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The Role of *Herpes simplex* virus type 2 (HSV-2) as a cofactor in HIV transmission

Thesis

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The Role of Herpes simplex virus type 2 (HSV-2) as a cofactor in HIV transmission

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Ph.D

2002

The Role of *Herpes simplex* virus type 2 (HSV-2) as a cofactor in HIV transmission

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A thesis submitted to the Open University in fulfilment of the conditions governing candidates for the degree of

Doctor of Philosophy

Discipline: Life Sciences

June 2002

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DATE OF SUBMISSION: 27 JUNE 2002
DATE OF AWARD. 19 SEPTEMBER 2002

A. Acknowledgements

Above all I thank God for his multiple blessings!

B. Dedication

To the memory of my father who gave me a very good start in life, the values which have driven me, and above all, the confidence to believe in my self.

To my mother for honesty and warmth,

To my large Family for love and support,
To Dame Claude, Marilyn, Amanda...just beyond words...

...and to all those who care to listen to the illnesses, whispering the tales of the voiceless - particularly the women - in the developing world.

C. Declaration

The work described in this thesis has not previously been submitted for a degree at the Open University or any other University.

Data in several sections have been published (see list of scholarly contributions-copies attached in appendix).

With the following exceptions, I have carried out all the work described in this thesis:
a) Dr P. Mayaud, Dr H. Weiss, Prof D. Mabey and Prof R. Hayes help with study design. b) Dr G. Gresenguet did the clinical evaluations of the patients in Bangui. c) Prof R. Ashley and J. Dalessio help design the HSV neutralizing antibody study. Dr L. Andreoletti, Dr. A. Si-Mohamed and M. Matta help design the herpes quantitation study. Prof J-L Paul's laboratory performed the Vitamin A & E evaluation. d) Dr D. Brown and prof L. Belec supervised the whole study.

I confirm that the above is a true statement and that, subject to any comment above, the thesis is my own original work.

Signed: London, February 15th, 2002

François-Xavier Mbopi-Kéou

D. Scholarly contributions from this thesis

D.1. Publications in peer review journals

Mbopi-Keou, F.X., Gresenguet, G., Mayaud, P., Weiss, H.A., Gopal, R., Brown, D., Hayes, R.J., Mabey, D.C.W. and Belec, L. (1999) Genital *Herpes simplex virus* type 2 shedding is increased in HIV-infected women in Africa. *AIDS* 4, 536-537.

Mbopi-Keou, F.X., Gresenguet, G., Mayaud, P., Weiss, H.A., Gopal, R., Brown, D., Hayes, R.J., Mabey, D.C.W. and Belec, L. (2000) Interactions between Herpes simplex virus type 2 and HIV infection in African women: opportunities for intervention.
J Infect Dis **182**, 1090-1096.

Belec, L., Gresenguet, G., Mbopi-Keou, F.X., Mayaud, P. (2000) High frequency of asymptomatic shedding of herpes simplex virus type 2 in African women.
Clin Microbiol Infect **6**, 56-57.

Bélec, L., Mbopi-Keou, F.X., Roubache, J.F., Mayaud, P., Paul, J.L., Gresenguet, G. (2002) Vitamin A deficiency and genital tract infections in women living in Central Africa. *J Acquir Immune Defic Syndr* **29**, 203-204.

Mbopi-Keou, F.X., Robinson, N.J., Mayaud, P., Belec, L. and Brown D.W. (2002) Herpes simplex virus type 2 and heterosexual spread of human immunodeficiency virus infection in developing countries: hypotheses and research priorities.
Clin Microbiol Infect **in press.**

Mbopi-Keou, F.X., Legoff, J., Gresenguet, G., Si-Mohamed, A., Matta, M., Mayaud, P., Andreoletti, L., Brown, D., Belec, L. (2002) Genital shedding of HSV-2 DNA and HIV-1 proviral DNA in HIV- and HSV- co-infected women. *JAIDS* **in press.**

D.2. Paper submitted for Publication in peer review journals:

Mbopi-Keou, F.X., Dalessio, J., Gresenguet, G., Brown, D.W.G., Belec, L. Ashley, R.L. (2002) Cervicovaginal neutralizing antibodies to Herpes simplex virus type-2 (HSV-2) in cervicovaginal secretions of African women. (Submitted)

D.3. Presentations at International meetings (presenting author name underlined):

Chair **of the herpesviruses session** at 41th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Chicago, IL, USA, December 16-19, 2001, and presenter of the paper :

Mbopi-Keou, F.X., Andreoletti, L., Gresenguet, G., Matta, M., Mayaud, P., Weiss, H.A., Gopal, R., Si-Mohamed, A., Lang, M.C., Hayes, R.J., Brown, D., Mabey, D.C.W., Belec, L. (2001) Association of genital replication of HSV-2 with increasing HIV-1 RNA, but not HIV-1 proviral DNA, genital shedding in HIV- and HSV- co-infected women [oral presentation]. 41th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA, December 16-19, Abstract no 1113.

Mbopi-Keou, FX. Interactions between HSV-2 and HIV-1 infection in African women: "Pefa me Si dasi meji" [oral presentation]. Annual Virology and Infectious Diseases Research Conference, University of Washington, Fred Hutchinson Cancer Research Center, Children's Hospital and Regional Medical Center, Seattle, USA, 9th March 2000.

Mbopi-Keou, FX, Gresenguet G, Mayaud P, Weiss HA, Gopal R, Matta M, Brown D, Hayes RJ, Mabey DCW, Belec L. Increased shedding of HSV-2 in HIV-1 infected women in Africa [oral presentation]. 39th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), San Francisco, California, USA, September 26-29, 1999, Abstract no 1407.

Mbopi-Keou, FX, Gresenguet G, Mayaud P, Weiss HA, Gopal R, Matta M, Brown D, Hayes RJ, Mabey DCW, Belec L. High seroprevalence and shedding of HSV-2 in HIV-1 infected women in Africa [oral presentation]. "Research in Progress" Royal Society of Tropical Medicine & Hygiene, London, UK, 17th November 1999 (highly commended presentation see *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2000, 94, 128-134).

Mbopi-Keou, F.X., Robinson, N.J., Gresenguet, G., Weiss, H.A., Mayaud, P., Gopal, R., Hayes, R.J., Mabey, D.C.W., Belec, L. and Brown, D. Case for control of genital herpes infection in developing countries [poster presentation]. Joint Meeting of the ASTDA and the MSSVD, Baltimore, MD, USA, May 3-6, 2000, Abstract no 117.

Mbopi-Keou F.X., Dalessio, J., Gresenguet, G., Gopal, R., Mayaud, P., Weiss, H.A., Hayes, R.J., Mabey, D.C.W., Corey, L., Brown, D.W.G., Belec, L. , Ashley RL. Relationship between HSV-2 shedding and cervical antibody responses in African women at risk of or with HIV [poster presentation]. 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada, September 17-20, 2000, Abstract No 771.

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F. Abbreviations

bp:	Base pair
BV:	Bacterial vaginosis
BHT:	Butylated hydroxy toluene
CAR:	Central African Republic
CNHU:	Centre National Hospitalier et Universitaire
CNRMST/SIDA:	Centre National de Reference des Maladies Sexuellement Transmissibles et du Sida
CPHL:	Central Public Health Laboratory, London
CVLs:	Cervico-vaginal lavage sample
CVS:	Cervicovaginal secretions
DEIA:	DNA enzyme immunoassay
DNA:	Deoxyribonucleic acid
ECL-WB:	Enhanced chemoluminescence Western Blot
EIA:	Enzyme immunosorbent assay
ELISA:	Enzyme-linked immunosorbent assay
g:	Gram
GUD:	Genital ulcer disease
Hb:	Haemoglobin
HSV:	*Herpes simplex* virus
HPLC:	High pressure liquid chromatography
HIV:	Human immunodeficiency virus
IgA:	Immunoglobulin A
IgG:	Immunoglobulin G
LSHTM:	London School of Hygiene and Tropical Medicine
Mab:	Monoclonal antibodies

min:	Minute
mL:	Millilitre
mM:	Millimolar
NAU:	Neutralizing activity units
μg:	Microgram
μl:	Microlitre
OD:	Optical densities
ONPG:	O-nitrophenyl β-D-galactopyranoside
PCR:	Polymerase chain reaction
RIA:	Radio-immunoassay
RNA:	Ribonucleic acid
RFLP:	Restriction fragment length polymorphism
RT:	Reverse transcription
RT-PCR:	Reverse transcription polymerase chain reaction
RtPCR:	Real time PCR
sec:	Second
STD:	Sexually transmitted disease
STI:	Sexually transmitted infection
SDS:	Sodium dodecyl sulfate
TBE:	Tris-borate EDTA buffer
Tm:	Melting temperature
TMB:	Tetra methyl benzadene
TPHA:	*Treponema pallidum* haemagglutination assay
WHO:	World Health Organization

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H. List of Figures

"Science knows no country because knowledge belongs to humanity, and is the torch which illuminates the world"

Louis Pasteur

"Concern for man himself and his fate must always form the chief interest of all technical endeavors...in order that the creations of our mind shall be a blessing and not a curse to mankind "

Albert Einstein

Chapter 1: General introduction to Herpes simplex virus type 2 and heterosexual spread of human immunodeficiency virus

1.1. The history of genital herpes

Although herpes labialis (herpes febrilis, "fever blisters") was well described as early as the first century AD, it was not until the eighteenth century that a clear description of genital herpes (herpes genitalis, herpes progenitalis) appeared [Hutfield, 1966]. This was published in 1736 by Jean Astruc, physician to the King of France, in his treatise on venereal diseases [Astruc, 1736]. Astruc did not give the disease a name, but he vividly described the lesions of genital herpes in males and females as well as those of rectal herpes in recipients of anal intercourse. Greek scholars, e.g, Hippocrates, used the word "herpes", meaning to creep or crawl, to describe spreading lesions [Roizman & Furlong, 1974; Whitley & Roizman, 2001]. Genital herpes was well recognized by nineteenth century venereologists, who described the disease and called attention to its generally benign nature, its tendancy to recur in the same anatomic area, and its association with previous history of a recognized sexually transmitted disease (STD) such as gonorrhea, syphilis, or chancroid [Bateman, 1813; Greenough, 1881]. Unna, a German Venereologist, described genital herpes as a "vocational disease" of prostitutes, reporting its occurrence in 4 to 9 per cent of prostitutes admitted to the Hamburg General Hospital from 1878 to 1881 [Unna, 1883]. Unna also provided the first description of the histopathology of the genital lesions [Unna, 1896]. It was not until the early part of the twentieth century that Gruter, Lipschutz and other European investigators established the infectious etiology of herpes labialis and genital herpes by transmitting infection to

experimental animals [Gruter, 1920; Lipschutz, 1921]. In 1946, Slavin and Gavett isolated Herpes simplex virus (HSV) from lesions of the vulva demonstrated that "herpetic vulvovaginitis" was a manifestation of primary HSV infection and described the conjugal transmission of genital herpes from a husband with penile herpes to his wife [Slavin & Gavett, 1946]. However, genital herpes was still not widely accepted as an STD until the rediscovery of the two HSV serotypes in the late 1960s, and the development of assays to distinguish between them permitted the different epidemiologies of HSV-1 and HSV-2 to be delineated [Plummer, 1964; Nahmias & Dowdle, 1968].

1.2. Herpes *simplex virus* structure and replication

HSV is a double-stranded DNA virus belonging to the *Alphaherpetovirinae* sub-family. Infection is characterised by both neural and cutaneo-mucous tropism. The herpes simplex virion consists of four components: an electron-dense core containing viral DNA, an icosahedral capsid, a layer of proteins (the tegument) which surrounds the capsid and an envelope [Roizman, 1979]. The capsid consists of 162 capsomeres and is surrounded by the tightly adhering tegument [Roizman, 1979]. The envelope surrounds the capsid-tegument structure and consists of at least 10 glycosylated and several nonglycosylated viral proteins, lipids and polyamines. Viral DNA is at least 152 kbp in length.

The variability in size is chiefly due to the variation in the number of reiterations of specific terminal and internal sequences. HSV-1 and HSV-2 DNAs consist of two covalently linked components, designated L (long) and S (short) [Roizman, 1979]. Each component is composed of unique sequences (UL or US, respectively) flanked by relatively large inverted repeats. The inverted repeat sequences flanking UL are in the ab and b'a' orders, whereas

those flanking US are a'c' and ca. The two components can invert relative to one another to yield four populations of DNA molecules differing solely in the relative orientation of their DNA sequences [Roizman & Sears, 1996].

Although the vesicular nature of lesions associated with herpetic infections has been well characterized, it was not until 1893 that Vidal specifically recognized person-to-person transmission of HSV infections [Wildy, 1973]. To initiate infection, HSV must attach to cell-surface receptors, fuse its envelope to the plasma membrane, and allow the de enveloped capsid to be transported to the nuclear pores. The DNA is released into the nucleus at the pore [Roizman & Sears, 1996].

The key events in viral replication have been summarised in a recent review by Whitley and Roizman [Whitley & Roizman, 2001]. Currently, at least 10 viral glycoproteins (designated gB, gC, gD, gE, gD, gH, gI, gK, gL, and gM) are required for attachment to the cell surface. gD is required for entry of the virus into cells, gE and gI form an Fc receptor and gE is also required for basolateral transmission of virus in polarized cells and for efficient expression of late gene.

Several aspects of viral replication cycle and of viral proteins that are relevant to the pathogenesis of human diseases and to present and future developments in antiviral chemotherapy have also been summarised by Whitley and Roizman. After a primary infection, neutralizing antibodies are evoked. Following entry and infection of nerve endings, HSV is transported by retrograde movement to the nuclei of sensory ganglia [Stevens & Cook, 1971]. Available evidence indicates that the virus multiplies in a small

number of sensory neurons and in the majority of infected neurons, the viral genome remains in an episomal state for the entire life of the individual. HSV has the ability to recur in the presence of humoral immunity by reactivation of latent infection. Consequently, seropositive individuals who develop recurrent labial or genital lesions experience clinically mild disease. Reactivation is not fully understood, it occurs following a variety of local or systemic stimuli such as physical or emotional stress, fever, exposure to ultraviolet light, tissue damage and immunosuppression.

The spectrum of disease caused by HSV includes primary and recurrent infections of mucous membranes, keratoconjunctivitis, neonatal HSV infection, visceral HSV infections in immunocompromised hosts, HSV encephalitis, Kaposi's varicella-like eruption and an association with erythema multiforme [Corey & Spear, 1986]. Animal vectors of human HSV have not been described, and humans remain the only reservoir for transmission to other humans. Virus is transmitted from infected to susceptible individuals during close personal contact, and there is no seasonal variation in the incidence of infection. HSV infection is rarely fatal. As mentioned above, an important recent advance in our knowledge of HSV infections comes from the ability to distinguish between HSV type 1 and HSV type 2 [Nahmias & Dowdle, 1968].

1.3. HSV-2 infection: a growing public health problem

HSV-2 infection is almost always sexually transmitted and causes genital ulceration. HSV is a persistent infection that may give rise to recurrent episodes. Significant progress in our understanding of HSV infection has occurred over the last decade, in part related to the development of accurate and sensitive laboratory tests to study HSV-2 [Munday et al, 1998;

Ashley & Wald, 1999; Ashley et al, 2000]. The application of PCR and type specific serology to individual cases and in population based studies have identified a potentially important role for HSV-2 infection as a cofactor in the sexual transmission of HIV. This is a particular issue in developing countries [Corey, 2000]. It has been established that genital HSV-2 infection is frequently unrecognized and that asymptomatic HSV-2 shedding may be important for transmission [Mertz et al, 1985; Barton et al, 1996; Wald & Corey, 1996; Wald et al, 1997]. Commercial type-specific serology assays have become available allowing population based serological surveys of HSV-2 infection which have shown that: (i) HSV-2 prevalences are high in some western countries and very high in developing countries [Fleming et al, 1997; Halioua and Malkin, 1999; O'Farrell, 1999] (Table 1.1); (ii) The global prevalence of genital HSV-2 has likely increased during the past 10 years ; (iii) HSV-2-seropositivity may be a useful marker of high risk sexual behaviour in some settings. In many countries in sub-Saharan Africa, the HSV-2 epidemic has spread in parallel with the HIV epidemic [Chen et al, 2000; Buve et al, 2001; Carael & Holmes, 2001].

1.3.1. Natural history of genital herpes

Most HSV infections are unrecognised. Susceptible individuals develop primary infection after their first exposure to HSV-1 or HSV-2. Genital herpes infections can be associated with serious morbidity. First episode genital herpes can cause painful ulcerative lesions and systemic manifestations, including headache, malaise, and fever lasting up to 3 weeks [Ashley & Wald, 1999].

Complications in men are rare; aseptic meningitis and urinary retention are more common in women [Whitley & Roizman, 2001]. In addition to the latent state, subclinical or

'asymptomatic' infectious viral shedding is frequent [Wald et al, 1997]. The median recurrence rate after a symptomatic first episode of genital herpes is 4 to 5 episodes per year. Patients who suffer severe first episodes are associated with even higher recurrence rates [Benedetti et al, 1994]. Most HSV-2 seropositive persons do have signs and symptoms associated with HSV-2 reactivation, but these are unrecognised without councelling [Langenberg et al, 1989; Koelle et al, 2000]. In most studies, the proportion of infections that are both symptomatic and recognised (by patient and clinician) vary between 13% and 37%. This proportion is higher among HIV-positive individuals.

Transmission and acquisition of HSV-2 infection.

HSV-2 infections are almost always sexually transmitted. Most genital HSV are caused by HSV-2; however, an increasing proportion is attributable to HSV-1 [Ashley & Wald, 1999]. Prospective studies of HSV-2 discordant partners have shown that most transmission events were not associated with a clinically recognised HSV-2 recurrence in the infected partner [Mertz, 1993; Koelle et al, 2000]. As for other sexually transmitted infections (STIs), the risk of acquisition of HSV-2 seems to be higher in women than in men [Benedetti et al, 1994; Mertz, 1993; Koelle et al, 2000]. This increased risk of acquisition of HSV-2 may relate to the higher number of HSV-2 recurrences in infected men (about 20% higher than in women) [Benedetti et al, 1999]. It may also be related to biological factors such as the larger and more vulnerable genital mucosal surface of women [Nicolosi et al, 1994; Carpenter et al, 1999].

Extent and frequency of subclinical shedding of HSV-2.

Data have recently been accumulating, mainly from the USA, emphasizing the frequency of subclinical HSV-2 shedding and its role in the transmission of HSV-2 infection to sexual partners and neonates [Whitley, 1994; Brown et al, 1997; Patel & Harper, 1998]. These studies have shown that the level of virus required to infect the neonate can sometimes be small [Arvin et al, 1986].

Although it is not known what proportion of new disease acquisitions (outside these groups) is related to subclinical shedding, it is likely to contribute to the spread of HSV-2 disease [Patel & Harper, 1998]. Reported rates of subclinical shedding vary widely between studies [Patel & Harper, 1998]. Shedding from internal and external genital, perineal, and perianal sites is frequent [Koelle et al, 1992; Augenbraum et al, 1995; Wald et al, 1997; Krone et al, 1998]. The frequency of clinical recurrence and asymptomatic shedding is significantly lower in HSV-1 genital infection than HSV-2 [Mertz et al, 1985]. There is no difference in shedding rates between those individuals who are seropositive for HSV-2 alone and those who are positive for both HSV-1 and HSV-2 [Patel & Harper, 1998].

The frequency of subclinical shedding rises with the number of clinical attacks, although this association may be lost with a low number of lesions [Wald et al, 2000]. Moreover, the frequency of subclinical shedding is highly dependent on the methodology used for detection. Multiple site swabbing and the application of highly sensitive methods, such as PCR assays, have increased the yield [Wald et al, 1997]. PCR detection has shown that the virus may be present on mucocutanous surfaces up to 3.5 times more often than previously found by culture [Strauss et al, 1993; Wald et al, 1997]. Such virus is predominantly associated with subclinical shedding [Patel & Harper, 1998]. Quantitative PCR shows that, overall, HSV DNA titre tends to be higher on lesional days compared to non-lesional days

[Wald et al, 1997; Wald et al, 2000]. In a study that used daily specimens collection, genital or perianal shedding was detected on 27.9% of days (range, 0-77.3% of days) among 20 women with recent onset, symptomatic genital HSV-2 infection [Wald et al, 1997].

Predictors of asymptomatic shedding in immunocompetent women

Virus type. The lower symptomatic recurrence rate for anogenital HSV-1 infection in comparison with HSV-2 is also applicable to asymptomatic shedding, reflecting a type-specific difference in pathogenesis [Benedetti et al, 1994]. The overall rate of cervical or external genital shedding is 1.2% for HSV-1 and 4.3% for HSV-2 during the first year after primary infection [Wald et al, 1997].

Duration of infection. The rate of recurrence and the rate of shedding of HSV-2 from genital and perianal areas both decrease over time [Benedetti et al, 1999]. In a study of 227 women with primary genital HSV-2 infection, the rate of shedding from the cervix or vulva decreased from 3.1% of days during the first year, to 2.3 % during the second year and 2.1% during the third year [Koelle et al, 1992].

Asymptomatic shedding in immunocompetent men

In homosexual men, the most common site of shedding was the perianal area followed by the penile skin and rarely the urethra and semen [Corey & Wald, 1999]. In heterosexual men, the penile skin was the most common site of asymptomatic shedding [Corey & Wald, 1999]. The subclinical shedding rate for men with a history of genital herpes was 2.0% of days using daily cultures. As in women, lesions and symptoms were absent on a third of the days with positive cultures. Among HSV-2 seropositive men without a history of genital herpes, subclinical shedding was detected on 3.9% of days [Wald et al, 1997]. HSV infection of men and women therefore appears to be similar with regards to the overall rate

of asymptomatic shedding and the presence of shedding in persons without a clinical history of genital herpes.

Host immune status and asymptomatic shedding

Little is known about viral or host factors controlling the development of lesions and symptoms when HSV reactivates. Immunity to HSV-1 does not confer protection against asymptomatic shedding of HSV-2. Indeed, two studies of women failed to show an association between HSV-1 serostatus and the rate of asymptomatic recurrent anogenital shedding of HSV-2 [Koelle et al, 1992; Wald et al, 1997]. In addition, recent data clearly showing that previous HSV-1 infection does not prevent HSV-2 acquisition has led to a reassessment of the overall concept of protective type-common immunity to HSV [Brown et al, 1997; Corey et al, 1999]. In a large prospective study (the Chiron vaccine study), the presence of previous HSV-1 infection did not protect against HSV-2 seroconversion. An immunological influence on the clinical severity of disease could be discerned, as asymptomatic seroconversion to HSV-2 was more common in people with pre-existing HSV-1 infection [Langenberg et al, 1999].

Asymptomatic shedding of HSV from the oropharynx and its role in genital HSV disease

It has been demonstrated that the proportion of newly acquired genital HSV infections caused by HSV-1 is increasing and is greater than 50% in some parts of the world [Corey & Wald, 1999]. As mentioned above, the asymptomatic shedding rates for HSV-1 from the genital tract of women appear to be lower than those for HSV-2. In addition, the rate of asymptomatic shedding of HSV-1 from the orolabial area may be of relevance in transmission to the anogenital area during oral sex. Indeed, while oral-genital transmission of HSV-1 has been documented [Dolin et al, 1975], few studies have addressed

asymptomatic shedding of HSV from the mouth (rate of up to 10%) [Scott et al, 1997; Spruance, 1984].

Effect of antiviral therapy on asymptomatic shedding

Antiviral therapy is known to reduce epithelial HSV replication and the symptoms of initial and recurrent genital HSV infection. The nucleoside analogues acyclovir, famciclovir and valaciclovir all suppress asymptomatic genital and perianal shedding of HSV-2. Acyclovir, for example, has being studied in a group of young, non pregnant, predominantly Caucasian, HSV-2 only seropositive women [Wald et al, 1997]. In this study, women who had a history of genital herpes of <2 years were selected, as the rate of asymptomatic shedding is higher at this stage of infection. Patients were randomised to receive oral acyclovir 400 mg or placebo for 70 days. Following a 14 days washout period patients were crossed over to the other arm and received 70 days of placebo or active drug. Measurements included daily cultures of the cervical, vulvar and perianal areas. Asymptomatic shedding accounted for nearly half of the total viral shedding noted during the trial period. Reduction of asymptomatic shedding was noted on all anatomic sites on treatment. When analysed by PCR, the reduction rates for individual women were reduced by a median of 80% (range, 34-91%). Among specimens that contained HSV DNA, treatment with acyclovir reduced the amount of DNA detected by 90% [Wald et al, 1997]. While the genital/perianal HSV asymptomatic shedding rate in HSV-2 seropositive women without a clinical history of genital herpes is similar to that in women with recognized recurrence disease [Lawrence Corey, personal communication], antiviral therapy has not been studied separately in this population.

1.3.2. Epidemiology of HSV and HSV-related genital ulcers

Developed countries.

It has been recognised for decades that HSV is transmitted sexually [Slavin & Gavett, 1946; Griffiths, 2000]. Both HSV-1 and HSV-2 can cause primary genital herpes, with HSV-1 accounting for nearly half of the cases in some countries [Barton et al, 1982]. In western countries, HSV-1 rates are dropping in childhood [Vyse et al, 2000] and many adolescents are infected with HSV-1 as a result of their first exposure to the virus during sexual activity (infection acquired by genital-to-genital contact or by oral-genital contact) [Nahmias et al, 1990]. Nevertheless, HSV-1 reactivates less frequently from latency in sacral ganglia than HSV-2, so most episodes of recurrent genital herpes are caused by HSV-2 [Barton et al, 1982; Lafferty et al, 1987; Nahmias et al, 1990; Griffiths, 2000].

A population based study in the USA, reported that, in 1996, 22% of adults were seropositive for HSV-2 [Fleming et al, 1997]. This figure had increased by about 30% since a previous similar survey a decade earlier [Johnson et al, 1989], despite widespread health education programmes in the 1980s and 1990s about the importance of 'safe-sex' in response to the HIV epidemic [Severson et al, 1999].

Between 1988 and 1994, 45 million people were estimated to have acquired HSV-2 infection [Fleming et al, 1997], which represents 1 in 5 people over the age of 12 years. The HSV-2 seropositivity rate in HIV-infected persons are even higher. Hook and colleagues in Baltimore, USA, showed an HSV-2 seroprevalence rate of 81% among HIV-positive homosexual or bisexual men [Hook et al, 1992]. Other countries have been slower to

document the extend of HSV exposure, but emerging results showed that the HSV-2 epidemic has reached pandemic proportions [Griffiths, 2000].

Developing countries.

In sub-saharan Africa (Figure 1.1), epidemiological studies of HSV-2 have recorded high seroprevalence rates in a range of adult populations : 40% in Kinshasa [Laga et al, 1994], 40% in Zimbabwe [Gwanzura et al, 1998], 30%-50% in South Africa [Auvert et al, 2001] 51% in Rwanda [O'Farrell, 1999], 67% in Tanzania [Obasi et al, 1999], 68% in Uganda

Published studies of HSV-2 seroprevalence in non high risk adult populations in Africa

Uganda
(68%)[5]

Benin
(30%)[3]

Cameroon
(51%)[3]

CAR
(82%)[1]

Congo
(71%)[2]

Dem. Rep. of Congo
(40%)[2]

Zambia
(55%)[3]

Republic of
South Africa
(51%)[7]

Kenya
(68%)[3]

Rwanda
(51%)[2]

Tanzania
(67%)[4]

Zimbabwe
(40%)[6]

1. Mbopi-Keou *et al*, 2000   5. Wagner *et al*, 1994
2. Nahmias *et al*, 1990   6. Gwanzura *et al*, 1998
3. Weiss *et al*, 2001   7. Auvert *et al*, 2001
4. Obasi *et al*, 1999

[O'Farrell, 1999] and even higher levels in the Central African Republic (82%) [Mbopi-Keou et al, 2000], demonstrating that HSV-2 is a common genital infection in Africa. Infection with HSV-1 is endemic in these populations, and is mostly acquired in childhood [Wagner et al, 1994; Obasi et al, 1999; Mbopi-Keou et al, 2000]. HSV-2 rises rapidly in adolescents who are sexually active [Nahmias et al, 1996] and increases with age [Obasi et al, 1999]. In Asia, HSV-2 seroprevalences range from 6% in general populations to 50% in high risk groups [Nahmias et al, 1996].

The low rate of HSV-2 in young versus old chinese women can be explained by the effective control of sexually-transmitted infections after the revolution [Nahmias et al, 1996] whereas, the low rates of HSV-2 seroprevalence in some isolated tribes in Brazil suggest a recent introduction of the virus [Nahmias et al, 1996]. Numerous epidemiological studies clearly demonstrate that chancroid, syphilis and genital herpes are all common causes of genital ulcer disease (GUD) in developing countries [Kamali et al, 1999; Htun, 2001].

However, there is some evidence that there have been changes in the aetiology of GUD during the past ten years in parallel with the spread of AIDS epidemic [O'Farrell, 1999], and this may effect the impact of current clinical management. A prospective study in South Africa from 1986 to 1998 showed that the proportions of GUD due to chancroid and syphilis decreased during the study period [Htun, 2001]. Of the 239 GUD diagnosed in 1986, 53% were chancroid and 12% syphilis. By 1998, 33% and 3% of 200 GUD cases were chancroid and syphilis respectively. In parallel, a rapid increase in the proportion of herpetic ulcers, in both HIV-positive and negative subjects, was noted in the same setting [3/239 (1%) in 1986 and 47/200 (24%) in 1998] [Htun, 2001].

1.3.3. HSV-2 as a marker for sexual behaviour

An association between HSV-2 infection and reported sexual behaviour has been observed in studies performed in industrialized countries [Johnson et al, 1989; Cowan et al, 1994; Nahmias et al, 1996]. Thus, a good correlation between the prevalence of HSV-2 antibodies and the number of life-time sexual partners, particularly in men, has been observed [Nahmias et al, 1996]. It also appears that with an equal number of sexual life-time partners, women will have a higher prevalence of HSV-2 antibodies than men [Nahmias et al, 1996].

In contrast to studies in industrialized countries, Obasi and colleagues found no significant association with age at first intercourse [Obasi et al, 1999]. In this study, HSV-2-infected persons were more likely to report higher numbers of sex partners. Furthermore, the trend for increasing prevalence of infection with increased number of lifetime partners was statistically significant for both men and women, even after adjustment for age and residence [Obasi et al, 1999].

HSV-2 serology may be a useful objective biological marker for changes in sexual behaviour in HIV intervention studies since HSV-2 is more readily transmitted sexually than HIV. However, given the chronic nature of the infection, seroprevalence will be more discriminating in younger age groups [Obasi et al, 1999]. Therefore, HSV-2 seroincidence would be a preferable marker of behaviour change, especially in countries in sub-Saharan Africa where there is high incidence among young people. Indeed, the recent study of Auvert and colleagues among youth in Carletonville (South Africa) demonstrated that HIV infection was associated with HSV-2 seropositivity and sexual behaviour [Auvert et al, 2001]. The strong association between HIV infection and HSV-2 seroprevalence, and the fact that HSV-2 infection was a frequent cause of genital ulcers indicated that HSV-2 could

play a major role in the spread of HIV in the population of young people of South Africa [Auvert et al, 2001].

I.4. HSV-2 type specific serology

Both HSV-1 and HSV-2 DNAs are linear double-stranded molecules, approximately 100 megadaltons in molecular mass, with a composition of 67 to 69 percent guanine and cytosine [Kieff et al, 1971]. The nucleic acids from the two viruses demonstrate approximately 50 percent base-sequence homology [Ludwig et al, 1972; Kieff et al, 1972; McGeoch et al, 1987], greater than that shown between any of the nucleic acids of other herpesviruses thus far studied [Roizman, 1979]. Extensive work by Roizman and colleagues [Roizman, 1980] has revealed the importance of virus-specific polypeptides or glycoproteins in HSV DNA synthesis. Thus, several investigators have developed tests based on the type-specific glycoprotein G from HSV-2 (gG-2) and either glycoprotein C (gC-1) or glycoprotein G (gG-1) from HSV-1. These Accurate type-specific serologic assays allow identification of silent carriers of HSV-2 in patients with or without pre-existing antibodies to HSV-1 [Ashley & Wald, 1999]. Many feel the commercial availability of these tests is a significant advance for patient care and for public health efforts to control genital herpes [Mindel, 1998; Ashley et al, 1999; Corey & Hadsfield, 2000]. Such tests can provide useful information in symptomatic patients, particularly when virological tests such as culture, antigen detection or PCR are not helpful. Conversely, these tests can be useful in ruling out genital herpes in uninfected patients who have symptoms suggestive of herpes [Ashley & Wald, 1999; Langenberg et al, 1999]. Tests based on glycoprotein G may also be essential to distinguish antibody responses to HSV infections from those to subunit vaccines containing other, unrelated HSV glycoproteins. However, recipients of other vaccine

formulations containing gG should be advised that a positive gG based type specific serology will not be useful in diagnosing HSV should they become infected. Type specific serology can also characterise the nature of risk that a pregnant woman has for exposing a neonate to genital HSV at term [Brown, 2000]. A more controversial use of serology is for screening women and their partners to identify those women at risk of acquiring genital HSV-1 or HSV-2 late in pregnancy. Indeed, Brown and colleagues reported that third trimester genital infections with HSV-1 or HSV-2 in the seronegative mother or HSV-2 in the HSV-1 seropositive mother pose a considerable risk of peripartum transmission to the infant [Brown et al, 1997]. Furthermore, studies showing an association between genital herpes and risk of HIV acquisition suggest another patient population that may benefit from diagnosing unrecognised genital HSV infection [Fleming & Wasserheit, 1999; Corey & Handsfield, 2000; Krone et al, 2000]. Controlling genital herpes may help slow the spread of HIV. As experts in the field have suggested, the public health benefits and psychological impact of widespread HSV antibody screening in low prevalence populations remain to be determined by further studies [Fairley & Monteiro, 1997; Kinghorn, 1998; Handsfield et al, 1999]. However, for the individual patient, accurate tests can provide the basis for a proper clinical management, timely treatment and appropriate treatment relating to the natural history of transmission risks of the disease [Kinghorn, 1998; Smith et al, 2000].

I.4.1. Gold standard non-commercial tests for HSV type specific antibody

Western Blot (WB). This assay was developed more than 10 years ago, and its performance characteristics was validated against culture in symptomatic patients in the United States and in Australia [Bernstein et al, 1983; Ho et al, 1993]. In WB, sera are reacted against separated, fixed protein arrays ("blots") from either HSV-1 or HSV-2 infected cell lysate.

When combined with a step to cross-absorb antibodies to HSV-1 and HSV-2 antigens, this test is highly accurate in differentiating HSV-1 and HSV-2 antibodies, even in HSV-2 patients with previous HSV-1 infections [Ashley & Wald, 1999]. However, Western blotting is time consuming, expensive and technically difficult to replicate on a large scale, whereas ELISA tests are more widely available and have been developed into commercial assays [Cowan, 2000].

Immunodot Enzyme Assay (IEA)

This test uses immunoaffinity purified gG-1 and gG-2 immobilized on nitrocellulose discs [Lee et al, 1985]. The EIA was validated against culture and the University of Washington Western Blot test (UW WB) [Ashley et al, 1988] and has been used to track HSV-2 seroprevalence trends in the USA between 1979 and 1990 [Nahmias et al, 1990; Fleming et al, 1997].

Monoclonal Antibody blocking Assays

This assays developed by the Central Public Health Laboratory in London gains it specificity for HSV-1 and HSV-2 from the use of monoclonal antibodies against type specific gG-2 and gG-1 epitopes. The original radioimmunoassay format was validated against culture and UW WB [Slomka et al, 1995]. The recent EIA version of the test [Gopal et al, 2000] has a high concordance with WB [Vyse et al, 2000] and is the major reference test in the United Kingdom [Scoular et al, 1999].

Indirect gG-2 ELISA

Lectin purified gG-2 is used as antigen for enzyme immunoassays developed in Australia and in Scandinavia [Ho et al, 1992; Person et al, 1995]. This format is highly sensitive and specific for HSV-2 antibodies against culture [Ho et al, 1992].

gG-2 Immunoblot

Baculovirus recombinant gG-1 and gG-2 that have been denatured and electrophoresed to separate the target proteins is used in this test [Sanchez-Martinez et al, 1997]. In this test, the sensitivity is somewhat less than IEA [Ashley, 1998].

gG-Capture ELISAs

In this tests, type specificity is conferred by monoclonal antibodies bound to microwell plates. Comparison studies showed slightly lower sensitivity for HSV-1 (89%) and HSV-2 (90%) than the IEA test [Hashido et al, 1997].

I.4.2. Commercially gG-based type-specific tests.

Three major companies, Meridian Bioscience Inc, MRL Diagnostics (now called "Focus Technology"), and Diagnology have received approval from the USA Food and Drug Administration (FDA) for six gG diagnostic tests; two for HSV-1, three for HSV-2 and one that combines HSV-1 and HSV-2 in one kit. Meridian Bioscience, until recently, offered a gG based HSV-1 and HSV-2 ELISAs, using affinity purified gG-1 and gG-2, originally developed by Gull Laboratories. These tests have been discontinued. Focus Technologies has three tests, HSV-1 and HSV-2 ELISAs and an immunoblot test combining HSV-1 and HSV-2 antibody detection. Diagnology (Belfast, Northern Ireland) offers the only point of care or "near patient" test for HSV-2 that is designed for clinic use, the "POCKit-HSV-2". The antigen for the POCKit is lectin affinity purified gG-2.

Sorin Diagnostics Biomedica (ETI-HSVK-G2), Centocor (Captia Select HSV-2 EIA; marketed by Trinity Biotech and by Wampole Laboratories), and Roche (Cobas Core HSV-2 IgG EIA) produced gG-2 based tests in formats that are cost effective and easy to perform but none is FDA approved. In addition, HSV-1 type specific detection is not offered by these companies.

I.4.3. Limitations of gG-based tests.

Determining duration of infection

Limitations include potential slow time to seroconversion to gG-2. Indeed, Ashley and colleagues reported a seconversion time by Gull gG-2 ELISA [Ashley, 1999]. In their study, only 38% of patients were positive by 3 months, whereas, non-commercial gold standard tests require a median of 2-6 weeks. In addition, "staging" the disease as being recently acquired cannot be accomplished reliably by serology. Indeed, nearly 20% of those reporting first episodes of genital symptoms are not, in fact, newly infected but are presenting with first clinically apparent recurrences [Diamond et al, 1999; Lowhagen et al, 2000].

HSV-1 genital herpes

HSV-1 is causing an increasing proportion of new genital herpes infections as indicated by recent studies from Scandinavia [Lowhagen et al, 2000], the United Kingdom [Vyse et al, 2000], and the USA [Lafferty et al, 2000]. The POCkit, Cobas, Captia Select, and ETI-HSVK-G2 tests do not detect type specific antibodies to HSV-1 [Ashley, 2001]. The Focus test can detect HSV-1 specific antibodies. However, no test can distinguish between between HSV-1 antibodies that are generated in response to oral infection and those arising after a genital HSV-1 infection [Ashley et al, 2001].Furthermore, type specific tests for HSV-1 tend to be 5-10% less sensitive than HSV-2 counterparts and may require longer to reflect seroconversion [Ashley, 2001].

Use of type specific tests in paediatric sera

Fleming and colleagues studies revealed an HSV-2 seroprevalence of 0.25% in children using immunodot enzyme assay [Fleming et al, 1997]. Eis-Hubinger and colleagues found

an HSV-2 seroprevalence of 4% in children with the Gull test and 3% with the Cobas test [Eis-Hubinger et al, 1999]. In this large comparison study, paediatric sera accounted for nearly all of the false positive results. Since performance in paediatric sera by the Focus, POCkit, and other commercial tests are not known, these tests should be used with caution, if at all, in children under 14 [Ashley, 2001].

"seroreversion" or loss of gG-2 antibodies

It has been reported that the outcome of glycoprotein G based type specific tests may change over time from positive to negative [Schmid et al, 1999; Arvaja et al, 1999]. This phenomenon has been term "seroreversion" and implies that the immune response to gG-2 wanes to undetectable levels over time. This possibility has caused concern about the long term reliability of these tests. Ashley and colleagues examined 300 sera from 32 patients with long term clinic follow up for HSV-2 genital herpes 96-22 years; median 12 years) by western blot and by the Gull gG based HSV-1 and HSV-2 ELISAs [Ashley et al, 2000]. They found no evidence in HSV-1 or HSV-2 western blot profiles that could suggest loss of antibody titre. In contrast, the Gull test gG-2 test resulted resulted in sporadic reversal from HSV-1 positive to negative in two subjects. Therefore, type specific test that turn negative over time should be questioned and the sera involved should be repeated.

Type specific IgM tests

Very few testing formats have been adapted to detect type specific IgM to gG-2 [Ho et al, 1992]. As previously described [Ashley, 1998], Gull Laboratories developed prototype gG based IgM that could detect seroconversion much faster than could the Gull type specific IgG tests. However, this IgM ELISA was not useful for discriminating primary episodes from recurrent episodes since 35% of recurrent HSV-2 episodes elicited IgM to HSV-2 [Ashley, 1998].

1.5. Genital HSV-2 as a possible cofactor in HIV infection

1.5.1. STI : Cofactors of sexual transmission of HIV

Epidemiological [Pepin et al, 1989; Cameron & Padian, 1990; Fleming & Wasserheit, 1999] and intervention studies [Laga et al, 1993; Grosskurth et al, 1995] in sub-Saharan Africa, have demonstrated the role of STIs in facilitating the acquisition and transmission of HIV. This has provided a strong argument for making STD control an integral part of HIV prevention strategies [Siegal et al, 1981; Stamm et al, 1988; Laga et al, 1994; Grosskurth et al, 1995; Wawer et al, 1999]. In fact, STDs that cause genital ulceration such as syphilis, chancroid and HSV-2 infection are particularly implicated in facilitating HIV transmission [Holmberg et al, 1988; Cameron & Padian, 1990; Hook et al, 1992; Quinn, 1992; Augenbraum et al, 1995; Gwanzura et al, 1998; Dada et al, 1998; Schacker et al, 1998]. Genital ulcer disease (GUD) is believed to increase the risk of HIV acquisition per sexual exposure by increasing the amount of HIV shedding through genital lesions and/or increasing susceptibility by providing an easier portal of entry for the virus into the host [Hayes et al, 1995; Gwanzura et al, 1998]. These findings have been supported by recent biological studies that have shown the role of certain STD conditions such as gonorrhoea and genital ulcers in men [Cohen & Miller, 1998] and in women [Ghys et al, 1997; Mostad et al, 1997] in enhancing HIV shedding [Cohen & Miller, 1998].

Although GUD is a common complaint at STD clinics in Africa, screening for and management of HSV-2 is rarely done [Gwanzura et al, 1998]. Nevertheless, studies have found that HSV-2 was common in STD clinic attendees in Africa [Langeland et al, 1998]. For example, in Kampala, HSV-2 was present in 36% of GUD patients [Kamya et al, 1995].

In a study that screened sera from Dakar, the prevalence of HSV-2 ranged from 20% among surgical patients to 96% among prostitutes [Nahmias et al, 1990]. High prevalences of HSV-2 were recorded also in rural populations of Uganda and Tanzania [Wagner et al, 1994; Obasi et al, 1999]. Taking into account the high prevalence levels of HSV-2, the increased shedding of HIV through genital herpes lesions, and the fact that persons with HSV-2 remain potentially infectious for life, HSV-2 may make an important contribution to HIV transmission in Africa [Gwanzura et al, 1998].

1.5.2. Bi-directional interaction between HSV-2 and HIV

In their review of available data on the role of STIs in the sexual transmission of HIV infection, Flemming and Wasserheit [Fleming & Wasserheit, 1999] group the evidence that STIs facilitate the transmission of HIV into three categories: a) biological plausibility studies; b) HIV seroconversion studies; and c) community-level intervention studies. Whilst there is ample evidence for the role of ulcerative and non-ulcerative bacterial STIs in enhancing HIV transmission and acquisition, little is known however, about the relationship between HIV and viral STIs such as HSV (Figure 1.2 & Figure 1.3).

Biological plausibility for the hypothesis that HSV-2 and HIV interact.

During sexual intercourse genital ulcers may bleed, leading to the increased risk of HIV transmission. Studies of HIV infected people with genital ulcer disease (GUD) suggest that GUD may increase infectiousness as HIV proviral DNA have been detected in genital ulcer exudates [Plummer et al, 1991]. Similarly, Schacker and colleagues [Schacker et al, 1998] provided further evidence to support the hypothesis that genital herpes infection increases

the efficiency of the sexual transmission of HIV-1. In their study, HIV RNA was consistently detected in genital ulcers caused by HSV-2 in HIV-1 seropositive men in Seattle [Schacker et al, 1998]. Moreover, treatment or healing of GUD was accompanied by a decrease in HIV shedding [Schacker et al, 1998]. An increased viral load and the proximity of virions to cutaneous surfaces are factors that may lead to the increased transmission of HIV in the presence of HSV [Schacker et al, 1998].

Among HIV negative people, GUD may increase susceptibility by disrupting mucosal integrity; by the recruitment and activation of HIV target cells. There is also evidence that (i) among 'asymptomatically' HSV-2 infected patients (ie in the absence of a visible ulcer) HSV-2 genital shedding is increased in HIV positives [Augenbaum et al, 1995; Mbopi-Keou et al, 2000]; (ii) both HIV RNA and HSV-2 DNA shedding are increased in the presence of the other virus [Mbopi-Keou et al, 2000].

Figure 1.2. illustrates the difficulties in establishing the role of STIs in facilitating HIV transmission, and highlights the potential confounding role of unprotected sexual intercourse and impaired immunity. HSV-2/HIV interactions may be studied in high risk groups (e.g., HIV-negative partners in serodiscordant couples, HIV-negative sex workers)
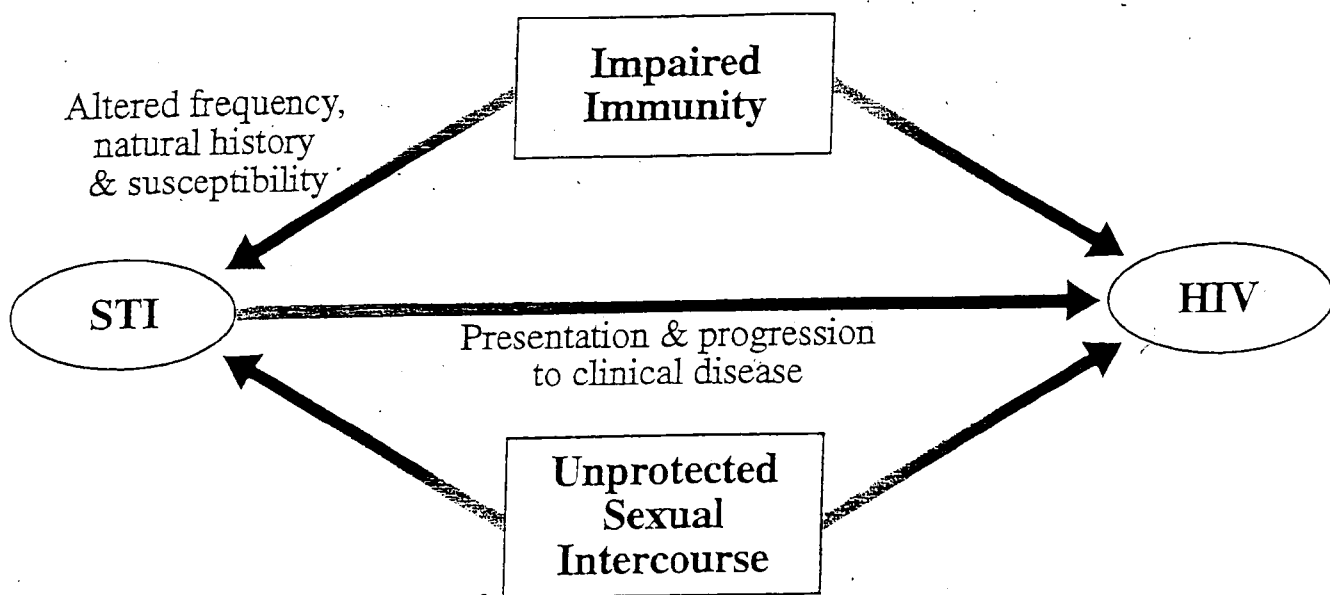
Figure 1.3. Bi-directional and synergistic interactions between HIV and HSV infections. In dually-infected individuals, the viruses may amplify each other's shedding at mucosal sites.
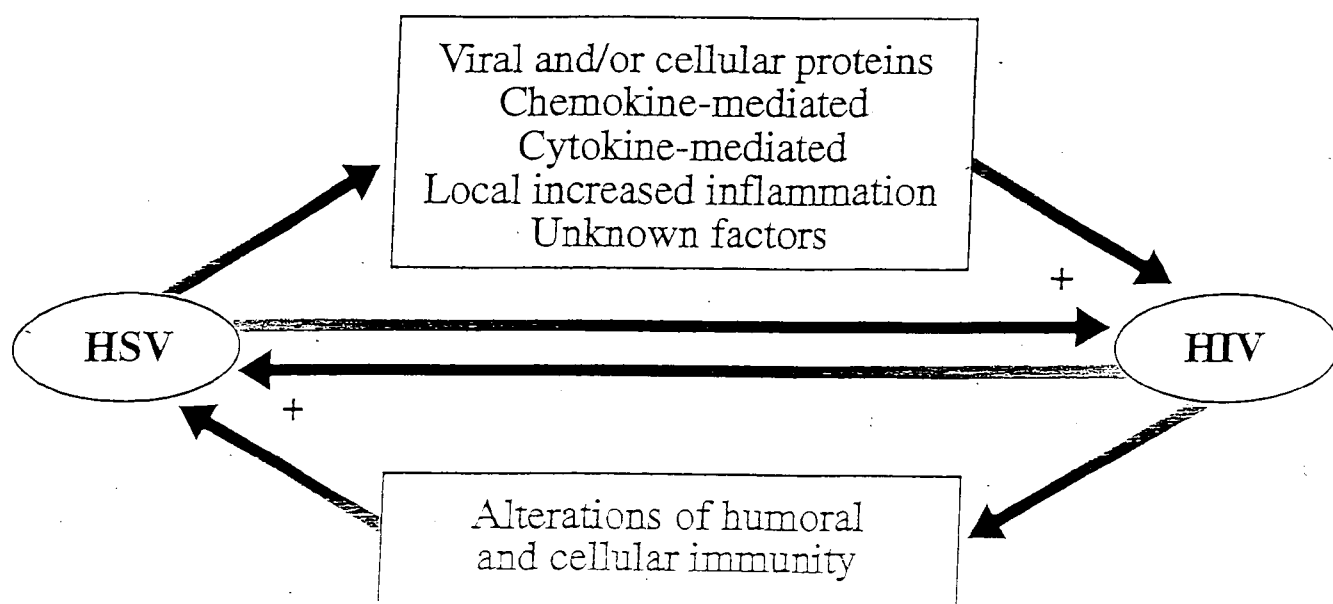
Another study also demonstrated that HSV-2 reactivation is associated with an increase in plasma HIV-1 RNA and intracellular gag mRNA and that plasma HIV-1 RNA level decreases significantly during treatment with acyclovir [Mole et al, 1997]. Kucera and colleagues [Kucera et al, 1990] reported that co-infection of human CD4+ cells with HSV and HIV result in an accelerated replication of HIV. Similarly, Heng and colleagues [Heng et al, 1994] found that the up-regulation of HIV-1 expression was induced by HSV, which may translate to increased plasma HIV-1 RNA levels. In addition, HSV-1 may pseudotype in humans with HIV glycoproteins gp120/gp41 [Heng et al, 1994]. Their study documented in vivo reciprocal enhancement of viral replication associated with the co-infection of keratinocytes and macrophages by HIV-1 and HSV-1 in patients with AIDS and HSV lesions.

Indeed, their observation may point to the importance of including antiherpetic drugs as part of the overall treatment of patients with AIDS to avoid the excessive replication of HIV-1 in both CD4+-dependent and -independent targets [Severson & Tyring, 1999]. In addition, a recent study reported that the inclusion of acyclovir together with antiretroviral therapy may prolong survival in HIV seropositive individuals [Severson & Tyring, 1999]. Furthermore, Rando and colleagues [Rando et al, 1987] showed that HIV Long Terminal Repeat (HIV LTR) is activated by herpesviruses as measured by HIV LTR directed expression of acetyltransferase gene (CAT). Other studies also demonstrated that alpha and beta genes of HSV are responsible for the transactivation of HIV gagLTR [Mosca et al, 1987; Ostove et al, 1987; Albrecht et al, 1989; Golden et al, 1992]. As a result, it is possible that HIV progresses more rapidly in untreated HSV-2 positive individuals. However, evidence is

inconclusive and more studies of the effect of episodic HSV-2 therapy on HIV are needed, especially in developing countries. The impact of HIV on HSV, with regards the clinical reactivation of HSV, the role of immunosuppression and the possible frequency of asymptomatic carriage, remains however largely undetermined.

Studies of HIV incidence in patients with genital ulceration due to HSV-2 and in HSV-2 seropositive individuals

Eight studies of HIV incidence in high risk individuals assesed the role of genital HSV-2 in the sexual transmission of HIV [Fleming & Wasserheit, 1999] including five studies on homosexual men [Holmberg et al, 1988; Keet et al, 1990; Kingsley et al, 1990; Kuiken et al, 1990; Craib et al, 1995].

Only three studies have investigated the role of HSV in heterosexual transmission of HIV infection [Telzack et al, 1993; Nelson et al, 1997; Nopkesorn et al, 1998]. In two studies, the rate of HIV seroconversion was higher among individuals who were HSV seropositive. In a case control study of Thai military conscripts, HSV antibodies were three times more common in HIV seroconverters than men who remained HIV negative (OR 3.1, 95% CI 1.2-7.9) [Nelson et al, 1997].

The second cohort study, also in Thai military conscripts, found a fourfold increase, which was statistically significant, in the relative risk of HIV seroconversion among those who were HSV seropositive [Nopkesorn et al, 1998]. However, the relative risk was 2.0 (95% CI 0.6 – 6.1) after adjustment for sexual behaviour. A study of STI patients in New York did not find an increased rate of HIV seroconversion associated with HSV infection [Telzack et al, 1993]. In this study, HSV infection was diagnosed by clinical examination and Tzanck cytodiagnosis, therefore many HSV infections are likely to have been missed.

There are three nested case control studies that have investigated the role of HSV infection in male to male transmission of HIV [Holmberg et al, 1988; Keet et al, 1990; Kingsley et al, 1990]. Holmberg and colleagues observed that the rate of HIV seroconversion was twice as frequent in men who were HSV-2 seropositive compared to those who were seronegative and that HSV-2 seroconverters were at even greater risk of HIV seroconversion (OR=6.0) [Holmberg et al, 1988]. Keet and colleagues also found an association between HIV acquisition and HSV-2 seropositivity [Keet et al, 1990]. However, no association was found in the study by Kingsley and colleagues [Kingsley et al, 1990]. Seroconversion studies are likely to underestimate the effect of HSV-2 on HIV transmission. First, HSV is a chronic disease and recurrences decrease over time. Second, HSV seropositivity has poor correlation with clinical episodes. Third, the methods used to measure HSV are often insensitive. And last, there is often failure to adjust for the usual confounders, such as sexual behaviour or other STIs.

Epidemiological data showing an association between HIV and HSV-2 seropositivity in Africa have been scarce [Gwanzura et al, 1998; Obasi et al, 1999; Mbopi-Keou et al, 1999; Mbopi-Keou et al, 2000]. A multicenter study assessed parameters which could explain the heterogeneity of HIV epidemics in four African cities characterized either by high HIV prevalences (20-30% in Kisumu, Kenya and in Ndola, Zambia) or by low HIV prevalences (3-8% in Cotonou, Benin and in Yaounde, Cameroon) [Weiss et al, 2001]. This study showed an association between HIV and HSV-2 infection [Weiss et al, 2001]. In fact, the authors of this study demonstrated that cities with high HIV prevalence also have high HSV-2 prevalence. This association is particularly strong among the youth population.

Secondly, Weiss and colleagues showed a strong association between HSV-2 prevalence and seropositivity for HIV (OR 5-10) after ajustment for sexual behaviour [Weiss et al, 2001].

Another study of 2397 adults in Harare, Zimbabwe, showed an HSV-2 prevalence of 39,8%, and seroconversion rate of HSV-2 of 6,2 person/year, without clear association to the risk of seroconversion for HIV [McFarland et al, 1999].

Lack of intervention studies focus on genital herpes

The demonstration of any bi-directional and/or synergistic interactions between STDs and HIV infection, has direct public health implications [Laga et al, 1994]. Intervention studies in Africa [Laga et al, 1994] and in Thailand [Khambooruang et al, 1996] combining preventive interventions and treatment of STDs demonstrated that it may be possible to reduce the incidence of HIV in the developing world. Furthermore, The Mwanza study in Tanzania demonstrated that improved STD case management was an important additional HIV prevention strategy [Grosskurth et al, 1995], resulting in a 40% reduction in HIV incidence in the general population. No such reduction was achieved by STD mass treatment in the Rakai study in Uganda [Wawer et al, 1999], and one reason postulated for this was a higher prevalence in Rakai of STDs such as genital herpes that were not targeted by the antimicrobial regimen [Hitchcock & Fransen, 1999].

Indeed, there are few intervention studies to date to document an impact of anti-HSV-2 treatment on HIV transmission. The Schacker study among 12 men in Seattle is the only study to have documented that treatment or healing of GUD caused by HSV-2 was

associated with a reduction of HIV shedding. Even in this instance, it was unclear whether such effect was spontaneous or due to any specific intervention [Schacker et al, 1998].

1.6. HSV and HIV interaction: the role of vitamin A

Vitamins A and E are micronutrients with anti-oxidant/anti-inflammatory properties, and deficiency of these vitamins has been associated with HIV-1 disease progression [Tang et al, 1997]. This section will focus on the role of vitamin A.

1.6.1. The role of vitamin A in immunity to infectious diseases

Many studies have suggested the important role of vitamin A as an immunoregulator during infectious diseases [Filteau et al, 1993; Semba, 1994a; Semba et al, 1994b; Mostad et al, 1997; John et al, 1997; Fawzi & Hunter, 1998a]. Vitamin A and its metabolites are immune enhancers that have been shown to potentiate antibody response to T cell-dependent antigens, increase lymphocyte proliferation responses to antigens and mitogens, inhibit apoptosis, and restore the integrity and function of mucosal surfaces [Semba, 1994a]. Vitamin A deficiency (xerophthalmia) is characterized by widespread alterations in immunity, including pathological alterations in mucosal surfaces, impaired antibody responses to challenge with protein antigens, changes in lymphocyte subpopulations, and altered T-and B-cell function [Semba, 1994a].

In the developing world, the mortality rate among otherwise well nourished children with xerophthalmia is several times higher than that among nonxerophthalmic children and correlate with the severity of xerophthalmia [Sommer et al, 1983; Milton et al, 1987; Nimmagadda et al, 1998]. Compared with other children, vitamin A deficient children are

two to four times more likely to develop respiratory diseases and diarrhea within three months of follow-up [Sommer et al, 1984; Milton et al, 1987; Bloem et al, 1990; Nimmagadda et al, 1998]. Meta-analyses of randomized clinical trials in Brazil, Haiti, Indonesia, India, and various African countries indicate that vitamin A supplementation given to children without clinical evidence of vitamin A deficiency reduces overall childhood mortality by approximatively 30% [Nimmagadda et al, 1998]. The most important part of this benefit stems from decreased mortality caused by infections, particularly those due to diarrheal illnesses, rather than from decreases in the incidence of infection [Fawzi et al, 1993; The Vitamin A and Pneumonia Working Group, 1995; Nimmagadda et al, 1998]. Vitamin A supplementation has also a beneficial effect on chilhood mortality due specifically to measles [Nimmagadda et al, 1998]. For example, randomized placebo-controlled prospective trials in Tanzania and South Africa clearly demonstrated that high dose vitamin A supplementation decreased by 50% the risk of death or major complication as well as the duration of illness in children who are hospitalized with measles [Barclay et al, 1987; Hussey and Klein, 1990; Coutsoudis et al, 1992]. Moreover, meta-analyses of these studies showed that vitamin A supplementation decreases overall death rate by 60%, deaths due to respiratory illness by 70% and deaths among infants by 90% [Glaziou & Mackerras, 1993; Fawzi et al, 1993]. Vitamin A supplementation is associated with increased concentrations of measles IgG antibodies and the total numbers of lymphocytes in patients with acute measles [Coutsoudis et al, 1992], whereas vitamin A deficiency is associated with decreased measles antibody concentrations following vaccination [Mahoney et al, 1995]. Therefore, these data are in accordance with the observations that decreased serum levels of retinol are linked with increased severity of measles [Butler et al, 1993].

1.6.2. Vitamin A deficiency in HIV seropositive persons

Although vitamin A deficiency is rare in developed countries, studies in the United States and Australia have found that the prevalence of hyporetinemia in HIV-seropositive patients is up to 29% [Bogden et al, 1990; Malcom et al, 1990; Beach et al, 1992; Semba et al, 1993; Semba et al, 1994b; Karter et al, 1995; John et al, 1997; Nimmagadda et al, 1998]. In contrast, the prevalence of hyporetinemia in HIV-seronegative control patients ranged from zero to 3.7% [Bogden et al, 1990; Malcom et al, 1990; Beach et al, 1992; Semba et al, 1993; Karter et al, 1995; Nimmagadda et al, 1998], and surveillance data indicate that less than 2% of the general adult population in the United States have serum retinol levels of <1.05 umol/L [Nimmagadda et al, 1998]. Large cohort studies of HIV-seropositive Intravenous Drug Users (IDUs) prospectively followed in Baltimore suggest that vitamin A deficiency may alter the clinical course of HIV disease [Semba et al, 1993; Semba et al, 1995]. While analysing a subgroup of this cohort, the relative risk of mortality was 4.3 times higher when the serum retinol level was <1.05 μmol/L [Semba et al, 1993]. Furthermore, in a follow-up case-control study, when assessing CD4 counts and wasting among patients who died of HIV infections, patients with baseline serum retinol levels of <1.05 μmol/L had a 3.5-5 fold increased risk of death [Semba et al, 1995].

Pregnant HIV-seropositive women, especially in developing countries are particularly at risk for vitamin A deficiency, most likely because of the increased nutritional demands of pregnancy [Nimmagadda et al, 1998]. In Nairobi, 58% of HIV-infected women attending a prenatal clinic had serum vitamin A concentrations of <1.05 μmol/L [Nduati et al, 1995].

Such maternal vitamin A deficiency is associated with increased risk of maternal HIV transmission [Nimmagadda et al, 1998]. In Malawi for example, Semba and colleagues found that the relative risk of perinatal HIV transmission was 4.38 times higher in mothers with serum vitamin A concentrations of <0.70 μmol/L than in mothers with serum vitamin A levels of >1.33 μmol/L [Semba et al, 1994a]. Studies showed that the effect of maternal vitamin A was independent of maternal age, CD4 lymphocyte percentage, or body mass index [Semba et al, 1994a]. Similarly, in the United States, Greenberg and colleagues recently showed that serum retinol levels of <0.70 μmol/L were associated with increased maternal transmission of HIV infection, after the duration of rupture of membranes and CD4 lymphocytes count were assessed [Greenberg et al, 1997].

The exact mechanism by which vitamin A deficiency increases mother-to-child transmission of HIV is still unclear [Fawzi & Hunter, 1998a; Fawzi et al, 1998b; Nimmagadda et al, 1998]. However, in Nairobi, John and colleagues found, among women during the third trimester of pregnancy, evidence of association between hyporetinemia (<0.70 umol/L) and vaginal HIV-1 shedding [John et al, 1997]. Moreover, the fact that vitamin A plays a role in maintaining mucosal surfaces suggest that HIV transmission could occur as a consequence of decreased placental integrity or increased susceptibility of the birth canal to trauma during delivery [Nimmagadda et al, 1998]. Furthermore, in women with a CD4 lymphocyte count <400/uL and vitamin A serum levels <0.70umol/L, the risk of detecting proviral DNA in breast milk is 20 times higher than in non hyporetinemic women [Nduati et al, 1995]. It has been postulated that there could be potential applications for vitamin A therapy or supplementation to alter the course of infectious diseases including HIV [Semba, 1994a]. In

addition, the role of vitamin A in cervico-vaginal HIV shedding has been recently demonstrated [Mostad et al, 1997].

1.7. Research priorities for the developing world

1.7.1. Biological interactions between HSV-2 and HIV

There is an urgent need for more studies to assess the burden of clinical disease and infection due to HSV-2 in different settings. Such studies may also help to better understand the biological mechanisms that underlie the HSV-2/HIV interaction (Figure 1.2 & Figure 1.3) and may also provide biological endpoints for future intervention trials.

1.7.2. HSV-2 treatment trials

In order to determine whether HSV-2 infection enhances HIV shedding and transmissibility, randomised intervention studies will be necessary, preferably in settings with high HSV-2 seroprevalence (and shedding) and high HIV seroprevalence and/or incidence. It is accepted that a randomised-controlled trial of interventions could provide the most convincing evidence to demonstrate this relationship.

The aim of such intervention studies should therefore be to decrease HIV shedding in individuals coinfected with HIV and HSV-2 (and ideally also HIV incidence in HIV negative partners). This decrease should in theory be mediated by a measurable decrease in HSV-2 shedding (i.e. prevalence or duration of shedding and/or quantity of virus particles shed) and lesions.

Two types of studies should be considered:

a) Episodic treatment of genital ulcer disease (GUD), by adding aciclovir/valaciclovir to the syndromic management vs. placebo and measuring the effect on HSV-2 and HIV shedding.

b) Suppressive therapy with aciclovir/valaciclovir (the latter drug preferable for its single daily dose) of dually HIV/HSV-2 infected individuals to prevent HSV replication. (e.g., HIV-negative partners in serodiscordant couples, HIV-negative sex workers)

Aciclovir is an antiviral drug, which has been the standard treatment for genital herpes for the past decade in developed countries [Whitley et al, 1998]. Two new drugs, valaciclovir and famciclovir have recently become available, and are also effective and safe treatments [Wald, 1999]. However, they are more expensive than aciclovir [WHO/UNAIDS/LSHTM International workshop, 2001].

HSV2 treatment trials and HIV-related outcomes:end points and possible study groups

There are few intervention studies to date to document an impact of anti-HSV-2 treatment on HIV shedding. The high prevalence rates of both HSV-2 and HIV in sub-saharan Africa, the high frequency of asymptomatic carriage of HSV-2, and the hypothesis of a synergistic interaction between these viral infections suggest that more research need to be done on HSV-2 to have a potential impact in the control of HIV infection.

The first type of intervention would be "the strategy of episodic treatment" added to syndromic management of genital ulcers desease (GUD)" as recommended by WHO. Thus, oral acyclovir or valaciclovir could be combined to the usual antibacterial treatment, and its impact in terms of HSV-2 and HIV genital shedding should be evaluated. This type of study

may be advisable for highly exposed individuals from core groups such as female sex workers.

The second type of intervention could consist of suppressing asymptomatic HSV shedding and clinical recurrences. Although it would seem unlikely that this type of intervention could be conducted as a public health intervention on a large scale, it may be advisable for HIV-negative partners in serodiscordant couples or HIV-negative sex workers. Ideally cohort studies of individuals whose serological status (HIV and HSV-2) are known and in whom the existence of HSV-2 shedding could be determined should be set up. Individuals in these groups with symptomatic or asymptomatic HSV shedding should be randomized to take long term suppressive therapy (intervention group), or placebo (control group), and followed up prospectively, e.g 6 months to one year. Measurable outcomes would be HIV incidence in those individuals who were HIV-seronegative, and HIV infectiousness, as measured by HIV shedding. It would be important to monitor the emergence of resistant HSV strains to aciclovir or valaciclovir in this situation, this being particularly a problem in long-term antiviral therapy of HIV-infected individuals.

Effects of HSV2 therapy on HIV shedding and transmission.

HSV2 antiviral therapy has been shown to decrease HIV shedding from herpetic lesions in individuals co-infected with HSV2 and HIV [Mbopi-Keou et al, 2002]. However, there are no studies demonstrating a reduction in the rate of HIV seroconversion.

Therapy to prevent herpetic disease.

Although currently there are no data available, there is a biological rationale for prophylactic therapy in HSV2-negatives [Mbopi-Keou et al, 2002]. This would aim at

preventing herpetic disease rather than infection. Because the first episode of disease would be aborted, fewer latent copies should in theory be present in the ganglia for reactivation, and this should limit the frequency of recurrences. A further consideration is that the practicalities of prophylactic therapy make it unlikely to be feasible on a large scale in developing countries. However, their use could be considered in specific groups at high risk of HIV infection, such as HIV-negative members of discordant couples, sex workers and, possibly, young women [Mbopi-Keou et al, 2002].

Viral resistance.

It has been reported that HSV can develop resistance to aciclovir through mutations in the viral gene that encodes thymidine kinase, by generation of thymidine-kinase-deficient mutants or by selection of mutants with a thymidine kinase unable to phosphorylate aciclovir [Whitley & Roizman, 2001]. Clinical isolates resistant to aciclovir are almost uniformly deficient in TK, although isolates with altered DNA polymerase have been recovered from HSV-infected patients [Whitley et al, 1998]. Drug resistance was considered rare, and resistant isolates were believed to be less pathogenic until a series of aciclovir-resistant isolates from patients with AIDS were characterized [Erlich et al, 1989]. These resistant mutants were deficient in TK. Although aciclovir-resistant HSV is susceptible to vidarabine and foscarnet in vitro, only foscarnet has been shown to be effective in the treatment of infection due to acyclovir-resistant HSV [Whitley et al, 1998]. Aciclovir-resistant HSV can cause pneumonia, encephalitis, esophagitis, and mucocutaneous infections in immunocompromised patients. Resistant HSV occurs more frequently among HIV-infected patients, probably due to increased replication of HSV and decreased immunity in these patients [Severson & Tyring, 1999].

Toxicity.

Continuous aciclovir therapy has not caused long-term toxicity in the past decade [Whitley & Roizman, 2001]. Renal dysfunction has been reported, particularly in patients given large doses of aciclovir by rapid intravenous infusion, but appears to be uncommon and is usually reversible [Whitley et al, 1998]. The risk of nephrotoxicity can be minimized by administering aciclovir by slow infusion and by ensuring adequate hydratation [Whitley et al, 1998]. A few reports have linked intravenous administration of aciclovir with disturbances of the CNS, including agitation, hallucinations, disorientation, tremors, and myoclonus [Whitley et al, 1998]. The Aciclovir in Pregnancy Registry has gathered data on prenatal exposure to acyclovir [Andrews et al, 1988]. No increase in the risk to mothers or fetuses has been documented, but the total number of monitored pregnancies is too small to detect any low-frequency events [Andrews et al, 1988].

1.7.3. Genital mucosal immunity to HSV-2 infection

The control of HSV latency is not understood but mucosal immune responses against HSV are likely to be important in the control of mucosal lesions [Kuklin et al, 1998]. Studies in a mouse model on host resistance to HSV-2 have shown that vaginal infection with an attenuated HSV-2 strain induces protective immunity to subsequent lethal challenge with wild-type virus. Both cell-mediated and humoral local immunity, mainly of the IgG or IgA isotype have been described [McDermott et al., 1990; Parr & Parr, 1997]. Furthermore, T-cell mediated immunity at the mucosal level is thought to play a major role in the resolution of recurrent HSV lesions in animal models [Posavad et al, 1998]. Cellular immunity to HSV is thought to be important in limiting recurrent genital HSV-2 infection [Koelle et al., 2000].

Antibodies to HSV of both IgA and IgG isotypes have been detected in cervicovaginal secretions of women with vaginal discharge [Persson et al, 1988] and in women suffering from primary genital herpes infection [Merriman et al., 1994; Ashley et al., 1994] or reactivation [Merriman et al, 1984]. However, little is know about their functional properties. Studies to localize neutralizing activity to purified cervical IgG, IgA, or IgM components would be useful.

1.7.4. Vaccination against HSV-2.

Given the high prevalence of HSV2 infection in many countries, and the fact that most infections are subclinical, the development of an effective HSV2 vaccine would provide a powerful control tool. Most vaccines strategies against herpes simplex virus have focused on the development of subunit vaccines that consist of one or more HSV glycoproteins [Bourne et al, 1996]. In fact, studies have shown that vaccination of mice with purified glycoprotein D (gD) provides protection against a lethal intraperitoneal challenge of HSV and vaccination of guinea pigs with recombinant gD or gB protects against intravaginal HSV-2 infection [Berman et al, 1985; Stanberry et al, 1987]. Clinical trials indicate that purified gD induce neutralizing antibody responses in previously uninfected persons and boosted the antibody response in patients with genital herpes [Strauss et al, 1993].

Three HSV-2 vacines have been recently evaluated in clinical trials:

Recombinant Glycoprotein Vaccine gB$_2$ and gD$_2$ with MF59.

This subunit HSV vaccine is no longer in commercial development. Phase I studies showed that it was well tolerated and induced specific neutralising antibody and T-cell lymphoproliferation responses comparable to or higher than those seen in HSV2 infected

subjects. Two phase III trials have assessed effectiveness of the vaccine in prevention of HSV2 infection. Survival analysis showed a short-term efficacy of 50% for the first five months of follow-up but, thereafter, the effect disappeared [Corey et al, 1999]. The overall efficacy was 9% (95% CI −29% to 36%), although substantial differences were observed in men and women (-4% in men and 26% in women). Vaccination had no significant influence on duration of the first clinical episode of genital HSV2, or on the subsequent frequency of recurrence. The authors concluded that efficient and sustained protection against sexual acquisition of HSV2 infection will require more than high titres of specific neutralizing anitbodies and, as the vaccine provided only transient protection against HSV2 infection, work has been halted [Corey et al, 1999].

Recombinant Glycoprotein Vaccine gD$_2$ with adjuvent SBAS4.

This subunit HSV vaccine is in commercial development and, again, induced HSV-specific antibody and cell mediated immune responses in phase I studies. Two phase III trials assessed vaccine efficacy in prevention of genital HSV disease with secondary assessment of prevention of HSV2 infection [Spruance et al, 2000]. The vaccine induced significant protection (approximately 70% efficacy) against genital herpes disease in women who were initially HSV1 and HSV2 seronegative. Trends towards protection of women against HSV infection were also seen in both studies (39-48% efficacy), although not statistically significant. In contrast, there was no evidence of protection in women who were initially HSV1 seropositive, or in men. The main disadvantages of this vaccine are the apparent failure to improve on protection provided by HSV1 infection and the need for frequent vaccine administration to boost host immunity.

Disabled Infectious Single Cycle (DISC) HSV2 Vaccine.

Phase I studies have shown that this vaccine is well tolerated and induces neutralising antibody and lymphoproliferative responses comparable to those seen in HSV2 infected subjects [WHO/UNAIDS/LSHTM International workshop, 2001]. 83% of vaccine recipients developed HSV-specific cytotoxic T lymphocyte responses. Phase II efficacy trials are underway in US and UK to assess efficacy of the DISC vaccine as a therapeutic vaccine for the treatment of frequently recurrent genital HSV2 infections. Because of its rich content of HSV2 virion, this product may improve on the natural protection provided by HSV1. However, its closeness to HSV2 means that it would be difficult to distinguish natural infection from vaccine-induced immunity. The main disadvantages of the DISC therapeutic vaccine are that it needs to be administered at least 6 monthly, is expensive and needs further development. Nevertheless, Partnerships should be sought to enable the DISC vaccine to be taken to the next stage of development [WHO/UNAIDS/LSHTM International workshop, 2001]. In addition, vaccine trials to evaluate the efficacy of a therapeutic HSV-2 vaccine should be part of today's research agendas [WHO/UNAIDS/LSHTM International workshop, 2001].

Table 1.1. HSV2 seroprevalence in general populations in developing countries

Country	Population	Year	Prevalence[1]
Uganda	Adults (rural)	1989	74% (f); 57% (m) [a]
Congo	Adults (urban)	1982	71% [b]
Kenya	Adults (urban)	1997	68% (f); 35% (m) [c]
Zambia	Adults (urban)	1997	55% (f); 36% (m) [c]
Rwanda	Hospital workers (rural)	1985	51% [b]
Cameroon	Adults (urban)	1997	51% (f); 27% (m) [c]
Costa Rica	Adult women	1985	43% (f) [b]
Tanzania	Adults (rural)	1993	42% (f); 19% (m) [2, d]
Zaire	Adults (urban)	1985	41% [b]
Rwanda	Adults (rural)	1985	33% [b]
Benin	Adults (urban)	1997	30% (f); 12% (m) [c]
Brazil	Blood donors (urban)	1994	29% [e]
Rwanda	Army Recruits (rural)	1985	28% (m) [b]
Senegal	Surgical patients (urban)	1985	20% [b]
China	Gynaecology clinic (urban)	1984-5	2% (f) [b]

[1] (f) females; (m) males
[2] Age-weighted sample: younger age-groups were over-represented
[a] Wagner et al, 1994
[b] Nahmias et al, 1990
[c] Weiss et al, 2001
[d] Obasi et al, 1999
[e] Da Rosa-Santos et al, 1996

Chapter 2. Aims and setting of the study

2.1. Aims of the study

□ Measure the HSV-2 seroprevalence and the prevalence of HSV-2 shedding in African women, with or without genital symptoms, living in Bangui, Central African Republic, a country of high HIV-1 prevalence area.

□ Investigate the relationship between HSV-2 and HIV-1 infection by comparing (i) the prevalence and quantity of HSV-2 genital shedding in HIV-1 positive and HIV-negative women; (ii) the association between HIV-1 and HSV-2 genital shedding among dually infected women.

□ Investigate the relationship between vitamin A deficiency and Sexually transmitted infections such as HSV-2.

□ Investigate the association between HSV-2 shedding and HIV-1 RNA, HIV-1 proviral DNA in HIV-and HSV-2 co-infected women.

□ Investigate the association between HSV-2 shedding and local immune response.

2.2. Setting of the study

2.2.1. Background information on Central African Republic

The Central African Republic (CAR) is bordered by five other African countries (Cameroon, Congo, Chad, Sudan and the Democratic Republic of Congo formerly Zaire) and is crossed by the trans-African highway which connects west and Central Africa with East Africa (Figure 2.1). Bangui, the capital city has a population of about 600,000 inhabitants. The CAR has rain forests in the south, small desert in the north, and the rest is mostly savanna. The country is hot and humid all the year round. The rainy season starts in April and continues until October. With a population of 3, 150, 000 inhabitants living in an area of 622, 980 km^2, the main ethnic groups are of Bantu origin (Figure 2.2 & Figure 2.3), and the rest consists of 150.000 Arab merchants, 20.000 Pygmies and 20.000 Mbororo (ancient Arab nomads) [Report, Japanese Embassy, Bangui, 1996]. Several thousand European expatriates also live in CAR. The people of Bantu tribes live in rural areas, practice mainly farming for a living and eat cassava as their staple food. Houses are made of dried bricks of laterite with a thatched roof. Pygmy tribes live in the southern rain forest areas near the border with Congo. They make simple houses with grass and live there for a while, then move, and repeat this cycle. Unlike the Pygmies, the Mbororos build simple houses with roofs. In the past, they led a nomadic life with cattle and sheep. Now most of them own their pastures, and have began to build houses and settle like the Bantu [Report, Japanese Embassy, Bangui, 1996].

Map of the Central African Republic

Central African Republic

CHAD

Abéché
Adré
Al Ju
Ati
Mongo
Melfi
Am Timan
Aoukalé
Bacha

Kélo
Koumra
Doba
Sarh
Bahr Salamat
Bahr Aouk
Bahr Kameur
Bahr Oulou
VAKAG

Moundou
Ouham
Logone

CAMEROON
Goré
Vina

Ndélé
BAMINGUI-
BANGORAN

HAUTE

Bocaranga
Batangafo
OUHAM
GRIBINGUI
Kaga
Bandoro

Bria
Kotto

OUHAM-
PENDE
Bouca

Ouham
Bossangoa

Bouar
KEMO-
GRIBINGUI
OUAKA
Bambari
Bakou
M

NANA-
MAMBÉRE
Bozoum
Sibut
Grimari

Mambéré

OMBELLA-
MPOKO
Damara
Ouaka
Alindao
BASSE-
KOTTO
Bangassou

Camot
HAUTE-SANGHA
Boda
Bangui
Bimbo
Mobaye
Kembé

Berberati
LOBAYE
Zongo
Mobayi-
Mbongo

Mbaïki
Ubangi
Mongafa

Nola
SANGHA
Yokaduma

Oubangui
Libangui
Gemena

CAMEROON

Ngoko
Lisala
Bur

Ouesso
Impfondo
Congo
Sangha

Figure 2.2 & Figure 2.3. People of Bantu origin

The principal features of the country are described in table 2.1 [Report Japanese Embassy, Bangui, 1996]. The CAR is one of the poorest countries in the world, but the self sufficiency rate in food is high. The literacy rate is 27%, the number of languages listed for CAR is 69. The official language is French but the most widely spoken language is the Bantu language called *Sango*. The main historical events are summarize in table 2.2.

2.2.2. HIV epidemic in Central African Republic

The CAR has experienced a significant epidemic with the human immunodeficiency virus (HIV), with the greatest burden of infection recorded in Bangui [Gresenguet et al, 1991; Massanga et al, 1996]. The first HIV seroprevalence surveys using randomized samples of Bangui's population were performed by the Pasteur Institute of Bangui between 1984-1988. These surveys clearly showed that the number of HIV cases increased rapidly each year in the population aged 15-45 years (it rose from 2.6% in 1984 to 4.6% in 1986 and 7.8% in 1987) [Prof. Alain Georges, personal communication]. Given this dramatic situation, the National AIDS Control Program was implemented in 1989, to look for efficient strategies in curbing the spread of AIDS. Sentinel surveillance based on anonymous testing started in Bangui and in other major cities of the country from 1990. Seroprevalence rates of HIV in pregnant women were 8.6% in Bangui, 7.1% in Bossangoa, and 7.3% in Bambari in 1990. During 1991-1993 an increase in HIV prevalence was recorded in Bangui as well as in the whole country, with seroprevalence rates ranging from 10-15% in pregnant women and blood donors to 35-45% in patients with STDs. In addition, researchers from Pasteur Institute of Bangui reported a seroprevalence rate of 55.7% among patients with tuberculosis in 1994 [Garin et al, 1995]. Recent data show that the growth of the HIV

epidemic is no longer exponential but, however, the epidemic continues to increase. In 1994, the seroprevalence rates of HIV infection in Bangui were 15.3% among pregnant women, 18% among blood donors 19.5% among factory workers and 31.6% among STD patients [Dr. Ph. Courtois, National HIV/AIDS Control Program, personal communication]. The main strategies of the National AIDS Control Program have largely focussed on behavioural interventions such as: increased use of condom use and education programmes on safer sex measures, particularly targeting the youth.

2.2.3. Sexually Transmitted Diseases in CAR

Sexually transmitted diseases are frequent in CAR. According to recent surveys in general population, the prevalence of the major STDs are: *N. gonorrhea* 3%, *Trichomonas vaginalis* 10%, *T. pallidum* 7%, and that of *C. trachomatis* 3% (STD/AIDS survey, Ministry of Health, CAR, 1996).

2.2.4. Health services in CAR

The main health services in CAR are in Bangui. There are primary health care centers and antenatal clinics (such as the clinics of Petevo, Castor and Mamadou). For all health services, patients pay a flat fee for a consultation. Patients attending health clinics have access to the Centre National Hospitalier et Universitaire (CNHU), The Pasteur Institute and the Centre National de Reference des MST et du SIDA (CNRMST/SIDA) where laboratory facilities are available. But the CNRMST/SIDA is the only centre in CAR capable of offering voluntary HIV testing, and capable of receiving, councilling and treating cases related to HIV infection or other STDs.

2.2.5. The CNRMST/SIDA of Bangui

The Centre National de Référence des MST et du SIDA of Bangui, Central African Republic has grown to become one of the major medical research centres in CAR and in Central Africa as a whole. The Centre was created in 1992, with funding from the European Community. Since then, it has been functioning with support from the CAR government and research grants from the European Community, Medecin du Monde, Pasteur Institute, and Georges Pompidou University Hospital in Paris.

The Centre comprises a clinical ward with a waiting and education room, computerised registration office, two consulting rooms (one for men, the other for women). The centre is also equiped with laboratory facilities comprising, a room for serology and a room for bacteriology, a store, offices for the doctors and for the accountant. Two doctors, several support staff and medical students (sent for elective attachment periods) work in the Center. Forty percent of the patients consult the Center for family planning advice or general gynaecology problems, 40% for sexually transmitted diseases, and 20% for HIV testing. Patients pay a general fee of 60 FF (£6) for all clinical services, laboratory investigations and treatment. These patients and their specimens are anonymised but linked by means of individual sticker numbers. Appropriate treatment and counselling are provided for any conditions diagnosed on the day of the visit and a follow-up appointment card is provided to collect the results of further laboratory investigations and receive further treatment if necessary. Patients who wish to know about their HIV test result are bled for a second named/numbered blood test and are referred to the counselling services within the building. The Center has long-standing links with major research centers in CAR (Pasteur Institute),

in France (Georges Pompidou University Hospital, Paris) and in the United States (Washington University, Seattle).

Table 2.1. Data about Central African Republic (CAR)*

Area	622,980 km^2
Population	3,150,000
Density of the population	5/km^2
Birthrate	44°/oo
Births per woman	5.6
Mortality	18°/oo
Population under 15 years	42%
Population above 65 years	3%
Life expectancy	49 years**
Infant mortality	14%
Consumption of calories	1,850 cal/P
Defense expenditure	8 US$/P
GNP per capita population	340 US$**
National expenditure	61 US$/P
Consumption of energy	50 kwh/P
Population engaged in Agriculture	62%
Production of potatoes and cereals	0.32 ton/P
Production of meat	0.02 ton/P

* Japanese Embassy, Bangui, CAR, 1996
** World Health report 1998

Table 2.2. The History of Central African Republic (CAR)*

Years	Events
~1750	Arabs and Europeans start the slave trade
1875	Egyptian rule
1885	Become French colony known as "Oubangui-Chari" in 1906
1911	German occupied part of the country
1919	Got back to French territory
1945	Boganda founded the Black African Social Labor Party
1957	Autonomous government is established
1960	Independence
1965	Revolution by Colonel Bokassa
1977	Declared as Central African Empire by Emperor Bokassa
1979	Back to a Republic
1981	General Kolingba, military regime by a bloodless coup d'etat
1985	Civil regime elected
1986	New Constitution agreed
1991	Multi party system is legalized
1994	Government of A. Patasse is established by a presidential election

* Japanese Embassy, Bangui, CAR, 1996

Chapter 3. Patients & Methods

3.1. Study population

Three hundred consecutive and consenting women attending the Centre National de Référence des Maladies Sexuellement Transmissibles et du SIDA, in Bangui, the capital city of the Central African Republic, were enrolled in the study after informed consent (*see patient information sheet and consent sheet in appendix*). The centre offers multipurpose reproductive health services including provision of STD services, as well as operating the main voluntary HIV testing and counselling centre in Bangui. Reasons for attendance, socio-demographic and behavioural data, obstetric and STD histories were elicited in a structured interview (*see questionnaire in appendix*). Women underwent general, genital and pelvic examination, during which serum, vaginal and cervical samples were collected. Clinical immuno-suppression was assessed using the WHO Bangui clinical case definition for AIDS in Africa [Mbopi Keou et al, 1992, Belec et al, 1994]. A seven-day follow-up appointment was arranged for all women and appropriate treatment was provided free of charge for any treatable STD syndrome or genital pathogen diagnosed. Patients who were willing to know about their HIV test result were bled for a second named/numbered blood test and were referred to the counselling services within the building. The study was approved by the Ethical Committees of the Central African Republic and the London School of Hygiene & Tropical Medicine, London, UK.

3.2. Sample size

Our initial sample size was calculated on the basis of an HIV prevalence rate of 25% in our study population. In order to detect a statistically significant difference of 10% in the prevalence of HSV-2 shedding among HIV positive compared to HIV negative, a sample

size of 288 would be necessary (80% power, 95% significance). We therefore decided to enroll 300 women. All these calculations were performed using Epi Info 6, Statcalc, 1993.

3.3. Samples taken and laboratory methods

3.3.1. Serology

A venous blood specimen was collected from each patient for serological testing and biochemistry. Serological diagnosis of syphilis was made using the Rapid Plasma Reagin (RPR) test (VD-25, Murex Diagnostics, Dartford, UK); any positive result was confirmed using the *Treponema pallidum* haemagglutination assay (TPHA, Fujirebio, Tokyo, Japan). HIV serology was performed following the WHO testing strategy, using two different enzyme-linked immunosorbent assays (ELISA, Genelavia-Mixt HIV-1/HIV-2, Sanofi-Diagnostics Pasteur, Marnes-la-Coquette, France and Wellcozyme HIV, Murex Diagnostics Ltd, Dartford, UK); discordant results were analysed by Western blot (New-LAV Blot I, Sanofi-Diagnostics Pasteur). Reactive samples by both ELISAs or those confirmed by Western blot were considered HIV-1 seropositive.

3.3.2. HSV-1 and HSV-2 type specific serology

Serum antibodies to HSV-1 and HSV-2 were measured using a validated competitive type-specific ELISA with a high sensitivity (93%) and specificity (91%) in comparison with Western blot (the gold standard test for HSV-2 antibodies) on African and UK sera [Gopal et al, 2000]. Briefly, plates (96 well maxisorp immunoassay plates) coated with antigen diluted in PBS at a specific reactivity (μg/ml) previously determined [antigen provided by Wendy Labbett, Enteric & Respiratory Virus Laboratory (Dr. D. Brown), CPHL, London] were stored overnight at 4°C (100μl per well of HSV-2 infected cell lysates). The coated

plates were then washed with PBS. Next, the plates were incubated for 2 hours at 37°C with

300μl of 5% non fat milk in PBS to inhibit non specific binding of the test serum and then

washed with PBS. One hundred microlitres test serum diluted 1:2 in diluent (PBS 0.05%

Tween 20) was added per well and incubated for 3 hours at 37°C. After washing with PBS

0.05% Tween 20, 100μl HSV-2 monoclonal antibodies (Mab) AP1 (Marsden et al, 1984)

was added and incubated for 1 hour at 37°C. Wells were washed with PBS 0.05% Tween

20 and incubated with 100μl of 1:10000 goat anti-mouse horseradish peroxidase conjugate,

for 1 hour. Finally after washing with PBS 0.05% Tween 20 and PBS, 100μl of tetra methyl

benzadene (TMB) was placed in each well and the plates were held in the dark at room

temperature for 30 minutes. The colour reaction was stopped as appropriate by addition of

50 μl 2M sulfuric acid (H_2SO_4). The wells' absorption was read at 450 nm and the results

used to calculate the percentage blocking of Mab AP1 binding for each test serum. Results

were quantified by calculating percentage inhibition of Mab binding using controls of strong

positive serum and negative diluent as previously described [Slomka et al, 1995]. Validation

criteria were as followed. (i) The absorbance value of any single control positive or negative

sera must not deviate by more than ± 20% from the mean of its corresponding four controls.

(ii) The mean absorbance of the negative controls must be greater than 0.7. (iii) The mean of

the negative absorbance values divided by the mean of the positive absorbance values must

be greater than 10.

3.3.3. Vitamin A and vitamin E

The serum concentrations of vitamin A and vitamin E measurements were performed by

Jean-Louis Paul and colleagues at Broussais University Hospital Paris, using high

performance liquid chromatography with UV reading at 325nm as previously described

[Bieri et al, 1979]. Briefly, plasma was deproteinized in the presence of ethanol-butylated hydroxy toluene (BHT) containing retinyl acetate as internal standard. Vitamin A was extracted with hexane and evaporated to dryness under a stream of nitrogen. The residues were redisolved in methanol-high pressure liquid chromatography (HPLC) containing $2\mu mol/l$ BHT and injected into the chromatographic column. The HPLC system consisted of a beckman model 126 pump, a model 406 interface, a Dell L computer and a diode array detection module 168. The column used was a C18 reversed-phase (Merck, Darmstadt, Germany). Mobile phase consisted of acetonitril/dichloromethane/methanol (75: 15: 10, by volume) with a flow rate of 1.3 ml/min and the detection was accomplished by measurement of absorbance at 325 nm. The micronutrient levels were compared to international reference values [Lentner, 1984], with vitamin A deficiency defined as levels below $30\mu g/dL$ and vitamin E deficiency defined as levels below 500 $\mu g/dL$. Vitamins A and E are micronutrients with anti-oxidant/anti-inflammatory properties, and deficiency of these vitamins has been associated with HIV-1 disease progression [Tang et al, 1997].

3.3.4. Vaginal and cervical infections

Standard tests were carried out for: bacterial vaginosis (BV) applying Nugent's scoring method of a Gram stained vaginal smear; *Trichomonas vaginalis* (TV) by direct microscopy of a wet mount of vaginal secretions; *Candida albicans* (CA) by culture of a vaginal swab on Sabouraud medium; *Neisseria gonorrhoeae* (NG) by cervical culture onto a modified Thayer-Martin agar plate; *Chlamydia trachomatis* (CT) by an enzyme immunoassay of an endocervical swab using the Syva Microtrak assay (Syva Co., Palo Alto, California, USA).

3.3.5. Cervico-vaginal lavages

Cervico-vaginal lavage (CVL) were collected by a standardised non-traumatic 60-second-vaginal washing with 3mL of phosphate-buffered saline (PBS). The cellular and cell-free fractions were separated by centrifugation and kept frozen at -30°C until processing. To avoid the possible misclassification of shedding results that could result from the presence of seminal plasma in the vagina, CVL samples were further evaluated for semen traces. Prostate specific antigen (PSA) was detected in the supernatant of CVL by immuno-enzymatic tests (PSA IMX® System, Abbott Laboratories, Abbott Park, Chicago, Illinois, USA). PSA-positive CVL were excluded from further analyses.

3.3.6. HIV-1 RNA in plasma and in cervicovaginal secretions

Evaluation of HIV-1 RNA levels in plasma and in the acellular fraction of CVL were measured using a sensitive commercial assay (Amplicor HIV Monitor® Test 1.5, Roche Diagnostics Systems, Inc., Branchburg, N.J., USA), with a detection threshold of 20 copies/mL and a upper limit of quantitation of 750,000 HIV RNA copies/mL. For this assay, the virus concentration was determined by centrifuging 0.5 mL of plasma or CVL at 23,600 x g for 60 min at 4°C. The pelleted virus particles were lysed by treatment with 600μL of lysis buffer, and the released RNA was precipitated with 600μL of 100% isopropanol. The precipitated RNA was recovered by centrifugation, washed with 1 mL of 70% ethanol, and resuspended in 100μL of Amplicor HIV-1 Monitor specimen diluent. Then, 50μL of the processed specimen was added to 50μL of the working Amplicor HIV-1 Monitor master mix for the RT-PCR amplification reactions. Further amplication, hybridization, and detection steps were performed according to the manufactuter's instructions. This assay has a sensitivity of 98.5% and a specificity of 100% for the detection of HIV-1 RNA subtype A [Nkengasong et al, 1998], which is the predominant clade in Central Africa.

3.3.7. Cell associated provirus DNA quantification in cervicovaginal samples

Cell-associated HIV-1 proviral DNA was quantified in peripheral blood mononuclear cells (PBMC) and paired cellular pellet of CVL. Briefly, cells were treated in lysis buffer containing RNAse A, DNAse-free reagents (Blood and Cell Culture DNA Mini Kit, Qiagen, Courtaboeuf, France) for 20 mn at 37°C, and total DNA was then extracted by fixation-elution using silicated column. DNA concentration was then measured using the Hoechst Dye 332.258 protocole (Dynaquant, Biorad, Marne-La-Coquette, France), as recommended by the manufacturer's instuctions. Quality of DNA and absence of substantial PCR inhibitors were assessed by positivity of ß-globin PCR detection on all extracted samples. 2.5 µg of extracted DNA were further diluted in 80 µL of specimen diluent. Then, 50 µL of DNA preparation were added to 30 copies of DNA internal standard (pSDL, provided by Roche Diagnostic Research and Development, Meylan, France), and amplified using the Amplicor HIV Monitor® Test 1.5, (Roche Diagnostics), as described [Burgard et al, 2000]. Sensitivity of this assay is estimated to 10 copies per amplification reaction [Christopherson et al, 2000], corresponding under our experimental conditions to a threshold of 4 copies of HIV DNA per µg of cellular DNA. Results were expressed as log of proviral DNA copies/µg of cellular DNA.

3.3.8. HSV in genital secretions

HSV DNA was detected in the acellular fraction of CVL by in-house PCR for a conserved 290-bp segment of the DNA polymerase gene of HSV-1 and HSV-2 [Epsy et al, 1993] (Figure 3.1). Hybridization of PCR products was further carried out by DNA enzyme immunoassay (Gen-Eti-K, Sorin Biomedica, Saluggia, VC, Italy), using a single-stranded

biotinylated HSV probe [biotinylated-5'-GTC CTC ACC GCC GAA CTG AG-3' (3109-3128)]. The quantity of amplicons for HSV was assessed by optical densities (OD) of hybridized products at 450 nm, as previously described [Mantero et al, 1991; Payan et al, 1995]. The combination of PCR and DEIA hybridization allowed semi-quantitative evaluation of HSV DNA target present in CVS [Mantero et al, 1991; Payan et al, 1995]. In this method, there was a linear proportionality between ODs from cut-off point (OD=0.150) to 1.5 and the number of HSV DNA copies, ranging from 1 to 1000 copies, respectively. ODs of 1.5 to 3 followed a non-linear relationship.

Figure 3.1. Map of HSV DNA polymerase gene, and schematic diagram of the products of PCR detection of HSV [Espy et al, 1993].
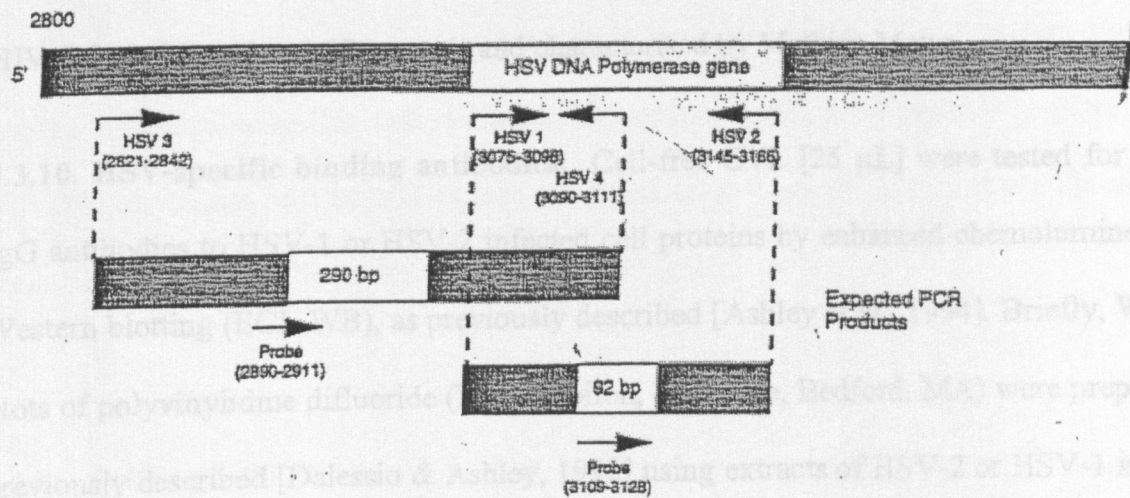
3.3.9. PCR controls

A reaction mixture without DNA was used as control for PCR contamination. DNA extracted from 10^5 MRC5 human fibroblasts constituted the negative control. DNA extracted from a culture on MRC5 fibroblasts of a primary clinical isolate of HSV-2 constituted the positive control. The strain was isolated from a genital herpetic lesion of an HIV-infected inpatient of Broussais and characterised by Mathieu Matta.

3.3.10. HSV-specific binding antibodies.

Cell-free CVL [25 μL] were tested for IgA or IgG antibodies to HSV-1 or HSV-2 infected cell proteins by enhanced chemoluminescence Western blotting (ECL-WB), as previously described [Ashley et al., 1994]. Briefly, Western blots of polyvinylidine difluoride (Immunobilon, Millipore, Bedford, MA) were prepared as previously described [Dalessio & Ashley, 1992] using extracts of HSV-2 or HSV-1 infected human diploid fibroblasts. CVL were diluted 1:40 (25μL) in PBS containing 4% solution of goat serum, then incubated with blots at room temperature overnight on a rocker platform. Blots were washed three times with PBS-Tween and once with PBS and then were incubated for 90 min with peroxidase-conjugated goat antibodies directed against either human alpha chain (1:800 Pierce, Rockford, IL) or human gamma chain (1:4000 Boehringer Mannheim, Indianapolis, IN). After thorough washing with PBS-Tween, blots were incubated for 1min with a commercial western blot detection system based on cheluminescence as directed by the manufacturer (Pierce, IL). Blots were then covered with plastic wrap and exposed to Hyperfilm-ECL (Amersham) for 10s to 5 min. Film was then developed in Kodak X-Omat processor. ECL-WB was used to evaluate major differences between the protein targets of isotype-specific antibodies and between proteins recognized

by CVL specimens and to study differences in responses among patients. The relative intensity of bands corresponding to HSV-2 proteins as detected by bound cervical antibodies provided an "antibody profile". The maximal antibody profile for each specimen and for each antibody isotype was obtained by extending the time that each blot was exposed to film until the maximum number of bands appeared without loss of resolution [Dalessio & Ashley, 1992; Mohamed et al, 1994]. Profiles of CVL HSV-2 blots were compared with profiles of the positive and negative controls sera given the same exposure time. The exposure time selected for analysis gave the most intense and extensive profiles against HSV-2 and minimal background with the negative control serum. In this study, the exposure times used were 10s to 2min for cervical IgA and IgG.

3.3.11. Total IgG and IgA in CVS.

Total IgA and IgG in CVS (not directed solely against HSV) were measured by a direct binding ELISA using goat anti-human IgA (alpha chain) conjugated with peroxidase (Pierce, Rockford, IL) or goat anti-human IgG conjugated with peroxidase and TMB (3,3', 5,5'-tetramethyl-benzidine, Kirkegaard & Perry, Gaithersburg, MD). CVS were diluted serially in twofold increments in sodium carbonate (PH 9.6) from 1:40 for IgA and from 1:200 for IgG and dispensed into the free portions of the respective plates containing human IgA and IgG standards. Purified human secretory IgA or IgG of known concentration (starting at 0.023 mg/ml for IgA and 0.037mg/ml for IgG) were used to generate standard curves. Plates were held at 4°C overnight, washed with PBS/Tween, then blocked with 4% goat serum in PBS for 1 hour at 37°C, then washed again. Bound CVS antibodies were detected with peroxidase-conjugated goat antibodies directed against either human alpha

chain (1:800 Pierce, Rockford, IL) or human gamma chain (1:4000 Boehringer Mannheim, Indianapolis, IN) and TMB substrate (3'.3', 5, 5'-tetramethylbenzidine; Kirkegaard & Perry, Gaithersburg, MD). Absorbance values at 450 nm were plotted for each dilution. Regression lines were calculated from the IgA and IgG standards. Two points were then chosen from the linear part of each specimen's titration curve within the range of the regression lines, and IgA and IgG concentrations were calculated by extrapolation. Levels >0.5 µg/100µl were considered positive. Levels below 0.5 µg/100µl give inconsistent results and those specimens were considered negative for total antibodies.

3.3.12. Neutralizing activity assay.

HSV-2 infectivity inhibition assay was performed as previously described (Ashley, 1997), except duplicate CVS were serially diluted two-fold in 100µl EMEM with 2% foetal bovine serum (FBS) from 1:4 to 1:128 in 96-well plates (Falcon, Becton Dickinson Labware; Lincoln Park, NJ). In brief, infected tissue culture fluid containing HSV-2 strain 333 was adjusted to $\approx 3.2 \times 10^3$ $TCID_{50}$ /ml and 100µl was added to each well. In addition, virus stock diluted 2-fold from $\approx 3.2 \times 10^3$ $TCID_{50}$ /ml to 1×10^2 $TCID_{50}$ /ml was added, in duplicate, to wells containing 100µl EMEM/2% FBS to generate a virus dilution curve. After 1 hour incubation at 37°C, the virus/CVS sample mixtures and the diluted virus were transferred to wells containing confluent monolayers of ELVIS™ cells (kindly provided by Dr. Paul Olivo, Washington University School of Medicine, and Diagnostics Hybrids, Inc., Athens, OH) [Stabell, 1992]. Plates were incubated overnight at 37° C with 5% CO_2. Cells were washed with phosphate buffered saline pH 7.2, then lysed with 100µl of a buffered 1% NP40 solution. Induced β-galactosidase activity secondary to HSV infection was detected

by adding 135µl of 3mM ONPG (o-nitrophenyl β-D-galactopyranoside, Sigma, St. Louis, MO) in 0.1M sodium Phosphate, pH 7.5 1mM $MgCl_2$, and 45mM β-mercaptoethanol. Absorbance was read as OD at 415 nM on a Biotek EL340 plate reader (Winooski, VT). OD values were further processed with Biotek KC4 software. To quantify the amount of HSV-2 infectivity inhibition by the diluted secretions, OD values were first adjusted for background by subtracting the mean OD from six uninfected cell wells. Then units of virus infectivity were interpolated from the virus curve and expressed as neutralizing activity units (NAU).

3.3.13. Specific activity.

The HSV-specific neutralizing activity was calculated from the ratio of HSV-2-NAU to the CVL concentrations of total IgA plus total IgG in 100µL of cell-free CVL. For that calculation, we made the assumption that the CVS concentration of total IgM is much lower than those of IgA plus IgG, as previously observed in HIV-negative as well HIV-infected African women [Belec et al., 1995].

3.3.14. Quantitation of HSV DNA in genital secretions by real time PCR

Quantitation of HSV DNA was performed using a real time PCR based on the LightCycler™ as previously described [Kessler et al, 2000]. Briefly, in a first step, a titrated plasmid (pS4) (kindly provided by K. W. Knopf, German Cancer Research Center, Heidelberg, Germany, containing a single copy of a SalI restriction fragment of the HSV polymerase gene from the HSV-1 strain Angelotti) served as a standard for the determination of the detection limit. Tenfold dilutions of the plasmid were prepared and tested with each of the following assays: SYBR®Green I and with TaqMan™ Probe. In a

second step, the first European Union Concerted Action HSV Proficiency Panel, which

contained different concentrations of HSV type 1 Samples were tested with TaqMan™

Probe. In a third step, clinical specimens were investigated. Oligonucleotides used were

deduced from the published sequence of the DNA polymerase gene-coding region from

HSV [Larder et al, 1987; Tsurumi et al, 1987]. This set of primers, which was chosen within

a highly conserved region of the DNA polymerase gene from the herpesvirus family, allows

amplification of a 92-bp fragment of the HSV-1 and HSV-2 DNA polymerase genes in

clinical samples [Kessler et al, 2000]. The primer and probe sequences and assays

characteristics have been previously published [Kessler, 2000]. The detection threshold for

real time PCR quantitation of HSV-2 shedding was 20 copies/mL. Individuals whose

samples contained quantity below this were deemed 'non-shedders'.

3.3.15. Statistical analysis

Data were double-entered and validated using the Epi-Info 6.0 statistical package (Centers

for Disease Control and Prevention, Atlanta, Georgia, USA, 1996). Analyses were carried

out using STATA 6 (Stata Corp., 1999). Viral loads below the detection threshold of 20

copies/mL for both HIV-1 RNA, HSV-2 PCR EIA and HIV-1 DNA were included in

analyses, and assigned an arbitrary value of 10 copies/mL. The detection threshold for real

time PCR quantitation of HSV-2 shedding was 20 copies/mL. Samples from individuals

with quantity below this were deemed 'non-shedders'. Geometric means of viral loads were

compared using a t-test on the log values, and correlations between viral loads were

assessed using Spearman's rank correlation coefficient. Comparisons of two proportions

were carried out using the chi-squared statistic or Fisher's exact test where appropriate.

Analyses of HIV-1 and HSV-2 shedding were conducted in the subgroup of women who did

not show semen traces in their CVL, so as to attribute the findings entirely to shedding of the viruses under study.

The non-parametric Mann-Whitney U test was used to compare the specific activity of neutralizing antibodies between HSV-2 shedders versus non-shedders. Correlation between the distribution of the specific activities of cervicovaginal HSV-specific neutralizing antibodies and CVL levels of HSV-2 DNA was assessed using the Spearman's rank order test.

Chapter 4. Results

4.1. Interactions between *Herpes simplex* virus type-2 and HIV-1 infection in African women: opportunities for intervention

4.1.1. Population characteristics

A total of 300 women were eligible for enrolment and none refused. The mean age of the study population was 27 years (range 15-48 years). Median age of first sexual intercourse was 16 years, with a median of two reported lifetime partners (range 1-8). The majority of women were married, either in monogamous (47%) or polygamous (13%) marriages. Most women (62%) had reached secondary education level, 40% were employed and 39% were housewives (Table 4.1).

4.1.2. Information on sexual and reproductive health

None of the participants were taking any antiretroviral drugs. Women attended the clinic for various reasons, including specific STD services (12%), antenatal care (15%) and family planning services (15%). However, the main category included women attending for "general gynaecological problems" (58%) (Table 4.1). Overall, 262 women (87%) reported a current vaginal discharge, and 37 (12%) reported current genital ulcers, although only 57 (19%) and 10 (3%) were found to have a genital discharge or genital ulcer on clinical examination, respectively.

Fifty one women were currently pregnant. The median age of first sexual intercourse for all participating women was 16 years (Table 4.2). All were sexually active. Over the last twelve months, 91% of the women reported having one sexual partner and over the last three months, 97% of the women reported having only one sexual partner (Table 4.2). Only

32% of the women reported at the time of the interview using a form of contraceptive and 54% had ever used condoms (Table 4.2).

4.1.3. Clinical findings and STD prevalence

Eighty nine percent of the women complained of problems related to the lower abdominal region and the private parts respectively. The vast majority of the women complained of vaginal itching (84%) or of vaginal discharge (71%). While 12% complained of genital ulceration, other genital problems were recognised by 7% and blisters on genitalia by 6%. It is noteworthy that only 44% of the women reported having used condoms during symptoms of genital tract infection (Table 4.3). Widespread herpes simplex infection accounted for 48% (Table 4.4).

The prevalence of the various STD pathogens and STD syndromes is shown in Table 4.5. Nearly 80% of women had an endogenous vaginal infection (*Candida albicans* and/or bacterial vaginosis), but only 4 women (1%) had *Trichomonas vaginalis*, and 12 (4%) had a cervical infection (*Neisseria gonorrhoeae* and/or *Chlamydia trachomatis*). Overall, 79 women were HIV-1 infected (26%), 24 (8%) had evidence of active syphilis, 297 (99%) had antibodies for HSV-1 and 247 (82%) had antibodies to HSV-2. Prevalences of these pathogens and syndromes changed little with regard to PSA status of the CVL (Table 4.5). Sixty-one women (20%) had traces of semen detected in their CVL and these subjects were excluded from further analyses involving results obtained on CVL samples.

HIV-1 seropositive women were significantly more likely to be HSV-2 seropositive than HIV-1 seronegative women (91% versus 78%, p=0.02) (Table 4.6). Of the 10 women with a

clinical genital ulcer disease, all were HSV-2 seropositive and 4 were co-infected with HIV. There were no significant differences between HIV-1 seropositive and seronegative women in terms of other STDs, BV, contraceptive use or pregnancy status.

The mean serum concentrations of vitamin A and vitamin E were 52μg/dL and 995μg/dL, respectively and few women were considered deficient in either micronutrient, 4% for vitamin A and 1% for vitamin E, respectively. There was a statistically significant difference in the levels of vitamin A between HIV-1-positive and HIV-negative individuals (p<0.001), but there was no significant difference in vitamin E levels (p=0.42) (Table 4.6).

4.1.4. HIV-1 viral loads in plasma and genital secretions

Among the 58 HIV-1 seropositive women without semen traces in their CVL, the geometric mean HIV-1 RNA plasma load was 3.24 (95% CI, 2.8-3.6) 10^3 copies/mL and the geometric mean of HIV-1 RNA genital load was 2.39 (95% CI, 2.1-2.7) 10^3 copies/mL. A positive association was observed between HIV-1 RNA levels in the plasma and genital tract, which was of borderline statistical significance (Spearman's rank correlation coefficient r=0.24; p=0.07; Figure 4.1). HIV-1 RNA was detected in the CVL of 31/41 (76%) women with detectable HIV-1 RNA in plasma, compared with 9/17 (53%) women without detectable plasma HIV-1 RNA (Fisher's exact test, p=0.12). There were no significant differences between the women with detectable HIV-1 RNA in their CVL and the others in terms of STD and BV prevalences, vitamin A levels, contraception use or pregnancy status (data not shown).

4.1.5. HSV-2 genital shedding

HSV-2 DNA was detected in the CVL. Among the 194 HSV-2 seropositive women without semen traces in their CVL, those also infected with HIV-1 were almost twice as likely to be shedding HSV-2 as HIV-1 negative women (23/53 [43%] vs. 31/141 [22%]; p=0.003) (Table 4.6). Among the 54 women shedding HSV-2, the estimated quantity of HSV-2 DNA in the CVL, as assessed by ODs of hybridized amplicons, was significantly higher among HIV-1 positive women (geometric mean of OD 1.85, 95% CI 1.46-2.36) compared with HIV-1 negative women (geometric mean of OD 1.16, 95% CI 0.89-1.52) (p=0.01; Table 4.6).

4.1.6. Association of genital shedding of HIV-1 and HSV-2

HSV-2 and HIV-1 genital viral loads for the 53 dually seropositive women are shown in Figure 4.2. Overall there was no significant correlation between the levels of HIV-1 RNA and HSV-2 DNA in CVL (Spearman's r=-0.18; p=0.21). HIV-1 RNA was detected in CVL from 24/30 (80%) women without detectable HSV-2 DNA in CVL, compared with 13/23 (57%) women with detectable HSV-2 DNA. This difference was of borderline statistical significance (P=0.07). There was some evidence that the 30 women not shedding HSV-2 DNA were at higher risk of HIV shedding due to presence of other STDs. Although they were less likely to have a genital ulcer (0/30), compared with those shedding HSV-2 DNA (3/23; P=0.08) they were more likely to have a cervical infection with *Neisseria gonorrhoeae* and/or *Chlamydia trachomatis* (3/30 vs. 0/23; P=0.25) or to have bacterial vaginosis (19/30 (63%) vs. 11/23 (48%); P=0.26), but none of these differences reached statistical significance. Levels of vitamin A were similar in both groups (geometric

mean=0.43 µg/dL among women without HSV-2 DNA vs. 0.45 µg/dL among women with

HSV2-DNA; P=0.80).

Despite the overall lack of association between genital shedding of HIV-1 and HSV-2, a

significant correlation was observed between genital HIV-1 RNA and genital HSV-2 DNA

among the subset of 23 HIV-1 seropositive women with HSV-2 shedding (Spearman's

r=0.47; P=0.02; Figure 4.2).

4.1.7. Discussion

The high HSV-2 seroprevalence found in this study is consistent with prevalences recorded

in general populations of Africa [Wagner et al, 1994; Gwanzura et al, 1998; Obasi et al,

1999]. Recent studies of patients attending STD services with GUD have recorded high

rates of HSV-2 isolation (20-30%) and an association with HIV-1 infection in African

settings [Htun et al, 1998; Malonza et al, 1999]. In a community-based trial of mass STD

treatment in the Rakai district, Uganda, 43% of subjects with genital ulcers were identified

with genital herpes by PCR [Wawer et al, 1999]. In contrast, there have been very few

studies of genital shedding of HSV-2 among non-GUD patients, particularly among women

outside industrialised countries. Our study is the first in sub-Saharan Africa to show both an

increased prevalence and quantity of genital HSV-2 shedding among HIV-1 infected women

and to show a correlation between the quantities of HIV-1 RNA and HSV-2 DNA in genital

secretions among HSV-2 shedders.

Two possibly coexisting mechanisms could explain such findings. Firstly, an increased

shedding of both viruses could be observed because of immunosuppression associated with

HIV-1 disease, or under the influence of other systemic or local cofactors of HIV-1 genital shedding. Secondly, HSV-2 may be an independent cofactor of HIV-1 shedding, and activation of HSV-2 shedding may be accompanied by increased HIV-1 shedding.

In support of the first mechanism, whereby HIV-1 enhances HSV-2 shedding, although anecdotal evidence supports the notion that clinical expression of HSV-2 infection is increased in the presence of advanced HIV-1 disease, there have been surprisingly few studies to examine the impact of HIV on the natural history of HSV infection [Corey & Wald, 1999]. One previous study demonstrated increased shedding of HSV-2 among HIV-1 seropositive women in New York City associated with declining CD4 T-cell counts [Augenbraun et al, 1995]. However, in a study of HIV-1 seropositive female sex workers in Mombasa, Kenya, only women with very low CD4 T-cell counts ($<200/\mu L$) showed increased HSV-2 DNA positivity in cervical swabs [Mostad et al, 2000]. We do not have data on CD4 T-cell counts in our study population, but the women attending this outpatient clinic did not show evidence of clinical immunosuppression as assessed by the WHO Bangui clinical case definition for AIDS in Africa [Belec et al, 1994]. Furthermore, micronutrient levels in this population were essentially within normal ranges according to reference values, which suggests that most of these women were not at an advanced stage of HIV infection [Tang et al, 1997].

In support of the second mechanism, whereby HSV enhances HIV-1 transmission, *in vitro* studies have suggested that HSV can activate latent HIV-1 or enhance its replication [Heng et al, 1994]. *In vivo* studies demonstrating a direct correlation between HSV infection and

increased HIV-1 burden have been rare. One small prospective study conducted among 16

patients demonstrated that an acute HSV episode can result in transient 3 to 4-fold increased

levels of plasma HIV-1 RNA [Mole et al, 1997]. In another study of 16 men with proven

herpetic ulcers in Seattle, Schacker et al found large amounts of HIV-1 DNA in the ulcers,

and treatment or healing of the ulcers was accompanied by a decrease in HIV-1 shedding

[Schacker et al, 1998].

An important determinant of HIV-1 shedding may be the HIV-1 plasma load. We found a

positive association, but of borderline significance, between HIV-1 RNA levels in plasma

and genital secretions. Studies conducted in industrialised countries have usually found a

clearer association [Hart et al, 1999]. The difference could be partly explained by different

sampling methods leading to the presence of blood in the genital tract sample in other

studies [Hart et al, 1999], or could be attributed to the relatively small sample size and low

power of our study. Alternatively, our data may support the notion of compartmentalisation

of HIV-1 replication, in keeping with previous studies demonstrating distinct viral HIV-1

variants, and immunological responses to HIV, between peripheral blood and the female

genital tract [Zhu et al, 1996]. Such data on women living in Africa are still scarce. It is

possible that other systemic or local factors, in addition to HIV-1 plasma viral load, may be

very important in determining genital shedding of HIV-1 in Africa, and these may include

HSV-2 and other STDs such as cervical infections or changes in the vaginal flora [Martin et

al, 1999], as was also observed in our study.

The interactions between HSV and HIV infections are not fully understood. Among HIV-

positive individuals, HSV-2 associated GUD may enhance HIV shedding and infectiousness

by disrupting genital mucosal integrity. Among HIV-negative individuals, HSV-2 associated GUD may increase susceptibility by disrupting mucosal integrity, but also by the recruitment and activation of HIV target cells, and possibly by HIV taking advantage of chemokine receptors [Fleming & Wasserheit, 1999]. A number of cohort studies have demonstrated that HSV-2 seroconversion, with or without obvious clinical disease, was a risk factor for HIV-1 seroconversion [Fleming & Wasserheit, 1999]. Studies to explain the mechanism by which the largely asymptomatic genital shedding of HSV-2 in females may act as a cofactor for HIV infection are lacking. Our cross-sectional study cannot demonstrate a causal role of HSV-2 in HIV-1 transmission and further longitudinal and intervention studies will be necessary to elucidate this point. However, in the face of the severe and worsening HIV-1 epidemic in Africa, the high prevalence of HIV-1 and HSV-2, and the high frequency of asymptomatic HSV-2 shedding, a possible synergistic effect between the infections suggested by this study, may be cause for concern. We have learned in the past decade that no single approach will contain the HIV/AIDS epidemic, and we need to consider all available means of control, however imperfect. The Mwanza study in Tanzania demonstrated that improved STD case management was an important additional HIV prevention strategy [Grosskurth et al, 1995], resulting in a 40% reduction in HIV incidence in the general population. No such reduction was achieved by STD mass treatment in the Rakai study in Uganda [Wawer et al, 1999], and one reason postulated for this was a higher prevalence in Rakai of STDs such as genital herpes that were not targeted by the antimicrobial regimen [Hitchcock & Frensen, 1999].

Our data prompt a number of challenges and potential avenues for further research on genital herpes and STD/HIV control in Africa. In order to demonstrate the enhancing effect of HSV-2 on HIV transmission or acquisition, it will be necessary to conduct randomised

intervention trials that specifically target HSV-2, measuring the outcome in terms of HIV shedding or HIV incidence, as well as HSV-2 shedding. Such interventions could include the addition of anti-herpetic treatment to syndromic management algorithms for STD patients in Africa, which may become feasible now that acyclovir is out of patent; the provision of suppressive therapy against HSV-2 for HSV-2/HIV dually infected individuals, an option not likely to be feasible on a large scale in most developing country settings, but which could be tried in high-risk groups such as commercial sex workers; and the development and testing of preventive interventions, particularly those targeting youth [Mindel, 1998]. However, it may be that the most realistic hope for control is the development of a safe and effective HSV-2 vaccine [Mindel, 1998].

Table 4.1. Demographic characteristics by HIV and HSV2 status among 300 consecutive women attending the National STD/AIDS Reference Centre of Bangui

	HIV +ve	HIV -ve	HSV2 +ve	HSV2 -ve	Total
Total	79	221	247	53	300
Age group					
<20	2 (8%)	23 (92%)	11 (44%)	14 (56%)	25
20-24	30 (29.7%)	71 (70.3%)	83 (82.2%)	18 (17.8%)	101
25-29	27 (34%)	52 (65.8%)	70 (88.6%)	9 (11.4%)	79
>=30	20 (21%)	75 (78.9%)	83 (87.4%)	12 (12.6%)	95
Religion					
Protestant	31 (29.2%)	75 (70.7%)	83 (78.3%)	23 (21.7%)	106
Traditional	35 (25.3%)	103 (74.6%)	120 (87%)	18 (13%)	138
Muslim	4 (14.3%)	24 (85.7%)	22 (78.6%)	6 (21.4%)	28
Catholic	9 (33.3%)	18 (66.6%)	22 (81.5%)	5 (18.5%)	27
Education					
No school	3 (18.7%)	13 (81.2%)	15 (93.7%)	1(6.2%)	16
Primary	29 (29.5%)	69 (70.4%)	85 (86.7%)	13 (13.3%)	98
Secondary	45 (25%)	134 (75%)	142 (79.3%)	37 (20.7%)	179
University	2 (33.3%)	4 (66.7%)	5 (83.3%)	1 (16.7%)	6
Other	0	1 (100%)	0	1 (100%)	1
Occupation					
Student	11 (22%)	39 (78%)	36 (72%)	14 (28%)	50
Unskilled man.	8 (25.8%)	23 (74.2%)	22 (70.9%)	9 (29%)	31
Skilled manual	27 (30.6%)	61 (69.3%)	78 (88.6%)	10 (11.3%)	88
Office/teacher	30 (25.4%)	88 (74.6%)	101 (85.6%)	17 (14.4%)	118
Business	3 (23.1%)	10 (76.9%)	10 (76.9%)	3 (23%)	13
Marital status					
Married mono	37 (26%)	105 (74%)	117 (82%)	25 (18%)	142
Married poly	8 (21%)	30 (79%)	31 (82%)	7 (18%)	38
Sep/Div.	1 (16.6%)	5 (83.3%)	6 (100%)	0	6
Single	33 (29%)	80 (71%)	92 (81%)	21 (19%)	113
Reason for coming					
Antenatal check-up	10 (23%)	34 (77%)	31 (70%)	13 (30%)	44
Family planning	6 (13%)	39 (87%)	32 (71%)	13 (30%)	45
STD	14 (39%)	22 (61%)	32 (89%)	4 (11%)	36
Other gyne problem	49 (28%)	126 (72%)	152 (87%)	23 (13%)	175

Table 4.2. Sexual behaviour among 300 consecutive women attending the National STD/AIDS Reference Centre of Bangui (by HIV and HSV2 status)

	HIV +ve	HIV -ve	HSV2 +ve	HSV2 -ve	Total
Total	79	221	247	53	300
Median age at first intercourse	16	16	16	16	16
Number of lifetime partners					
1	27 (23%)	92 (77%)	91 (76%)	28 (24%)	119
2	43 (28%)	111 (72%)	132 (86%)	22 (14%)	154
3	8 (35%)	15 (65%)	20 (87%)	3 (13%)	23
>=4	1 (25%)	3 (75%)	4 (100%)	0	4
Number of partners in last 12 months					
0	0	2 (100%)	2 (100%)	0	2
1	70 (25%)	204 (75%)	224 (82%)	50 (18%)	274
2	9 (43%)	12 (57%)	19 (90%)	2 (10%)	21
3	0	3 (100%)	2 (67%)	1 (33%)	3
Number of partners in last 3 months					
0	0	3 (100%)	3 (100%)	0	3
1	79(27%)	213 (73%)	239 (82%)	53 (18%)	292
2	0	4 (100%)	4 (100%)	0	4
3	0	1 (100%)	1 (100%)	0	1
Ever used condoms	48 (29%)	115 (71%)	133 (82%)	30 (18%)	163
Used condom in last casual intercourse	11 (21%)	39 (79%)	40 (80%)	10 (20%)	50
Used condom in last regular intercourse	11 (22%)	37 (78%)	37 (78%)	11 (22%)	48
Currently pregnant	9(18%)	42 (82%)	38 (74.%)	13 (26%)	51
Number of children					
0	27 (28%)	70 (72%)	75 (77%)	22 (23%)	97
1	26 (33%)	54 (67%)	71 (89%)	9 (11%)	80
2	15 (33%)	31 (67%)	33 (72%)	13 (28%)	46
3	8 (24%)	26 (76%)	30 (88%)	4 (12%)	34
>=4	3 (7%)	40 (93%)	38 (88%)	5 (12%)	43
Current contraceptive use none	61 (30.50%)	139 (69.50%)	166 (83%)	34 (17%)	200
Oral	10 (20%)	41(80%)	38 (74.5%)	13 (25.5%)	51
Injection	4 (50%)	4 (50%)	7 (87.5%)	1 (12.5%)	8
Traditional	3 (10%)	28 (90%)	28 (90%)	3 (10%)	31
Other	1 (20%)	4 (80%)	3 (60%)	2 (40%)	5
Condom	0	4 (100%)	4 (100%)	0	4

Table 4.3. Prevalence of symptoms of STDs by HIV status among 300 consecutive women attending the National STD/AIDS Reference Centre of Bangui

	HIV positive	HIV negative	Total	p-value
Total	79	221	300	
Lower abdominal pain	74 (27.61%)	194 (72.39%)	268 (89%)	0.14
Private parts	50 (28.09%)	128 (71.91%)	178 (59%)	0.40
Tickling/itching	69 (27.49%)	182 (72.51%)	251 (84%)	0.30
Vaginal discharge	68 (29%)	167 (71%)	235 (78%)	0.052
Foul smell	59 (31%)	133 (69%)	192 (64%)	0.02
Dysuria/pain	47 (30%)	111 (70%)	158 (53%)	0.157
Pain on intercourse	46 (29%)	112 (71%)	158 (53%)	0.24
Genital ulceration	13 (35%)	24 (65%)	37 (12%)	0.19
Blisters on genitalia	6 (33%)	12 (77%)	18 (6%)	0.48
Other problems	6 (26%)	17 (74%)	23 (7%)	0.97
Condom use during symptoms	40 (31%)	91 (69%)	131 (44%)	0.34

Table 4.4. Prevalence of clinical signs by HIV status among 300 consecutive women attending the National STD/AIDS Reference Centre of Bangui

	HIV positive	HIV negative	Total	p-value
Total	79	221	300	
Weight loss >10% of baseline weight	52 (33%)	104 (67%)	156 (52%)	0.004
Diarrohea > 1 month	15 (33%)	31 (67%)	46 (15%)	0.29
Fever > 1 month	40 (33%)	82 (67%)	122 (41%)	0.036
Chronic fatigue	45 (35%)	83 (65%)	128 (43%)	0.003
CGL	13 (30%)	30 (70%)	43 (14%)	0.53
Herpes zoster in last 5 yrs	39 (29%)	95 (71%)	134 (45%)	0.32
Recurrent herpes zoster	43 (30%)	102 (70%)	145 (48%)	0.20
Herpes zoster > 1 month	43 (30%)	102 (70%)	145 (48%)	0.20
Widespread herpes simplex infection	41 (28.47%)	103 (71.53%)	144 (48%)	0.41
Pruritic dermatitis	24 (27%)	64 (73%)	88 (29%)	0.81
Oropharyngeal candidiasis	4 (24%)	13 (76%)	17 (6%)	0.78
Cough > 1 month duration	1 (8%)	12 (92%)	13 (4%)	0.11

Table 4.5. Prevalence of STD pathogens and STD syndromes among 300 consecutive women attending the National STD/AIDS Reference Centre of Bangui, and in 239 women with PSA-negative cervico-vaginal secretions

	ALL WOMEN (N=300) n (%)	WOMEN WITH PSA-NEGATIVE CVS (N=239) n (%)
CERVICO-VAGINAL STD		
N. gonorrhoeae	3 (1%)	2 (1%)
C. trachomatis	9 (3%)	5 (2%)
T. vaginalis	4 (1%)	4 (2%)
C. albicans	128 (43%)	97 (41%)
Bacterial vaginosis	199 (66%)	143 (60%)
Any cervico-vaginal STD	238 (79%)	181 (76%)
STD syndromes		
Genital discharge syndrome*	57 (19%)	42 (18%)
Genital ulcer syndrome**	10 (3%)	8 (3%)
SEROLOGICAL STD		
Syphilis (TPHA+/RPR+)	24 (8%)	18 (8%)
HIV-1	79 (26%)	58 (24%)
HSV-1	297 (99%)	237 (99%)
HSV-2	247 (82%)	194 (81%)

* Symptoms of vaginal discharge, plus vaginal or cervical discharge on clinical examination.
** Genital ulcer or erosion on clinical examination.

Table 4.6. Prevalence of HSV-2 markers and other indicators by HIV-1 serostatus, in 239 women with PSA-negative cervico-vaginal secretions

INDICATORS	N	HIV-1 positive n (%)	N	HIV-1 negative n (%)	p value
HSV–2 markers					
- Serum HSV-2 IgG	58	53 (91%)	181	141 (78%)	**0.02**
- Cervico-vaginal HSV-2 DNA*	53	23 (43%)	141	31 (22%)	**0.003**
- Cervico-vaginal levels of HSV-2 DNA (optical density) **	23	1.85 (1.46-2.36)	31	1.16 (0.89-1.52)	**0.014**
STDs					
- *N gonorrhoeae* and/or *C trachomatis* and/or *T vaginalis*	58	3 (5%)	181	8 (4%)	0.81
- Bacterial vaginosis	58	33 (57%)	181	110 (61%)	0.60
- *C albicans*	58	24 (41%)	181	73 (40%)	0.89
- Genital ulcer	58	3 (5%)	181	5 (3%)	0.41
- Syphilis	58	5 (9%)	181	13 (7%)	0.78
Contraceptive use					
- Oral contraceptive	58	8 (14%)	181	33 (18%)	0.44
- Injectable contraceptive	58	3 (5%)	181	3 (2%)	0.14
Pregnant	58	8 (14%)	181	35 (19%)	0.34
Micronutrients *					
- Serum vitamin A (µg/dL)	49	47 (43-52)	168	57 (54-59)	<0.001
- Serum vitamin E (µg/dL)	49	988 (930-1040)	168	1020 (980-1050)	0.42

* Among HSV-2 seropositive women.

** Among HSV-2 shedders. The geometric mean of optical densities, and 95% confidence intervals in brackets, are shown.

*** Micronutrient data are missing for 9 HIV seropositive and 13 HIV seronegative women. The mean, and 95%confidence interval in brackets, are shown.

Figure 4.1: Distribution of HIV-RNA levels in paired plasma and cervico-vaginal samples in HIV-1 seropositive women with PSA negative cervicovaginal secretions
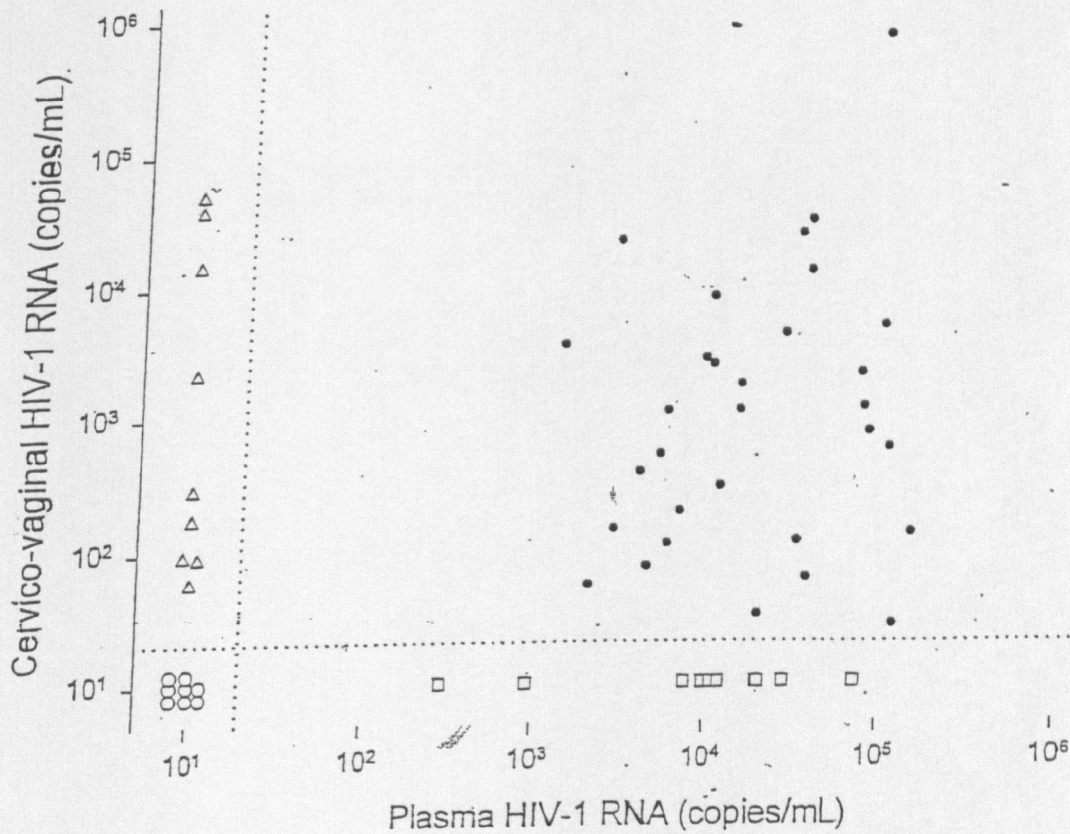
• Detectable in plasma and cervico-vaginal secretions
△ Undetectable in plasma, detectable in cervico-vaginal secretions
□ Detectable in plasma, undetectable in cervico-vaginal secretions
o Undetectable in plasma and cervico-vaginal secretions
······ Detection threshold of 20 copies/mL for HIV-1 RNA

Figure 4.2: Distribution of HIV-RNA and HSV-2 DNA in cervico-vaginal secretions of 53 dually HIV-1 and HSV-2 seropositive women with PSA negative cervicovaginal secretions

- ● HSV-2 shedders, detectable cervico-vaginal HIV-1 RNA
- △ HSV-2 non-shedders, detectable cervico-vaginal HIV-1 RNA
- □ HSV-2 shedders, undetectable cervico-vaginal HIV-1 RNA
- ○ HSV-2 non-shedders, undetectable cervico-vaginal HIV-1 RNA

.. Detection threshold of 20 copies/mL for cervico-vaginal HIV-1 RNA

— — Positivity threshold for HSV-2 DNA detection

* Two individuals at this point
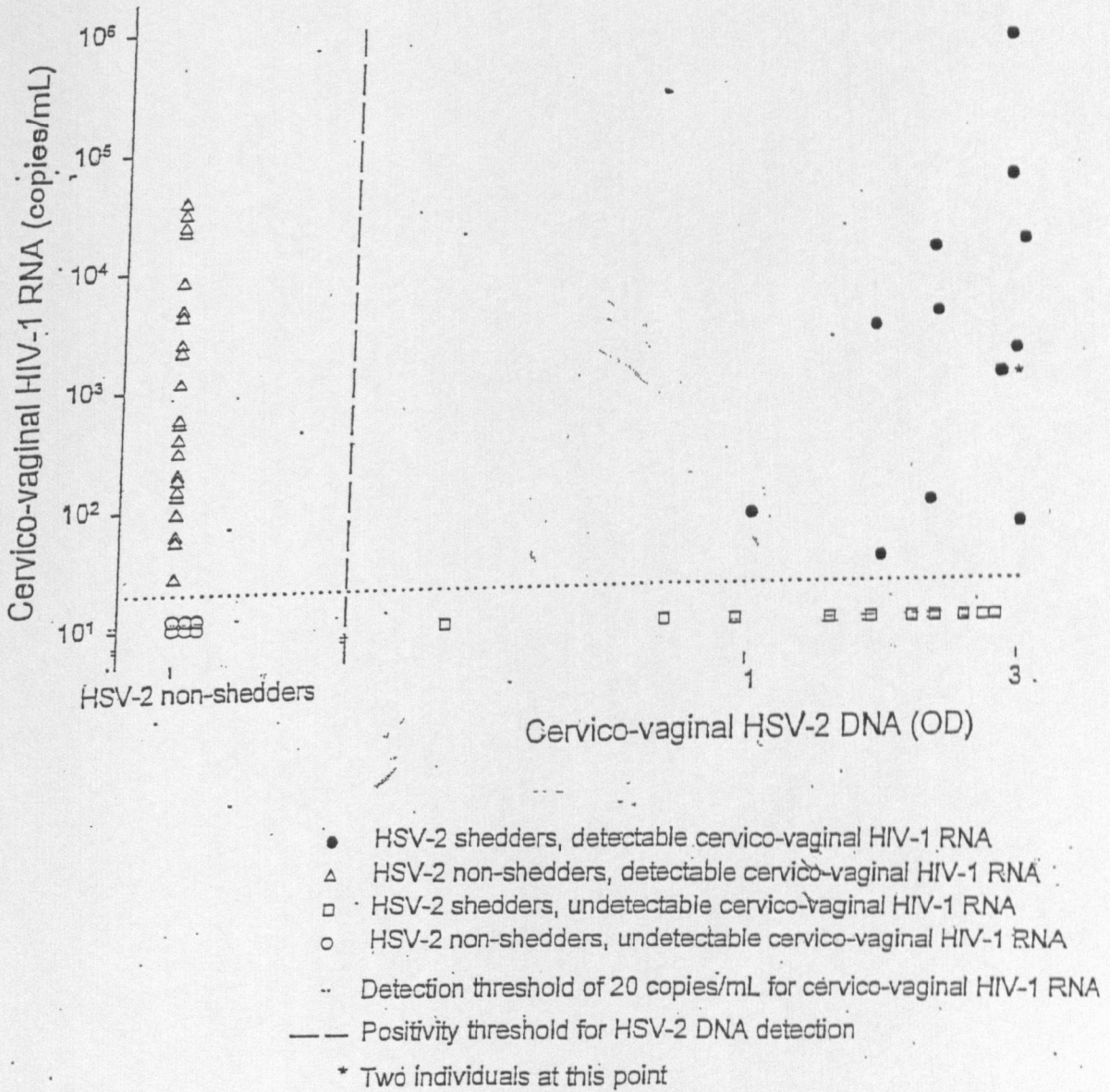
4.2. Vitamin A deficiency and genital tract infection in women living in Central African Republic

The mean serum concentrations of vitamin A was 52 μg/dL and only 15 (5.5%) women were considered deficient. There was a statistically significant difference in the levels of vitamin A between HIV-1-seropositive and HIV-negative individuals (p<0.001), and between syphilis-seropositive and syphilis-seronegative individuals (p<0.01) (Table 4.7). The proportion of HIV-infected patients with serum vitamin A deficiency was higher than that of HIV-negative patients (p<0.005). In patients harboring only one genital tract pathogen, the proportion of patients with vitamin A deficiency was higher in HIV-infected patients than in HIV-negative patients (p<0.01) (Table 4.8). The proportion of patients with serum vitamin A deficiency increased in parallel with the number of diagnosed genital tract infections or seropositivity for active syphilis, both in HIV-negative and in HIV-infected patients (p<0.0001 and p=0.0002, respectively, using the X_2 test for trend).

4.2.1. Discussion

In the present study, vitamin A deficiency was associated in an additive fashion with the number of genital tract infections diagnosed or seropositivity for active syphilis in women attending the main STI clinic of Bangui. These findings suggest that patient vitamin A deficiency favours genital tract infections. This is likely because vitamin A deficiency *per se* may increase the susceptibility to genital tract infections [Semba, 1994a]. In HIV-infected

women, genital tract infections are associated with increased infectiousness of genital secretions [Mostad & Kreiss, 1996], thus providing increased risk to transmit HIV infection in an exposed sexual male partner. In HIV-seronegative women with genital tract infections such as syphilis, *Trichomonas vaginalis*, *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, higher susceptibility to heterosexual acquisition of HIV has been demonstrated [Fleming & Wasserheit, 1999]. In women suffering from *Candida albicans* genital infection, the inflammatory conditions affecting the cervicovaginal mucosa may also lead to increased risk for HIV acquisition or transmission, especially when other cofactors of transmission exist [Cohen et al, 1999; Vernazza et al, 1999]. Finally, the findings in the present report raise the possibility that vitamin A deficiency may increase the risk of female-to-male as well as male-to-female HIV transmission, because it indirectly increases the probability of female genital tract pathogens, which act as major cofactor for HIV transmission. Further prospective studies should focus on vitamin A supplementation in individuals attending STD clinics in Africa, both to assess its potential for curing and prevent genital tract infections, and also its use in reducing HIV sexual transmission.

Table 4.7. Vitamin A serum levels according to infection status in 275 childbearing-aged women consulting the main STI clinic of Bangui, Central African Republic.

Infections	Vitamin A levels (µg/dL)				P
	Negative		Positive		
	N	Mean (SD)	N	Mean (SD)	
HIV	204	2.02 (0.61)	71	1.72 (0.62)	<0.001
Syphilis*	251	1.97 (0.64)	24	1.60 (0.43)	<0.01
Bacterial vaginosis**	110	1.95 (0.61)	165	1.82 (0.49)	NS
Candida albicans	147	1.95 (0.62)	128	1.94 (0.65)	NS
Trichomonas vaginalis	271	1.94 (0.63)	4	2.11 (0.38)	NS
Neisseria gonorrhoeae	271	1.95 (0.63)	4	1.52 (0.66)	NS
Chlamydia trachomatis	266	1.94 (0.64)	9	2.00 (0.49)	NS

* Serological diagnosis of active syphilis was defined by a positive Rapid Plasma Reagin test (VD-25, Murex Diagnostics, Dartford, UK), with positive confirmatory Treponema pallidum haemagglutination assay (TPHA, Fujirebio, Tokyo, Japan).
** Bacterial vaginosis was diagnosed by the Nugent scoring method of a Gram stained vaginal smear.

SD: Standard deviation
NS: Not significant
Statistical analyses were carried out using InStat™ software (GraphPad, Inc., San Diego, CA, USA).

Table 4.8. Vitamin A deficiency and HIV serostatus according to the number of genital tract infections* or seropositivity for syphilis in 275 childbearing-aged women living in Central Africa.

Number of infections	Vitamin A deficiency**				
	N (%)	n (%)	HIV⁺ (%)	HIV⁻ (%)	p
0	129 (47)	1 (0.7)	1/34 (<1)	0/95 (0)	NS
1	128 (46)	6 (6.3)	4/29 (14)	2/99 (2)	0.008
2	13 (5)	4 (15.4)	2/4 (50)	2/9 (21)	NS
3	5 (2)	4 (80.0)	3/4 (75)	1/1 (100)	NS
Total	275	15	10/71 (14)	5/204 (2.5)	0.002

* Genital tract infections were *Candida albicans*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.
** Vitamin A deficiency is defined as levels below 30μg/dL.

NS: Not significant
Statistical analyses were carried out using InStat™ software (GraphPad, Inc., San Diego, CA, USA).

4.3. Genital shedding of HSV-2 DNA and HIV-1 proviral DNA

in HIV- and HSV- coinfected African women

A total of 239 (79.6%) women had no detectable PSA in their CVL. Among those, 63

(26.3%) women were seropositive for both HIV and HSV-2. Plasma or genital loads of

HIV-1 and HSV-2 were measured and analyzed in this latter population. Cell-free HIV

RNA was detectable in the plasma samples of 50 of 63 (80%) patients, and in the vaginal

lavage supernatants of 46 (73%) women. Levels of free HIV-1 RNA load in plasma

(mean±standard error for the mean : 19,816±4,626 copies/mL) were higher than those in

CVL (5,987±2,071 copies/mL); the plasma HIV-1 RNA levels correlated weakly with

genital HIV-1 RNA loads, but the association did not reach statistical significance

(Spearman's r =0.25; P=0.085). In the vaginal lavage samples, no correlation was observed

between cell-free HIV-1 RNA loads and HIV-1 proviral DNA integrated cellular loads

(Spearman's r=0.07; P=0.64). Twenty-seven (47%) HIV-positive women were shedding

HSV, in all cases type 2, as assessed by RLFP analysis. Among the 63 dually seropositive

women, there was no difference between median genital HIV-1 RNA load in HSV-2

shedders (3,152±2,770 copies/mL) and in non-shedders (4269±1557 copies/mL) (P=0.76);

similarly, there was no difference in median genital HIV-1 DNA load between HSV-2

shedders (101±36 copies/mL) and in non-shedders (92±35 copies/mL) (P=0.53). Overall,

there was no significant correlation between the levels of HSV-2 DNA and HIV-1 RNA in

CVL (Spearman's r=0,08; P=0.61) ; similarly, there was no significant correlation between

the levels of HSV-2 DNA and HIV-1 DNA in CVL (Spearman's r=0,15 ; P=0.32). Despite

the overall lack of association between genital shedding of HIV-1 and HSV-2, a significant

correlation was observed between genital HIV-1 RNA and genital HSV-2 DNA among the subset of 23 HIV-1-seropositive women with HSV-2 shedding (Spearman's r=0.56 ; P=0.0025). In contrast, there was no significant association between HIV DNA and HSV-2 DNA (Spearman's r=0.34 ; P=0.09). Only 3 HIV-infected women shedding HSV-2 had genital ulceration, all others being clinically asymptomatic.

In the present study, the levels of HSV-2 DNA in CVL did not correlated to levels HIV-1 proviral DNA.

4.3.1. Discussion

Two possibly coexisting mechanisms could explain the association we observed between HIV-1 RNA levels and HSV-2 shedding. First, increased shedding of both viruses could be associated with the immunosuppression due to HIV-1 disease, or with systemic or local cofactors of HIV-1 genital shedding. Second, HSV-2 may be an independent cofactor of HIV-1 shedding, activation of HSV-2 shedding being accompanied by increased HIV-1 shedding. Thus, asymptomatic HSV-2 genital replication may constitute a cofactor in female-to-male sexual transmission of HIV, by increasing the genital HIV-1 infectivity. HIV transactivation by HSV at the genital level is plausible and is supported by in vitro experimentations demonstrating that HSV proteins may increase HIV replication [Mosca et al, 1987; Albrecht et al, 1989; Maregolis et al, 1992], and that both viruses may form pseudotypes after co-infection of the same cellular target [Griffiths, 1996]. The levels of cervicovaginal HIV-1 proviral DNA, in contrast to HIV-1 RNA, were not associated with those of HSV-2 DNA, even in women shedding HSV-2 DNA in their genital secretions [Mbopi-Keou et al, 2001]. These observations suggest that HSV-2 genital replication may influence the genital production of HIV-1 RNA, rather than that of HIV-1 DNA. The

existence of a cellular reservoir of productively infected cells with rapid turnover within the female genital tract has been previously hypothesized based on the marked and rapid effect of antiretroviral treatment in decreasing the quantity of HIV-1 RNA in CVL [Si Mohamed et al, 2000]. We can hypothesize that HSV-2 genital replication positively influences the genital reservoir of productively infected cells with rapid turnover. The apparent lack of effect of genital HSV-2 replication on the level of cervicovaginal HIV-1 DNA suggests the existence of a cellular genital reservoir of HIV-infected cells with low turnover, similar to that previously recognized in the systemic compartment [Ho et al, 1995; Perelson et al, 1997]. Finally, HIV-1 replication in the female genital tract may be subcompartmentalized in distinct cellular reservoirs each with its turnover, and which may be influenced differently under the effect of genital HSV-2 replication. These findings then provide the basis for differential influence of genital cofactors on the cervicovaginal reservoirs of free and cell-associated HIV-1, and finally for possible different involvement of free and cell-associated HIV-1 in female-to-male sexual transmission of the virus.

4.4. Cervicovaginal neutralizing antibodies to Herpes simplex virus type-2 (HSV-2) in cervicovaginal secretions of African women

4.4.1. Study populations. Of the original 84 women enrolled, 7 had neither HSV-2 antibody nor HSV-2 DNA and were, therefore considered HSV-2 uninfected and not studied further. Finally, 77 women were selected for cervicovaginal HSV-specific antibody analysis

if they had evidence of HSV-2 infection by either HSV-2 type specific serum antibodies or HSV-2 DNA in their CVL, or both HSV-2 antibodies and HSV-2 shedding.

Three groups of women were classified according to their HIV serostatus and their HSV-2 genital shedding. Group I comprised 34 HIV-seronegative women who all had HSV-2 DNA in their genital secretions; 30 of them were HSV-2-seropositive; the 4 remaining women shed HSV-2, but were HSV-2 seronegative by both the competitive ELISA and the highly sensitive ECL-WB. These subjects, most likely, had primary HSV-2 infection.

Group II comprised 20 HIV-1-seropositive women who were shedding HSV-2 in their genital secretions. All were HSV-2 seropositive.

Group III comprised 23 HIV-seronegative women without detectable HSV-2 DNA in their genital secretions; all were HSV-2 seropositive by both ELISA and ECL-WB. All selected women were seropositive for HSV-1.

4.4.2. Cervicovaginal total IgG and IgA.

Total IgA and IgG cervicovaginal levels were measured partly as a means to obtain specific activities of neutralizing activity in CVL, but also as a possible marker of the effects of either HIV-1 infection or HSV-2 shedding on local antibody concentrations. Total IgA and total IgG were less likely to be detected in Group I HIV-seronegative women who were shedding HSV-2 (20 of 34; 59%) than in Group II women who were HIV infected and shedding HSV-2 (17 of 20; 85%), but the difference was only marginally significant (P=0.068; Fisher's exact test) (Table 4.9). Of Group III (HSV-2 non-shedders who were HIV seronegative) 22 of 23 (96%) had IgG in CVL. IgA was detected in 12 of 34 Group I women (35%) and 13 of 20 (65%) Group II women. CVLs from thirteen of 23 (57%) Group

III women contained IgA. IgG was present at higher concentrations than IgA in CVL from women regardless of HIV status or HSV-2 shedding. In the present series, median concentrations of IgG and IgA were not affected by either HIV infection or by HSV-2 shedding.

4.4.3. Detection of cervicovaginal HSV-2-Specific IgG and IgA.

ECL-WB for IgG to HSV-1 and HSV-2 antibody was performed on 75 of the 77 HSV-2 infected subjects; 69 subjects had sufficient sample to perform ECL-WB testing for both IgG and IgA antibody to HSV-2. Fifty-two subjects had HSV-specific IgG in CVL. Seventeen of 34 (50%) Group I (only HSV-2 shedders) subjects had IgG. Seventeen of 19 (89%) of Group II (HIV+ and HSV-2 shedders), subjects had IgG. Eighteen of 22 (82%) of Group III (HSV non shedders) had IgG (Table 4.10). While it might be predicted that HSV-2 shedding would be accompanied by a local antibody response, only 20 of 53 HSV-2 shedders (38%) had HSV-2 IgG in CVL compared with 11 of 22 non-shedders (50%). Thus, presence versus absence of detectable IgG antibodies did not appear to be closely linked to HSV-2 shedding.

Thirty-five of 69 tested subjects (51%) had IgA to HSV. Eleven of 32 (34%) of Group I had IgA; 11 of 17 (64%) of Group II had IgA, and 12 of 21 (57%) of Group III had IgA. As with IgG, HIV-seronegative HSV-2 shedders were less likely to have detectable HSV IgA and a slightly lower proportion of HSV-2 shedders had HSV-2 IgA in their CVL (31%) than non shedders (48%).

It was somewhat surprising that even in the presence of HSV-2 shedding, a substantial number of subjects had local IgG to only HSV-1 (10 in Group I and 4 in Group II). A total of 7 HSV-2 shedders had cervicovaginal IgA to only HSV-1. HSV-1 local antibodies can be present in high concentrations in genital secretions of subjects with only oral herpes infections [Ashley et al, 1994]. Thus, these findings could reflect longer-standing HSV-1 infection and more efficient binding antibodies to HSV-1 than to HSV-2.

The IgA and IgG profiles in any single CVL sample were seldom the same. The most obvious differences were in detection of antibodies to HSV-1 versus detection of antibodies to HSV-2 (Table 4.11). Several women had IgG to only HSV-1, but IgA to only HSV-2 (n=3), or IgG to both HSV-1 and HSV-2 but IgA to only HSV-1 (n=2). Fifteen women had detectable IgG, but not IgA to either virus.

4.4.4. HSV neutralizing activity.

HSV-specific neutralizing activity was detected in the CVL of 13 women including 10 of 54 (19%) HSV-2 shedders, and 3 of 23 (13%) non-shedders. Twenty-five percent (5 of 20) of dually HIV- and HSV-2-seropositive women had detectable neutralizing antibodies in CVL as compared to 14% (8 of 57) of HIV-seronegative/HSV-2-seropositive women (Table 4.12). Thus, presence of neutralizing activity against HSV was not associated with HIV status or HSV-2 DNA shedding. Neutralizing activity most often occurred in CVL that had detectable binding antibody to HSV, of 11 CVL with neutralizing activity that had at least HSV IgG binding antibody tests, only 1 lacked detectable IgG or IgA to HSV (Table 4.11). Seven of 11 CVL with neutralizing activity had both IgG and IgA to both HSV-1 and HSV-

2. Two other subjects had both IgG and IgA to HSV-2, one had IgG to HSV-1 and HSV-2, but was not tested for IgA (Table 4.11).

These data provide suggestive evidence that HSV-2 neutralizing activity resides in the HSV-specific antibody fraction of CVL. Among those with positive tests for HSV-2 neutralization, the specific activity of HSV neutralization in CVL was substantially higher in both HIV-seronegative and HIV-seropositive HSV-2 shedders than in non-shedders (Table 4.11) (P<0.04). Moreover, the distribution of the specific activities of cervicovaginal HSV-specific neutralizing antibodies and cervicovaginal levels of HSV-2 DNA (as estimated from the OD at 450 nm of hybridized HSV PCR products) in the 10 women shedding HSV-2 in their CVL showed a highly significant correlation (r^2=0.91; P<0.007). The higher the relative level of DNA, the higher the specific activity of neutralizing activity to HSV-2.

4.4.5. Discussion

Vaccine strategies for sexually transmitted infections such as HSV may depend upon understanding the mechanisms by which host immune functions protect against infection of the genital mucosa [Ashley et al, 1994]. Studies have shown that even partial local immunity to HSV is valuable because it diminishes the extent of challenge infections and the severity of lesions due to recurrent infections [Parr & Parr, 1997]. In the present study, we used a highly sensitive ECL Western blot method to detect HSV-specific-IgG and IgA in cervicovaginal fluids of African women HSV-2-seropositive and/or with HSV-2 DNA genital shedding, and a novel infectivity assay to evaluate the neutralizing activity of HSV-specific cervicovaginal antibodies.

HSV-2-specific antibody was found in the cervicovaginal secretions of a high proportion of HSV-2-seropositive women shedding the virus. Furthermore, cervicovaginal HSV-specific antibodies were functional, demonstrating neutralizing in vitro activity. These findings suggest a role for the mucosal immune response to HSV in the control of HSV-2 replication within the female genital tract. These data are in agrement with an earlier study by Pass and colleagues, demonstrating a high prevalence (90%) of neutralizing antibody to HSV in recipients of renal allografts as well as an association between antibodies titers before transplantation and HSV lesions after transplantation [Pass et al, 1979].

The following hypotheses may be of relevance. First, HSV-2 shedding in the genital tract may trigger HSV-specific neutralizing antibodies that could serve to control the genital replication of the virus. If this is true, we would predict that neutralizing activity is higher in the genital tract during times of HSV-2 shedding. Second, neutralizing activity resides with IgA or IgG antibodies against HSV. If this is true, we should observed that cervicovaginal fluids showing neutralizing activity against HSV-2 infectivity of ELVIS™ cells should also contain HSV-specific IgG and/or IgA.

To evaluate these two hypotheses, 77 women with serologic or virological evidence of HSV-2 infection were tested for HSV-specific IgA and IgG and for neutralizing activity against HSV. Although local neutralizing activity was found in only 10 of 54 women with HSV-2 in their CVL, the mean specific activity of HSV-specific antibodies was about 5-fold higher in women who were shedding HSV-2 DNA as compared to non shedding women. Furthermore, a direct relationship could be observed between the specific activity of

cervicovaginal neutralizing antibodies to HSV and the level of genital shedding of HSV-2 DNA, as assessed by semi-quantitative assay.

These data clearly support the first hypothesis that HSV-2 neutralizing activity is higher in HSV-2 shedders than in HSV-2 non-shedders. However, the relatively low prevalence of HSV neutralizing activity in cervicovaginal secretions of HSV-2 shedding women (18%) as well as non shedding women (13%) was unexpected, as preliminary analyses of cervicovaginal secretions collected using tear flow indicator strips (to wick secretions, avoiding collecting accumulated secretions or mucus) predicted at least half of HSV-2-seropositive women would have cervicovaginal neutralizing activity against HSV-2 (Ashley et al, unpublished observations). Such discrepancies between study populations may be due to selection bias of patients and to methodological artifacts in cervicovaginal secretions collection and processing. In particular, the sampling method for cervicovaginal fluids may be crucial in detecting mucosal antibody responses to genital HSV. Then, the vaginal washing procedure we used in the present population of African women introduces a dilution factor of about 1:10 [Bélec et al, 1995], that could hamper the detection of low cervicovaginal levels of HSV-2-specific antibodies.

The second tested hypothesis was that neutralizing activity would be associated with the demonstration of binding antibodies to HSV-2 in CVL. Ten samples showing HSV neutralizing activity had also been evaluated for both IgA and IgG binding antibody assays. Nine of 10 contained cervicovaginal HSV-2-specific IgA and IgG; only one had no binding antibody against HSV antigens. Two of 41 (5%) CVL samples without HSV-2-specific

binding activity had neutralizing activity. In contrast, 11 of 35 (31%) of CVL with at least IgA or IgG (or both) to HSV-2 had neutralizing activity.

Thus, neutralizing activity in this small number of CVL samples was far more likely to be expressed in CVL with HSV-type specfic IgA or IgG than in those without HSV-2 binding antibody. Taken together, these observations suggest a possible role for HSV-specific cervicovaginal antibodies in *in vitro* neutralization of HSV-2, and for their participation to the host control of the genital replication of HSV-2 during asymptomatic recurrences. Futher evaluations of virus shedding, genital humoral as well as cellular immune responses to HSV-2, and of immunochemical characterization of cervicovaginal HSV-2-specific antibodies would be useful to assess the importance of local immune factors to control HSV-2 genital reactivation during the natural history of genital herpes in women.

Table 4.9

Total IgG and IgA in Cervicovaginal Lavage samples

	IgG*	IgA*	CVL Positive for IgG**	CVL positive for IgA
HSV-2 Shedders Group 1 HIV–seronegative N=34	16.1 ± 7.2 (0-227.5)	1.4 ± 0.5 (0-13.0)	20 (59%)	12 (35%)
Group 2 HIV-seropositive N=20	17.7 ± 5.3 (0.2 – 96.5)	2.2 ± 0.5 (0 – 7.6)	17 (85%)	13 (65%)
HSV-2 non shedders Group 3 HIV–seronegative N=23	13.1 ± 0 (0 – 59 1.0)	1.2 ± 0 (0.07 – 42.0)	22 (96%)	13 (57%)

* (µg/100µl)

** (>.5 µg IgG/100µl (%))

Table 4.10

Presence of HSV-Specific IgG or IgA In Cervicovaginal Samples as Detected by ECL–Western Blot

Group	Tested for IgG	IgG to HSV	% with IgG to HSV	% with IgG to HSV-2	Tested for IgA	IgA to HSV	% with IgA to HSV	% with IgA to HSV-2
I* HIV Negative	34	HSV-1 $\frac{}{10}$ HSV-2 $\frac{}{1}$ Both $\frac{}{6}$	50	21	32	HSV-1 $\frac{}{6}$ HSV-2 $\frac{}{1}$ Both $\frac{}{4}$	34	16
II* HIV Positive	19	HSV-1 $\frac{}{4}$ HSV-2 $\frac{}{4}$ Both $\frac{}{9}$	89	68	17	HSV-1 $\frac{}{1}$ HSV-2 $\frac{}{2}$ Both $\frac{}{8}$	64	62
III** HIV Negative	22	HSV-1 $\frac{}{7}$ HSV-2 $\frac{}{3}$ Both $\frac{}{8}$	82	50	21	HSV-1 $\frac{}{2}$ HSV-2 $\frac{}{4}$ Both $\frac{}{6}$	57	48

* HSV-2 Shedders
** Non Shedders

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Table 4.11

HSV Type Specific IgG and IgA in Cervicovaginal lavage samples (N=77)

Subjects with IgG Sorted by HSV Antibody Type

Subjects with IgA sorted by HSV Antibody Type	Neg	HSV-1	HSV-2	HSV-1&2	ND	Total
Neg	19* (1)	8	4	3	3	34
HSV-1	0	9	0	0	0	9
HSV-2	0	3	2	2*	2*	7
HSV-1&2	0	0	1* (1)	17** (7)	0	18
ND	4	1* (1)	0	2* (1)	2* (1)	9
Total	23	21	7	24	2	77

* 1 Neut positive in each group

** 7 Neut positive

Table 4.12

Specific Activity Neutralization of HSV-2 in Cervicovaginal samples N=77

Group	Number with Neutralizing Activity (%)	Neutralizing Units	Specific Activity[1] Median (range)
HSV-2 Shedders HIV-seronegative Group 1 N=34	5 (15)	252 - 1935	17.30 1.10 – 171.3
HSV-2 Shedders HIV-seropositive Group 2 N=20	5 (25)	503 - 2117	33.62 17.90 – 100.43
HSV-2 Non-Shedders HIV-seronegative Group 3 N=23	3 (13)	435 - 522	3.10 0.78 – 3.6

[1] Neutralizing activity units (NAU)/total IgG + total IgA in µg/100µl. Median calculated on only positive samples.

Chapter 5. General discussion, concluding remarks and future studies

Through a study on women in Bangui, the capital city of Central African Republic, I have investigated the role of HSV-2 as a cofactor in HIV transmission. In this final chapter of the thesis, I will try to summarise the findings and conclusions of the previous chapters. **Section 5.1** focuses on the burden of HIV infection, a range of sexually transmitted infections, HSV-2 seroprevalence, sexual behaviour, HSV-2 genital shedding and vitamin A results. **Section 5.2** discusses the methodology and the algorithm of HSV DNA quantitation within the female genital tract. The main results of my thesis are discussed in **section 5.3**, in the context of original broad set of data and providing explanatory hypotheses on the interactions between HSV-2 and HIV within the female genital tract as well as on the relationship between HSV shedding and the production of HSV neutralising antibodies. Given the increasing awareness of the link between HSV-2 and HIV, I discuss in **section 5.4** the public health implications of HSV-2 and HIV interactions and future research studies.

5.1. The burden of HIV infection, sexually transmitted infections, HSV-2 seroprevalence, sexual behaviour, HSV-2 genital shedding and the vitamin A range

5.1.1. Seroprevalence of HIV infection and other sexually transmitted infections

This study confirms the high prevalence of HIV infection in the sexually active population of Bangui [Massanga et al, 1996], a rate that is comparable to other neighbouring countries [Mbopi-Keou et al, 1998b]. The prevalence of cervical infections e.g *Chamydia trachomatis*, overall was surprisingly low (3%). It is possible that women coming primarily

for antenatal check-up and family planning and who can afford relatively high fees may have lower STD rates. In some of the countries in the region, prevalence rates of cervical infections were found to be high: 8% in urban [Mayaud et al, 1998] and rural Tanzania [Mayaud et al, 1995], 10.8% in urban Kenya [Thomas et al, 1996] and 10% in urban Cameroon [Mbopi-Keou, personal communication]. This study also confirms the low prevalence of Chlamydia trachomatis generally found in Central/East Africa. Nevertheless, women in this study have higher rates of infection with Trichomonas vaginalis and some of the endogenous infections such as bacterial vaginosis and candidiasis which are all treatable conditions and which may increase HIV transmission [Laga et al, 1994; Sewankambo et al, 1997]. The prevalence of syphilis, which is high in this study (8%) confirm other data obtain in Central African Republic [Gerard Gresenguet, personal communication], however, higher rates of 17% have been found in neighbouring Cameroon [Mbopi-Keou et al, 1998a]. These findings support the urgent need for taking appropriate measures to detect and manage STDs in Bangui and in the developing world [Mayaud et al, 1998; Gerbase et al, 1998]. Moreover, my findings of an early commencement of sexual activity, together with results of low contraceptive utilisation rate when having symptoms of STDs, and a high prevalence of STI confirm earlier findings in Central African Republic [Gresenguet et al, 1991; Martin et al, 1992; Massanga et al, 1996] and support results of studies on young women in both rural and urban African communities [Mayaud et al, 1995; Leroy et al, 1995; Mayaud et al, 1998; Mbopi-Keou et al, 1998a].

5.1.2. HSV-2 seroprevalence and sexual behaviour

In sub-saharan Africa, studies of HSV-2 are scarce, but a high seroprevalence (>40%) have been recorded in East Africa [Wagner et al, 1994; Obasi et al, 1999; Langeland et al, 1998],

demonstrating that HSV-2 is a significant genital infection in this part of the world. My findings indicate an even higher prevalence of HSV-2 infection in the population of women attending the National Reference STD Center of Bangui (82%) compared to 40.8% reported for Kinshasa, 51.3% for Rwanda [Nahamias et al, 1990] and 44.6% for Zimbabwe [Gwanzura et al, 1998]. I also found that HSV-2 seroprevalence was more prevalent in HIV-infected women (91% vs. 78%, P=0.02). In addition, the fact that a very high proportion of women in my study were young calls for the urgent need to develop effective STD control interventions targeted at this highly vulnerable population. Such programmes should include education on condom use for contraception and be targeted at girls before they become sexually active [Mbopi-Keou et al, 1998a].

5.1.3. HSV-2 shedding

There have been very few studies of genital shedding of HSV-2 among non-GUD patients, particularly among women outside industrialised countries. My study is the first in sub-Saharan Africa to show both an increased prevalence and quantity of genital HSV-2 shedding among HIV-1 infected women, and the first to show a correlation between the quantities of HIV-1 RNA and HSV-2 DNA in genital secretions among HSV-2 shedders. Indeed, HSV-2 shedding (particularly among asymptomatic individuals) may be a major reservoir for ongoing transmission. Numerous investigations, mainly in developed countries, show that genital herpes is often acquired from individuals who are unaware that they were infected [Mertz et al, 1992]. Other studies also reveal, through viral culture from individuals without typical symptoms, that asymptomatic shedding is extremely common in both men and women and occurs from multiple anatomical sites [Wald et al, 1997; Mindel, 1998]. I did not perform viral culture in this study because of logistical constraints imposed

by working with field samples. Nevertheless, my PCR methods clearly demonstrated that asymptomatic shedding may be even more common than previously thought. It is yet to be determined whether the detection of HSV DNA by PCR can be correlated with infectivity [Mindel, 1998].

5.1.4. Serum level of vitamin A

In this study, the serum level of vitamin A was significantly different between HIV positive and HIV negative women (p<0.001). As this is one of the first studies to measure vitamin A in Central Africa, it is difficult to ascertain normal levels in the absence of control subjects. Many studies have suggested the important role of vitamin A as an immunoregulator during infectious diseases [Semba, 1994a; Semba et al, 1994b; Mostad et al, 1997; John et al, 1997]. Vitamin A deficiency is characterized by widespread alterations in immunity, including pathological alterations in mucosal surfaces, impaired antibody responses to challenge with protein antigens, changes in lymphocyte subpopulations, and altered T-and B-cell function [Semba, 1994a]. However, the very high rates of cervicovaginal infections and high levels of HSV-2 shedding both in HIV positive and HIV negative individuals in this study precluded the possibility that low levels of vitamin A would have led to enhanced acquisition of HSV-2 and HIV [Belec et al, 2002].

5.2. Methodology and algorithm of HSV DNA quantitation within the female genital tract

5.2.1. Methodology

Our study population is representative of the population of women attending the CNRMST/SIDA of Bangui. The CNRMST/SIDA is a heavily used Center for reproductive health. Access is not restricted to referrals, hence our study individuals are likely to represent the general female population. However, the high level of clinic attendance fees may be selecting only the wealthier section of the population. As women who are able to afford contraceptives may differ from the general population in sexual behaviour and HIV/STD risks, some degree of bias in the patient selection could have been operative in this study.

5.2.2. HSV DNA quantitation

I chose to amplify target HSV DNA from the acellular compartment of cervicovaginal secretions because HSV-2 is not principally cell-associated during productive infection, in contrast to HIV, for example [Lawrence Corey, personal communication]. The PCR strategy I used was not type-specific: it amplified a conserved fragment of the HSV DNA polymerase gene of HSV, common to type 1 and 2 [Espy et al, 1993]. The PCR needed to be exquisitely sensitive for at least two reasons: (i) the genital shedding of HSV-2 is probably low in asymptomatic carriers; (ii) cervicovaginal fluid may contain Taq polymerase inhibitors [Laurent Belec, unpublished data]. I used the DEIA hybridization approach both to increase the sensitivity and to assess the specificity of amplification [Mantero et al, 1991; Payan et al, 1995]. The use of a probe specific of the HSV DNA polymerase gene ensured the specificity of the amplification process. The herpes type was subsequently characterised using an RFLP procedure [Vogel et al, 1994]. All genital HSV were confirmed to be type 2. The evaluation the level of amplicons by DEIA hybridization enabled semi-quantitative estimation of the level of genital HSV-2 shedding. HSV-2 in the

vagina reflects, local HSV-2 production from the lower genital tract of an HSV-2-positive woman, or from semen-associated HSV-2 passively deposited in the female genital tract after sexual intercourse with an HSV-2 male carrier. In the present study, the majority of women shedding HSV-2 in their genital secretions were also HSV-2-seropositive, so it was likely HSV-2 was being produced by their own genital tract. About a quarter of HSV-2-seronegative women had HSV-2 in their cervicovaginal secretions; in these women, HSV-2 may originate from a male HSV-2 carrier. One may also speculate that in the HSV-2 seronegative woman, genital HSV-2 corresponds to local viral production accompanying the invasive phase of "primary" HSV-2. These considerations led me to screen my specimens first for the presence of prostatic specific antigen, and also to use a real-time quantitation method [Kessler et al, 2000] to assess the amount of total HSV DNA shed from the female genital tract.

5.2. 3. Algorithm for HSV DNA Quantitation within the female genital tract

Little information is available about the accuracy of different nucleic acid or signal amplification techniques in quantifying HSV DNA in the female genital tract [Hobson et al, 1997; Ryncarz et al, 1999; Mbopi-Kéou et al, 2000]. The ability to assess viral burden in genital secretions is essential to permit understanding the pathophysiology of sexual as well as mother-to-child HSV transmissions, and in predicting the effect of antiviral therapy at the level of the genital compartment [Corey & Handsfield, 2000]. This will require the availability of standardized assays for the quantification of HSV DNA which should be sensitive, specific and reproducible. To my knowledge, quantitative molecular assays for HSV DNA in female genital secretions include: competitive PCR in HSV-2 glycoprotein B

gene [Hobson et al, 1997]; real-time quantitative-based PCR assay amplifying a type-common region of the HSV glycoprotein B (gB) [Ryncarz et al, 1999]; and PCR detection in the HSV DNA polymerase gene followed by DNA enzyme immunoassay hybridisation with a specific probe [Mbopi-Kéou et al, 2000]. Although the real-time PCR assay constitutes a real progress (high throughput; lack of contamination during processing; and high reproducibility), there remains numerous pitfalls that should be more systematically addressed particularly when studying HSV DNA in the female genital secretions. First, the best way to collect genital secretions for the detection of HSV DNA may need to be evaluated. Thus, others have attempted systematic swabbing of the cervix or of the perigenital region [Hobson et al, 1997; Ryncarz et al, 1999]. However, this procedure may lead to low sensitivity in detecting HSV DNA. In this thesis, HSV DNA in cervicovaginal lavage samples was quantified, using a previously described method [Belec et al, 1995]. This collection procedure may introduce mucosal factors that inhibit or decrease the throughput of the PCR. Thus, false-negative PCR can result from complete or partial inhibition of Taq polymerase by tissue inhibitors, which may reach high concentrations in body fluids [Ochert et al, 1995]. Indeed, one report on the quantitation of HIV in female genital tract demontrated that collection by a swab is more suitable than collection by vaginal lavage [Baron et al, 2000], probably because the cervix is a site of highly active replication for HIV. No comparison of collection procedures is yet reported for genital herpes. Second, processing of female genital tract samples should focus on the presence of semen-traces, because HSV DNA may be found in post-intercourse male genital secretions [Centifanto, 1972]. In this study, the presence of prostatic soluble antigen in genital secretions was checked for. However, this method may lack sensitivity. Systematic

detection of Y DNA chromosome may be more suitable when the frequency of women with semen-positive genital secretions is less that 60%, as recently demonstrated by Chomont and colleagues [Chomont et al, 2001]. Third, extraction procedures for nucleic acids contained in the female genital tract should also be optimized. The many steps involved in the purification of nucleic acids from clinical specimens by classical procedures (involving detergent-mediated lysis, proteinase treatment, extractions with organic solvant, and ethanol precipitation) increase the risk of transfer of nucleic acids from one sample to another, therefore leading to false positive results. The silica-based extraction procedure is one of the most convenient to extract both DNA and RNA in corporeal fluids, and may overcome the presence of PCR inhibitors [Boom et al, 1990; Ochert et al, 1995; Boom et al, 1999]. Furthermore, prior virus precipitation has been shown to increase the sensitivity of RNA detection and quantitation in cervicovaginal secretions of HIV-positive women [Si-Mohamed et al, 2001]. Finally, an automated method could be useful to process a great number of sample with increased reliability. In these studies, I used the Qiagen method for DNA extraction and also did further evaluations which demonstrated that this method was as sensitive as the automated extration (Roche MagnaPure) (data not presented).

5.3. Interaction between HSV and HIV within the female genital tract

Synergistic interactions have been demonstrated between herpesviruses and HIV [Webster et al, 1989; Mbopi-Keou et al, 2002]. HIV and HSV2 interact bi-directionally: HSV2 increases the efficiency of HIV acquisition and transmission whereas HIV may increase susceptibility to HSV2 and HSV2 shedding. HSV2 reactivation is significantly increased in

immunosuppressed HIV infected individuals [Augenbraun et al, 1995]. The frequency and severity of recurrences increase as CD4 cell count decreases [Augenbraun et al, 1995; Shacker et al, 1998].

Effect of HIV infection on HSV2 transmission. HIV infection is also likely to increase transmission of HSV2, and these studies provide evidence that the prevalence and quantity of genital HSV-2 shedding is significantly increased among HIV seropositive individuals.

Effect of HSV2 on the natural history of HIV disease. There is some evidence that inclusion of acyclovir in antiretroviral therapy may prolong survival in HIV seropositive individuals [Severson and Tyring, 1999]. One study has shown that HSV2 reactivation is associated with increases in plasma HIV1 RNA levels and intracellular gag mRNA and that plasma HIV1 RNA level decreases significantly during treatment with acyclovir [Mole et al, 1997]. As a result, it is possible that HIV progresses more rapidly in untreated HSV2 positive individuals. However, the evidence is inconclusive at present and more studies of the effect of HSV2 therapy on HIV are needed, especially in developing countries.

Effect of HSV2 on HIV transmission. In these studies, I found an association between genital HIV RNA and HSV-2 DNA levels only among dually HIV and HSV-2 shedders [Mbopi-Keou et al, 2000]. A study of 12 men in the US infected with both HSV2 and HIV showed that HIV RNA was present in almost all HSV2 lesions, suggesting that HIV transmission is enhanced in the presence of HSV2 lesions [Shacker et al, 1998]. Others also reported that HIV RNA is often present at high titres in genital lesions (independent of plasma RNA levels), but titres of both HIV1 and HSV2 fall rapidly on treatment with acyclovir, supporting the hypothesis that HSV2 reactivation may play an important role in upregulation of HIV1 on mucosal surfaces [Lawrence Corey, personal communication].

Few data are available on the effect of subclinical HSV2 on HIV shedding and viral load. It is plausible that the interaction between the two viruses differs in developed and developing countries, as well as between developing countries, due to the role of other factors (such as prevalence of other STDs and concurrent partnerships). For these reasons, similar prospective studies of dually infected individuals are needed in developing countries.

5.4. HSV-1 and HSV-2 binding antibodies and HSV-specific neutralizing activity within the female genital tract

The role of local antibodies in the control of infection is poorly understood. In this thesis, I tested the genital secretions of HSV-2-seropositive women for HSV-2 DNA, binding antibody to HSV-1 and HSV-2 proteins, and neutralizing activity against HSV-2. HSV-specific neutralizing activity was found in cervicovaginal lavage samples both in women shedding HSV-2 and in those not shedding the virus. However the specific activity of the neutralization was much lower in women not shedding HSV-2. Presence of HSV-specific IgG and IgA binding antibodies was correlated with presence of neutralizing activity [Mbopi-Keou et al, 2000b], which suggests that neutralizing activity is effected by HSV-specific IgG and IgA antibodies. HSV shedding events may induce antibody responses that are associated with specific HSV-2 neutralizing components in the genital tract. However, more direct studies to localize neutralizing activity in purified cervical IgG, IgA, or IgM components are necessary.

5.5. Concluding remarks and future studies

In the face of the severe and worsening HIV-1 epidemic in Africa, the high prevalence of HIV-1 and HSV-2, and the high frequency of asymptomatic HSV-2 shedding, a possible synergistic effect between the infections as demonstrated in my thesis, is a cause for concern [Mbopi-Keou et al, 2000a]. We have learned in the past decade that no single approach will contain the HIV/AIDS epidemic, and we need to consider all available means of control, however imperfect. The Mwanza study in Tanzania demonstrated that improved STD case management was an important additional HIV prevention strategy [Grosskurth et al, 1995], resulting in a 40% reduction in HIV incidence in the general population. No such reduction was achieved by STD mass treatment in the Rakai study in Uganda [Wawer et al, 1999], and one reason postulated for this was a higher prevalence in Rakai of STDs such as genital herpes that were not targeted by the antimicrobial regimen [Hitchcock & Frensen, 1999]. There is an urgent need for further research on genital herpes and STD/HIV control in Africa. These studies should help to better understand the biological mechanisms that underlie the HSV-2/HIV interaction and provide biological endpoints for further intervention trials. Further studies on patients with genital ulcer disease and HIV will be necessary to examine if episodic or suppressive therapy with acyclovir or another prodrug can hasten the healing process, thereby decreasing HIV and HSV-2 shedding [Fife, 1996; Fife et al, 1997; Reitano et al, 1998].

In developing countries, the focus of such trials should be on the effect of therapy on HIV transmission. Indeed, although there is some evidence that herpetic treatment decreases HIV shedding, further studies in developing world settings are needed to measure the impact of both HIV transmission and acquisition. In addition, evaluation of the effect of therapy on HIV is required before recommending the widespread use of therapy for mild

herpetic ulcers. This may lead to revision of current syndromic management practices in developing world settings. In trials of episodic therapy, randomization could be carried out at either the individual or community level [WHO/UNAIDS/LSHTM International workshop, 2001]. Community randomization would allow measurements of effects on HIV transmission and acquisition. However, the substantial geographical variations in the epidemiology of HIV and STIs, and in access to health care services, may limit the generalizability of such trials. Trials of suppressive therapy will help determine the impact on HIV shedding, and to study the natural history of HSV2 in treated and untreated patients.

We also need to develop and test preventive interventions, particularly those targeting youth. As with other sexually transmitted infections, behavioural interventions are needed. The format of these interventions will depend on the HSV-2 epidemiology currently prevailing in the region in question. In countries with high HSV-2 prevalence in the general population, such interventions will need to target the general population. In other countries where the epidemic is at an early stage, interventions may be focused on 'high-risk' groups. Control measures should include the proclamation of prevention messages with the aim of delaying sexual debut and reducing rates of partner change. It is reported that male condoms protect against HSV-2 less effectively than against HIV, although a recent study of discordant couples by Wald and colleagues found that consistent condom use significantly protected women, but not men, from acquiring HSV-2 infection [Wald et al, 2001]. Given the efficacy of condoms in protecting against HIV infection, promotion of condom use should always be included in any STI prevention intervention [WHO/UNAIDS/LSHTM International workshop, 2001]. Preventions strategies for herpes

transmission should focus on individuals with genital herpes lesions. These will be mainly individuals who are HSV-2 seropositive, but are likely to be the ones at highest risk of transmitting the virus. Indeed, health education for such patients should promote abstinence during symptomatic period, and consistent condom use with regular partners [WHO/UNAIDS/LSHTM International workshop, 2001].

However, it may be that the most realistic hope for control is the development of a safe and effective HSV-2 vaccine [Stanberry, 1998]. During a recent WHO workshop in London, there was strong commitment to lobby for increased investment in the development and evaluation of prophylactic vaccines [WHO/UNAIDS/LSHTM International workshop, 2001]. This should include evaluation of the DISC vaccine for use as a prophylactic vaccine. A major priority is to ensure that any vaccine produced is available and affordable in the developing countries. Vaccine trials should initially be conducted in HSV2-seronegative women. These women would be stratified by HSV-1 serostatus, though in some settings, as it is the case of Central Africa, there may be few HSV-1 negative individuals. Individual randomization of young people would minimize sample size, because of the higher seroconversion rates in this age group. The primary endspoints would be prevention of HSV-2 disease, because of the impossibility of distinguishing between natural infection and vaccine-induced immunity, with a secondary endpoint of the prevention of HIV infection [WHO/UNAIDS/LSHTM International workshop, 2001].

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Appendix

The Role of *Herpes simplex* virus type 2 (HSV-2)
as a cofactor in HIV transmission

Participant information sheet

Purpose of the Research

The aim of this study is to improve services for people suffering from STDs. Women with and without STDs are being studied. The health authorities in Bangui have approved this study which is being conducted to find out more about reported health problems and sexual behaviour of women suffering from STDs. With this information, treatment services can be improved. Women are at greater disadvantage of contracting STDs and often suffer from complication for themselves and their offsprings. This is partly the case because often do not realise that they have an STD or report their STD condition to health workers.

Procedures

You will be asked questions by a trained doctor, and you will be offered a full gynaecological examination (including genital +cervical swabs) and specimens will be taken to diagnose STDs. Treatment will be provided immediatly according to some of the findings, but you will be asked to come after 2 weeks to collect all your results and to get further treatment if necessary. A blood sample will be collected to diagnose some STDs like syphilis and also tested for HIV. I remind you that your name will not appear. If you want to know the result of your HIV test, I will direct you to the appropriate service for counselling, in this building, after the interview.

As a participant of the study, you will be doing so on a voluntary basis. You may decide to withdraw from the study at any point. This will not affect your further care or treatment. All interviews will be conducted at the Centre National de reference des MST et du SIDA (CNRMST/SIDA) of Bangui. If you are interested in participating, or you have any questions, please contact the staff of the (CNRMST/SIDA).

Risk and Discomforts

You will not be placed at risk during the study. The questionnaire will be conducted in a safe environment with a trained interviewer. The examination is standard practice in this clinic. Only stickers with a number will be used to identify your responses. All your answers will be kept secret.

Benefits

You will benefit directly and indirectly because: 1) any STD infection that you may have will be diagnosed and treated; 2) your participation may provide useful insights to the role of some STDs in the STD/HIV epidemic in Africa and may help improve STD services.

The Role of *Herpes simplex* virus type 2 (HSV-2)
as a cofactor in HIV transmission

Participant consent sheet

Interviewer: Before conducting this interview, cary out the following procedures:

a)   Read the Information Sheet to the respondent
b)   Read the following statement to the respondent

"The aim of this study is to improve services for people suffering from STD. The health authorities in Bangui have approved this study, as well as the ethical committees of the London School of Hygiene and Tropical Medicine. Your answers will be confidential. Today, you will be asked some questions on your health problems and about your sexual partners. You will be asked to undergo a gynaecological examination and some samples will be collected. You will be treated for any condition you may have. Tests for HIV will be performed but these results will be confidential and will only be given to you if you want to know them. If you would like to know the results of your HIV test, pre-and post-test counselling will be provided at the clinic."

Do you have any questions?

Do you want to know the result of your HIV test?

Do you consent to participate in this study?

Name of respondent:

Respondent's adress:

Interviewer's name:

Date of interview:

Sign to confirm that you have explain the study and obtained
the respondent agreement.

Place a sticker on the cover sheet and tear it off, put it in a safe place, then use the same sticker number for the rest of the questionnaire and for the samples.

The Role of *Herpes simplex* virus type 2 (HSV-2) as a cofactor in HIV transmission

(Questionnaire)

Main reason for coming:
1. Antenatal checkup
2. Family planning
3. STD
4. Other gynaecological problem

I- *Demographic parameters*

1.What is your age?   years

3. Where do you live?   code

    A=   B=   C=
    D=   E=   F=

4. What is your religion?   code

    A=Protestant   B=Traditional   C=Muslim
    D=Catholic   E=Other

5. What is your level of education?   code

    A=Never went to school
    B=Primary education   D=Secondary education
    C=University background   E=Other

6. What kind of work do you do?   code

    A=Student/School pupil   B=Unskilled manual
    C=Skilled manual   E=Office work/teacher/nurse
    E=Business/Marketeer   F=Housewife
    G=Farming only   H=Other

7. Marital status   code

    A=married monogamous   B=married polygamous   C= separated/divorce
    D= Widowed   E= single   F=other

II- *Obstetrical data*

8. Are you pregnant now?   Y/N

8.1.If yes how many weeks?   NW

(calculate Last Menstruation Period)
NW=Number of Weeks

8.2. How many pregnancies have you had in your lifetime   No

including this one?

9. In total how many times have you delivered?   No

    9.1.These deliveries included:

      a. Delivery of a stillborn baby   Y/N
      b. Have a miscarriage (abortion)   Y/N
      c. Signs of baby born with congenital herpes   Y/N

10. How many children do you have?   No

11. Are you taking any contraceptive?   Y/N

    11.1. If yes which contraceptive?   code
    A=Oral   B=Injection   C=Traditional
    D=Traditional   E=Other   F=Use of condom
    11.2. Duration?   code
    A= <3 month   B=3-6 months   C=>6 months

III- *History of Sexually Transmitted Diseases* :

12. Today do you have any problem in your :
      Lower abdominal region?   Y/N
      Privates parts?   Y/N

13. Do you have any of the following symptoms ?

a. Tickling sensation/itching vulva   Y/N

b. Vaginal discharge/   Y/N

c. Foul smell   Y/N

d. Dysuria/Pain/Urinary discomfort   Y/N

e. Irregular menstrual flow   Y/N

f. Pain when having intercourse   Y/N

g. Genital ulceration   Y/N

h. Blisters on genitalia   Y/N

i. Rashes/Itching   Y/N

j. Other genital problems   Y/N

14.Did your partner use a condom while you had symptoms?   Y/N

IV. *General Health problems*

I would like to ask you further questions about your general health

15. Do you have any of the following symptoms

a. Weight loss > 10% of the baseline weight   Y/N

b.Diarrhea >1 month's duration   Y/N

c.Chronic or intermittent fever > 1 month's duration   Y/N

d.Chronic fatigue   Y/N

e.Chronic generalized   lymphadenopathy   Y/N

f.Herpes zoster in last 5 years   Y/N

g.Reccurent herpes zoster   Y/N

h.Herpes zoster > 1 month's duration   Y/N

i.Chronic and disseminated herpes simplex infection   Y/N

j.Generalized pruritic dermatitis   Y/N

k.Oropharyngeal candidiasis   Y/N

m.Cough > 1 month's duration   Y/N

(Total number of symptoms mentioned)   Number

V- *Sexual Behaviour*

I would like to ask you some questions about your sexual behaviour. May I remind you that your name is not written on this form, and everything you tell me will be kept confidential.

16. How old where you when you had sexual   age in years
intercourse for the first time ?

17. How many men have you had sex with (including regular and casual partners):
17.1   17.2   17.3
LifetimeNo   last year No   last 3 months   No

18. Condoms:
       1. Have you ever heard about condoms?   Y/N
       2. Have you ever seen condoms?   Y/N
       3. Have you ever used condoms?   Y/N
       4. Did you used condoms during
       your last intercourse with casual partner?   Y/N
       5. Did you used condoms during
       your last intercourse with regular partner?   Y/N

VI- *Examination*

19. General Examination

    a. Signs of severe weight loss   Y/N

    b. Generalized lymphadenopathy   Y/N
    (>2 sites extrainguinal)

    c. Signs of herpes zoster infection   Y/N
        1. old   2. recent   code
    d. Generalized pruritic dermatitis   Y/N
    e. Oropharyngeal candidiasis   Y/N

    f. Other signs
        1. Kaposi's sarcoma   Y/N
        2. pneumopathy   Y/N
        3. other   Y/N

20. Signs of secondary syphilis

    1. generalised or localised skin rash   Y/N
    2. rash of palms and/or soles   Y/N
    3. hair loss in patches   Y/N
    4. condylomata lata   Y/N

21. Genital Examination

21a. Vulva inflamed   Y/N
21b. Genital warts   Y/N
21c. Vaginal discharge   Y/N

21d. Cervix:
       1. Cervical discharge   Y/N
       2. Cervical dysplasia / ectropion   Y/N
       3. Cervical inflammation (cervicitis)   Y/N
       4. Herpes blister (s)   Y/N

21e. Genital ulcer   Y/N
      a. localisation
      b. number

21f. Inguinal region:
        a. isolated bubo   Y/N
        b. lymphnodes affected   Y/N

21g. Sign of PID
        a. pain at mobilisation of cervix   Y/N
        b. pain in fornixes   Y/N
        c. lower abdominal tenderness   Y/N

22. Clinician's diagnostic: code

        A=Genital discharge   D=PID
        B=Genital ulcer   E=Other STD
        C=Herpes   D=No STD

VII. *Specimen taking*

23. Vaginal samling for

        a. Gram stain   Y/N
        b. Wet preparation results for :   code
        1=*Trichomonas vaginal (TV)*
        2= *Candida (CA)*
        3= *TV+ CA*
        4=Nil

24. Cervical sampling for

        a. GC culture   Y/N
        b. Chlamydia   Y/N

25. Cervico-vaginal lavage with 3ml PBS   Y/N

26. Serum sample   Y/N
27. Plasma sample   Y/N

Clinician Initials: Clinician's Code

Copies of publications

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Genital herpes simplex virus type 2 shedding is increased in HIV-infected women in Africa

The enhancing effect of bacterial sexually transmitted diseases (STD) on HIV transmission is 'no longer a hypothesis' [1]. Little is known, however, about the interrelationship of HIV and viral STD, such as genital herpes simplex virus (HSV)-2, although a recent study among male STD patients in Seattle (Washington, USA) has documented the role of HSV-2 in enhancing sexual HIV transmission [2]. STD, including HIV, are a major public health problem in Africa [3], and studies have shown much higher STD prevalence and incidence in East Africa compared with many industrialized countries [4]. We report preliminary findings from a study that aimed to determine the seroprevalence and genital shedding of HSV-2 and to study factors influencing HSV-2 shedding among women in Bangui, Central African Republic.

Ninety-seven consenting women (median age, 26 years) attending the National STD Reference Centre, a multipurpose reproductive health clinic in Bangui, were enrolled during the period June–July 1998. Only six of these women presented specifically for STD treatment. HIV serology was performed following the World Health Organization testing strategy using two different enzyme-linked immunosorbent assays (ELISA; Genelavia-Mixt HIV-1/HIV-2, Diagnostic Pasteur, Marnes-la-Cocquette, France; and Wellcozyme HIV-1, Murex Diagnostics, Dartford, Kent, UK). Discordant results were confirmed by Western blot (New-LAV Blot 1, Diagnostic Pasteur, Paris, France). Type-specific serum antibodies to HSV-2 were assessed by an ELISA developed from a radioimmunoassay [5], and validated against Western blotted sera from the United Kingdom and Africa. Shedding of extracellular HSV in cervicovaginal secretions was assessed by polymerase chain reaction (PCR). Analysis of all PCR products was carried out using DNA enzyme immunoassay (DEIA), and the level of HSV-2 amplicons assessed by optical density (OD) of hybridized products at 450 nm. All cervicovaginal samples that were positive for HSV on agarose gel or after DEIA hybridization were further assessed using the restriction fragment-length polymorphism technique to confirm HSV type. Tests were also carried out for bacterial vaginosis, *Trichomonas vaginalis*, *Candida albicans*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and serological syphilis.

Our findings showed a high seroprevalence of HSV-2 (80%), a high prevalence of HSV-2 genital shedding (32%), and a high prevalence of HIV (21%) and other STD (87%). HSV-2 seroprevalence did not vary significantly by HIV serostatus (Fig. 1). However, 13 (65%) out of 20 HIV-positive women were shedding HSV-2, compared with 18 (23%) out of 77 HIV-negative women [odds ratio (OR), 6.1; 95% confidence interval (CI), 2.1–17.6; $P < 0.001$]. This association changed little after adjustment for age, other STD or pregnancy status by logistic regression (adjusted OR, 5.7; 95% CI, 1.8–18.3). Furthermore, the quantity of HSV-2 shed was significantly higher in HIV-positive shedders (geometric mean OD, 0.28; 95% CI, 0.10–0.77) than in HIV-negative shedders (OD, 0.04; 95% CI, 0.03–0.06; $P < 0.001$).

Amongst the 13 HIV-positive women with HSV-2 genital shedding, 11 (85%) were HSV-2-seropositive. Similarly, amongst the 18 HIV-negative women with HSV-2 shedding, 13 (72%) were HSV-2-seropositive ($P = 0.42$). The recovery of HSV-2 DNA from HSV-2-seronegative women may represent primary HSV-2 infection, or may be due to the presence of semen from an HSV-2-shedding male partner.
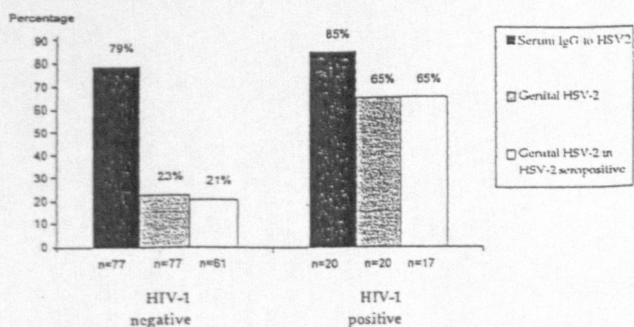

Fig. 1. HSV-2 antibody prevalence and HSV-2 shedding by HIV status among 97 women living in Bangui, Central African Republic.

In summary, this report describes increased prevalence and quantity of HSV-2 shedding amongst HIV-infected compared with HIV-uninfected women. If HSV-2 shedding is in turn associated with increased shedding of HIV, these results would indicate that HSV-2 genital shedding may play an important role in HIV transmission in this part of Africa. Given the high prevalence and incidence of HSV-2 in this region, intervention measures would be urgently warranted. Potential candidates include (i) HSV-2 screening and suppressive antiviral therapy amongst HIV-positive individuals (probably not feasible in Africa); (ii) revision of current guidelines for genital ulcer management to include antiviral treatment for herpes; (iii) enhancement of preventive measures for safer sex, particularly among young people; and (iv) primary prevention with a safe and effective HSV-2 vaccine.

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Interactions between Herpes Simplex Virus Type 2 and Human Immunodeficiency Virus Type 1 Infection in African Women: Opportunities for Intervention

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Sexually transmitted diseases (STDs) are cofactors for human immunodeficiency virus (HIV) transmission, but the specific role of herpes simplex virus type 2 (HSV-2) is unclear. This study aimed to examine the in vivo relationships between HSV-2 and HIV-1 in 300 women in Bangui, Central African Republic. Sera were tested for syphilis, HIV-1, HSV-2 antibody, and levels of vitamins A and E. Genital specimens were tested for other STDs. HSV-2 DNA and HIV-1 RNA were quantified in cervicovaginal lavage. The prevalences of HSV-2 antibody (91% vs. 78%, $P = .02$), HSV-2 shedding (43% vs. 22%, $P = .003$), and levels of HSV-2 DNA ($P = .01$) were all significantly higher among HIV-1–seropositive than among HIV-1–seronegative women. There was a significant correlation between genital HIV-1 RNA and HSV-2 DNA levels ($P = .02$) among the 23 women who were shedding HSV-2 DNA. If confirmed, such associations highlight the urgent need for HSV-2 control measures in populations at high risk of both infections.

The human immunodeficiency virus (HIV)/AIDS pandemic continues to worsen, particularly in many developing countries where heterosexual transmission predominates. Many cofactors for HIV genital shedding have been suggested, including systemic factors, such as immunosuppression, hormonal contraceptive use, or micronutrient deficiencies [1, 2], and local factors, such as the presence of other sexually transmitted diseases (STDs), particularly those causing genital ulceration [3]. Herpes

simplex virus type 2 (HSV-2), a major cause of genital ulcer disease (GUD), is highly prevalent in human populations worldwide [4]. The high prevalence of infection, its associated morbidity, and the frequent recurrence of clinical episodes render this disease of great concern to patients and health care providers [5]. Increased genital shedding of HIV reported in men with genital ulcers caused by HSV-2 [6] has further emphasized the importance of this infection. Recent findings clearly indicate that seropositivity for HSV-2 is associated with viral shedding in the genital tract, even in subjects with no reported history of genital herpes [7].

In sub-Saharan Africa, studies of HSV-2 have been scarce, owing to the lack of available laboratory tests and facilities. However, high seroprevalence rates in young adults (60%–80%) have been recorded in population-based studies [8–10]. Recent evidence suggests that an increasing proportion of genital ulcers may be attributed to HSV-2 in many parts of Africa [11–13]. In fact, HSV-2 infection and recurrences would be expected to increase in frequency and severity in areas with high HIV prevalence, because of immunosuppression [14].

The present study aimed to measure the HSV-2 seroprevalence and the prevalence of HSV-2 shedding in African women, with or without genital symptoms, living in an area with high HIV-1 prevalence. We sought to investigate the relationship between HSV-2 and HIV-1 infection by comparing the prevalence and quantity of HSV-2 genital shedding in HIV-1–positive

and HIV-negative women, as well as the association between HIV-1 and HSV-2 genital shedding among dually infected women.

Subjects and Methods

Three hundred consecutive and consenting women attending the Centre National de Référence des Maladies Sexuellement Transmissibles et du SIDA in Bangui, the capital city of the Central African Republic, were enrolled in the study. The center offers multipurpose reproductive health services, including provision of STD services, and operates the main voluntary HIV testing and counseling center in Bangui. Reasons for attendance, sociodemographic and behavioral data, and obstetric and STD histories were elicited in a structured interview. Women underwent general, genital, and pelvic examination, during which serum, vaginal, and cervical samples were collected. Clinical immunosuppression was assessed by using the World Health Organization (WHO) Bangui clinical case definition for AIDS in Africa [15]. A 7-day follow-up appointment was arranged for all women, and appropriate treatment was provided free of charge for any treatable STD syndrome or genital pathogen diagnosed.

Samples Taken and Laboratory Methods

Serology. A venous blood specimen was collected for serological testing and biochemistry. Diagnosis of serological syphilis was done by using the rapid plasma reagin test (VD-25; Murex Diagnostics, Dartford, UK): any positive result was confirmed by the *Treponema pallidum* hemagglutination assay (Fujirebio, Tokyo). HIV serology was performed according to the WHO testing strategy, using 2 different ELISAs (Genelavia-Mixt HIV-1/HIV-2; Sanofi–Diagnostics Pasteur. Marnes-la-Coquette, France, and Wellcozyme HIV; Murex Diagnostics Ltd, Dartford, UK); discordant results were analyzed by Western blot (New-LAV Blot I; Sanofi–Diagnostics Pasteur). Reactive samples by both ELISAs or those confirmed by Western blot were considered to be HIV-1 seropositive. Serum antibodies to HSV-1 and HSV-2 were measured with a competitive type-specific ELISA with a high sensitivity (93%) and specificity (91%) in comparison with Western blot (the gold standard test for HSV-2 antibodies) on African and UK sera [16].

Vitamins A and E. The serum concentrations of vitamin A and vitamin E were determined by high-performance liquid chromatography, with the UV reading at 325 nm [17]. The micronutrient levels were compared with international reference values [18], with vitamin A deficiency defined as levels <30 µg/dL and vitamin E deficiency defined as levels <500 µg/dL. Vitamins A and E are micronutrients with antioxidant/anti-inflammatory properties, and deficiency of these vitamins has been associated with HIV-1 disease progression [19].

Vaginal and cervical infections. Standard tests were carried out for the following: bacterial vaginosis (BV), by applying Nugent's scoring method of a Gram-stained vaginal smear; *Trichomonas vaginalis.* by direct microscopy of a wet mount of vaginal secretions; *Candida albicans*, by culture of a vaginal swab on Sabouraud medium; *Neisseria gonorrhoeae*, by cervical culture onto a modified Thayer-Martin agar plate; and *Chlamydia trachomatis*, by an EIA

of an endocervical swab, using the Syva Microtrak assay (Syva Co., Palo Alto, CA).

Cervicovaginal lavages. Cervicovaginal secretions (CVSs) were collected by a standardized, nontraumatic 60-s vaginal washing with 3 mL of PBS. The cellular and cell-free fractions were separated by centrifugation and were kept frozen at $-30°C$ until processing. To avoid the possible misclassification of shedding findings that could result from the presence of seminal plasma in the vagina, CVS samples were further evaluated for semen traces. Prostate-specific antigen (PSA) was detected in the supernatant of CVSs by immunoenzymatic tests (PSA IMX System; Abbott Laboratories, Abbott Park, IL). PSA-positive CVSs were excluded from further analyses.

HIV-1 RNA in plasma and in CVSs. HIV-1 RNA levels in plasma and in the acellular fraction of CVSs were measured by using a commercial hypersensitive assay (Amplicor HIV Monitor Test 1.5; Roche Diagnostics Systems, Branchburg, NJ), with a detection threshold of 20 HIV RNA copies/mL and an upper limit of quantitation of 750,000 copies/mL. This polymerase chain reaction (PCR) analysis has a sensitivity of 98.5% and a specificity of 100% for the detection of HIV-1 RNA subtype A [20], which is the predominant clade in Central Africa.

Statistical Analysis

Data were double-entered and validated by using the Epi-Info 6.0 statistical package (Centers for Disease Control and Prevention, Atlanta). Analyses were carried out by using Stata 6 (Stata Corp., College Station, TX). Virus loads below the detection threshold of 20 copies/mL for both HIV RNA and HSV-2 PCR EIA were included in analyses with a value of 10 copies/mL. Geometric means of virus loads were compared by using a t test on the log values, and correlations between virus loads were assessed by using Spear-

Table 1. Prevalence of sexually transmitted disease (STD) pathogens and STD syndromes among 300 consecutive women attending the National STD/AIDS Reference Centre of Bangui.

STD pathogen or syndrome	All women (N = 300), no. (%)	Women with PSA-negative CVSs (N = 239), no. (%)
Cervicovaginal STD		
Neisseria gonorrhoeae	3 (1)	2 (1)
Chlamydia trachomatis	9 (3)	5 (2)
Trichomonas vaginalis	4 (1)	4 (2)
Candida albicans	128 (43)	97 (41)
Bacterial vaginosis	199 (66)	143 (60)
Any cervicovaginal STD	238 (79)	181 (76)
STD syndrome		
Genital discharge syndrome[a]	57 (19)	42 (18)
Genital ulcer syndrome[b]	10 (3)	8 (3)
Serological STD		
Syphilis (TPHA⁻/RPR⁻)	24 (8)	18 (8)
Human immunodeficiency virus type 1	79 (26)	58 (24)
Herpes simplex virus type 1	297 (99)	237 (99)
Herpes simplex virus type 2	247 (82)	194 (81)

NOTE.   PSA. prostate-specific antigen: CVS. cervicovaginal secretion: TPHA. *Treponema pallidum* hemagglutination assay; RPR. rapid plasma reagin.

[a] Symptoms of vaginal discharge. plus vaginal or cervical discharge on clinical examination.

[b] Genital ulcer or erosion on clinical examination

Table 2. Prevalence of herpes simplex virus type 2 (HSV-2) markers and other indicators, by human immunodeficiency virus (HIV) 1 serostatus, in 239 women with prostate-specific antigen–negative cervicovaginal secretions.

Indicator	N	HIV-1 positive	N	HIV-1 negative	P
HSV-2 markers					
Serum HSV-2 IgG	58	53 (91)	181	141 (78)	.02
Cervicovaginal HSV-2 DNA[a]	53	23 (43)	141	31 (22)	.003
Cervicovaginal levels of HSV-2 DNA, optical density[b]	23	1.85 (1.46–2.36)	31	1.16 (0.89–1.52)	.014
STDs					
Neisseria gonorrhoeae and/or *Chlamydia trachomatis* and/or *Trichomonas vaginalis*	58	3 (5)	181	8 (4)	.81
Bacterial vaginosis	58	33 (57)	181	110 (61)	.6
Candida albicans	58	24 (41)	181	73 (40)	.89
Genital ulcer	58	3 (5)	181	5 (3)	.41
Syphilis	58	5 (9)	181	13 (7)	.78
Contraceptive use					
Oral contraceptive	58	8 (14)	181	33 (18)	.44
Injectable contraceptive	58	3 (5)	181	3 (2)	.14
Pregnant	58	8 (14)	181	35 (19)	.34
Micronutrients[c]					
Serum vitamin A, μg/dL	49	47 (43–52)	168	57 (54–59)	<.001
Serum vitamin E, μg/dL	49	988 (930–1040)	168	1020 (980–1050)	.42

NOTE. Unless otherwise indicated, values are no. (% of *N*) for each indicator.

[a] Among HSV-2–seropositive women.

[b] Among HSV-2 shedders. Values are geometric mean of optical densities (95% confidence interval).

[c] Micronutrient data are missing for 9 HIV-seropositive and 13 HIV-seronegative women. Values are mean (95% confidence interval).

man's rank correlation coefficient. Comparisons of 2 proportions were carried out by using the χ^2 statistic or Fisher's exact test, where appropriate. Linear regression was used to adjust the log virus load associations for potential confounders. Analyses of HIV-1 and HSV-2 shedding were conducted in the subgroup of women who did not show semen traces in their CVSs, so as to attribute the findings entirely to shedding of the viruses under study.

Results

Population characteristics and STD prevalence. A total of 300 women were eligible for enrollment, and none refused. The mean age of the study population was 27 years (range, 15–48 years). Median age at time of first sexual intercourse was 16 years, with a median of 2 reported lifetime partners (range, 1–8). The majority of women were married, either in monogamous (47%) or polygamous (13%) marriages. Most women (62%) had reached secondary education level; 40% were employed, and 39% were housewives. None of the participants was taking any antiretroviral drugs.

Women attended the clinic for various reasons, including specific STD services (12%), antenatal care (15%), and family planning services (15%). However, the main category included women attending for "general gynecological problems" (58%). Overall, 262 women (87%) reported a current vaginal discharge, and 37 (12%) reported current genital ulcers, although only 57 (19%) and 10 (3%) were found to have a genital discharge or genital ulcer on clinical examination, respectively (table 1).

The prevalence of the various STD pathogens and STD syn-

dromes is shown in table 1. Nearly 80% of women had an endogenous vaginal infection (*C. albicans* and/or BV), but only 4 women (1%) had *T. vaginalis* infection, and 12 (4%) had a cervical infection (*N. gonorrhoeae* and/or *C. trachomatis*). Overall, 79 women (26%) were HIV-1 infected, 24 (8%) had evidence of active syphilis, 297 (99%) had antibodies to HSV-1, and 247 (82%) had antibodies to HSV-2. The prevalences of these pathogens and syndromes changed little with regard to PSA status of the CVSs (table 1). Traces of semen were detected in the CVSs of 61 women (20%), and these subjects were excluded from further analyses involving results obtained on CVS samples.

HIV-1–seropositive women were significantly more likely to be HSV-2 seropositive than were HIV-1–seronegative women (91% vs. 78%, $P = .02$; table 2). Of the 10 women with a clinically defined ulcer, all were HSV-2 seropositive and 4 were coinfected with HIV. There were no significant differences between HIV-1–seropositive and –seronegative women in terms of other STDs, BV, contraceptive use, or pregnancy status.

The mean serum concentrations of vitamin A and vitamin E were 52 and 995 μg/dL, respectively, and few women were considered to be deficient in either micronutrient: 4% for vitamin A and 1% for vitamin E, respectively. There was a statistically significant difference in the levels of vitamin A between HIV-1–seropositive and HIV-negative individuals ($P < .001$), but there was no significant difference in vitamin E levels ($P = .42$; table 2).

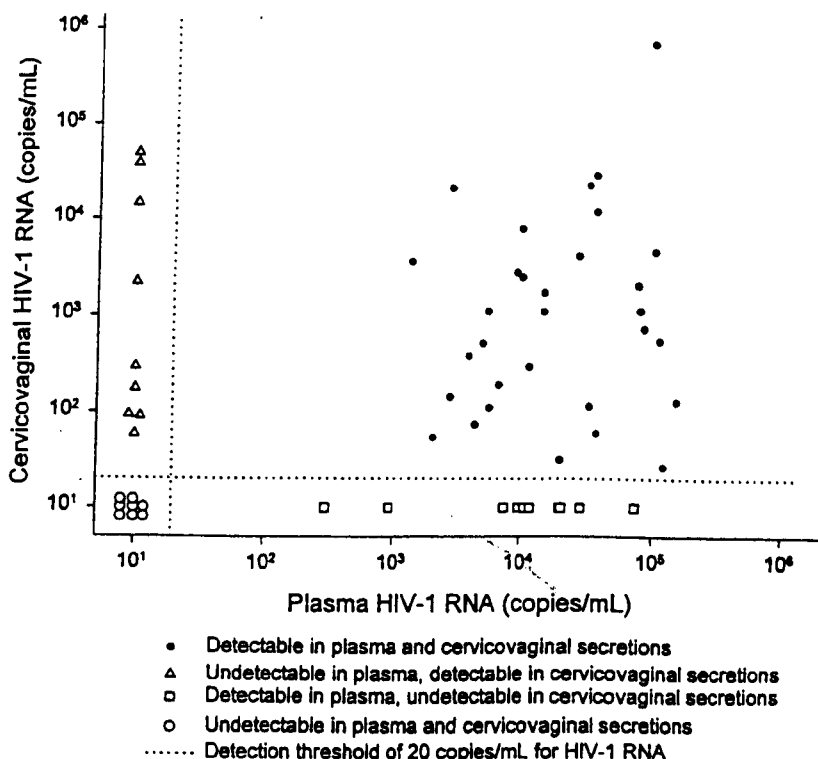*HIV-1 loads in plasma and CVSs.* Among the 58 HIV-

Figure 1. Distribution of human immunodeficiency virus (HIV)-1 RNA levels in paired plasma and cervicovaginal samples among 58 HIV-1-seropositive women with prostate-specific antigen–negative cervicovaginal secretions.

1-seropositive women without semen traces in their CVSs, the geometric mean of HIV-1 RNA plasma load was 3.24 (95% confidence interval [CI], 2.8–3.6) log copies/mL, and the geometric mean of HIV-1 RNA genital tract load was 2.39 (95% CI, 2.1–2.7) log copies/mL.

A positive association was observed between HIV-1 RNA levels in the plasma and genital tract, which was of borderline statistical significance (Spearman's rank correlation coefficient $r = .24$; $P = .07$; figure 1). HIV-1 RNA was detected in the CVSs of 31 (76%) of 41 women with detectable HIV-1 RNA in plasma, compared with 9 (53%) of 17 women without detectable plasma HIV-1 RNA (Fisher's exact test, $P = .12$). There were no significant differences between the women with detectable HIV-1 RNA in their CVSs and the others in terms of STD and BV prevalences, vitamin A levels, contraception use, or pregnancy status (data not shown).

HSV-2 genital shedding. Only HSV-2 DNA was detected in the CVSs. Among the 194 HSV-2–seropositive women without semen traces in their CVSs, those also infected with HIV-1 were about twice as likely to be shedding HSV-2 as were HIV-1-negative women (23/53 [43%] vs. 31/141 [22%]; $P = .003$; table 2). Among the 54 women who were shedding HSV-2, the estimated quantity of HSV-2 DNA in their CVSs, as assessed by optical densities (ODs) of hybridized amplicons, was significantly higher among HIV-1–positive women (geometric mean of OD, 1.85; 95% CI, 1.46–2.36) than among HIV-1-neg-

ative women (geometric mean of OD, 1.16; 95% CI, 0.89–1.52; $P = .01$; table 2).

Association of genital shedding of HIV-1 and HSV-2. HSV-2 and HIV-1 genital virus loads for the 53 dually seropositive women are shown in figure 2. Overall, there was no significant correlation between the levels of HIV-1 RNA and HSV-2 DNA in CVSs (Spearman's $r = -.18$; $P = .21$). HIV-1 RNA in CVSs was detected in 24 (80%) of 30 women without detectable HSV-2 DNA in their CVSs versus 13 (57%) of 23 women with detectable HSV-2 DNA, and this difference was of borderline statistical significance ($P = .07$). There was some evidence that the 30 women without HSV-2 DNA were at higher risk of HIV shedding, due to the presence of other STDs. Although they were less likely to have a genital ulcer than were those with HSV-2 DNA (0/30 vs. 3/23, respectively; $P = .08$), they were more likely to have a cervical infection with *N. gonorrhoeae* and/or *C. trachomatis* (3/30 vs. 0/23, respectively; $P = .25$) or to have BV (19/30 [63%] vs. 11/23 [48%], respectively; $P = .26$), but none of these differences reached statistical significance. Levels of vitamin A were similar in both groups (geometric mean, 0.43 µg/dL among women without HSV-2 DNA vs. 0.45 µg/dL among women with HSV-2 DNA; $P = .80$).

Despite the overall lack of association between genital shedding of HIV-1 and HSV-2, a significant correlation was observed between genital HIV-1 RNA and genital HSV-2 DNA
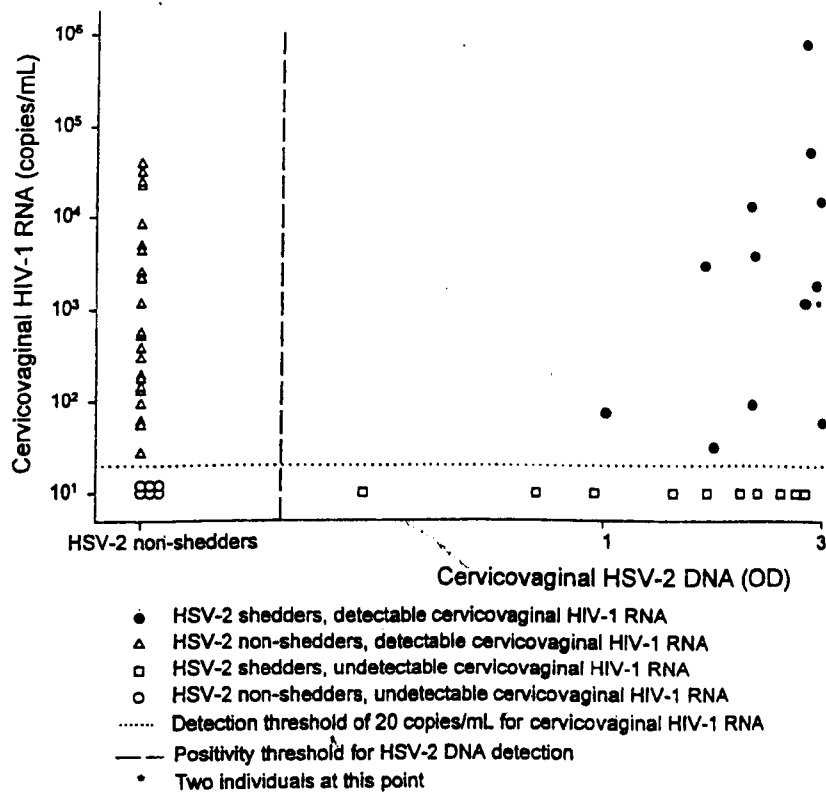
Figure 2. Distribution of human immunodeficiency virus (HIV)–1 RNA and herpes simplex virus (HSV)–2 DNA in cervicovaginal secretions of 53 dually HIV-1– and HSV-2–seropositive women with prostate-specific antigen–negative cervicovaginal secretions

among the subset of 23 HIV-1–seropositive women with HSV-2 shedding (Spearman's $r = .47$; $P = .02$; figure 2).

Discussion

The high HSV-2 seroprevalence found in this study is consistent with prevalences recorded in general populations of Africa [8–10]. Recent studies of patients attending STD services with a complaint of GUD have recorded high rates of HSV-2 isolation (20%–30%) and an association with HIV-1 infection in African settings [11, 12]. In a community-based trial of mass STD treatment in the Rakai district, Uganda, 43% of subjects with genital ulcers were identified with genital herpes by PCR analysis [13]. In contrast. there have been very few studies of genital shedding of HSV-2 among non-GUD patients, particularly among women outside industrialized countries. Our study is the first in sub-Saharan Africa to show both an increased prevalence and an increased quantity of genital HSV-2 shedding among HIV-1–infected women and to show a correlation between the quantities of HIV-1 RNA and HSV-2 DNA in genital secretions among HSV-2 shedders.

Two possibly coexisting mechanisms could explain such findings. First, an increased shedding of both viruses could be observed because of immunosuppression associated with HIV-1 disease, or it could occur under the influence of other systemic

or local cofactors of HIV-1 genital shedding. Second, HSV-2 may be an independent cofactor of HIV-1 shedding, and activation of HSV-2 shedding may be accompanied by increased HIV-1 shedding.

In support of the first mechanism, whereby HIV-1 enhances HSV-2 shedding, although anecdotal evidence supports the notion that clinical expression of HSV-2 infection is increased in the presence of advanced HIV-1 disease, there have been surprisingly few studies to examine the impact of HIV infection on the natural history of HSV infection [5]. One previous study demonstrated increased shedding of HSV-2, associated with declining CD4 T cell counts, among HIV-1–seropositive women in New York City [25]. However, in a study of HIV-1–seropositive female sex workers in Mombasa, Kenya, only women with very low CD4 T cell counts (<200/μL) showed increased HSV-2 DNA positivity in cervical swabs [26]. We do not have data on CD4 T cell counts in our study population, but the women attending this outpatient clinic did not show evidence of clinical immunosuppression, as assessed by the WHO Bangui clinical case definition for AIDS in Africa [15]. Furthermore. micronutrient levels in this population were essentially within normal ranges, according to reference values, which suggests that most of these women were not at an advanced stage of HIV infection [19].

In support of the second mechanism, whereby HSV enhances

HIV-1 transmission, in vitro studies have suggested that HSV can activate latent HIV-1 or enhance its replication [27]. In vivo studies demonstrating a direct correlation between HSV infection and increased HIV-1 burden have been rare. One small prospective study conducted among 16 patients demonstrated that an acute HSV episode can result in transient 3–4-fold increases in levels of plasma HIV-1 RNA [28]. In another study of 16 men with proven herpetic ulcers in Seattle, Schacker et al. [6] found large amounts of HIV-1 DNA in the ulcers, and treatment or healing of the ulcers was accompanied by a decrease in HIV-1 shedding.

An important determinant of HIV-1 shedding may be the HIV-1 plasma load. We found a positive association, but of borderline significance, between HIV-1 RNA levels in plasma and in genital secretions. Studies conducted in industrialized countries have usually found a clearer association [29]. The difference could be partly explained by different sampling methods, which might have led to the presence of blood in the genital tract sample in other studies [29], or could be attributed to the relatively small sample size and low power of our study. Alternatively, our data may support the notion of compartmentalization of HIV-1 replication, in keeping with previous studies demonstrating distinct viral HIV-1 variants, and immunological responses to HIV, between peripheral blood and the female genital tract [30]. Such data on women living in Africa are still scarce. It is possible that other systemic or local factors, in addition to HIV-1 plasma virus load, may be very important in determining genital shedding of HIV-1 in Africa, and these may include HSV-2 and other STDs, such as cervical infections or changes in the vaginal flora [31], as was also observed in our study.

The interactions between HSV and HIV infections are not fully understood. Among HIV-positive individuals, HSV-2–associated GUD may enhance HIV shedding and infectiousness by disrupting genital mucosal integrity. Among HIV-negative individuals, HSV-2–associated GUD may increase susceptibility by disrupting mucosal integrity, but also by the recruitment and activation of HIV target cells, and possibly by HIV taking advantage of chemokine receptors [3]. A number of cohort studies have demonstrated that HSV-2 seroconversion, with or without obvious clinical disease, was a risk factor for HIV-1 seroconversion [3]. Studies to explain the mechanism by which the largely asymptomatic genital shedding of HSV-2 in women may act as a cofactor for HIV infection are lacking. Our cross-sectional study cannot demonstrate a causal role of HSV-2 in HIV-1 transmission, and further longitudinal and intervention studies will be necessary to elucidate this point. However, in the face of the severe and worsening HIV-1 epidemic in Africa, the high prevalence of HIV-1 and HSV-2, and the high frequency of asymptomatic HSV-2 shedding, a possible synergistic effect between the infections, as suggested by this study, may be cause for concern. We have learned in the past decade that no single approach will contain the HIV/AIDS epidemic, and

we need to consider all available means of control, however imperfect. The Mwanza study in Tanzania demonstrated that improved STD case management was an important additional HIV prevention strategy [32], resulting in a 40% reduction in HIV incidence in the general population. No such reduction was achieved by STD mass treatment in the Rakai study in Uganda [13], and one reason postulated for this was a higher prevalence in Rakai of STDs, such as genital herpes, that were not targeted by the antimicrobial regimen [33].

Our data prompt a number of challenges and potential avenues for further research on genital herpes and STD/HIV control in Africa. To demonstrate the enhancing effect of HSV-2 on HIV transmission or acquisition, it will be necessary to conduct randomized intervention trials that specifically target HSV-2, measuring the outcome in terms of HIV shedding or HIV incidence, as well as HSV-2 shedding. Such interventions could include the addition of antiherpetic treatment to syndromic management algorithms for STD patients in Africa, which may become feasible now that acyclovir is out of patent; the provision of suppressive therapy against HSV-2 for individuals dually infected with HSV-2 and HIV, an option not likely to be feasible on a large scale in most developing countries, but which could be tried in high-risk groups, such as commercial sex workers; and the development and testing of preventive interventions, particularly those targeting youth [34]. However, it may be that the most realistic hope for control is the development of a safe and effective HSV-2 vaccine [34].

Acknowledgments

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High frequency of asymptomatic shedding of herpes simplex virus type 2 in African women

Clin Microbiol Infect 2000: 6: 56–57

Recently, the Rakai study tested the hypothesis that community-level control of sexually transmitted disease (STD) would result in lower incidence of HIV-1 infection in comparison with control communities [1]. This randomized, controlled, single-masked, community-based trial of intensive STD control, via home-based mass antibiotic treatment, took place in Rakai District, Uganda. Ten community clusters were randomly assigned to intervention or control groups. All consenting residents aged 15–59 years were enrolled, visited in the home every 10 months, interviewed, asked to provide biological samples for assessment of HIV-1 infection and STD, and were provided with mass treatment (azithromycin, ciprofloxacin, metronidazole in the intervention group, vitamins/anthelmintic drug in the control group). However, no effect of the STD intervention was observed on the incidence of HIV-1 infection. In the Rakai population, a substantial proportion of HIV-1 acquisition appears to occur independently of treatable STD cofactors. Untreated genital infections such as genital herpes might overwhelm any effects associated with treatment of classic STD. In the Rakai study, serological testing for herpes simplex virus type 2 (HSV-2) revealed a high prevalence of 31% in men and 61% in women. Furthermore, 43% of self-reported genital ulcers were for HSV-2 by multiplex polymerase chain reaction (PCR). Asymptomatic genital shedding of HSV-2 may be a cofactor of heterosexual transmission of HIV that was not investigated in the study population

We have evaluated the frequency of asymptomatic genital shedding of HSV-2 in women of childbearing-aged who were attending the National Reference Center for Sexually Transmitted Diseases of Bangui, the capital of the Central African Republic. In this population, 80% of women were seropositive for HSV-2 using a type-specific enzyme-linked immunosorbent assay [2]. Cervicovaginal lavage samples were prospectively obtained from 280 women without clinical evidence of genital ulcers, of whom 78 (27.8%) were seropositive for HIV-1. HSV-2 DNA sequences were detected in the acellular part of cervicovaginal secretions by PCR followed by DNA enzyme immunoassay hybridization. Seventy-nine (28.2%) women were shedding HSV-2. The HSV-2 shedding was nearly two-fold higher in HIV-infected women [41.0% (32 of 78)] in comparison with HIV-negative women [23.3% (47 of 202)] ($P = 0.0001$). These findings clearly demonstrate that asymptomatic genital carriage of HSV-2 may be very common in an African population that is highly seropositive for HSV-2.

Co-infection by HIV and HSV-2 increases the rate of asymptomatic genital shedding of HSV-2. Asymptomatic replication of HSV-2 in the genital tract of HIV-infected women may increase the genital replication of HIV [3,4], resulting in

both increased infectivity and female-to-male transmission of HIV. Thus, bidirectional interaction between HIV and HSV-2 may act synergistically in a vicious circle, each infection enhancing the genital replication of the other. In a population such as the Rakai population, where seroprevalence for HSV-2 is high, symptomatic HSV-2 infection (genital ulcer) as well as asymptomatic genital HSV-2 shedding may constitute major cofactors for HIV transmission. In areas of high prevalence for HSV-2 infection, intervention measures should include: (i) revision of current guidelines for genital ulcer management to include antiviral treatment for herpes, and (ii) HSV-2 screening and suppressive antiviral therapy among HIV and HSV-2 co-infected individuals (possibly difficult to realize in Africa).

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Letters to the Editor

Vitamin A Deficiency and Genital Tract Infections in Women Living in Central Africa

To the Editor: Low vitamin A and carotenoid levels could increase the risk of sexual HIV acquisition by altering the integrity of the genital epithelium or by providing immunologic dysfunction (1). The impact of vitamin A deficiency on HIV-1 acquisition remains, however, conflicting. Mac Donald et al. (2) recently reported the lack of relationship between vitamin A deficiency and an increased risk of HIV-1 infection among men with concurrent sexually transmitted infections (STI) in Nairobi, Kenya, suggesting that vitamin A deficiency is not associated with increased susceptibility to HIV. In the same way, a transversal study of HIV-1 seroconverting women in Rwanda (3) showed no difference in the mean serum retinol levels between HIV-1-seroconverting and HIV-1-seronegative women. In contrast, low serum provitamin A carotenoid levels were associated with an increased risk for heterosexual HIV acquisition in male or female patients suffering from STI in Pune, India (4). We herein report that vitamin A deficiency may favor the receptivity to genital tract infections, including STI, which are major cofactors for both HIV transmission and acquisition via sexual intercourse (5).

We enrolled 275 consecutive and consenting women attending the Centre National de Reference des Maladies Sexuellement Transmissibles et du SIDA, in Bangui, the capital city of the Central African Republic. The center offers multipurpose reproductive health services including provision of STI services, as well as operating as the main voluntary HIV testing and counseling centre in Bangui, as previously described (6). Women underwent general, genital, and pelvic examination, during which serum, vaginal, and cervical samples were collected. A 7-day follow-up appointment was arranged for all women and appropriate treatment was provided free of charge for any treatable STI syndrome or genital pathogen diagnosed. All women were tested for HIV and syphilis serologies. Standard tests were carried out for bacterial vaginosis (BV), *Trichomonas vaginalis* (TV), *Candida albicans* (CA), *Neisseria gonorrhoeae* (NG), and *Chlamydia trachomatis* (CT) by an enzyme immunoassay of an endocervical swab, and *Haemophilus ducreyi*, as previously described (6). Vitamin A concentrations in serum was determined by high performance liquid chromatography with UV reading at 325 nm (7). The micronutrient levels were compared to international reference values, with vitamin A deficiency defined at levels <30 μg/dL (8). Statistical analyses were carried out using InStat software (GraphPad, Inc., San Diego, CA, U.S.A.).

The mean age of the study population was 27 years (range 15–48 years). Median age of first sexual intercourse was 16 years, with a median of two reported lifetime partners (range 1–8). The majority of women were married, either in monogamous (47%) or polygamous (13%) marriages. The prevalence of the various genital pathogens is shown in Table 1. Nearly 80% of women had an endogenous vaginal infection (CA

TABLE 1. *Vitamin A serum levels according to infection status in 275 women of childbearing age consulting the main STI clinic of Bangui, Central African Republic*

| | Vitamin A levels (μg/dL) | | | | |
| | Negative | | Positive | | |
Infections	N	Mean (SD)	N	Mean (SD)	*p* value
HIV	204	2.02 (0.61)	71	1.72 (0.62)	<.001
Syphilis[a]	251	1.97 (0.64)	24	1.60 (0.43)	<.01
Bacterial vaginosis[b]	110	1.95 (0.61)	165	1.82 (0.49)	NS
Candida albicans	147	1.95 (0.62)	128	1.94 (0.65)	NS
Trichomonas vaginalis	271	1.94 (0.63)	4	2.11 (0.38)	NS
Neisseria gonorrhoeae	271	1.95 (0.63)	4	1.52 (0.66)	NS
Chlamydia trachomatis	266	1.94 (0.64)	9	2.00 (0.49)	NS

[a] Serologic diagnosis of active syphilis was defined by a positive rapid plasma reagin test (VD-25 Murex Diagnostics, Dartford, UK), with positive confirmatory Treponema pallidum hemagglutination assay (TPHA, Fujirebio, Tokyo, Japan).

[b] Bacterial vaginosis was diagnosed by the Nugent scoring method of a Gram-stained vaginal smear.

SD, standard deviation; NS, not significant.

and/or BV), but only 4 women (1.4%) had TV, and 13 (4.7%) had a cervical infection (NG and/or CT). Overall, 71 women were HIV infected (26%) and 21 (8%) had evidence of active syphilis.

The mean serum concentration of vitamin A was 52 μg/dL and only 15 (5.5%) women were considered deficient. There was a statistically significant difference in the levels of vitamin A between HIV-1-seropositive and HIV-negative individuals (*p* < .001), and between syphilis-seropositive and syphilis-seronegative individuals (*p* < .01) (Table 1). The proportion of HIV-infected patients with serum vitamin A deficiency was higher than that of HIV-negative patients (*p* < .005). In patients harboring only one genital tract pathogen, the proportion of patients with vitamin A deficiency was higher in HIV-infected patients than in HIV-negative patients (*p* < .01) (Table 2). The

TABLE 2. *Vitamin A deficiency and HIV serostatus according to the number of genital tract infections[a] or seropositivity for syphilis in 275 women of childbearing age living in Central Africa*

| Infections, n | Vitamin A deficiency[b] | | | | *p* value |
	N (%)	n (%)	HIV+ (%)	HIV− (%)	
0	129 (47)	1 (0.7)	1/34 (<1)	0/95 (0)	NS
1	128 (46)	6 (6.3)	4/29 (14)	2/99 (2)	.008
2	13 (5)	4 (15.4)	2/4 (50)	2/9 (21)	NS
3	5 (2)	4 (80.0)	3/4 (75)	1/1 (100)	NS
Total	275	15	10/71 (14)	5/204 (2.5)	.002

[a] Genital tract infections were *Candida albicans*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis*.

[b] Vitamin A deficiency is defined as levels <30 μg/dL.

NS, not significant.

proportion of patients with serum vitamin A deficiency increased in parallel with the number of diagnosed genital tract infections or seropositivity for active syphilis, both in HIV-negative and in HIV-infected patients ($p < .0001$ and $p = .0002$, respectively, using the χ_2 test for trend).

In the present study, vitamin A deficiency was associated in an additive fashion with the number of genital tract infections diagnosed or seropositivity for active syphilis in women attending the main STI clinic of Bangui. These findings suggest that patent vitamin A deficiency favors genital tract infections, likely because vitamin A deficiency per se may increase the susceptibility to genital tract infections (1). In HIV-infected women, genital tract infections are associated with increased infectiousness of genital secretions (9), thus providing increased risk to transmit HIV infection to an exposed sexual male partner. In HIV-seronegative women with genital tract infections such as syphilis, T. vaginalis, N. gonorrhoeae and C. trachomatis, higher susceptibility to heterosexual acquisition of HIV has been demonstrated (5). In women suffering from C. albicans genital infection, the inflammatory conditions affecting the cervicovaginal mucosa may also lead to increased risk for HIV acquisition or transmission, especially when other cofactors of transmission exist (10,11). Finally, the findings in the present report raise the possibility that vitamin A deficiency may increase the risk of female-to-male as well as male-to-female HIV transmission, because it indirectly increases the probability of female genital tract pathogens, including STI, which act as major cofactors for HIV transmission. Further prospective studies should focus on vitamin A supplementation in individuals attending STD clinics in Africa, both to assess its potential for curing and preventing genital tract infections, and also its use in reducing HIV sexual transmission.

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Herpes simplex virus type 2

and heterosexual spread of human immunodeficiency

virus infection in developing countries :

hypotheses and research priorities

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Introduction

Herpes simplex virus type 2 (HSV-2) is a double-stranded DNA virus belonging to the *Alphaherpesvirinae* sub-family [1]. Infection is characterised by both neural and cutaneo-mucous tropism [1]. HSV-2 infection is almost always sexually transmitted and causes genital ulceration. HSV-2 causes a persistent infection which may give rise to recurrent clinical episodes. Significant progress in our understanding of HSV infection has occurred over the last decade, in part related to the development of accurate and sensitive laboratory tests to study HSV-2 [2]. The application of PCR and type specific serology to individual cases and in population based studies have identified a potentially important role for HSV-2 infection as a cofactor in the sexual transmission of HIV. This is a particular issue in developing countries. It has been established that genital HSV-2 infection is frequently unrecognized and that asymptomatic HSV-2 shedding may be important for transmission [3]. Commercial type-specific serology assays have become available allowing population based serological surveys of HSV-2 infection which have shown that: (i) HSV-2 prevalences are high in some western countries and very high in developing countries ; (ii) The global prevalence of genital HSV-2 has probably increased during the past 10 years ; (iii) HSV-2-seropositivity may be a useful marker of high risk sexual behaviour in some settings. In many countries in sub-Saharan Africa, the HSV-2 epidemic has spread in parallel with the HIV epidemic. This review will describe the epidemiology of HSV-2 infection in the HIV era, the hypotheses for HSV/HIV interactions, and research priorities for the developing world.

I. HSV-2 infection: a growing public health problem

I-1. Natural history of genital herpes

Most HSV infections are unrecognised. Susceptible individuals either develop primary infection after their first exposure to HSV-1 or HSV-2. Genital herpes infections can be associated with serious morbidity. First-episode genital herpes can cause painful ulcerative lesions and systemic manifestations, including headache, malaise, and fever lasting up to 3 weeks [2]. Complications in men are rare; aseptic meningitis and urinary retention are more common in women [1]. In addition to the latent state, subclinical or 'asymptomatic' infectious viral shedding is frequent [3]. The median recurrence rate after a symptomatic first episode of genital herpes is 4 to 5 episodes per year. Patients who suffer severe first episodes have even higher recurrence rates [4]. Studies have demonstrated that many or most seropositive persons shed HSV-2 that is detectable by culture from the genital tract, and many have symptoms, such as itching and discharge, that are directly referable to HSV-2 detectable by culture [3-4]. Such symptoms, however, are not often recognised as indicating an infection [5-6]. In most studies, the proportion of infections that are both symptomatic and recognised (by patient and clinician) vary between 13% and 37%. This proportion is higher among HIV-positive individuals.

Transmission and acquisition of HSV-2 infection. HSV-2 infections are almost always sexually transmitted. Most genital genital HSV infections are caused by HSV-2; however, an increasing proportion is attributable to HSV-1 [2]. Prospective studies of HSV-2 discordant partners have shown that most transmission events were not associated with a clinically recognised HSV-2 recurrence in the infected partner [5,7]. As for other sexually transmitted infections (STIs), the risk of acquisition of HSV-2 seems to be higher in women than in men [5,7-8]. This increased risk of acquisition of HSV-2 may relate to the higher number of HSV-2 recurrences in infected men (about 20% higher than in women) [9]. It may also be related to biological factors such as the larger and more vulnerable genital mucosal surface of women [10,11].

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Extent and frequency of subclinical shedding of HSV-2. Data have recently

been accumulating, mainly from the USA, emphasizing the frequency of subclinical

HSV-2 shedding and its role in the transmission of HSV-2 infection to sexual partners

and neonates [12-14]. These studies have shown that the level of virus required to infect

the neonate can sometimes be small [15]. Although it is not known what proportion of

new disease acquisitions (outside these groups) is related to subclinical shedding, it is

likely to contribute to the spread of HSV-2 disease [14]. Reported rates of subclinical

shedding vary widely between studies [14]. Shedding from internal and external genital,

perineal, and perianal sites is frequent [3,16-17].

The frequency of clinical recurrence and asymptomatic shedding is significantly

lower in HSV-1 genital infection than HSV-2 [14]. There is no difference in shedding

rates between those individuals who are seropositive for HSV-2 alone and those who are

positive for both HSV-1 and HSV-2 [14]. The frequency of subclinical shedding rises

with the number of clinical attacks, although this association may be lost with a low

number of lesions [18]. Moreover, the frequency of subclinical shedding is highly

dependent on the methodology used for detection. Multiple site swabbing and the

application of highly sensitive methods, such as PCR assays, have increased the yield

[3]. PCR detection has shown that the virus may be present on mucocutanous surfaces

up to 3.5 times more often than previously found by culture [3,19]. Such virus is

predominantly associated with subclinical shedding [14]. Quantitative PCR shows that,

overall, HSV DNA titre tends to be higher on lesional days compared to non-lesional

days [3].

I-2. Epidemiology of HSV and HSV-related genital ulcer

Developed countries. It has been recognised for decades that HSV is transmitted sexually [20]. Both HSV-1 and HSV-2 can cause primary genital herpes, with HSV-1 accounting for nearly half of the cases in some centers [21]. In western countries, HSV-1 rates are dropping in childhood [22] and many adolescents are infected with HSV-1 as a result of their first exposure to the virus during sexual activity (infection acquired by genital-to-genital contact or by oral-genital contact) [23]. Nevertheless, HSV-1 reactivates less frequently from latency in sacral ganglia than HSV-2, so most episodes of recurrent genital herpes are caused by HSV-2 [20, 24].

A population-based study in the USA, reported that, in 1996, 22% of adults were seropositive for HSV-2 [25]. This figure had increased by about 30% since a previous similar survey a decade earlier [26], despite widespread health education programmes in the 1980s and 1990s about the importance of 'safe-sex' in response to the HIV epidemic [27]. Between 1988 and 1994, 45 million people were estimated to have acquired HSV-2 infection [25], which represents 1 in 5 people over the age of 12 years. The HSV seropositivity rate in HIV-infected persons are even higher. Hook and colleagues in Baltimore, USA, showed an HSV-2 seroprevalence rate of 81% among HIV-positive homosexual or bisexual men [28]. Indeed, genital herpes transmission appears to be epidemic within the USA. Other countries have been slower to document the extend of HSV exposure, but emerging results showed that the HSV-2 epidemic has pandemic proportions [20].

Developing countries. In sub-saharan Africa, epidemiological studies of HSV-2 have recorded high seroprevalence rates in a range of adult populations : 40% in Kinshasa [29], 40% in Zimbabwe [30], 30%-50% in South Africa [31] 51% in Rwanda [32], 67% in Tanzania [33], 68% in Uganda [32] and even higher levels in the Central African Republic (82%) [34], demonstrating that HSV-2 is a common genital infection

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in Africa. Infection with HSV-1 is endemic in these populations, and is mostly acquired in childhood [33-35]. HSV-2 prevalence rises rapidly in adolescents who are sexually active [36] and increases with age [33]. In Asia, HSV-2 seroprevalences range from 6% in general populations to 50% in high risk groups [36]. The low rate of HSV-2 in young versus old Chinese women can be explained by the effective control of sexually-transmitted infections after the revolution [36] whereas, the low rates of HSV-2 seroprevalence in some isolated tribes in Brazil suggest a recent introduction of the virus [36].

Numerous epidemiological studies clearly demonstrate that chancroid, syphilis and genital herpes are all common causes of genital ulcer disease (GUD) in developing countries [37]. However, there is some evidence that there have been changes in the aetiology of GUD during the past ten years in parallel with the spread of AIDS epidemic [32], and this may effect the impact of current syndromic management. A prospective study in South Africa from 1986 to 1998 showed that the proportions of GUD due to chancroid and syphilis decreased during the study period [37]. Of the 239 GUD diagnosed in 1986, 53% were chancroid and 12% syphilis. By 1998, 33% and 3% of 200 GUD cases were chancroid and syphilis respectively. In parallel, a rapid increase in the proportion of herpetic ulcers, in both HIV-positive and negative subjects, was noted in the same setting [3/239 (1%) in 1986 and 47/200 (24%) in 1998] [37].

I-3. HSV-2 as a marker for sexual behaviour

An association between HSV-2 infection and reported sexual behaviour has been observed in studies performed in industrialized countries [26,36,38]. Thus, a good correlation between the prevalence of HSV-2 antibodies and the number of life-time sexual partners, particularly in men, has been observed [36]. It also appears that with an equal number of sexual life-time partners, women will have a higher prevalence of HSV-2 antibodies than men [36].

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In contrast to studies in industrialized countries, Obasi and colleagues found no significant association with age at first intercourse [33]. In this study, HSV-2-infected persons were more likely to report higher numbers of sex partners. Furthermore, the trend for increasing prevalence of infection with increased number of lifetime partners was statistically significant for both men and women, even after adjustment for age and residence [33]. HSV-2 serology may be a useful objective biological marker for changes in sexual behaviour in HIV intervention studies since HSV-2 is more readily transmitted sexually than HIV. However, given the persistent nature of the infection, seroprevalence will be more discriminating in younger age groups [33]. Therefore, HSV-2 seroincidence would be a preferable marker of behaviour change, especially in countries in sub-Saharan Africa where there is high incidence among young people.

Indeed, the recent study of Auvert and colleagues among youth in Carletonville (South Africa) demonstrated that HIV infection was associated with HSV-2 seropositivity and sexual behaviour [31]. The strong association between HIV infection and HSV-2 seroprevalence, and the fact that HSV-2 infection was a frequent cause of genital ulcers indicated that HSV-2 could play a major role in the spread of HIV in the population of young people of South Africa [31]. -

II. Genital HSV-2 as a possible cofactor of HIV infection

II-1. STI : Cofactors of sexual transmission of HIV

Epidemiological [39-41] and intervention studies [42-43] in sub-Saharan Africa, have demonstrated the role of STIs in facilitating the acquisition and transmission of HIV. This has provided a strong argument for making STD control an integral part of HIV prevention strategies [29,43-44]. In fact, STDs that cause genital ulceration such as syphilis, chancroid and HSV-2 infection are particularly implicated in facilitating HIV

transmission [17,28,30,41,45-47]. Genital ulcer disease (GUD) is believed to increase the risk of HIV acquisition per sexual exposure by increasing the amount of HIV shedding through genital lesions and by providing an easier portal of entry for the virus into the host [30,48]. These findings have been supported by recent biological studies that have shown the role of certain STD conditions such as gonorrhoea and genital ulcers in men [49] and in women [50-51] in enhancing HIV shedding [49].

Although GUD is a common complaint at STD clinics in Africa, screening for and management of HSV-2 is rarely done [30]. Nevertheless, studies have found that HSV-2 was common in STD clinic attendees in Africa [52]. For example, in Kampala, HSV-2 was present in 36% of GUD patients [53]. In a study that screened sera from Dakar, the prevalence of HSV-2 ranged from 20% among surgical patients to 96% among prostitutes [23]. High prevalences of HSV-2 were recorded also in rural populations of Uganda and Tanzania [33,35]. Taking into account the high prevalence levels of HSV-2, the increased shedding of HIV through genital herpes lesions, and the fact that persons with HSV-2 remain potentially infectious for life, HSV-2 may make an important contribution to HIV transmission in Africa [30].

II-2. Bi-directional interaction between HSV-2 and HIV

In their review of available data on the role of STIs in the sexual transmission of HIV infection, Flemming and Wasserheit [39] group the evidence that STIs facilitate the transmission of HIV into three categories: a) biological plausibility studies; b) HIV seroconversion studies; and c) community-level intervention studies. Whilst there is ample evidence for the role of ulcerative and non-ulcerative bacterial STIs in enhancing HIV transmission and acquisition, through the three lines of evidence, little is known about the relationship between HIV and viral STIs such as HSV.

Biological plausibility suggesting interactions between HSV-2 and HIV

During sexual intercourse genital ulcers may bleed, leading to the increased risk of HIV transmission. Studies of HIV infected people with genital ulcer disease (GUD) suggest that GUD may increase infectiousness as HIV proviral DNA have been detected in genital ulcer exudates [54]. Similarly, Schacker and colleagues [47] provided further evidence to support the hypothesis that genital herpes infection increases the efficiency of the sexual transmission of HIV-1. In their study, HIV RNA was consistently detected in genital ulcers caused by HSV-2 in HIV-1 seropositive men in Seattle [47]. Moreover, treatment or healing of GUD was accompanied by a decrease in HIV shedding [47]. An increased viral load and the proximity of virions to cutaneous surfaces are factors that may lead to the increased transmission of HIV in the presence of HSV [47].

Among HIV-negative people, GUD may increase susceptibility by disrupting mucosal integrity; by the recruitment and activation of HIV target cells. There is also evidence that (i) among 'asymptomatically' HSV-2 infected patients (ie in the absence of visible ulcer) HSV-2 genital shedding is increased in HIV positives [17,34]; (ii) both HIV RNA and HSV-2 DNA shedding are increased in the presence of the other virus [34]. Another study also demonstrated that HSV-2 reactivation is associated with an increase in plasma HIV-1 RNA and intracellular gag mRNA and that plasma HIV-1 RNA level decreases significantly during treatment with acyclovir [55]. Kucera and colleagues [56] reported that co-infection of human CD4+ cells with HSV and HIV result in an accelerated replication of HIV.

Similarly, Heng and colleagues [57] found that the up-regulation of HIV-1 expression was induced by HSV, which may translate to increased plasma HIV-1 RNA levels. Their study documented in vivo reciprocal enhancement of viral replication associated with the co-infection of keratinocytes and macrophages by HIV-1 and HSV-1 in patients with AIDS and HSV lesions. Indeed, their observation may be evidence of the importance of including antiherpetic drugs as part of the overall treatment of patients with AIDS to avoid the excessive replication of HIV-1 in both CD4+-dependent and -

independent targets [27]. In addition, a recent study reported that the inclusion of acyclovir together with antiretroviral therapy may prolong survival in HIV seropositive individuals [27]. Furthermore, Rando and colleagues [58] showed that HIV Long Terminal Repeat (HIV LTR) is activated by herpesviruses as measured by HIV LTR directed expression of acetyltransferase gene (CAT). Other studies also demonstrated that alpha and beta genes of HSV are responsible for the transactivation of HIV gagLTR [59-62].

As a result, it is possible that HIV progresses more rapidly in untreated HSV-2 positive individuals. However, evidence is inconclusive and more studies of the effect of episodic HSV-2 therapy on HIV are needed, especially in developing countries. The impact of HIV on HSV, with regards the clinical reactivation of HSV, the role of immunosuppression and the possible frequency of asymptomatic carriage, remains however largely undetermined.

Studies of HIV incidence in patients with genital ulcerations due to HSV-2 or HSV-2 seropositive

Eight studies of HIV incidence in high risk individuals assesed the role of genital HSV-2 in the sexual transmission of HIV [39] including five studies on homosexual men [45,63-66].

Only three studies have investigated the role of HSV in heterosexual transmission of HIV infection [67-69]. In two studies, the rate of HIV seroconversion was higher among individuals who were HSV seropositive. In a case control study of Thai military conscripts, HSV antibodies were three times more common in HIV seroconverters than men who remained HIV negative (OR 3.1, 95% CI 1.2-7.9) [68].

The second cohort study, also in Thailand military conscripts, found a fourfold increase, which was statistically significant, in the relative risk of HIV seroconversion among those who were HSV seropositive [69]. However, the relative risk was 2.0 (95% CI 0.6 – 6.1) after adjustment for sexual behaviour. A study of STI patients in New York did not find an increased HIV seroconversion associated with HSV infection [67]. In this latter study, HSV infection was diagnosed by clinical examination and Tzanck cytodiagnosis, therefore many HSV infections may have been missed.

There are three nested case control studies that have investigated the role of HSV infection in male to male transmission of HIV [45,63-64]. Holmberg and colleagues observed that the risk of HIV seroconversion was two times greater in men who were HSV-2 seropositive compared to those who were seronegative and HSV-2 seroconverters were at even greater risk of HIV seroconversion (OR=6.0) [45]. Keet and colleagues also found an association between HIV acquisition and HSV-2 seropositivity [63]. However, no association was found in the study by Kingsley and colleagues [64].

Seroconversion studies are likely to underestimate the effect of HSV-2 on HIV transmission. First, HSV is a chronic disease and recurrences decrease over time. Second, HSV seropositivity has poor correlation with clinical episodes. Third, the methods used to measure HSV are often insensitive. And last, there is often failure to adjust for the usual confounders, such as sexual behaviour or other STIs.

Epidemiological data showing an association between HIV and HSV-2 seropositivity in Africa have been scarce [30,33-34]. A multicenter study assessed parameters which could explain the heterogeneity of HIV epidemics in four African cities characterized either by high HIV prevalences (20-30% in Kisumu, Kenya and in

Ndola, Zambia) or by low HIV prevalences (3-8% in Cotonou, Benin and in Yaounde, Cameroon) [70]. This study showed an association between HIV and HSV-2 infection [70]. In fact, the authors of this study demonstrated that cities with high HIV prevalence also have high HSV-2 prevalence. This association is particularly strong among the youth population. Secondly, Weiss and colleagues showed a strong association between HSV-2 prevalence and seropositivity for HIV (OR 5-10) after ajustment for sexual behaviour [70]. Another study of 2397 adults in Harare, Zimbabwe, showed an HSV-2 prevalence of 39,8%, and an incidence of seroconversion for HSV-2 of 6,2 person/year, without clear association to the risk of seroconversion for HIV [71].

Lack of intervention studies focus on genital herpes

The demonstration of any bi-directional and/or synergistic interactions between STDs and HIV infection, added to the fact that HIV is an STD, has direct public health implications [29]. Intervention studies in Africa [29] and in Thailand [72] combining preventive interventions and treatment of STDs demonstrated that it may be possible to reduce the incidence of HIV in the developing world. Furthermore, the Mwanza study in Tanzania demonstrated that improved STD case management was an important additional HIV prevention strategy [43], resulting in a 40% reduction in HIV incidence in the general population. No such reduction was achieved by STD mass treatment in the Rakai study in Uganda [44], and one reason postulated for this was a higher prevalence in Rakai of STDs such as genital herpes that were not targeted by the antimicrobial regimen [73].

Indeed, there are few intervention studies to date to document an impact of anti-HSV-2 treatment on HIV transmission. The Schacker study among 12 men in Seattle is the only study to have documented that treatment or healing of GUD caused by HSV-2 was associated with a reduction of HIV shedding. Even in this instance, it was unclear whether such effect was spontaneous or due to any specific intervention [47].

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III. Research priorities for the developing world

Biological interactions between HSV-2 and HIV

There is an urgent need for more studies to assess the burden of clinical disease due to HSV-2 in different settings. These studies may also contribute to the better understanding of the biological mechanisms that underlie the HSV-2-HIV interaction and may also provide biological endpoints for further intervention trials.

HSV-2 treatment trials

In order to determine whether HSV-2 enhances HIV shedding and transmissibility, randomised intervention studies will be necessary, preferably in settings with high HSV-2 seroprevalence (and shedding) and high HIV seroprevalence and/or incidence. It is accepted that a randomised-controlled trial of interventions could provide the most convincing evidence to demonstrate this relationship.

The aim of such intervention studies should therefore be to decrease HIV shedding in individuals coinfected HIV/HSV (and ideally also HIV incidence in HIV negative partners). This decrease should in theory be mediated by an observed decrease in HSV-2 shedding (i.e. prevalence or duration of shedding and/or quantity of virus particles shed) or lesions apparent or inapparent.

Two types of studies should be considered:

a) Episodic treatment of genital ulcer disease (GUD), by adding aciclovir/valaciclovir to the syndromic management vs. placebo and measuring the effect on HSV-2 and HIV shedding.

b) Suppressive therapy with aciclovir/valaciclovir (the latter drug preferable for its single daily dose) of dually HIV/HSV-2 infected individuals to prevent HSV reactivation.

Effects of HSV2 therapy on HIV shedding and transmission. HSV2 antiviral therapy has been shown to decrease HIV shedding from herpetic lesions in individuals co-infected with HSV2 and HIV. However, there are no studies demonstrating a reduction in the rate of HIV seroconversion.

Therapy to prevent herpetic disease. Although currently there are no data available, there is a biological rationale for therapy in HSV2-negatives. This would aim at preventing herpetic disease rather than infection. Because the first episode of disease would be aborted, fewer latent copies should in theory be present in the ganglia for reactivation, and this should limit the frequency of recurrences. A further consideration is that the practicalities of prophylactic therapy make it unlikely to be feasible on a large scale in developing countries. However, its use could be considered in specific groups at high risk of HIV infection, such as HIV-negative members of discordant couples, sex workers and, possibly, young women.

Viral resistance. It has been reported that HSV can develop resistance to aciclovir through mutations in the viral gene that encodes thymidine kinase, by generation of thymidine-kinase-deficient mutants or by selection of mutants with a thymidine kinase unable to phosphorylate aciclovir [1]. Resistant HSV occurs more frequently among HIV-infected patients, probably due to increased replication of HSV and decreased immunity in these patients [27].

Toxicity. Continuous aciclovir therapy has not caused long-term toxicity in the past decade [1].

Genital mucosal immunity to HSV-2 infection

The control of HSV latency is not understood but mucosal immune responses against HSV are likely to be important in the control of mucosal lesions. Studies in a mouse model on host resistance to HSV-2 have shown that vaginal infection with an attenuated HSV-2 strain induces protective immunity to subsequent lethal challenge with wild-type virus. Both cell-mediated and humoral local immunity, mainly of the IgG or IgA isotype have been described [74-75]. Furthermore, T-cell mediated immunity at the mucosal level is thought to play a major role in the resolution of recurrent HSV infection in animal models [76]. The cellular immunity to HSV is thought to assist in limiting secondary genital HSV-2 infection [5]. Antibodies to HSV of both IgA and IgG isotypes have been detected in cervicovaginal secretions of women with vaginal discharge [77], and in women suffering from primary herpetic genital infection [78-79] or reactivation [78]. However, little is know about their functional properties. Studies to localize neutralizing activity to purified cervical IgG, IgA, or IgM components would be useful.

Vaccination against HSV-2.

Given the high prevalence of HSV2 infection in many countries, and the fact that most infections are subclinical, the development of an effective HSV2 vaccine would provide a powerful control tool.

Most vaccines strategies against herpes simplex virus have focused on the development of subunit vaccines that consist of one or more HSV glycoproteins [80]. In fact, studies have shown that vaccination of mice with purified glycoprotein D (gD) provides protection against a lethal intraperitoneal challenge of HSV and vaccination of guinea pigs with recombinant gD or gB protects against intravaginal HSV-2 infection [81-82]. Clinical trials indicate that purified gD has induced neutralizing antibody responses in previously uninfected persons and boosted the antibody response in patients with genital herpes [83].

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Three HSV-2 vacines have been recently evaluated in clinical trials:

Recombinant Glycoprotein Vaccine gB$_2$ and gD$_2$ with MF59. This subunit HSV vaccine is no longer in commercial development. Phase I studies showed that it was well tolerated and induced specific neutralising antibody and T-cell lymphoproliferation responses comparable to or higher than those seen in HSV2 infected subjects. Two phase III trials have assessed effectiveness of the vaccine in prevention of HSV2 infection. Survival analysis showed a short-term efficacy of 50% for the first five months of follow-up but, thereafter, the effect disappeared [84]. The overall efficacy was 9% (95% CI –29% to 36%), although substantial differences were observed in men and women (-4% in men and 26% in women). Vaccination had no significant influence on duration of the first clinical episode of genital HSV2, or on the subsequent frequency of recurrence. The authors concluded that efficient and sustained protection against sexual acquisition of HSV2 infection will require more than high titres of specific neutralizing anitbodies and, as the vaccine provided only transient protection against HSV2 infection, work has been halted [84].

Recombinant Glycoprotein Vaccine gD$_2$ with adjuvent SBAS4. This subunit HSV vaccine is in commercial development and, again, induced HSV-specific antibody and cell mediated immune responses in phase I studies. Two phase III trials assessed vaccine efficacy in prevention of genital HSV disease with secondary assessment of prevention of HSV2 infection [85]. The vaccine induced significant protection (approximately 70% efficacy) against genital herpes disease in women who were initially HSV1 and HSV2 seronegative. Trends towards protection of women against HSV infection were also seen in both studies (39-48% efficacy), although not statistically significant. In contrast, there was no evidence of protection in women who were initially HSV1 seropositive, or in men. The main disadvantages of this vaccine are the apparent failure to improve on

protection provided by HSV1 infection and the need for frequent vaccine administration to boost host immunity.

Disabled Infectious Single Cycle (DISC) HSV2 Vaccine. Phase I studies have shown that this vaccine is well tolerated and induces neutralising antibody and lymphoproliferative responses comparable to those seen in HSV2 infected subjects [86]. 83% of vaccine recipients developed HSV-specific cytotoxic T lymphocyte responses. Phase II efficacy trials are underway in US and UK to assess efficacy of DISC as a therapeutic vaccine, in the treatment of frequently recurrent genital HSV2 infections. Because of its rich content of HSV2 virion, this product may improve on the natural protection provided by HSV1. However, its closeness to HSV2 means that it would not be possible to distinguish natural infection from vaccine-induced immunity. The main disadvantages of the DISC therapeutic vaccine are that it needs to be administered at least 6 monthly, is expensive and needs further development. Nevertheless, partnerships should be sought to enable the DISC vaccine to be taken to the next stage of development [86]. In addition, vaccine trials to evaluate the efficacy of a therapeutic HSV-2 vaccine should be part of today's research agendas [86].

Conclusion

In the face of the severe and worsening HIV-1 epidemic in Africa, the high prevalence of HIV-1 and HSV-2, and the high frequency of asymptomatic HSV-2 shedding, a possible synergistic effect between the infections is cause for concern. We have learned in the past decade that no single approach will contain the HIV/AIDS epidemic, and we need to consider all available means of control, however imperfect. There is an urgent need for further research on genital herpes and STD/HIV control in Africa. These studies may also help in the better understanding of the biological mechanisms that underlie the HSV-2-HIV interaction and may also provide biological endpoints for

further intervention trials. Studies on patients with genital ulcer disease and HIV will be necessary to see if episodic therapy with aciclovir or another prodrug hasten the healing process, thereby decreasing HIV and HSV-2 shedding. This may lead to revision of current syndromic management practices in developing world settings. We also need to develop and test preventive interventions, particularly those targeting youth. However, it may be that the most realistic hope for control is the development of a safe and effective HSV-2 vaccine.

Acknowledgments

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