INTERACTION OF HIV AND SYPHILIS
AT THE KERATINOCYTE LEVEL

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Submitted in fulfilment of the requirements for the degree of

Master of Medical Science

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2014
DECLARATION

I Relebohile Lylian Moeketsi declare that

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Prof. A.W. Sturm
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This work is dedicated to my parents

Mr. and Mrs Thelejane

for their constant love and support
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List of Abbreviations

AF-ELISA: axial filament antigen enzyme linked immunosorbent assay
ARV: antiretroviral
CCR5: C-C chemokine receptor 5
CD4: cluster of differentiation 4
CDC: centres for disease control and prevention
CSF: cerebrospinal fluid
DFA-TP: direct fluorescent antibody staining for Treponema pallidum
DFAT-TP: direct fluorescent antibody tissue staining for Treponema pallidum
DNA: deoxyribonucleic acid
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme linked immunosorbent assay
EMEM: eagle’s minimum essential medium
FBS: fetal bovine serum
FTA: fluorescent treponemal antibody test
FTA-abs: fluorescent treponemal antibody- absorption test
FTA-abs (DS): fluorescent treponema; antibody-absorption double staining test
Galcer: galactosylceramide
gp120: glycoprotein 20
HIV-1: human immunodeficiency virus
HSPGs: heparin sulphate proteoglycans
IPC: infection prevention control
kDa: kilo daltons
MHA-TP: microheamagglutination assay for antibodies to Treponema pallidum
ml: milli litres
PBS: phosphate buffered saline
PBMC: peripheral blood mononuclear cells
PCR: polymerase chain reaction
PTFE: polytetrafluoroethylene
RPMI: Roswell Park Memorial Institute
RPR: rapid plasma reagin
T. pallidum: Treponema pallidum
TP-ELISA: Treponema pallidum enzyme linked immunosorbent assay
TPHA: Treponema pallidum haemagglutination assay
TRUST: toluidine red unheated serum test
UKZN: university of KwaZulu-Natal
USA: United States of America
USR: unheated serum reagin
VDRL: venereal disease research laboratory test
ABSTRACT

Introduction

Syphilis is a sexually transmitted disease, and once acquired, progresses through a series of overlapping stages. Data from a series of surveillance studies indicate that the acquisition of syphilis may be altered by co-infection with HIV, suggesting that HIV infection decreases the chances for infection with Treponema pallidum at the muco-cutaneous level.

This study, through the inoculation of keratinocytes with HIV and/or T.pallidum, investigated the effect of HIV infection of keratinocytes, on the transmigration abilities of T.pallidum across a keratinocyte monolayer.

Methods

Using magnetically labelled antibodies specific for antigens in the viral envelope, infectious HIV virions were isolated from blood. T.pallidum was harvested from the testes of rabbits in which the organism was propagated. HaCaT cells were cultured on collagen-coated transwell inserts, in 24-well tissue culture plates. Upon confluency, cells in one experiment were inoculated first with HIV, and three days later with T.pallidum; cells in a second experiment were exposed to T.pallidum first, and three days later inoculated with HIV; and cells in the third experiment were exposed to both HIV and T.pallidum at the same time. The media below the inserts, which contained the treponemes that passed through, was harvested at
different time points (24, 48, and 72 hours). For experiments one and two, post-inoculation time points only took effect after inoculation with the second organism. DNA was extracted using Probetec lysis buffer and quantitation was done by real-time PCR.

Results

The number of treponemes that passed through prior HIV and *T.pallidum* infected monolayers indicated little difference between the two culture conditions. The treponeme numbers indicated an initial drastic decline, followed by a remarkable increase between 24 and 48 hours and a plateauing at 72 hours. However, transmigration through *T.pallidum* and HIV exposed keratinocytes (experiment three), displayed a slight initial decline followed by a drastic continuous increase in quantity till 48 hours post-infection, reaching significantly higher levels, compared with experiment 1 and 2.

Conclusion

The results suggest that at the time HIV enters the keratinocytes, changes in the cell membrane structure occur thus allowing for better adhesion and intake of *T.pallidum*; therefore a higher transmigration rate. This observation may explain both a decrease in primary syphilis in HIV endemic areas as well as the reported rapid progression to secondary syphilis in patients with concurrent HIV infection.
CHAPTER 1

INTRODUCTION

Syphilis remains a major health problem, and its association with other sexually transmitted diseases, especially HIV, increases the complexity of syphilis control programmes.

Treponema pallidum, a helically coiled bacterium, is a member of the order Spirochaetes. T. pallidum carateum, T. pallidum subspecies pertenue, and endemicum are aetiological agents of the non-venereal diseases pinta, yaws and endemic syphilis respectively. T. pallidum subspecies pallidum is the causative organism of venereal syphilis (Singh, 1999).

Syphilis undergoes a series of stages and presents itself as an ulcer in the primary stage, systemic infection with skin rash in secondary disease, and cardiovascular and neurological manifestations in the tertiary stage.

Previous studies to maintain T. pallidum in continuous in vitro culture systems have been unsuccessful, thus in vivo passage of treponemes in laboratory animals, is how treponemal cultures are currently maintained. T. pallidum has been maintained in rabbits since 1912, and this has been achieved by intra-dermal or intra-testicular injection (Nichols Hj, 1913). T. pallidum has been shown to eagerly adhere to mammalian cells, and survival of the bacterium in culture is prolonged by presence of tissue culture cells (Fieldsteel, Becker, & Stout, 1977; Fitzgerald, Johnson, Miller, & Sykes, 1977; Fitzgerald, Miller, & Sykes, 1975). Attachment of pathogenic treponemes to cultured cells, and the absence of this with non-pathogenic treponemes, suggests that attachment is an important part of treponemal
pathogenesis. When visualized by scanning electron microscopy, as many as 70-100 treponemes were observed attached to each cell, and the cultured cells retained their morphological integrity and viability (Fitzgerald, Cleveland, Johnson, Miller, & Sykes, 1977). However, a few years later, using a variety of cells, including keratinocytes, morphological damage to cells, indicated by rounded appearance and cellular detachment to solid surfaces, was detected (Fitzgerald, Repesh, & Oakes, 1982).

Fibronectin is a filamentous glycoprotein with an array of cellular functions, among which is strengthening of the cell surface. It has been shown to function as an affinity matrix for adherence of motile Treponema pallidum to host cells (cytadherence) (Peterson, Baseman, & Alderete, 1983). Studies done have shown that, in the presence of antiserum to fibronectin, treponemal attachment to cultured cells is hindered and virulence inhibited (Fitzgerald & Repesh, 1985). This glycoprotein has distinct domains with which it binds to the cell surface, and T.pallidum recognises the domain reserved for this cell-binding (Hayashi & Yamada, 1983; Thomas, Baseman, & Alderete, 1985).

Based on a 28-41% blockage of attachment of T.pallidum to cultured cells in the presence of soluble fibronectin, Fitzgerald et al postulated that not all organisms attach to host cells by interaction with fibronectin (Fitzgerald & Repesh, 1985); but could instead attach to laminin, collagens and glycosaminoglycans which may be present in different cultured cell types (Fitzgerald, Repesh, Blanco, & Miller, 1984).

Initial lesions (chancres) occur at the site of inoculation during transmission. These lesions are thought to follow T. pallidum infiltration of intact mucous membranes or entry through minor skin abrasions.
Dissemination of the organism into internal tissues was found to be through paracellular transport, and the presence of hyaluronidase associated with the surface of *T. pallidum* plays a pivotal role in facilitating dissemination in connective tissue (Fitzgerald & Repesh, 1987). When *T. pallidum* was added to endothelial cells grown on membrane filters under conditions in which tight intercellular junctions had formed, *T. pallidum* was found in the intercellular junctions, between the cells and the membrane, as well as in the pores of the filter (Thomas, Fogelman, Miller, & Lovett, 1989; Thomas et al., 1988). However, in work focusing on the mechanism through which *T. pallidum* enters the human body, Govender et al. studied the interaction between a human keratinocyte cell line and a Nichols strain of *T. pallidum*. The treponemes were observed adherent to the cell surface of keratinocytes, in membrane bound compartments, and, underneath the cells. These observations lead to the authors’ conclusion that *T. pallidum* has the ability to migrate through an intact layer of keratinocytes without disrupting the tight junctions (intracellular migration) (Sturm and Govender, 2006).

In an investigation to develop an *in vitro* model with which invasion of tissues by pathogenic *T. pallidum* could be studied, culture chambers were created using complete abdominal walls excised from mice. The top chambers were then inoculated with both motile *T. pallidum* and non-pathogenic *T. phagedenis* biotype Reiter; under observation using dark-field microscopy and scintillation counting, only *T. pallidum* was detected as having crossed the barrier. (Riviere, Thomas, & Cobb, 1989).

*T. pallidum* was found to be microaerophilic in nature, with the highest multiplication and survival rate observed in an environment with 1.5-5% oxygen (Cover, Norris, & Miller, 1982; D. L. Cox et al., 1990; Fieldsteel, Cox, & Moehl, 1982). During the natural course of infection, *T. pallidum* attaches to the keratinocytes and disseminates to other tissues via bloodstream and lymphatic system inducing symptoms of secondary disease. This indicates
that *T. pallidum* is competent of growth and proliferation at 37°C, the host temperature. However, multiplication in low temperature areas of the body, most notably the skin, appears optimum (Fieldsteel et al., 1982).

Upon infection, the skin is the first line of defence, and keratinocytes, the predominant cell type in the dermis, act to impede pathogen entry. The close propinquity of Langerhans cells to the mucosal surface make them among the first cells to be recruited upon HIV-1 infection. Experiments to determine entry of HIV-1 into human foreskin utilized inner and outer foreskin explants placed on top of a permeable membrane, in a two-chamber system. HIV-1 was found to enter inner foreskin explants, but was trapped within the thick layer of keratin of the outer foreskin explants. The authors suggested that the thick keratin layer in the outer foreskin provides a physical impediment to HIV-1 entry, thus possibly preventing internalization of the virus by Langerhans cells (Ganor et al., 2010).

Data of a large number of surveillance studies on the aetiology of genital ulcer disease have shown that HIV patients have a significantly decreased chance of having primary syphilis (Table 1). One of the possible reasons for this is that HIV infection protects against *T. pallidum* infection at the level of the keratinocyte. This is indicated by a study done by Sturm *et al* that suggests that HIV infection decreases the chance for infection with *T. pallidum* at the muco- cutaneous level (Sturm, Moodley, & Sturm, 2003).
Table 1: Unpublished data, Sturm et al.

This study aims to investigate whether migration through a keratinocyte layer is influenced by prior infection with HIV.

The hypothesis of the study states that *T.pallidum* passes more rapidly through HIV infected keratinocytes.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Prevalence (HIV infected n=331)</th>
<th>Prevalence (HIV uninfected n=314)</th>
<th>RR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T.pallidum</em></td>
<td>5</td>
<td>10</td>
<td>0.78 (0.65 - 0.78)</td>
<td>0.001</td>
</tr>
<tr>
<td><em>H.ducreyi</em></td>
<td>6</td>
<td>6</td>
<td>1.01 (0.88 - 1.15)</td>
<td>0.9</td>
</tr>
<tr>
<td>HSV</td>
<td>50</td>
<td>44</td>
<td>1.05 (0.99 - 1.13)</td>
<td>0.08</td>
</tr>
<tr>
<td><em>C.trachomatis</em> (LGV)</td>
<td>12</td>
<td>8</td>
<td>1.09 (1.00 - 1.20)</td>
<td>0.07</td>
</tr>
<tr>
<td>no aetiology</td>
<td>30</td>
<td>34</td>
<td>0.95 (0.89 - 1.02)</td>
<td>0.2</td>
</tr>
</tbody>
</table>
2.1 Historical perspective

Venereal syphilis is listed, by biologist Irwin Sherman, as one of the twelve diseases that changed the world (Sherman, 2007). However, the origin of syphilis is one of the most disputed subjects in medical history.

Of the origin of syphilis in Europe, three hypotheses were advanced: the Pre-Columbian, Columbian and Unitarian hypothesis (Harper, Zuckerman, Harper, Kingston, & Armelagos, 2011; Nunn & Qian, 2010).

The pre-Columbian theory proposes that syphilis is the result of a mutation in the genome of the causative agent of another treponemal disease already present in Europe. This theory alleges that syphilis originated in central Africa and its introduction to Europe was prior to the voyage to the ‘new world’ by Columbus (Bullen, 1972). Skeletal characteristics of syphilis were found missing in human bones from pre-Columbian Europe, Africa and Asia, thus instigating doubt to this theory (B. M. Rothschild, 2005).

The Columbian theory is the most popular and best supported of the three. This theory suggests that syphilis originated in the new world and Christopher Columbus and his crew brought it back to Europe (Crosby, 1969; Harrison, 1959; Weisman, 1966). Historical, archaeological and molecular phylogenetic evidence suggests that Columbus and his soldiers
got infected in the Dominican Republic, and transmitted the disease to the old world in 1642 upon their return (Harper et al., 2011; B. M. Rothschild, 2005; B. M. Rothschild, Calderon, Coppa, & Rothschild, 2000; C. Rothschild & Rothschild, 2000).

The Unitarian theory, postulated by Hudson, proposed that what we now call syphilis is in fact evidence of treponemal infection. The Unitarians debated that clinical manifestations in man were different in diverse geographical areas because of climate and cultural differences: yaws in the tropics, bejel in the Middle East and pinta in Central America (Crosby, 1969; Hudson, 1958, 1964).

In South Africa, syphilis was unknown to the Africans. The disease is said to have been introduced by sailors, army troops and white settlers (Europeans) into the cape region, and was therefore primarily confined to coastal areas. In 1802, no evidence of syphilis was found in the AmaXhosa of the Eastern cape (Lichtenstein H (1812): quoted by Sax, 1952), little syphilis was detected among the Batswana people in 1857 (Livingstone D (1857): quoted by Murray, 1957), but by 1885, Warren noted substantial infection in the ‘natives’ of Bechuanaland (Warren’s expedition. Official report. Quoted by McArthur, 1922).

Sydney L Kark propounded that the spread of syphilis in South Africa was as a result of the discovery of diamonds at Kimberly in 1867, and further spread of the disease was upon discovery of gold at the Witswatersrand (Kark, 2003).

### 2.2 Characteristics of *T. pallidum*

Organisms of the genus *Treponema* are strictly anaerobic or microaerophilic, and are chemoorganotrophs utilising a variety of amino acids or carbohydrates as energy and carbon
sources. In secretions from lesions in any phase of the disease, treponemes can be viewed by dark-field microscopy in unstained wet preps (Norris, Cox, & Weinstock, 2001).

The cells of the species *T. pallidum* range in length from 6-20 µm as measured along the axis of the helix and 0.10-0.18 µm in diameter (Norris & Larsen, 1995; Singh, 1999). The organisms are highly motile. Different from other motile bacteria, the flagella of treponemes do not traverse the outer membrane but emerge from each end of the organism and run in the periplasmic space parallel to the longitudinal axis of the cell in the direction of the other pole (periplasmic or endoflagellae). (Norris et al., 2001). When these flagellae rotate, the bacterial cylinder is wounded around the bundle of flagellae resulting in the helix with flagella as an axis in the centre.

The genus *Treponema* includes at least ten species and subspecies associated with men. Four of these are pathogenic in human and six live on mucosal surfaces of human but have not clearly been associated with disease.

Syphilis is a sexually transmitted disease caused by *T. pallidum subsp. pallidum*. The other non-venereal treponematoses are endemic syphilis (bejel) caused by *T. pallidum subsp. endemicum*, yaws, caused by *T. pallidum subsp. pertenue* and pinta, caused by *T. pallidum. carateum* (Lukehart, 2012). Recent studies have defined a genetic signature in the 5’-flanking region of the 15-kDa lipoprotein gene (*tpp15*) that distinguishes *T. pallidum subsp.pallidum* from *T. pallidum subsp. pertenue* and *endemicum* (Centurion-Lara et al., 1998).

2.3 **Clinical features of syphilis**
Once acquired, the disease progresses through a series of overlapping stages: primary, secondary, latent and tertiary syphilis. Each stage has unique signs and symptoms and different levels of infectivity.

Infection with *T. pallidum* results in a chancre at the site of inoculation. This stage of the disease is known as primary syphilis. The chancre is usually between 0.3-3.0 cm in diameter, and is described as painless with an indurated base. It develops approximately three weeks after exposure. (Chapel, 1978; DiCarlo & Martin, 1997; Singh, 1999). In heterosexual men the chancre are mainly located on the glans, the prepuce and the shaft of the penis. Additional locations in homosexual men are the rectum, the anal canal or oral cavity (Lafond & Lukehart, 2006). In women the lesions are found on the vaginal wall, labia or cervix of the uterus (Chapel, 1978; Lafond & Lukehart, 2006; Singh, 1999). If untreated, the chancre spontaneously heals within three to eight weeks, and disappears (Kent & Romanelli, 2008; Lafond & Lukehart, 2006).

Secondary lesions appear during or after primary ones (Baughn & Musher, 2005). In early secondary syphilis, a relapse of primary lesions can occur.

Malaise, sore throat, headache, weight loss, low-grade fever and muscle aches are some of the symptoms associated with secondary syphilis. The most common and earliest finding however, is the macular rash. The signs and symptoms are usually observed four to ten weeks after the appearance of the chancre (Baughn & Musher, 2005; Chapel, 1980; Golden, Marra, & Holmes, 2003; Kent & Romanelli, 2008; Lafond & Lukehart, 2006; Singh, 1999). If not treated, the disease then progresses to latent and possibly late stages of the disease (Baughn & Musher, 2005; Hall, Klausner, & Bolan, 2004).
A *T. pallidum* infection is referred to as latent syphilis between disappearance of secondary symptoms and appearance of tertiary manifestations or cure. The CDC described the period within a year of the onset of latency as early latency, and late latency as the period untreated persons enter after a year without disease reappearance, and before tertiary syphilis onset. The clinical difference between the two is that in early latency relapse of secondary syphilis can occur while that does not happen in late latency. Latent syphilis is said to have ended when tertiary disease symptoms have developed, or, when the patient with or without the treatment has been cured (Baughn & Musher, 2005; Singh, 1999).

The three main forms of organ associated or tertiary syphilis are: late benign, cardiovascular and neurosyphilis. Gummata, a localised form of tissue destruction rarely causes serious disease, but when it occurs in vital organs, complications may occur. Cardiovascular disease is commonly evidenced by aortitis, followed by aortic aneurysm or aortic insufficiency. Neurosyphilis has been divided into 5 major categories: asymptomatic, meningeal, meningeovascular, parenchymatous and gummatous neurosyphilis (Singh, 1999).

Co-infection of syphilis with other sexually transmitted diseases, such as HIV, increases the complexity of the problem. Of late, it has been hypothesised that infection with HIV may alter the presentation and natural history of syphilis. This is due to reports of unusual presentations and rapid progression of syphilis in patients with concurrent HIV infection (Golden et al., 2003; Hutchinson, Hook, Shepherd, Verley, & Rompalo, 1994; Karp, Schlaeffer, Jotkowitz, & Riesenberg, 2009; Lynn & Lightman, 2004).

*T. pallidum* infection in the new born is called congenital syphilis, and the treponemes are transmitted transplacentally from mother to foetus (Norris & Larsen, 1995). Early post-natal
manifestations are infectious and appear in the first two years of life; late manifestations appear after two years. Stillbirth, prematurity and low birth weight are some of the manifestations observed as a result of congenital infection (Lafond & Lukehart, 2006; Singh, 1999).

2.4 Diagnosis of syphilis

2.4.1 Detection of *T. pallidum* in syphilitic lesions

2.4.1.1 Dark-field microscopy

The use of dark-field illumination for the microscopical visualisation of *T. pallidum* was first described by Coles in 1909. He noted especially the specific motility of the organism (CDC, 1998; Coles, 1909). To perform the examination, microscopes equipped with a single reflecting or double reflecting dark-field condenser are needed (Kennedy & Creighton, 1998a). Accuracy of the interpreted results is dependent on the experience and expertise of the microscopist (Ratnam, 2005).

2.4.1.2 Visuwell® reagin test

The Visuwell® reagin test is a solid-phase enzyme linked immunosorbent assay for the qualitative detection of *T. pallidum* antigen directly from syphilitic lesions. In a comparison study between the Visuwell®, monoclonal antibody staining and dark-field microscopy, the authors concluded that the Visuwell® test was an alternative method for evaluating genital ulcers, but they also found it to be less sensitive and specific than dark-field microscopy and immunofluorescent staining. (Cummings et al., 1996).
2.4.1.3  Immunofluorescent staining techniques

The direct fluorescent-antibody staining for \textit{T. pallidum} (DFA-TP) was first described by Yobbs et al., in 1964 (CDC, 1998). Specimens collected from oral, rectal or intestinal lesions can be used, and, different from darkfield microscopy, the organisms are not required to be alive (Ratnam, 2005).

The DFA tissue-TP (DFAT-TP) test is a combination of the DFA-TP test and histological stains, and is used to detect presence of pathogenic treponemes in biopsy and autopsy material (Norris & Larsen, 1995).

2.4.2  Specific treponemal antibody tests

In 1957, Deacon et al. developed the fluorescent antibody (FTA) technique used as a confirmatory test for syphilis (CDC, 1998). By preparing a sonicate from cultures of the Reiter spirochete, Deacon et al. removed, by absorption, the antigens shared with other treponemes found in human, thus eliminating false positive results. This led to the development of a more sensitive and specific fluorescent treponemal antibody absorption (FTA-abs) test in 1964 (George, Hunter, & Fears, 1998a). The FTA-abs double-staining (DS) is a modification of FTA-abs (George, Hunter, & Fears, 1998b).

Rathlev developed the hemagglutination test (TPHA) for syphilis in 1965 (CDC, 1998), and in an attempt to automate the test, a microtiter version was developed: microhemagglutination assay for antibodies to \textit{T. pallidum} (MHA-TP) (P. M. Cox, Logan, & Norins, 1969).

The enzyme linked immunosorbent assay (ELISA) was first applied to syphilis serology in 1975 by Veldkamp and Visser (Veldkamp & Visser, 1975), and subsequently, several tests (TP-ELISA; AF-ELISA) have been reported (van Eijk, Menke, Tideman, & Stolz, 1986).
2.4.3 Non-treponemal antibody tests

These widely available tests include: venereal disease research laboratory (VDRL) test (Kennedy & Creighton, 1998b); unheated serum reagin (USR); rapid plasma reagin (RPR) (Larsen & Creighton, 1998); and toluidine red unheated serum test (TRUST) (Ratnam, 2005). These are relatively inexpensive and are useful for screening and establishing disease activity in patients not on treatment.

2.4.4 PCR

In recent years, polymerase chain reaction (PCR) has been used to successfully detect *T.pallidum* DNA in primary lesions, CSF, amniotic fluid and sera of patients with syphilis (Burstain, Grimprel, Lukehart, Norgard, & Radolf, 1991; Centurion-Lara et al., 1997; Grimprel et al., 1991).

2.5 Treatment of syphilis

After many years of use, penicillin still remains the drug of choice in the treatment of syphilis. A single intramuscular dose of 2.4 mega units of benzathine penicillin is effective in treating primary, secondary and early latent syphilis. Three intramuscular doses of 2.4 mU of benzathine penicillin, given at weekly intervals are effective in treating late latent and tertiary syphilis. In patients allergic to penicillin, tetracycline derivatives are used as alternatives (CDC, 2010).
Although studies have been reported on the interaction and subsequent effect HIV and *Treponema paliidum* have on each other, to our knowledge, none of the studies reported on the *in vitro* interaction between the two organisms, on a keratinocyte level.
CHAPTER 3

METHODOLOGY

3.1 Infection of keratinocytes with HIV

3.1.1 Processing of HIV containing blood

HIV containing blood samples were obtained from antiretroviral (ARV)-naive patients enrolled in an on-going study conducted in the Department of Medicine, Nelson R Mandela School of Medicine, UKZN. Upon receipt, the blood was centrifuged at 2000 g for 10 minutes, and the plasma was aliquoted and stored at -80ºC for further use.

3.1.2 Isolation of infectious virions

It is established that host cell proteins are either actively or passively incorporated into the retroviral envelope as HIV-1 buds from the cell membrane. This fact has enabled the ability to distinguish host cell types supporting viral replication by a targeted capture of virions directly from HIV- containing plasma. This can be done by using magnetic beads in combination with antibodies as markers against viral envelope antigens.

Isolation of infectious HIV virions from plasma was done by means of a formerly published method (Albert et al., 1987; Lupo & Butera, 2004, vol 8( 1)). To this extend the μMACS™ VitalVirus HIV isolation kit was used following manufacturer’s instructions. Three antibodies (CD26, 36 and CD 44) against host cell specific antigens in the viral envelope were used as markers of the cellular origin of the viral particles. All antibodies were provided in biotinylated form.
3.1.2.1 Sample preparations and magnetic labelling

Stored plasma samples were thawed and centrifuged at 13 000 g for 30 seconds to remove particulate matter. The supernatant was then transferred to a clean sterile tube. Isolation of T-cell and macrophage derived HIV was performed using the biotinylated CD26 and CD36 antibodies, respectively, in combination with the µMACSTM streptavidin beads: 200µl of virus containing plasma was labelled with 1µg of biotinylated CD26 or CD36 antibody and incubated for 30 minutes at room temperature. Thereafter, 50µl of µMACS streptavidin MicroBeads were added to the sample and incubated for an additional 10 minutes.

For isolation of virions with CD44 marker, 50µl of anti-CD44 MicroBeads were added to the virus-containing sample and incubated for 30 minutes at room temperature.

3.1.2.2 Magnetic separation

This is done using µColumns and µMACS separator; the µColumns are placed in the µMACS magnetic separator attached to a multi-stand. The column is first prepared by applying a 100 µl of equilibration buffer for protein application, on top of the column. They are then rinsed three times with 100 µl of virus wash buffer containing 2% Fetal Bovine Serum (FBS). Next the magnetically labelled sample is loaded onto the column and the column further washed.

3.1.2.3 Elution of intact virions

To obtain viable and infectious virus, the column is removed from the magnetic separator and placed over a 2ml eppendorf tube. Next, 500 µl of Roswell Park Memorial Institute (RPMI) 1640 media is added to the column, and the solution containing magnetically labelled virions is collected. Viral stocks were aliquoted for single use and stored at -80°C.
3.1.3  p24 antigen ELISA

p24 is a structural protein that makes up majority of the HIV viral core proteins. Studies have indicated that the protein is detectable in blood, is found in early HIV infection and, is associated with high levels of infectious virions in both plasma and peripheral blood mononuclear cells (PBMCs) (Daar, Moudgil, Meyer, & Ho, 1991). p24 antigen ELISA detects the p24 antigen in serum and does not specifically identify infectious virions; virus isolation and culture is the only means to evince that a sample contains infectious virus (Constantine, September 2001).

To determine the infectivity of the isolated virions, a total of $1 \times 10^6$ HaCaT cells were infected with HIV and 3 days later, the HIV p24 antigen was quantified by the Quicktiter™ lentivirus-associated HIV p24 titer kit (Cell Biolabs, San diego, CA). Infection was monitored in culture supernatants.

3.2  Tissue culture: HaCaT cells

The spontaneously immortalized HaCaT cells are a widely employed keratinocyte model, and have been shown to retain all the functional differentiation properties of normal keratinocytes (Boukamp et al., 1988). The cell line used in this study was donated by Prof NE Fusinig of the Cancer Research Centre in Germany, to the Department of Medical Microbiology, Nelson R Mandela School of Medicine, Durban.

3.2.1  Resuscitation and propagation of cells

A vial of frozen cells from previous harvests was thawed in a 37°C water bath and the cells reconstituted in a 25cc tissue culture flask containing 5 mls of RPMI 1640 supplemented with
10% FBS. The cells were incubated at 37°C and culture media was changed every three days or earlier as indicated by the media pH indicator.

The cells were grown to ~ 80 - 90% confluency and the spent cell culture media discarded. The monolayer was rinsed thrice with warm phosphate buffered saline (PBS), pH 7.0, to remove any traces of FBS that would inhibit the action of trypsin/EDTA. One ml of 0.05% EDTA was added and distributed across the monolayer, and the flask incubated at 37°C for five minutes. The excess EDTA was discarded and 1 ml of trypsin-EDTA was added and the flask swirled to distribute the trypsin to cover the entire cell surface. The flask was further incubated for a minute or until the cells detached when gently tapped. The action of trypsin was inactivated by addition of 500 µl of FBS dispersed by pipetting over the cell monolayer a few times. This cell viability was determined by the trypan blue exclusion test using a haemocytometer (Černe, Erman, & Veranič, 2013). Viable cell suspensions were either further propagated or cryo-preserved to maintain frozen stocks.

### 3.2.2 Cryopreservation of cells

For long term storage and preservation of cells, the cell suspension obtained above was transferred to a fresh sterile tube and an equal volume of cryo-preservation fluid (RPMI 1640, 20% FBS and 20% glycerol) was added drop by drop with gentle mixing to maintain a homogeneous cell suspension. The suspension was then aliquoted into sterile cryovials and stored at -70°C.

### 3.3 Treponema pallidum

The virulent Nichols strain of *T.pallidum* used throughout this work was kindly donated by Dr. Allan Pillay of the CDC, Atlanta, GA, USA.
3.3.1 Infection of Rabbits

Prior to infection, an aliquot of the treponemal suspension was viewed under dark field microscopy to determine motility and viability of the organism. The suspension was carried on ice from the department of Infection Prevention and Control (IPC) to the Biomedical Research Unit where the New Zealand white rabbits were housed. Under supervision of trained personnel, the rabbits were inoculated intra-testicularly with 0.4 mls (~1x10^8) of the treponemal suspension. Preceding testes inoculation, the rabbits were sedated with a combination of ketamine (20mg/ml) and xyline (100mg/ml) administered intramuscularly. The rabbits were observed daily for any drinking and eating behavioural changes. To decrease their immune response, the rabbits were administered a daily injection of 6mg/kg hydro-cortisone on day 4 - 11 post inoculation. Day 12 post infection, the time of peak orchitis, the animals were sedated and euthanized by an intra-cardiac injection with pento-sodium barbitone (200mg/ml). The rabbit testes were aseptically removed and each testis was transported in ~10 mls of PBS back to the department of IPC for further processing.

3.3.2 Storage and recovery of T.pallidum

On arrival at the IPC department, the unused testes were placed in a bottle containing 1 ml FBS and stored at -70°C. One testicle was used to extract T.pallidum. To recover T.pallidum, the testicle was rinsed in the PBS, and trimmed of any fatty tissue and minced in a petri dish. The minced tissue was place into an Erlenmeyer flask containing five ml EMEM 12-662F media supplemented with 10% FBS. The flask was then placed in an anaerobic jar containing a campygen gas pack, to induce a microaerophilic atmosphere. The jar was placed on a shaker for 30 minutes in the hot room. The tissue suspension was then centrifuged for five minutes at 500 g to remove debris. To assess treponemal viability, an aliquot of the
supernatant was viewed under dark field microscopy. The supernatant was aliquoted into one ml eppendorf tubes and stored at -70°C until further use.

3.4 Transmigration assay

To determine the effect of HIV infection of the keratinocytes on the transmigration ability of *T.pallidum* across the keratinocyte monolayer, four experiments were performed. The keratinocytes were exposed to (i) *T.pallidum* alone, (ii) *T.pallidum* and HIV together, (iii) *T.pallidum* first and HIV 3 days later, and (iv) HIV first and *T.pallidum* 3 days later.

HaCaT cells were cultured and trypsinized as previously described (heading 3.2). The cells were enumerated and 1x10⁶ cells/ml were seeded on collagen-coated polytetrafluoroethylene (PTFE) 24-well tissue culture inserts, with a 0.3 µM pore size. Upon 80-90% confluency, the monolayer was rinsed with PBS and the transwell transferred to a new tissue culture plate well, containing 1 ml of EMEM supplemented with 20% FBS. The cells were then inoculated with 100 µl microbial suspension, depending on the experiment. For combination parameters (HIV and *T.pallidum*), 3 day periods were given between inoculation of the cells with the first organism, to inoculation with the second organism, and only then would the post inoculation time points take effect. At 24, 48 and 72 hours post inoculation, the inserts were placed into another well and the media below the inserts, which contained all the treponemes that passed through the membrane, was transferred into an eppendorf tube.

To rule out damage to the monolayer, trypan blue exclusion was used. This was done before the monolayer was inoculated and at 72hrs post inoculation. The insert was transferred to a well containing PBS; 4% (w/v) trypan blue (was then added to the top chamber and incubated for 1 minute. The PBS remained clear thus indicating an intact monolayer.
Quantitation of transmigrated treponemes was done using Real-time PCR, preceded by extraction of treponemal DNA.

3.5 DNA extraction

The harvested fluid from under the keratinocyte monolayer, with or without treponemes, was centrifuged at 12,000 g for 15 minutes. The supernatant was decanted and 200 μl of Probetec® lysis buffer was added and the pellet was reconstituted. To isolate the DNA, the eppendorf tube was placed in a heating block (113.2°C) for 30 minutes, vortexed and stored at -20°C or -70°C (Caliendo et al., 2005).

3.6 Real-time PCR

To quantitate the treponemes real-time PCR was performed in 50 μl reaction volumes containing 5 μl of extracted DNA, 25 μl of 2x universal master mix, 2.5 μl of 18 μM forward and reverse primers, 2.5 μl of 5 μM probe and 12.5 μl water. The primers were synthesized by Applied Biosystems, and they were targeting the polymerase A gene of *T.pallidum*, as described by Chen *et al*, 2006 (Chen et al., 2006). Real-time PCR was run in duplicate in a 96 well optical reaction plate using the ABI SDS 7000™ system.

3.7 Ethics statement

Approval to used stored down isolates (class ethics) was obtained (BCA 27/409). Ethics approval for use of the rabbits was obtained from the UKZN Animal Ethics subcommittee of the University Research and Ethics Committee (028/12/Animal). Ethics approval was also obtained for the HIV isolation from patients’ blood by the principal investigator of the main
study in HIV infected patients (BFC 151/07 and BF 096/09). Written informed consent was provided by all study participants.
CHAPTER 4

RESULTS

4.1 Transmigration of *Treponema pallidum*
Confluent HaCaT cell monolayers grown on collagen-coated PTFE inserts (Transwell, Corning, NY) were inoculated with *T.pallidum* and/or HIV. The transmigration assays were performed three times in duplicate. Figure 1 shows the number of transmigrated treponemes when the monolayer was inoculated with *T.pallidum* only.
Figure 1: Migration of *T. pallidum* through a confluent keratinocyte layer

This graph shows that *T. pallidum* does migrate through the keratinocyte monolayer, with the highest number of transmigrated treponemes observed at 48 hours post-inoculation. At 72 hours post-inoculation a small number of treponemes passed through the monolayer.
Figure 2: Migration through the monolayer when the monolayer is inoculated with *T.pallidum* first and three days later HIV.

Figure 2 shows the number of transmigrated treponemes when the monolayer is inoculated with *T.pallidum* first, and then 3 days later with HIV.
Figure 3: Migration of *T.pallidum* through a keratinocyte monolayer infected with HIV for a period of three days.

Figure 3 shows the number of transmigrated treponemes when the monolayer is inoculated with HIV first, and then 3 days later with *T.pallidum*.

Figure 2 and 3 appear to follow a similar pattern. However, by 72 hours post inoculation, there appears to be a continuous increase in number of treponemes passing the barrier when the monolayer is inoculated first with HIV (Fig.3), as compared to the plateauing observed at the same time point, when the monolayer is inoculated with *T.pallidum* first.
Figure 4: Migration through a keratinocyte monolayer infected with HIV and *T.pallidum* at the same time.

Figure 4 shows the number of transmigrated treponemes when the monolayer is inoculated with both HIV and *T.pallidum* at the same time. The drastic decline observed at 72 hours can be explained by the fact that at 48 hours, the majority of the treponemes had crossed the membrane barrier, and thus leaving a minimal number of treponemes to migrate through the keratinocyte layer.
Figure 5: Summary of migration experiments with *T.pallidum* through a keratinocyte monolayer.

Figure 5 shows the number of migrated treponemes through a keratinocyte monolayer over time, for all four experiments.

The results indicate that during simultaneous exposure, *T.pallidum* transmigrates at the highest rate.
CHAPTER 5

DISCUSSION

It is well established that HIV and syphilis have an effect on each other. This effect starts at the site of transmission since both infections are sexually transmitted. In addition, the effect that HIV has on the human immune response affects the presentation of concomitant syphilis. Epidemiological studies of syphilis in a HIV setting have observed that the course of syphilis is altered by co-infection with HIV. A decreased risk of primary syphilis in the HIV infected, an increased number of ulcers in those HIV infected subjects that do develop primary disease, and, a more rapid development of secondary syphilis, have been observed (Lynn & Lightman, 2004).

The work presented here aims to investigate whether migration through a keratinocyte layer is influenced by prior infection with HIV. The keratinocyte layer is the first barrier that pathogens that cause genital ulcers have to pass into the host. It has been demonstrated that syphilitic ulcers disrupt epithelium and mucosa, thus providing a port of entry for HIV virus (Karp et al., 2009). Additionally, T. pallidum and its constituent lipoprotein were found to induce in vitro the expression of CCR5 on macrophages, thus potentially enhancing transmission of macrophage-tropic HIV-1 (Sellati et al., 2000).

HIV efficiently enters permissive cells which express CD4, using gp120 mediated membrane binding. In keratinocytes which do not express CD4, HIV entry has been suggested to occur in an envelope-independent manner, using galactosylceramide (Galcer) and heparin sulphate proteoglycans (HSPGs) as receptors (Vacharaksa et al., 2008). In our study, in determining
the infectivity of freshly isolated virions, keratinocytes (HaCaT cells) were successfully infected with HIV. This was quantified using HIV p24 titre kit (unpublished results).

In line with previous studies (Sturm and Govender, 2006), data from the transmigration assays reveals that *Treponema pallidum* is capable of transversing a monolayer of human keratinocytes. However, the *in vitro* migration of *Treponema pallidum* through a prior HIV infected keratinocyte layer has not been documented.

In this study, we observed that during simultaneous exposure of the keratinocytes to both HIV and *Treponema pallidum*, *Treponema pallidum* migrates at the highest rate. One possible explanation is that HIV enters the keratinocytes and alters the cell membrane structure, thus allowing for enhanced adhesion and subsequent entry of *Treponema pallidum*. If this explanation holds true, it would mean the spirochetes do not remain in the epithelial layer but move rapidly under the epithelium. This observation would then potentially explain the recorded decrease in primary syphilis prevalence in HIV endemic areas, as well as the rapid progression to secondary syphilis in patients with concurrent HIV infection.

Further studies involving *in vitro* work, focusing on what happens to the keratinocytes on a cellular level, when they are exposed to HIV are needed to expound on this explanation.
REFERENCES


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