. Neutrophils migrating into the epidermis can give rise to intra-epidermal microabscesses (Fig. 6). The stage is accompanied by a dense dermal perivascular infiltrate, consisting mainly of plasma cells. Blood vessels are affected usually only mildly in yaws, in contrast to venereal syphilis. Little proliferation of endothelial cells can be found. Epidermal changes can occur, as in condylomata lata, but the infiltrate sometimes is distributed in a diffuse rather than in a perivascular way. Hyperkeratotic lesions show nonspecific characteristics: acanthosis, hyperkeratosis, and parakeratosis with only a mild infiltrate.[39,40]

With silver impregnation (Fig. 7) or immunofluorescent staining techniques, large numbers of treponemes can be observed between epidermal cells, particularly above the tips of the dermal papillae, in the early stage.

Characteristic for yaws is the epidermotropic character of *T. pertenue*.[39,40]

Laboratory Tests

Tests originally designed for the serodiagnosis of venereal syphilis also are used in yaws where available.[41,42] These include the nontreponemal serology tests (rapid plasma reagin [RPR] test and Venereal Disease Research Laboratory [VDRL] test), and the treponemal serology tests: the *T. pallidum* hemagglutination assay (TPHA), the microhemagglutination assay-*T. pallidum* (MHA-TP), and the fluorescent treponemal antibody absorption (FTA-ABS) test. In remote regions the nontreponemal tests are the only practical ones. In reference laboratories the treponemal tests can be used to confirm reactive nontreponemal tests. In the field the most practical method for collecting and transporting blood for serologic tests is the filter paper method.[42]

Sera from patients with yaws react similarly to sera from syphilitic patients. The beginning of reactivity after infection and the persistence of positive serological test results after treatment are similar in yaws and venereal syphilis.

Dark-field examination of exudates from early stage skin lesions can reveal the presence of treponemes.

Diagnosis

A diagnosis of yaws can be made on clinical and epidemiological grounds. Positive serological tests in all stages except the very early stage, the presence of treponemes in dark-field examination of exudates of cutaneous lesions, and examination of skin biopsies confirm the diagnosis. In regions where yaws and other treponematoses coexist, the disease cannot always be differentiated from venereal syphilis, pinta, and endemic syphilis, particularly in latency and in the late stage. Thus far it has not been possible to distinguish between the causative agents of different treponemal diseases on serological, morphological, or biochemical grounds.

Apart from the other treponematoses several other diseases in the tropics can resemble yaws. Skin lesions should be differentiated from impetigo, ecthyma, leprosy, tungiasis (jiggers), tropical ulcers, chromomycosis, cutaneous leishmaniasis, sarcoidosis, psoriasis, vitamin deficiencies, scabies, and viral infections such as mollusca contagiosa or plantar warts. Bone lesions in yaws can resemble those of venereal syphilis, endemic syphilis, tuberculosis, bacterial osteomyelitis, and sickle cell anemia. Rhinopharyngeal lesions can resemble those of espundia (mucocutaneous leishmaniasis), rhinosporidiosis, rhinoscleroma, tuberculosis, leprosy, and South American blastomycosis (paracoccidioidomycosis).

Therapy

The 1980 World Health Organization Scientific Group recommends treating yaws with 600,000 units of benzathine penicillin for all patients and contacts (for those aged under 10 years) and 1,200,000 units (for those aged over 10 years) by single intramuscular injection. Contacts of patients and patients with latent yaws should receive the same doses as those suffering from active yaws.[43]

In case of penicillin allergy tetracycline and erythromycin have been recommended as alternatives.[44]

After single-shot therapy has been given, the classical early infectious lesions will heal within 7 to 10 days. Changes in titers determined by quantitative nontreponemal tests are the major parameters used to measure response to therapy. Treponemal tests remain positive despite treatment.

Benzathine penicillin given prophylactically will prevent infection for several weeks.

Outside the endemic regions patients with (a history of) yaws are probably overtreated regularly,[45] since serological reactions cannot differentiate between yaws and syphilis, which requires a higher dosage.

Resistance of *T. pertenue* to penicillin has not yet been demonstrated clearly.[44] Recently Stapleton et al. reviewed this potential threat,[46] concluding that it is impossible to predict the chance of developing resistance in the future. A safe and effective vaccine has not been developed.

Discussion

Yaws is becoming a serious public health problem again. Due to migration and travelling, cases of yaws can turn up anywhere in the world and confront the medical profession with a diagnostic dilemma, especially in latency or in the late stage.

Continuing surveillance by seroepidemiological evaluation is urgently needed. As we know from the past, treatment campaigns, health education, and improvement of medical and living conditions can contribute to lower the incidence of yaws. Integration of "anti-yaws" programs into primary health care systems is required.[47–49] Again, vigilance more than ever is needed urgently to call the resurgence of the endemic treponematoses and yaws in particular to a halt.

Several questions remain to be answered. Since it has not been possible to cultivate *T. pertenue in vitro* for sustained periods, hamsters and rabbits are used most frequently in experimental studies for propagation of the treponemes.[2,50] Turner and Hollander described a slightly different type of reaction of *T. pertenue* in hamsters, compared with *T. pallidum*.[2] Available genetic evidence indicates that *T. pallidum* and *T. pertenue* are indistinguishable.[51] However, in a recent study it has been shown that *T. pertenue* and *T. pallidum* differ in at least one nucleotide in a homologous antigen.[52]

It is hoped that further basic research will lead to the development of a specific test for yaws in the future, for the diagnosis of this disease in the individual and for epidemiological purposes, especially in rural circumstances. More detailed study of the epidermotropic character of *T. pertenue* and characterization of the inflammatory infiltrate could be helpful in gaining more insight into the course of this infection.

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1.5.

THE RESURGENCE OF YAWS
WORLD-WIDE CONSEQUENCES

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The Resurgence of Yaws
World-Wide Consequences

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In developing countries the burden of infectious diseases is of the utmost importance. Diseases such as yaws (framboesia tropica), leprosy, malaria, tuberculosis, trachoma, and leishmaniasis, among others, still are responsible for severe handicaps. Treponemal diseases still are widespread in developing countries.[1] Not only infectious syphilis, but now also yaws (causative agent *Treponema pallidum* subspecies *pertenue*), are once again spreading rapidly, mainly in Africa, Southeast Asia, and South America.[2–4] WHO- and UNICEF-assisted mass campaigns against the endemic treponematoses (yaws, endemic syphilis, and pinta) still remain important success stories in the history of medicine.[1] In many endemic areas the prevalence of yaws has been greatly reduced (Figs. 1,2). Unfortunately, however, in several countries transmission has persisted, and in some areas the number of infectious cases surpasses even the precampaign data.[2,3]

Diagnosis

Yaws, an infectious disease of childhood, is prevalent in very warm, humid tropical regions, frequently inaccessible and underserved by primary health care. Transmission is facilitated under overcrowding conditions where open lesions on the skin are common and where no protective clothing is worn. The infection is spread under humid tropical circumstances. In the late stage of yaws, mutilations can result in severe handicaps, which can be prevented if yaws is detected and sufficiently treated in an early stage. Early detection is crucial in yaws treatment and control.

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Air travel easily can transport migrating people with yaws outside the endemic regions. In nonendemic countries the medical profession, often unfamiliar with yaws, must be alerted, as it can be confronted with this disease. For example, in The Netherlands we recently have diagnosed early yaws in a girl originating from Ghana.[5] The danger of the spread of yaws outside the tropical regions is small, due to climatologic and hygienic circumstances.[1,4]

Latent yaws often is not recognized as such. A reactive serologic test in this stage is only indicative. Recently, atypical forms of yaws (attenuated yaws) have been recognized,[3,6] which can obscure the diagnosis. Furthermore, late stage manifestations may imitate many other diseases and as a consequence the diagnosis of yaws is not always considered.

A specific test to differentiate between the different treponematoses is not yet available. None of the tests commonly used for diagnosing treponematoses is subspecies-specific. For diagnosing yaws in the individual patient and for epidemiologic surveillance, a specific test would be of utmost importance.

Treatment

Therapeutic recommendations differ between different authors. It would be of great importance to use standardized schedules for treatment of the different treponematoses. For public health reasons, persons with a positive serology originating from endemic regions mostly receive the treatment regimen recommended for sexually transmitted syphilis. In case of a nonvenereal treponematosis, this probably means overtreatment; in contrast with venereal syphilis, cardiovascular and neurologic involvement in nonvenereal treponematosis is considered to be extremely rare or absent. Moreover, it is assumed that these diseases do not carry the risk of transplacentary connatal infection of the fetus. These matters are not yet fully understood and

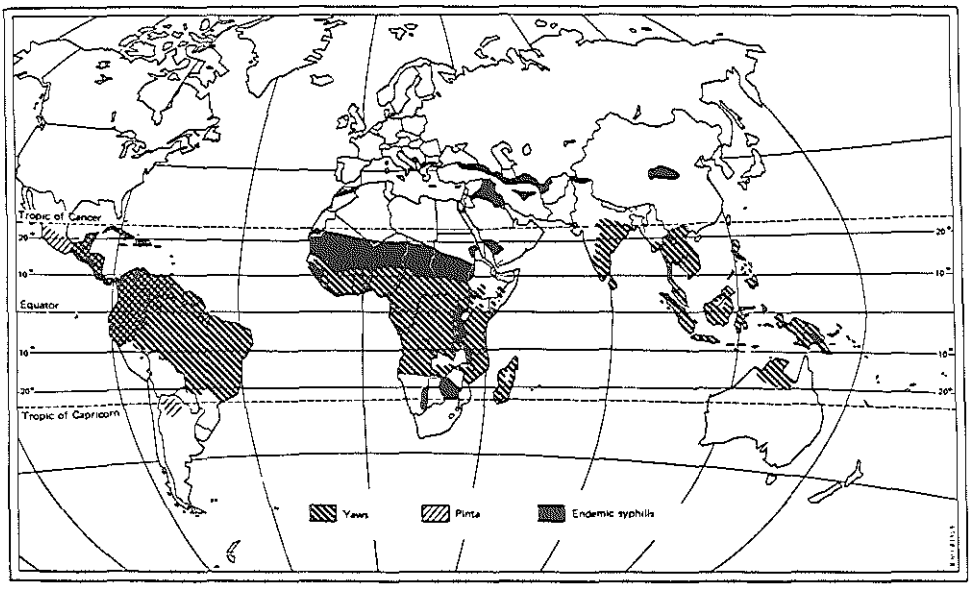

Figure 1. Geographical distribution of the endemic treponematoses in the early 1950s. Reproduced with permission from Perine PL, et al. Handbook of Endemic Treponematoses: Yaws, Endemic Syphilis, and Pinta. Geneva: World Health Organization, 1984.

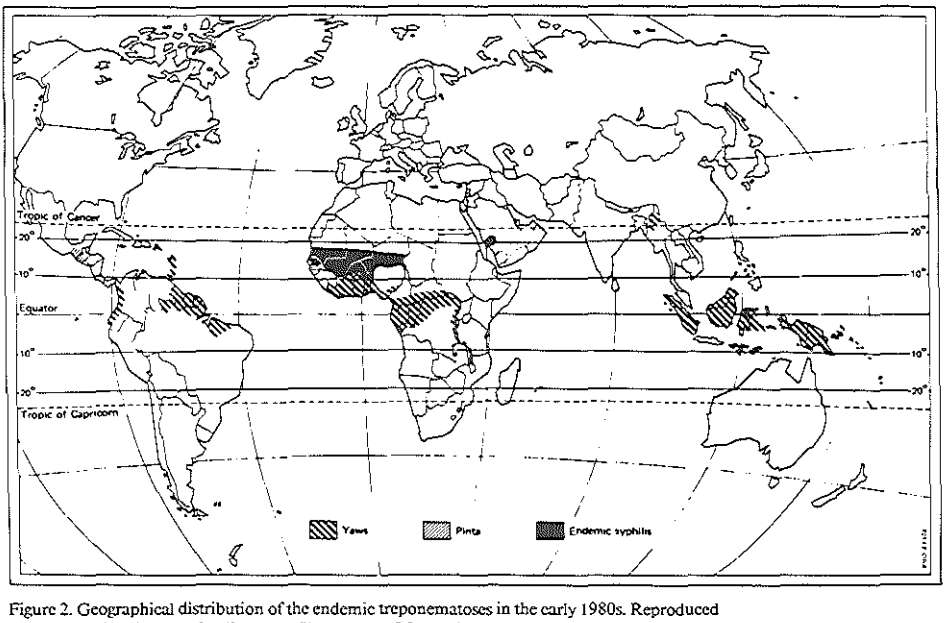

Figure 2. Geographical distribution of the endemic treponematoses in the early 1980s. Reproduced with permission from Perine PL, et al. Handbook of Endemic Treponematoses: Yaws, Endemic Syphilis, and Pinta. Geneva: World Health Organization, 1984.

further study of the possible (late stage) consequences of yaws has been recommended recently.[7]

Reactive treponemal and/or nontreponemal serologic test results in patients originating from parts of the world where the endemic treponematoses are still prevalent, should arouse our suspicion. Not every reactive test in these expatriates is due to a sexually transmitted disease. Special care must be taken in the exploration of positive results. Mistaking a nonvenereal treponematosis for venereal syphilis can have very serious social implications for the person and his family involved.

Conclusion

Renewed attention to and continuing education on the nonvenereal treponematoses in developed and developing countries is required to alert all workers in the medical field to a proper recognition of these almost forgotten diseases. Presumably, in the 1990s attention will be focused on the AIDS epidemic, but this must not make us forget the important common infectious diseases of years gone by.

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1.6.

ENDEMIC TREPONEMATOSES
PART II. PINTA AND ENDEMIC SYPHILIS

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Endemic Treponematoses
Part II. Pinta and Endemic Syphilis

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Treponemal infections occurring in humans comprise venereal syphilis and the endemic treponematoses yaws, pinta, and endemic syphilis. The endemic treponematoses have comparable natural histories. Young children are at the highest risk to acquire the nonvenereal treponematoses. Yaws was recently reviewed in the Journal.[1] This paper will focus on the other two nonvenereally transmitted treponematoses, pinta and endemic syphilis. Sexually transmitted syphilis will not be reviewed in this context. The causative organism of pinta is *Treponema carateum* (*T. carateum*), and that of endemic syphilis is *Treponema pallidum* subspecies *endemicum*.[2] At present the causative agents of the different treponematoses cannot be distinguished from each other serologically, morphologically, or by other means.[3–7]

In the 1950s an estimated one million cases of pinta were present in Central and South America. Another million or more persons were suffering from endemic syphilis in the Middle East, with several other foci, particularly in Africa.[4,8,9] As a result of penicillin treatment of nearly 50 million people in mass campaigns, the incidence of the endemic treponematoses has been greatly reduced.[4,5,8,10] However, these diseases have not been eradicated. Transmission has only been suppressed, and nowadays increasing numbers of infected persons are being reported from endemic regions.[11,12]

History

It has been postulated that treponematoses originated some 22,000 years ago in the Eurasian-African landmass.[13,14] Pinta is considered the first treponematosis to occur in humans. Reports on pinta had already appeared on the American continent in the early years of the 16th century, described as *carate*, an affection of the skin, especially in Aztec and Carib Amerindians.[5,6]

The causative agent of pinta was first described as being of mycotic origin.[4] Armenteros and Triana (Cuba, 1938) and Blanco (Mexico, 1942), recognized a spirochaete, indicated as *T. carateum*, as the causative organism.[5]

In the 1950s, Colombia and Mexico were the countries where pinta was highly endemic.[15] After the treatment campaigns, pinta was considered extinct in these areas.

Endemic syphilis is assumed to have existed before 8,000 BCE, probably as a result of a mutation of the (ancestor) treponeme, or as an adaptation to differing epidemiologic influences.[13,14,16,17] The sexually transmitted form of treponematosis (venereal syphilis) emerged in a later millennium, when bodily contact among adults was more restricted to sexual contact.[13,17]

Outside the tropics, endemic syphilis also occurred in scattered communities in other regions of the world. In the 18th and 19th centuries, indications of endemic syphilis were found in many European countries, for example in Norway (*radesyge*), in Scotland (*sibbens*), and in Ireland (button scurvy).[3,8,16] In Yugoslavia the disease was prevalent in Bosnia-Herzegovina. Between 1948 and 1953 mass campaigns were set up in Yugoslavia.[18] Follow-up surveys led to the conclusion that the transmission of endemic syphilis had ceased in this country.[19] From the 1950s on, isolated cases occasionally were reported from Europe.[20] Due to mass treatment campaigns, the disease was considered eradicated in several regions of the world (for example in Australia and Central Asia)[4,7]; however, latent cases still are highly prevalent, and millions of people continue to be at risk for acquiring the endemic treponematoses from relapsing cases.[4,7,11,12] In latent treponematosis, recurrent infectious episodes may spread the disease and so entail the risk of continuation of transmission.

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Pinta

The causative organism of pinta is *Treponema carateum*, closely related to the causative agents of yaws, venereal, and endemic syphilis.[4] Several names have been used for pinta: *carate* (Venezuela and Colombia), *mal del pinto* (Mexico and Cuba), and *azul* (Chile and Peru), among others.[4,5] *T. carateum* is considered to be a separate species, not a subspecies of *Treponema pallidum*, because an animal model for pinta has not yet become available and *T. carateum* cannot be cultured continuously,[2,4,5,21] in contrast with the other pathogenic treponemes found in humans. In recent studies it has been demonstrated that a very high degree of antigenic relatedness exists between *T. carateum* and *T. pallidum* subsp. *pallidum*.[21] The microorganism is considered to be pathogenic only in humans and the higher apes.[4,7]

Epidemiology of Pinta

Distribution. Pinta nowadays is still endemic in remote rural regions. This chronic treponemal infection is prevalent in tropical Central and South America among exposed persons in endemic regions.[4,7,11,12,15,22,23] From Mexico and Colombia cases of infectious pinta have been reported recently.[23] A 1982 to 1983 serosurvey in a remote region in Panama revealed 20% seropositivity. Two to 3% of the population, mainly children younger than 5 years, was found to suffer from skin lesions of pinta.[11] Unfortunately, no precise figures on the current prevalence of pinta are available. Serologic surveys in these areas for the detection of treponemal activity are essential in estimating the current magnitude of this treponemal reservoir.

Age and sex. Most patients acquire the infection during childhood. Sexes are equally affected.[5]

Transmission. The mode of transmission is not entirely clear.[4] Transmission most probably occurs through direct skin or mucous membrane contact. The role of insects in the transmission of pinta has been considered, but has never been proven.[6] The disease is transmitted among children before the sexually active age; sexual transmission plays no essential role.[4–6]

Clinical Picture of Pinta

In pinta, as in the other treponematoses, an early and a late stage are recognized. The early stage is characterized by the initial lesions and more generalized lesions, while the late stage comprises the tertiary and the late latent phase. Stages often show considerable overlapping.[4,5] After an incubation period of some weeks to several months, the initial lesion, a papule or an erythematosquamous plaque (Fig. 1), is formed. Sites of predilection are the uncovered parts of the body. The initial lesions may become pigmented, hyperkeratotic, and scaly, accompanied by local lymphadenopathy. These lesions disappear spontaneously after a period of time. After several months or even years more extensive and often smaller lesions may appear, comparable to the initial lesions: so-called "pintids," which may remain present for years or reappear in recurrences.[4–7] Remarkable changes in skin pigmentation can be the result of these eruptions (Fig. 2). In late (tertiary) pinta, disfiguring pigmentary changes, achromia, skin atrophy, and hyperkeratoses are the main features (Figs. 3, 4). The degree of pigmentation of skin lesions can be different in one and the same patient, resulting in a mottled aspect of the skin that often persists lifelong. Skin lesions may turn red, white, bluish, violet, or brown. No severe mutilations occur; it is assumed that the skin is the only organ affected by this chronic treponemal disease.[4,5,7] Pinta is considered the most benign of the endemic treponematoses, with a good prognosis. No cardiologic or neurologic symptoms have been described.[4,6,24] A congenital form is not known.[4–6]

Figure 1. Violaceous psoriatic plaque of early pinta on the forearm. Reproduced, by permission, from: Perine PL, et al. Handbook of Endemic Treponematoses: Yaws, Endemic Syphilis, and Pinta. Geneva: World Health Organization, 1984 (Fig. 61).

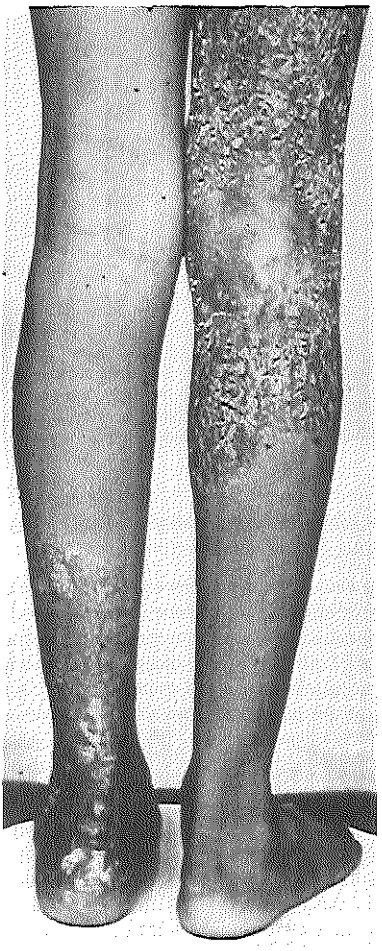

Figure 2. Marked color changes in pinta. Reproduced, by permission of the Programme of Sexually Transmitted Diseases, Geneva, World Health Organization.

Histopathology of Pinta

The histopathologic changes in pinta are largely similar to those in yaws,[1,25–28] except that ulcer formation comparable to that in friable yaws lesions does not occur.[25] In the early lesion only mild acanthosis is present, with migration of some lymphoid cells into the epidermis. Basal cells show loss of melanin and liquefaction degeneration. In early stage lesions melanophages can be present in the upper dermis.[6,26] A moderate dermal inflammatory infiltrate consists mainly of plasma cells and lymphocytes. Histiocytes and neutrophils can be seen.[26] Occasionally, slight swelling of endothelial cells may be present.[25] In the late stage irregular acanthosis or epidermal atrophy can occur. Pigmentary changes vary with the color of skin lesions.[6] A lymphocytic infiltrate and many melanophages may be present in the dermis. In early and late stages treponemes sometimes can be visualized in the epidermis by means of silver staining methods or immunofluorescent techniques. In general, dyschromic late skin lesions still can contain treponemes and show an inflammatory infiltrate, but achromic late skin lesions do not contain treponemes, and an inflammatory infiltrate is no longer present.[26]

Differential Diagnosis of Pinta

Pinta must be distinguished from the other treponematoses: venereal syphilis, yaws, and endemic syphilis. Pinta also must be differentiated from many skin diseases characterized by changes in pigmentation, such as erythema dyschromicum perstans, vitiligo, pityriasis versicolor, pityriasis alba, Riehl melanosis, chloasma,

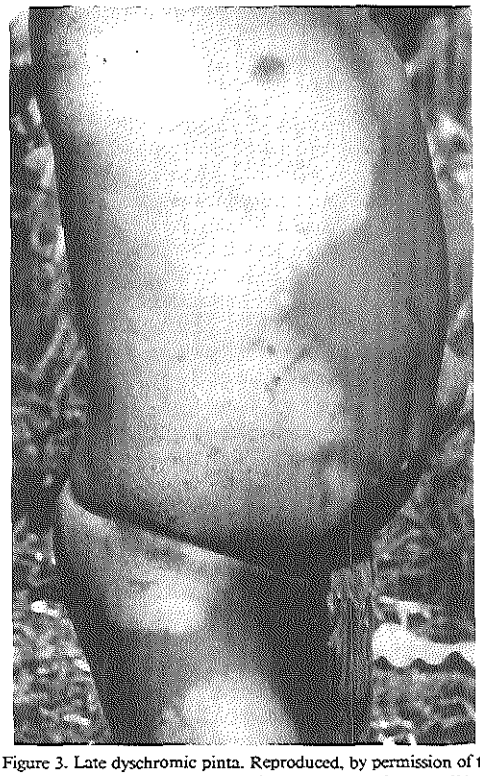

Figure 3. Late dyschromic pinta. Reproduced, by permission of the Programme of Sexually Transmitted Diseases, Geneva, World Health Organization.

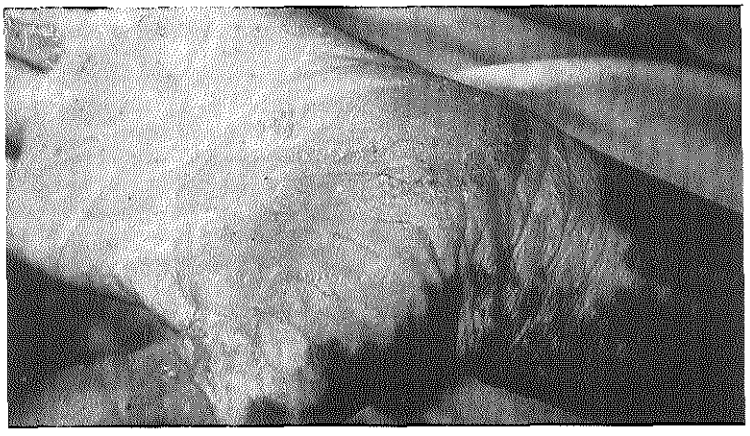

Figure 4. Late achromic pinta. Achromic triangle on the inside of the wrist. Reproduced, by permission of the Programme of Sexually Transmitted Diseases, Geneva, World Health Organization.

discoid lupus erythematosus, and, furthermore, eczema, psoriasis, tinea corporis, chronic pellagra, and tuberculoid leprosy.

Endemic Syphilis

The causative organism *T. pallidum* subspecies *endemicum* is closely related or perhaps identical to the agent of venereal syphilis.[4,7] Many names have been given to this chronic infectious disease. Among Bedouin Arabs in Syria and Iraq, the disease is named *bejel*; elsewhere in the Middle East it is called *firjal* or *loath*, among other names.[8] Endemic syphilis still occurs in some isolated communities as a nonvenereal endemic disease. Contrary to general belief, endemic syphilis continues to be a significant problem, specifically in dry, hot climatic zones.[29]

Epidemiology of Endemic Syphilis

Distribution. Endemic syphilis still exists, especially among isolated, closed communities under primitive, crowded, and unhygienic conditions.[4,7,11,12,23,30–35] Infectivity is present particularly under dry, arid circumstances in the Eastern hemisphere, among nomads and seminomads in Saudi Arabia and in Sahel countries in Africa.[11,30–38] From the Sahelian regions in Africa a dramatic increase in cases of *bejel* has been reported recently, with 15% to 40% of children showing serologic evidence of treponemal infection and 2% to 20% with clinical infectious lesions of early endemic syphilis.[11] It has been documented that among Bedouin tribes in the Middle East endemic syphilis is prevalent in up to 27% of those born and bred in the desert.[38] *Bejel* still is highly prevalent in several rural areas of Saudi Arabia, in the Turaiba area, and among nomadic and seminomadic Bedouins.[32,36,38]

Age and sex. The main reservoir of endemic syphilis consists of children aged from 2 to 15 years,[4] within family groups, with no clear sex preponderance. The disease also can be found in older members in infected families. Adults who have escaped the infection in childhood are at risk of contracting the disease later, particularly from their own children.[4]

Transmission. The disease is transmitted nonvenereally. This familial treponemal disease is most probably transmitted directly or indirectly by skin-to-skin or mouth-to-mouth contacts with infectious lesions, and by contaminated fingers.[4,7] The role of flies and other insects in the transmission of the disease is unclear.[7] Improvement of hygienic circumstances and housing conditions is essential in arresting the spread of endemic syphilis.[8]

Clinical Picture of Endemic Syphilis

Endemic syphilis can be divided into an early and a late stage, the early stage comprising primary and secondary symptoms, and the late stage comprising tertiary disease and the late latent stage. In contrast to the other treponematoses, primary lesions frequently remain unobserved in endemic syphilis due to the small inocula involved.[7] Especially the oropharyngeal mucosa is involved in the primary phase.[4] The initial presentation of the disease frequently occurs in the secondary stage, which resembles secondary venereal syphilis. Patches on the mucous membranes, angular stomatitis (Fig. 5), nonitchy skin eruptions, and generalized lymphadenopathy are the most important early

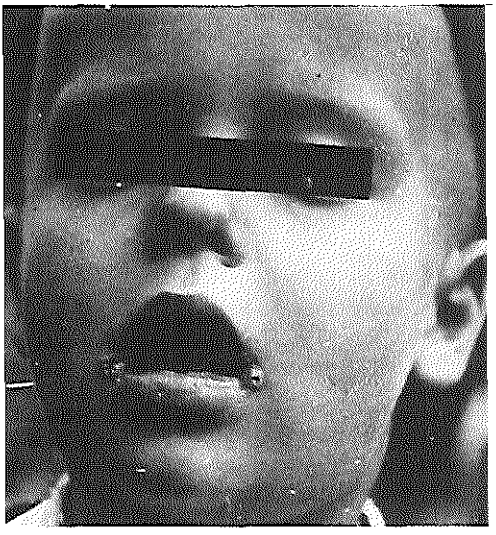

Figure 5. Angular stomatitis of early endemic syphilis. Reproduced, by permission of the Programme of Sexually Transmitted Diseases, Geneva, World Health Organization.

manifestations.[7] Condylomata lata frequently occur, comparable to those in yaws and sexually transmitted syphilis. Mothers may develop a nipple chancre from suckling a child with infectious lesions.[18] In the early stage a painful osteoperiostitis may occur, similar to the osteoperiostitis in yaws.[4] After early lesions have subsided, the patient enters latency. In this stage treponemal infection can be detected only by reactive serologic tests.

In some patients the late stage may develop even at a young age (Fig. 6).[31,37] In this stage, affection of skin, bones, and cartilage may lead to severe destruction, especially of the nose and palate.[4,7] The larynx may be solitarily affected.[38] Jones[39] in Iraq described a solitary case of "malignant" tertiary *bejel*, with serious gross tissue destruction. In cases of nasopharyngeal involvement, gummata may progress to destructive chronic ulcerations, producing lesions comparable to gangosa (rhinopharyngitis mutilans) (Fig. 7).

According to Pace and Csonka,[32] the predominant form of endemic syphilis is the late latent stage. Recently the classic clinical form of endemic syphilis has been replaced by an "attenuated" form or by atypical forms.[7,32,33]

Neurologic and cardiologic involvement is extremely rare or even absent in infection with *T. pallidum* subspecies *endemicum*.[4,8,37,38,40] Recently, ocular manifestations associated with endemic syphilis have been described by Tabbara et al.[41] In a study of 17 patients it was reported that uveitis was the most frequent ocular finding.

Congenital infection rarely, if ever, occurs.[4,8,38,40-42] It has been speculated that this is due to the considerable time span between the early age of contracting the disease and the actual age of becoming pregnant.[43]

Histopathology of Endemic Syphilis

The histopathologic picture closely resembles that of venereal syphilis.[7] In the early stage, the dermal infiltrate is located mainly perivascularly, while plasma cells and lymphocytes are most prominent. Slight vascular changes may be present. In early endemic syphilis, granulomas consisting of epithelioid cells and multinuclear giant cells may be present.[26] Silver staining methods or immunofluorescent staining methods may assist in visualization of the treponemes in the early stage. In the late stage this is extremely difficult.

Differential Diagnosis of Endemic Syphilis

Endemic syphilis must be differentiated from venereal syphilis, yaws, and pinta. The clinical picture, patient's history and country of origin are important factors in the diagnosis. Essential morphologic or serologic differences have not yet been recognized between the different treponematoses. No specific serologic test is available. Diagnosis can be difficult in regions where venereal syphilis and/or yaws are simultaneously prevalent.

Other dermatoses resembling early endemic syphilis include psoriasis, eczema, pityriasis rosea, lichen

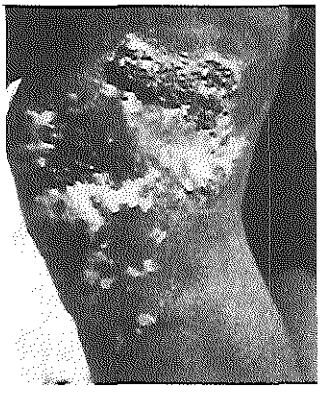

Figure 6. Chronic ulceration and depigmentation of late cutaneous endemic syphilis. Reproduced, by permission, from: Perine PL, et al. Handbook of Endemic Treponematoses: Yaws, Endemic Syphilis, and Pinta. Geneva: World Health Organization, 1984 (Fig. 59).

Figure 7. Late tertiary endemic syphilis—rhinopharyngitis mutilans. Reproduced, by permission, from: Perine PL, et al. Handbook of Endemic Treponematoses: Yaws, Endemic Syphilis, and Pinta. Geneva: World Health Organization, 1984 (Fig. 58).

planus, leprosy, mycoses, herpes simplex, condylomata acuminata, perlèche, and aphthae, and many diseases presenting with a generalized rash. Late-stage manifestations may imitate malignancies such as carcinoma, mycosis fungoides, leukemias, and Bowen disease, and, furthermore, lupus vulgaris, toxicodermias like bromoderma and iododerma, infiltrated types of rosacea and lupus erythematosus, and facial granuloma. Late-stage lesions like gangosa (destructive ulceration of the nose, nasopharynx, and hard palate) or osteoperiostitis often cannot be differentiated from the late manifestations of yaws. Apart from yaws, the differential diagnosis of nasopharyngeal lesions includes leishmaniasis, histoplasmosis, rhinoscleroma, and rhinosporidiosis.

Laboratory Tests for Pinta and Endemic Syphilis

The same nontreponemal and treponemal tests originally designed for the serodiagnosis of venereal syphilis are used for pinta, endemic syphilis, and yaws.[1,3,4,44] Recent progress in DNA technology with *Escherichia coli*-derived recombinant *T. pallidum* proteins have led to the development of tests that can be used for sexually transmitted syphilis as well as for the endemic treponematoses.[45–47] Shared antigens give rise to cross-reactive antibodies common to all treponemal diseases, thus so far precluding a differential diagnosis on the basis of serologic tests.

Demonstration of treponemes by dark-field examination of exudates from early-stage skin lesions can assist in diagnosing treponemal disease, but no differentiating morphologic criteria are available.

Diagnosis

A diagnosis of pinta or endemic syphilis must be based on clinical, geographic, epidemiologic, and laboratory findings. Positive serologic tests, and the presence of treponemes at dark-field examination or in skin biopsies confirm the diagnosis. So far it has not been possible to distinguish between the causative agents of the different treponemal diseases on serologic, morphologic, or biochemical grounds. Geographic data, the clinical picture, and the patient's history assist in typification of the disease.

Therapy

The drug of choice is a long-acting penicillin preparation. Treatment of pinta and endemic syphilis requires the same dosage as described for yaws, namely 600,000 units of benzathine penicillin for all patients and contacts (for those aged under 10 years), and 1,200,000 units (for those aged over 10 years) by a single intramuscular injection.[48,49] Family members, all other contacts of patients, and patients with latent infection should receive the same dosages as those suffering from active disease. No other drug is effective in a single dose against the endemic treponematoses. Tetracycline and erythromycin have been recommended as alternatives in cases of penicillin allergy.[49]

After successful treatment, titers in nontreponemal tests show a gradual decline and become negative after a period of time. Changes in titers determined by quantitative nontreponemal tests are the major parameters used to measure response to therapy. Treponemal tests probably remain reactive for life after treatment has been given. Thus far, no vaccines have been developed. Skin lesions of pinta heal apparently more slowly after treatment than skin lesions of the other treponematoses; however, the same treatment schedule as for yaws and endemic syphilis suffices.[48–50]

Discussion

Endemic syphilis and pinta still are prevalent in different parts of the world, especially among people living in unhygienic circumstances in remote, often inacces-

sible regions where no regular adequate medical care can be provided.[4–7,11,12] WHO- and UNICEF-assisted treatment campaigns in the 1950s led to a sharp decline in the number of infectious cases and even to the eradication of endemic syphilis in some regions, for example in Bosnia.[4,7,19] Alertness for the endemic treponematoses diminished after these campaigns, as many other often life-threatening diseases were given priority. A resurgence of endemic treponematoses has been documented.[11,12] Millions of people are again at risk for contracting these infections. Due to the current modes of transportation and the trends in migration, nonvenereal treponematoses easily can be transported to any place in the world, and so confront the local medical examiner with a diagnostic dilemma. A positive treponemal test result in a person originating from an endemic country must arouse the suspicion that the cause is one of the nonvenereally transmitted treponematoses. In these circumstances, an endemic treponematosis should obligatorily be considered in the differential diagnosis with venereally transmitted syphilis. In several countries, prejudice against venereal disease exists. When a nonvenereally transmitted treponematosis is diagnosed mistakenly as venereal syphilis, this may have catastrophic social consequences for the patient and his or her family.

Endemic syphilis nowadays may present a milder, attenuated form,[7,32,33] or an atypical form, comparable to observations in yaws.[51–55] Possibly, this phenomenon could have been induced by the widespread use of antibiotics, by improvement of social conditions, by a mutation or an adaptation of the causative organism, or by altered immune responses of the host.[55] A modified form of pinta has not yet been reported.

A most intriguing aspect of the endemic treponematoses originates from the observation that, in contrast to venereal syphilis, congenital infection and neurologic and cardiovascular involvement are assumed to be absent or extremely rare.[1,4–8,24,37,40,56] This remains an enigma to be solved.

It is hoped that further research will lead to the development of specific tests to differentiate morphologically or serologically between the treponematoses. Reliable serologic screening in field circumstances is of utmost importance. Serologic tests for treponematoses must answer to the following requirements: high sensitivity combined with high specificity, simplicity of performance (also in large series of tests), and they must be economically practicable, with low cost and simple, sturdy equipment for use in field conditions. Sample collection and transport should be simple and practicable. Standardized sera and control sera must be available readily and regularly. Control of test results should be performed regularly in a local or regional reference laboratory. Local manpower, staff, and laboratories (counterparts) must participate in the programs.

During relapsing infectious periods, persons with latent treponematosis will present periodically with infectious lesions. All contacts exposed are at risk for contracting the disease and so may contribute to resurgence of the infectious cycle.

Early detection and treatment campaigns are crucial in the control of the treponematoses. Because no precise data on pinta and endemic syphilis are currently available, continuing surveillance by clinical and serologic follow-up studies is of the utmost importance. As we know from past experiences, treatment campaigns, continuing health education, and improvement of social and medical conditions all will contribute to halt the spread, and, it is hoped, in the end will eradicate the nonvenereal treponematoses.[57–59] Eradication programs should be integrated into other existing health programs, for example vaccination programs, mother-and-child health clinics, and tuberculosis and leprosy programs. Together with existing primary health care services, these measures certainly will offer new possibilities of interfering with the spread of the treponematoses.[4,55,57,60]

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1.7.

ASPECTS OF *TREPONEMA PALLIDUM* RESEARCH

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Submitted for publication

1.7. ASPECTS OF *TREPONEMA PALLIDUM* RESEARCH

Introduction

After the studies of the classic treponematoses, important to mankind, which were reviewed in the previous chapters, many questions still remain to be answered. Hitherto, the pathogenesis of treponematoses is only partially understood. During recent years, intensive efforts in research have dealt with several aspects of treponemal infection and new information has been gained. Some important recent aspects and problems in treponemal research are described below.

Culturing pathogenic treponemes

Because treponemal research is greatly hampered by the fact that pathogenic treponemes cannot be cultured *in vitro* for prolonged periods, developing a culture system has been an objective of research. Some advances have recently been made; single-passage survival and limited propagation have been achieved in tissue culture (1-4). However, laboratory animals, of which the most usable are described below, are still indispensible for the propagation of these bacteria.

The animal model

The different treponemes pathogenic for mankind cannot be cultured continuously *in vitro*. An animal model remains necessary to obtain large numbers of bacteria for experiments. Unfortunately, only a narrow host range is available for experimental infection. Experiments with many different species of animals, for example monkeys or apes, mice, rats and guinea pigs, have been performed (5,6). It became clear that most species are unsuitable for laboratory experiments, because clinical manifestations cannot be regularly induced, microorganisms cannot be obtained in large numbers, or handling of the animals is too expensive or too laborious. So far, for propagation of *T.pallidum* the rabbit has been the most appropriate and most frequently used; in rabbits the best- accessible tissue lesions can be induced. Methods used to establish experimental syphilis in the rabbit include the intratesticular, intradermal, and intravenous route, depending upon the design of the

experiments (6). Intratesticular injection of *T.pallidum* usually causes an orchitis, with diffuse swelling and enlargement of the testicle. After intratesticular inoculation of *T.pertenue* a different reaction pattern has been observed: very small miliary nodules appear on the tunic of the testis, a socalled granular periorchitis (5, own observations). Intradermal injection gives rise to accessible lesions on the skin within a few days. Histopathological findings are similar to those in human infection. Intravenous injection of treponemes causes generalised lesions after a few weeks.

Experimental infection in the rabbit results in rapid dissemination to blood and regional lymph stations. The intradermal and intravenous routes are mainly used for immunological studies. To obtain many *T.pallidum* organisms, intratesticular inoculation is preferred, because large numbers of microorganisms can be obtained from the testicles after a few days (7,8). Numbers of microorganisms in rabbit testicles reach a peak 10-15 days after injection of 2-5 x 10^7 *T.pallidum* (9). The course of rabbit orchitis has been described in detail by several groups (5,8-16). However, even in the rabbit model the complex disease in the human is only partially imitated. For example no tertiary stage manifestations can be observed (6). Only recently was a rabbit model for congenital syphilis described (17).

As a consequence of the use of rabbits for the multiplication of treponemes, the treponeme suspensions are contaminated with host components and possibly with substances produced during the infection in the rabbit. To obtain pure intact bacteria without host contamination, several purification methods have been studied. Some of these methods include membrane filtration or use of gradient centrifugation on gradients of different inorganic or organic chemicals (18). It was observed that these techniques cause damage to the microorganisms. In 1984 it was demonstrated that density gradient centrifugation on gradients of polyvinylpyrrolidone-coated silica particles (Percoll) yielded suspensions of motile and virulent treponemes that were relatively free of host proteins (19,20). It was reported that the morphology, motility and virulence of treponemes remained unaltered (19). In this thesis experiments are described using Percoll purified *T.pallidum*.

Mechanisms in the pathogenesis of treponemal infection

To obtain a better insight into the precise mechanisms of treponeme-host interactions, special attention has been paid to attachment of treponemes to host cell surfaces, which is considered as an important initial step in pathogenicity (21-23), and *in vitro* parallels of

treponemal dissemination (23-25). Pathogenic treponemes possess the ability to bind to a wide range of cell types *in vitro*, perhaps via specific attachment ligands (8,26-28). Nonpathogenic *T.phagedenis* biotype Reiter lacks the capacity to attach, which suggests the presence of specific qualities of pathogenic treponemes. It is hypothesised that establishment of infection could possibly be prevented by interference with the mechanisms of adherence. In the infected host, rapid dissemination of treponemes to remote anatomical regions occurs. In experiments designed to study the dissemination of *T.pallidum* throughout the tissues, it was shown that *T.pallidum* is able to pass through a monolayer of endothelial cells *in vitro* by actively moving between cells with tight intercellular junctions (24). In more recent work Riviere *et al.* (25) showed the ability of *T.pallidum*, contrary to *T.phagedenis*, to pass through more complex tissue (murine abdominal wall) in *in vitro* experiments using double-sided culture chambers. It was concluded that *T.pallidum* requires an epithelial surface to traverse the tissue barrier. However, in the *in vivo* situation, the internal environment in the infected host is much more complicated. Elucidation of the precise mechanisms and consequences of spread of treponemes throughout tissues and organs of infected hosts remains difficult. So far, late stage disease manifestations cannot be reproduced in the animal model.

Evasion of the immune system

Treponemes are assumed to have a number of special properties, of importance in the complex pathogenesis of treponemal infection. Treponematoses are chronic diseases, and late mutilating manifestations are notorious. The host does not always succeed in complete eradication of the treponemes, despite the arousal of a seemingly vigorous response of the immune system. The mechanism(s) responsible for the survival of treponemes in a host for many years have not been fully elucidated. Several speculations exist which attempt to explain the manner in which treponemes could evade the host defences:

I: Antigenic variance

It has been hypothesised that *T.pallidum* may be able to induce changes in its morphology, for example, induce changes on the outer surface, changes in attachment characteristics or a change in the state of virulence. Baseman *et al.* described the presence of two different "populations" of *T.pallidum* (Nichols strain) after Hypaque centrifugation. He discussed the possible existence of interacting types of treponemes, necessary for pathogenesis (18).

Subgroups of treponemes may exist *in vivo* that are more resistant to host defence mechanisms than other subgroups. Recently, minor differences in polypeptide profiles were detected in studies of different strains of *T.pallidum*. In this work it was suggested that some limited genetic diversity amongst clinical isolates might exist (29-31). The significance of these findings remains to be established.

II: Immunoprotective niches

Rapid dissemination via blood or lymphatics to remote locations may result in foci of potentially harmful treponemes, which are inaccessible to the immune system. It was postulated that *T.pallidum* may temporarily escape attack from the immune system by localisation in protective niches (32).

III: Intracellularity

In tissue, treponemes are located almost exclusively extracellularly (33). However, some reports have described intracellularity in human tissue (34), and in rabbit tissue (35,36). It has been postulated that the intracellularity was associated with the phenomenon of latency and that intracellular survival of treponemes may provide an opportunity to evade immune surveillance.

IV: Affection of the immune system

The humoral immune response is supposed to be of major importance in treponemal disease. Even in the early stage of infection the immune system is already involved: polyclonal activation of B-lymphocytes occurs. Antitreponemicidal effects, however, are not complete. The role of the cellular immune response is still unclear. Possible affection of the immune response in syphilitic infection has been deduced from results of *in vitro* lymphocyte stimulation studies. However, the meaning of these results in actual infection remains unclear (8,37). The role of the humoral and cellular immune response in treponemal infection has been thoroughly reviewed elsewhere (8,37-39).

V: Coverage by a protective layer

As early as in 1963, Christiansen hypothesised coverage of the treponeme with a layer offering protection against the host defences (40). The presence of mucopolysaccharides on treponemes *in vitro* was shown by Fitzgerald *et al.* (41) and in infected rabbit testicles by Zeigler *et al.* (42). Alderete and Baseman reported that several serum proteins are in close association with the outer membrane of *T.pallidum* isolated from infected rabbit testes (43). However, in electron-microscopic studies the outer membrane presented itself as a

symmetrical structure that shows no signs of extra-membrane components. Moreover, Hovind-Hougen argued that the mere presence of substances on the outer surface of *T.pallidum* does not prove that they play a part in providing protection to the organisms against the host defences (44).

VI: Structure of the outer membrane

A recent hypothesis focused on the role of the structure of the outer membrane of *T.pallidum* in the evasion of the treponemes from the immune response. By freeze fracture and deep etching techniques it was recently shown that the outer membrane of *T.pallidum* contains only a small number of integral membrane proteins that can serve as targets for specific antibodies. These studies did not provide evidence that the surface of *T.pallidum* is covered by an outer coat (45,46).

VII: Autoimmune phenomena

Autoimmune reactions may be responsible for the induction of late stage disease (47). The presence of microorganisms is possibly no prerequisite for late sequelae of treponemal infection to occur (8,48,49).

Application of molecular biology techniques

Recombinant technology (39,50-52) has made it possible to obtain antigens of *T.pallidum* on a large scale. The adoption of modern methods such as use of monoclonal antibodies, gene cloning technology and immunoblot analysis may be useful, for example, in studies to improve laboratory tests for the diagnosis and the treatment of treponemal infections, and in search of a reliable vaccine (39,50,51,53). Cloning of *T.pallidum* DNA and expression of *T.pallidum* proteins in *E.coli* has become possible in several laboratories (39). Until now, over twenty different *T.pallidum* proteins have been expressed in *Escherichia coli* (54,55). The *E.coli*-derived recombinant *T.pallidum* proteins have been used for the development of new serological tests for the serodiagnosis of syphilis (as well as for the endemic treponematoses) (51,56,57). Some of these modern tests, for example ELISAs with TmpA and the 4D antigen, may have approximately the same sensitivity and specificity as the TPHA test (51). Unfortunately, until now the precise function of, or location in the intact microorganisms of many antigenic polypeptides has remained unclear (53). Furthermore, with the use of molecular biology techniques, some progress has been made in the study of the differentiation between *T.pallidum* and *T.pertenue*. A minimal difference (one nucleotide)

was demonstrated between genes coding for homologous proteins of *T.pallidum* and *T.pertenue* by Noordhoek *et al.* (52,58). Initial results of the use of the polymerase chain reaction (PCR) and tpf1- en tyf1-specific DNA probes have recently been published (52). A subspecies-specific test would be of major importance for the diagnosis in an individual patient. Also, for epidemiological surveillance a differentiating test would be useful in outlining future treatment strategies.

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CHAPTER 2. PURPOSES OF STUDY

Nowadays the treponematoses are still prevalent worldwide. Although seldom if ever fatal, these infections cause an important public health, social and economic problem in affected populations. In the AIDS era, venereal syphilis poses serious problems in surveillance, diagnosis and treatment throughout the world. Unfortunately, after intensive efforts to eradicate the endemic treponematoses several decades ago, a resurgence of these diseases, especially of yaws, is observed in various large tropical regions. In this thesis, the current worldwide status of these infections is described. The major results of a small survey on yaws, held in 1988 in West Sumatra are presented, with special emphasis on clinical, serological, histopathological and ultrastructural features of yaws. The results of histopathological and ultrastructural studies of *T.pertenue* are compared with findings on *T.pallidum*.

In the laboratory part of this thesis emphasis was put on the study of the question how treponemes may escape host defence mechanisms. So far, the ability of treponemes to escape the host defences is not fully understood. In Chapter 1.7. several speculations were enumerated. It has been suggested that incomplete eradication of the microorganisms is caused for example by either presence of a protective cover or scarcity of epitopes on the treponemal surface. However, it remains intriguing how treponemes manage to survive among a host of anti-treponemal antibodies. In our experiments we studied the immobilisation of rabbit-derived *T.pallidum* (Nichols), which had been purified by Percoll gradient centrifugation, in an attempt to gain insight into the role of host factors in the immobilisation process.

CHAPTER 3. CLININAL ASPECTS OF SYPHILIS AND YAWS

3.1. RECENT EPIDEMIOLOGICAL ASPECTS OF SYPHILIS

During several centuries sexually transmitted syphilis has been present as a pandemic. The pandemic character has been greatly restricted, due to the widespread use of penicillins after World War II, the introduction of large serological screening programmes, health education and sex-partner notification. However, syphilis is far from eradicated (1-5) and is still an important health problem. In the United States and Europe the prevalence of syphilis is rising again, especially in some core groups. In the second half of the 1980s the disease gained renewed world-wide interest, due to the epidemic of another sexually transmitted disease (STD), the acquired immunodeficiency syndrome (AIDS). In chapter 1.3, the interaction between infection with *T.pallidum* and infection with the immunodeficiency virus was discussed. There is now evidence that syphilis, like several other STDs, and especially the ulcerative STDs, may facilitate the spread of HIV infection (6-9). The current situation in developing countries, the United States and the Netherlands is briefly discussed below.

Developing countries

Several recent studies have shown that in many developing countries, among the huge burden of STDs, syphilis still is highly prevalent (10-15). Prostitutes are a major reservoir of syphilis in many African countries, for example in Kenya, Burkina Faso, and Somalia (16-18). As a consequence, congenital syphilis is not a rarity in these countries (19,20).

United States of America

In the early 1980s the overall incidence of syphilis declined slightly in the United States, almost certainly due to changes in sexual behaviour because of the AIDS epidemic (21). This decline continued until 1986. In 1987, however, syphilis reemerged nationally in epidemic proportions, with the highest incidence since 1950. Compared with 1986, for 1987, 30% more cases of primary and secondary syphilis were reported to the Centers for Disease Control (2,4). Highest incidence rates were found in New York, Florida and California. The ratio of male-to-female cases changed considerably during the 1980s, with a strong increase in cases among females, representing a shift from homosexual to heterosexual transmission (22). Furthermore, a shift from white homosexual males to black heterosexual males and females became clear (23). The practice of exchanging drugs for sex, and drug usage (especially of crack/cocaine, which became available in major US cities in 1986) have contributed to the spread of syphilis, and have hampered the control of the spread of infection (24-25). As a consequence, an increased incidence of cases of congenital syphilis has been reported (2,26,27). In figures 1 and 2, the numbers of cases of early syphilis in the United States and New York City during the 1980s are shown.

Source of figure 1: Centers for Disease Control, Atlanta, U.S.A. (see reference 4).
Source of figure 2: New York State Department of Health, Center for Community Health, Albany, NY, U.S.A. (see reference 23).

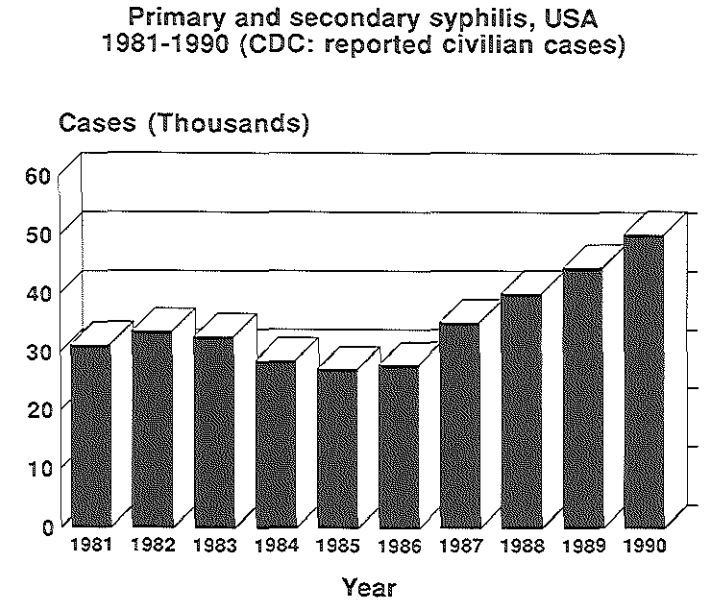

Figure 1

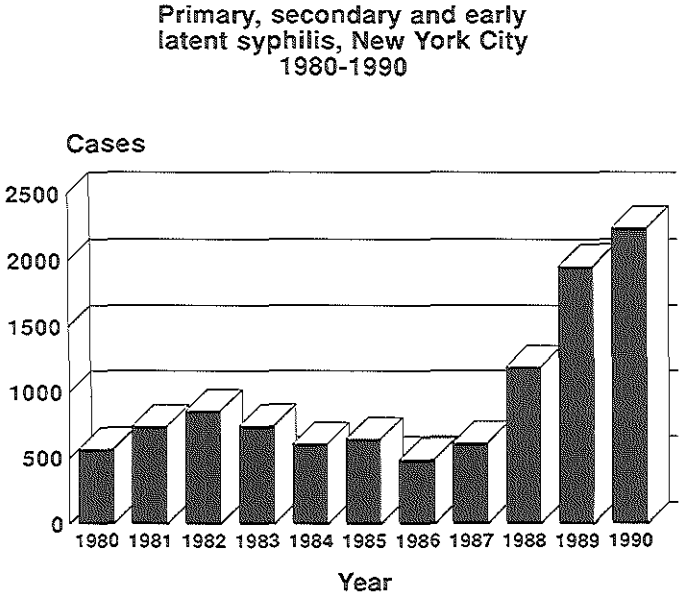

Figure 2

The Netherlands

In the early 1980s a decrease in the incidence of early syphilis in the Netherlands was reported (28). Recently however, an increase of cases, especially among heterosexuals, has been reported from Amsterdam, resembling the increase among heterosexuals in the United States. Van den Hoek et al. have recently reported that the proportion of syphilis patients who reported using hard drugs, especially prostitutes, increased substantially from 1985 to 1988 (29). In figure 3, numbers of cases of primary and secondary syphilis in the Netherlands in the 1980s are shown. These figures are based on the "Tables for Infectious Diseases", published by the Medical Chief Inspector of Public Health.

Numbers of patients suffering from primary or secondary syphilis between 1980 and 1991, who attended the University Hospital Rotterdam-Dijkzigt are shown in figures 4 and 5 [source: Central Coding System, University Hospital Rotterdam-Dijkzigt, the Netherlands, with special thanks to Mr.Vink]. These numbers should be interpreted with due reserve, however, because changes have occurred in the encoding rules, the pattern of coding and the personnel involved, during these years.

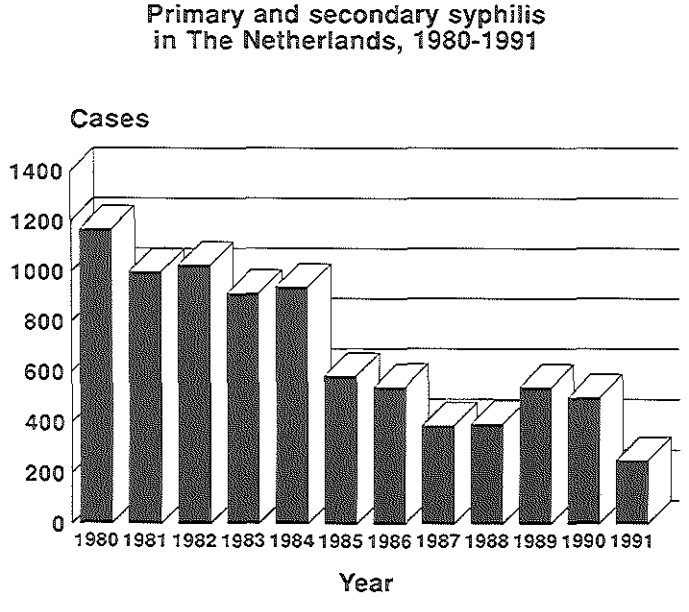

Figure 3

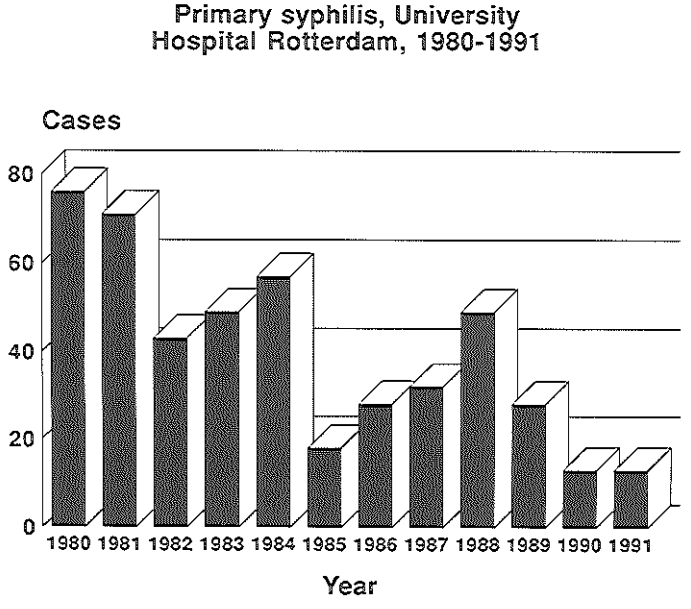

Figure 4

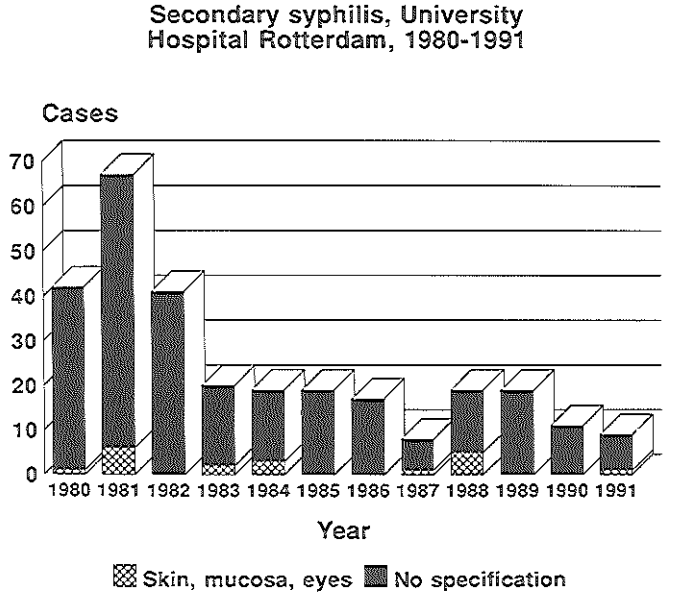

Figure 5

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3.2.

YAWS IN WEST SUMATRA, INDONESIA: CLINICAL MANIFESTATIONS, SEROLOGICAL FINDINGS, AND CHARACTERISATION OF NEW TREPONEMA ISOLATES BY DNA PROBES

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Yaws in West Sumatra, Indonesia: Clinical Manifestations, Serological Findings and Characterisation of New Treponema Isolates by DNA Probes

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The results of a yaws survey on the island of Sumatra in Indonesia are presented. The prevalence of yaws in the investigated region was found to be very high, a minimum of 300 cases per 100,000 individuals, which indicates that yaws is far from being eradicated and that campaigns for treatment are necessary. Patients suffering from early infectious yaws showed florid skin lesions. Of 101 serum samples from such patients, 100 had a positive reaction in one or more treponemal tests. The *Treponema pallidum* haemagglutination assay was found to be the most sensitive test (97 % positive) in detecting antibodies against *Treponema pallidum* subsp. *pertenue*, followed by the fluorescent treponemal antibody absorption test (94 %), the Venereal Disease Research Laboratory test and the TmpA enzyme immunoassay (91 %), and analysis by Western blot using *Treponema pallidum* antigens (88 %). Of 42 asymptomatic contacts of yaws patients 32 showed positive reactions in one or more tests, indicating that many people in the investigated region have been infected with treponemes. Eight new *Treponema pallidum* subsp. *pertenue* strains were isolated from yaws skin lesions. In vitro amplification of treponemal DNA and hybridisation with specific DNA probes showed that all eight strains were identical with *Treponema pallidum* subsp. *pertenue* CDC 2575, with regard to the subsp. *pertenue* specific *tyfl* gene.

The recently observed resurgence of yaws in the tropics has revived interest in this non-venereal treponematosis which is caused by *Treponema pallidum* subsp. *pertenue* (1). The manifestations of the disease have been described (2, 3) but only a few recent reports have been published on the clinical aspects in combination with a detailed serological analysis (4, 5).

Due to the great similarity of this microorganism to *Treponema pallidum* subsp. *pallidum*, the causative agent of syphilis, it has not been possible to distinguish venereal and non-venereal treponematoses by classical serological tests (2). Also, purified recombinant DNA derived *Treponema pallidum* subsp. *pallidum* antigens, such as TmpA and the 4D antigen, are not useful for this purpose (6–8). However, classical serological tests as well as tests based on recombinant DNA derived antigens are excellent tools for diagnosing venereal as well as non-venereal treponematoses.

Up to now only one molecular difference between *Treponema pallidum* subsp. *pallidum* (the Nichols strain) and *Treponema pallidum* subsp. *pertenue* (strain CDC 2575) has been described (9). In these strains the homologous genes *tpfl* and *tyfl* were found to differ in a single base pair. With oligonucleotide probes specific for either *tpfl* or *tyfl*, it has been possible to distinguish *Treponema pallidum* subsp. *pallidum* from *Treponema pallidum* subsp. *pertenue* strains after in vitro amplification of these genes by the polymerase chain reaction (PCR) (10).

This study was undertaken to investigate the current extent and the clinical manifestations of present day yaws in Indonesia in combination with a detailed serological investigation, the isolation of new *Treponema pallidum* subsp. *pertenue* strains and their analyses by PCR and *tpfl/tyfl* specific DNA probes.

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Materials and Methods

Subjects. In November 1988 a small yaws survey in West Sumatra was undertaken by a Dutch-Indonesian team. Six different rural locations, near the coast and inland, in the district of Pariaman were chosen because yaws was known to be endemic in this region and patients generally were not treated with antimicrobial drugs. With the cooperation of the local authorities 214 persons were examined clinically. After photography, blood samples were collected from 190 persons by venipuncture and dark field microscopy examination was performed on exudate of skin lesions. Biopsies were taken from yaws lesions and immediately frozen on dry ice or prepared for histological examination (11). According to clinical findings and personal information the subjects were divided into five groups: 1) yaws patients with clinical signs of the disease (n = 101); 2) persons who were suspected of having yaws, but showing other dermatological aberrations (n = 16); 3) family members of the yaws patients with no clinical signs and no known history of yaws, contacts A (n = 28); 4) family members who remembered having had yaws previously, from 2 months to 40 years ago, contacts B (n = 14); 5) healthy employees of health centres with no history of yaws, controls (n = 27). The investigations took place with consent of the persons or parents of children involved. Standard therapy was given to all patients and contacts, according to WHO guidelines (2).

Serological Investigations. After clotting, the blood samples were centrifuged, and the sera were frozen and transported in dry ice to The Netherlands where serological tests were carried out. The *Treponema pallidum* haemagglutination assay (TPHA) was performed using a serum dilution of 1/80; haemagglutination was scored from – to 2+ (TPHA-Test, Medac, FRG). The fluorescent treponemal antibody absorption test (FTA-Abs) was performed semi-quantitatively with a serum dilution of 1/5 and the results were scored from – to 4+ (12). The Venereal Disease Research Laboratory test (VDRL) was performed quantitatively using two-fold serum dilutions (12). The reciprocal value of the highest dilution showing a positive reaction was recorded. An enzyme immunoassay (EIA) with the recombinant TmpA antigen derived from *Treponema pallidum* subsp. *pallidum* was performed as described (7) using a serum dilution of 1/100. The EIA was calibrated with serum samples from healthy blood donors and syphilis patients. A pool of syphilitic serum samples with strong positive results in all classical syphilis tests was arbitrarily taken to contain 100 units (U)/ml. Serum samples containing more than 6 U/ml were considered positive (+). The OD corresponding with 6 U/ml was 2.5 times the average background OD value of the donor sera (data not shown). Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were done as described previously (9). *Treponema pallidum* subsp. *pallidum* strain Nichols was cultivated by serial passage in rabbit testes (13). Treponemes were purified by urografin gradient centrifugation. *Treponema pallidum* subsp. *pal*- samples were applied to 13 % acrylamide gels and after electrophoresis and blotting, the nitrocellulose membranes were incubated with 1/200 dilution of serum samples. The results were scored as done by Fohn et al. (14), who used pinta sera: 0, antibody reaction equal to normal serum; 1, antibody reaction only with a few bands; 2, antibody reaction with the complete *Treponema pallidum* subsp. *pallidum* antigenic pattern; 3, very heavy reaction, smear.

Culture of Treponema pallidum subsp. pertenue Pariaman. For transport of live treponemes overseas, we used the method described by Liska et al. (15). Syrian Golden hamsters, ten male and ten female, were obtained from Biopharma, Bandung, Indonesia and kept air-conditioned whenever possible. Skin biopsies, taken from yaws lesions, were immediately homogenised in phosphate buffered saline (PBS) and examined by dark field microscopy. Homogenates containing treponemes were intradermally inoculated in the shaved inguinal areas of the hamsters. The hamsters were transported to The Netherlands. The inguinal lymph nodes of hamsters that developed skin lesions were homogenised in PBS and injected into rabbit testicles. The treponemes were further cultured by serial passages in rabbits as described for *Treponema pallidum* subsp. *pallidum* (13).

Polymerase Chain Reaction. Treponema pallidum subsp. *pertenue* chromosomal DNA was purified from crude rabbit testicle extracts. The polymerase chain reaction (PCR) was performed with about 100 ng DNA as described (10). Primers to amplify a 299 bp fragment of *tpfl/tyfl* gene from *Treponema pallidum* subsp. *pallidum* and *Treponema pallidum* subsp. *pertenue* have been described (10). After amplification the PCR product was analysed by electrophoresis in ethidium bromide-containing agarose gels and by Southern blotting. Hybridisation was performed with ^{32}P-labelled *tpfl* and *tyfl* specific synthetic DNA probes. These probes enable distinction of *Treponema pallidum* subsp. *pallidum* strain Nichols from *Treponema pallidum* subsp. *pertenue* strain CDC 2575 (10).

Results

Prevalence of Yaws and Clinical Symptoms. During this survey 114 individuals with a diagnosis of yaws based on the clinical manifestations were found among a population of about 37,000 people. From these data we estimate a prevalence of yaws in the region of Pariaman at 0.3 %. Ninety-six percent of the yaws patients were under the age of 15, and 40 % were between the ages of six and ten.

The most frequently encountered clinical manifestation observed in the yaws patients was crustopapillomatous skin lesions (Figure 1). The size of the lesions ranged mainly from lenticular to nummular. The most commonly affected parts of the body were the lower legs. Usually one or only a few lesions were found, however occasionally patients with widespread skin lesions were encountered (Figure 2). In several patients small satellite lesions were found around the primary skin lesion (Figure 3). Oc-

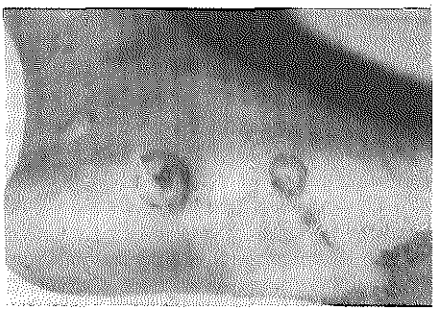


Figure 1: Yaws lesions. Three crustopapillomatous lesions on the left foot of an 11-year-old girl.

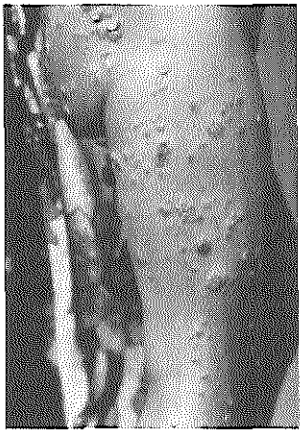


Figure 2: Yaws lesions. Disseminated crustopapillomatous skin lesions on a 5-year-old girl.

Figure 3: Yaws lesions. Papulosquamous plaque with three nummular satellite lesions on the knee of a 1 1/2-year-old boy.

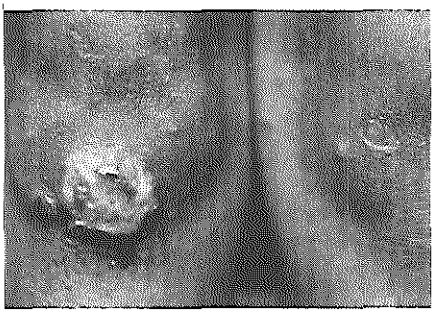


Figure 4: Yaws lesions. Nummular ulceropapillomatous and lenticular crustopapillomatous lesion on the right knee of an 8-year-old boy; the left knee shows a scar.

casionally we observed skin scars and pigmentary aberrations, presumably the remnants of healed yaws lesions (Figure 4). A penile lesion was found in one boy. In two patients with typical yaws lesions we found swollen finger joints and bone abnormalities were subsequently shown on X-ray photographs. A detailed description of the latter three cases has been published elsewhere (16, 17).

Dark Field Microscopy and Serological Investigations. Exudates from skin lesions of 54 patients were examined by dark field microscopy. In 34 cases spirochaetes were seen. The presence of erythrocytes and/or numerous other bacteria in the exudates hindered microscopic detection of the treponemes. This might have led to an underestimation of the number of positive exudates. For serological investigation of patients with positive clinical manifestations of yaws, serum samples were obtained from 101 individuals and analysed by various methods. The results are presented in Table 1. TPHA was found to be the most sensitive test for detecting antibodies (97 % positive), followed by FTA-Abs (94 %), VDRL and TmpA EIA (91 %), and Western blot (88 %). In two exudates from six patients, whose sera were either reactive solely in the TPHA or in the FTA-Abs, treponemes were found by dark field microscopy. This indicates that these patients must have acquired the *Treponema pallidum* subsp. *pertenue* infection very shortly before blood sampling. Only one serum sample was nonreactive in all tests. This sample originated from a 2 1/2-year-old girl who had solitary skin lesions on the back and face. However, no dark field examination was performed in this patient. This patient could have had very early yaws, or a faulty clinical diagnosis due to similarities between yaws lesions and impetiginous skin lesions.

The band patterns on Western blots incubated with yaws sera (Figure 5, lanes 6 and 7) were indistinguishable from the patterns obtained with syphilis sera (Figure 5, lane 8). Of the yaws sera 88 % showed reactivity with score 2 or 3 on the Western blot.

We also investigated 16 individuals suspected by the local health workers of having yaws. Careful clinical examination of these 16 patients subsequently excluded a clinical diagnosis of yaws, but other types of skin lesions were present, e.g. impetiginous or ecthymiform skin lesions, leprosy, verrucae, mollusca contagiosa or some other skin abnormalities. Although ten of these 16 patients with non-yaws skin manifestations were found to be reactive in the TPHA and/or the FTA-Abs test, only four individuals were positive by VDRL or TmpA EIA. This suggests that only four persons in this group could have suffered from an active *Treponema pallidum* subsp. *pertenue* infection, concurrent with other infections (Table 1).

To determine whether family members who had no clinical symptoms of the disease could have been infected in the distant or recent past, serological investigations were performed on samples from members of the households of the yaws patients. Only nine of the household members with no known history of yaws from contacts were non-reactive in all of the serological tests. Others were reactive in one or more tests (Table 1). The majority (84 %) of these positively reacting sera were from individuals older than 15 years. Therefore, it is likely that these persons had a history of yaws which had not been noticed previously. From the contacts with a known history of yaws nearly all individuals were reactive in the TPHA and/or FTA-Abs and about half of these were also reactive in the VDRL and TmpA EIA (Table 1). This indicates that the latter individuals had recently acquired an infection with *Treponema pallidum* subsp. *pertenue,* or had persistent antitreponemal antibodies after treatment. The VDRL and/or TmpA non-reactors probably were individuals who had been infected in the past, who had responded to treatment many years ago and who were probably cured. The Western blot data confirm this supposition, as antibodies against only a few treponemal polypeptides were found in sera from these apparently healthy individuals (Figure 5, lanes 4 and 5).

Finally, a control group of healthy employees was investigated. None of the sera of these controls were reactive in VDRL or TmpA EIA. However, 17 control sera were found to contain anti-treponemal antibodies, as assayed by TPHA and/or FTA-Abs. This suggests a previous infection with *Treponema pallidum* subsp. *pertenue* which had not been remembered or noticed. From these data we could not distinguish latent yaws from previously healed or asymptomatic infections.

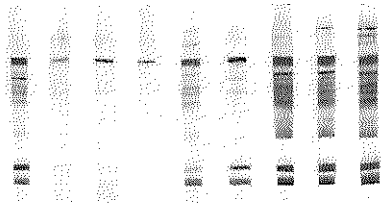
Table 1: Results of serological tests in sera from yaws patients, contacts and controls.

	Reactivity pattern										
TPHA[a]	+	+	+	+	+	+	–	–	+	+	–
FTA-Abs[b]	+	+	+	+	+	–	+	–	0[f]	0	0
VDRL[c]	+	+	+	–	–	–	–	–	0	0	0
TmpA EIA[d]	+	+	–	–	–	–	–	–	+	–	–
Western blot[e]	+	–	–	+	–	–	–	–	+	–	–
Yaws patients[g] (n = 88)	77	3	0	0	1	4	2	1	0	0	0
Yaws patients (n = 13)	0	0	0	0	0	0	0	0	12	1	0
Other skin diseases (n = 12)	1	0	0	2	2	2	0	5	0	0	0
Other skin diseases (n = 4)	0	0	0	0	0	0	0	0	3	0	1
Contacts A[h] (n = 28)	0	1	1	1	8	6	2	9	0	0	0
Contacts B[i] (n = 14)	6	0	0	0	6	1	0	1	0	0	0
Controls (n = 27)	0	0	0	1	5	9	2	10	0	0	0

[a] TPHA = *T. pallidum* haemagglutination assay; –, if the result was – or +/–; +, if result was +, 2+, 3+ or 4+.
[b] FTA-Abs = fluorescent treponemal antibody absorption test; –, if the result was – or +/–; +, if result was +, 2+, 3+ or 4+.
[c] VDRL = Venereal Disease Research Laboratory test; –, if titer was < 2.
[d] TmpA EIA = enzyme immunoassay with TmpA antigen; +, if EIA result was > 6 Units.
[e] Western blot with *T. pallidum* subsp. *pallidum* as antigen; +, if serum was reacting with all antigenic bands (score 2 or 3).
[f] 0 = not done, because sufficient serum was not available.
[g] Yaws patients, diagnosis by clinical symptoms only.
[h] Contacts A: family members with no history of yaws.
[i] Contacts B: family members with a known history of yaws.
n = total number of sera tested.

Culture and Preliminary Characterisation of Clinical Isolates of Treponema pallidum subsp. pertenue. We tried to culture *Treponema pallidum* subsp. *pertenue* from 19 individuals who had dark field-positive skin lesions. This was done by inoculation of 20 hamsters with homogenised skin biopsies from these patients. Fourteen hamsters developed skin lesions in the inguinal area four to eight weeks after inoculation. The duration of the lesion development varied when male hamsters and female hamsters were compared. Eight male hamsters developed lesions after 25 to 31 days, with one exception of 57 days, and six female hamsters needed 46 to 58 days before lesions developed. The other hamsters died before inguinal lesions were noted. The inguinal lymph nodes of the hamsters with lesions were considerably enlarged and all of them contained treponemes when examined by dark field microscopy. To further propagate the treponemes, eight rabbits were inoculated with homogenates from the hamster lymph nodes. All rabbits showed seroconversion after periods varying from three to eleven weeks. A very mild orchitis developed slowly in only three rabbits, and the testicular extracts of seven rabbits contained a small number of treponemes. Passage of testicular extracts to fresh rabbits resulted in seroconversion and mild orchitis in all rabbits. During further passages a gradual increase in the numbers of treponemes occurred, but the number of treponemes harvested never exceeded 10^8 treponemes per rabbit. The eight strains regularly produced granular periorchitis, which is considered to be typical for *Treponema pallidum* subsp. *pertenue.*

To investigate whether the strains isolated in this study contained the *tyf1*-specific DNA sequence, we amplified in vitro by PCR a 299 bp DNA fragment containing the putative *tyf1* gene. All eight trepo-



lane no.:	1	2	3	4	5	6	7	8	9
Diagnosis:	L2	NHS	CI	CI	CtA	CtA	Y	Y	Y
TPHA :	2+	0	0	0	2+	2+	2+	2+	2+
FTA-Abs :	3+	0	0	0	2+	1+	3+	3+	4+
VDRL :	128	0	0	0	0	0	16	64	64
TmpA :	nd	2	1	1	7	3	94	46	125
Age:	nd	nd	22	20	48	35	11	8	11

Figure 5: Representative immunoblots and results of serological tests. Western blots with lysates of *Treponema pallidum* subsp. *pallidum* were incubated with sera from different test groups. Lane 1, secondary syphilis (L2); lane 2, normal human serum (NHS); lane 3 and 4, Indonesian controls (CI); lanes 5 and 6, sera from contact group A with a history of yaws (CtA); lanes 7, 8 and 9, yaws patients (Y). The results of the treponemal and non-treponemal tests and the age of the individuals are shown below the lanes. nd, not done or unknown. Numbers to the left indicate molecular sizes (in kDa) of various *Treponema pallidum* subsp. *pallidum* antigens.

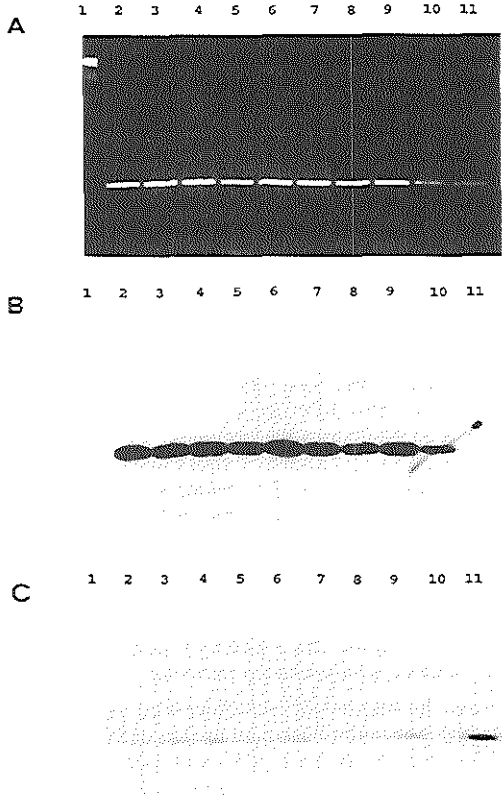


Figure 6: Amplification of the 299 bp fragment of the *tpf1/tyf1* gene and hybridisation with *tpf1*- and *tyf1*-specific DNA probes of different *Treponema pallidum* subsp. *pertenue* patient isolates. Panel A, ethidium bromide-stained agarose gel; panels B and C, blots hybridised with *tyf1*- and *tpf1*-specific probes, respectively. Lane 1, *Hin*dIII-digested lambda DNA; lane 2 to 9, DNA from 8 different patient isolates; lane 10, *tyf1* control *Treponema pallidum* subsp. *pertenue* strain CDC 2575; lane 11, *tpf1* control *Treponema pallidum* subsp. *pallidum* Nichols strain.

nemal isolates from the Pariaman region were found to contain an amplifiable fragment that hybridised with the *tyf1*-specific synthetic DNA probe and did not hybridise with the *tpf1* specific oligomer (Figure 6).

Discussion

About 90 % of the individuals diagnosed clinically by the local health authorities as having yaws were serologically positive in the VDRL and TmpA EIA, indicating that these persons had recently acquired a treponemal infection. According to the Centres for Disease Control in Padang (Sumatra), in recent years venereal syphilis has not been encountered in the rural regions where our investigations took place.

The clinical manifestations in the patients were typical of those described previously for yaws. We found no direct indications in the present study that the signs and symptoms observed differed in any way from the classical description of the disease (2, 3). Although some investigators have suggested that yaws is becoming a disease with milder symptoms and fewer lesions than the classical description (18), we saw several patients with florid disseminated skin manifestations, even after prolonged dry periods in this region. We observed various types of skin lesions, greatly differing in severity, of which the crustopapillomatous lesions were the most prevalent. No late manifestations of yaws were found in this survey. Most of the lesions seemed to have started on the extremities, especially the lower legs. The children in the Pariaman district usually walk either barefoot or with simple slippers, and wear shorts or skirts. Therefore they usually have many scratches and other skin injuries, particularly on the lower legs, which are a potential port of entry for *Treponema pallidum* subsp. *pertenue*.

From the number of well-diagnosed yaws cases a prevalence in the Pariaman district of Sumatra was calculated of at least 300 cases per 100,000 inhabitants. This figure is undoubtedly an underestimation of the real prevalence. During the short period of the survey we examined only clinically clearly manifest cases of yaws. The proposed underestimation is consistent with the high rate of seropositive apparently healthy individuals found in the same region (Table 1). Other studies indicate that in endemic areas the number of seropositive individuals is three to four times greater than the number of yaws patients with clinical manifestations (18).

In 1954 it was estimated that there were 10 million cases of yaws among a total population in Indonesia of approximately 75 million (19). The WHO supported yaws control programmes, and a recent survey in Indonesia suggested a very low incidence of yaws on Java, South Kalimantan and Sulawesi. However, as a consequence of the lack of finance in the last decade, only limited campaigns were undertaken to eradicate yaws in other districts. As a result yaws is still prevalent in eastern and western parts of Kalimantan, the whole island of Sumatra, the Moluccas, and Irian Jaya. The Treponematosis Control Programme in Indonesia reported for West Sumatra a prevalence of 100 cases per 100,000 in 1982 (19, 20). In the years 1987 and 1988 a prevalence of 20 to 50 cases per 100,000 was reported by the Centres for Disease Control, Jakarta. The data obtained in our study suggest a much higher prevalence of yaws. It is not known whether this is due to underestimation of the prevalence by the health authorities or to large local differences in prevalence.

Virtually all patients with clinical manifestations had antibodies to *Treponema pallidum* subsp. *pallidum* as measured by the TPHA or FTA-Abs tests. In contrast to the latter tests, which remain positive for years after successful drug treatment, the VDRL and TmpA EIA (at least in the case of syphilis) become negative after successful treatment (7). The high percentage (91 %) of VDRL and TmpA EIA positive patients is consistent with an active infection, as established by clinical diagnosis and/or dark field microscopy.

Certain spirochaetal infections like borrelioses, leptospiroses and infections with cultivable treponemes such as *Treponema denticola* and *Treponema hyodysenteria* sometimes lead to false positive results in serological *Treponema pallidum* tests, probably due to the presence of cross-reactive antigens such as endoflagella and heat shock proteins (21–24). The Western blot patterns obtained in this study with sera from yaws patients were invariably characteristic for an infection with *Treponema pallidum* subsp. *pallidum* or *Treponema pallidum* subsp. *pertenue*, indicating that none of the positive reactions observed in the serological tests were false positive.

After amplification by PCR of a fragment of the *tyf1* gene and hybridisation with specific probes, the Indonesian strains were found to be the same as *Treponema pallidum* subsp. *pertenue* strain CDC 2575 (9) which means they have a G as residue 123 in the *tyf1* gene. Thus, treponemes isolated from eight different patients living in six different areas in the region of Pariaman were all shown to be of the same *Treponema pallidum* subsp. *pertenue* type with regard to the *tpf1/tyf1* gene. Most *Treponema pallidum* subsp. *pallidum* isolates tested thus far are of the Nichols type with an A as residue 123 in the *tpf1* gene, and most *Treponema pallidum* subsp. *pertenue* isolates are of the CDC 2575 type (10). This suggests

that *Treponema pallidum* subsp. *pallidum* and *Treponema pallidum* subsp. *pertenue* strains tend to differ in this gene and that this method could be used for distinguishing between yaws and syphilis strains.

DNA amplification by PCR is a sensitive method for the detection of microorganisms and studies are in progress to detect by PCR treponemes in frozen skin biopsies collected from the yaws patients in this study. The percentages of TPHA and/or FTA-Abs positive household contacts and health workers, both with no previous history of yaws, were 68 % and 50 %, respectively. These individuals were mainly negative in the VDRL or TmpA test, indicating that they acquired yaws years before our investigation and were cured, or alternatively that they might have a latent form of yaws. Further investigation would be necessary to establish whether secondary yaws develops in these individuals.

The high rates of seropositivity among healthy persons reinforce the idea that yaws is a common disease in this remote region of Indonesia. A general conclusion from this survey, as from recent investigations conducted in West and Central Africa, Papua New Guinea and the western Pacific (25–27), is that yaws is far from being eliminated and that, despite anti-yaws campaigns in the 1950s and 1960s, the disease is on the increase again.

Acknowledgements

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3.3.

EARLY YAWS, IMPORTED IN THE NETHERLANDS

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Early yaws, imported in The Netherlands

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SUMMARY Early yaws in a 9 year old girl from Ghana, diagnosed as imported disease in The Netherlands is reported. She had lived in The Netherlands for six months. Tropical non-venereal treponematoses are rarely seen in Europe, and only a few case reports have been published. Migration and travelling may confront the medical profession with cases of tropical diseases such as yaws. Positive serological reactions in non-venereal tropical or venereal treponematoses cannot be distinguished at present.

Tropical non venereal treponematoses are rarely seen in Europe. Only a few cases of early yaws have been recently reported in Europe.[1–6] Owing to migration and travelling it is expected that more cases of this disease will be seen in Europe. Yaws is transmitted by non-venereal bodily contact, especially among school children under 15 years of age. Minor traumata (bites, wounds, abrasions) play an important role.[7] The microorganism causing this disease, *Treponema pallidum* subsp *pertenue (T pertenue)*, was discovered by Aldo Castellani in 1905 (Latin: pertenuis = very weak).[8] However, the close relationship of the causative agents of the treponematoses has so far made it impossible to distinguish them by serological means. The diagnosis is made on clinical and epidemiological grounds. Especially people living in the humid rural regions in the tropical zones around the world are at risk. After an incubation period of 9–90 days initial lesions appear. These are generally located extragenitally, often on the lower extremities, and have a (ulcero-) papillomatous appearance. These primary stage lesions are very rich in treponemes. Secondary lesions can appear after a latent period, but the primary and secondary stages can overlap. The period of latency may last the lifetime of the patient. However, some patients enter a destructive tertiary (late) stage after a variable period of time. Severe mutilations, such as gangosa or sabre tibia may develop in the late stage. No congenital form of yaws (as observed in syphilis) has been recognised.

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Case report

In March 1988 a 9 year old girl from Ghana was referred to the Paediatric Dermatological Out-Patient Department, Sophia Children's Hospital, Rotterdam, with swelling of the right half of the face and pustules on the body. She had previously been seen by the neurologist, in view of suspected paresis of the right facial nerve. Six months before, she had migrated from Ghana to The Netherlands. Two months after her arrival the skin lesions on her right lower arm were recognised.

On examination the patient was well nourished and apparently in good health. Generalised strawberry-like red-yellowish papulosquamous painless lesions, either moist or covered with crusts, were present (fig 1). On the right arm some remarkable, partly confluent, papillomata were observed (fig 2). The surface of the lesions was partly covered by yellowish scabs. Gentle scraping on the surface caused a friable haemorrhagic exudative lesion. On the right half of the tongue and face, a flat vascular naevus was seen.

The differential diagnosis included sporotrichosis, mycoses, leprosy, treponematoses, cutaneous leishmaniasis and sarcoidosis. Additional laboratory studies were performed. Dark-field examination of the exudate of the lesion on the right arm disclosed the presence of many motile treponemes. Serological tests gave positive results: TPHA positive, FTA-ABS 3+, VDRL positive, titre 1/16. Furthermore the ESR was 64 mm in the first hour, and eosinophilia was present ($0{\cdot}45 \times 10^9$/l).

Routine histopathology of a biopsy specimen taken from a papillomatous lesion on the right arm showed pronounced oedema of the epidermis, papillomatosis and intra-epidermal microabcesses. The silver impreg-

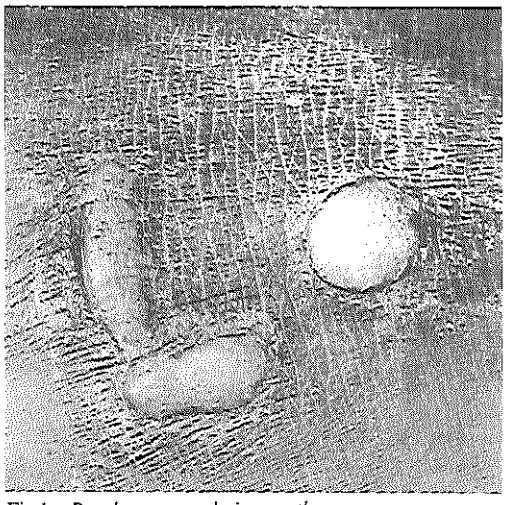

Fig 1 *Papulosquamous lesions on the arm.*

nation technique according to Steiner[9] revealed the presence of many treponemes. Blood vessels were not affected.

Radiographs of the right tibia and left fibula revealed periosteal new bone formation.

An extract was obtained from a second biopsy specimen and the extraction fluid, which contained motile treponemes, was injected into the testes of a New Zealand white rabbit with a negative syphilis serology. Serological screening of this rabbit gave positive results after three weeks. The blind passage of the testicular extract of this rabbit to a fresh rabbit with a negative serology produced mild orchitis with the presence of treponemes (positive dark-field examination) and seroconversion.

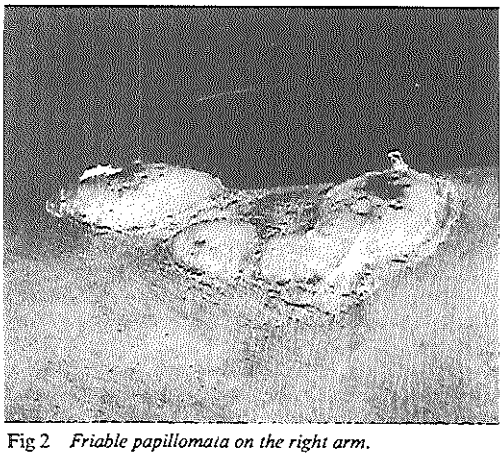

Fig 2 *Friable papillomata on the right arm.*

On clinical and epidemiological grounds and on the basis of the laboratory findings, the diagnosis of early yaws was made. The patient was given daily intramuscular injections of 900 000 units of procain benzylpenicillin (Bicillin®) for fourteen days, in view of possible neurological complications. Under this regimen all lesions rapidly disappeared. Examination of her contacts followed. Only her mother and brother were in Holland.

Of the contacts examined, her older brother was the only one with a positive serology. Prophylactic penicillin was given to brother and mother (single dose 2·4 million units benzathine benzylpenicillin (Penidural®)).

Discussion

This girl developed yaws two months after arriving in The Netherlands. Four months later she consulted us when she was probably in the secondary stage. It seems most probable that she contracted yaws in Ghana shortly before she left that country. This case is fully comparable with the case described by Fry and Rodin in 1966.[2] The World Health Organisation recommends treating yaws with 600 000 units of benzathine benzylpenicillin for all cases and contacts aged under 10 years, and 1 200 000 units for those aged over 10 years.[7] The penicillin therapy given to our patient was therefore probably excessive, but we were confused by a possible facial nerve paresis and in doubt about neurological complications. In general it is assumed that no neurological abnormalities occur in early yaws. But in late yaws there are indications that, as in syphilis, neurological involvement may be encountered, as has been suggested by some authors.[10,11] Late yaws has been associated with asymptomatic CSF abnormalities, reactive CSF-VDRL tests, spinal meningovascular lesions, Jamaican neuropathy and tropical spastic paraparesis.[11] Besides neurological abnormalities, abnormal pupils, perivascular sheathing and optic atrophy have also been observed in late yaws.[10] We are not sure that there is no confusion between yaws and the other treponematoses in these publications.

The incidence of endemic treponematoses in the world was greatly reduced after successful mass penicillin treatment campaigns. In many countries yaws seemed to be eradicated but resurgence of the disease has been observed. In Africa, several reservoirs of yaws can be found. From many African countries no data are available at present.

In Ghana, treatment campaigns were held between 1956 and 1972.[12,13] A 12-fold increase of yaws in Ghana was described between 1968 and 1981.[14] A new campaign followed but still endemic foci exist. Also the disease is still endemic in other African countries and

may be more prevalent. In view of increasing migration one should be aware of the possible importation of yaws into Europe. Recognition of this infectious disease is important, but infectivity is only present in humid tropical areas. Still, millions of people living in endemic areas are susceptible to yaws, and transmission continues. In combination with campaigns against other diseases, treponematoses need attention.[15]

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CHAPTER 4. LIGHT MICROSCOPY: SYPHILIS AND YAWS (PATIENT MATERIAL)

4.1. EARLY YAWS: A LIGHT MICROSCOPIC STUDY

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Early yaws: a light microscopic study

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Abstract

This paper presents the light microscopic findings in biopsies of skin lesions from 45 patients, in whom a diagnosis of early yaws was suspected. In 27 cases typical light-microscopic features of yaws were observed, consisting of parakeratosis or crust containing exudate, marked acanthosis with widening and elongation of the rete ridges or pseudocarcinomatous hyperplasia and spongiosis. Intraepidermal microabscesses consisting of polymorphonuclear leucocytes were frequently encountered. In a large majority a moderate to dense infiltrate was present, composed mainly of lymphocytes and plasma cells. Vascular changes consisted of only slight endothelial cell proliferation and thickening of vessel walls. Steiner staining revealed the presence of treponemes in the epidermis in 23 of 27 cases. Remarkably, clusters of treponemes were also seen in the papillary dermis in three out of 23 cases. Seven other cases were strongly suggestive of yaws. Other histopathological diagnoses were made in 6 patients, due to the simultaneous occurrence of other skin diseases. The remaining five specimens did not contain enough tissue to allow conclusions to be made.

Introduction

Yaws, caused by *Treponema pallidum* subsp. *pertenue* (*T. pertenue*), is a chronic infectious disease found in rural areas with tropical, very warm, humid climates. The disease is transmitted nonvenereally by skin-to-skin contact, mainly among toddlers and infants.[1-2] Early lesions are mainly restricted to the skin and mucous membranes. In some patients the disease progresses to the late stage, in which destructive changes of skin, bone, cartilage and soft tissues are notorious.

An alarming resurgence of yaws has been reported,[3] specifically in West and Central Africa. In Southeast Asia there are also many residual foci.[4-5] During investigations in 1988 cases of early infectious yaws were observed in West Sumatra, Indonesia.[6]

In 1957 Hasselmann pointed to the remarkable epidermiotropic character of *T. pertenue*.[7] Only a limited number of studies on the microscopic aspects of yaws had been published before 1940. Conclusions were based on material obtained from a limited number of patients. During the last 30 years the histological features of early yaws have not been studied intensively.

In this article we discuss the histological features of early yaws. A light-microscopic study of skin biopsies from 45 patients was performed.

Materials and methods

Subjects of this study were 45 patients, 11 females and 34 males, all presenting with skin lesions. In all persons a diagnosis of early infectious yaws was suspected on clinical and epidemiological grounds. Mean age of the patients was 7·6 years (range: 1–25 years). They were examined between 18 November and 25 November, 1988, in six different regional Health Centres in rural areas in West Sumatra, Indonesia. According to the Centre for Disease Control (CDC) in Padang, yaws is hyperendemic and venereal syphilis is not encountered in these remote regions in Sumatra (personal communication).

After clinical examination, dark-field examination of exudates of skin lesions of 41 patients was performed and blood samples were collected by venipuncture from 42 patients. In The Netherlands the Venereal Disease Research Laboratory (VDRL) test, the *Treponema pallidum* haemagglutination assay (TPHA) and the fluorescent treponemal antibody absorption (FTA-ABS) test were performed.

Biopsy specimens were taken from suspect skin lesions. A part of the biopsy tissue was fixed in phosphate-buffered 4% formaldehyde solution. Another part was frozen immediately and specimens were kept in dry ice. They were transported in dry ice from Indonesia to The Netherlands. In Rotterdam,

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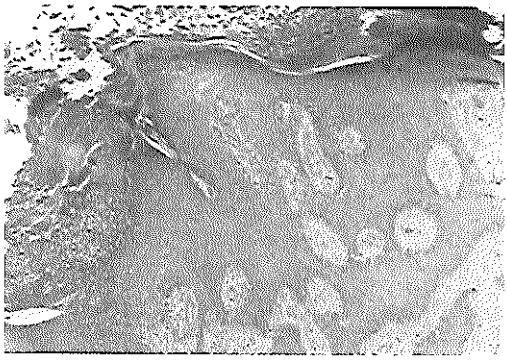

Figure 1 Papillomatous lesion: marked acanthosis and spongiosis. Infiltrate consisting mainly of lymphocytes and plasma cells (haematoxylin-azophloxin, 120 ×).

routine staining was performed with haematoxylin-azophloxin. To study the presence and localisation of treponemes in the biopsies, the histochemical silver staining method according to Steiner[6] was applied.

Results

The 45 patients presented with skin lesions characteristic of yaws. Papillomatous and papulosquamous skin lesions, often ulcerated and covered with crusts, were by far the most frequent. The large majority of all skin lesions were located on the lower extremities (especially the lower legs and arms), which often form the portal of entry for *T pertenue*.[1-2]

Of 42 persons tested serologically, all blood samples except one reacted positive in one or more of the VDRL, TPHA and FTA-ABS tests. The sero-negative patient presented with an ulcer on the left leg (dark-field examination negative, Steiner staining negative) for which no cause could be detected.

Dark-field examination showed the presence of treponemes in 24 out of 41 cases.

In 23 patients light microscopy revealed the unmistakable presence of epidermal changes, consisting of parakeratosis or crust-containing exudate, inflammatory cells and fibrin. There was marked acanthosis with widening and elongation of the rete ridges or pseudocarcinomatous hyperplasia and spongiosis (fig 1). Collections of polymorphonuclear leucocytes were present within the epidermis in all 23 cases. The upper dermis showed marked oedema, dilatated capillaries and cellular infiltration in all cases. An infiltrate composed of plasma cells and lymphocytes was observed in all 23 cases; histiocytes were present in four of 23 cases and polymorphonuclear leucocytes were seen in two. In addition, eosinophils were present in the infiltrate in three cases. The cellular infiltrate in the deeper dermis consisted mainly of plasma cells and was located around the blood vessels. Of two of 23 cases a mild infiltrate was observed; a moderate infiltrate was seen in eleven and a heavy infiltrate in ten cases. The capillaries in all dermal layers revealed only slight endothelial cell proliferation and thickening of vessel walls (fig 2) in all except one case, in which no vascular changes were present. No thrombosis or rupture of vessels was observed.

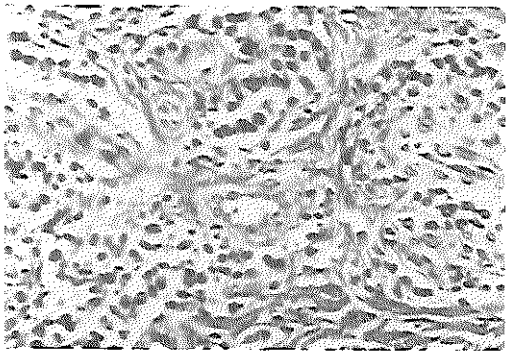

Figure 2 Only mildly affected blood vessels: slight endothelial cell proliferation and thickening of vessel walls (h-a, 420 ×).

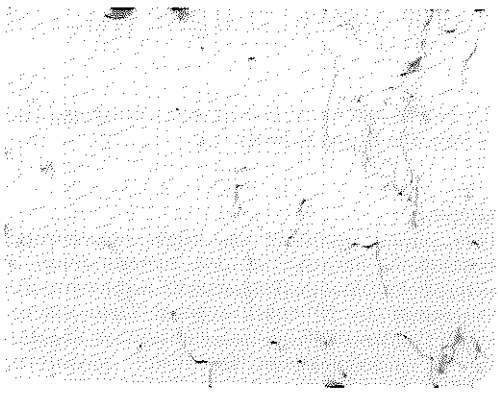

Figure 3 Numerous treponemes are visualised in the epidermis (Steiner staining method, 580 ×).

In all 23 cases foci of treponemes could be demonstrated by the Steiner staining method in the epidermis (8 in the upper epidermis and 15 throughout the epidermis) and in only three cases microorganisms were also seen in the papillary dermis. In several cases extensive clusters of treponemes were noticed in the epidermis (fig 3).

In four cases the histological picture strongly suggested yaws, but this was not confirmed by silver staining. Scar tissue was found in one case, in which dermal fibrosis with a slight inflammatory infiltrate was observed. This could have been the remnant of a healed yaws lesion, since dark-field examination of the exudate of other skin lesions revealed the presence of many motile treponemes and serological tests were strongly positive. In one case an atypical picture was seen: irregular acanthosis, only slight

hyperkeratosis and no microabscesses; in the dermis a slight perivascular infiltrate consisting of lymphocytes and histiocytes was present. Steiner staining revealed the presence of many treponemes in the upper epidermis. Other histopathological diagnoses were made in six specimens: verruca vulgaris, eczematous changes or non-specific infection.

From ten patients insufficient tissue was obtained, but nevertheless treponemes were detected by Steiner staining in the epidermis in four of ten preparations, and multiple microabscesses were noticed in the epidermis of one case, suggesting yaws. No conclusions could be drawn from the remaining five specimens, which only showed necrotic material.

Discussion

In yaws, a primary stage and a secondary stage were discerned, although these stages can overlap. Nowadays the term early yaws is used, comprising the primary and secondary stage.[9]

According to the old literature on the histopathology of yaws, in the early stage lesion epidermal hyperplasia and papillomatosis are pronounced, often with focal spongiosis.[10–12] Frequently microabscesses are present within the epidermis (neutrophils, migrating into the epidermis). A dense dermal infiltrate consists mainly of plasma cells,[12] but can also contain lymphocytes, neutrophils, histiocytes, and eosinophils. Little or no proliferation of endothelial cells is present, and obliterative changes in the vessels are not encountered. This is in contrast with the picture in venereal syphilis, in which involvement of blood vessels is much more pronounced, although not consistently present.[11]

The pathological findings in the early yaws lesions we describe, are largely similar to those described many years ago. However, in our material blood vessels were involved in most cases, but endothelial proliferation was only slight. Intraepidermal microabscesses were present in most cases. The infiltrate was moderate to dense, and consisted mainly of plasma cells and lymphocytes, and occasionally of neutrophils, histiocytes and eosinophils. The epidermiotropic character of *T pertenue* was obvious, as studied using Steiner silver staining. In most cases the microorganisms were detected between epidermal cells, in the upper regions of the epidermis or throughout the epidermis, although in three preparations treponemes were present in the upper dermis too. In some of our preparations of early yaws a microscopic diagnosis was doubtful. Other bacterial (super-) infections made the picture of yaws less clear. In a few cases skin lesions strongly suggested yaws clinically, but microscopically another diagnosis was made, such as eczema, verruca vulgaris or non-specific bacterial infection, probably due to the simultaneous occurrence of other skin infections.

In conclusion, in this study the microscopic picture of early stage skin lesions was frequently characteristic. Owing to the variety in the clinical presentation of yaws, the friable character of skin lesions and the frequent occurrence of other bacterial infections simultaneously affecting the skin, a clear histological picture is not always seen. However, in a large majority of patients the clinical diagnosis of yaws was confirmed by histopathological examination.

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4.2.

PRIMARY AND SECONDARY SYPHILIS: A HISTOPATHOLOGICAL STUDY

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ORIGINAL ARTICLE

Primary and secondary syphilis: a histopathological study

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Summary: We present a study of biopsies taken from skin lesions of 44 patients presenting with primary or secondary syphilis. In most primary lesions erosion or, more often, ulceration was present, with a dense inflammatory infiltrate. In secondary syphilis a wide variety of histological changes was present. Blood vessels were frequently involved, with marked endothelial swelling and often proliferation. Treponemes were demonstrated with the Steiner staining method in all investigated cases of primary syphilis and in 71% of secondary syphilis cases. Treponemes were present throughout the dermis, particularly perivascularly, and in the dermal–epidermal junction zone. In two specimens of secondary syphilis treponemes were located predominantly in the epidermis, but there were always some microorganisms demonstrable in the dermis. The inflammatory infiltrate was often located in a perivascular coat-sleeve-like arrangement. In this study plasma cells and lymphocytes were present in all specimens of primary and secondary syphilis. Syphilitic lesions differed from yaws lesions mostly in the location of treponemes and the affection of blood vessels. In this histopathological study of early syphilis, treponemes did not show the epidermiotropic character of yaws, and blood vessel changes were more pronounced than in yaws. Unfortunately, due to the protean histopathological manifestations described in venereal syphilis and in yaws, these two treponemal diseases cannot always be differentiated on histological grounds alone.

Keywords: Syphilis, *Treponema pallidum*, yaws, histopathology

INTRODUCTION

In the AIDS era new problems have arisen in the diagnosis, treatment and follow-up of syphilis and the disease again receives well-deserved attention[1–3]. Unfortunately, the histopathological aspects have been the subject of few studies recently. Only sparse light-microscopic studies on syphilis have been published in the 1980s, all focused on the secondary stage of syphilis[4–6]. The major finding was that syphilis, more than ever, is the 'great imitator'.

In this article we discuss the histological features of the primary and secondary stages of syphilis. A light-microscopic study of skin biopsies from 44 patients is described. We compared our findings with earlier studies and with our recent study on early yaws[7].

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PATIENTS AND METHODS

Patients

Biopsy specimens were taken from skin lesions of 44 patients. They presented with clinical symptoms of venereal syphilis at the STD Clinic, University Hospital Rotterdam-Dijkzigt, The Netherlands, between August 1984 and January 1990. Dark-field examination of exudates of skin lesions was performed, and blood samples were collected by venipuncture at the same visit, if possible. The Venereal Disease Research Laboratory (VDRL) test, Rapid Plasma Reagin (RPR) test, Fluorescent Treponemal Antibody-Absorbed (FTA-ABS) test and *Treponema Pallidum* Haemagglutination Assay (TPHA) were performed. One or more of the VDRL, RPR, FTA-ABS and TPHA tests were positive in all patients tested at the first visit, except for one patient, in whom treponemal serology became positive at the second visit to our clinic.

On clinical and serological grounds a diagnosis of primary syphilis was made in 29 patients (3 women and 26 men). In 15 patients (8 women and

Table 1. History of sexually transmitted diseases in 44 patients, prior to, or at time of biopsy

	No of patients
Negative history	14
Positive history	22
Syphilis	3
Gonorrhoea	12
Chlamydial urethritis	1
Non-gonococcal urethritis	5
Bacterial vaginosis	1
Genital herpes simplex	3
Condylomata acuminata	4
Pediculosis pubis	1
History unknown	8

7 men) a diagnosis of secondary syphilis was made. The mean age of our patient group (11 females and 33 males) at the time of diagnosis was 34.3 years (range 21–54 years) for primary syphilis patients and 34.1 years (range 18–55 years) for secondary syphilis patients.

Of 29 patients suffering from primary syphilis, 25 males presented with an ulcer on the penis, in one male patient an ulceration was noticed in the corner of the mouth and the three female patients presented with ulcers on the perineum, vulva and labia majora, respectively. Of 15 patients suffering from secondary syphilis 13 had generalized skin lesions, in 2 women the only clinical manifestation of syphilis consisted of condylomata lata on the labia. Typical syphilitic skin lesions of hand palms and foot soles were seen in 7 patients. In 28 of 29 patients with primary syphilis, dark-field examination of the exudate of skin lesions was performed with positive results in 20 cases. Dark-field examination was performed in 8 of 15 cases of secondary syphilis, revealing the presence of treponemes in 3 out of 8 cases.

Patient charts were carefully studied. Sexually transmitted diseases which had been diagnosed at our department before and at time of biopsy taking are listed in Table 1. At the time of the biopsy, in 4 patients another genital infection was diagnosed. In one female patient a diagnosis of bacterial vaginosis was made. Three males were suffering simultaneously from chlamydial urethritis or non-gonococcal urethritis.

Histopathology

Specimens were fixed in phosphate-buffered 4% formaldehyde solution. Routine staining was performed with haematoxylin–azophloxin (H&A). To study the presence and localization of treponemes in the biopsies, the histochemical silver staining method according to Steiner[8] was applied.

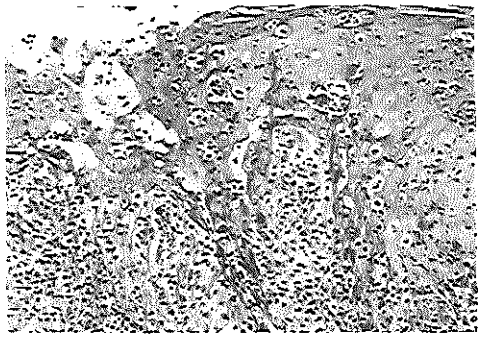

Figure 1. Border of ulceration in primary syphilis, showing irregular hyperplasia. A dense inflammatory infiltrate consisting of lymphocytes, plasma cells and neutrophils is present (haematoxylin and azophloxin stain, ×72)

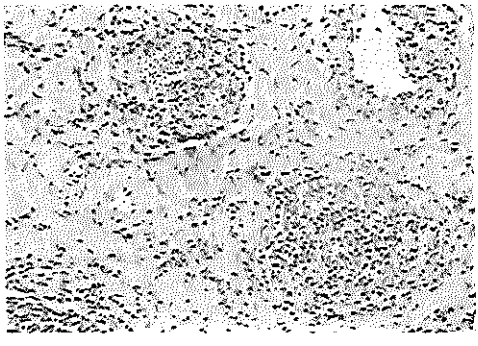

Figure 2. Perivascular inflammatory infiltrates in primary syphilis (H&A stain, ×72)

RESULTS

Primary stage

In this stage erosion or absence of the epidermis in ulcerated regions was observed. Adjacent to the ulceration the epidermis showed hyperplasia (Figure 1), with widening and elongation of the rete ridges. Spongiosis with vesicle formation was observed in some cases. The ulcerative surface was frequently covered by an exudate, which consisted of fibrin, necrotic tissue fragments, and polymorphonuclear leucocytes. In 28 biopsy specimens, the dermal inflammatory infiltrate was composed of lymphocytes and plasma cells (in highly variable numbers) in all cases of primary syphilis, with histiocytes in 9 and polymorphonuclear leucocytes in 18 cases. The inflammatory infiltrate was located throughout the dermis, particularly perivascularly (Figure 2). The intensity of the infiltrate was described as dense in 21, moderate in 5 and sparse in 2 specimens. The perivascular infiltrate was associated with endothelial

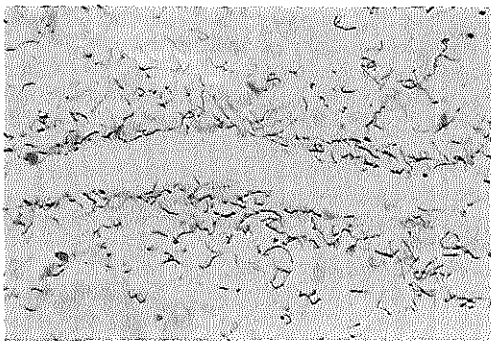

Figure 3. Treponemes around a blood vessel, visualized by the silver staining method according to Steiner. Primary syphilis (×284, oil immersion)

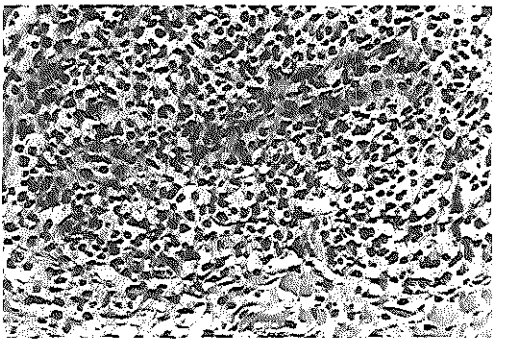

Figure 5. Perivascular arrangement of the inflammatory infiltrate in secondary syphilis. The infiltrate consists of lymphocytes and plasma cells, and sporadically histiocytes. Note the striking swelling of the endothelial cells and invasion of the capillary walls by inflammatory cells. Same case as Figure 4 (H&A stain, ×247)

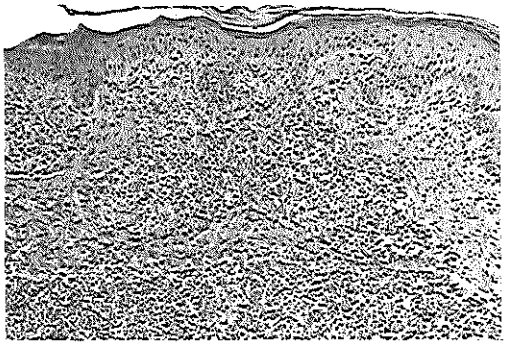

Figure 4. Slight irregular epidermal hyperplasia with elongation of the rete ridges and hyperkeratosis in a case of secondary syphilis. There is a dense inflammatory infiltrate in all dermal layers (H&A stain, ×65)

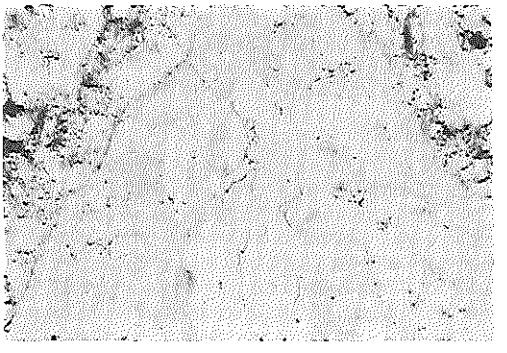

Figure 6. Secondary syphilis. Silver staining (Steiner) shows treponemes located around blood vessels in the dermis (×284, oil immersion)

swelling (marked in 15 cases, slight in 12 cases). In rare cases even obliteration of the vascular lumen was present, due to endothelial swelling or endothelial proliferation. One biopsy showed fibrinoid vessel wall necrosis. One biopsy, taken from an ulcer in the corner of the mouth, showed granulation tissue only.

The Steiner silver staining method demonstrated the presence of treponemes in all biopsies examined (28). One biopsy did not contain enough tissue to allow Steiner staining to be performed. Only a few treponemes were detected in 2 cases, both in the dermis. A moderate number of treponemes were observed in 14 cases, mainly located in the dermal–epidermal junction zone, most perivascularly, sometimes in the walls of papillary blood vessels; in 6 of these 14 cases treponemes were located in the dermis only. Large numbers of treponemes (Figure 3) were demonstrated in 12 specimens, again mostly in the dermal–epidermal junction and perivascularly.

Secondary stage

In 7 cases the epidermis showed acanthosis, with irregular elongation and sometimes widening of the rete ridges, and hyperkeratosis (5) and parakeratosis (3). Focal ulceration or atrophy of the epidermis were the only changes noted in 2 other specimens. In 6 biopsies the epidermis was not affected. The intensity of the inflammatory infiltrate and the distribution of cell types were highly variable, dense in 9, moderate in 4 and sparse in 2 specimens. Lymphocytes and/or plasma cells predominated in all biopsies of secondary syphilis (Figure 4). However, plasma cells were sparse in 2 biopsies. The predominantly lymphoplasmacellular infiltrate was admixed with histiocytes, neutrophils, and in one case eosinophils. The infiltrate was located perivascularly (Figure 5) in a coat-sleeve-like arrangement throughout the dermis in 8 specimens, and in the papillary dermis only in 3 cases. A diffuse infiltrate in the upper dermis with a perivascular infiltrate in the deeper layers of the dermis was seen

3 times. A diffuse infiltrate was present throughout the dermis in one case. In some cases the infiltrate was observed in close relationship with adnexal structures. Spread of inflammatory cells into the epidermis occurred in 9 specimens, sometimes associated with spongiosis. The dermis showed mild oedema in 5 cases.

Endothelial swelling was very prominent in 6 cases, mild in 5 cases and absent in 4 cases. Small vessels throughout the dermis showed endothelial proliferation in only a few cases. Fibrinoid vessel wall necrosis and intravascular thrombus formation were seen in one case. In the two condylomata lata cases, rete ridges were widened and elongated and a dense inflammatory infiltrate was present.

Steiner staining revealed the presence of treponemes in 10 out of 14 cases studied (71%). In 2 specimens treponemes were observed mainly in the epidermis and in 8 specimens treponemes were mainly in the dermal–epidermal junction zone or throughout the dermis (Figure 6). In the dermis treponemes were frequently located perivascularly. The two condylomata lata cases were very rich in treponemes.

DISCUSSION

In most primary stage lesions erosion or more often ulceration was present, together with a dense inflammatory infiltrate in the dermis. Light-microscopic findings showed a wide range of changes in the secondary stage lesions. Many different histological features were observed. In the primary and secondary stage lesions, lymphocytes and plasma cells were prevalent in all cases, in highly variable numbers. This is in contrast to findings in a study of secondary syphilis by Abell *et al.* who found that plasma cells were absent or inconspicuous in 25% of biopsies[9]. In their study blood vessels were unaffected in more than half the biopsy specimens of secondary syphilis. In our study, in the large majority of biopsies, small blood vessels showed endothelial swelling which was often marked. This is comparable with other studies, in which vascular changes were also found to be very common[4,5,10]. Silver staining demonstrated treponemes in a higher percentage of cases than dark-field examination. Dark-field examination, however, remains an important technique in the diagnosis of venereal syphilis.

In comparison with our previous light-microscopic findings in yaws[7] caused by *Treponema pallidum* subspecies *pertenue*, this study of syphilis revealed some striking differences. The causative agents of venereal syphilis and nonvenereally transmitted yaws cannot yet be differentiated routinely on morphological or serological grounds. In syphilitic lesions it was particularly the location of treponemes and the affection of blood vessels that showed a different character compared with yaws. In our study of early yaws, treponemes showed a characteristic epidermiotropic character, and were rarely found in the dermis. Moreover, blood vessel changes were slight or absent[7]. In this series of biopsies of syphilitic skin lesions, the Steiner silver staining method showed treponemes most frequently throughout the dermis or epidermal–dermal junction, frequently perivascularly. In two specimens of secondary syphilis treponemes were predominantly located in the epidermis, but there were always some microorganisms demonstrable in the dermis. Blood vessels frequently showed striking endothelial swelling. Furthermore, the coat-sleeve-like arrangement of the inflammatory infiltrate was a more frequent finding in syphilitic lesions. Unfortunately, due to the protean histopathological manifestations described in venereal syphilis and in yaws, these 2 treponemal diseases cannot always be differentiated on histological grounds alone.

In the AIDS era the importance of genital ulcerations in the spread of HIV infection has recently been stressed. Genital ulcer disease has been described as a risk factor for transmission of the human immunodeficiency virus type-1 (HIV-1)[11]. HIV-testing in The Netherlands has caused and is still causing debate[12]. Written informed consent is needed before HIV-testing can be performed. Retrospectively it became clear that until February 1991, the HIV serostatus was known in only 6 out of 44 patients biopsied (14%). With written informed consent 2 patients were tested at the time of diagnosis of the syphilitic infection and 2 patients were tested approximately 3 years later, all 4 with a negative result. Two patients, who had been tested after the biopsy was taken, after 7 months and 5 years respectively, turned out to be HIV-seropositive, confirmed by Western blotting. In 86% of patients in this study, often frequent visitors to our STD clinic, no testing for HIV antibodies was performed. As a consequence, information on the spread of HIV infection in this important patient group is scarce.

In this histopathological study no remarkable forms of syphilis such as lues maligna were encountered. No histological changes suggested an associated STD or complication of immunosuppression.

The variety in the histological picture of secondary syphilis observed in this study is in agreement with findings of others[4,5,9,10]. Jordaan *et al.* have given diagnostic guidelines, with a list of histological features, which either singly or in combination strongly point to syphilis[5]. However, the pattern of changes observed in our study was not always specific of syphilis. Light-microscopic findings in syphilis may mimic many other skin disorders. High suspicion of syphilis and close communication between clinician and pathologist remain of the utmost importance.

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4.3.

THE LOCALISATION OF TREPONEMES AND CHARACTERISATION OF THE INFLAMMATORY INFILTRATE IN SKIN BIOPSIES FROM PATIENTS WITH PRIMARY OR SECONDARY SYPHILIS, OR EARLY INFECTIOUS YAWS

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Genitourinary Medicine (in press)

Abstract

Objective - To study the localisation of treponemes and to analyse the inflammatory infiltrate in biopsies from patients suffering from primary or secondary syphilis, or early infectious yaws.

Materials and methods - Skin biopsies originating from human lesions of primary (29x) or secondary (15x) syphilis (Rotterdam), or early yaws (18x) (West Sumatra) were studied. Different histochemical and immunohistochemical detection methods were used in this study.

Results and conclusion - The histochemical silver staining method according to Steiner revealed the presence of *T.pallidum* in all cases of primary syphilis studied. In 10 out of 14 cases of secondary syphilis, treponemes were demonstrated. With an immunofluorescence staining technique (IF) using anti-*T.pallidum* antiserum raised in rabbits (a-Tp), *T.pallidum* was demonstrated in 28 out of 29 cases of primary syphilis, and in 14 out of 14 studied cases of secondary syphilis. The silver staining method and IF showed identical localisations of *T.pallidum* (mainly in the dermal-epidermal junction zone or throughout the dermis). Using a-Tp antiserum in the indirect immunofluorescence technique, *T.pertenue* could be demonstrated in the dermis more often than with Steiner silver staining. However, epidermotropism of *T.pertenue* in yaws specimens was remarkable, compared with more mesodermotropism of *T.pallidum*; numbers of *T.pertenue* in the dermis were limited in all specimens. The dermal inflammatory infiltrate in primary and secondary syphilis was composed mainly of lymphocytes and plasma cells. In most cases more T (CD3 positive) cells than B (CD22 positive) cells were present. Regarding T cell subpopulations, in primary syphilis, T helper/inducer (CD4 positive) cells predominated in 86% of cases. In secondary syphilitic lesions, numbers of T helper/inducer cells were less frequent than or equal to T-suppressor/cytotoxic (CD8 positive) cells in 60% of cases. Remarkably, in yaws specimens the inflammatory infiltrate consisted mainly of IgG, but also IgA and IgM producing plasma cells. T or B lymphocytes were scarce, which is in sharp contrast with findings in syphilitic lesions.

Introduction

Sexually transmitted syphilis, caused by *Treponema pallidum* subspecies *pallidum* (*T.pallidum*) is still a major worldwide threat. In Europe and the United States syphilis has not yet been eradicated (1-3). In many developing countries this treponemal infection is highly prevalent (4). In the AIDS era, the classical sexually transmitted ulcerative diseases such as syphilis deserve renewed interest, because these may facilitate the transmission of HIV infection (5-7). In the 1980s only a few articles were published on the histopathological aspects of syphilis. The wide variety of possible microscopical changes in secondary syphilis, analogous to the variety seen in the clinical expression of the disease, was stressed (8-11). One recent study by Tosca *et al.* focused on composition of the inflammatory infiltrate before and after antibiotic treatment was given (10).

Yaws (framboesia tropica), caused by *Treponema pallidum* subspecies *pertenue* (*T.pertenue*), is a chronic nonsexually transmitted treponematosis of childhood occurring in rural remote regions with tropical, humid climates (12). Specifically in West and Central Africa, a dramatic re-emergence of yaws was recently reported (13-15). Yaws has also become resurgent in several countries in Southeast Asia (16-18). From the 1950s onwards, studies of the histopathological findings in early yaws have been scarce (19,20). Conclusions of older studies were based on material obtained from a limited number of patients. To our knowledge, analysis of the inflammatory infiltrate in yaws has never been addressed before. In this article we investigate and compare the localisation of spirochetes in skin biopsies from patients suffering from primary or secondary syphilis, or from early infectious yaws. An analysis of the inflammatory infiltrate in these specimens was performed.

Materials and methods

Syphilis

Biopsy specimens were taken from untreated skin lesions of 44 patients, who presented with clinical symptoms of early infectious syphilis at the Department of Dermatology and Venereology, University Hospital Rotterdam-Dijkzigt, the Netherlands, between August 1984 and January 1990, and gave permission for a biopsy. Blood samples of all patients reacted positively in one or more of the following tests: the Venereal Disease Research Laboratory

(VDRL) test, Rapid Plasma Reagin (RPR) test, fluorescent treponemal antibody-absorbed (FTA-ABS) test and *Treponema pallidum* haemagglutination assay (TPHA) (at the first or second visit) and/or dark-field examination of exudates of lesions showed the presence of treponemes. A diagnosis of primary syphilis was made in 29 patients (3 women and 26 men). The mean age was 34.3 years (range 21-54 years). In 15 patients (8 women and 7 men) a diagnosis of secondary syphilis was made. In this group the mean age was 34.1 years (range 18-55 years). Clinical manifestations have been described previously (11). The biopsies were sent immediately to the Department of Pathology. All samples were cut into two equal parts. One was fixed in phosphate-buffered 4% formaldehyde solution, and paraffin-embedded. The remaining part was snap-frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen for immunohistochemical studies. At the time of the biopsy, the serostatus for antibodies against HIV was unknown in all patients. To study the presence and localisation of treponemes in the biopsies, the histochemical silver staining method according to Steiner (21) and immunohistochemical methods were used, and analysis of the inflammatory infiltrate was performed.

Yaws

Skin biopsies from 18 patients, five girls and 13 boys, suffering from yaws in rural regions in West Sumatra, Indonesia (November 1988) were studied. The mean age of the patients was 7.4 years (range: 1.5 - 13 years). An overlap between the primary and secondary stages of yaws was typical (12), and no distinction between these stages could be made. In all patients biopsied, a diagnosis of early infectious yaws was made on clinical and epidemiologic grounds, proven by reactive serologic tests (VDRL, TPHA, FTA-ABS) and / or positive dark-field examination of the exudate of skin lesions, and positive Steiner silver staining. One part of biopsied tissue was fixed in phosphate-buffered 4% formaldehyde solution. For immunohistochemical studies, another part was frozen immediately, kept in dry ice and transported in dry ice from Indonesia to the Netherlands. To study the localisation of treponemes, results of Steiner silver staining were compared with immunohistochemical methods. Analysis of the inflammatory infiltrate was performed.

Routine staining of all specimens was performed with haematoxylin-azophloxin (H&A). In the H&A slides, depending on the total number of inflammatory cells, the infiltrate was graded as mild (a small number), as dense (a large number), or as moderate (when the total

number of inflammatory cells was in between the other two categories).

The composition of the inflammatory infiltrate was analysed on slides from frozen parts of the biopsies, using commercially available polyclonal and monoclonal antibodies against immunoglobulins, T-lymphocytes, T-lymphocyte subpopulations and B-cells.

The sections were tested with commercially available polyclonal antibodies against immunoglobulins (IgG, IgA, IgM, (Immuno de Beer Medicals B.V., Kallestad)), C1q, and β_1a (Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis). Furthermore, an antiserum against the pathogenic Nichols strain of *T.pallidum*, raised in rabbits, was used (rabbit anti-*T.pallidum* antiserum (a-Tp)). Positive and negative controls were included.

The indirect immunoperoxidase (IIP) method was performed with an optimal dilution of the monoclonal antibody; the presence of T-lymphocytes (CD3, pan-T), helper/inducer T-lymphocytes (CD4 (T_h)), suppressor/cytotoxic T-lymphocytes (CD8 (T_s)) and B-lymphocytes (CD22, pan-B) (Becton Dickinson) was studied. Positive and negative controls were included.

Results

Localisation of treponemes

Primary syphilis

The Steiner silver staining method demonstrated the presence of treponemes in 28 out of 29 biopsy samples from patients with primary syphilis; one specimen did not contain enough tissue to allow silver staining to be performed. In two cases only a few treponemes were detected, in both cases located in the dermis. A moderate number of treponemes were observed in 14 cases, in most cases located in the dermal-epidermal junction zone. In the dermis most treponemes were seen perivascularly, sometimes in the walls of papillary blood vessels; in six out of these 14 cases treponemes were located in the dermis only. Large numbers of treponemes were demonstrated in 12 specimens, again mostly in the dermal-epidermal junction and the perivascular areas (fig 1). With immunofluorescence using a-Tp (fig 2), treponemes were visualised in 28 out of 29 specimens, mostly in the dermis (perivascularly) or dermal-epidermal junction. In one case a-Tp did not show treponemes: however, in the formaldehyde-fixed counterpart of this biopsy many treponemes were demonstrated in the dermis with Steiner silver staining. Only occasionally were

microorganisms visualised with anti-IgG, C1q, and β_1a.

Secondary syphilis

Steiner silver staining revealed the presence of treponemes in 10 out of 14 cases studied, with only a few treponemes in five, a moderate number in two, and a large number in three cases. In eight specimens treponemes were mainly observed in the dermal-epidermal junction zone or throughout the dermis, frequently located perivascularly. In two specimens treponemes were mainly observed in the epidermis, reaching the surface.

With a-Tp antibodies, treponemes were visualised in all 14 specimens studied, again mostly in the dermis (perivascularly) or dermal-epidermal junction. In the four Steiner-negative cases, few treponemes were detected using a-Tp, in the dermis (2x) or in the dermal-epidermal junction (2x). In one case *T.pallidum* was observed in the epidermis with anti-IgG.

Yaws

In 18 of 18 cases Steiner staining revealed the epidermal location of *T.pertenue*. In two of 18 biopsy specimens no dermal tissue was present. In only one of the other 16 cases, in which a large number of treponemes were present in the epidermal layers, a few microorganisms were also noted focally in the papillary dermis. In the epidermis the microorganisms were regularly observed in a band-like pattern, sometimes in clusters (fig 3).

In the frozen material used for immunofluorescence detection techniques, the epidermis was absent in one biopsy, the dermis in another one. In all biopsies covered by an intact epidermis (17x), many *T.pertenue* organisms were detected in the epidermis. In 16 out of 17 biopsies treponemes were found in the dermis. Numbers in the dermis were small: *T.pertenue* was distributed focally, solitarily or in small clusters. Treponemes were observed most frequently using a-Tp (fig 4). Besides with a-Tp, treponemes were also frequently observed with C1q. With anti-IgG, IgM, IgA and β_1a, the presence of treponemes was observed only occasionally. In some slides the presence of *T.pertenue* was doubtful, owing to strong background staining of other structures resembling treponemes.

Inflammatory infiltrate

Primary syphilis

In 28 H&A specimens, the inflammatory infiltrate was located in all layers of the dermis,

particularly perivascularly. The infiltrate was described as dense in 21, moderate in five and mild in two specimens. The infiltrate consisted of lymphocytes and plasma cells (in highly variable numbers) in all cases, admixed with histiocytes in nine and polymorphonuclear leucocytes in 18 cases.

With anti-IgG, anti-IgM and anti-IgA conjugates, plasma cells stained positively in 17 of 29 (59%), 20 of 29 (69%) and 21 of 29 (72%) of cases, respectively; numbers of positive cells were highly variable. No plasma cells were observed in four cases. In most cases there was a preponderance of T lymphocytes in the dermal inflammatory infiltrate: more T (CD3 positive) cells than B (CD22 positive) cells were present in 25 of 29 biopsies of primary syphilis. In four cases this ratio was unknown (see table 1). Study of T cell subpopulations demonstrated that in 20 out of 29 biopsies (69%), helper/inducer T cells (CD4) were more numerous than suppressor/cytotoxic T cells (CD8) (fig 5). In six cases T helper/inducer cells were about equal to T suppressor/cytotoxic cells (21%), and in three, T helper/inducer cells were less common than T suppressor/cytotoxic cells (10%). See table 1.

Secondary syphilis

In secondary syphilis, the density of the inflammatory infiltrate and the distribution of inflammatory cell types were highly variable, dense in nine, moderate in four and mild in two specimens. In all biopsies of secondary syphilis lymphocytes and plasma cells predominated. However, plasma cells were only scarce in two biopsy specimens. The predominantly lymphoplasmacellular infiltrate was admixed with small amounts of histiocytes, neutrophils, and in one case with eosinophils.

Plasma cells stained positively in six out of 15 (40%) with anti-IgG antiserum, in 12 of 15 (80%) with anti-IgM antiserum, and in 12 out of 15 (80%) with anti-IgA antiserum, numbers of plasma cells being highly variable. In two cases immunofluorescence did not reveal the presence of plasma cells. It was demonstrated that in biopsies from secondary syphilis lesions also, T cells predominated in the infiltrate (fig 6) in most (13 out of 15) cases (table 1). However, numbers of positive cells were highly variable. Study of T cell subsets showed that in six out of 15 biopsies (40%) T helper/inducer cells were more numerous than T suppressor/cytotoxic cells. In four cases T helper/inducer cells were about equal to T suppressor/cytotoxic cells (27%), and in five cases, T helper/inducer cells were fewer in number than T suppressor/cytotoxic cells (33%) (see table 2 and figure 7).

Table 1 Results of immunoperoxidase staining of primary (n=29) and secondary (n=15) syphilis biopsy specimens; CD3 (pan-T) versus CD22 (pan-B) positive cells.

	Primary syphilis	Secondary syphilis
Pan-T > Pan-B	25	13
Pan-B > Pan-T	0	0
Pan-T = Pan-B	0	1
Unknown	4	1

Table 2 Results of immunoperoxidase staining of primary (n=29) and secondary (n=15) syphilis biopsy specimens: T cell subpopulations (CD4(T_h) versus CD8 (T_s) positive cells).

	Primary syphilis	Secondary syphilis
$T_h > T_s$	20	6
$T_h < T_s$	3	5
$T_h = T_s$	6	4
Unknown	0	0

Yaws

With H&A staining, in the yaws specimens an infiltrate composed of plasma cells and lymphocytes was observed in all 16 cases studied. Histiocytes were present in five cases and polymorphonuclear leucocytes were seen in two. In addition, eosinophils were present in the infiltrate in four cases. The inflammatory infiltrate in the deeper dermis consisted mainly of plasma cells. In one case a mild infiltrate was observed; a moderate infiltrate was seen in seven and a dense infiltrate in eight cases.

With anti-IgG, anti-IgM and anti-IgA conjugates, plasma cells stained positively (fig 8) in specimens of all 18 cases. The numbers of plasma cells were highly variable. In most cases it was obvious that with anti-IgG the largest number of plasma cells gave a positive reaction. Only in a few cases was a weak staining of small groups of CD3, CD22, CD4, and CD8 positive cells observed in the dermis. In most cases no B- or T-lymphocytes were observed.

Legends

Figure 1. The silver staining according to Steiner demonstrates the presence of *T.pallidum*, located perivascularly (primary syphilis).

Figure 2. Primary syphilis. Many treponemes in the dermis (rabbit anti-*T.pallidum* antiserum (a-Tp)).

Figure 3. Many *T.pertenue* organisms are detected (Steiner staining method).

Figure 4. Yaws: numerous treponemes are visualised in the epidermis, using a-Tp.

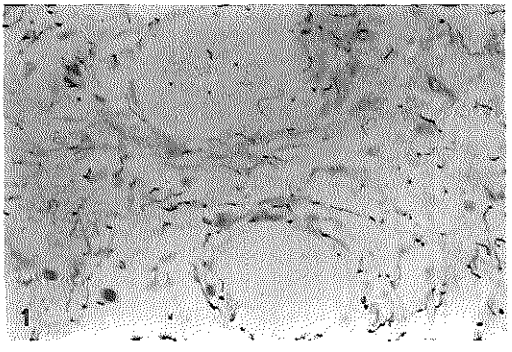
Figure 5. Primary syphilis: helper/inducer T cells (CD4) (fig 5a) are more numerous than suppressor/cytotoxic T cells (CD8) (fig 5b). Immunoperoxidase staining, 160 x

Figure 6. Secondary syphilis: more T (CD3 positive) cells (fig 6a) than B (CD22 positive) cells (fig 6b) are present in the infiltrate. Immunoperoxidase staining, 160 x

Figure 7. Secondary syphilis: helper/inducer T cells (fig 7a) are less numerous than suppressor/cytotoxic T cells (fig 7b). Immunoperoxidase staining, 250 x

Figure 8. With immunofluorescence, using anti-IgA, many plasma cells are demonstrated in a biopsy of yaws.

Figure 9. Many plasma cells in the inflammatory infiltrate of yaws (methyl green pyronin staining).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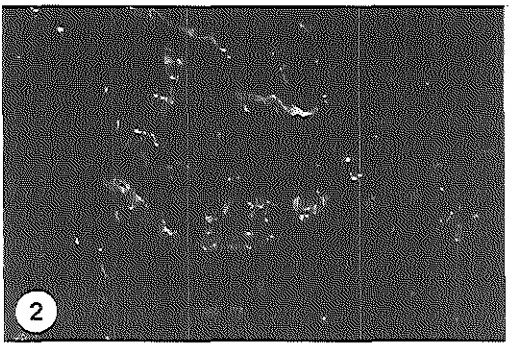
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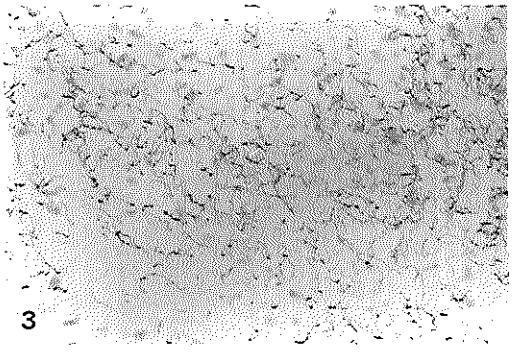
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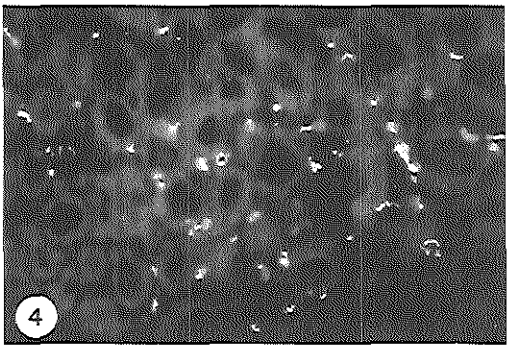
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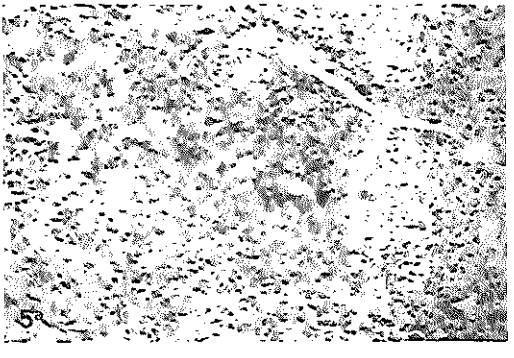
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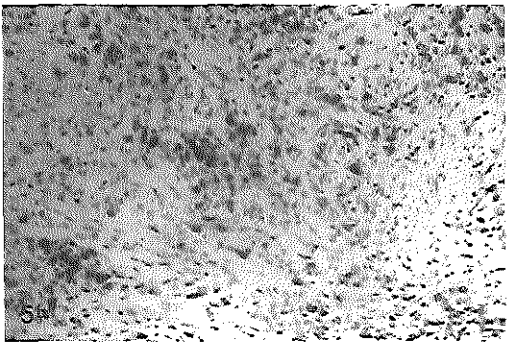
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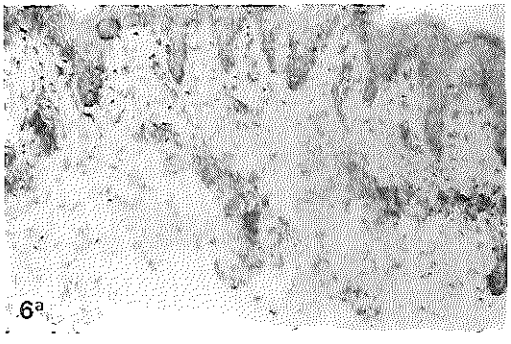
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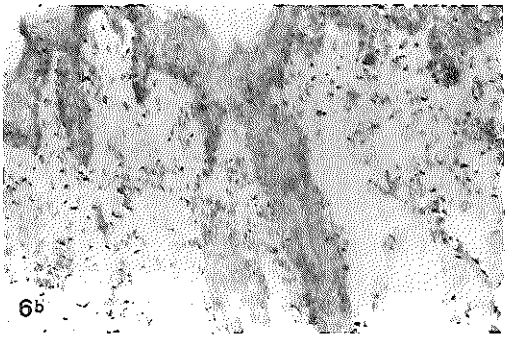
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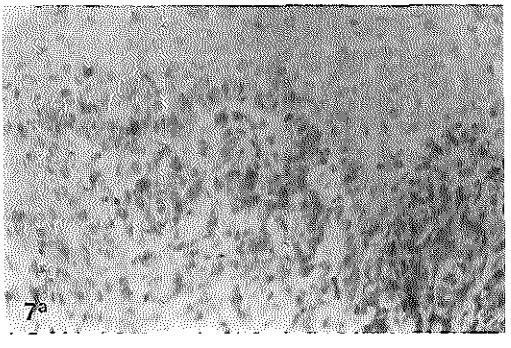
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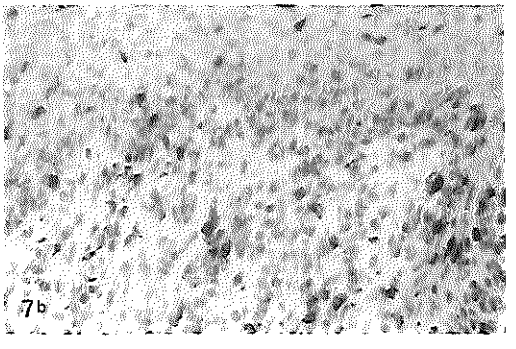
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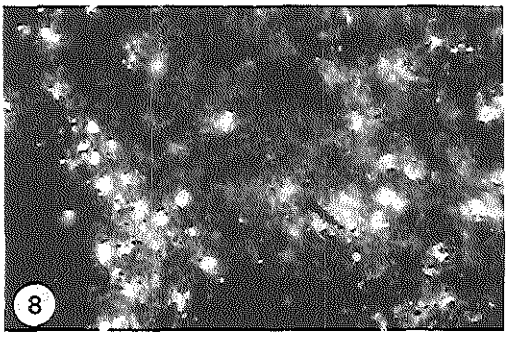
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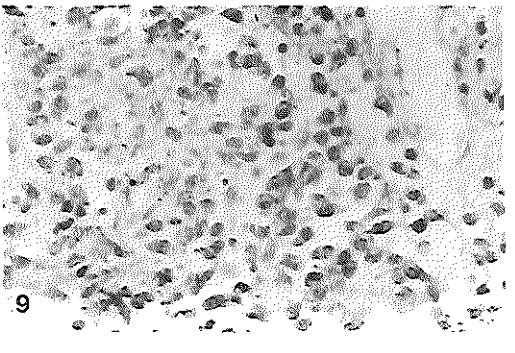
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Discussion

Hitherto no rigorous morphological, serological, immunological or genetic differences have been described to distinguish between the causative agents of yaws and syphilis. Distinctive clinical, epidemiological and geographical features, however, exist (12,14). In previous work, the epidermotropic character of *T.pertenue* was obvious, in contrast to the more mesodermotropic character of *T.pallidum* (11,20). It was hypothesised that this difference could be held responsible for the different clinical and pathological findings in lesions of venereal syphilis and yaws, and the milder course of the latter disease (11,19). In this study, with Steiner silver staining as well as with a-Tp using immunofluorescence, it was demonstrated that *T.pallidum* was primarily located in the dermis or dermal-epidermal junction. We noticed that with the Steiner silver staining method, the epidermotropism of *T.pertenue* was obvious. Hardly any treponemes could be demonstrated in the dermis. However, using a-Tp antibodies, *T.pertenue* could also be demonstrated in the dermis in nearly all specimens. These microorganisms located dermally were nevertheless only scarce, in contrast to the large numbers of treponemes in the epidermal layers, thus confirming the epidermotropic character of *T.pertenue*. Immunoglobulins or complement components could rarely be demonstrated in relation to the treponeme organisms.

Histopathologically, in many studies the inflammatory infiltrate in early syphilis and yaws has been characterised as mainly lymphoplasmacellular. Nowadays, the availability of specific monoclonal antibodies can be used to characterise exactly the composition of the infiltrate. In this study it became clear that T cells were the predominating cells in the infiltrate in a large majority of cases of primary as well as of secondary syphilis: B lymphocytes and plasma cells were less frequent. In most cases of primary syphilis, in this predominantly T cell infiltrate, the T helper/inducer cells were more frequent than T suppressor/cytotoxic cells. However, in secondary syphilis biopsy samples the ratio of T helper/inducer and T suppressor/cytotoxic cells was approximately equal or reversed in more than half of the cases. Our findings are comparable with those of Tosca *et al.*, who demonstrated that in primary lesions T-helper lymphocytes were more numerous than T-suppressor lymphocytes, while in secondary lesions T-suppressor lymphocytes predominated (10). The increased numbers of T-suppressor lymphocytes in the secondary stage lesions of syphilis may result in the natural shutdown of the early vigorous immune response following

clearance of most of the treponemes from the lesions, ushering in the latent stage.
In contrast with these findings, however, in yaws specimens hardly any T and B lymphocytes were detected. We found that plasma cells were by far the most important constituent of the inflammatory infiltrate in early infectious yaws. With immunofluorescence it was observed in the yaws cases that IgG producing plasma cells were the most numerous, but IgA and IgM producing plasma cells were also present in all specimens. Afterwards, these findings in yaws were histochemically confirmed with methyl green pyronin staining, which again demonstrated the presence of an abundance of plasma cells (fig 9).

In syphilis patients no overlap between the primary and secondary stage lesions, as in yaws, was observed. Unfortunately, the precise duration of the presence of lesions at the time of biopsy, and the HIV serostatus were unclear. Furthermore, to allow a more accurate interpretation of our results, serial sectioning of biopsy material may offer additional information, to eliminate the one-dimensional view of a single section.

A remarkable difference in the inflammatory reaction of yaws and venereal syphilis is presented here. Whether this represents a typical feature of yaws, regional differences of *T.pertenue* strains or influence of transport remains to be seen. Therefore, further study of biopsy samples from patients suffering from syphilis or endemic treponematoses in other regions of the world is recommended for a better understanding of treponemal infection.

Acknowledgements

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CHAPTER 5. EXPERIMENTAL TREPONEMAL INFECTION (SYPHILIS AND YAWS)

5.1. RAPID IN VITRO IMMOBILISATION OF PURIFIED *TREPONEMA PALLIDUM* (NICHOLS STRAIN), AND PROTECTION BY EXTRACTION FLUIDS FROM RABBIT TESTES

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E Stolz, and J J van der Sluis.

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Rapid in vitro immobilisation of purified *Treponema pallidum* (Nichols strain), and protection by extraction fluids from rabbit testes

H J H Engelkens, M Kant, P C Onvlee, E Stolz, J J van der Sluis

Abstract

The use of Percoll-purified treponemes in an assay similar to the *Treponema pallidum* Immobilisation test demonstrated that immobilisation of purified treponemes by seronegative normal human serum proceeded at a much higher rate than that of unpurified treponemes. This suggests that the removal of the testicular extract makes the treponemes more vulnerable to this action. A preincubation of the purified treponemes with the testicular extract from infected or uninfected testes delayed their rate of immobilisation to that demonstrated by the unpurified treponemes. This showed that substances produced during the infection are probably not responsible for the delay in immobilisation. Discrimination between the classical and the alternative pathway of complement activation, studied by the ethylene glycol-bis (beta-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) method, showed that the classical pathway was responsible for the rapid immobilisation of the purified treponemes. However, the slow immobilisation in the EGTA-serum samples suggested a minor role of the alternative pathway in the immobilisation of the purified treponemes. Since the testicular extracts exerted an anti-complement activity, it needs to be investigated whether the protection offered to the purified treponemes by the testicular extracts is based on their deteriorating effect on the classical complement pathway or is due to a re-establishment of the protective cover around the treponemes.

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Introduction

Syphilis caused by *Treponema pallidum* subspecies *pallidum* (*T pallidum*) can result in chronic disease. If left untreated, syphilis may result in disabling or even life-threatening situations during the tertiary stage, indicating that the host does not succeed in complete eradication of the treponemes. The mechanisms of the survival of treponemes in a host for many years are not yet fully understood. Besides a possible affection of the immune response,[1] there are several other hypotheses on how treponemes could possibly evade the host defence.

One hypothesis points to coverage of the treponeme with a protective layer, consisting of mucopolysaccharides and host proteins. The presence of mucopolysaccharides on treponemes in vitro was shown by Fitzgerald *et al*[2] and in rabbits by Zeigler *et al*.[3] Several serum proteins have been shown to be in close association with the outer membrane of *T pallidum*, isolated from infected rabbit testes.[4] However, in electron-microscopic studies the outer membrane presents itself as a symmetrical structure that shows no signs of extra-membrane components. Moreover, it was argued that the mere presence of substances on the outer surface of *T pallidum* does not prove that they play a role in providing protection to the organisms against the host defences.[5]

A more recent hypothesis focuses on the role of the structure of the outer membrane of *T pallidum* in the evasion of the treponemes from the immune response. It was shown by freeze fracture and deep etching techniques that the outer membrane contains only a small number of integral membrane proteins that can serve as targets for specific antibodies. These studies did not provide evidence that the surface of *T pallidum* is covered by an outer coat.[6]

As a consequence of both hypotheses it is assumed that treponemes can survive despite the sensitisation of the immune system, as demonstrated for instance by the presence of antitreponemal antibodies.

Treponemes for laboratory use are usually extracted from rabbit testes. As a consequence the treponeme suspensions are contaminated with rabbit components and possibly with substances produced during the infection. In 1984 it was shown by Hanff

et al[7] that density gradient centrifugation on Percoll gradients yielded suspensions of motile and virulent treponemes that were relatively free of host proteins.[7 8] The availability of this technique prompted us to compare the susceptibility to complement-dependent immobilisation of purified and unpurified treponemes, and to study the influence of the testicular extract on this susceptibility.

Material and methods

Propagation and extraction of T pallidum. Propagation and extraction of *T pallidum* was performed as previously described.[9] Briefly, the testes were minced and 1 ml of serum free basal reduced medium (BRM)[10] was added per gram of wet testicular tissue. The mixture was shaken for 45 minutes at room temperature in an atmosphere of 5% carbon dioxide and 95% nitrogen, and centrifuged for 10 minutes (800 × *g*) to sediment gross particulate matter. The fluid layer containing the treponemes was collected and part of it was used to prepare suspensions of "fresh" treponemes. The other part was centrifuged at 12000 × *g* at 4°C for 10 minutes to pellet the treponemes. The supernatant was carefully removed and saved. This centrifugation step reduced the number of treponemes in the supernatant to less than one per microscopic darkfield, a number that did not interfere with the final results of immobilisation experiments. The treponemes were resuspended in fresh BRM and subjected to Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation (43% Percoll in BRM) for 30 minutes at 37000 × *g* according to Hanff *et al.*[7] The layer containing the treponemes was collected and used to prepare suspensions of "Percoll" treponemes.

Enumeration of treponemes. The treponemes were counted using microslides (path length 0·05 mm, Camlab Limited, Cambridge, England, ref:5005) and the density of treponemes was calculated as previously described.[9]

Serum. One pool of human serum served as a complement source throughout this study. This pool was prepared from blood samples obtained from 50 donors all with a negative *T pallidum* Haemagglutination Assay (TPHA) test result. It was stored in small aliquots at −70°C. Samples used in the experiments were thawed only once. Serum from a patient with secondary syphilis (TPHA +, Venereal Disease Research Laboratory test 1:64, Fluorescent Treponemal Antibody-Absorbed test 3+) was used to isolate antitreponemal IgG (IgG(SII)), by DEAE-Sephadex-A 50 chromatography as described elsewhere.[11]

Modification of complement. Heat-inactivated serum was prepared by heating samples of the serum pool at 56°C for 30 minutes. Discrimination between the classical and the alternative pathway of complement activation was made by blockade of the classical pathway by the ethylene glycol-bis (beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) method for human sera. The efficacy of this procedure was demonstrated by the absence of lysis of optimally sensitised sheep red blood cells.[12] Proper functioning of the alternative pathway in the serum/EGTA buffer mixtures was verified by lysis of rabbit red blood cells as described by Platts-Mills and Ishizaka.[13]

Erythrocyte suspensions. Rabbit erythrocytes were washed three times in isotonic veronal buffer pH = 7·5 (VBS-buffer) and were used to prepare suspensions containing 1·5 × 10^8 erythrocytes per ml VBS buffer. Sheep erythrocytes suspended in Alsever's solution (Centraal Diergeneeskundig Instituut, Lelystad, The Netherlands) were washed three times in VBS-buffer containing 0·15 mM $CaCl_2$ and 1·0 mM $MgSO_4$ (VBS^{++}-buffer) and suspended at a density of 1 × 10^9 erythrocytes per ml. Optimal sensitisation was achieved with a 1:800 dilution of rabbit anti-sheep erythrocyte antibodies (Amboceptor, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands).

Immobilisation of treponemes. Suspensions of fresh treponemes were adjusted to a final density of 2 × 10^7 treponemes/ml, a final content of 10% v/v testicular extract and a final content of 25% v/v serum, by adding appropriate amounts of BRM and supernatant and serum pool. Mixtures containing 2 × 10^7 Percoll treponemes/ml and 5%, 10% and 20% v/v supernatant respectively were preincubated for 15 minutes. Subsequently 25% v/v serum was added. Aliquots of 0·5 ml of these mixtures were placed in small tubes, which were loosely plugged with cotton-wool and incubated in a reduced oxygen atmosphere at 34°C.[14] The percentage of mobile treponemes was determined in wet mounts after 0, 1, 2, 3·5, and 5·5 hours by observing at least 100 treponemes in randomly selected microscopic darkfields.

The immobilisation of Percoll treponemes by the classical and/or the alternative pathway of complement was studied as follows: mixtures of 25% v/v serum pool and 75% v/v VBS^{++} buffer and of 25% v/v serum pool and 75% v/v EGTA buffer were prepared. Similar mixtures were prepared using heat-inactivated serum samples. After 20 minutes at room temperature, an appropriate amount of Percoll treponemes was added to obtain a final density of 2 × 10^7 treponemes/ml. In control experiments it was verified that the Ca^{2+} ions present in the aliquot of Percoll treponemes did not abolish the blockade of the classical pathway by EGTA. Aliquots of these mixtures were stored and the percentage of mobile treponemes was determined after 0, 1, 2, 3, and 4 hours as described above.

Estimation of anti-complement capacity. The possible anti-complement activity of supernatants was inves-

tigated in a set-up analogous to the CH50 method for human sera.[12] Since BRM had been used in all immobilisation experiments, this buffer was used in these estimations. One ml of a 10-fold dilution of the serum pool caused approximately 50% haemolysis. This 10-fold dilution was used in the analysis of the effect of the supernatants on the complement-dependent haemolysis: 100 μl serum was mixed with 80 μl of the various supernatants and adjusted to 1·0 ml. This resulted in a serum:supernatant ratio of 5:4, representing the ratio that was used in the immobilisation experiments with the largest amount of supernatant. The haemolysis in these tubes was expressed as a percentage of the haemolysis obtained in the tubes with the 10-fold diluted serum.

Immunofluorescence. Immunofluorescence studies were performed on Percoll treponemes, which stick spontaneously to clean glass surfaces. The integrity of the outer membrane could be preserved by the addition of 0·025% v/v fetal calf serum (FCS) to the suspensions. Slides were prepared as follows: suspensions of Percoll treponemes were adjusted to 2×10^6 treponemes/ml and 0·025% (v/v) FCS was added. One ml was placed in a 35 mm plastic Petri dish (Costar) equipped with a clean coverglass. After centrifugation at $800 \times g$ for 10 min, the coverglasses were rinsed in BRM and were overlaid with two drops of IgG(SII) and incubated at room temperature for 30 min. After rinsing the coverglasses were overlaid with two drops of goat anti-human IgG, working dilution 1:50, labelled with fluorescein isothiocyanate (FITC, Nordic, Tilburg, The Netherlands) and incubated for 30 min at room temperature. Coverglasses were rinsed again and then placed upside down on microscopic slides and sealed with nail polish. They were read immediately. This procedure allowed for the adherence of approximately 70% of treponemes originally present. Absence of fluorescence with the monoclonal antibody (MoAb) CC 9,[15] which is directed against the axial filaments of the treponemes, was used as a control on the integrity of the outer membrane.[16] Microscopic equipment was as described previously.[11]

SDS-PAGE and Western Blotting. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting were performed using a mini-apparatus (Biorad, Richmond, California) according to the instructions of the manufacturer. Stacking and separating gels consisted of 5% and 12% acrylamide respectively. Approximately 4×10^7 Percoll-purified treponemes were suspended in sample buffer composed of 0·0625 M Tris-HCl (pH 6·8), containing 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and bromophenol blue as a tracking dye, and were heated in a boiling water bath for 4 min. These were electrophoresed until the dye front reached the bottom of the gel and the polypeptides were transferred to Immobilon PVDF membranes (Millipore, Bedford, Massachusetts) during 1 h. The membrane was cut into 20 strips, resulting in approximately 2×10^6 solubilised treponemes per strip. Strips were incubated for 2 hours in a 200-fold dilution of the serum pool and a 50-fold dilution of the supernatants respectively to detect IgG anti-treponemal antibodies in these fluids. Anti-treponemal antibodies associated with the treponemes were detected with affinity-purified goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, Alabama). Immunochemical staining was visualised by incubation for 2 h with the appropriate gold-labelled conjugates, followed by silver enhancement. Direct staining of the polypeptides was performed with Aurodye Forte. Blocking of non-specific protein-binding sites of the membranes, preparation of primary antibody and conjugate dilutions and of the washing solutions were done as recommended by the manufacturer (Janssen Life Sciences Products, Beerse, Belgium). Non-reactivity of the conjugates with the treponemal polypeptides was controlled by incubation in PBS/Tween 20 (0·05%) instead of primary antibody, followed by the appropriate conjugate. The low-molecular weight standards from Sigma (St Louis, USA) were used in estimating the size of the treponemal polypeptides.

Statistical analysis. In the evaluation of results, Spearman's correlation coefficient was used.

Results

Investigations into the complement-dependent immobilisation of the fresh treponemes using human serum showed a gradual time-dependent decline in the mobility of the treponemes (fig 1). After 5·5 h of incubation, approximately 40% of the treponemes was still mobile. On the other hand, the Percoll treponemes had lost their mobility almost completely after 2 h of incubation. The controls with heat inactivated serum showed a good mobility of the treponemes: the mobility of the fresh and Percoll purified treponemes was more than 96% after incubation for 5·5 h (not shown). This demonstrates the complement-dependent nature of the immobilisation.

Pre-incubations of the Percoll treponemes with autologous supernatants of rabbit testes caused a rescue in their complement-dependent immobilisation. As shown in fig 1, immobilisation of the treponemes in the supernatant-containing samples progressed at a similar rate as that of the fresh treponemes. After the incubation period of 5·5 h the mean percentages of mobile treponemes were 24%, 30% and 32% in the samples containing 5%, 10% and 20% supernatant respectively. This indicates a slight dose-dependent influence of the supernatants.

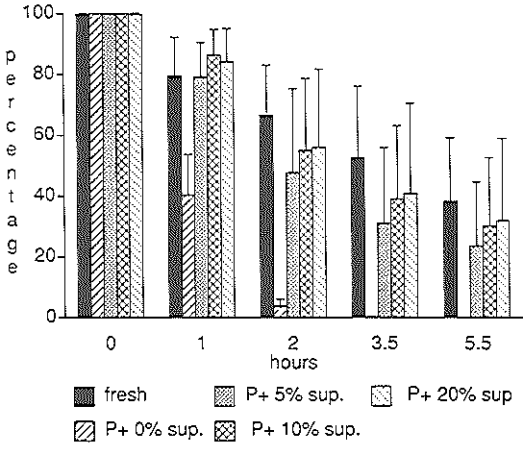

Figure 1 Complement-dependent immobilisation of fresh and Percoll-purified (P + 0%) treponemes and the effect of 5% (P + 5%), 10% (P + 10%) and 20% (P + 20%) supernatant from infected testes on the immobilisation of the Percoll-purified treponemes. Human serum was used as a source of complement. Results are expressed in percentage mobile treponemes. Means and standard deviations of six treponeme suspensions isolated from different rabbits are shown.

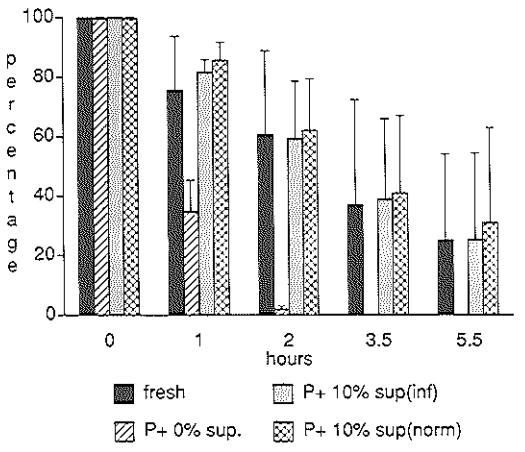

Figure 2 Complement-dependent immobilisation of Percoll-purified treponeme suspensions containing 10% supernatant from infected (P + 10% sup (inf)) or uninfected (P + 10% sup (norm)) rabbit testes and Percoll-purified (P + 0% sup) and fresh treponemes. Results are expressed in percentage mobile treponemes. Means and standard deviations of three treponeme suspensions isolated from different rabbits are shown.

Again, the treponemes incubated with heat-inactivated serum had a mobility of at least 96% in all supernatant-containing mixtures after 5·5 h of incubation.

Experiments in which the preincubations were performed with supernatants prepared from uninfected rabbit testes (fig 2) demonstrated that these supernatants interfered similarly with the immobilisation of the Percoll treponemes as did the autologous supernatants from infected rabbit testes. The Percoll treponemes pre-incubated with supernatants from uninfected rabbit testes showed at all times a slightly higher percentage of mobile treponemes than Percoll treponemes pre-incubated with supernatants from infected testes.

The results after blocking the classical complement pathway are shown in fig 3. When untreated serum was used as complement source the Percoll treponemes were rapidly immobilised. However, when EGTA-serum was used the immobilisation of the Percoll treponemes progressed at a much slower rate. When heat-inactivated serum or heat-inactivated EGTA-serum was used, the Percoll treponemes survived well during the observation period of 4 h. This demonstrates that removal of Ca^{2+}-ions was not responsible for the immobilisation of the treponemes during the observation period. Since an effective blockade of the classical pathway and a proper functioning of the alternative pathway in EGTA-serum was verified, the absence of rapid immobilisation of the Percoll treponemes demonstrates that the classical pathway is the major route by which the treponemes are immobilised. However, the slow immobilisation in EGTA-serum samples indicates that the alternative pathway can also contribute to the immobilisation of the Percoll treponemes.

It was realised that the delay in immobilisation caused by the supernatants could be based on anti-complement action of the latter. The addition of supernatants used in fig 1 to the complement source lowered its haemolytic capacity to 77·8, SD 4·4% of its capacity without supernatant. With the supernatants from uninfected testes the result was 84·6, SD 6·0%. However, the expectation that a less effective complement cascade would result in less rapid immobilisation of the Percoll treponemes could not be proven by statistical analysis: no correlation was found between these two parameters at any of the times indicated in fig 1.

In immunofluorescence investigations with MoAb

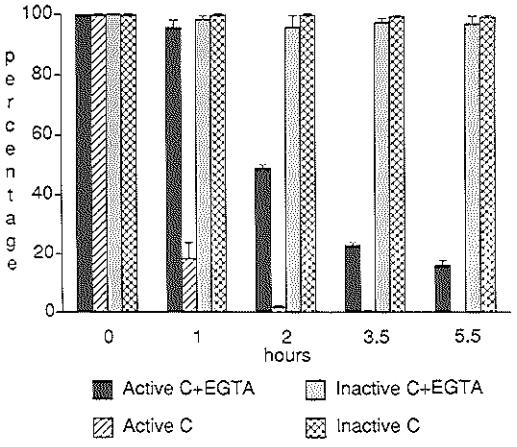

Figure 3 Complement-dependent immobilisation of Percoll-purified treponemes in untreated serum (active C), serum treated with EGTA to block the classical pathway (active C + EGTA) and the heat-inactivated counterparts (inactive C and inactive C + EGTA respectively). Results are expressed in percentage mobile treponemes. Means and standard deviations of three suspensions isolated from different rabbits are shown.

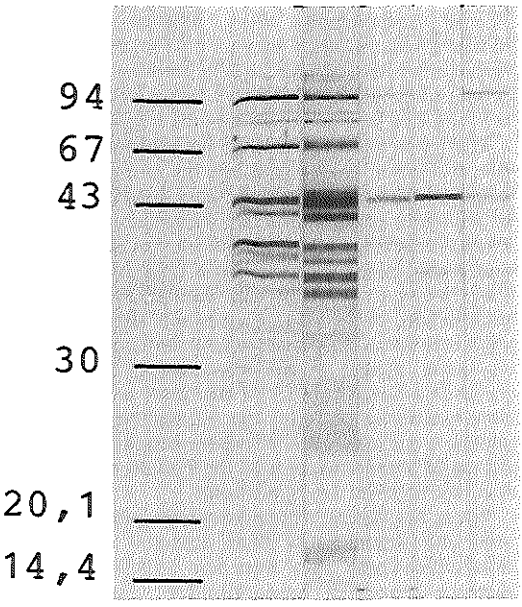

Figure 4 Western immunoblot analysis of antibodies against treponemal antigens in the serumpool and testicular supernatants. Solubilised treponemes purified by Percoll density centrifugation were used. From left to right: lanes 1–5. Lane 1: Treponemal polypeptides stained with Aurodye to visualise all polypeptides. Lane 2: Treponemal polypeptides stained with IgG (SII), followed by gold-labelled goat anti-human IgG and silver enhancement. Lane 3: Treponemal polypeptides stained with 1:200 diluted human serum pool, and stained with gold-labelled goat anti-human IgG, followed by silver enhancement. Lanes 4 and 5: Two representative examples of treponemal polypeptides incubated with 1:50 diluted supernatants from infected rabbit testes and stained with gold-labelled goat anti-rabbit IgG, followed by silver enhancement. The location of the molecular mass markers is indicated on the left.

CC 9, no fluorescence was observed. This points to an intact outer membrane. With the polyclonal IgG (SII) preparation, over 95% of the Percoll treponemes showed a weak positive fluorescence, often of a speckled character. This demonstrates the accessibility of the outer membrane of the Percoll treponemes to antibodies.

The presence of IgG class antibodies in the supernatants, in the human serum pool and on the Percoll-purified treponemes was investigated by means of SDS-PAGE and Western Blotting (fig 4). Antitreponemal IgG antibodies were readily detected in the serum pool and the supernatants, of which two examples are shown (lanes 4,5). IgG antibodies strongly reactive to 44 kD treponemal polypeptides were common in these fluids. In addition, IgG antibodies to 94, 78, 67, and 39 kD polypeptides were detected in the human serum pool. The supernatants contained IgG antibodies to 94, 39 and 36 kD polypeptides. Detection of antibodies on the Percoll treponemes was less clearcut. Only occasionally very faint indications of anti-IgG conjugate-stained polypeptides of molecular mass of 50 kD, corresponding to the heavy chain of IgG, were observed (not shown).

Discussion

A large part of the hypothesis that *Treponema pallidum* is covered by a protective extra-cellular layer has been deduced from serological reactions in which live treponemes are used, for example the *Treponema Pallidum* Immobilisation (TPI) reaction. During the long incubation times needed to immobilise the treponemes, the microorganisms are supposed to lose their protective cover, making them susceptible to the combined action of anti-treponemal antibodies and complement. The availability of a technique to purify the treponemal suspensions from the rabbit components offered the opportunity to compare the periods of time needed for the immobilisation of purified and unpurified microorganisms. The purification of the treponemes drastically changed their behaviour towards complement-dependent immobilisation: after 2 h the

Percoll treponemes were almost completely immobilised, while a mean of 67% of the fresh treponemes still showed good mobility. A slowly progressing immobilisation of fresh treponemes in seronegative sera had been demonstrated before.[17]

However, to our knowledge the rapid immobilisation of Percoll treponemes has not been previously demonstrated. Our results indicate that after removal of testicular components the treponemes are much more vulnerable to the combined action of antibodies and complement. Some authors have hypothesised that, owing to some special features innate to the architecture of the outer membrane, there is only a sparse presence of proteinaceous antigenic sites on the outer surface of the treponemes. This would result in a limited ability of the outer membrane to bind antibodies and give the treponemes resistance against a complement-dependent attack.[6] However, the rapid immobilisation of the Percoll treponemes does not support this hypothesis.

When the Percoll treponemes were pre-incubated with the supernatants from infected as well as uninfected testes, they were less sensitive to complement-dependent immobilisation. This demonstrates: firstly that a delay in immobilisation caused by supernatants is possible, and secondly that this delay is not dependent on the presence of infection in the testes from which the supernatant was derived. This rules out a role of components present in the testicular tissue, produced as a result of the treponemal infection. Previously, it was demonstrated that a treponemal infection in the rabbit testes is accompanied by the production of a large amount of hyaluronic acid, which is easily extracted from the minced testicular tissue.[9] This compound, together with other acid mucopolysaccharides is often regarded as a substance which could possibly provide protection to the treponemes.[2-3 9 18-20] The present experiments make a major role of hyaluronic acid in the delay of complement-dependent immobilisation unlikely.

This delay caused by the supernatants could be accomplished in several ways:

Firstly, the supernatants could have an anti-complement action and, particularly relevant to our studies, lower the effectivity of the classical pathway of the activation of complement. A limited reduction in the capacity of the serum pool to haemolyse optimally sensitised sheep erythrocytes in the presence of supernatants was demonstrated. This shows an anti-complement capacity of the supernatants used. However, it is doubtful whether this reduction in complement level can explain the delay in immobilisation caused by the supernatants. It would be expected that, the more markedly the complement level is affected, the larger the delay in immobilisation of the treponemes should be. However, there was no correlation between the data to support this hypothesis. Therefore, a definite conclusion has to await experiments which demonstrate that it is possible to separate the component(s) delaying the immobilisation of the treponemes from those affecting the complement level. A second possibility is that treponemal antigens present in the supernatants obtained from infected rabbit testes can occupy binding places of antibodies present, thereby inhibiting the binding to the treponemes, thus influencing immobilisation characteristics. However, this possibility seems unlikely because in experiments in which supernatants from uninfected rabbit testes were used a similar level of delay in immobilisation was observed. A third possibility is that the delay in immobilisation of the purified treponemes is accomplished by the occupation of relevant antigenic sites on the treponemal outer membrane by components present in the supernatants. This would prevent formation of antigen-antibody complexes on the treponemal surface and in turn prevent the initiation of the classical complement cascade. The accessibility of the outer membrane of the Percoll treponemes to the anti-treponemal IgG (SII) antibodies was demonstrated by immunofluorescence studies. This is in contrast with fresh treponemes: earlier findings on the accessibility of the outer membrane of the fresh treponemes adhering to cultured fibroblast or layers of fibronectin, showed a fluorescence with a similar IgG preparation of only a limited percentage.[16] This difference in access of antibodies is compatible with the rapid, complement-dependent immobilisation of the Percoll treponemes as compared to that of the fresh treponemes.

As was discussed before by Fitzgerald, it is difficult to indicate which antibodies take part in the in vitro complement-treponeme interactions.[18] Freshly harvested, Percoll-purified treponemes still carry rabbit IgG class antibodies on their surface.[8] These antibodies might already have been present before the treponemes were harvested or have been collected during the extraction procedure. Our demonstration of anti-treponemal IgG class antibodies in Western blots of solubilised Percoll treponemes was doubtful. However, among the IgG class antibodies common to the supernatants and the human serum pool, there were antibodies directed to the treponemal polypeptides contained in the 44 kD region. Polypeptides of this size have been implicated as the surface antigens involved in the immobilisation of the treponemes. Murine MoAbs against 44 kD polypeptides have been shown to be capable of immobilisation.[21] The participation of the antibodies to the remaining polypeptides in this respect remains to be established.

In conclusion, it has been demonstrated that Percoll treponemes are rapidly immobilised by activation of the classical complement pathway.

Delay in this immobilisation can be provided by rabbit testicular components present in the extraction fluids. Further experiments will have to elucidate whether this delay is based on an anti-complement capacity of these extraction fluids or whether components in these fluids combine with the outer membrane of the purified treponemes in reconstructing a protective extra-cellular layer. In the latter case the present experimental design will permit an analysis of the substances that participate in the formation of this layer.

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5.2.

THE INFLUENCE OF DIFFERENT SERA ON THE IN VITRO IMMOBILISATION OF PERCOLL PURIFIED *TREPONEMA PALLIDUM*, NICHOLS STRAIN

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The influence of different sera on the in vitro immobilisation of Percoll purified *Treponema pallidum*, Nichols strain

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Abstract

***Objectives*—Investigation of sera, especially rabbit serum, in preventing in vitro immobilisation of Percoll purified *T. pallidum*.**

***Materials and methods*—The immobilisation of Percoll purified *T. pallidum* (Nichols) was studied after pre-incubations with basal reduced medium (BRM), heat-inactivated serum of seven different species of animals, heat-inactivated normal human serum (NHS) and rabbit sera containing a different level of antitreponemal antibodies. Also increasing percentages of heat-inactivated normal rabbit serum (NRS) were studied.**

***Results*—The rapid immobilisation of purified treponemes by NHS is delayed by pre-incubation with NRS in a dose-dependent manner. The treponemes from 5-day infections were immobilised significantly more slowly than treponemes from 7- and 8-day infections. Compared with NRS, pre-incubations with a high-titred, low-titred and "autologous" serum resulted in significantly more rapid immobilisation of the treponemes. With most other animal sera resistance to immobilisation was slight compared with that produced by NRS. Immunofluorescent studies revealed that the treponemes were covered with a layer of the human third complement factor (C3b), within an hour of incubation. With two sequential pre-incubations, a delay of the immobilisation was only noted in those test mixtures in which NRS had been present in both pre-incubations.**

***Conclusion*—Rabbit serum delays the rapid in vitro immobilisation of Percoll purified treponemes by normal human serum. There was no evidence that this was caused by preventing access of antibodies (in vivo as well as in vitro) to, or preventing the activation of complement on, the treponemal surface. The evidence points to a mechanism in the fluid phase, suggesting participation of a third factor in the immobilisation process, for instance an enzyme, which can be partially inhibited by rabbit serum component(s).**

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Introduction

In vitro immobilisation of treponemes by antibodies and complement takes a long time: complete killing of treponemes by high-titred immune serum requires 16 h.[1] Treponemes may escape rapid destruction by the presence of a protective cover around the microorganisms. This cover may consist of mucopolysaccharides[2 3] or several serum proteins[4] and may prevent access of antibodies to the antigens located on the treponemal outer membrane. A paucity of antigens on the treponemal surface, as was recently demonstrated[1 5] may add to the ability of the treponemes to survive.

Treponemes for laboratory use are usually extracted from rabbit testes. As a consequence the treponeme suspensions are contaminated by rabbit components and possibly by substances produced during the infection. With Percoll density gradient centrifugation it is possible to obtain suspensions of motile and virulent treponemes relatively free of host proteins.[6 7] It was observed that Percoll purified treponemes were immobilised more quickly than unpurified treponemes in the presence of antibodies and complement.[8] After the addition of testicular extracts, purified treponemes became more resistant to immobilisation. Addition of testicular extracts originating from infected or uninfected rabbits made no difference, so that a possible role of substances produced under the influence of the infection was ruled out. However, it was not possible to determine whether this delay in immobilisation was due to an anti-complement activity of testicular extracts, or to a re-establishment of a protective cover around the treponemes.[8]

The ability of the treponemes to resist rapid immobilisation in the presence of rabbit testicular extracts on the one hand,[8] the presence of serum proteins in these extracts[9] and the close association of some of these proteins with the treponemal surface[4] on the other, prompted us to study a possible inhibitory effect of sera, particularly rabbit serum, on the immobilisation of purified *T. pallidum*, Nichols strain.

Material and methods

Propagation and extraction of T. pallidum. *T. pallidum* (Nichols) was maintained by intratesticular transfer in male New Zealand White rabbits.[9] At the time of inoculation, all animals had a negative Venereal Disease Research Laboratory (VDRL) test, *Treponema pallidum* Haemagglutination Assay (TPHA) and Fluorescent Treponemal Antibody-Absorbed (FTA-Abs) test. Treponemes were harvested 7

or 8 days after inoculation, unless otherwise stated. Extraction, Percoll purification and enumeration of *T. pallidum* were performed.[9]

Human serum pool. A pool of normal human serum (NHS) served as a complement source throughout this study. This pool was prepared from blood obtained from 150 donors with negative TPHA test results.

Rabbit serum pool. A pool of rabbit serum (NRS) was derived from 15 rabbits with negative VDRL, TPHA and FTA-Abs test results.

Both pools were stored in small aliquots at −70°C. Samples used in the experiments were thawed only once. Heat-inactivated samples were also used for the pre-incubation step of the treponeme suspensions.

Other animal sera. A serum sample with a TPHA titre of 1:10240, a VDRL titre of 1:8 and a 3+ FTA-Abs test result was obtained from a rabbit previously infected with *Treponema pallidum* (high-titred serum); a serum sample with a TPHA titre of 1:80 and negative VDRL and FTA-Abs results was obtained from a rabbit suffering from a *Treponema cuniculi* infection (low-titred serum). "Autologous" serum samples were also taken from the infected rabbits, on the day they were killed for extraction of the treponemes.

The following sera were also tested; sera from a pig (cross-breeding of Danish country pig and Yorkshire pig), a Syrian golden hamster, a goat (outbred goat), a mouse (C 57 black mouse), a dog (Beagle), a rat (Wistar) and a guinea-pig (Dunkin-Hartley). To exclude possible variation between animals of the same species, blood was taken from five representatives of four species of animals (pig, rabbit, dog and rat).

Heat inactivation of sera. The total complement activity of the sera, used for pre-incubation was destroyed by heating samples at 56°C for 30 minutes.

Estimation of complement activity. Optimally sensitised sheep erythrocytes were prepared. Before use in the immobilisation experiments, mixtures of NHS and NRS were tested for their haemolytic capacity in a set-up analogous to the CH_{50} method, using BRM as diluent.[8] The ratio of NRS to NHS in these mixtures was 2:2·5, being twice the ratio that was used in the immobilisation experiments (see below). The haemolytic capacity of these mixtures was compared with similar mixtures of BRM and NHS. The presence of residual haemolytic complement capacity in 22 h samples used in the immobilisation experiments was again tested by their ability to lyse the sensitised sheep erythrocytes.[10]

Immobilisation of treponemes in the presence of pre-incubation serum. Percoll treponemes were used in a final density of 2×10^7 treponemes per ml. A sufficient number of treponemes were pre-incubated with basal reduced medium (BRM),[11] dithiothreitol being omitted, or with the heat-inactivated human or various animal sera (final content 10%, unless otherwise stated) for 15 minutes. Subsequently NHS was added to a final content of 25% (v/v). Aliquots of 0·5 ml of these mixtures were placed in small tubes, which were loosely plugged with cottonwool and incubated in a reduced oxygen atmosphere (4%) at 34°C.[12] The percentage of mobile treponemes was determined in wet mounts after 0, 1, 2, 3·5 and 5·5 hours by observing at least 100 treponemes in randomly selected microscopic darkfields.

Immobilisation of treponemes after removal of pre-incubation serum. Two sequential pre-incubations of 15 min each of the Percoll purified treponemes were performed. After pre-incubation with NRS or BRM, the suspensions were centrifuged at 12 000 × *g* at 4°C for 10 min. The supernatant was removed. A part of the treponemes, which had been pre-incubated with NRS were further pre-incubated with NRS, the other part with BRM. Furthermore, treponemes were sequentially pre-incubated in BRM. After adding NHS as the complement source, the mixtures were processed as described above.

Fluorescence. After incubation periods of one and two hours in the immobilisation experiments, 10 ml phosphate-buffered saline (PBS) was added to 0·5 ml of the test mixtures. After centrifugation at 12 000 × *g* at 4°C for 10 minutes, the supernatant was removed and the pellet was suspended in 1 ml BRM. This suspension was pipetted into a 35 mm plastic Petri dish (Costar) equipped with a clean cover glass. After centrifugation at 800 × *g* for 5 minutes, the cover glasses were rinsed in BRM and overlaid with two drops of the FITC labelled IgG-fraction of a goat anti-human C3b antiserum (working dilution 50×) (Centraal Laboratorium van de Bloedtransfusiedienst, Amsterdam) and incubated at room temperature in a clean Petri dish for 30 min. After rinsing in BRM the cover glasses were placed upside down on microscopic slides and sealed with nail polish. They were read immediately. The optical system has been described.[13]

Statistical analysis. Wilcoxon's tests for paired and unpaired observations was used in the evaluation of results.

Results

Effect of NRS and NHS on immobilisation. Pre-incubation of the Percoll purified 7- and 8-days treponemes with BRM and subsequent addition of NHS as complement source resulted in rapid immobilisation. As shown in fig 1, almost all treponemes had lost their mobility after two hours. After pre-incubation with heat-inactivated NRS followed by addition of NHS, two-thirds of the treponemes retained their mobility after 2 hours, and after 5·5 hours over one-half of the treponemes showed good mobility in the presence of complement. In contrast, after pre-incubation with heat-inactivated NHS, followed by incubation with NHS, only 1·5% of the treponemes retained their mobility after 2 h (fig 1). Longer pre-incubation periods of up to three hours with heat-inactivated NHS did not change this result. The haemolytic capacity of the complement source was not impaired by addition of heat-inactivated rabbit serum: the presence of double the amount used in the immobilisation experiments did not change the CH_{50} level of

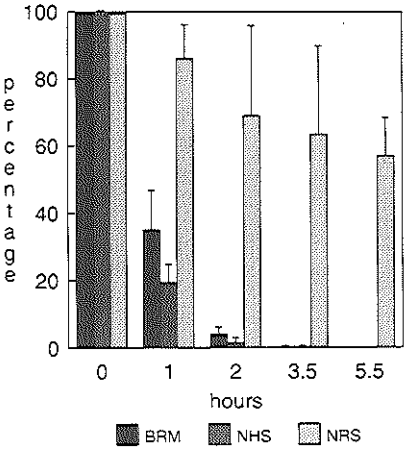

Figure 1 Immobilisation of Percoll purified treponemes; characteristics of preincubations with BRM, and inactivated normal human serum (NHS) versus inactivated normal rabbit serum (NRS). Normal human serum was used as a complement source throughout these experiments. Results are expressed in percentage of mobile treponemes. Means and standard deviations of the results of experiments with six treponemal suspensions originating from six different rabbits are shown.

the NHS pool in the lysis of optimally sensitised sheep erythrocytes (not shown).

Influence of the dose of NRS on immobilisation. Figure 2 shows the mean percentages of mobile treponemes at the various times after the treponemes had been pre-incubated with different amounts of NRS. Again, the treponemes pre-incubated in the absence of NRS had lost their mobility almost completely after 2 h. At this time the mean percentages of mobile treponemes in the NRS-containing reaction mixtures varied from 17% in mixtures containing 0·3% (v/v) NRS to 97% in mixtures containing 20% (v/v) NRS.

Influence of other animal sera on immobilisation. Pre-incubations of purified treponemes with the heat-inactivated sera from single representatives of seven different animal species all resulted in a delay of the immobilisation as compared with pre-incubations with heat-inactivated NHS. The effects of the hamster, goat, mouse, rat and guinea pig sera were slight. The dog serum had a stronger effect. The strongest effect was displayed by the pig serum (table 1). Results observed in experiments with sera from groups of five rabbits, five pigs, five dogs and five rats were the following. All five rabbit sera gave a strong inhibition of the rapid immobilisation, while the inhibition provided by the five rat sera was weak. The dog sera demonstrated an intermediate result. However, the pre-incubations with the sera of a group of five pigs gave varying results: the sera from two pigs provided strong resistance to immobilisation of the treponemes and with the three remaining sera the inhibition of immobilisation varied from moderate to very weak. All test mixtures in this set of experiments contained residual haemolytic complement activity. After 22 h of incubation the test mixtures were still able to haemolyse sensitised sheep erythrocytes.

Mobility of the treponemes and the presence of C3b on their surface. Immunofluorescent studies revealed that all treponemes in the experiments with the sera from the groups of animals were covered with a layer of the human third complement factor (C3b) within an hour of incubation. No relation was found between the rate of immobilisation and the presence of C3b on their surface after one or two hours.

Influence of duration of infection on immobilisation. As is shown in fig 3, Percoll-purified treponemes harvested after 5 days of infection and pre-incubated in BRM were immobilised significantly less rapidly than similarly treated treponemes, harvested after 7 or 8 days ($2\alpha \leq 0{\cdot}05$). There was no difference in rate of immobilisation of purified treponemes harvested after 7 or 8 days at any of the times indicated in fig 3. Pre-incubation of the 5-, 7- and 8-day treponemes with heat-inactivated NRS diminished their rate of immobilisation as compared with their counterparts which had been pre-incubated with BRM. The strongest effect was noted with 5-day treponemes. At all time points a significant difference was observed between 5-day treponemes on the one hand, and 7- and 8-day treponemes on the

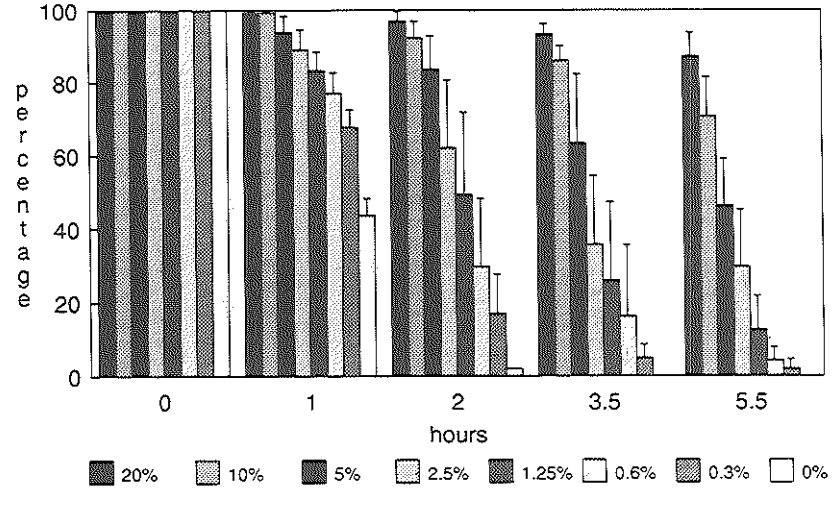

Figure 2 Treponemal mobility after preincubation with eight different percentages of NRS (20%–0%) are demonstrated. Results are expressed in percentage of mobile treponemes. Means and standard deviations of the results of experiments with three suspensions are shown.

Table 1 Immobilisation of Percoll purified Treponema pallidum after pre-incubation with sera from different species of animals. The mean percentages of mobile treponemes with the serum of one representative of each species, tested against three different treponeme suspensions are shown (range in parentheses)

	2 hours	5·5 hours
Pig	72·3 (55–89)	54·7 (34–71)
Hamster	5·7 (2–11)	1·0 (0–3)
Goat	18·0 (15–21)	4·3 (4–5)
Mouse	6·3 (3–10)	0·3 (0–1)
Dog	29·3 (19–44)	11·3 (5–17)
Rat	11·3 (2–25)	0·7 (0–1)
Guinea-pig	10·3 (8–18)	0·3 (0–1)
Human	2·0 (2–2)	0·0

other ($2\alpha \leq 0{\cdot}05$). No differences were seen between the 7- and 8-day treponemes (fig 3).

The effect of the level of anti-treponemal antibodies in the pre-incubation sera on immobilisation. Figure 4 shows that all sera used caused a delay in the immobilisation of the treponemes as compared with BRM. However, compared with the normal rabbit serum pool, the pre-incubations with the high-titred-, low-titred- and "autologous" serum resulted in a significantly more rapid immobilisation of the treponemes ($2\alpha \leq 0{\cdot}05$). The treponemes were immobilised significantly more slowly in low-

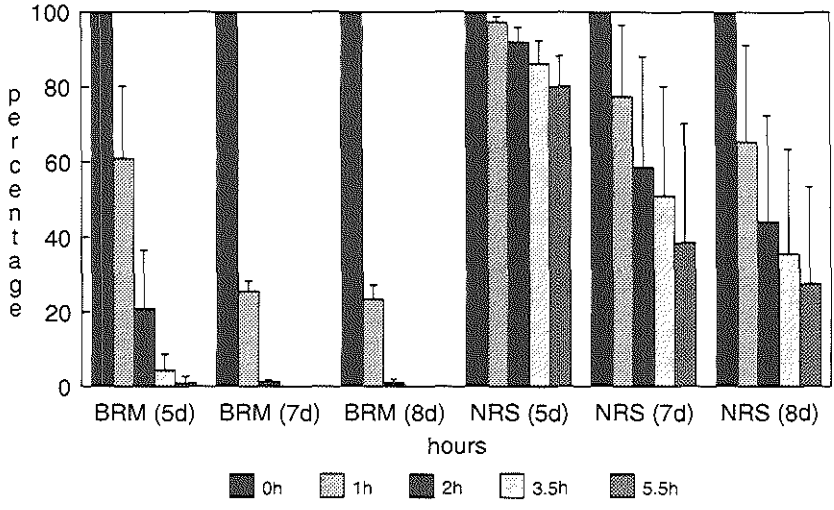

Figure 3 Immobilisation of Percoll purified treponemes using T pallidum harvested after different periods of infection (5, 7 or 8 days). Results are expressed as percentage of mobile treponemes. Means and standard deviations of the results of experiments with five suspensions obtained from different rabbits for each period of infection are shown.

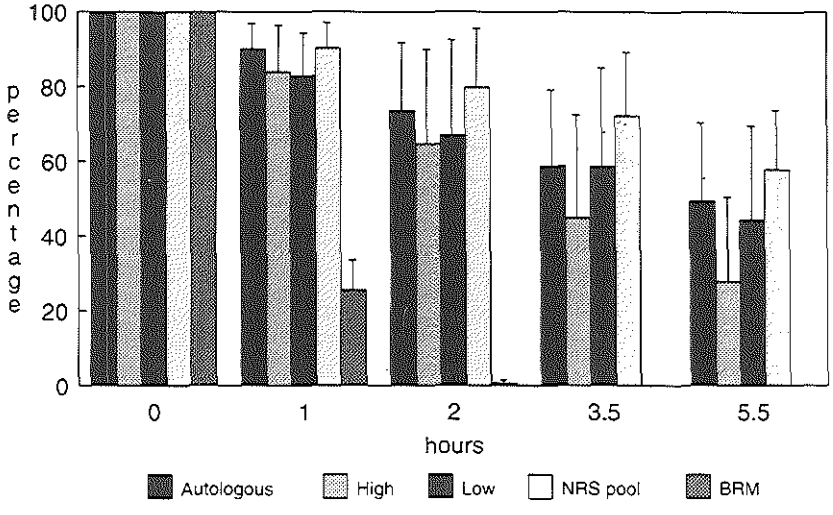

Figure 4 Percentage of mobile treponemes after preincubation with different heat-inactivated rabbit sera or BRM only, before addition of normal human serum as a complement source. Means and standard deviations of results of experiments with six treponeme suspensions isolated from different rabbits are shown.

Table 2 Mean percentage (range in parentheses) of mobile treponemes after two sequential preincubations with NRS followed by NRS; with NRS followed by BRM; and BRM followed by BRM, before the incubation with NHS, as determined with 5 different treponeme suspensions

	2 hours	5·5 hours
NRS/NRS	50·0 (25–81)	20·8 (7–34)
NRS/BRM	2·2 (0–9)	0
BRM/BRM	0·4 (0–2)	0

titred serum and in "autologous" serum than in high-titred serum ($2\alpha \leq 0{\cdot}05$).

Influence of removal of pre-incubation serum on immobilisation. In table 2 it is shown that the treponemes which were pre-incubated in BRM in both pre-incubation steps were immobilised after 2 h. The same result was noted for treponemes which were pre-incubated with NRS in the first and with BRM in the second pre-incubation step. However, after pre-incubation of the purified treponemes with heat-inactivated NRS in both pre-incubations a delay in the immobilisation was observed. After 2 h a mean mobility of 50% was noted.

Discussion

In a previous study we demonstrated that the rapid immobilisation of Percoll purified treponemes follows activation of complement along the classical pathway.[8] This suggests the participation of antibodies in the form of antigen-antibody complexes on the outer surface of the treponemes, which then initiates the classical complement cascade. The complement level of the human serum pool, measured as its haemolytic capacity by the CH_{50} method, was not changed by adding heat-inactivated NRS. Furthermore, the test mixtures used to study the immobilisation of the treponemes contained residual complement after 22 h of incubation, as was shown by their capacity to lyse sensitised sheep erythrocytes. As a consequence, neither an anti-complement effect nor a lack of complement was responsible for the delay in immobilisation of the treponemes pre-incubated in NRS, as compared with those pre-incubated in BRM or NHS. The inability to form antigen-antibody complexes may relate to a protective cover on the outer surface of the treponemes, preventing access of antibodies. The antibodies involved in immobilisation are derived from cross-reacting antibodies in the human serum pool used as the complement source, antibodies present on the surface of the treponemes, which were not removed by the purification procedure and/or antibodies present in the sera used in the pre-incubation steps.

In studying the possible role of antibodies that are carried over, we hypothesised that a shorter period of infection would lead to fewer antibodies on the treponemal surface, since Hanff *et al* have demonstrated that after infection of the rabbits, serum antibodies with anti-treponemal specificities increase from day 3 onwards.[14] Indeed, we noted that Percoll purified treponemes from 5-day infections were immobilised significantly more slowly than the purified treponemes from 7- and 8-day infections in otherwise identical reaction mixtures. This makes it likely that "carry over" antibodies may participate in the in vitro immobilisation of the purified treponemes and suggests that in the infected rabbit the newly formed antibodies may have access to the treponemal surface. This accords with the findings of Blanco *et al*.[15]

The study of the role of antibodies in the sera used for pre-incubation showed that pre-incubations with all three types of anti-treponemal antibody containing sera resulted in a significant delay in the immobilisation as compared with pre-incubations with BRM, but produced a significant acceleration of the immobilisation as compared with pre-incubations with the seronegative rabbit serum pool. This shows that anti-treponemal antibodies in the serum used for pre-incubation can participate in the immobilisation of the treponemes and demonstrates that the surface of the Percoll treponemes is accessible to antibodies, despite the presence of rabbit serum proteins. Taken together, these results indicate that it is unlikely that the rabbit sera provide a resistance to in vivo or in vitro immobilisation by formation of a physical barrier to antibodies.

Immunofluorescence revealed that irrespective of the sera used for pre-incubation, the treponemes were covered with a layer of human C3b after an hour of incubation with NHS. This shows firstly that the antibodies on the treponemal surface can act as initiators of the complement cascade despite the presence of rabbit serum proteins. Secondly this shows that a considerable part of the treponemes, pre-incubated in a serum able to provide resistance against rapid immobilisation, can survive in vitro for at least several hours despite the activation of complement on their surface. These findings are partly in agreement with those of a recent study in which it was demonstrated that antibody binding and complement-mediated immobilisation of unpurified treponemes were not the rate-limiting steps in the immobilisation of treponemes.[15] In that study the prolonged time required for in vitro immobilisation of the unpurified treponemes was related to the limited rate of complement activation. Aggregation of the treponemal rare outer membrane protein (TROMP) occurred in the presence of antibodies during in vitro incubation and was thought to be necessary to make binding of the first complement component by the antibodies possible.[15] The Percoll purified treponeme suspensions we used are relatively free of host proteins and rapidly activated complement, as was demonstrated by the presence of a C3b layer around the treponemes after an hour of incubation with NHS. It appears, therefore, that the purification procedure modifies the treponemal surface in such a way that it becomes capable of rapid binding of the first complement component, possibly by allowing a more rapid aggregation of the TROMP. Although this may explain the rapid activation of complement, it fails to explain the prolonged time needed for immobilisation of the purified treponemes after

a pre-incubation with sera, which delay the immobilisation.

After performing two sequential pre-incubations of the purified treponemes a decrease of the immobilisation rate was noted only in the test mixtures in which NRS had been present in both pre-incubations. The treponemes in those test mixtures in which BRM had been present in both pre-incubations or only in the second one were immobilised at the same (rapid) rate. This shows the necessity of presence of rabbit serum proteins in the reaction mixture to obtain a delay in the immobilisation and suggests that the inhibition of the immobilisation is caused by some mechanism that operates in the fluid phase. One possibility is that the rabbit serum proteins interact with the late-acting human complement components to prevent either their formation or their lytic effect. However, this seems unlikely, since sensitised sheep erythrocytes were lysed by the NHS/NRS mixtures. A second possibility is that an enzyme system needed for the immobilisation is competitively inhibited by the combination of human and rabbit serum. The participation of an enzyme in the immobilisation is not unprecedented. Müller *et al* have shown that in vitro immobilisation of unpurified treponemes does not occur in the complete absence of lysozyme.[16] It is not known whether additional enzymes play a role in the immobilisation of the treponemes. It might be possible that the dose dependent delay of immobilisation by NRS originates from an inhibitory effect on such (an) enzyme(s).

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5.3.

THE IMPORTANCE OF DIFFERENT COMPONENTS OF NORMAL HUMAN SERUM AND LYSOZYME IN THE RAPID IMMOBILISATION OF PURIFIED *TREPONEMA PALLIDUM*, NICHOLS STRAIN

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E Stolz, and J J van der Sluis

Submitted for publication

Abstract

Objectives - To study the role of different components in normal human serum and of lysozyme in rapid immobilisation of Percoll purified *T.pallidum*.

Materials and methods - The immobilisation of Percoll purified *T.pallidum* was studied after pre-incubations with different serum fractions (Fr) of normal human serum (Fr 1, containing IgM; Fr 2, containing IgG and a low level of haemolytic complement, and Fr 1 (abs), depleted of IgG). A guinea-pig serum pool was used as a complement source in the immobilisation experiments. The influence was studied of removal of lysozyme from guinea-pig serum on the immobilisation reaction. Further experiments were performed, using a fluorescence technique, to detect C3b depositions on fixed treponemes and treponemes in suspension.

Results - Rapid immobilisation of Percoll-purified treponemes by the NHS serum fractions occurred only after pre-incubation with Fr 1 and Fr 2 simultaneously. This was largely dependent on the presence of a small amount of haemolytic C in Fr 2. Removal of lysozyme reduced this rapid rate of immobilisation. In fluorescence experiments it was demonstrated that C3b deposition on fixed (i.e. damaged) treponemes occurred upon their incubation with Fr 2 or the combination of Fr 1 and 2. However, on treponemes in suspension C3b deposition occurred only after incubation with the combination of Fr 1 and 2.

Conclusion - The rapid immobilisation of Percoll purified treponemes by serum fractions from normal human serum requires antibodies of the IgM and IgG class, together with complement and lysozyme. Omission of one of these reactants slows immobilisation. Our experiments suggest that the reactants act in sequence: the loss of integrity of the outer membrane by an attack by IgM and C offers the opportunity for lysozyme to hydrolyse the peptidoglycan layer surrounding the cytoplasmic membrane of the treponemes, which then is accessible for attack by antibodies and C.

Introduction

The eradication of *Treponema pallidum* subspecies *pallidum* (*T. pallidum*), the causative organism of syphilis, in patients is often incomplete. This may lead to chronic infection and in some cases to tertiary syphilis. In experimental syphilis in rabbits the persistence of treponemes has also been documented, despite the development of chancre immunity.

The mechanisms of survival of the treponemes for many years in the host are not yet fully understood. Several hypotheses have been proposed. One of these suggests a possible affection of the immune response, but the experimental results are contradictory. Recently, however, these findings have been reviewed in relation with the up- and down-regulation of the immune response. It was hypothesized that an early down-regulation of the immune response could allow the survival of a small number of treponemes (1). In other hypotheses it was postulated that coverage of the treponemal outer membrane with mucopolysaccharides (2,3) or host serum proteins (4) confers protection against attack by the host's defences upon the treponemes. Although this might explain the presence of pathogenic treponemes together with antitreponemal antibodies of high titre in a host, evidence for the presence of a cover offering protection to the treponemes is not available. More recently, it was hypothesized that a lack of antigenicity, due to the presence of scarce transmembrane particles in the treponemal outer membrane, may provide a mechanism by which the treponemes evade the host immune response (5,6).

In vitro immobilisation of rabbit-derived treponemes by antibodies and complement takes a long time. It was hypothesised that treponemes had to lose their protective cover before antibodies could gain access to the treponemal surface and complement could be activated to eventually lyse the treponemes. Lysozyme has been implicated in this process, because its presence accelerated the immobilisation. However, the location of the substrate of this enzyme has been a subject of discussion (7-9). Blanco *et al.* recently demonstrated that not the antibody-binding step but the complement-activation step was rate limiting in the immobilisation process (10). The latter correlated with the antibody-mediated aggregation of the rare outer membrane protein. Recently, we demonstrated that treponemes, which were harvested from rabbit testicles and purified by Percoll centrifugation, were quickly immobilised by normal human serum (NHS) (11). The immobilisation could be inhibited by fluids from infected and non-infected rabbit testicles. As a part of our investigations into this

issue, the results of the study of the components of NHS which participate in the rapid immobilisation of purified treponemes are presented.

Materials and Methods

Propagation and extraction of T.pallidum. Propagation and extraction of *T.pallidum* (Nichols) were performed as previously described (11). Briefly, the testes were minced and 1 ml of serum free basal reduced medium (BRM) without dithiothreitol was added per gram of wet testicular tissue. The mixture was shaken for 45 min at room temperature in an atmosphere of 5% carbon dioxide and 95% nitrogen, and centrifuged for 10 min (800 g) to sediment gross particulate matter. The fluid layer containing the treponemes was collected and centrifuged at 12000 g at 4°C for 10 min to pellet the treponemes. The supernatant was removed and the treponemes were resuspended in fresh BRM and subjected to Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation (43% Percoll in BRM) for 30 min at 37000 g according to Hanff *et al.* (12). The layer containing the treponemes was collected and used for further experiments.

Enumeration of treponemes. The treponemes were counted using microslides (path length 0.05 mm, Camlab Limited, Cambridge, England, ref: 5005) and the density of treponemes was calculated as previously described (13).

Human serum pool. A serum pool was prepared from blood samples from 150 blood donors with negative TPHA results. The pool was stored in aliquots at -70°C. Portions of 10 ml were used to prepare antibody-containing serum fractions.

Guinea-pig serum pool. Guinea-pigs were bled and their serum was tested individually for the capacity to sustain the viability of Percoll purified treponemes in an assay similar to the immobilisation assay described below. Only those specimens in which treponemal viability was better than 70% after 22 h were used to prepare a serum pool. This pool was stored in aliquots at -70°C and was used as the complement source in immobilisation experiments. Samples used in these experiments were thawed only once.

Preparation of serum fractions from normal human serum (NHS). Approximately 10 ml portions from the human serum pool were subjected to Sephadex G-200 gel filtration. The IgM-containing 19S and the IgG-containing 7S fractions (designated Fr 1 and Fr 2 respectively) were collected and concentrated to the volume of serum initially applied to the

column in Amicon concentration cells equipped with PM10 membranes. After dialysis for 24 h against Earles balanced salt solution (one change), the fractions were stored in small portions at -70°C until use.

Estimation of complement activity. Complement activity of sera and serum fractions was determined as previously described (14).

SDS-PAGE and Western blotting. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, including the blocking and staining procedures of the strips were performed as described previously (11). Ten μl of Fr 1, Fr 1 (abs)(see below), and human IgG (Nordic, Tilburg, the Netherlands, lot nr. 1-169) as a reference, were appropriately diluted in sample buffer and applied to different slots of the gel after heating in a boiling water bath for 4 min. After completion of the electrophoresis run and the blotting procedure the strips were incubated with conjugate, consisting of the affinity purified gold-labeled IgG fraction from a goat antiserum against heavy and light chains of human IgG (Janssen Life Sciences Products, Beerse, Belgium) for 2 h, followed by silver enhancement. Separate strips were stained according the Auroprobe staining procedure (Janssen Life Sciences Products) to visualise the polypeptides. The low-molecular weight standards from Pharmacia (Uppsala, Sweden) were used in estimating the size of the visualised polypeptides.

Depletion of IgG. Depletion of IgG from Fr 1 was accomplished by absorption with Staphylococcus aureus, strain Cowan 1. (Serva, Heidelberg, Germany, lot nr. 16082c). 2.5 ml of Fr 1 was incubated with 0.05 g dry weight of pre-washed bacteria for 1 h at 4°C. The bacteria were pelleted by centrifugation at 27000 g at 4°C. The supernatant, designated Fr 1 (abs) was collected and stored in aliquots at -70°C. The efficacy of the absorption procedure was controlled by SDS-PAGE electrophoresis and immunoblotting.

Estimation of lysozyme. The lysozyme content of guinea-pig serum and bentonite-absorbed cavia serum, expressed as units/ml and of chicken egg white lysozyme (Sigma, St Louis, USA, lot nr. 89F8275), expressed as units/mg, was determined by the lysis of *Micrococcus lysodeikticus* (Sigma, lot nr. 109F68081) according to the instructions of the supplier. The latter results were used to calculate the amounts of chicken egg white lysozyme to be added to bentonite-absorbed serum to attain the specified number of units finally present in the mixtures for the study of immobilisation.

Depletion of lysozyme. Guinea-pig serum was depleted of lysozyme by absorption with ben-

tonite-SF (Serva, Heidelberg, Germany, cat.nr. 14515) according to Wardlaw (15). After completion of the absorption procedure the absorbed serum was checked for its lysozyme and complement contents. Lysozyme could not be detected any more. The complement level had decreased by 25% as compared with pre-absorption levels.

Immobilisation of treponemes. The Percoll purified treponemes were used in a final density of 2 x 10^7 treponemes/ml. A sufficient number of treponemes were mixed with Fr 1 or Fr 2 (final content 10% v/v) or with a 1:1 mixture of Fr 1 and Fr 2 (final content 20% v/v). These mixtures were supplemented to three-quarters of the final volume with BRM and pre-incubated for 15 min at room temperature. Finally, pooled guinea-pig serum or bentonite-absorbed pooled guinea-pig serum was added to a final content of 25% (v/v). Aliquots of 0.5 ml of these mixtures were placed in small tubes, which were loosely plugged with cottonwool and incubated in a reduced oxygen atmosphere (4%) at 34°C (16). The percentage of mobile treponemes was determined in wet mounts after 0, 1, 2, 3.5 and 5.5 h by observing at least 100 treponemes in randomly selected microscopic darkfields. Control tubes were set up by adjusting a volume containing 4 x 10^7 treponemes from the various suspensions to 1.5 ml with BRM. After the pre-incubation period 0.5 ml pooled guinea-pig serum was added. These tubes were treated further as described above. A similar set-up was used to study the capacity of serum from individual guinea-pigs to support the mobility of the treponemes. Here, 4 x 10^7 treponemes, suspended in 1.5 ml BRM, were pre-incubated and mixed with 0.5 ml of individual guinea-pig serum. Aliquots of these mixtures were stored and read as described above with additional readings after 22 h.

Fluorescence. Three types of fluorescence experiments were performed to detect C3b depositions on treponemes. In the first type 50 μl from a Percoll purified treponeme suspension (density 2 x 10^7) was applied to glass slides, which were air-dried and heat-fixed. It was demonstrated previously that this procedure damages the treponemal outer membrane (11). These slides were overlaid with two drops of Fr 1, Fr 2 or a 1:1 mixture of Fr 1 and Fr 2 for 30 min at room temperature. The second type of experiments was performed on treponemes which had been incubated in suspension with Fr 1, Fr 2 or their 1:1 mixture. In the third type of experiments the treponemes in the tubes with the reaction mixtures for immobilisation were used. In the type 2 and type 3 experiments the contents of the tubes were supplemented with 10 ml BRM and centrifuged at 12000 x g at 4°C for 10 min. The supernatant was removed and the pelleted treponemes were resuspended in 1 ml BRM. The

suspension was pipetted into a Petri dish equipped with a coverglass. After centrifugation at 800 g for 5 min, the coverglasses were rinsed with BRM. In type 1 and type 2 experiments the treponemes were incubated with two drops of a FITC labelled IgG fraction from a goat anti-human C3b antiserum (Centraal Laboratorium van de Bloedtransfusiedienst, Amsterdam), in type 3 experiments the treponemes were incubated with a similar fraction from a goat anti-guinea-pig C3b antiserum (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, USA) for 30 min at room temperature. After rinsing, the slides were covered with a coverglass. The coverglasses with adhering treponemes from the Petri dishes were laid upside down on microscopic slides. The preparations were sealed with nail polish and read within 3 h as previously described (17).

Results

A pre-incubation of Percoll purified treponemes with the IgG-containing Fr 2 resulted, after the addition of guinea-pig serum, in a survival of 88% of the treponemes after two hours and of 50% after 5.5 h. In similar experiments with the IgM-containing Fr 1 a mean of 88% survived after 2 h and 22% after 5.5 h. A rapid immobilisation of the treponemes was noted only when the treponemes had been pre-incubated with the combined Fr 1 and Fr 2. Two hours after the addition of guinea-pig serum almost all treponemes had been immobilised (Fig 1). In the control tubes, a mean of 97% of the Percoll purified treponemes survived after 5.5 h.

SDS-PAGE electrophoresis and immunoblotting showed that Fr 1 contained some IgG, which was no longer detectable after absorption of this fraction with *Staphylococcus aureus*, strain Cowan 1. The immobilisation of the purified treponemes after pre-incubation with Fr 1(abs) occurred more slowly than the immobilisation after pre-incubation with Fr 1. After 5.5 h, 51% of the treponemes that had been pre-incubated with Fr 1(abs) survived; after pre-incubation with Fr 1 this was 8%. This indicates a role for IgG in the immobilisation process.

An analysis of the complement content of Fr 1 and Fr 2 showed that only Fr 2 produced a haemolysis of sensitised sheep erythrocytes just above control values, which demonstrated that Fr 2 contained a low level of haemolytic complement. A 1:1 mixture of Fr 1 and Fr 2 did not increase the haemolysis of sensitised erythrocytes, indicating that no separation of

complement components had occurred during the preparation of serum fractions.
Percoll purified treponemes which had been pre-fixed onto glass slides showed a deposition of human C3b after they had been overlaid with Fr 2 or the combination of Fr 1 and Fr 2, followed by conjugate. No C3b deposition was observed on pre-fixed treponemes that had been overlaid with Fr 1 and conjugate, emphasizing the absence of C from this fraction and demonstrating the non-reactivity of the conjugate with the treponemes. After incubation of purified treponemes in suspension with each fraction or their combination a deposition of C3b was observed only after incubation with the combined Fr 1 and Fr 2 (Table 1). These results show firstly the different reactivities of the serum fractions towards damaged and intact treponemes and secondly that in addition to Fr 2, the presence of Fr 1 is essential for the deposition of C3b on treponemes which had been incubated in suspension.
Parallel with their immobilisation, the C3b deposition on the treponemes was studied. All treponemes which had been pre-incubated with the mixture of Fr 1 and Fr 2 showed a deposition of guinea-pig C3b of a 3+ to 4+ (strong to very strong) intensity, as early as 1 h after incubation. At later times the number of treponemes decreased sharply in the fluorescence preparations. After pre-incubation with Fr 1 or Fr 1(abs) all treponemes showed a very weak fluorescence for C3b of a speckled character, which did not change towards the end of the experiments. The same was true of a majority of approximately 60% of the treponemes that had been pre-incubated with Fr 2.

It became clear that the rate of immobilisation of the Percoll purified treponemes after pre-incubation with the mixture of Fr 1 and Fr 2 was greatly reduced when Fr 2 was replaced by heat-inactivated Fr 2. Replacement of Fr 1 by heat-inactivated Fr 1 in this mixture hardly influenced the rapidity of the immobilisation as compared with the immobilisation after pre-incubation with the mixture of Fr 1 and Fr 2.

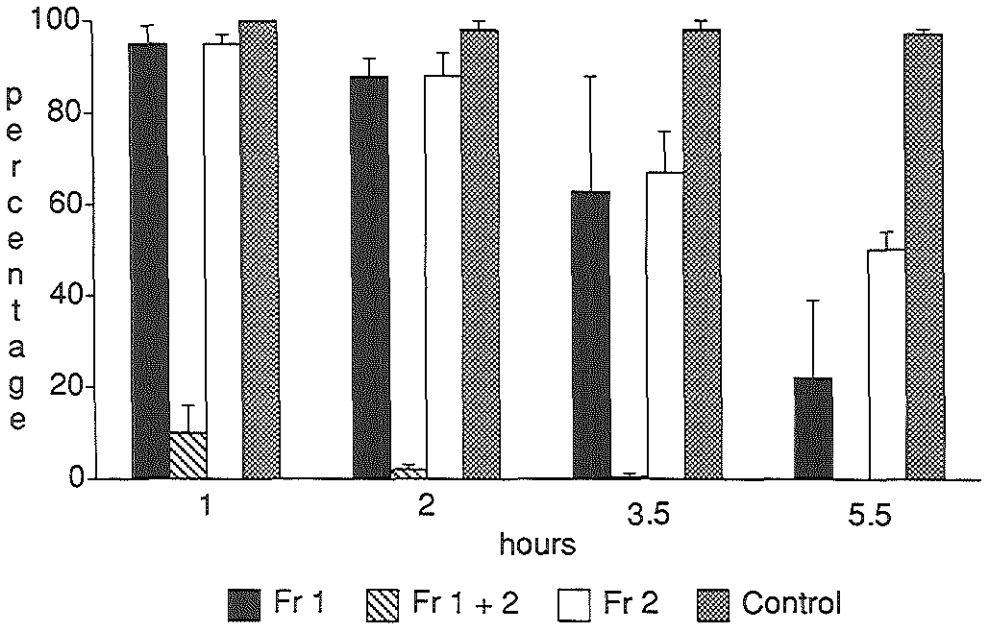

Figure 1. Immobilisation of Percoll purified treponemes. Treponemal mobility after preincubation with fraction 1, fraction 2, the mixture of fractions 1 and 2, or BRM (= control). Pooled guinea-pig serum was used as a complement source throughout these experiments. Results are expressed in percentages of mobile treponemes. Means and standard deviations of the results of experiments with 6 treponemal suspensions originating from different rabbits are shown.

Table 1 Percentage of differently treated treponemes showing deposition of C3b after incubation with Sephadex G-200 separated fractions from NHS and their mixture (Fr 1: 19S fraction, Fr 2: 7S fraction; int. = intensity of fluorescence)

Antigen	Fr 1	Fr 2	Fr 2	Fr 1+2	Fr 1+2
	% pos	% pos	int.	% pos	int.
Fixed treponemes	0	100	1-2	100	2-3
Treponemes in suspension	0	0	-	100	1-2

The role of lysozyme is demonstrated in figure 2. As before, the immobilisation of purified treponemes which had been pre-incubated with the mixture of Fr 1 and Fr 2 proceeded rapidly after the incubation with guinea-pig serum. However, when bentonite-absorbed guinea-pig serum was used the immobilisation proceeded much more slowly: after 5.5 h 51% of the treponemes still showed good mobility. The participation of lysozyme in the rapid immobilisation was further demonstrated by the reconstitution of the absorbed guinea-pig serum with graded amounts of chicken egg white lysozyme. In the presence of the lowest amount of lysozyme added (5 U) almost no mobile treponemes were left after 5.5 h (Fig 2).

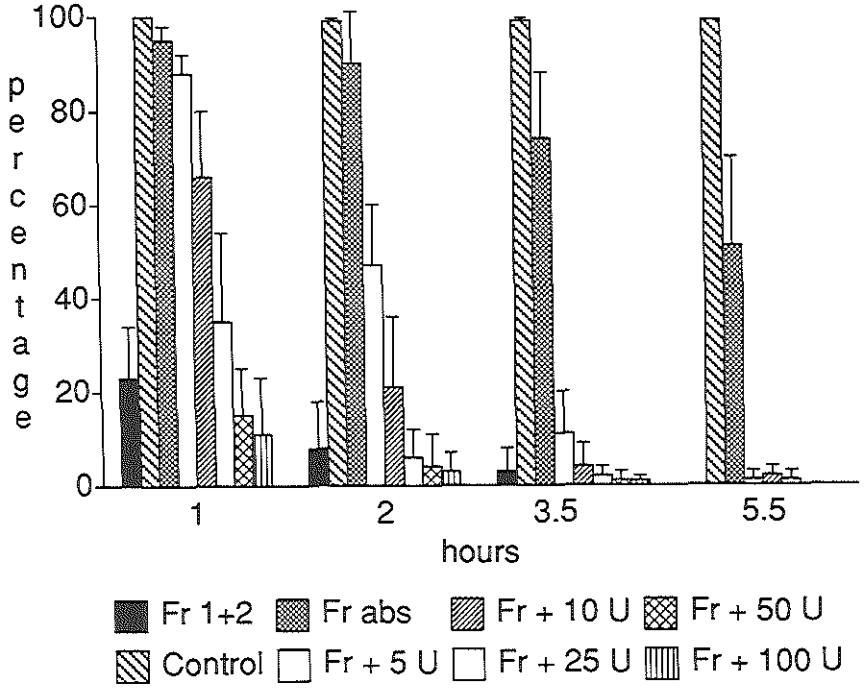

Figure 2. The role of lysozyme in the immobilisation of Percoll purified *T.pallidum*. Treponemal mobility after preincubation with the mixture of fractions 1 and 2 (Fr 1+2), (or BRM = control), incubated with guinea-pig serum or bentonite-absorbed (Fr abs) guinea-pig serum. Reconstitution of the absorbed guinea-pig serum with lysozyme was performed by addition of graded amounts of chicken egg white lysozyme (from 5 U to 100 U). Results are expressed in percentages of mobile treponemes. Means and standard deviations of the results of experiments with 3 treponemal suspensions originating from different rabbits are shown.

Reconstitution of the absorbed guinea-pig serum with 25 U of lysozyme resulted in an immobilisation as rapid as with unabsorbed guinea-pig serum. Higher amounts of lysozyme did not influence the rate of immobilisation any further.
Moreover, these results indicate that the slow immobilisation with bentonite-absorbed guinea-pig serum was indeed due to the depletion of lysozyme and not to a loss of complement from the guinea-pig serum.

To establish a possible reaction sequence of the fractions, pre-incubations were performed with Fr 1(abs) or Fr 2 and after 1 h of incubation with guinea-pig serum, the missing fraction was added to half of the reaction mixtures. These results are demonstrated in figure 3.

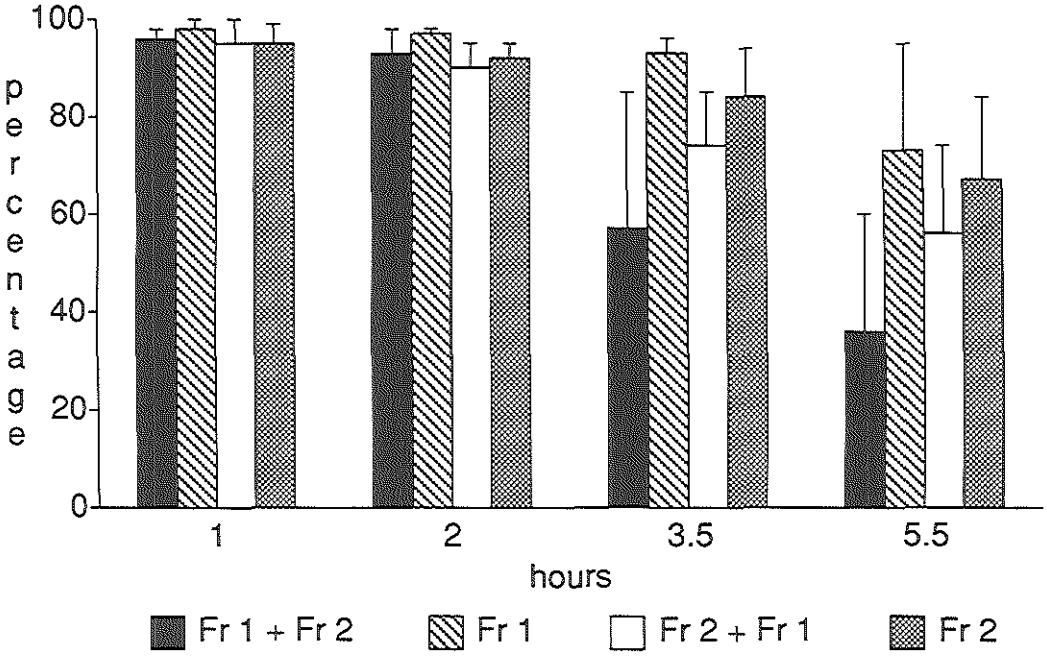

Figure 3. Experiments to study a possible reaction sequence of the fractions. Preincubations were performed with Fr 1(abs) or Fr 2. After one hour of incubation with guinea-pig serum the missing fraction was added to one-half of the reaction mixtures (Fr 1(abs) + Fr 2, or Fr 2 + Fr 1(abs)), or incubation was continued with one fraction only (Fr 1(abs) or Fr 2). Results are expressed in percentages of mobile treponemes. Means and standard deviations of the results of experiments with 5 treponemal suspensions originating from different rabbits are shown.

In all four types of reaction mixtures the mobility of the treponemes hardly changed during the first hour when one or both fractions were present. At the end of the experiments the immobilisation was greatest in the tubes which had been pre-incubated with Fr 1(abs) and to which Fr 2 was added later (survival 36%). In the tubes which had been pre-incubated with Fr 2, with Fr 1(abs) added later, the survival of the treponemes was 56%. The survival of the treponemes in the tubes containing only one fraction was approximately 70%. In parallel experiments bentonite-absorbed guinea-pig serum was used. Here, in all four types of reaction mixtures approximately 90% of the treponemes survived after 5.5 h.

Discussion

The rapid immobilisation of purified treponemes by the serum fractions from the NHS pool occurred only when the treponemes had been pre-incubated with Fr 1 and Fr 2 simultaneously and was accompanied by a strong deposition of C3b on the treponemal surface. The rapid immobilisation was largely dependent on presence of a small amount of haemolytic complement in Fr 2. Remarkably, only a minor part of the treponemes were immobilised within 5.5 h after pre-incubation with Fr 2, despite the presence of anti-treponemal IgG and C in this fraction. This can possibly be explained by the results of the experiments in which the C3 depositions on fixed treponemes and on treponemes in suspension were compared. It was shown that C3b deposition on fixed treponemes occurred upon their incubation with Fr 2 or the combined Fr 1 and 2. However, C3b deposition on treponemes in suspension occurred only when they were incubated with the combined Fr 1 and 2. These different phenomena may be a reflection of the different requirements for various classes of antibodies, which lead to the activation of the classical complement pathway: a single IgM molecule present in an antigen-antibody complex can bind C1q to start the C-cascade. For IgG, however, at least a doublet of IgG molecules, sufficiently near to each other is needed for C1q binding (18). Apparently, the fixed treponemes (i.e. damaged treponemes) offer a sufficiently close packing of the epitopes to allow C-activation by IgG. These results show that C-activation "within Fr 2" is possible. This does not occur with treponemes in suspension. Here, the IgM containing fraction is needed to accomplish C3b deposition. As previously demonstrated by others (5,6,19), the

outer membrane of *T.pallidum* shows a scarcity of epitopes. This may be the reason why IgM, but not IgG, can form antigen-antibody complexes on the treponemal surface, which are capable of C-activation. This may be due to the larger size of this class of antibody molecules, which will allow them to bridge larger distances between epitopes. A preference for the binding of IgM antibodies to adherent treponemes has been demonstrated previously (20).

This makes it plausible that the first step in the rapid immobilisation of the treponemes is an attack by IgM and C. However, the results of the experiments performed with Fr 1(abs) demonstrate that presence of IgM alone does not result in rapid immobilisation. For this to occur IgG is also needed, as emphasised by the rapid immobilisation produced by the mixture of Fr 1 and Fr 2 and the reduction in the immobilisation rate after removal of IgG from Fr 1. The results of the study of the reaction sequence of IgM and IgG antibodies largely agree with this. In this set of experiments more rapid immobilisation was noted when the preincubation was performed with Fr 1(abs) as compared with a preincubation with Fr 2. These results might point to an inhibition of IgM by IgG, due to a competition for epitopes on the treponemal surface.

A role of lysozyme in the immobilisation of treponemes was recognized three decades ago (7-9). From our experiments it is clear that removal of lysozyme reduces the rate of immobilisation. Reconstitution of the guinea-pig serum with graded amounts of lysozyme restores the capacity of rapid immobilisation (Fig 2). The results so far obtained demonstrate that rapid immobilisation can be accomplished without presence of lysozyme during the pre-incubation steps. This shows that the action of lysozyme does not occur during the initial steps of immobilisation, but probably only after the outer membrane of the treponemes has been damaged by a complement dependent immunological reaction. This is in agreement with the conclusions of Müller *et al* (9).

Our results demonstrate that the rapid immobilisation of purified treponemes by serum fractions from NHS requires antibodies of the IgM and IgG classes, together with complement and lysozyme. Omission of one of these reactants inhibits the rapid immobilisation. All available evidence indicates that these reactants act in sequence in the rapid immobilisation: the integrity of the outer membrane is first attacked by IgM and C. The loss of integrity of this membrane provides the opportunity for lysozyme to hydrolyse the peptidoglycan layer surrounding the cytoplasmic membrane of the treponemes. The

latter is then accessible for attack by antibodies and C. Presumably IgG plays a major role in this process, since rapid immobilisation without IgG did not occur.

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5.4.

ULTRASTRUCTURAL ASPECTS OF INFECTION WITH *TREPONEMA PALLIDUM* SUBSPECIES *PERTENUE* (PARIAMAN STRAIN)

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Ultrastructural aspects of infection with *Treponema pallidum* subspecies *pertenue* (Pariaman strain)

H J H Engelkens, V D Vuzevski, F J W ten Kate, P van der Heul, J J van der Sluis, E Stolz

Abstract

***Objective*—To study ultrastructural aspects of infection with *Treponema pertenue* (Pariaman strain), originating from West Sumatra, Indonesia.**

***Materials and methods*—Biopsy material originating from skin lesions in ten young children suffering from early infectious yaws in Indonesia, and rabbit testicular tissue inoculated with *T pertenue*. Human skin as well as rabbit testicular tissue was examined by means of conventional electron microscopy.**

***Results*—In human skin, treponemes were found in interepidermal spaces in 5 out of 10 specimens. In two of five positive specimens, treponemes were also seen in the dermis. In one out of five specimens from rabbit testicular tissue a profusion of treponemes was found lying in the interstitial myxomatous tissue. Microorganisms showed no adhesion to fibroblasts.**

***Conclusion*—This ultrastructural study of *T pertenue* demonstrated the scarcity and focal distribution of treponemes in tissue and did not reveal any morphological differences from the Gauthier strain of *T pertenue*. No differences from the ultrastructure of *T pallidum* were observed either.**

Introduction

Yaws (framboesia tropica, buba, pian, patek) was not eradicated after mass treatment campaigns in the 1950s and 1960s. This led to the persistence or resurgence of endemic foci in tropical regions, from where the disease is spreading rapidly.[1-3] The causative agent of yaws is *Treponema pallidum* subspecies *pertenue* (*T pertenue*), which cannot be distinguished morphologically or serologically from *Treponema pallidum* subspecies *pallidum* (*T pallidum*), the causative agent of venereal syphilis. However, there are clinical, geographic and epidemiologic differences between the different treponematoses.[1 4] *T pertenue* cannot be cultured *in vitro* for prolonged periods of time. For laboratory investigations, treponemes are preferably propagated in rabbits or hamsters.

Literature on the ultrastructure of *T pertenue* is extremely scarce.[5-8] Most recent work has been restricted to the study of the Gauthier strain, which was isolated from a patient in Nigeria in 1960.[7 8] During investigations in 1988 cases of infectious yaws were detected in West Sumatra, Indonesia.[9 10] In Rotterdam, a strain of *T pertenue* (Pariaman strain) has recently been adapted to the rabbit. We investigated this strain in patient as well as in rabbit material by means of conventional electron microscopy and compared our findings with earlier publications on the morphology of *T pertenue*.

Material and methods

Biopsy material was studied, originating from skin lesions of ten patients. All patients were young children, presenting with florid skin lesions of the early stage of yaws in West Sumatra, Indonesia.[9 10] Six children presented with crusto-papillomatous skin lesions, two with ulcero-papillomatous and two with ulcero-crusto-papillomatous skin lesions. Treponemal and non-treponemal serologic tests and darkfield examination of exudates from skin lesions were positive in all patients. Immediately after obtaining the specimens, fixation of small fragments was performed in a solution of glutaraldehyde-formaldehyde (4CF-1G). The specimens were transported from Sumatra to Rotterdam.

Further, treponemes were propagated in laboratory animals. In Indonesia, treponeme-containing suspensions obtained from patients were inoculated into Syrian Golden hamsters, which were transported to the Netherlands. After several weeks, when skin lesions had developed in the inguinal region, lymph nodes were removed. Treponeme-containing suspensions obtained from the lymph nodes (checked by darkfield examination) were injected intratesticularly in New Zealand white rabbits.

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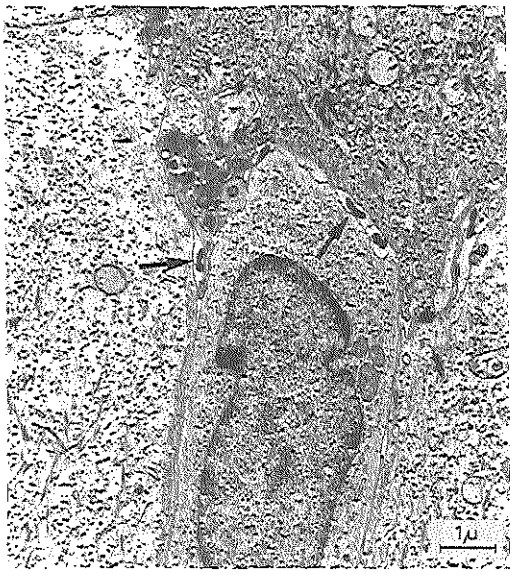

Figure 1 Human tissue. In the interepidermal space adjacent to inflammatory cells there are numerous treponemes (arrows) lying free or lying close to the membrane of inflammatory cells (magnification 7000 ×).

These animals, about four kg weight and about 6 to 8 months old, were obtained from a commercial breeding farm. All rabbits developed a positive treponemal serology after two to four weeks. The testes were removed aseptically as soon as palpable changes were present. Compact nodules of only a few mm in diameter appeared on the testis in most cases. Multiple small fragments from these small nodules and the other testicular tissue were fixed immediately in glutaraldehyde-formaldehyde. After fixation, all tissue was postfixed with 1% (w/v) osmium tetroxide at 4°C. After acetone dehydration, the specimens were embedded in LX 112 (Epon). Semi-thin plastic sections for light microscopy were stained with a freshly prepared 1% toluidine blue solution in distilled water. Ultrathin sections (LKB ultratome IV) were mounted on copper grids (300 mesh) and contrasted with uranyl acetate (10 minutes at 45°C) and lead citrate. They were examined with a Zeiss 902 electron microscope. In all specimens *T pertenue* were detected by silver impregnation.

Results

Ultrastructural features

Electron microscopy showed an undulating appearance of *T pertenue*, changing in amplitude and frequency (fig 1). Treponemes had a width of approximately 0·11 to 0·17 μm. They were surrounded by a cytoplasmic membrane and an outer membrane, both consisting of three layers. In only a few treponemes a clear space was observed between the outer membrane and the cytoplasmic membrane. Flagella (axial filaments) were clearly visible, lying between the outer membrane and peptidoglycan layer (fig 2).

Treponemes were detected in five out of ten specimens of human tissue, in the intercellular spaces of the epidermal layer and among the inflammatory cells (fig 3). In some lesions the microorganisms were also found in the cytoplasm of the macrophages. These treponemes were observed in a membranous sac within the cytoplasm, a phagosome-like structure. The cellular membrane was clearly preserved. In the epidermis treponemes were lying in intercellular spaces, between the keratinocytes. At some locations *T pertenue* appeared to be in close contact with the epidermal cells, which ultrastructurally showed no signs of damage (fig 3). In two cases only a few treponemes were observed in the dermis (fig 4). In most positive samples treponemes were scarce, located in clusters.

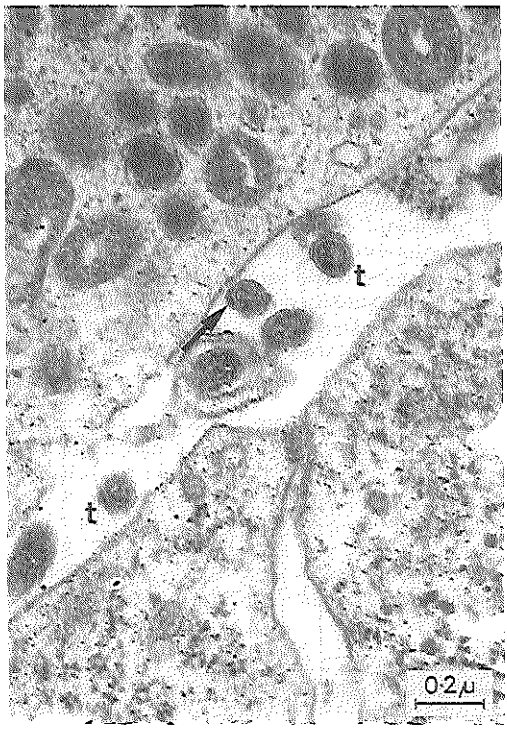

Figure 2 Transversally cut treponemes (t) near inflammatory cells, showing their normal architecture. The membrane has a lamellar structure and axial filaments (arrow) are clearly visible (human skin, magnification 30 000 ×).

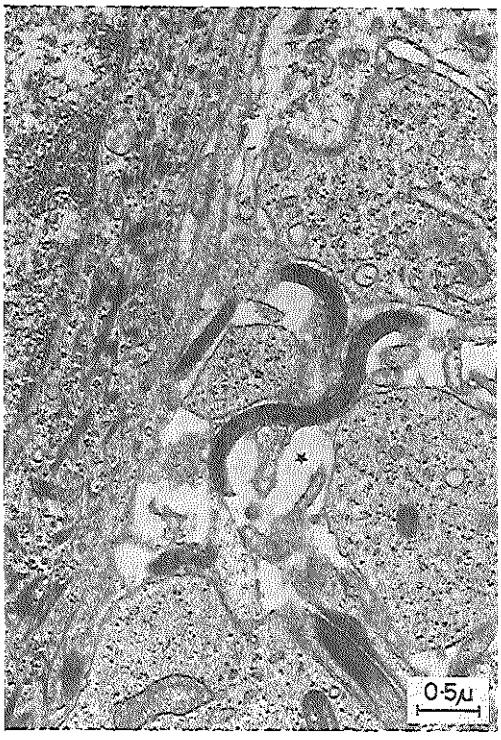

Figure 3 The epidermis shows spongiotic (asterisk) changes (dilatation of intercellular spaces). In these areas there are numerous treponemes. Epidermal cells show no disruption of cytoplasmic membranes. Human tissue, magnification 12 000 ×).

In the rabbit specimens, in one out of five specimens many treponemes were seen among inflammatory cells (figs 5, 6). In the other four rabbit samples no treponemes could be detected by electron microscopy. In the only positive specimen, many treponemes were found in the interstitial spaces of the testis. They were concentrated around fibroblasts, mesenchymal cells, the interstitial cells of Leydig and small blood vessels. Some treponemes were observed in close contact with mononuclear cells. Some treponemes were seen in cellular invaginations, without evidence of membrane disruption or fusion of the microorganisms with the cellular membrane. No differences could be detected between the morphology of treponemes in rabbit and human tissue.

Discussion

Ultrastructural studies have been of great significance for the classification of spirochetes.[11 12] The presence of axial filaments is characteristic of this species of microorganisms.[13]

Most ultrastructural studies on pathogenic treponemes have been done on *T pallidum*, compared with the very few on *T pertenue*,[5–8] in which only the Gauthier strain of *T pertenue* was used.[11] Hovind-Hougan *et al* pointed out a subtle morphologic difference between the causative agents of yaws and syphilis, namely the presence of thin fibrils at the periphery of negatively stained *T pertenue* (Gauthier strain) in skin lesions and lymph node biopsies of experimentally infected hamsters. These structures termed "fimbriae" were only detectable in optimally stained preparations.[8 11 12] This finding has not been confirmed by other workers.

In our study *T pertenue* showed an ultramicroscopic morphology identical to *T pallidum* (Nichols strain), a strain previously studied in our laboratory.[14] It became clear that *T pertenue* was very scarce, and primarily located in clusters. No morphologic differences were observed between the treponemes in human and rabbit tissue. Our observations of the Indonesian strain of *T pertenue* were similar to those reported for the Gauthier strain.[7 8]

Intracellularity of *T pallidum* has been proposed as a means to evade the immune response.[15] Although it was shown that *T pallidum* was located almost exclusively in the extracellular ground substance, some investigators nevertheless demonstrated the intracellular presence of treponemes in a variety of cells.[15–18] Intracellularly located treponemes were

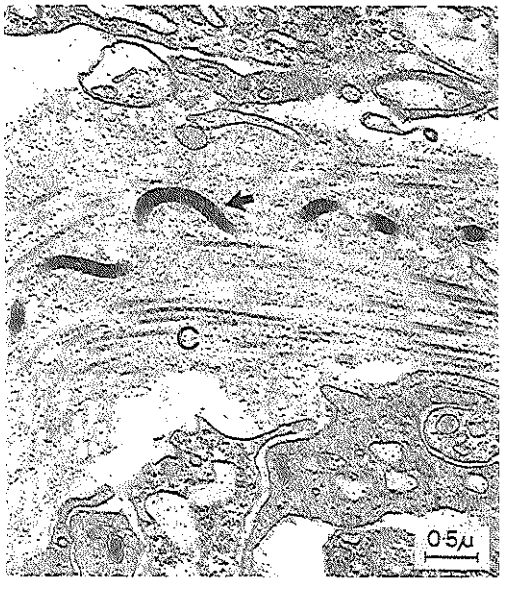

Figure 4 In the dermis between two fibroblasts among the collagen (C) bundles treponemes (arrow) are visible (magnification 12 000 ×).

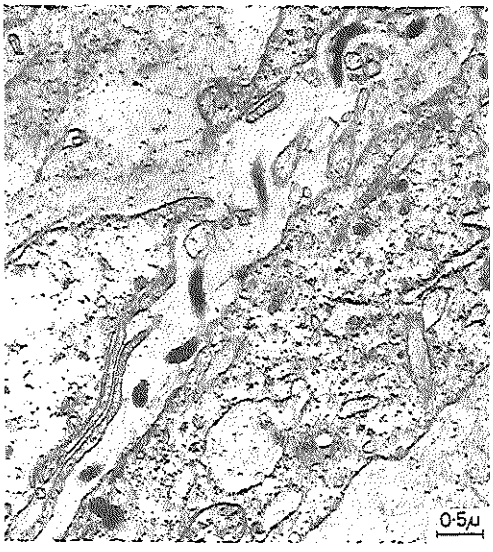

Figure 5 Interstitial tissue of the testes, with several fibroblasts lying in myxomatous stroma. Between the cells there are convoluted profiles of treponemes. Treponemes are lying in the myxomatous tissue, showing no adhesion to fibroblasts. Rabbit tissue, magnification 12 000 ×).

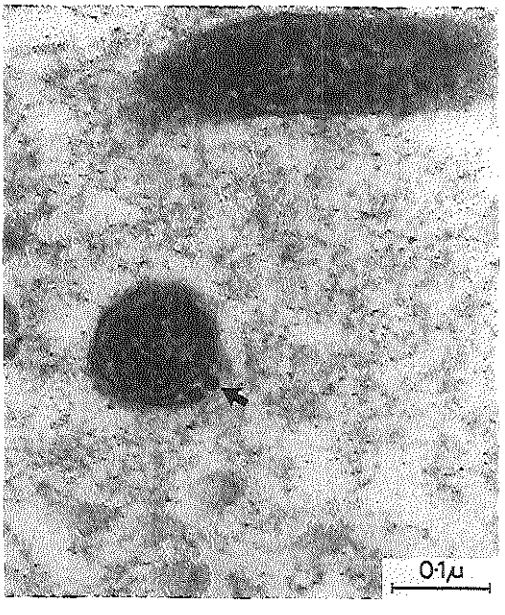

Figure 6 Higher magnification (85 000 ×) of two treponemes cut tangentially and longitudinally. Note their architecture: the outer membrane, axial filaments (arrow) and inner membrane are clearly visible. These T pertenue microorganisms ultrastructurally show no differences from T pallidum.

assumed to be smaller, thicker and more electron dense. In our study the treponemes appeared to be located extracellularly, sometimes in close proximity to epidermal cells. It was observed that in some cases bacteria were engulfed by the cell membrane of phagocytic cells, and so came to lie in a membranous sac within the cytoplasm (a phagosome-like structure) with clearly preserved cellular membranes. These findings resemble those found with *T pallidum*.[7,15] This may represent intracellularity of *T pertenue*. Much of the biology of yaws still remains unclear. Further study of this intriguing re-emerging disease is recommended.

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CHAPTER 6. DISCUSSION

CLINICAL AND (HISTO-)PATHOLOGICAL STUDIES

This thesis focused on treponemal disease important to mankind, namely venereal syphilis and the non-venereal endemic treponematoses yaws, endemic syphilis and pinta. Several studies of the treponematoses are presented.

Venereal syphilis still is a major worldwide threat. Lately, this disease has received renewed attention because of an increase in infectious cases and cases of congenital syphilis in the United States (chapter 3.1), and because it became clear that sexually transmitted diseases (STD), with ulcerative STD such as syphilis, may facilitate the spread (chapter 1.3) of the acquired immunodeficiency syndrome (AIDS). This means a bad look-out, since in many countries in the world ulcerative STD are extremely common and hard to combat.
Intensive efforts were made to eradicate the endemic treponematoses in the 1950s and 1960s. Mobile teams were formed to give penicillin injections to patients and their contacts (1). These mass campaigns were very successful and in some countries endemic treponematoses were eradicated (chapter 1.6). However, yaws for example has never been eradicated in any of the nations of the African continent. Recently a dramatic resurgence was described, especially of yaws and endemic syphilis. Unfortunately, in some countries the present situation resembles the precampaign situation (chapter 1.4 and 1.5), and continuing surveillance is urgently required.

Although after the mass treatment campaigns yaws disappeared from large parts of Indonesia, some foci of yaws still exist. In a small survey in the Pariaman region in West Sumatra by Stolz *et al.*, it was observed that yaws still is an important health problem in this region (chapter 3.2). Many children were suffering from early yaws, and consequently at risk of developing the mutilating late stage manifestations. Although the people investigated in this remote humid region in Sumatra were not selected at random and represented only a small select group of the total population of West Sumatra, an estimation was made of the prevalence of early infectious yaws in this region. This was found to be high, a minimum of 300 cases of yaws per 100,000 individuals. Many asymptomatic persons in the same region gave positive test results in treponemal and/or nontreponemal serological tests. This meant that a very large proportion of these inhabitants had been in contact with treponemal

infection. It was not possible to estimate the potential risks for development of late stage sequels in this region. Certainly yaws still exists in this part of Indonesia, and requires renewed attention.

Recently results of other surveys on yaws in other parts of the world, for example in Zaire (2), Papua New Guinea (3), the Solomon Islands (4), and Ecuador (5) were published. In these studies and in ours it is concluded that continuing and renewed attention remains urgently needed. Unfortunately, because of so many other public health problems in the same regions of the world eradication of yaws seems unrealizable. No reports about coexistence of HIV infection and endemic treponematoses have been published yet (see chapter 1.3).

A differentiation between the different treponematoses cannot be made on morphological or biochemical grounds. DNA homology studies did not reveal any differences (6). Furthermore, development of a serological test to differentiate between yaws and syphilis has so far not been possible (7). Until now only one Scandinavian group of investigators described a subtle morphological difference between *T.pallidum* and *T.pertenue*, namely the presence of thin fibrils at the periphery of negatively stained *T.pertenue* (Gauthier strain) in skin lesions and lymph node biopsies of experimentally infected hamsters. These structures termed "fimbriae" were only detectable in optimally stained preparations (8,9). Until now, this finding has not been confirmed by other workers. Most ultrastructural studies of pathogenic treponemes have been performed on *T.pallidum*, compared with the very few of *T.pertenue*, in which only the Gauthier strain of *T.pertenue*, originating from a patient suffering from yaws in Nigeria in 1960 was used (8-10). We did not find any difference in ultrastructure between our *T.pertenue* strain (Pariaman strain) and the Gauthier strain of *T.pertenue*. Furthermore, no differences from the ultrastructure of *T.pallidum* were seen (9). Unfortunately the research group of Hovind-Hougen *et al.* could not help us find "fimbriae" in the Pariaman strain [personal communication]. Study of the presence of "fimbriae" in our strain and several other *Treponema* strains would be interesting; until now, no morphological differences have been found which can be used for routine differentiation of the different treponematoses.

In our studies of biopsies from patients suffering from yaws or syphilis the histopathological findings were largely comparable with older studies (11-13). Syphilitic lesions differed from yaws lesions mostly in the location of treponemes and the affection of blood vessels. With the silver staining method according to Steiner and using an

immunofluorescence staining technique, it was demonstrated that *T.pertenue* shows an epidermotropic character, in contrast with *T.pallidum*, which shows a more mesodermotropic character. In biopsies from patients suffering from early syphilis, blood vessel changes were more pronounced than in yaws biopsies. In yaws specimens, vascular changes consisted of only slight endothelial cell proliferation and thickening of vessel walls, compared with the marked endothelial swelling and often proliferation in biopsies from syphilis patients. Furthermore, in a study of the inflammatory infiltrate in early syphilitic lesions it was evident that in most cases more T (CD3 positive) cells than B (CD22 positive) cells were present. Regarding T cell subpopulations, in primary syphilis, T helper/inducer (CD4 positive) cells predominated in 86% of cases. In secondary syphilitic lesions, numbers of T helper/inducer cells were smaller than or equal to T-suppressor/cytotoxic (CD8 positive) cells in 60% of cases. Similar observations were made by Tosca *et al* (14). Remarkably, in yaws specimens the inflammatory infiltrate consisted mainly of IgG, but also IgA en IgM producing plasma cells. T or B lymphocytes were scarce, which is in sharp contrast with findings in syphilitic lesions, in which lymphocytes and plasma cells were present (in variable numbers). To our knowledge, this has never been reported elsewhere, and further study is needed to establish whether this represents a unique feature of *T.pertenue* or a special character of the Pariaman strain only. Unfortunately, due to the protean histopathological manifestations, these two treponemal diseases cannot always be differentiated on histological grounds alone.

At present, for differentiation, the different clinical findings and epidemiology of the treponematoses (see chapters 1.2, 1.4 and 1.6) are most important. It is postulated that the differences observed in the histopathology of yaws and syphilis (see chapters 4.1 and 4.2) may represent the milder course of the former infection compared with the latter; in syphilis neurological and cardiovascular involvement are notorious and the congenital form is responsible for early death or mutilation of the young. In yaws and the other endemic treponematoses no neurological or cardiovascular sequels or a congenital form exist. Moreover, severe mutilation may occur in yaws and endemic syphilis, but (apart from the extreme skin colour changes) no serious late stage disease exists in the third endemic treponematosis, pinta, in which involvement of internal organs does not occur. Hitherto, these matters have not been fully elucidated.

LABORATORY STUDIES

In this part of the thesis it was demonstrated that a rapid *in vitro* immobilisation of *T.pallidum* (Nichols strain) can be accomplished after Percoll purification. The rapid immobilisation can be delayed by pre-incubation of the treponemes with the testicular extracts, which had been removed during the purification process.

A slowly progressing *in vitro* immobilisation of *T.pallidum* isolated from infected rabbit testicles in the presence of immune serum is well known (15) and has often been related to the survival of the treponemes in the host for many years. With the Percoll purification method mobile and virulent treponemes can be obtained, which are relatively free of host proteins (16). It was demonstrated that purification of *T.pallidum* by the Percoll purification method drastically changed their behaviour towards complement (C)-dependent immobilisation; purified *T.pallidum* was rapidly (almost within 2h) immobilised by normal (seronegative) human serum (NHS), while unpurified *T.pallidum* was immobilised slowly under the same circumstances. This demonstrates that a rapid *in vitro* immobilisation of the treponemes is possible.

In chapter 5.1 it was found that the classic pathway of C activation was responsible for this rapid immobilisation. In the same chapter it was shown that after the addition of testicular extracts, purified treponemes again became more resistant to immobilisation. Since testicular extracts from uninfected testicles displayed the same effect, any role of substances produced under the influence of the infection was ruled out. Moreover, in chapter 5.2 it was demonstrated that the addition of heat-inactivated serum from other animal species provoked a similar effect.

In chapter 5.3 the importance of the participation of IgM in the immobilisation process *in vitro* was described. It was suggested that the loss of integrity of the outer membrane by an attack by IgM and C offers the opportunity for lysozyme to hydrolyse the peptidoglycan layer surrounding the cytoplasmic membrane of the treponemes, which then is accessible for attack by antibodies and C. The cause of the importance of IgM may be twofold. Firstly, in view of the hypothesis of a scarcity of epitopes on the treponemal surface (17-19) IgM, but not IgG, may be capable of forming antigen-antibody complexes on the treponemal surface. This may be due to the larger size of this class of antibody molecules, which will allow them to bridge larger distances between epitopes. Secondly, a single IgM molecule, once bound in

an antigen-antibody complex is capable of C activation, while for the C activation by IgG at least a doublet of IgG molecules is required at a sufficiently short distance to allow the binding of C1q to initiate the C cascade.

In considering the delay in the rapid immobilisation, it is convenient to distinguish three steps in the immobilisation process, i.e. the antibody binding step, the C activation step, and finally lysis of the microorganisms. It is not known which step(s) in the rapid immobilisation are affected by the testicular extracts or the immobilisation-inhibiting sera and which components may be held responsible. Inhibition of the antibody-binding step may happen by the binding of substances from testicular extracts at the surface of the treponemes in such a way that access to treponemal epitopes is blocked. This would mean the re-establishment of a protective cover around the treponemes. However, the existence of such a cover is not supported by experimental evidence (20).

A second possible manner of inhibition of antibody-binding may be related to the method used to prevent false-positive reactions in the FTA-ABS reaction. Here, it was shown that the binding of cross-reacting antibodies to the treponemes can be circumvented by solutions of widely varying composition (21,22). Among these are solutions with a high protein content. However, the mechanism of blocking of the cross-reacting antibodies is unknown and does not seem to rely on a simple absorption process.

Blanco *et al.* have demonstrated that the C activation step was rate-limiting in the immobilisation of unpurified treponemes (23). C activation was accompanied by an antibody-driven aggregation of the *T.pallidum* rare outer membrane protein (TROMP). Therefore, it is feasible that the rapid immobilisation of the purified treponemes is a result of a more facilitated aggregation of the outer membrane proteins. It may also be imagined that interference with this aggregation by components in the testicular extract will reduce the speed of immobilisation.

When antibody-binding and C-activation are intact, the delay in immobilisation might result from interference with late-acting C components by the testicular extracts. This does not seem likely, since reaction mixtures that were used to study the immobilisation of the treponemes still contained residual C, as demonstrated by their capacity to lyse sensitised sheep erythrocytes. Effective lysis of the treponemes might be prevented by substances from the testicular extracts, which may adhere to the treponemal surface and absorb the attack of the lytic C-complexes on the treponemal outer membrane. However, from the observation

of the absence of C on the large majority of the treponemes in skin biopsies from syphilitic lesions of patients (chapter 4.3), it seems likely that C-activation *in vivo* does not occur. This phenomenon favours a lack of antibody-binding or of C-activation.

Whatever (combination of) factor(s) mentioned above might play a part, it will be clear that a slow immobilisation *in vivo*, especially during the initial phase of infection, will offer at least temporary protection, which will allow the treponemes to spread through the body tissues.

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CHAPTER 7. SUMMARY

Treponematoses important to mankind are venereal syphilis and the endemic treponematoses (yaws, endemic syphilis and pinta). The causative agents are *Treponema pallidum* subspecies *pallidum* (*T.pallidum*), *Treponema pallidum* subspecies *pertenue* (*T.pertenue*), *Treponema pallidum* subspecies *endemicum* (*T.endemicum*), and *Treponema carateum*, respectively.

Chapter 1

In chapter one the treponematoses are reviewed. Just after the beginning of the AIDS epidemic, syphilis received renewed attention. It became clear that classical STD and especially ulcerative STD such as syphilis may facilitate the transmission of HIV infection. Recent problems in the diagnosis and treatment of syphilis in HIV-seropositive or AIDS patients are described in a separate paragraph.

Recently a resurgence of the endemic, nonsexually transmitted treponematoses has been reported. Especially where yaws and endemic syphilis are concerned, this resurgence is dramatic, particularly in several parts on the African continent. The endemic treponematoses are reviewed and in a separate paragraph special attention is given to the world-wide consequences of the resurgence of yaws. Some developments in treponemal research are briefly discussed.

Chapter 2

In this chapter the aims of the study are enumerated.

Chapter 3

In the first part of chapter three, the current world-wide spread of sexually transmitted syphilis is discussed, with its serious consequences. Current trends in the epidemiology of syphilis in developing countries, the United States of America and the Netherlands, respectively, are briefly discussed.

The results of a small yaws survey on the island of Sumatra in Indonesia are presented next. The prevalence of yaws in the investigated region in West Sumatra was found to be

high, a minimum of 300 cases per 100.000 individuals, which indicates that yaws is far from eradicated and that continued screening and treatment are necessary. Patients suffering from early infectious yaws showed florid classical skin lesions. Of 101 serum samples from such patients, 100 had a positive reaction in one or more treponemal tests. The *Treponema pallidum* haemagglutination assay was found to be the most sensitive test (97% positive) in detecting antibodies against *T.pertenue*, followed by the fluorescent treponemal antibody absorption test (94%), the Venereal Disease Research Laboratory test (90%), the TmpA ELISA (91%), and analysis by Western blot using *T.pallidum* antigens (88%). Of 42 asymptomatic contacts of yaws patients 32 showed positive reactions in one or more tests, indicating that many people in the region investigated have been infected with treponemes. New *T.pertenue* strains were isolated from yaws skin lesions. *In vitro* amplification of treponemal DNA and hybridization with specific DNA probes in Bilthoven, the Netherlands, showed that the strains were identical with *T.pertenue* CDC 2575, with regard to the subspecies *pertenue* specific tyfl gene.

Migrating people may transport yaws out of endemic regions. In the last part of chapter three this is illustrated by a case report of a 9-year-old girl originating from Ghana suffering from (early infectious) yaws, diagnosed as imported disease in the Netherlands. This girl had lived in the Netherlands for six months. Endemic treponematoses are rarely seen in Europe, and only a few case reports have been published. Migration and travelling may confront the medical profession with cases of tropical diseases such as yaws. Positive serological reactions in non-venereal tropical or venereal treponematoses cannot be distinguished at present.

Chapter 4

Extensive studies of human skin biopsies, taken from patients suffering from early yaws or syphilis, performed in co-operation with the Department of Pathology, are presented in chapter four.

Firstly, the light-microscopic findings in biopsies of skin lesions from 45 patients, in whom a diagnosis of early yaws was suspected in rural areas in West Sumatra, Indonesia, are presented. Results of this study were the following. In 27 cases typical light-microscopic features of yaws were observed, consisting of parakeratosis or crust containing exudate, marked acanthosis with widening and elongation of the rete ridges or pseudocarcinomatous hyperplasia and spongiosis. Intraepidermal microabscesses consisting of polymorphonuclear

leucocytes were frequently encountered. In a large majority a moderate to dense infiltrate was present, composed mainly of plasma cells and lymphocytes. Vascular changes consisted of only slight endothelial cell proliferation and thickening of vessel walls. Steiner staining revealed the presence of treponemes in the epidermis in 23 of 27 cases. Remarkably, clusters of treponemes were also seen in the papillary dermis in three out of 23 cases. Seven other cases were strongly suggestive of yaws. Other histopathological diagnoses were made in six patients, due to the simultaneous occurrence of other skin diseases. The remaining five specimens did not contain enough tissue to allow conclusions.

Secondly, a study of biopsies taken from skin lesions of 44 patients presenting with clinical symptoms of primary or secondary syphilis at the Clinic for sexually transmitted diseases, University Hospital Rotterdam-Dijkzigt, the Netherlands is presented. In most primary stage lesions erosion or, more often, ulceration was present, with a dense inflammatory infiltrate. In secondary syphilis a wide variety of histological changes was present. Blood vessels were frequently involved, with marked endothelial swelling and often proliferation. Treponemes were demonstrated with the Steiner staining method in all investigated cases of primary syphilis and in 71% of secondary syphilis cases. Treponemes were present throughout the dermis, particularly perivascularly, and in the dermal-epidermal junction zone. In two specimens of secondary syphilis treponemes were located predominantly in the epidermis, but there were always some microorganisms demonstrable in the dermis. The inflammatory infiltrate was often located in a perivascular coat-sleeve-like arrangement. In this study plasma cells and lymphocytes were present in all specimens of primary and secondary syphilis. Syphilitic lesions differed from yaws lesions mostly in the location of treponemes and the affection of blood vessels. In this study, treponemes did not show the epidermotropic character of yaws, and blood vessel changes were more pronounced than in yaws. Unfortunately, due to the protean histopathological manifestations described in venereal syphilis and in yaws, these two treponemal diseases cannot always be differentiated on histological grounds alone.

Thirdly, we studied the localisation of treponemes and analysed the inflammatory infiltrate in biopsies from patients suffering from primary or secondary syphilis (Rotterdam, the Netherlands), or early infectious yaws (West Sumatra, Indonesia). Skin biopsies originating from human lesions of primary (29x) or secondary (15x) syphilis, or early yaws (18x) were studied. Different histochemical and immunohistochemical detection methods were used in

this study. The histochemical silver staining method according to Steiner revealed the presence of *T.pallidum* in all cases of primary syphilis studied. In 10 out of 14 cases of secondary syphilis, treponemes were demonstrated. With an immunofluorescence staining technique (IF) using anti-*T.pallidum* antiserum raised in rabbits (a-Tp), *T.pallidum* was demonstrated in 28 out of 29 cases of primary syphilis, and in 14 out of 14 studied cases of secondary syphilis. The silver staining method and IF showed identical localisations of *T.pallidum* (mainly in the dermal-epidermal junction zone or throughout the dermis). Using a-Tp antiserum in the indirect immunofluorescence technique, *T.pertenue* could be demonstrated in the dermis more often than with Steiner silver staining. However, epidermotropism in yaws specimens was remarkable, compared with more mesodermotropism of *T.pallidum*; numbers of *T.pertenue* in the dermis were limited in all specimens. The dermal inflammatory infiltrate in primary and secondary syphilis was composed mainly of lymphocytes and plasma cells. In most cases more T (CD3 positive) cells than B (CD22 positive) cells were present. Regarding T cell subpopulations, in primary syphilis, T helper/inducer (CD4 positive) cells predominated in 86% of cases. In secondary syphilitic lesions, numbers of T helper/inducer cells were smaller than or equal to T-suppressor/cytotoxic (CD8 positive) cells in 60% of cases. Remarkably, in yaws specimens the inflammatory infiltrate consisted mainly of IgG, but also IgA en IgM producing plasma cells. T or B lymphocytes were scarce, which is in contrast with findings in syphilitic lesions.

Chapter 5

In the first part of chapter five laboratory experiments with *T.pallidum* are described. A strong limitation of treponemal research is the impossibility of culturing pathogenic treponemes *in vitro* for prolonged periods of time. For our immobilisation experiments, which require large numbers of treponemes, numbers of *T.pertenue* and *T.endemicum* obtained after many rabbit passages were inadequate, so only *T.pallidum* could be used.

In the first study the use of Percoll-purified treponemes in an assay similar to the *Treponema pallidum* Immobilisation (TPI) test demonstrated that immobilisation of purified treponemes by seronegative normal human serum (NHS) proceeded at a much higher rate than that of unpurified treponemes. This suggests that the removal of the testicular extract makes the treponemes more vulnerable to this action. A preincubation of the purified

treponemes with the testicular extract from infected or uninfected testes reduced their rate of immobilisation to that demonstrated by the unpurified treponemes. This showed that substances produced during the infection are probably not responsible for the delay in immobilisation. Discrimination between the classical and the alternative pathway of complement activation, studied by the ethylene glycol-bis (beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) method, showed that the classical pathway was responsible for the rapid immobilisation of the purified treponemes. However, the slow immobilisation in the EGTA-serum samples suggested a minor role of the alternative pathway in the immobilisation of the purified treponemes. Since the testicular extracts exerted an anti-complement activity, it should be investigated whether the protection offered to the purified treponemes by the testicular extracts is based on their deteriorating effect on the classical complement pathway or is due to a re-establishment of the protective cover around the treponemes.

In the next part of chapter five an investigation is described of sera, especially rabbit serum, in preventing *in vitro* immobilisation of Percoll purified *T.pallidum*. The immobilisation of Percoll purified *T.pallidum* (Nichols) was studied after preincubations with basal reduced medium (BRM), heat-inactivated serum of seven different species of animals, heat-inactivated NHS and rabbit sera containing different levels of antitreponemal antibodies. Increasing percentages of heat-inactivated normal rabbit serum (NRS) were also studied. It became clear that the rapid immobilisation of purified treponemes by NHS is slowed down by preincubation with NRS in a dose-dependent manner. The treponemes from 5-day infections were immobilised significantly more slowly than treponemes from 7- and 8-day infections. Compared with NRS, preincubations with a high-titred, a low-titred and an "autologous" serum resulted in significantly more rapid immobilisation of the treponemes. With most other animal sera resistance to immobilisation was slight compared with that produced by NRS. Immunofluorescent studies revealed that the treponemes were covered with a layer of the human third complement factor (C3b), within an hour of incubation. With two sequential preincubations, a slowing down of the immobilisation was only noted in those test mixtures in which NRS had been present in both preincubations. In conclusion, it was observed that rabbit serum slows down the rapid *in vitro* immobilisation of Percoll purified treponemes by NHS. No evidence was present that this was caused by preventing access of antibodies (*in vivo* as well as *in vitro*) to, or preventing the activation of complement on the treponemal surface. The evidence points to a mechanism in the fluid phase, suggesting

participation of a third factor in the immobilisation process, for instance an enzyme, which can be partially inhibited by rabbit serum component(s).

In the third part of chapter five the importance of different components in NHS and the role of lysozyme in the rapid immobilisation were investigated. The immobilisation of Percoll purified *T.pallidum* was studied after preincubations with different serum fractions (Fr) of NHS (Fr 1, containing IgM; Fr 2, containing IgG and a low level of haemolytic complement, and Fr 1 (abs), depleted of IgG). A guinea-pig serum pool was used as a complement source in the immobilisation experiments. The influence of removal of lysozyme from guinea-pig serum on the immobilisation characteristics was studied. Furthermore, experiments were performed, using a fluorescence technique, to detect C3b depositions on fixed treponemes and treponemes in suspension. It was shown that the rapid immobilisation of Percoll-purified treponemes by the NHS serum fractions occurred only after preincubation with Fr 1 and Fr 2 simultaneously. This was largely dependent on the presence of a small amount of haemolytic C in Fr 2. Removal of lysozyme reduced this rapid rate of immobilisation. In fluorescence experiments it was demonstrated that C3b deposition on fixed (i.e. damaged) treponemes occurred upon their incubation with Fr 2 or the combination of Fr 1 and 2. However, on treponemes in suspension C3b deposition occurred only after incubation with the combination of Fr 1 and 2. We concluded that the rapid immobilisation of Percoll purified treponemes by serum fractions from NHS requires antibodies of the IgM and IgG class, together with complement and lysozyme. Omission of one of these reactants inhibits the rapid immobilisation. All available evidence indicates that these reactants act in sequence: the loss of integrity of the outer membrane by an attack by IgM and C offers the opportunity for lysozyme to hydrolyse the peptidoglycan layer surrounding the cytoplasmic membrane of the treponemes, which then is accessible for attack by antibodies and complement.

The last part of chapter five gives the results of a study of the ultrastructural aspects of infection with *T.pertenue* (Pariaman strain) originating from West Sumatra, in rabbit testicular tissue. Furthermore, electron microscopical study was performed of skin biopsies (human skin) originating from patients suffering from early infectious yaws in Sumatra. In one out of five specimens from rabbit testicular tissue a profusion of treponemes were found lying in the interstitial myxomatous tissue. Microorganisms showed no adhesion to fibroblasts. In human skin, treponemes were found in interepidermal spaces in five out of 10 specimens. In two of five positive specimens, treponemes were also seen in the dermis. This

ultrastructural study of *T.pertenue* demonstrated the scarcity and focal distribution of treponemes in tissue and did not reveal any morphological differences from the Gauthier strain of *T.pertenue*. No differences from the ultrastructure of *T.pallidum* were observed either.

CONCLUSION

Renewed attention and continuing education on venereal syphilis and the nonvenereal, endemic treponematoses are required to alert all workers in the medical field and to facilitate proper recognition of these important diseases. Presumably, in the 1990s, attention will be focused on the AIDS epidemic, but this must not make us forget the important classical infectious diseases.

CHAPTER 8. SAMENVATTING

De voor de mens van belang zijnde treponematosen zijn venerische syfilis en de endemische treponematosen (framboesia tropica, endemische syfilis en pinta). De verantwoordelijke ziekteverwekkers zijn respectievelijk *Treponema pallidum* subspecies *pallidum* (*T.pallidum*), *Treponema pallidum* subspecies *pertenue* (*T.pertenue*), *Treponema pallidum* subspecies *endemicum* (*T.endemicum*) en *Treponema carateum*.

Hoofdstuk 1

In dit hoofdstuk wordt een overzicht gegeven van de treponematosen. Na het begin van de AIDS epidemie kwam syfilis opnieuw extra in de belangstelling. Het werd steeds duidelijker dat de klassieke sexueel overdraagbare aandoeningen (SOA) en in het bijzonder ulceratieve SOA zoals syfilis de kans op overdracht van het humaan immunodeficiëntie virus (HIV) zouden kunnen vergroten. Enkele van de huidige problemen betreffende de diagnostiek en behandeling van syfilis in HIV-seropositieve en AIDS patiënten worden in een aparte paragraaf beschreven.

De laatste jaren wordt melding gemaakt van een terugkeer in de wereld van de endemische, niet venerische treponematosen. Er is met name sprake van een terugkeer van framboesia tropica en endemische syfilis, vooral in grote delen van Afrika. De verschillende endemische treponematosen worden uitgebreid behandeld en speciale aandacht wordt geschonken aan de consequenties van de terugkeer van framboesia tropica in de wereld.

Tot slot worden enige ontwikkelingen in treponemaal onderzoek beschreven.

Hoofdstuk 2

In dit hoofdstuk wordt het doel van het onderzoek beschreven.

Hoofdstuk 3

Het eerste deel van hoofdstuk drie bevat enige epidemiologische opmerkingen over syfilis, met speciale aandacht voor ontwikkelingslanden, de Verenigde Staten en Nederland.

Vervolgens worden resultaten van een klein onderzoek naar framboesia tropica op Sumatra in Indonesië besproken. Framboesia tropica kwam in de onderzochte gebieden in West

Sumatra nog veelvuldig voor (naar schatting tenminste 300 patiënten per 100.000 personen), hetgeen het belang van het continueren van screening en behandeling onderstreept. Bij patiënten met vroege framboesia werden klassieke huidlaesies aangetroffen. Bij 100 van 101 serum monsters van de patiënten werd een positieve reactie in één of meer treponemale testen gevonden. De *Treponema pallidum* haemagglutination assay bleek de meest sensitieve test (97% positief) te zijn voor het aantonen van antilichamen tegen *T.pertenue*, gevolgd door de fluorescent treponemal antibody absorption test (94%), de Venereal Disease Research Laboratory test (90%), de TmpA ELISA (91%), en analyse door middel van Western blotting waarbij van *T.pallidum* antigenen gebruik werd gemaakt (88%). 32 van 42 asymptomatische contacten van framboesia patiënten vertoonden een positieve reactie in één of meer testen. Dit toonde aan dat veel mensen in het bestudeerde gebied reeds in aanraking waren gekomen met een treponemale infectie. Nieuwe *T.pertenue* stammen werden geisoleerd van framboesia huidlaesies. Met behulp van *in vitro* amplificatie van treponemaal DNA en hybridisatie met specifieke DNA probes werd in Bilthoven aangetoond dat de stammen identiek waren aan *T.pertenue* CDC 2575, wat betreft het subspecies *pertenue* specifieke tyfl gen.

Migrerende mensen kunnen framboesia tropica buiten de endemische gebieden brengen. In het laatste deel van hoofdstuk drie wordt dit geïllustreerd aan de hand van een case report. Bij een 9 jarig meisje, afkomstig uit Ghana, werd de diagnose vroege (infectieuze) framboesia tropica gesteld. Dit meisje woonde reeds zes maanden in Nederland. Endemische treponematosen zijn een zeldzaamheid in Europa; slechts enkele case reports werden de laatste jaren gepubliceerd. Door migratie en het intensieve reizigersverkeer kan men geconfronteerd worden met gevallen van tropische ziekten zoals framboesia tropica.

Hoofdstuk 4

Uitgebreide studies van huidbiopten van patiënten lijdend aan vroege framboesia tropica of syfilis werden uitgevoerd (hoofdstuk vier).

In het eerste gedeelte van hoofdstuk vier wordt een lichtmicroscopische studie beschreven van huidbiopten, afgenomen bij 45 patiënten in verafgelegen streken in West Sumatra, Indonesië, bij wie de diagnose framboesia tropica werd vermoed. In 27 gevallen werden de typische licht-microscopische verschijnselen van framboesia gezien, bestaande uit parakeratose of crusta bevattend exsudaat, uitgesproken acanthose met verbreding en verlenging van de rete lijsten, of pseudocarcinomateuze hyperplasie en spongiose. Vaak

werden intraepidermale microabcessen, welke polymorfkernige leucocyten bevatten, aangetroffen. In een meerderheid der gevallen bestond een matig sterk tot sterk ontstekingsinfiltraat, voornamelijk opgebouwd uit plasma cellen en lymfocyten. Bloedvatafwijkingen bestonden uit een geringe endotheelcelproliferatie en verdikking van de wanden van de bloedvaten. Met behulp van de Steiner zilverkleuring kon de aanwezigheid van treponemen worden aangetoond in de epidermis van 23 van de 27 gevallen. Opmerkelijk was de aanwezigheid van kleine groepjes treponemen in de papillaire dermis in drie van de 23 gevallen. De bevindingen bij zeven andere gevallen waren sterk verdacht voor framboesia. In zes gevallen werd een andere histopathologische diagnose gesteld. De resterende vijf biopten bevatten onvoldoende materiaal.

Vervolgens wordt een studie beschreven van biopten afgenomen bij 44 patiënten met klinische verschijnselen van het primaire of secundaire stadium van syfilis (polikliniek voor sexueel overdraagbare aandoeningen, Academisch Ziekenhuis Rotterdam-Dijkzigt). In de meeste gevallen van primaire syfilis werd microscopisch een erosie of nog vaker een ulceratie gezien, gepaard gaande met een sterk ontstekingsinfiltraat. In gevallen van secundaire syfilis werd een grote variatie aan afwijkingen gezien. De bloedvaten waren frequent aangedaan. Dit ging vooral gepaard met endotheel zwelling en proliferatie. Treponemen konden met behulp van de Steiner zilverkleuringsmethode worden aangetoond in alle gevallen van primaire syfilis en in 71% van de gevallen van secundaire syfilis. *T.pallidum* bevond zich verspreid door de gehele dermis en met name perivasculair, en in het dermale-epidermale overgangsgebied. In twee specimen van secundaire syfilis werd *T.pallidum* voornamelijk in de epidermis aangetroffen, echter in beide gevallen bevonden zich tevens enige microorganismen in de dermis. Het ontstekingsinfiltraat was frequent in een perivasculair "coat-sleeve" patroon gerangschikt. In deze studie werden in alle gevallen van primaire en secundaire syfilis plasma cellen en lymfocyten aangetroffen.

De microscopie van syfilis laesies verschilde van framboesia laesies qua localisatie van de treponemen en qua mate van aantasting van de bloedvaten. *T.pallidum* toonde niet het epidermotrope karakter van *T.pertenue* en de bloedvatafwijkingen bij syfilis waren meer uitgesproken dan bij framboesia. Het werd duidelijk dat onderscheid tussen venerische syfilis en framboesia tropica evenwel niet uitsluitend met behulp van lichtmicroscopisch onderzoek kan worden gemaakt.

In het derde deel van hoofdstuk vier wordt een studie van de localisatie van treponemen

en een analyse van de ontstekingsinfiltraten in biopten van huidlaesies van patiënten lijdend aan primaire (29x) of secundaire (15x) syfilis (Rotterdam, Nederland), of vroege framboesia tropica (18x) (West Sumatra, Indonesië) behandeld. Verschillende histochemische en immunohistochemische detectie-methoden werden toegepast. Met behulp van de histochemische zilverkleuringsmethode volgens Steiner werd *T.pallidum* in alle bestudeerde gevallen van primaire syfilis aangetoond. Dit was het geval bij 10 van de 14 gevallen van secundaire syfilis. Met behulp van een immunofluorescentie kleuringstechniek (IF) waarbij gebruik gemaakt werd van anti-*T.pallidum* antiserum opgewekt in konijnen (a-Tp), werd *T.pallidum* aangetoond in 28 van de 29 gevallen van primaire syfilis, en in 14 van de 14 bestudeerde gevallen van secundaire syfilis. Een identieke localisatie van *T.pallidum* werd aangetoond met behulp van de zilverkleuringsmethode en IF. De treponemen waren voornamelijk gelocaliseerd in het dermale-epidermale overgangsgebied of verspreid door de gehele dermis. *T.pertenue* werd vaker in de dermis aangetoond met a-Tp antiserum dan met de Steiner zilverkleuring. Het epidermotropisme van *T.pertenue* was echter zeer uitgesproken, vergeleken met het meer mesodermotrope karakter van *T.pallidum*; indien *T.pertenue* in de dermis werd aangetroffen betrof dit slechts een klein aantal bacteriën. Het dermale ontstekingsinfiltraat bij primaire en secundaire syfilis bestond voornamelijk uit lymfocyten en plasma cellen. In de meeste gevallen waren er meer T (CD3 positieve) cellen dan B (CD22 positieve) cellen aanwezig. Studie van de T cel subpopulaties maakte duidelijk dat bij primaire syfilis, T helper/inducer (CD4 positieve) cellen in 86% van de gevallen de overhand hadden. In laesies van secundaire syfilis bleek in 60% van de gevallen het aantal T helper/inducer cellen geringer of gelijk te zijn aan het aantal T-suppressor/cytotoxic (CD8 positieve) cellen. Een opmerkelijke bevinding was dat in de framboesia coupes het ontstekingsinfiltraat hoofdzakelijk uit IgG, en ook, zij het in geringere mate, uit IgA en IgM producerende plasma cellen bestond. Slechts een enkele T of B lymfocyt was aanwezig, dit in tegenstelling tot de bevindingen bij syfilis.

Hoofdstuk 5

In de eerste twee delen van hoofdstuk vijf worden experimenten met *T.pallidum* beschreven. Het gedurende langere tijd *in vitro* kweken van pathogene treponemen is vooralsnog niet mogelijk. Dit vormt een grote belemmering voor het onderzoek. Het aantal *T.pertenue* en *T.endemicum* microorganismen dat werd verkregen na vele overentingen in het konijn bleek

te gering voor onze experimenten, waarvoor grote hoeveelheden microorganismen benodigd zijn. Deze experimenten werden dan ook alleen uitgevoerd met *T.pallidum* (Nichols).

In de eerste studie, waarbij gebruik werd gemaakt van Percoll-gezuiverde treponemen in een opzet gelijk aan de *Treponema pallidum* Immobilisatie (TPI) test, werd aangetoond dat immobilisatie van gezuiverde treponemen door (seronegatief) normaal humaan serum (NHS) veel sneller verliep dan immobilisatie van ongezuiverde treponemen. Dit suggereert dat het verwijderen van het testiculaire extract de treponemen kwetsbaarder maakt voor de invloed van het NHS. Een preincubatie van de gezuiverde treponemen met het testiculaire extract afkomstig van geïnfecteerde of niet-geïnfecteerde testes vertraagde de immobilisatie snelheid tot die van de ongezuiverde treponemen. Dit toonde aan dat producten, welke tijdens de infectie geproduceerd zouden kunnen worden, waarschijnlijk niet verantwoordelijk zijn voor de vertraging van de immobilisatie. Onderscheid tussen de klassieke en de alternatieve weg van complement activatie, bestudeerd middels de ethyleen glycol-bis (beta-aminoethyl ether) N,N,N',N'-tetraazijnzuur (EGTA) methode, liet zien dat de klassieke weg verantwoordelijk was voor de snelle immobilisatie van de gezuiverde treponemen. De langzame immobilisatie welke optrad in de EGTA serum monsters suggereerde echter dat een kleine rol was weggelegd voor de alternatieve weg bij de immobilisatie van de gezuiverde treponemen. Aangezien de testiculaire extracten een anti-complement activiteit uitoefenden, werd geconcludeerd dat het noodzakelijk is om te bestuderen of de bescherming welke door de testiculaire extracten aan de gezuiverde treponemen werd geboden gebaseerd is op een schadelijk effect van deze extracten op de klassieke complement weg, of op een hernieuwde formatie van de beschermende laag rond de treponemen.

In de volgende studie werd de rol van sera, speciaal sera afkomstig van het konijn, bestudeerd bij het verhinderen van de *in vitro* immobilisatie van Percoll gezuiverde *T.pallidum*. De immobilisatie van Percoll gezuiverde *T.pallidum* (Nichols stam) werd bestudeerd na preincubaties met basaal gereduceerd medium (BRM), hitte-geïnactiveerd serum afkomstig van zeven verschillende diersoorten, hitte-geïnactiveerd NHS en hitte-geïnactiveerde sera afkomstig van konijnen welke een verschillend niveau van antitreponemale antilichamen bevatten. Tevens werden oplopende percentages van hitte-geïnactiveerd normaal konijneserum (NRS) bestudeerd. Het werd duidelijk dat de snelle immobilisatie van gezuiverde treponemen onder invloed van NHS werd vertraagd door preincubatie met NRS op een dosis-afhankelijke wijze. De treponemen afkomstig van 5-

daagse infecties werden significant langzamer geïmmobiliseerd dan treponemen afkomstig van 7- en 8-daagse infecties. Bij vergelijking met een preincubatie met NRS, resulteerden preincubaties met een serum met een hoge titer, een lage titer en een "autoloog" serum in een significant snellere immobilisatie van de treponemen. Vergeleken met preincubatie met NRS bleek met de meeste andere dierensera de weerstand tegen immobilisatie slechts gering. Immunofluorescentie studies toonden aan dat binnen een uur van incubatie de treponemen bedekt waren met een laag van de humane derde complement factor (C3b). Met twee opeenvolgende preincubaties trad een vertraging van de immobilisatie alleen op in die test mengsels waarin NRS in beide preincubaties aanwezig was. Geconcludeerd werd dat konijneserum de snelle *in vitro* immobilisatie van Percoll gezuiverde treponemen door NHS kan vertragen. Er bestond geen duidelijkheid of dit werd veroorzaakt door het beletten van de toegang van antilichamen (*in vivo* evenals *in vitro*) tot, of het beletten van complement activatie op het oppervlak van de treponemen. Deze gegevens wijzen in de richting van een mechanisme in de vloeistoffase, en suggereren de participatie van een derde factor in het immobilisatie proces, bijvoorbeeld van een enzym, dat deels kan worden geinhibeerd door één of meerdere componenten uit het konijneserum.

In het derde deel wordt een studie beschreven van het aandeel van verschillende bestanddelen in NHS en van lysozyme in de snelle immobilisatie. De immobilisatie van Percoll gezuiverde *T.pallidum* werd bestudeerd na pre-incubaties met verschillende serum fracties (Fr) van NHS (de IgM bevattende Fr 1; Fr 2, welke IgG en enig haemolytisch complement bevat, en Fr 1 (abs), waaruit IgG is verwijderd). Een pool van cavia serum werd als complement bron gebruikt. De invloed van verwijdering van lysozyme uit het cavia serum op de immobilisatie werd bestudeerd. Met behulp van fluorescentie experimenten werd gekeken naar C3b deposities op gefixeerde treponemen en treponemen in suspensie. Het werd duidelijk dat de snelle immobilisatie van Percoll-gezuiverde treponemen door de verschillende serum fracties van het NHS alleen plaatsvond na gelijktijdige pre-incubatie met Fr 1 and Fr 2. Dit was grotendeels afhankelijk van de aanwezigheid van een geringe hoeveelheid haemolytisch complement in Fr 2. Verwijdering van lysozyme vertraagde de snelle immobilisatie. Met behulp van de fluorescentie experimenten werd aangetoond dat C3b depositie op gefixeerde, d.w.z. beschadigde treponemen plaatsvond na incubatie met Fr 2 of de combinatie van Fr 1 en Fr 2. C3b depositie op treponemen in suspensie vond alleen plaats na incubatie met de combinatie van Fr 1 and 2. Geconcludeerd werd dat de snelle

immobilisatie van Percoll gezuiverde treponemen door de verschillende NHS-fracties antilichamen vereist van de IgM en IgG klasse, samen met complement en lysozyme. Het weglaten van een dezer remt de snelle immobilisatie. Het lijkt erop dat de reactie in een bepaalde volgorde verloopt. Het verlies van integriteit van de buiten membraan door een aanval van IgM en complement biedt lysozyme de mogelijkheid om de peptidoglycaan laag, welke de cytoplasmamembraan van de treponemen omringt, te hydrolyseren. Deze wordt dan toegankelijk voor de aanval van antilichamen en complement.

Het laatste gedeelte van dit hoofdstuk betreft een electronen-microscopische studie van materiaal geïnfecteerd met *T.pertenue*. Dit betrof patiënten materiaal (huidbiopten) afkomstig uit West Sumatra, en materiaal afkomstig van konijnentestes na inoculatie met *T.pertenue* (de Pariaman stam). In één van de vijf specimen van konijnenmateriaal werden vele treponemen aangetroffen in interstitieel myxomateus weefsel. Er werd geen adhesie van treponemes aan fibroblasten bemerkt. In vijf van de tien huidbiopten van patiënten werden treponemen aangetroffen in de interepidermale ruimtes. In twee van de vijf positieve monsters werden tevens treponemen in de dermis bemerkt. Deze electronenmicroscopische studie van *T.pertenue* toonde de schaarsheid en de focale distributie van treponemen in het onderzochte weefsel aan. Morfologische verschillen konden niet worden aangetoond ten opzichte van de Gauthier stam van *T.pertenue*. Tevens werden geen verschillen bemerkt ten opzichte van de ultrastructuur van *T.pallidum*.

CONCLUSIE

Hernieuwde aandacht en voortgaande educatie inzake syfilis en de endemische treponematosen zijn van groot belang om deze belangrijke ziekten te bestrijden. De laatste jaren gaat de meeste aandacht uit naar AIDS, maar de klassieke infectieziekten mogen niet worden vergeten !

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