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MECHANISMS OF SEXUAL TRANSMISSION OF ENVELOPED VIRUSES AND A NOVEL STRATEGY TO PREVENT INFECTION

by

Vanaja R. Zacharopoulos

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1998

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ABSTRACT

MECHANISMS OF SEXUAL TRANSMISSION OF ENVELOPED VIRUSES AND A NOVEL STRATEGY TO PREVENT INFECTION

by

Vanaja R. Zacharopoulos

Advisor: Dr. Richard Coico

Heterosexual transmission of HIV accounts for the vast majority of AIDS cases in the world. An important factor compounding this problem is the occurrence of sexually transmitted diseases which facilitate the transmission of HIV. There has been increasing interest in the development of female-controlled prevention methods especially since the likelihood of a vaccine against HIV seems to be remote and probably beyond the reach of people who are at greatest need for protection.

In this thesis I have approached the issue of developing a vaginal microbicide by using both in vitro systems and animal models. Initial experiments were performed to understand how viral pathogens can be transmitted in the female genital tract, specifically by addressing the question of whether infected cells originating from semen can penetrate the mucosal barrier of the vagina. By tracking the migration of labelled autologous blood cells from the mouse vagina, cells were found beneath the epithelium and in neighboring lymph nodes a few hours after vaginal inoculation. Using an in vitro model for the sexual

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transmission of the human retrovirus, Human T Lymphotropic Virus Type I, mechanisms of cell to cell adhesion and infection were studied. Certain sulfated polysaccharides were found to block both adhesion of infected cells to target cells as well as subsequent infection. In order to test the efficacy of these compounds in blocking genital infection in vivo, I developed a mouse model for the vaginal transmission of HTLV-I. Although mice were infected by this route, as evidenced by the presence of proviral DNA in spleen cells, the model is not ready for efficient testing of potential antiviral compounds. The mouse model for genital herpes was used to test the ability of candidate compounds to block infection of Herpes Simplex Virus-2 via the vaginal route. The sulfated polysaccharide Carrageenan was highly effective in protecting mice from infection. Antimicrobial peptides were also found to block infection.

The research performed here shows that the development of a vaginal microbicide to protect women against HIV and possibly other sexually transmitted viruses is a feasible and realistic goal. Such a formulation should be able to block free virus as well as virus-infected cells from contact with the target cells in the vagina and/or cervix. More animal models are needed to further verify the antiviral activity of potential compounds.

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I would like to acknowledge the special encouragement and support of Dr. David M. Phillips: if he had not believed I should get a Ph.D, this dissertation would not have been written. Dr. Richard Coico made my experience at the Graduate Center very positive and rewarding. To both, I am deeply grateful.

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INTRODUCTION

The overall objective of my thesis work has been to carry out research which will lead to the development of a topical product (microbicide) which will protect women and men from AIDS and other sexually transmitted diseases (STDs). The product would be in the form of a formulation which would be inserted into the vagina before coitus. The search for a microbicide has led me to several related avenues of work. First I have studied basic mechanisms that are involved in the initial infection during sexual intercourse. This was undertaken because strategies for developing a microbicide are likely to depend on understanding how the Human Immunodeficiency Virus (HIV) is transmitted during sexual intercourse.

I have also worked with other human viruses. HIV infects only man and chimpanzees. A related African Green monkey virus, Simian Immunodeficiency Virus (SIV) infects Rhesus monkeys. However, given the costs associated with testing microbicides in Rhesus monkeys, it would be prohibitively expensive to use this animal model. I have, therefore, concentrated on viruses which can infect laboratory rodents. Much of the work which is described below was carried out with Human T Cell Leukemia Virus type I (HTLV-I). An important human pathogen in its own right, HTLV-I is similar to HIV in that both HIV and HTLV-I are retro-viruses although the relationship between HIV and SIV is closer. I chose HTLV-I because the literature suggested that rodents could be

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infected when they were inoculated with HTLV-I infected cells (119,158). I reasoned that if I could infect mice by vaginal inoculation I would be able to test potential microbicides in animals that are hundreds of times less expensive than monkeys. Before carrying out animal experiments, I studied infection *in vitro* to determine if the infection process during sexual transmission was similar to HIV and to determine if microbicides would block HTLV-I transmission in an *in vitro* model.

Although I have been able to infect mice by the vaginal route, the model is complex and cumbersome as a model for screening potential microbicide formulations. I have therefore carried out much of my thesis research on Herpes Simplex Virus 2 (HSV-2). This sexually transmitted human pathogen is similar to HIV in that they are both enveloped viruses. This is important because the microbicidal compounds that I am interested in act on the viral envelope. However, in other aspects of their biology, HIV and HSV-2 are very different. The advantage of HSV-2 is that mice are easily and reliably infected by vaginal inoculation and infection can be quickly detected. These characteristics of the HSV-2 model have facilitated identification of a very promising new microbicide formulation.

In the remainder of this introductory section I will briefly discuss why there is a need for a microbicide product. Because much of this thesis deals with HTLV-I, I will describe some of the properties of this virus. The rationale of using mice will be discussed briefly because all the animal work has been carried out with mice. Lastly I will briefly describe the organization of the thesis.

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Microbicides

It is estimated that 26 million adults and 2.4 million children in the world are infected with HIV. By the year 2000 more new cases of HIV infection are expected to occur in Asia than anywhere else in the world. India alone is expected to have 5 million cases by the turn of the century, making it the world's most infected country (72). In 1996, it was reported that in South and South-East Asia and sub-Saharan Africa more than 50% of those infected were women and the main mode of transmission was by heterosexual intercourse (57).

The current global strategy for preventing AIDS includes: 1) reducing the number of sex partners, 2) condom use and 3) treating concurrent STDs in people at risk for HIV. These are not options for many women who rely on partners for economic support and cannot negotiate condom use. In addition, it is unrealistic to expect people to avoid sex, and condoms are unacceptable for most men. Finally the infrastructure that is required to provide treatment for other STDs is not available in many parts of the developing world.

Early predictions that a vaccine would soon bring AIDS under control were overly optimistic in light of the complexities of HIV infection. Even if a vaccine is found, it is likely to be many years before it would be available to most of those who are at high risk of infection, the large majority of whom live in the developing world (174). There is, therefore, a pressing need for strategies other than

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vaccines that can protect individuals at risk from infection by STDs including HIV. As gender inequities limit the ability of women to protect themselves from HIV infection, especially in the developing world, a female-controlled prevention method is essential. Such a product would enable women to defend themselves against infection without the knowledge or consent of their male partners.

As mentioned above, a large majority of women in the world acquire HIV infection through heterosexual intercourse. Prevention strategies targeted to this route of infection must be able to establish a protective barrier between the virus present in the semen and the target cells present in the female genital tract. One such approach would be a microbicide present in a vaginal formulation, which would provide a chemical barrier against HIV either by blocking entry of the virus into host cells or by inactivating the virus in the vagina (36). If the formulation had wide spread activity against other STDs it could provide additional protection against sexually transmitted pathogens like HSV-2, human papilloma virus (HPV) and HTLV-I, which are also known to be cofactors of HIV (32,80).

The overall objective of my thesis research has been to develop such a vaginal microbicide. Ideally such a barrier would selectively provide effective protection against HIV and other STDs without impairing a woman's ability to conceive (37). Although the major emphasis of STD research has been on AIDS in recent years, other STDs have a serious impact on health - especially on reproductive health. Individuals who are at risk of infection by HIV are also at risk for a

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number of other STDs. A product that inhibits sexual transmission of one pathogen could enhance the transmission of another. Most of the viruses, bacteria and protozoa that are transmitted sexually are unrelated to one another and rely on markedly different mechanisms to adhere to and gain entry into target cells. Therefore, it is entirely possible that a formulation which inhibits transmission of one pathogen could enhance transmission of another. Thus, we cannot assume that a formulation which has not been tested against a sexually transmitted pathogen will necessarily have no effect or inhibit it. This makes it especially important to test potential vaginal microbicides in as many infection systems as possible, both *in vitro* and in animal models for vaginal infection. In addition, research on similar pathogens like HSV-2 and HTLV-I, both of which are enveloped viruses, may give some information about the efficacy of potential antimicrobial compounds on HIV. A microbicide which inactivates HSV-2 or HTLV-I by acting on the viral envelope may also be similarly active against HIV.

Biology of HTLV-I

HTLV-I was the first retrovirus to be linked to disease in humans, although prior to 1980, animal retroviruses causing leukemias and lymphomas were well known (55,135). HTLV-I belongs to a unique group of retroviruses called onco-retroviruses. In addition to the customary retroviral sequences *gag*, *pol* and *env*, the genome has an extra sequence named Px whose function is thought to be regulatory.

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HTLV-I causes a lymphoproliferative disorder called adult T cell leukemia (20,55,135,166) and has also been associated with neurological syndromes like tropical spastic paraparesis and myelopathy (HAM/TSP) (44,123,143). HAM/TSP is a slow neurodegenerative disorder resulting in paralysis of the lower limbs. HTLV-I is also associated with an increasing number of less severe syndromes, including immunosuppression, polymyositis, arthropathy, infectious dermatitis in children and uveitis (31). Evidence from both the Caribbean and the United States shows that dual infection with HTLV-I and HIV accelerate the progression to AIDS (15,134).

Japan, and later the Caribbean, were originally identified as endemic regions but HTLV-I is now quickly spreading throughout the world. It is estimated that 10 to 20 million people are infected with HTLV-I and although there has been a lot of emphasis on developing a vaccine against this virus none are available today (22,31). The presence of HTLV-I antibodies is now routinely tested in the blood supplies in U.S. and European blood banks as well as in developing countries. HTLV-I has a latency period ranging from 2 to 20 years which makes it an important pathogen to screen for. It has been proposed that HTLV-I has existed among human populations since prehistoric times, since there is a high incidence of HTLV-I in ancient, isolated populations like the Inuit, native Americans and the Ainu in Japan.

HTLV-I is transmitted during sexual intercourse (69, 109,111,142), from mother to child through breast feeding (74), needle sharing during IV drug use (17) and by blood transfusion (121). The routes of HTLV-I

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transmission were first suggested by familial studies in Japan. It was found that HTLV-I carriers were often clustered within families indicating two routes of transmission: mother to child and sexual contact (160). Kajiyama et al estimated that sexual transmission of HTLV-I in Japan overwhelmingly favors the male to female route (70). However in the Caribbean, where HTLV-I is also endemic, female to male transmission also occurs. Subsequently, other modes like blood transfusion and needle sharing during IV drug use were identified.

Although sexual transmission of HTLV-I is not as prevalent as the breast-feeding route, it is becoming an important route of transmission in certain parts of the world. Among heterosexuals, a correlation has been found between the acquisition of the viral infection and the number of sex partners (73,81,110).

Small animal models for testing microbicides

I have developed a mouse model for the sexual transmission of HTLV-I. Such an *in vivo* model would allow us to screen for compounds that may inhibit infection via this route. In addition a mouse model for genital HSV-2 has been used to test the efficacy of different compounds on the prevention of infection by this enveloped virus.

There are now a dozen or so different classes of compounds that show *in vitro* efficacy to either inactivate HIV or other sexually transmitted pathogens or to block infection *in vitro*. Some of these compounds are being formulated and plans are underway for Phase I safety

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trials. Human trials for efficacy testing are extremely costly and, at least for HIV, problematic (37). Thus, there is a great need for a next step after *in vitro* testing to select the best and eliminate the worst compounds.

There are numerous important differences between epithelial monolayers *in vitro* and the intact genital tract which could give different results *in vitro* and *in vivo*. The environment that we can create in tissue culture can never approximate the conditions in which epithelial cells exist *in vivo*. Epithelial cells are undoubtedly altered in many ways by the *in vitro* conditions. In addition, the genital tract is covered with vaginal and cervical mucus which has important roles in prevention of infection by microorganisms. There are likely to be other factors such as immunoglobulins, complement, pH and vaginal flora which play a role in preventing infection.

Work on the primate SIV/macaque model from several different laboratories has provided basic information relevant to mechanisms of sexual transmission of retroviruses (102,103). The Rhesus monkey model has also shown that the spermicide nonoxynol 9 partially inhibits infection by SIV (100,101). Because only few animals can be used for practical and economic reasons, the monkey system is extremely useful only when the vaginal product is highly efficacious. However, when the difference between numbers of control and experimental animals is not very large, experimental results are difficult to interpret and are often not statistically significant. As a consequence of small numbers mouse models do not suffer from these limitations since large numbers of

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animals can be used in each group tested. This is important when developing formulations, comparing formulations or whenever the efficacy is relatively low.

This thesis has been divided into 4 chapters. The first chapter deals with the basic question of the fate of infected cells in the vagina. Cells were placed in the vagina of mice and their ability to migrate between the epithelial cells of the genital tract was examined.

The second chapter deals with the question of whether human cervix-derived epithelial cells can be infected by HTLV-I. A cell culture system has been used to study the mechanisms of infection and a group of compounds, sulfated polysaccharides have been assayed for their ability to inhibit infection.

The third chapter deals with the development of a mouse model for the sexual transmission of HTLV-I. This is the first demonstration that mice can be infected by the vaginal route.

The final chapter deals with the use of a mouse model for genital transmission of HSV-2. Sulfated polysaccharides and other compounds have been screened for their ability to protect mice from vaginal challenge with the virus. In addition mice were also found to be infectable via the rectal route and this infection could be inhibited by prior exposure to sulfated polysaccharides.

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CHAPTER 1

A role for cell migration in the sexual transmission of HIV

ABSTRACT

It has been suggested that sexual transmission of human immunodeficiency virus (HIV) could be mediated by HIV-infected lymphocytes or macrophages in semen and female genital tract secretions rather than free virus. To test the hypothesis that mononuclear blood cells traffic from the vaginal vault through intact epithelia, double-stained, activated mononuclear blood cells were placed in the vagina of mice. Four hours later animals were euthanized and tissues prepared for examination by fluorescence microscopy. Numerous double-stained cells were observed in the connective tissue beneath the vaginal epithelium and in the iliac lymph nodes. We speculate that cell trafficking could be involved in sexual transmission of HIV.

INTRODUCTION

There is considerable controversy concerning how HIV enters the body following sexual contact. Several hypotheses have been proposed, including infection via; 1) microulcerations in the genital tract epithelium (172); 2) CD4+ Langerhans cells in genital and digestive

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tract epithelia (42); 3) direct infection of epithelial cells of the vagina, cervix or gut (161), and 4) virions associated with spermatozoa (14). In addition, the anatomical site(s) of infection is in question and there is controversy concerning whether infection is mediated via free virus or HIV-infected mononuclear cells (lymphocytes and in monocyte/macrophages) (131).

There can be little doubt that HIV is frequently transmitted through ulcerations in the genital tract epithelia. STDs that cause genital ulcers are associated with increased risk of infection and treatment protocols reduces the incidence of HIV infection (32,80). However, it is not known to what extent infection occurs in men and women without obvious lesions through intact epithelia or microlesions. Theoretically, breaks in the epithelium too small to be symptomatic could still allow viral entry. However, direct evidence suggests that HIV can be transmitted through intact epithelia: Rhesus macaques with apparently intact mucosal epithelia have been infected by virus placed on the cervix or vagina (99,104,152,153) or penile urethra (104, 153), or via the oral route (12,13), and chimpanzees have been infected by placing HIV-infected peripheral blood mononuclear cells (PBMCs) on the cervix (45). However, it would be premature to conclude from these studies that undetected microlesions do not play a role in sexual transmission of HIV.

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Langerhans cells and other dendritic cells

Other than the theory of transmission via lesions, probably the most currently accepted theory of HIV entry holds that Langerhans cells are the initial targets of infection. Langerhans cells are specialized dendritic cells present in skin, vaginal and cervical epithelia and submucosa. These CD4-positive cells can extend long thin processes to the surface of stratified squamous epithelia in the vagina and ectocervix (18,108). Langerhans cells sample antigen and subsequently migrate to lymph nodes where they present antigens to lymphocytes (141). A consideration which argues against the Langerhans cell as the initial target of infection is that these cells extend very thin processes and thus occupy only a tiny area of the surfaces of the vagina and cervix.

Pope et al have presented evidence that cultured skin dendritic cells form conjugates with lymphocytes isolated from human skin (42,137,156). When inoculated with HIV, the conjugates produce significant amounts of virus. However, pure cultures of Langerhans cells cannot be productively infected. This suggests that the role of the Langerhans cell may involve transporting HIV to lymph nodes (136,156). Marx and coworkers (153) studied the role of Langerhans cells in SIV infection at various times after vaginal exposure to SIV. The earliest time that infected cells were noted in the connective tissue beneath the vaginal epithelium was 2 days post-inoculation. At this time, infected cells were identified by *in situ* hybridization for SIV mRNA. Immunocytochemical staining of the positive cells in adjacent sections revealed that the infected cells were positive for what are

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believed to be dendritic cell markers. However, there is some controversy concerning the available immunocytochemical markers for dendritic cells. Although these findings implicate dendritic cells in the spread of HIV they do not establish that dendritic cells are the first cells to become infected because infection had already spread to the inguinal lymph nodes by 2 days following inoculation.

Further evidence implicating Langerhans cells in sexual transmission of HIV comes from the work of Essex and coworkers (150). HIV-1 has been categorized in different clades based on the variations in the molecular sequence in the *gag* and *pol* regions of the viral genome. These workers have presented epidemiological and *in vitro* evidence suggesting that Clade E of HIV-1, the predominant clade in Thailand, is more likely to be sexually transmitted then Clade B, the type found in N. America. *In vitro* experiments indicated that dendritic cells were more susceptible to infection by Clade E than Clade B, suggesting a potential role of dendritic cells in sexual transmission. These observations were used to explain the apparent prevalence of Clade E in the heterosexually infected population in Thailand (150).

"M" Cells

Membranous cells (M cells) are specialized epithelial cells found among absorptive and goblet cells in the gut. These cells, which are especially numerous in the epithelium over Peyer's patches, function in the process of antigen sampling. Unlike the adjacent epithelial cells of the gut, which are covered with a prominent glycocalyx to prevent

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adhesion of pathogens, the M cells lack a glycocalyx. Pathogens and inert particles readily adhere, are taken up, and pass though the M cell to a very large involution in the base of the cell where macrophages and lymphocytes reside. The M cell can be exploited; some pathogenic bacteria such as *Salmonella* use the M cell to enter and initiate infection in macrophages that lie in the pocket of the M cell (98). Neutra and colleagues have shown that in organ cultures of rabbit gut, HIV virions readily adhere to and are taken up by M cells (117). Although M cells are not present in the reproductive tract, they are reported to be present in human rectal epithelium (50). Thus, the theory that M cells may be involved in initiating infection following anal intercourse seems credible.

Semen

Semen from healthy men typically contains many leukocytes, including CD4+ mononuclear cells (6,8,175). Using direct immuno-fluorescence, we analyzed the number of CD4+ and CD8+ cells in semen of healthy, uninfected men once a week for 10 consecutive weeks (Zacharopoulos and Phillips, unpublished results). The number of these cell subsets varied widely, but the differences were so great between consecutive samples from the same subject that no significant differences between men were discernable. Thus, it appears that the number and type of mononuclear cells in the semen of a healthy man differs considerably from day to day.

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If CD4+ cells (lymphocytes and macrophages) are the vectors for HIV transmission, then reducing their numbers in semen could decrease the chances of infection. Lymphocytes and macrophages can originate from the testis, epididymis, seminal vesicles, or prostate (35), and inflammation of the genital tract results in an increased number of these cells in semen (176). Very little information is available, however, concerning the relative percentages of mononuclear cells that originate from the different organs of the male reproductive tract, what factors control their numbers, or what function(s) these lymphocytes and macrophages perform in semen. It has been suggested that vasectomy could reduce the infectivity of HIV-infected men as this procedure would eliminate mononuclear cells or cell-free virus in semen which had originated from the testis and epididymis (146). Although vasectomy has been reported to reduce the number of white blood cells in semen (122), HIV infected mononuclear cells have been detected in semen of seropositive vasectomized men (8). Further studies will need to be carried out before making a judgement concerning the efficacy of vasectomy for AIDS prevention.

In the first studies which addressed the problem of HIV-infected cells in semen, Ho et al (58) reported the recovery of HIV from the mononuclear fraction of semen of a healthy seropositive patient by coculture, but were unable to detect virus in the cell-free fraction of this patient. Similar results were reported at the same time by Zagury et al (181) who studied the cell fraction of semen from two AIDS patients. Borzy et al (24) reported reverse transcriptase activity in

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the cell-free fraction in semen of several seropositive men. Although the interpretation of the data presented in this paper has been questioned, subsequent reports using virus co-culture techniques, DNA-PCR and RNA-PCR have confirmed the presence of HIV-infected cells and cell-free virus in semen, although the percentage of samples which tested positive varied widely among the studies (7, 82,97,164,169). Ilaria et al (65) have found that pre-ejaculatory fluid from 6 of 13 men contained HIV-1 DNA as measured by PCR. Thus, pre-ejaculatory fluid as well as semen may be a source of HIV-infected cells.

Fate of cells in the vagina

The mucosal epithelium that lines the inside of the body is a protective barrier that is the first line of defence against invading pathogens. Although the female genitourinary tract is lined with such a barrier, pathogens are nevertheless able to breach it and cause infection. To do this they must either directly infect the epithelial lining or find a way past this barrier.

The concept that HIV transmission is mediated by HIV-infected lymphocytes and/or monocyte/macrophages was proposed nearly 10 years ago by Anderson (5) and by Levy (87). Since then it has been shown that HIV-infected mononuclear blood cells are present in both semen and cervical/vaginal secretions (82,144). Levy has emphasized that HIV-infected cells may be more resistant to the hostile environment of the vagina and that one HIV-infected mononuclear cell could produce thousands of new virions. Anderson used the term "Trojan horse" to

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describe the phenomenon. Like the Trojan horse, which enters the intact walled city through the gate and releases troops inside the city, the HIV-producing mononuclear cell would somehow pass through the intact epithelial barrier to the connective tissue where CD4-positive lymphocytes, macrophages and dendritic cells reside; once inside, it would release virions. Dr. David Phillips, under whose supervision most of the research for this thesis has been done, has recently reported that in confluent monolayer cultures of cervix-derived epithelia, HIV-infected, activated primary monocytes migrated between epithelial cells (162). Mayer and Anderson (95) recently presented ultrastructural evidence that T cells can migrate through cervical epithelia, further supporting the Trojan horse concept. The theory that mononuclear blood cells could be capable of exiting the vaginal vault through an intact epithelium is not unreasonable since many cells of the immune system traffic through the body to perform their normal functions.

As described above, there are a number of possible ways that HIV can enter the body following sexual contact. These include initial infection via microulcerations in the genital tract epithelium (172), Langerhans cells in the genital mucosa (42), direct infection of epithelial cells of the cervix or vagina (161) and virions associated with spermatozoa (14). Lymphoid cells originating from semen have been implicated in carrying pathogens like HIV (58,133). If these infected T cells and/or macrophages are able to invade the cervicovaginal mucosa, they have the potential for transporting the pathogen past the mucosal barrier (139). Some evidence exists for such a hypothesis. As

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mentioned earlier semen from HIV-infected men contains infected lymphoid cells (58). In addition, *in vitro* studies have shown that HIV-1 infected lymphocytic cells can migrate from the culture medium into an explant of human cervical mucosa (139,179).

The following experiments were carried out in mice to test the hypothesis that mononuclear blood cells are capable of trafficking through intact epithelia. Although these findings fall short of demonstrating that migration of mononuclear blood cells can mediate HIV-infection in humans, these observations suggest that trafficking of mononuclear cells from the vaginal vault can occur in mice.

RESULTS

In order to detect mononuclear blood cells which migrated from the vagina, activated peritoneal macrophages from female BALB/c mice were harvested using a procedure which is commonly used to attract and activate macrophages (29). In order to determine the exact phenotype of the peritoneal macrophages elicited by the thioglycolate broth treatment, the cells were labelled with fluorescent antibodies to anti-F4/80, a marker for resident and elicited mouse macrophages, anti CD-3, a pan-T cell marker and anti CD-45R, a B cell marker. It was determined that 85 to 95% of these cells were positive for the macrophage marker, 3

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to 11% were positive for the T cell marker and 2 to 3% were positive for the B cell marker. Others have also reported that the large majority of peritoneal cells obtained under similar conditions are macrophages (29). The cells were stained with supravital dyes which are known to be non-cytotoxic and have been previously shown to be suitable for following cell migration (25,33,145). Two supravital dyes were used so that cells could be identified with greater confidence: Hoechst 33342, a blue fluorochrome that binds DNA in the nucleus and PKH26-GL, a red fluorescent probe that is incorporated into the plasma membrane. All cells were found to be stained by both dyes.

Progestin-treated recipient mice (both inbred BALB/c and outbred CD1) were vaginally inoculated with the double-stained cells after the mice had been sedated. To determine if inoculation of cells had produced trauma, some mice were euthanized and sections of vaginas were examined using phase microscopy. No evidence of trauma was seen. Four hours later, the iliac lymph nodes were removed and cells were extracted from the lymph nodes. Cells that had red cytoplasmic stain and blue nuclei were observed in these preparations of the dissociated iliac lymph nodes of the 4 inoculated and two control animals. These double-stained cells were easily distinguishable in the inoculated mice (Fig 1g). The experimental animals had an average of about 50 cells per animal whereas no double-stained cells were seen in lymph node cells of the control mice. In a subsequent experiment, outbred CD-1 mice were used as recipients. Results were similar to those obtained using the inbred BALB/c mice as donor and recipient (Table 1).

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Sections of mouse vaginas, frozen 4 h following inoculation, were also examined to determine if cells had migrated from the vaginal vault to the connective tissue below the epithelium. Numerous double-stained cells were observed in the connective tissue (lamina propria) below the vaginal epithelium in each of the 6 experimental animals (Fig 1). In a number of cases, several cells were seen in the same section (Fig 1e). Sections were also examined from the vaginas of two uninoculated, progestin-treated mice to be confident that fluorescence was not due to autofluorescence. No double-stained cells were seen in tissues of these animals.

Additional experiments were carried out to determine the immunophenotype of the mononuclear cells that had reached the lymph nodes as well as that of the double-stained cells found beneath the vaginal epithelium. Of 202 PKH26-GL and Hoechst-stained cells observed, 154 (76%) were positive for the macrophage marker ,32 (16%) for the lymphocyte marker and 16 (8%) were inconclusive. In sections of vaginal tissue, some double-stained cells were seen to be labelled with the macrophage marker and some with the T cell marker.

DISCUSSION

As noted earlier the concept that HIV transmission is mediated by HIV-infected lymphocytes and/or monocyte/macrophages was proposed nearly 10 years ago by Anderson (5), and Levy (87). Since then it has been shown that HIV-infected mononuclear blood cells are present in both

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semen and vaginal mucus (82,144). However, even if HIV infection is cell-mediated, the mechanism is unclear. One obvious mechanism would be the entrance of an infected cell through breaks in the genital tract epithelium. Direct infection of epithelial cells is another possibility. In this regard, it has been demonstrated by Dr. Phillips' group that HIV-infected, T-lymphoma cells adhere to epithelia derived from the human cervix *in vitro* (127), and that adherence triggers directional secretion of HIV to the surface of the epithelium. Epithelial cells subsequently take up virus and become productively infected (128,161). A third possible mechanism involves trafficking of HIV-infected mononuclear cells between cells of intact epithelia. The behavior of HIV-infected primary activated monocytes was studied by Dr. Phillips. Monocytes typically crawl along surfaces, putting forward a leading pseudopod from which they secrete HIV (130). When added to monolayers of epithelial cells derived from the human cervix, monocytes migrated between epithelial cells, leading to the speculation that cell trafficking could be involved in HIV transmission (162). This chapter presents evidence that mononuclear blood cells are also capable of traversing intact epithelia in vivo. Immunophenotyping revealed the presence of cells positive for the F4/80 monoclonal antibody as well as cells positive for the antibody to CD3, indicating that both macrophages and lymphocytes can reach the lymph node. Although it seems that more macrophages than lymphocytes can reach the lymph nodes, more macrophages than lymphocytes were placed in the vagina, so they may have similar intrinsic capacities for migration.

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It is not surprising that mononuclear blood cells are capable of exiting the vaginal vault. Mononuclear as well as polymorphonuclear blood cells migrate from the base of the epithelia to the vaginal vault, uterine lumen (63,118) and intestine (112). Furthermore, migration of polymorphonuclear and mononuclear blood cells from the apical surface to the base of capillary endothelia is a basic element of the immune response.

However, at this point it can only be speculated that cell trafficking is involved in the sexual transmission of HIV. *In vivo* evidence in support of cell-mediated transmission comes from the observation by Girard et al. (45) that HIV was transmitted by a few hundred cells placed on the intact cervix of three chimpanzees. However, in macaques, SIV can be transmitted through an apparently intact epithelia by inoculation of free virus (152). In the present study, an artificial system was employed to increase the likelihood of finding cells. To raise the chances of observing the phenomenon, mice were treated with progestin which is known to cause thinning of the vaginal epithelium (126,165). Approximately the same number of mononuclear cells as would be in an entire human ejaculate (177) was placed in the vagina of an animal which weighs about 2000 times less then a human. However, when one considers that relatively few acts of intercourse result in an infection, and that theoretically it only takes a single HIV-secreting cell to initiate an infection, the hypothesis that infection is initiated by trafficking of an HIV-infected, activated mononuclear cell seems plausible.

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Figure 1:Immunofluorescent (a,c,e) and matching phase contrast (b,d,f) photomicrographs of the vaginal vault (V), epithelium (E) and lamina propria (LP) four hours following inoculation of stained peritoneal cells into the vagina. The vaginal epithelium is autofluorescent blue. Double-stained cells are observed in the vaginal vault (*) and in the lamina propria (arrow). The last two panels show fluorescent (g) and matching phase contrast (h) photomicrographs of dissociated cells from an iliac lymph node; a double-stained cell is indicated by the arrow. (Magnification X 600)

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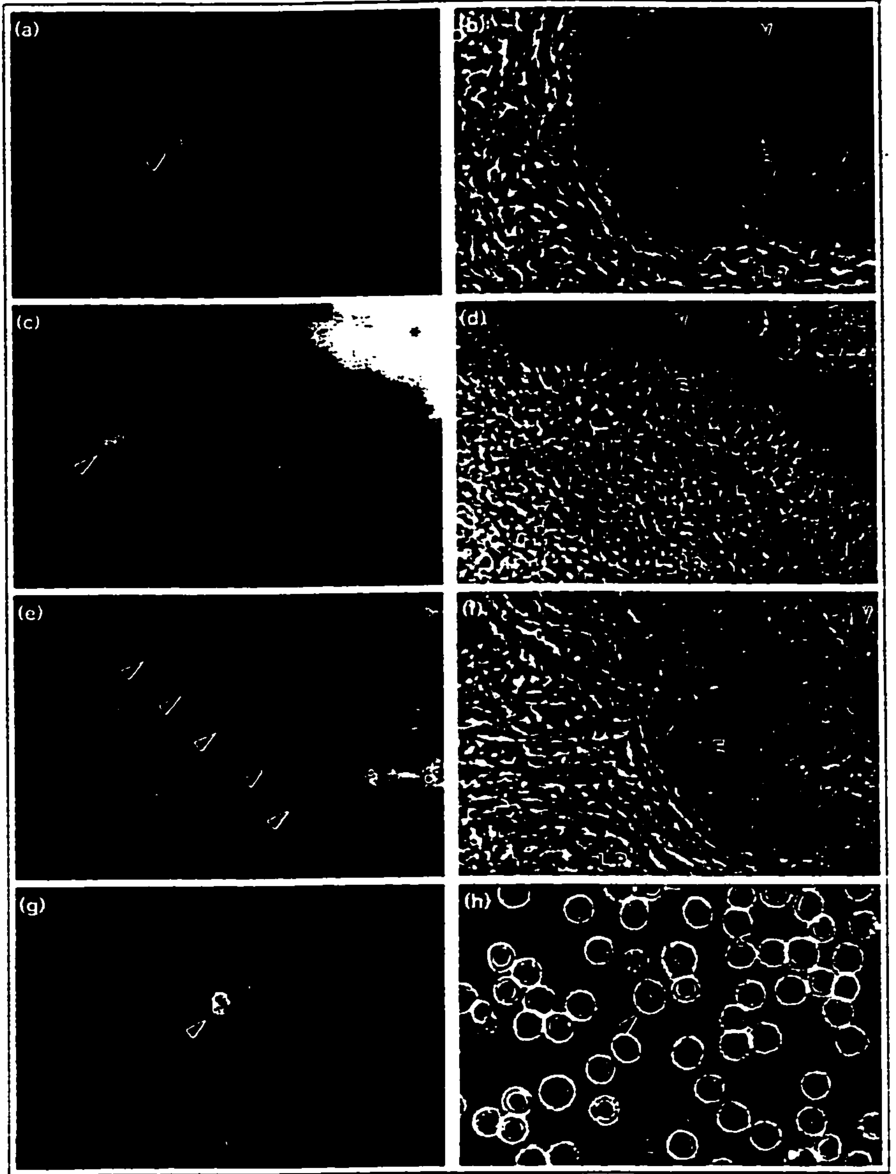

Fig. 1

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Table 1: Number of double-stained cells in dissociated iliac lymph nodes of 6 experimental and 4 control mice. Fluorescence microscopy was used to examine cells with both blue nuclei and red cytoplasm. BALB/c mice donors and recepients were used in #'s 1-4. BALB/c donors and outbred CD1 recepients were used in #'s 5,6,9 and 10. These mice were inoculated through a silastic tube attached to a pipet tip. Controls 7 and 8 were untreated. Controls 9 and 10 were inoculated with freeze-thawed double stained cells.

Mouse #	Total # cells observed	Double-stained cells
1	4.4×10^5	49
2	3×10^5	30
3	5.4×10^5	68
4	1.9×10^5	54
5	4.1×10^5	39
6	4.4×10^5	27
7 (control)	2.7×10^5	0
8 (Control)	2.5×10^5	0
9 (Control)	3.5×10^5	0
10 (Control)	3.5×10^5	0

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CHAPTER 2

Cell-mediated HTLV-I infection of a cervix-derived cell line

ABSTRACT

There is considerable evidence that sexual transmission of human T-cell leukemia virus-I (HTLV-I) is mediated by virus-infected lymphocytes in genital tract secretions. However, it is not clear whether infection occurs through lesions in the genital tract epithelium or takes place via an intact epithelium. I have carried out experiments to test the hypothesis that sexual transmission of HTLV-I is initiated by lymphocyte-mediated infection of intact genital tract epithelia. To examine this question either free virus or HTLV-I producing MT-2 T lymphoma cells were added to cultures of a cervix-derived epithelial cell line, MS751. Although free virus did not infect MS751 cells, MS751 cells which had been coincubated with MT-2 cells became infected. These cultures produced about 50 pg/ml of HTLV-I p24 antigen per 10^5 cells over a 24-h period on the sixth day following exposure to donor T-cells. Proviral DNA could be detected in target MS751 epithelial cells by PCR. Infection of epithelia could be blocked, in a dose-dependent manner, by the sulfated polysaccharides dextran sulfate, heparin, and fucoidan, and by the enzymes fucosidase and mannosidase, but not by a number of other agents that were tested.

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Since MT-2 cells were observed to attach to the epithelial monolayer, the ability of agents to inhibit adhesion was examined. Adherence was inhibited by the same agents that inhibited infection. Based on these findings, it can be concluded that sexual transmission of HTLV-I may involve lymphocyte-mediated infection of genital tract epithelia and lymphocyte adhesion to the epithelium is a critical event in transmission of HTLV-I. A sugar moiety on the epithelium, possibly mannose or fucose, may be involved in adhesion of T-cells to epithelial cells. As sulfated polysaccharides block both adhesion and productive infection of the epithelium, these compounds might be used as active ingredients in a vaginal formulation to help prevent HTLV-I transmission.

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-I) was the first retrovirus to be linked to disease in humans (55,135). HTLV-I shares many features with HIV. These human retroviruses are structurally quite similar and are transmitted by common routes. In addition they are particularly tropic to CD4+ T lymphocytes as well as other non lymphoid cells. In this respect, HTLV-I has a much broader range of target cells.

In addition to T helper cells, HTLV-I can also infect CD8+ T cells (159), B cells (91) as well as human endothelial cells (58,62) and human

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intestinal epithelial cells (180). Although most pathogens have mechanisms that promote infection of target cells without employing host cells, considerable evidence suggests that many viruses infect and spread via cell to cell mechanisms. Cell to cell spread has been described in paramyxoviruses (38,54), vaccinia (120), foamy virus (60), herpesvirus (19,59,90) and retroviruses including bovine leukemia virus (40) and visna virus (49, 71). *In vitro* studies suggest that infection is easier with HTLV-I-infected lymphocytes than free virus. HTLV-I has been isolated from mononuclear cells in semen, suggesting that sexual transmission of HTLV-I may be cell-mediated (114). A number of *in vitro* studies have concluded that cell-mediated transmission of this virus is more efficient than transmission via cell-free virus (62,144,180). As mentioned in the previous chapter, virus-infected lymphocytes from semen, could infect the mucosal cells lining the lower female genital tract. Once infected, these epithelial cells could productively shed virus basolaterally and further spread the infection to the underlying connective tissue target lymphocytes. Such a mode of transmission was demonstrated *in vitro* with HIV and human colon epithelial cells (39) and cervix-derived epithelial cells (132). Macrophages and Langerhans cells, present in the vaginal mucosa, may also be targets of the virus (85,92). In this way, the virus could infect healthy, intact cervix or vaginal epithelium without the need for abrasions or the presence of microulcerations. If cell-mediated transmission predominates with this virus (62,144) HTLV-I-infected white blood cells in semen could be a major source of the virus during sexual transmission.

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Previously I utilized an *in vitro* model to show that HTLV-I can be passed to intestine-derived, epithelial cells from chronically infected lymphocytic cells, such as those found in the breast milk of infected mothers, across a narrow space formed between the two interacting cell types. This work was part of the requirements for completion of a master's degree at CCNY and has been published (180). Upon addition to enterocyte (I-407) monolayers, HTLV-I-infected lymphocytic cells (MT-2) adhered to the epithelium via microvilli extending from both cell types. Simultaneously, the previously round lymphocytes developed polarity, with a smooth surface facing the epithelium and a ruffled surface distal to the epithelium. Within 20 minutes of addition of MT-2 cells to I-407 monolayers, virus was shed by the lymphocytes and was seen in the spaces between the lymphocyte and the epithelial cells. Assembly of virions along the lymphocyte surface facing the epithelium was demonstrated by postembedding EM immunogold labeling with anti-HTLV-I core antigen. EM immunocytochemistry demonstrated that small dense protrusions on the enterocyte plasma membrane represented sites where virions had fused with the epithelial cell plasmalemma. Analysis of epithelial cells by *in situ* hybridization showed that HTLV-I RNA could be detected in the cytoplasm of epithelial cells which had been incubated with virus-infected lymphocytes. Analysis by dot and Southern blot hybridization showed integrated HTLV-I provirus in epithelial cells after 4 months of subculture. From these studies we postulated that HTLV-I infection involving rapid polar budding of virus and uptake within the space between the lymphocyte and enterocyte might explain how this virus can

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escape inactivation by anti-viral antibodies present in milk and the gut and thereby successfully infect enterocytes in vivo.

Evidence from the previous chapter indicates that infected blood cells originating from semen may be able to traverse the vaginal epithelium and infect other cells on their way to the neighboring lymph nodes. In this chapter an *in vitro* cell to cell infection model is utilized to see if cervix-derived epithelia can be infected directly.

An *in vitro* system to mimic the manner in which human immunodeficiency virus (HIV) may be delivered from HIV-infected mononuclear cells in semen to the epithelial cells which line the reproductive tract and serve as a barrier to protect the underlying tissue from infection has been developed in Dr. Phillips' laboratory. In this model infection involves adhesion of HIV-infected mononuclear cells to a cultured, cervix-derived epithelial monolayer. Cell-cell adhesion triggers rapid secretion of HIV from the infected mononuclear cells towards the epithelium, leading to productive infection of epithelial cells (133,161). Although epithelial cells derived from the human gut and cervix can clearly be infected *in vitro*, there is no direct evidence that they become infected during sexual transmission. However, *in situ* hybridization studies using autopsy and biopsy tissue from AIDS patients suggest that HIV can infect epithelia (52,88,94,116). Recently, Howell et al (61) found that vibratome sections of living tissue and cell cultures from human oviduct, uterus, cervix, and vagina could be infected by primary isolates of HIV. Immunofluorescence and confocal microscopy revealed that a variety of cell types were infected,

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including CD4 negative epithelial cells. They concluded that "HIV-1 can infect cells and tissues from different sites within the female reproductive tract" and suggested "that multiple cell types, including epithelial cells may be targets of initial infection by HIV."

Therefore the first event in sexual transmission could be the infection of an epithelial cell. Infection may spread to contiguous cells but would be transient because the low-affinity receptors of CD4 negative cells probably would not permit a sustained infection. In addition, epithelial cells are constantly sloughed.

The experiments described in the present study were performed to determine whether a similar mechanism could be involved in the sexual transmission of HTLV-I. MT-2 cells were added to monolayer cultures of cells derived from the human cervix to examine the possibility that HTLV-I-infected donor T-cells might be capable of infecting epithelia derived from the genital tract. In addition, this *in vitro* system has been used to study the mechanism of infection. The findings provide some clues to the molecules that may be involved in sexual transmission of HTLV-I and suggest a strategy that might be used to prevent infection.

Cell Adhesion

In the experiments described below, adhesion between the infected T cells and the cervix-derived epithelial monolayer was the first step in the infection process. Adhesion between cells is necessary in cellular communication in the immune system. In order to fight

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infection, cells of the immune system must both circulate as non-adherent cells in the blood and lymph and migrate as adherent cells in tissue. The mechanism of cell adherence to epithelia has been studied primarily in endothelia. During the inflammatory response, blood flow through the capillaries slows down. The leukocytes roll over the endothelium and gradually the endothelial wall becomes paved with adherent leukocytes. At the junctions of the endothelial cells the adherent leukocytes crawl through to the connective tissue beneath the endothelium. Cell adhesion molecules are known to mediate this process of adhesion and crawling.

There are four families of molecules that are involved in these adhesive interactions. They are the immunoglobulin superfamily, cadherins, integrins and selectins. The Ig supergene family includes the immunoglobulins, T cell receptors, MHC molecules, CD4, CD8, intercellular adhesion molecules (ICAMS), vascular adhesion molecules (VCAMS) and platelet endothelial adhesion molecule (PECAM). The cadherins are found both within and outside the nervous system and they are calcium-dependant adhesion molecules. The integrins are a large family of heterodimeric transmembrane glycoproteins consisting of α and ß subunits. They have a diverse range of functions including platelet aggregation, inflammation, wound healing as well as cell signaling. The selectins are a small group that include endothelial leucocyte adhesion molecule (E-selectin), leucocyte adhesion molecule (L-selectin), and platelet activation-dependant granule-external-membrane protein (P-selectin or CD62). This family plays a major role in leucocyte

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migration into tissues and the inflammatory response. They mediate rolling and adhesion of leukocytes to endothelia. These molecules function in other capacities as well, like signal transduction and in many cases are used by bacteria and viruses to enter cells. HIV has exploited the CD4 molecule as a receptor on T cells (30,77) and the rhinoviruses use ICAM-1 (48). SV40 and Semliki forest virus use the MHC class I molecule (53).

In addition to the CAMs, other groups of molecules are known to be involved in cell adhesion. Lectins and the related carbohydrates they recognize play a part in diverse functions such as the attachment of sperm to an egg (171) and the migration of lymphocytes through endothelia (89). Since all cells have surface carbohydrates, there is potential for lectin involvement in adhesion and blocking.
Adhesion blocking experiments have been used to test the possible involvement of this superfamily in cell to cell interactions between infected T cells and cervix-derived epithelia. If adhesion is the initial step for cell to cell infection, then understanding the distribution of the surface molecules involved and the mechanisms of adhesion will provide insights into blocking infection for therapeutic use.

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RESULTS

Adhesion of MT-2 Cells to MS751 Epithelium

Using a fluorescence-based adhesion assay, four human cervix-derived epithelial cell lines were tested for the ability of HTLV-I-infected MT-2 cells to adhere to them. Of the four cell lines tested, MT-2 was found to adhere to MS751 and C33A better than the other two (Fig. 1). Since C33A grew as uneven monolayers compared to the flat monoolayers of MS751, the latter was used in all further *in vitro* experiments.

When MS751 cells were cocultured with MT-2 cells, the T-cells adhered to the monolayer of MS751 cells. When the culture was shaken while being observed in an inverted microscope, the T-cells remained adherent to the epithelium.

An adhesion assay was used to examine the effect of agents on adhesion. The most effective sulfated polysaccharide of those tested was fucoidan. This reagent blocked adhesion by 50% at around 15 to 30 μg/ml (Fig 2). Dextran sulfate and heparin blocked adhesion about 50% at concentrations of less than 0.1 mg/ml (Fig 3). Other sulfated polysaccharides that we tested had no effect (Table I). In order to determine which cell type fucoidan was affecting—the T-cells or the epithelial cells—we treated either MT-2 cells or the MS751 monolayer with fucoidan for 1 h, washed the cells, and subsequently carried out the adhesion assay. Pretreating MT-2 cells inhibited adhesion, whereas pretreating MS751 cells did not (Fig 4).

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To examine the possible role of sugars in adhesion, the adhesion assay was carried out in the presence of a number of glycosidases including fucosidase and mannosidase. Both of these enzymes blocked adhesion in a dose-dependent manner (Figs 5a & 5b), whereas other glycosidases tested had no effect (Table II). Antibodies to the adhesion molecules ICAM-I, LFA-1, LFA-3, and L-selectin had no effect, although all of the antibodies that were employed have been shown to block cell adhesion in other adhesion systems (34,76,154).

Blocking adhesion with lectins

Concanavalin A (Con A), succinylated Con A and lectins from *Pisum sativum* and *Lens culinaris* were used in the adhesion assay. All these plant lectins significantly enhanced adhesion between the MT-2 cells and the cervix-derived epithelia (Data not shown).

Expression of cell surface carbohydrates on MT-2 cells

MT-2 cells were stained with FITC-conjugated sugars or lectins to identify surface carbohydrates and carbohydrate ligands. Mannose and β galactose did not bind to the surface of these cells however fucose binding was observed. The cells were positive for all the other carbohydrates tested (Table III and IV).

Immunofluorescence and FACS analysis of CAMS

Seven fluorescent antibodies to cell adhesion molecules were used to detect these molecules on the surface of MT-2 cells using

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immunofluorescence techniques. These included antibodies to CD4, CD58, ICAM-1, CD11a, CD26, Lecam-1 (B-D) and Lecam-1 (AMAC). MT-2 cells were positive for all of these cell adhesion molecules except for the antibodies to L-selectin.

The same panel of antibodies were used in FACS analysis. The majority of the cells were strongly positive for all the antibodies tested except for Lecam-1 (Fig 6).

Infection of MS751 Cells

To detect infection, HTLV-I-infected MT-2 cells were added to the MS751 monolayer. After a 4 h incubation period, T-cells were removed by washing. Under the inverted microscope, adherent MT-2 cells were no longer observed. However, to be certain that any remaining T-cells were killed by treatment with mitomycin C, we waited for 4 days or more before measuring p24 by ELISA. The amount of p24 that had accumulated in the medium was several times above the background of the assay. The concentration of p24 in the medium reached 50 pg/10^3 cells over a 24-h period a week after coculture and subsequently declined (Fig 7).

One week after they had been exposed to MT-2 cells for 4 h, MS751 epithelial cells were assessed for virus production using a p24 ELISA assay. Fucoidan and dextran sulfate, which blocked adhesion, also reduced p24 production by the epithelium. The appearance of p24 was inhibited at roughly the same dose that blocked adhesion (Fig 8a & 8b). Other sulfated polysaccharides tested were not as effective (Table V).

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To confirm that the MS751 monolayer was productively infected and that infection could be blocked by sulfated polysaccharides, Southern hybridization of PCR-amplified DNA was carried out. DNA was extracted and amplified to determine whether the HTLV-I proviral DNA was present in MS751 cells which had been incubated with MT-2 cells. Uninfected MS751 cells were used as a negative control and MT-2 cells as a positive control. Agarose gel electrophoresis and Southern blot hybridization show that viral nucleic acids were present in the host nuclear extract following infection. DNA from cells infected in the presence of 1 mg/mL of fucoidan was also amplified and run on the same gel. Fucoidan greatly inhibited viral integration (Fig 9). PCR amplification of epithelial cells infected in the presence of a range of concentrations of dextran sulfate was carried out. With increasing concentrations of the sulfated polysaccharide, a reduction in the amount of proviral DNA in the epithelial cells was observed (Fig 10).

***In situ* hybridization**

In situ hybridization was carried out on MS751 cells which had been cultured after incubation with MT-2 cells. Uninfected epithelial cells were used as negative controls and MT-2 cells served as a positive control. Hybridized probe was not detected in the infected epithelial cell cultures.

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DISCUSSION

Although there is convincing evidence that HTLV-I is sexually transmitted (69,110,142), the receptor(s) and target cells of HTLV-I during initial infection are not known. The *in vitro* evidence that HTLV-I is capable of infecting many different cell types (27,58,62,79,91,159, 180), suggests that this virus might utilize multiple cell types as targets. Additional evidence that HTLV-I may use receptors and target cells other than CD4+ T-cells comes from studies in which it has been demonstrated that HTLV-I can infect a number of animal species including rats (64, 119), rabbits (28,148), and marmosets (75,178).

This report presents evidence that an epithelial cell line derived from the human cervix can be productively infected with HTLV-I. The fact that peak infection was found a week after the donor HTLV-I infected T-cells were added leaves little doubt that the virus that was measured by p24 ELISA was secreted by the MS751 cells rather than contaminating donor cells. Infection may have spread by cell to cell contact or by successive cell divisions of the host cell with the integrated virus. Six days after infection there is a drop in p24 concentration. This has also been observed in the infection of cervix-derived epithelia by HIV-infected T cells. At six days, the culture had reached confluence and thus there was no increase in cell number. The cells in the confluent monolayer probably undergo changes which result in less viral production.

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No detectable MT-2 cells remained in the culture after a week. Cultures were washed each day and no lymphoma cells were observed. In addition the MT-2 cells had been pretreated with 200 µg/mL mitomycin C before adding them to the MS751 cells. It has been previously shown that T-cells are killed within 3 days by this treatment (132). Cytotoxicity of mitomycin C on MT-2 cells also indicate that no live MT-2 cells remain 48h after mitomycin C treatment. PCR and filter hybridization confirmed these observations. Judging from the density of the bands, infected MS751 cells had a similar amount of proviral DNA as the donor MT-2 cells.

Exposure to fucosidase and mannosidase during the incubation period inhibited both adhesion to and infection of the epithelium. This suggests that adhesion and infection may involve fucose and/or mannose. Fucoidan has been shown to be effective in blocking adhesion of lymphocytes to endothelial cells by binding to the lectin domain of L-selectin on the lymphocyte surface (89). As antibodies to L-selectin, known to block adherence of lymphocytes to endothelial cells, were not able to block adhesion, another mechanism may be involved. Alternatively, adhesion may involve more than one type of adherence mechanism. Blocking one of several types of molecules that mediate adhesion may not be sufficient to inhibit MT-2 cells from adhering to the epithelial monolayer. This idea is supported by recent studies of adherence of HIV-infected T-cells to epithelia in which the authors attempted to block adhesion and infection with many antibodies to CAMs.

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Although all of the antibodies chosen have been shown to block in other systems, none had an effect on adhesion of T-cells to epithelia (128).

All the plant lectins tested enhanced adhesion suggesting that cross linking of carbohydrate ligands expressed on the two cell types facilitates cell-cell attachment. Immunofluorescence and FACS analysis further confirmed the distribution of certain carbohydrates on the surface of the cells.

The lack of a hybridized product in the *in situ* hybridization procedure may be a result of a low copy of viral transcripts per cell. *In situ* PCR would be a more sensitive technique, however there is some controversy regarding the reliability of this procedure.

It has been known for nearly 30 years that sulfated polysaccharides can inhibit herpes virus infection *in vitro* (113). More recently sulfated polysaccharides have been shown to inhibit infection by a number of enveloped viruses, including HIV (67,86,105,124,168). Dr. Phillips' laboratory has also reported that a number of sulfated polysaccharides block infection of cervix-derived epithelia by HIV-infected lymphocytes (127,128), as well as chlamydia (182). The results presented here suggest that it may be possible to use a sulfated polysaccharide in a vaginal product to prevent infection by HTLV-I.

FIGURE CAPTIONS

Fig. 1: Adhesion of MT-2 cells to cervix-derived epithelia : HT-3 (solid bars), C33A (narrow hatched bars) and MS751 (wide hatched bars).

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Fluorescence values represents a mean of 3 wells +/- S.D. Experiments were repeated 3 times with similar results.

Fig. 2: Two-fold serial dilutions of fucoidan were added to BCECF-AM-labelled MT-2 cells in the adhesion assay. Fucoidan was seen to block adhesion of MT-2 cells to MS751 epithelial monolayers by 50% at a concentration of 15 to 30 μg/mL. Fluorescence values represent the mean of 3 wells +/- S.D. Experiments were repeated 3 times with similar results.

Fig. 3: Lymphocytes were coincubated with epithelial monolayers in the presence of serial dilutions of iota carrageenan (solid bars), heparan sulfate (notched bars), heparin (crosshatched bars), or dextran sulfate (dotted bars) for 90 min. Fluorescence values represent the mean of 3 wells +/- SD. Experiments were repeated 3 times with similar results.

Fig. 4: To see if fucoidan was blocking adhesion by binding to the surface of the T-cells or the epithelial cells, parallel experiments were carried out in which either the MT-2 cells or the epithelial monolayer were pretreated with fucoidan for 60 min, washed, and subsequently incubated with the epithelial monolayer or T-cells. Fucoidan blocked adhesion when MT-2 cells were pretreated (solid bars). In contrast, no blocking was observed when the monolayer was pretreated (hatched bars).

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Fig. 5: Serial dilutions of a) α-L-fucosidase and b) α- (solid bars) and ß- (hatched bars) mannosidase were coincubated for 90 min with the T-cells and the epithelial cells. All three inhibit adhesion.

Fig. 6: Single parameter fluorescence histograms of MT-2 cells that were labelled with the respective monoclonal antibody and fixed prior to analysis. Increasing fluorescence intensity is plotted on the x axis in log fluorescence vs cell number on the y axis. The Control plot represents unlabelled MT-2 cells.

Fig. 7: To study the course of infection, culture medium was collected every 24 h, cultures were washed, and fresh medium was added. Viral productivity was measured by p24 ELISA for days 4 to 8 postinfection (pi). Peak productivity was reached on day 6 pi (50 pg/10^5 cells) after which there was a decline in viral output by the infected epithelial cells.

Fig. 8: During the infection assay, 5-fold dilutions of a) dextran sulfate and b) fucoidan were added to cocultures of MT-2 cells and epithelial cells in 6-well plates for 4 h. After a week of culture, a 24 h accumulation of p24 in the medium was measured by ELISA. Infection was blocked at between 8 and 40 μg/mL.

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Fig. 9: Southern blot hybridization of PCR amplified HTLV-1 DNA sequences of about 158 bp from uninfected MS751 (lane 1), infected MS751 (lane 2), MS751 infected in the presence of 1 mg/mL fucoidan (lane 3), and MT-2 cells (lane 4). Fucoidan inhibited the incorporation of proviral DNA in MS751 cells.

Fig. 10: Effect of dextran sulfate on viral infection and integration. DNA was extracted from MS751 cells cocultured in the presence of 5-fold dilutions of dextran sulfate. PCR amplification of HTLV-I DNA sequences (158 bp) showed that there was a decrease in the level of viral incorporation with increasing concentrations of dextran sulfate. MT-2 (lane 1), infected MS751 (lane 2), 1.6 μg/ml dextran sulfate (lane 3), 8 μg/ml (lane 4), 40 μg/ml (lane 5), 200 μg/ml (lane 6), 1000 μg/ml (lane 7) and uninfected MS751 (lane 8).

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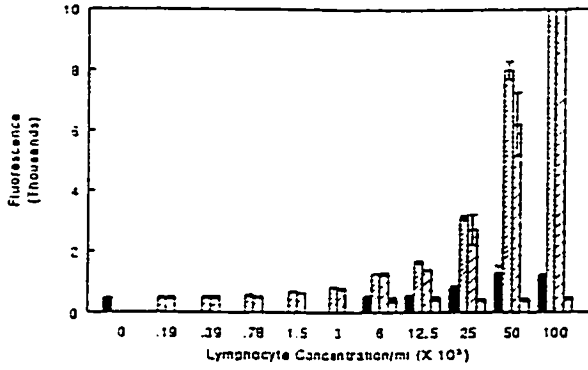

Fig. 1

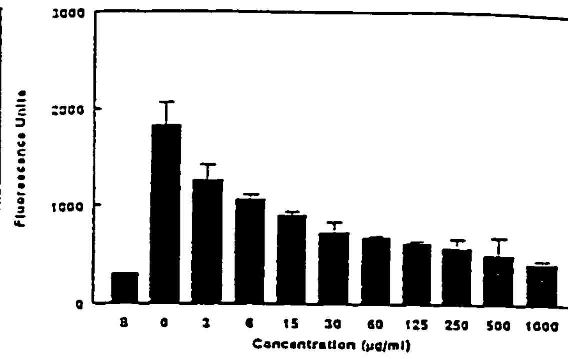

Fig. 2

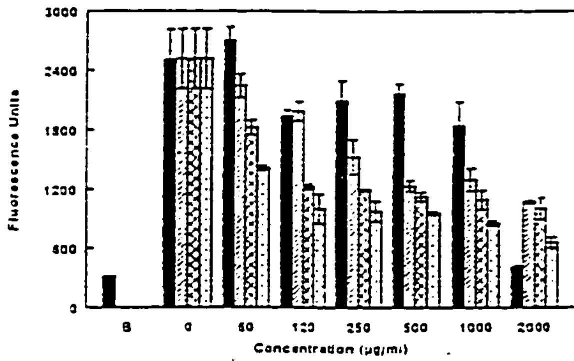

Fig. 3

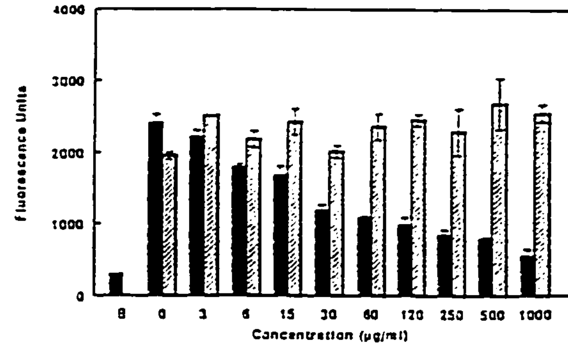

Fig. 4

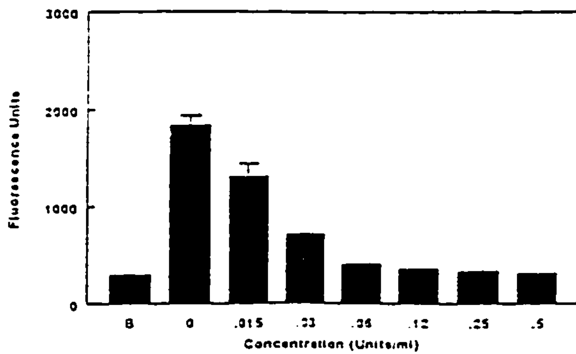

Fig. 5a

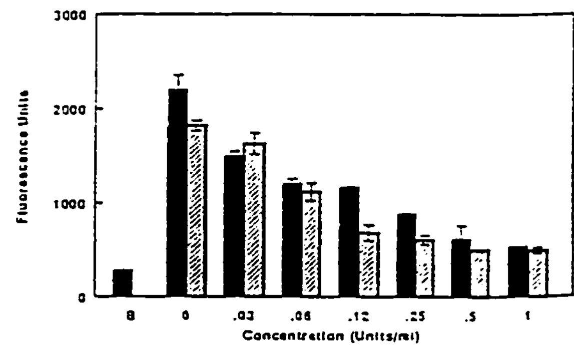

Fig. 5b

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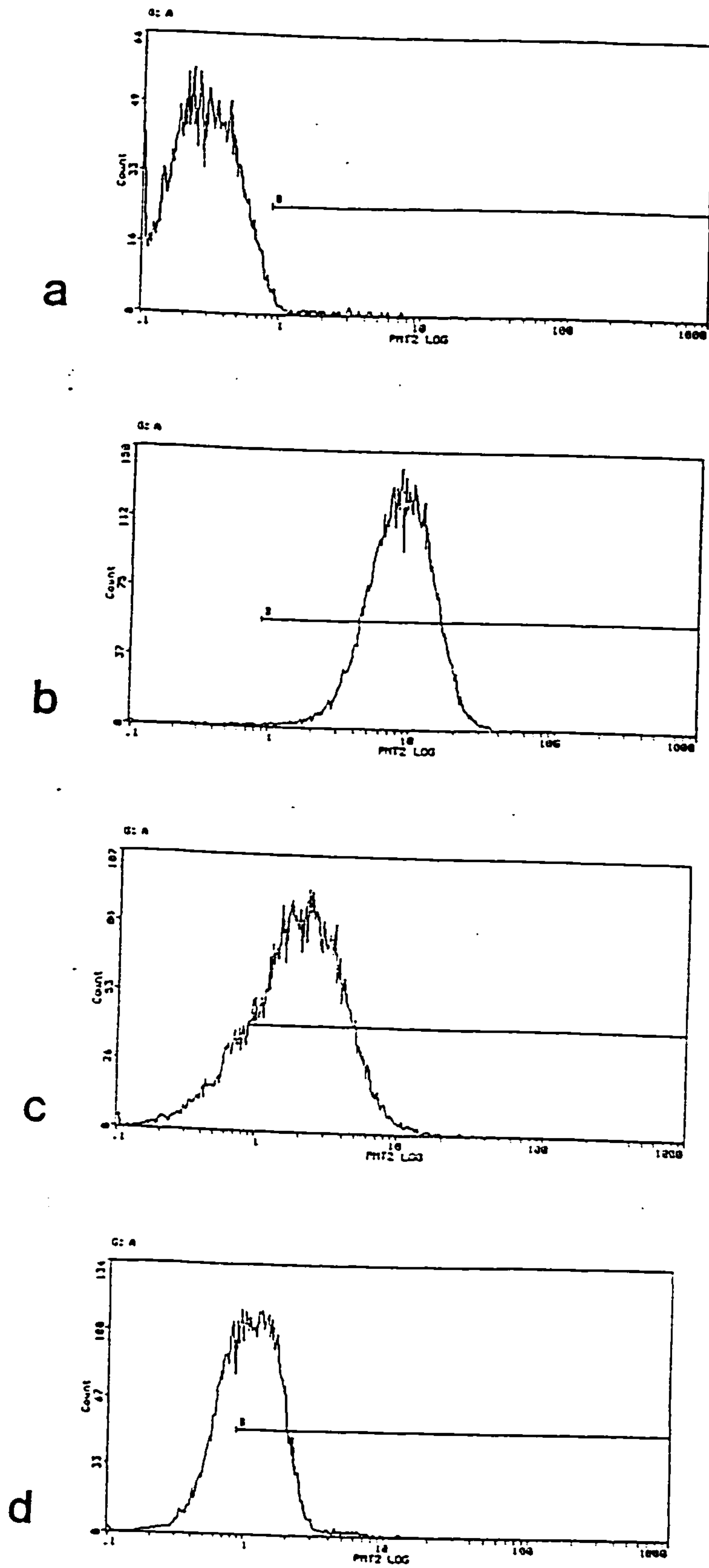

Fig. 6

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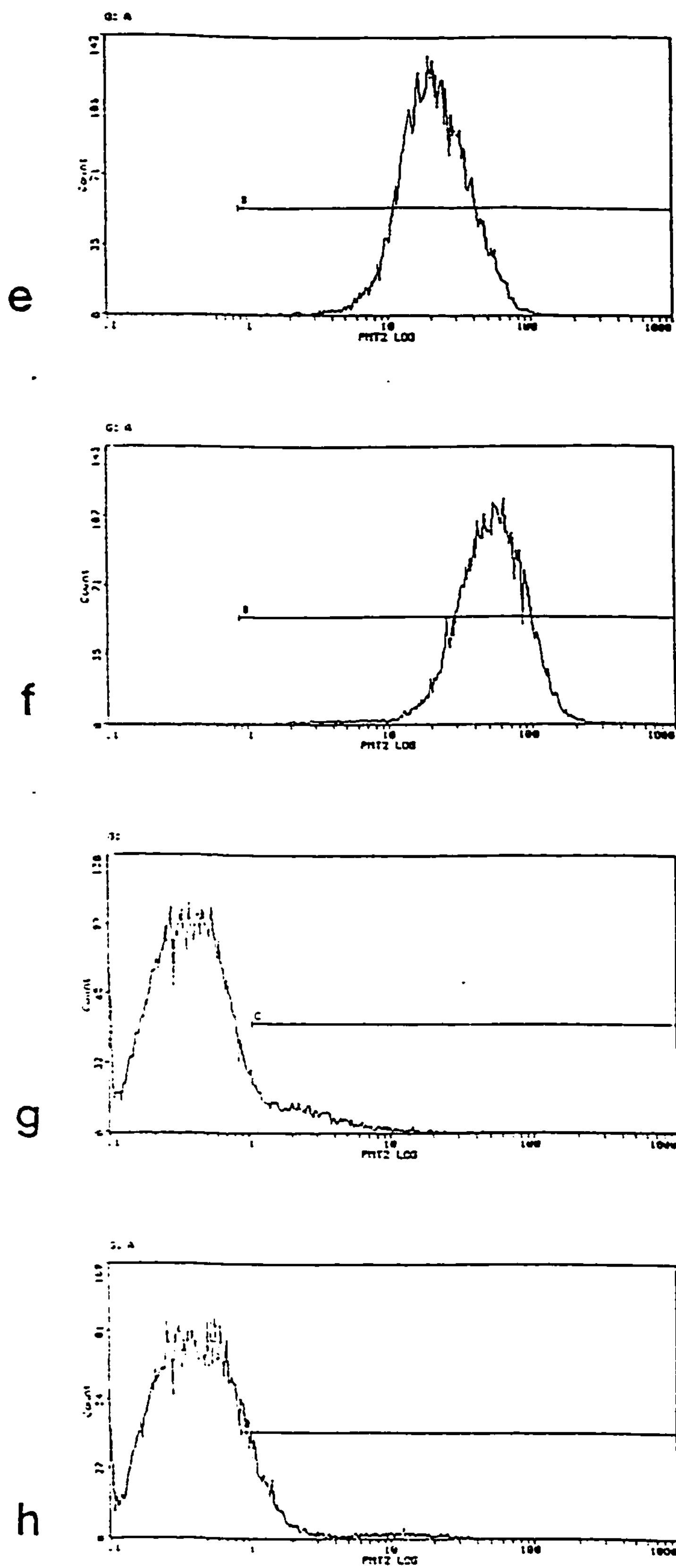

Fig. 6

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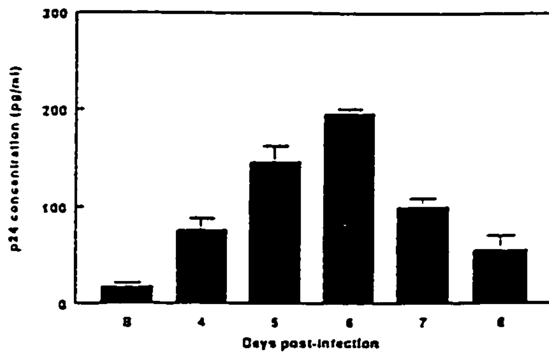

Fig. 7

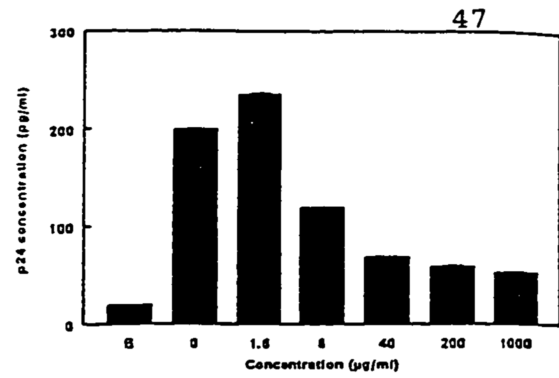

Fig. 8a

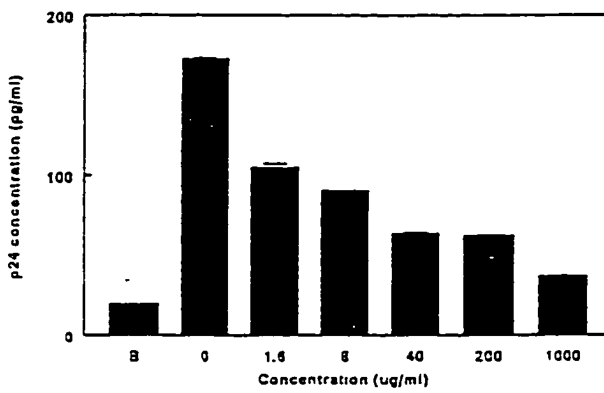

Fig. 8b

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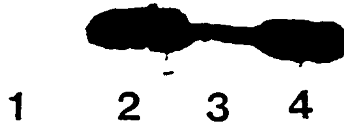

Fig. 9

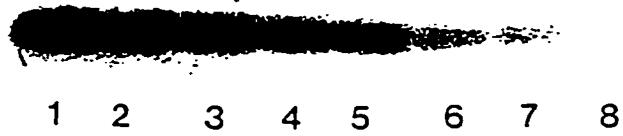

Fig. 10

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Table I: Effect of sulfated polysaccharides on adhesion of MT-2 cells to MS751 epithelia.

SULFATED POLYSACCHARIDES	ADHESION BLOCKING (IC^{50} µg/ml)
Fucoidan	15-30
Heparin	60-120
Dextran Sulfate	60-120
Heparan Sulfate	250-500
Carrageenan i	<1000
Carrageenan k	No effect
Carrageenan λ	No effect

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Table II: Effect of glycosidases on lymphocyte to epithelial cell adhesion.

ENZYMES	ADHESION BLOCKING (IC_{50} U/ml)
α-mannosidase	0.3-0.6
ß-mannosidase	0.1-0.2
α-L-fucosidase	0.015-0.03
neuraminidase	No effect
N-glycosidase	<5

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Table III : Immunofluorescent staining of lectins on MT-2 cells

Lectins	**Specificity**	**Staining**
Lotus purpurea	fucose	+
Concanavalin A	mannose	+
E. crystagalli	galactose	+
Wheat germ agglutinin	N-acetylglucosamine	+
Peanut agglutinin	ß-galactose	+
L. polyphemus	N-acetylneuraminic acid	-
R. communis	galactose	+

TABLE IV : Immunofluorescence staining of Carbohydrates on MT-2

Carbohydrate	Staining
α-galactose	-
ß-galactose	-
α-mannose	-
lactose	-
fucose	+

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Table V: Effect of sulfated polysaccharides on infection of MS751 epithelia.

SULFATED POLYSACCHARIDES	DOSE REQUIRED TO BLOCK INFECTION (IC_{50} ug/ml)
Fucoidan	8-40
Dextran Sulfate	8-40
Heparin	<1000
Heparan Sulfate	<1000
Carrageenan i	<1000
Carrageenan k	No effect
Carrageenan λ	No effect

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CHAPTER 3

Small Animal Model for Sexual Transmission of HTLV-I

ABSTRACT

The use of small animal models for studying retrovirus infections can provide critical information for developing HIV prevention strategies. In this study it has been demonstrated that mice inoculated intravaginally with HTLV-I-infected human T cells, MT-2, could be infected via this route. In addition to behavioral abnormalities observed 4 weeks postinoculation, HTLV-I proviral DNA was detected in spleen cells of inoculated mice by PCR amplification techniques. These results indicate that a mouse model for the sexual transmission of HTLV-I is feasible. Such a model would be useful in screening potential microbicidal agents for their ability to protect mice against HTLV-I via the vaginal route. Agents that act nonspecifically on the retroviral envelope may also be effective against HIV.

INTRODUCTION

The first two chapters have shown that exogenous cells can traverse the vaginal epithelium of the mouse, that genital tract epithelia can be infected by HTLV-I, and that infection can be blocked by sulfated polysaccharides *in vitro*. It may be that a vaginal formulation containing a sulfated polysaccharide may be protective against retroviral infections in the female genital tract.

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To develop alternatives for protection from pathogens during sexual intercourse it is essential that an animal model be available to study the process of sexual transmission. As HIV infects only humans and chimpanzees, it is not feasible to develop an animal model with HIV. Although the SIV/macaque model has provided important information for many aspects of pathogenesis and transmission of HIV (102), this model is impractical due to the number of animals which would be required in these types of studies. Cats can be infected with FIV by intravaginal inoculation (107). This model is an alternative but not very appropriate since FIV is naturally transmitted by biting and not by sexual contact. Another retroviral animal model is Murine Leukemia Virus (MuLV) in mice (138). In this case a sexually transmitted retrovirus infects it's natural host, however it is not as appropriate as a model using a human retrovirus. HTLV-I has the advantage that it is a human retrovirus which is relatively similar to HIV, although not similar as SIV. In addition, sexual transmission is one of the major routes of infection (69,110,142). An animal model mimicking sexual transmission of HTLV-I, resulting in a diseased state, would be a more suitable model for this mode of retroviral transmission. Compounds could be screened for their ability to prevent the vaginal transmission of human retroviruses in vivo.

Although there are no reports of infecting animals by the intravaginal route, there is evidence suggesting that it would be feasible. A number of studies of HTLV-I have been carried out in rats, rabbits and marmosets. In these studies, animals were injected

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intravenously or intraperitoneally with MT-2 cells or other HTLV-I secreting cells (119). These animals were found to raise antibody responses to HTLV-I antigens (64,106), express the virus genome in peripheral blood as well as some organs (83), and develop neurological symptoms akin to Tropical Spastic Paraparesis/HTLV-I Associated Myelopathy (TSP/HAM) (66,119). Rabbits which ingested HTLV-I infected lymphoid cells or were inoculated IV, seroconvert and develop similar symptoms (28,106,148,158,167). Marmosets have been infected by oral administration of infected T cells (75,178).

RESULTS

In a study using 14 mice, four weeks after intravaginal inoculation of MT-2 cells, mice began to show symptoms not observed in the control mice. These symptoms ranged from hair loss from the back and around the nose, sleeping separately, and nervous repetitive behavior. Within 2 months, symptoms progressed to include aggressive behavior towards cage mates and sensitive and inflamed limbs.

Using commercially available HTLV-I antibody ELISA kits, sera from MT-2-inoculated or control RPMI 1640-inoculated mice were tested for the presence of antibodies specific to the virus. ELISAs were also carried out using a synthetic gag peptide of HTLV-I (84). Although some mice had sera that was slightly more reactive compared to the controls, no significant differences were seen between the sera from control and experimental mice using either protocol. No significant reactivity was found when vaginal washes were assayed for antiviral antibodies either.

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A Western blot kit for detecting antibodies to HTLV-I was used to see if low level antibodies to specific proteins could be detected in mouse sera. Bands were seen corresponding fairly closely to p24, p19 and gp46 and these were not observed in the RPMI 1640 controls (Fig 1). In a subsequent experiment mice were inoculated either with infected MT-2 cells or with uninfected CEM cells. Western Blots performed on this group of mice showed that sera from both groups, MT-2-inoculated (#2-#11) and CEM-inoculated (#12-#21) showed reactivity to some of these proteins as well (Fig 2).

In order to determine if HTLV-I provirus could be found incorporated into host tissue, spleenic DNA from inoculated and control mice, was amplified by polymerase chain reaction using a primer pair, SK43 and SK44, specific for the Px region of the HTLV-I genome as well as SK54 and SK55, specific for the pol region. Since neither pair were sensitive enough to detect incorporated provirus, I amplified splenic DNA using two pairs of primers, Tr101/TR102 and TR103L/TR104 (also in the Px region), in a nested primer protocol provided by Dr. William Hall at the Rockefeller University. This procedure consists of initially amplifying a 264bp fragment in the pX region followed by a second amplification of the initial product for a smaller fragment (158bp). Bands were seen in DNA from spleens of mice infected with HTLV-I that corresponded to 159bp that was also seen in the DNA extracted from MT-2 cells used as a positive control (Fig 2). No corresponding bands were seen in spleenic DNA from uninfected, control mice.

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DISCUSSION

In this study mice were infected with HTLV-I through intravaginal inoculation with HTLV-I-secreting human T-cells (MT-2). Several methods were used to detect virus-specific antibodies in sera of inoculated mice, however the data obtained were not conclusive since sera from control mice showed similar reactivity. It is not surprising that the mice were raising antibodies to the human cells used for inoculation. It may be that these antibodies to the human T cell lines, MT-2 and CEM, were cross reacting with the HTLV-I proteins in the ELISA and Western blot protocols used. Unfortunately these serological techniques were not able to distinguish between antibodies to the virus and those that might have been directed against the human T cell lines used. One way around this problem would be to use mouse cells infected with HTLV-I. Although no such cell line exists, the current data indicates that it is possible to infect mouse cells since proviral sequences were found in the spleen.

Another issue to consider is the methods used in serological analysis. The commercial kits used to detect the mouse antibodies have been developed for human clinical diagnostic use although results from the synthetic peptide ELISAs were similarly dissappointing. Although these methods were modified for detection of mouse immunoglobulins there may be other factors affecting the assays. Finally it may be that mice do not have a strong antibody response to HTLV-I. In addition the presence of antibodies to murine endogenous retroviruses may be interferring with these detection protocols.

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DNA-PCR detected proviral sequences in spleen cells and inoculated mice displayed symptoms similar to those seen in humans infected with HTLV-I. Since PCR amplification of splenic DNA showed the presence of HTLV-I proviral sequences, this indicates that mice inoculated intravaginally with MT-2 cells can be infected via this route and that infection results in the incorporation of provirus in the mouse tissue.

In order to be able to use this system as a working model for screening compounds for prevention, a dose study will be required to determine the concentration of infected T cells in the inoculum that results in 50% or 100% of mice becoming infected. In addition a time study will be needed to determine the earliest time point at which infection can be reliably assessed.

It is very encouraging that mice can be infected by HTLV-I via the vaginal route. This is the first demonstration of a small animal model for the sexual transmission of a human retrovirus. Such a model would be extremely useful not only to look at prevention mechanisms but to study several questions: 1) what are the target cells in the vagina? and 2) can infected T cells penetrate the vaginal/cervical epithelial barrier and infect the underlying target cells? Finally such a model would permit the screening of potential antiretrovital compounds that have the ability to protect mice from infection via the vaginal route.

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FIGURE CAPTIONS

Figure 1a

Western Blot of sera from mice inoculated vaginally with MT-2 cells (#2-#5) or tissue culture medium (#6-#9). #22 is positive control serum from a HTLV-I positive patient.

Figure 1b

Western Blot of sera from mice inoculated intravaginally with infected MT-2 cells (#1-#11) or uninfected CEM cells (#12-#21). #22 is positive control serum from an HTLV-I positive patient.

Figure 2

Southern Blot hybridization of DNA extracted from spleen of 7 mice innoculated intravaginally with 10^5 MT-2 cells per mouse, 5 weeks after innoculation. A nested PCR protocol using the primer pairs TR101/TR102 and TR103L/TR104 was performed. This amplifies a 158bp segment in the pX region of the HTLV-I genome. Amplified DNA was electrophoresed in a 3:1 Nusieve agarose gel along with a 100bp DNA ladder. The DNA was transferred to a nitrocellulose membrane and subsequently hybridized to a DIG-conjugated DNA probe, SK 45. Lanes 1 to 7 correspond to DNA from mice innoculated with MT-2 cells. Lanes 8, 9 and 10 are splenic DNA from uninfected control mice. As a positive control, DNA from MT-2 cells was also amplified (lane 11).

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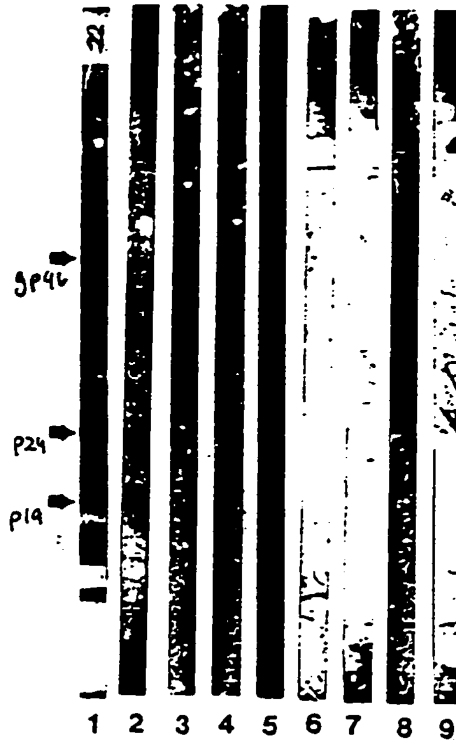

a

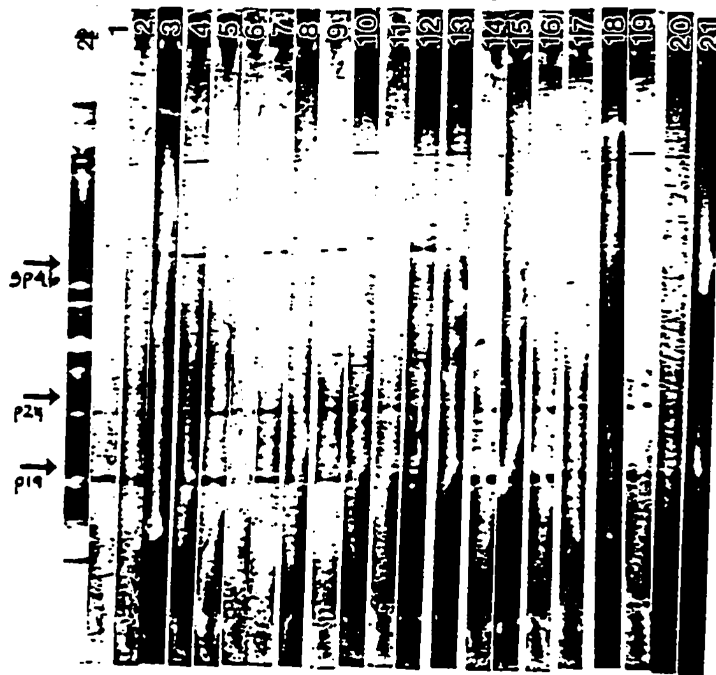

Fig. 1 b

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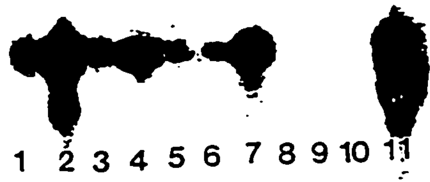

Fig. 2

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CHAPTER 4

Vaginal formulations of carrageenan protect mice against HSV-2 infection

ABSTRACT

The observations presented in this chapter show that vaginal formulations of the sulfated polysaccharide carrageenan are highly effective in protecting mice from herpes simplex virus-2 (HSV-2) infection. Test formulations were placed in the vagina of progestin treated mice prior to inoculation with HSV-2. Infection was determined by the presence of inflammation in the genital region and subsequent death. At a dose that infected half of the control animals, 1% solutions of either lambda, kappa, or iota carrageenan prevented infection of almost all of the animals. Concentrations as low as 0.5 mg/ml protected a large majority of the mice. At a dose that infected all of the controls, 1% solutions of carrageenans protected 85% of the inoculated mice. Other sulfated polysaccharides were less effective or showed no efficacy in preventing HSV-2 infection. Formulations of carrageenan also protected naturally cycling mice at the diestrus stage of their reproductive cycle as well as mice inoculated via the rectal route. These findings suggest that a vaginal formulation of carrageenan may be effective in blocking sexual transmission of HSV-2 in women. In addition the antimicrobial wound peptide PR-39 and it's truncated derivative PR-26 were both found to inhibit infection at a concentration of 20mg/ml indicating that other classes of compounds may also be effective in preventing vaginal infection of HSV-2.

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INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects the human genital tract. It is a large, enveloped virus (150Kbp) and initiates infection by attachment to cell surface receptors (151). The virus is able to infect mucosal epithelium in the genital tract and spread to the central nervous system in the absence of viremia resulting in a latent infection and there is no cure for infection.

The majority of genital herpes infections are caused by HSV-2. This virus infects many millions of people, causing persistent and recurrent painful genital lesions. Despite the efforts of numerous workers there is no vaccine for any sexually transmitted pathogen except hepatitis B virus. Thus, prevention strategies are limited to behavior modification and condoms. Results of recent *in vitro* and animal studies suggest that it may be feasible to develop a topical formulation that would prevent infection by various sexually transmitted pathogens (127,128, 161).

Although humans are the natural host for HSV-2, infection can be experimentally induced in many species including mice and guinea pigs, which can be infected through vaginal inoculation (99,126,173).

A mouse model to study vaginal infection by HSV-2 has been developed (96). Mice inoculated intravaginally with HSV-2 develop visible inflammation and hair loss in the perineal region accompanied by

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mucus discharge and local hemorrhage a week after inoculation (96). This is followed by hind limb paralysis and death a few days later. These mice elicit a strong immune response to the infection (125) and virus can be found in epithelial cells from vaginal swabs taken from infected mice a few days after inoculation.

Spear et al have identified cell surface heparan sulfate as the major factor in the binding of HSV to the cell surface (151). Since sulfated polysaccharides have been reported to inhibit infection of Green Monkey kidney cells (Vero) *in vitro* (93), it would be of significant importance to see if this group of carbohydrates is able to inhibit infection *in vivo*, perhaps by interfering with the attachment of the virions to the surface of the target cell.

In the previous chapter I developed a mouse model for the sexual transmission of HTLV-I. This model has the potential of being extremely useful not only in studying the mechanisms of infection via the genital tract but also for screening candidate antiretroviral compounds. However it will be some time before this animal model will be ready for reliably and efficiently testing compounds to block infection. In order to be able to test the efficacy of sulfated polysaccharides in an animal model, I have used the mouse model for HSV-2. It has been previously shown that certain sulfated polysaccharides can block infection of cultured cells by HSV and other enveloped viruses (11,93,113). In the present study the possibility that sulfated polysaccharides can block HSV-2 infection in mice has been explored. The results suggest that certain of these compounds are very effective. These compounds may

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prove useful in a topical formulation to prevent the sexual transmission of HSV-2 and possibly other enveloped viruses, including human immunodeficiency virus (HIV).

Wound Peptide PR-39 and Prophenin

Antimicrobial peptides are widely distributed in nature in both the plant and animal kingdoms. They are thought to be a part of a primitive immune system eliciting antibacterial activity at the mucosal surface. Peptides which kill microbes have been isolated from the skin and lumen (respiratory and gastrointestinal tracts) of vertebrate animals (16). Mammalian neutrophils produce a number of antimicrobial peptides. Recently there has been interest in developing these peptides for therapeutic applications. Some examples are the defensins (43), magainins (26) and proline and arginine-rich peptides (4,51). The wound peptide PR-39 and prophenin-1 have a unique amino acid composition in that proline and arginine represent more than 60% of the constitutive amino acids, and the other amino acids present are mainly polar (4). The fact that PR-39 is found in circulating phagocytes in the intestinal epithelium and is secreted in skin wounds supports the hypothesis that PR-39 is involved in the host defense on the mucosal surface. Although the existence of anti-microbial peptides has been known for more than ten years there has been relatively little progress in using these molecules as therapeutics, presumably because they are inactivated by serum (129). However, intradermal injection of defensins into rabbits has been shown to eradicate or delay development of cutaneous lesions

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caused by *Treponema palladium* (23) suggesting that anti-microbial peptides may be efficacious in some topical applications. Aboudy et al demonstrated that two synthetic amphiphillic peptides and magainin-2 were active against both HSV-1 and HSV-2 *in vitro* (3). Defensins have also been shown to inhibit HIV replication *in vitro* (115). The HSV-2/mouse model was used to test the efficacy of PR-39 and prophenin in preventing HSV-2 infection when applied vaginally. The results indicate that this family of compounds has potential in an antimicrobial vaginal formulation.

RESULTS

Determining Infectious Dose

In preliminary experiments the course of infection of animals that displayed vaginal inflammation at 6 days postinfection and those who did not was followed. In all cases mice with vaginal inflammation developed more severe symptoms, including hair loss and paralysis. Inoculated and uninoculated animals were caged together to determine whether HSV-2 was transmitted among cagemates. Vaginal inflammation was never observed in uninoculated animals.

A dose-range study was performed to determine a dose of virus for blocking studies (Table 1). A dose of 10^3 plaque forming units (pfu) was chosen, as this dose resulted in infection of about half the animals, reasoning that an infection rate of 50% would allow the detection of both inhibition and enhancement.

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Blocking Studies

Separate experiments were carried out with different vaginal formulations. In each case 20 animals were used per treatment group. As there could be unknown variables, a control of 20 animals treated with PBS were included in each of the nine experiments. Controls were very consistent; the number of animals infected was about 50% in all nine experiments. This data has been combined in Table 2.

Nonoxynol-9

Nonoxynol-9 (N-9) is the active ingredient in all vaginal spermicide products in the USA. A 4% N-9 product has been reported to block HSV-2 infection in mice (173). Since N-9 had been shown to protect mice from infection, this surfactant was employed as a positive control in the blocking experiments. It was observed that 0.5% N-9 slightly inhibited infection, 1% N-9 prevented infection in half the animals, and 2% N-9 completely blocked infection (Table 2).

Carrageenan

It has previously been found that a number of polysaccharides are effective in blocking cell-mediated infection of cervix-derived epithelial cells by HIV (128,161, 162). The most effective of these was dextran sulfate. Heparin, fucoidan, and carrageenan were also very

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effective. The effect of 0.5% and 1% aqueous solutions of these compounds was tested on HSV-2 infection in mice.

Carrageenans were by far the most effective in blocking HSV-2 infection. Of the first 120 animals that were tested with iota, lambda, or kappa carrageenan, two mice became infected, whereas the infection rate of control mice was about 50% (Table 2).

A study was carried out with decreasing doses of iota and lambda carrageenan. Iota carrageenan prevented infection in all of 60 animals at concentrations of 1%, 0.5%, and 0.25% and was highly effective at a concentration as low as 0.05% (Table 3). Results of the dosage study with lambda carrageenan were very similar (Table 4).

A similar experiment was carried out using low molecular weight dextran sulfate. Concentrations of 2.5% to 5% were required to protect the mice. Lower concentrations were ineffective (Table 5).

To determine if carrageenan would protect animals from high doses of HSV-2, the dose of HSV-2 was increased to 10^4 pfu; a ten fold higher dose. Different dosages of lambda and iota carrageenans were compared to PBS controls. In this experiment animals were not sacrificed after the first week, but each surviving mouse was examined once a week on days 14, 21, 35 and 42 post inoculation. All of the control mice died from the infection. However, both 1% lambda and iota carrageenan protected 85% of the animals. Doses of 0.1% also afforded some protection (Table 6).

Of the total of 140 animals used in this experiment infection was detected in 74 on day seven. Eleven more infected mice were detected on

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day 14 and 4 more on day 21. All animals judged to be infected died. No further infected animals were detected on days 35 or 42 (Table 6).

Dextran Sulfate

Dextran sulfate is the most commonly employed sulfated polysaccharide in *in vitro* blocking studies of enveloped viruses and usually the most efficacious of the sulfated polysaccharides (128,162). Because of its toxicity when administered systemically, dextran sulfate will probably not have a practical role in the treatment of HIV infection (41). Several groups are interested in using this compound for a vaginal "microbicide" for preventing HIV infection since its large molecular weight makes systemic absorption following intravaginal application theoretically improbable. A phase I safety trial has been carried out on a low dose formulation of this sulfated polysaccharide (2,155). Although high MW dextran sulfate did not inhibit HSV-2 infection, low MW dextran sulfate prevented infection in half of the animals (Table 2). A dose study to determine if higher doses of low MW dextran sulfate would block infection was carried out. In this experiment there was no significant effect at doses of 0.5% and 1%. However, doses of 2.5% and 5% protected the majority of the animals (Table 6).

Other sulfated polysaccharides

The sulfated polysaccharide heparin and fucoidin are efficacious in blocking infection, but not as effective as carrageenan. Chondroitin sulfate did not protect animals from infection (Table 2).

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Wound Peptide

The wound peptide PR-39 was found to inhibit HSV-2 infection when placed in the vagina prior to virus inoculation. Using the high dose of virus, 20mg/ml solutions in PBS resulted in 10/20 infected mice compared to 19/20 in the PBS control group. The activity of the peptide was dose dependant. Experiments in which the virus was pretreated with the peptide prior to inoculation showed enhanced protection (2/20 at 20 mg/ml) as shown in Table 7. PR-26, the truncated fragment of PR-39, showed a similar dose response (Table 8).

HSV-2 in vaginal vault and ganglia

Although HSV-2 infection in mice is generally fatal, it has been shown that HSV-2 can spread from the vagina and cervix to the lumbosacral ganglia and establish a latent infection (78,157,170). To determine if the vaginas of inoculated mice contained infectious virus or if dorsal root ganglia were latently infected, control (PBS) and 0.1% lambda carrageenan protected animals were inoculated with a high dose (10^4 pfu/mouse) of HSV-2.

To detect virus in the vagina, vaginal swabs were cultured on days 2, 5, 7, 9, 14 and 28 postinoculation on Vero cell monolayers. All cultures of Vero cells which were inoculated with vaginal contents from 10 control animals were positive for HSV-2 plaques on days 2 and 5. No further swabs were taken because 9 of the 10 control animals were dead on day seven and the tenth was very sick. Of the carrageenan treated animals 7 of 10 were positive on day 2; six of 10 on day 5. On day 7

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post infection two of the animals with positive assays had died and one of the remaining animals tested positive. This animal died on day 9. Cultures from the 6 remaining animals were negative on days 9, 14 and 28.

As a positive control, explants of dorsal root ganglia were cultured from 10 PBS-treated animals 7 days after inoculation. All cultures showed viral plaques. Explanted ganglia were cultured from 6 protected animals 28 days following inoculation. All of these cultures were negative for viral plaques.

Infection of Diestrus Mice

Although progestin treatment of mice make them more susceptible to infection this results in a less realistic model. In order to verify the results obtained with carrageenan in the hormone treated mice, female mice were allowed to cycle naturally. They were grouped according to their stage in the estrous cycle, which was determined by cytological observations of vaginal lavages. Mice at the diestrus stage were found to be most susceptible to vaginal infection and a virus dose of 2×10^4 pfu/mouse resulted in 7/20 diestrus mice being infected (Table 9). This dose was two times greater than what is required to infect about 90-100% of progestin-treated mice. In a subsequent experiment, diestrous mice were inoculated with a higher dose of virus in the presence of 1% or 0.1% λcarrageenan. Half of the PBS controls got infected while in the 1% group only 10% of the mice became infected (Table 10).

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Rectal Inoculation of Male Mice

Male mice were inoculated with 10^4 to 2 X 10^5 pfu of HSV-2 to see if they could be infected via this route. At the lowest dose 20% of mice became infected however 75% of mice became infected at the highest dose (Table 11). In a subsequent experiment a higher dose of HSV-2, 4 X 10^5 pfu/mouse resulted in 95% infection. 1% λ carrageenan administered via the rectal route resulted in 35% infection. 0.1% λ carrageenan still afforded some protection. (Table 12).

DISCUSSION

Sulfated polysaccharides have been shown to block infection of cultured cells by enveloped viruses. The work described in this chapter is the first evidence that a sulfated polysaccharide is highly effective in inhibiting HSV-2 infection in an animal model. Solutions of the sulfated polysaccharide carrageenan ranging from 0.25% to 1% are nearly 100% efficacious in preventing infection in mice at a virus dose that infects half of the control mice. Furthermore, the three types of carrageenan, kappa, iota, and lambda all appear to have a similar ability to block infection. When used at concentrations as low as 0.05%, carrageenan still protects the large majority of animals that were inoculated with HSV-2. At doses that infect all of the control animals 1% lambda or kappa carrageenan continued to protect 85% of the animals.

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It should be noted, that in the low dose experiments, infection was monitored only up to day seven. When animals were examined on later days in a subsequent experiment, infection was detected on day 14 or 21 in about 20% of the animals that had not shown symptoms on day 7. Thus, the per cent of animals infected in the low dose experiments is likely to be somewhat higher than would be the case if the animals had been followed further.

HSV-2 typically causes latent infections in man, although in very rare cases HSV can infect the CNS (56). In mice, the same virus typically kills the host although it can cause latent infections in ganglia (78,157,170). It is unlikely that the carrageenan-protected animals were latently infected because virus was not detected in cultures of ganglia or in vaginal smears. In addition, in the experiment where the animals were followed for 42 days, all of the 51 mice that did not display signs of infection on day 21 post infection still had no sign of infection on day 42.

The carrageenans are large galactose-linked polysaccharides that form a natural structural component of red seaweed. They are particularly attractive candidates for a vaginal formulation to prevent HSV-2 infection because they are considered GRAS (generally regarded as safe) by the US Food and Drug Administration. In fact, they are used as thickeners in soups and ice cream at a concentration of about 1%, the same concentration used in these experiments. These compounds are very stable, not substrates for bacteria, not absorbed, inexpensive, and used as inert ingredients in pharmaceutical products. The observations that

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carrageenans block HIV infection of cervix-derived epithelial cells (128) suggests that they may also find utility in protecting women from infection by HIV.

The efficacy of sulfated polysaccharides was compared to N-9. Carrageenan at 0.5 to 1% is considerably more effective than N-9 in blocking infection. N-9 has been shown to be highly effective *in vitro* in inactivating enveloped viruses including HSV (9), HIV (68), and feline leukemia virus (107). In comparison carrageenan is far less cytotoxic than N-9. *In vitro*, N-9 is toxic to lymphoma cells at a concentration similar to the effective dose (127), whereas carrageenan is effective at over 1000 times lower than the cytotoxic dose (128). A phase I safety trial of a 2% formulation of carrageenan was recently completed by the Population Council. No damage to the vaginal or cervical epithelia was observed by colposcopic examination (unpublished observations).

The antimicrobial peptides PR-39 and prophenin are generally active in the range of a few μg/ml against gram negative bacteria (21,149), and at much higher concentrations against gram positive bacteria (51). It is not surprising that higher concentrations are required to inactivate HSV-2 since it is an enveloped virus. PR-26 is a synthetic peptide consisting of the NH_2-terminal segment of PR-39. This truncated functional domain has been shown to be as effective against gram negetive bacteria as its parent molecule, suggesting that the anti-bacterial domain of PR-39 is within the first 26 amino acid residues of the NH_2 terminus (149). In this study PR-26 is as effective at inhibit-

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ing HSV-2 infections in mice as PR-39, further confirming that these 26 residues confer it's antimicrobial activity.

It has been previously shown that pregnant mice and mice at the diestrus stage of their cycle are more susceptible to HSV-2 infection (126,163). The results obtained in this study with cycling mice show that a higher dose of virus is required to infect mice at diestrus compared to progestin-treated mice. This is not surprising when one considers the extent to which the hormone treatment thins the vaginal epithelial barrier (165). However it is encouraging that λ carrageenan can still afford considerable protection to naturally cycling mice at a stage of their reproductive cycle when they are most susceptible to infection even though they were inoculated with a much higher concentration of virus.

Anorectal sex is not limited to the homosexual population. About 25% of heterosexuals in the U.S. have participated in receptive anal intercourse (147). Acute HSV-2 infection has been detected in the rectal mucosa of both men and women (46). These infections result in HSV proctitis and are accompanied by severe anorectal pain, difficulty in urinating, and diffuse ulcerations in the distal rectal mucosa. In this study male mice were inoculated with HSV-2 to see if mice could be infected via the rectal route. Male mice were chosen to eliminate the possibility of leakage of virus inoculum from the rectum to the vagina in females. External symptoms like swelling and inflammation of the anus and perineal region were observed as early as 4 days post inoculation. This was followed by more severe symptoms and hind leg

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paralysis, similar to vaginally inoculated mice, by 2 weeks post inoculation. Even though a high dose of virus was needed to infect a majority of untreated animals, 1% and 0.1% λ carrageenan were still protective. This suggests that λ carrageenan formulations may be able to protect people from HSV-2 during anorectal sex as well.

Although the results presented here are encouraging, it is premature to conclude that carrageenan will block infection of HSV-2 in women. In addition the evidence presented here and in *in vitro* studies with HIV is not sufficient to conclude that carrageenan will block HIV infection in women. As human efficacy trials for prevention of infection are demanding and expensive, the prudent approach in developing a vaginal formulation for prevention of STDs is to develop more animal models for the testing of carrageenan as well as other candidate compounds.

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TABLES

Table 1: Number of mice that were infected by various doses (plaque forming units) of HSV-2.

Pfu/mouse	N infected/total (%)
7×10^4	10/10
10^4	9/10
10^3	5/10
10^2	1/10
0	0/10

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Table 2: Number and percent of mice that became infected when the test formulation was placed in the vagina prior to inoculation with HSV-2.

Treatment	N inf./tot. (%)
PBS	44/100 (44%)
0.5% N9	7/20 (35%)
1% N9	5/40 (12.5%)
2% N9	0/20 (0%)
1% Chondroitin Sulfate A	9/20 (45%)
1% Dextran Sulfate (high MW)	9/20 (45%)
0.5% Dextran Sulfate (high MW)	8/20 (40%)
1% Dextran Sulfate (low MW)	4/20 (20%)
0.5% Fucoidan	5/20 (25%)
1% Heparin	7/20 (35%)
0.5% Heparin	3/20 (15%)
1% k Carrageenan	1/40 (2.5%)
1% i Carrageenan	1/40 (2.5%)
1% λ Carrageenan	0/40 (0%)

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Table 3: Number and percent of mice that became infected after different doses of iota carrageenan were placed in the vagina prior to inoculation with HSV-2.

i Carrageenen	N infected/total (%)
None (PBS)	9\20 (45%)
1%	0\20 (0%)
0.5%	0\20 (0%)
0.25%	0\20 (0%)
0.1%	2\20 (10%)
0.05%	2\20 (10%)

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Table 4: Number and percent of mice that became infected after different doses of lambda carrageenan were placed in the vagina prior to inoculation with HSV-2.

λ Carrageenan	N infected/total (%)
None (PBS)	9\20 (45%)
1%	0\20 (0%)
0.5%	0\20 (0%)
0.25%	2\20 (10%)
0.1%	2\20 (10%)

Table 5: Number and percent of mice that became infected after different doses of dextran sulfate (MW 8000) were placed in the vagina prior to inoculation with HSV-2.

Dextran Sulfate	N infected/total (%)
None (PBS)	13\20 (65%)
5%	5\20 (25%)
2.5%	3\20 (15%)
1%	10\20 (50%)
0.5%	9\20 (45%)

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Table 6: Number (%) of mice that became infected after PBS, lambda or iota carrageenan were placed in the vagina prior to inoculation with HSV-2. In this experiment the dose of was increased to 10^4pfu. Animals were examined on days 7,14,21,35 and 42 to determine if more infected animals would be detected after the first week. On day 7 we determined that 74 of the 140 animals in the experiment were infected. Eleven more infected animals were detected on day 14 and 4 more on day 21. All of the 51 carrageenan treated animals that did not show signs of infection on day 21 showed no signs of infection on days 35 or 42. "NC" indicates no change in the number of animals infected from the previous week.

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Treatment	Day 7 pi inf/tot (%)	Day 14 pi inf/tot (%)	Day 21 pi inf/tot (%)	Day 35 pi inf/tot (%)	Day 42 pi inf/tot (%)
PBS	17/20 (85%)	20/20 (100%)	NC	NC	NC
1% λ	2/20 (10%)	3/20 (15%)	NC	NC	NC
0.1% λ	9/20 (45%)	NC	11/20 (55%)	NC	NC
0.01% λ	14/20 (70%)	17/20 (85%)	18/20 (90%)	NC	NC
1% iota	3/20 (15%)	NC	NC	NC	NC
0.1% iota	13/20 (65%)	16/20 (80%)	NC	NC	NC
0.01% iota	16/20 (80%)	17/20 (85%)	18/20 (90%)	NC	NC

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Table 7: Number and percentage of mice that became infected after different doses of PR-39 was placed in the vagina. The table shows results of no pretreatment of the virus and a 30min pretreatment of the virus with the peptide before intravaginal inoculation.

PR-39	No Pretreat. # inf/total (%)	Pretreat. # inf/total (%)
None (PBS)	19/20 (95%)	17/20 (85%)
20mg/mL	10/20 (50%)	2/20 (10%)
10mg/mL	16/20 (80%)	2/18 (11%)
5mg/mL	17/20 (85%)	3/20 (15%)
2.5mg/mL	17/20 (85%)	4/20 (20%)
1% N-9	9/20 (45%)	-

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Table 8: Number and percentage of mice that became infected after different doses of PR-26 was placed in the vagina prior to HSV-2 inoculation.

PR-26	# infected/total (%)
None (PBS)	18/20 (90%)
20mg/ml	8/20 (40%)
10mg/ml	10/20 (50%)
5mg/ml	17/20 (85%)
2.5mg/ml	17/20 (85%)

Table 9: Number and percentage of mice at different stages of their estrous cycle that became infected after inoculation with 2 X 10[illegible] pfu of HSV-2.

Estrus Stage	# infected/total (%)
Diestrus	7/20 (35%)
Proestrus	2/18 (11%)
Estrus	0/14 (0%)
Metestrus	0/20 (0%)

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Table 10: Number and percentage of diestrous mice that became infected after λ Carrageenan was placed in the vagina prior to inoculation with 3.83 X 10^5pfu of HSV-2.

TREATMENT	# INFECTED/TOTAL (%)
PBS	5/10 (50%)
1% λ Carrageenan	1/10 (10%)
0.1% λ Carrageenan	2/10 (20%)

Table 11: Number and percentage of male mice that became infected after different doses of HSV-2 was inoculated in the rectum.

HSV-2 dose/mouse	# infected/total (%)
2 X 10^5 pfu	15/20 (75%)
10^5 pfu	7/20 (35%)
5 X 10^4 pfu	5/20 (25%)
10^4 pfu	4/20 (20%)

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Table 12: Number and percentage of male mice that became infected after λ Carrageenan was placed in the rectum prior to rectal inoculation of HSV-2.

TREATMENT	# infected/total (%)
PBS	19/20 (95%)
1% λ Carrageenan	7/20 (35%)
0.1% λ Carrageenan	12/20 (60%)

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CONCLUSIONS

The main goal of the research in this dissertation has been to develop a vaginal formulation that women can use to protect themselves from sexually transmitted viruses during intercourse. To fully understand how such a preventive formulation may be successful in a practical setting it was necessary to pursue certain aspects of sexual transmission in the female genital tract.

As described in chapters 1 and 2, there are many possible ways that HIV can infect a host. I have shown that cells can migrate through the vaginal mucosa and make their way to neighboring lymph nodes in a short period of time. Immunophenotyping of these cells indicates that both T cells and macrophages are capable of migrating. The data implicate infected cells in semen as having an important role in disseminating the virus to target cells within the female host. Although these observations were made using a mouse model, it is likely that this entry mechanism is possible in the human genital tract as well.

Further evidence implicating the role of cells in infection comes from the fact that many pathogens use cell to cell infection mechanisms to gain entry into the body. In the case of HTLV-I, which is predominantly cell associated, I have shown that infected T cells are able to adhere to cervix-derived epithelia and subsequently infect the epithelial cells by directional secretion of the virus onto the epithelial surface. Some sulfated polysaccharides were found to inhibit

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cell to cell adhesion as well as infection of the epithelial cell line by HTLV-I.

To be effective, a vaginal formulation must be able to protect a women against free viruses as well as cell associated viruses. The broad ranging activity of this large family of carbohydrates makes them attractive candidates for screening in *in vivo* models for sexual transmission. In chapter 3 mice were found to be infectable with HTLV-I when virus carrying cells were placed in the vagina. This is the first demonstration of a small animal model for the sexual transmission of a human retrovirus. Once this mouse model is standardized and quick and reliable methods of detection are established, this animal model will be extremely useful in studying viral pathogenesis and prevention mechanisms.

An excellent alternative has been the mouse model for genital HSV-2. Using this model I have screened compounds for their ability to protect mice against vaginal inoculation of HSV-2. Carrageenan was found to be highly effective against HSV-2 when compared to nonoxynol 9. I have also shown that mice can be infected rectally with HSV-2. This is the first demonstration of rectal transmission of HSV-2 in an animal model. Evidence presented here shows that carrageenan is effective in protecting mice against rectal inoculation with HSV-2 as well. Since the use of N-9 in an antimicrobial formulation is somewhat controversial owing to its detergent properties, less toxic alternatives are highly desirable. It is encouraging that antimicrobial peptides like PR-39 also show some activity against HSV-2 *in vivo*. Since its mechanism of

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action is not known further studies will have to be performed to understand how this short, proline-rich peptide works in this model.

The research performed in this thesis shows that an antimicrobial vaginal formulation based on carrageenan as its active ingredient is a very attractive possibility. The compound is cheap and safe, has a broad range of activity, and seems to be effective against infection via the vaginal and anorectal routes. These studies are preliminary and further work is needed using other *in vivo* model systems - perhaps the SIV/macaque model. Such a simple prevention strategy has the potential of saving many lives while the world waits for future vaccines.

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MATERIALS AND METHODS

CHAPTER 1

Donor cells

Female BALB/c mice (Harlan Sprague Dawley, Indianapolis, In) were inoculated intraperitoneally with 1 ml of Brewer's thioglycollate broth (Difco, Detroit, Mn.) to activate peritoneal cells.

Recipient mice

Six to 8 week old female BALB/c mice or outbred CD-1 mice were injected subcutaneously with 0.1 ml of 25mg/ml Depo Provera (Upjohn, Kalamazoo, Mi.) in PBS and used on the fifth day after progestin treatment.

Staining donor cells

Peritoneal cells were collected three days after activation by abdominal lavage of each mouse with 5ml of serum free RPMI1640 and centrifuged at 400 x g for 5 min and resuspended in PBS. The cells were counted and washed again in preparation for staining with PKH26-GL (Sigma, St Louis, Mi.). Briefly 2 X 10^7 cells were resuspended in 1 ml of Diluent C (provided in the staining kit) and the cells were added to 1 ml of 4 X 10^{-6}M of PKH26-GL dye and immediately mixed. The cells were incubated for 4 min at room temperature with periodic gentle mixing. The reaction was stopped by adding an equal volume of heat inactivated fetal bovine serum (FBS) (Gibco, Gaithersburg, Md.) for 1 min. and then washed 4 times in RPMI 1640 supplemented with 10% FBS at 400 x g for 10 min.

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The stained cell pellet was resuspended in PBS containing 3μg/ml Hoechst 33342 (bisbenzimide, Sigma, St Louis, Mo.) and incubated in the dark for 30 min at room temperature with occasional mixing. After the cells were double-stained they were then washed in PBS and resuspended in RPMI1640 supplemented with 10% FCS.

Inoculation of double-stained cells

Five days after progestin treatment mice were sedated with 0.1 ml of a mixture of ketamine HCl (Ketaset, Fort Dodge Labs., Fort Dodge, Ia.) and xylazine (Rompun, Miles Inc., Shawnee Mission, Ka.) in PBS. Twenty to 30 μl of double-stained cell suspension was inoculated into the vaginas of the mice using a P-20 Pipetman (Rainin, Woburn, Ma.) with or without a sheath of latex tubing. The number of cells that each mouse received was about 4×10^5 cells. Four hours later mice were sacrificed and the iliac lymph nodes removed. Cells were extracted from the lymph nodes by mincing and passing the minced tissue through a 100μm mesh insert (Falcon, Becton Dickenson, Lincoln Park, NJ) (1). Cells were extracted from lymph nodes of 2 untreated mice for negative controls, in addition to lymph nodes from mice inoculated with freeze-thawed double-stained cells. Vaginas and cervices were removed from all experimental and control mice and frozen in OCT compound.

Lymph node cells were washed in PBS at 400 x g for 5 min and the cells counted. They were then fixed in 2% paraformaldehyde for 30 min. followed by two washes in PBS. The cells were resuspended in 15 to 20 μl of PBS and 4 μl aliquots were spotted onto 8-ring slides (Carlson

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Scientific) and carefully coverslipped. The slides were sealed with clear nail polish before viewing in a Nikon epifluorescence microscope equipped with an Omega cube. Cells that had a red cytoplasmic stain and blue nuclei were counted.

Ten μm serial sections of frozen tissue were air dried. The sections were then fixed in 2% paraformaldehyde for 20 min. and washed twice in PBS for 5 min. and mounted using an aqueous mounting medium. As described above the sections were screened for double stained cells below the epithelium.

Immunophenotyping

Immunophenotyping of activated peritoneal cells was carried out by labeling the cells, after pre-incubation in rat IgG, with the following FITC-Mabs: anti-F4/80, a marker for resident and elicited macrophages (47) (Serotec, Oxford, England), anti-CD3 (clone 29B), a pan-T cells marker (Sigma, St.Louis,Mo) and anti-CD45R (clone RA3-6B2), a B-cells marker (Sigma, St.Louis, Mo).

Cells extracted from the lymph nodes were also stained with either the macrophage marker or the T cell marker. Briefly, lymph node cells from each mouse were washed and fixed in 4% paraformaldehyde. The cells were divided into two parts and one part was incubated with anti-mouse F4/80, diluted 1:100 in PBS and the other with undiluted anti-mouse CD3 for 1h at room temperature. The cells were washed and incubated in anti-rat IgG2b conjugated to FITC (Sigma, St. Louis, MO) similarly. The

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cells were washed and resuspended in a drop of PBS and observed in a fluorescence microscope as described above.

Sections of frozen vaginal tissue were air dried and fixed in 2% paraformaldehyde and washed. To block free aldehyde groups the sections were incubated in 10mg/ml ammonium chloride in PBS for 10min, rinsed in PBS and followed by a further blocking step in 10% mouse IgG (Sigma) for 20 min. Primary and secondary antibodies were diluted as described above. The slides were washed and mounted in PBS.

CHAPTER 2

Cell Culture

The human cervix-derived epithelial cell line, MS751, C33A, ME180 andf HT-3 were purchased from American Type Culture Collection (ATCC, Rockville, MD). The following reagent was obtained from AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: MT-2 from Douglas Richman. All cell lines were cultured in RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum. Cultures were maintained at 37°C and 5% CO_2.

Reagents

N-glycosidase, α-L-fucosidase, neuramidase, fucoidan, dextran sulfate, heparin, heparan sulfate, iota, kappa, and lambda carrageenan were purchased from Sigma (St.Louis, Mo), and α- and ß-mannosidase from

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Boehringer Mannheim (Indianapolis, IN). The mouse monoclonal antibodies to cell-adhesion molecules ICAM-1 (clone 84H11), LFA-1 (clone 25.3), LFA-3 (clone AICD58) and L-selectin (clone DREG56) were purchased from AMAC/Immunotech (Westbrook, ME). Another antibody to L-selectin (clone SK11), was purchased from Becton Dickenson (San Jose, CA). The lectins Lens culinaris, Con A, succinylated Con A and Pisum sativum were also purchased from Sigma.

Infection Assays

MS751 cells were seeded at a concentration of 2 X 10^5 cells/well into 6-well plates (Becton Dickenson, Lincoln Park, NJ) and cultured for 48-h. For infection with cell-free virus, we employed supernates of MT-2 cell cultures. For cell-mediated infection, MT-2 cells were pretreated with 200 μg/ml mitomycin C (Sigma, St. Louis, MO) for 1-h to destroy their ability to divide and washed three times with culture medium. Each well of MS751 cells then received 1 ml of MT-2 supernate (cell free) or 2 X 10^3 MT-2 cells in 1 ml of fresh medium. After 4-h of coculture, the wells were washed three times with serum-free medium to remove adherent and nonadherent MT-2 cells. The epithelial cells were washed daily for 6 days after which a 24-h accumulation of medium was collected and assayed for the presence of HTLV-1 p24 antigen by ELISA according to the manufacturer's instructions (Coulter Corp., Hialeah, FL).

Adhesion Assay

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The BCECF adhesion assay was employed as described previously (127). To study the effect of compounds on adhesion, serial dilutions of the compound to be tested were made in RPMI 1640 with 10% serum in 96-well U-bottomed plates. BCECF-labelled MT-2 cells were added (2 X 10^5/well) to these plates, and the cells and medium were mixed briefly with an eight-channel pipet. The cells in medium containing the test compound were immediately transferred to confluent monolayers of MS751 cells grown in 96-well plates. In some experiments we pretreated either MT-2 or MS751 cells for 1 h with the test compound before coincubation. After 90 min, nonadherent cells were washed off and fluorescence was read using a Cytoflour 2300 fluorescence reader (Millipore, Bedford, MA).

Immunofluorescence and FACS analysis

Cells were incubated with FITC-conjugated lectins (100 µg/ml) or carbohydrates (500 µg/ml) in PBS with 0.1% BSA, at 37°C for 30 min. acccording to the manufacturer's instructions. Cells were washed in PBS and resuspended in a small drop of Vectashield Mounting Medium (Vector Laboratories, Burlingame, Ca.) and viewed in a fluorescence microscope.

T cells and adherent epithelia were incubated with monoclonal antibodies directed at cell adhesion molecules at 4°C in PBS with 0.1% BSA and 0.01% sodium azide for 30 min. Cells were washed twice in cold PBS by centrifugation at 400 X g for 2 min. and a goat anti mouse FITC-conjugated secondary antibody (Boehringer-Mannheim, Indianapolis, In.) was added. After 30 min. in the cold the cells were washed as before

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and resuspended in a drop of Vectashield Mounting Medium. The labelled cells were visualized in a florescence microscope. Both fixed and unfixed cells were screened.

To more precisely quantitate expression of these molecules, I have used FACS analysis. Cells were stained as described above and fixed in 0.4% formalin in PBS. Fixed cells were taken to the FACS core facility at the City College of New York, NY where they were analysed.

PCR and Filter Hybridization

DNA (2 μg) isolated from 3 X 10^5 cells—either infected epithelial cells, uninfected epithelia, or MT-2 cells—using a nonorganic extraction kit (Oncor, Gaithersburg, MD) was amplified in a 100 μl PCR amplification buffer as described by Ausubel (10). The buffer contained 1 μM of each primer, 200 μM of each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$ and 2.5 units of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). The oligonucleotide primer pairs, SK43 (position 7358-7377) and SK44 (position 7516-7468), generic for the *tax* region of both the HTLV-I and HTLV-II genome, were synthesized in a Gene Assembler II (Pharmacia, Piscataway, NJ). A cycle consisted of three steps: 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min; each cycle was repeated 40 times.

The amplified product was initially electrophoresed in a 4% Nusieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME) and subsequently transferred onto a Genescreen Plus nylon membrane (NEN Research Products, Boston, MA) according to the manufacturer's recommendations.

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The air-dried membrane was prehybridized for 2-h at 65°C in an aqueous solution containing 1% SDS, 1 M sodium chloride, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. The membrane was hybridized overnight at 65°C in the prehybridization solution containing 10 pmol/ml of a digoxygenin-labelled HTLV-1 DNA probe (SK45, position 7447-7468). The 40mer oligonucleotide probe was synthesized in a Gene Assembler II (Pharmacia, Piscataway, NJ) and tail labelled according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Following hybridization, the membrane was washed twice in 2 X SSC for 10 min, twice in 2 X SSC and 1% SDS for 30 min at 65°C and twice in 0.1 X SSC for 30 min. Hybridized probe was detected using an anti-DIG alkaline phosphatase conjugate followed by an enzyme-catalysed color reaction (Boehringer-Mannheim, Indianapolis, IN). Amplifications and Southern Blotting were repeated 3 times with similar results.

In situ hybridization

Infected and uninfected MS751 were trypsinized, washed and seeded in 4-chambered Lab-Tek slides (Nunc Inc, Naperville, Il.) and cultured at 37°C and 5% CO_2 till confluent. As a positive control, MT-2 cells were dropped onto glass slides and allowed to air dry. The slides were fixed in cold 4% paraformaldehyde for 30min. The slides were processed for *in situ* hybridization using the primer pairs SK43 and SK44, according to the protocol provided by Boehringer Mannheim for the detection of digoxigenin (DIG)-labelled DNA probes using the Genius

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System. The probe used was the same as that used in the above Southern Blot protocol.

CHAPTER 3

Cells and Cell Culture

MT-2 cells and CEM cells were obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and maintained at 37°C and 5% CO_2.

Mice

Six to 8 week old, female BALB/-cbyj mice obtained from Jackson Laboratories were injected subcutaneously with 0.1 ml of 25 mg/ml medroxyprogesterone acetate (Depo-Provera, Upjohn Company, Kalamazoo, MI) in PBS. One week later mice were inoculated with 30 µl of tissue culture medium containing 10^5 MT-2 cells or CEM cells via the intravaginal route. As additional controls mice were inoculated with 30 µl of medium only.

Mice were monitored every 2-3 days for behavioral or physical changes. Blood was collected from the tail vein every 2-3 weeks, and serum separated and stored at -20°C. To obtain vaginal secretions 50-100 µl of PBS was flushed into the mouse vagina and the secretions were centrifuged to separate particulate matter. The supernatants were stored at -20°C.

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Detection of Antibodies

Commercially available enzyme immunoassay (EIA) kits (Cambridge Biotechnology Corp., Worcester, Ma.) for the detection of HTLV-I antibodies in human serum were modified to screen for HTLV-I antibodies in mouse sera. Briefly, mouse sera, separated from whole blood or vaginal washes was diluted with the kit diluent (1:20 to 1:100) and incubated in antigen coated wells for an hour at 37°C. The wells were washed six times in an automated ELISA plate washer (Dynateck) and the secondary antibody to human IgG was substituted with a goat anti mouse antibody to IgG conjugated to horseradish peroxidase (Sigma, St. Louis, Mo), diluted 1:3000.

ELISA was carried out on vaginal washes as well, as described above. To determine the isotype of the immunoglobulin in the vaginal washes secondary antibodies to IgA and IgM were used as well.

An alternate ELISA protocol was carried out using a synthetic peptide-based enzyme immunoassay as described by Lal et al (84). The HTLV-I gag peptide (Gag-1a$^{102-117}$) was obtained from the NIH AIDS Research and Reference Reagent Program.

Western Blots

Using a commercially available kit for the detection of anti-HTLV-I antibodies in human sera (Cambridge Biotech, Worcester, Ma) serum from mice inoculated with MT-2 cells, CEM cells or tissue culture media were screened for anti-HTLV-I antibodies. The manufacturer's anti-human

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IgG conjugate was replaced by an alkaline phosphatase-conjugated anti-mouse IgG (Sigma, St.Louis, Mo).

Detection of proviral DNA in mouse cells

DNA was extracted from mouse spleen or peripheral blood lymphocytes using a non-organic DNA extraction kit (Oncor, Inc., Gaithersburg, Md.). As a positive control, DNA was extracted from MT-2 cells. One to 2 µg of DNA were amplified using a panel of primer pairs specific for HTLV-I gene sequences. These included SK43/SK44 (positions given above) and SK54 (position 3365-3384) and SK55 (position 3483-3465). The probe used for SK54/SK55 was SK56 (position 3460-3426). In order to increase sensitivity, a nested PCR protocol was used to further amplify specific segments. The oligonucleotide primers (provided by Dr. William Hall at the Rockefeller University) were TR101 (position 7308-7327) and TR102 (position 7572-7553), TR103L (position 7337-7357) and TR104 (position 7495-7475). One µg of splenic DNA was amplified in a 50µl PCR amplification reaction mixture consisting of 20 pmol/µl of each oligonucleotide primer pair (TR101/TR102), 125 µM of each dNTP, 10 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$ and 5 units/µl Taq polymerase (Perkin-Elmer Cetus, Norwalk, Ct.). After initial heating for 5 min at 94°C, 35 cycles of 94°C for 40 sec., 53°C for 30 sec. and 72°C for 40 sec were run. After 10 min at 72°C the amplified products were stored at 4°C. A second round of amplification was carried out with 5 µl of the first product in a 50 µl reaction volume. Twenty pmol/µl of the second primer pair (TR103L/TR104) were used in a similar amplification protocol. The

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amplified product were initially electrophoresed in an agarose gel and then stained with ethidium bromide to visualize the DNA bands. For hybridization, DNA was transferred onto a Genescreen Plus nylon membrane (NEN Research Products, Boston, MA) according to the manufacturer's recommendations. The air-dried membrane was prehybridized for 1h at 65°C in a hybridization buffer containing 5X SSC, 1% blocking solution (Boehringer-Mannheim, Indianapolis, In.), 0.1% N-lauroylsarcosine, and 0.02% SDS. The membrane was hybridized overnight at 57°C in the pre-hybridization solution containing 10 pmol of a digoxygenin-labelled HTLV-I specific oligonucleotide probe (SK45) which recognizes a sequence within the pX region of HTLV-I. Following hybridization, the membrane was washed twice in 2 X SSC with 0.1% SDS for 10 min, twice in 0.1X SSC with 0.1% SDS for 10 min at 65°C. The hybridized probe was detected using an anti-DIG alkaline phosphatase conjugate followed by an enzyme-catalyzed color reaction (Boehringer-Mannheim, Indianapolis, IN). Amplifications were repeated 3 times with similar results.

CHAPTER 4

Mice

Six- to 8-week-old female BALB/c mice (Harlan, Sprague Dawley Inc., Indianapolis, IN) were maintained on a 12-h light cycle at The Rockefeller University laboratory animal care facility. Animals were

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injected subcutaneously, 5 days prior to inoculation, with 0.1 ml of medroxyprogesterone acetate (Depo-Provera, Upjohn, Kalamazoo, MI), 25 mg/ml in phosphate-buffered saline (PBS). Treatment with medroxyprogesterone acetate has been shown to increase the susceptibility of mice to HSV-2 infection (126,173). Six to 8-week old male BALB/c mice were also maintained in the same facility. Twenty-four hours prior to inoculation mice were deprived of food to clear the rectum of fecal matter.

In order to stimulate a natural estrous cycle, 6-to 8-week old female mice were maintained in a facility with a 14-h light period followed by a 10-h dark period for 2 weeks during which male urine-soaked bedding was added once a week to further stimulate cycling. On the day of infection, mice were assessed for the stage of estrous cycle by cytologic evaluation of vaginal lavages and grouped accordingly.

HSV-2

HSV-2 strain G obtained from American Type Culture Collection (ATCC, Rockville, MD) was propagated in Vero cells (ATCC) as described by McDermott et al (96). Virus titer was assayed by plaque formation on Vero cells as described by Rawls et al (140). Virus stock was aliquoted into 0.8 ml Eppendorf tubes and stored at -70°C. The same stock virus was used for all experiments.

Compounds

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Sulfated polysaccharides and N-9, purchased from Sigma (St Louis, MO), were diluted in phosphate buffered saline (PBS) (Gibco BRL). The sulfated polysaccharides used were 1.25mM of 8,000 MW dextran sulfate (D-4911), 0.02mM of 500,000 MW dextran sulfate (D-6001), 0.62mM of heparin (H-3393), 0.17mM of fucoidan (F-5631), 0.04mM of iota carrageenan (C-4014), 0.03mM of lambda carrageenan (C-3889), 0.06mM of kappa carrageenan (C-1263) and 0.2mM of chondroitin sulfate A (C-8529). PR-39, PR-26 and prophenin were synthesized at Dr. Phillips laboratory by FMOC peptide synthesis.

Infection

Mice were inoculated 5 days after progestin treatment. Naturally cycling mice were inoculated on the day that cytology was performed on the vaginal lavages. Twenty µl of the test compound was carefully instilled into the vagina using a P20 "Pipetman" (Rainin, Woburn, MA). For 1% formulations of iota carrageenan and kappa carrageenan, which are somewhat viscous, the end of the pipet tip was cut off to a diameter of approximately 1 mm for vaginal delivery. Five min after receiving the formulation, mice were inoculated intravaginally with 10^3 pfu or 10^4 pfu of HSV-2 in a volume of 10 µl. Six to 7 days after inoculation, mice were scored as infected or uninfected depending on the presence or absence of inflammation in the genital region. Mice were sacrificed after being examined. In subsequent experiments higher doses of virus were used and mice were scored on day 14 post infection.

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For rectal inoculation 50ul of PBS was flushed in and out of the rectum of mice that had been starved for 24h, to eliminate any remaining fecal material. Twenty µl of the test compound was instilled into the rectum followed 5 min. later by the virus inoculum in a 10 µl volume. Mice were maintained for 2 weeks and scored on day 14 post inoculation.

Assay for HSV-2 in vaginal secretions

Two groups of 10 mice were used for studying vaginal shedding and latency: Ten PBS control mice inoculated with 10^4 pfu of HSV-2 and 10 mice treated with 0.1% λ carrageenan prior to HSV-2 inoculation. Samples of vaginal secretions were collected by swabbing the vagina as described by Richards et al (96). HSV-2 was detected using a plaque assay on Vero cell monolayers (140).

Assay for latency

Four dorsal root ganglia were removed from each mouse as described by Waltz et al (170). Explanted ganglia were rinsed in medium and cultured on Vero cell monolayers in 12 well plates for 2 weeks. HSV-2 was detected by a plaque assay (140).

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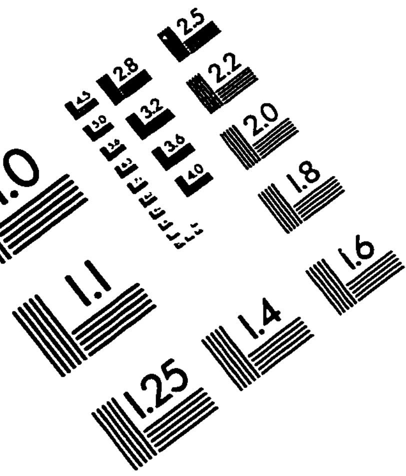

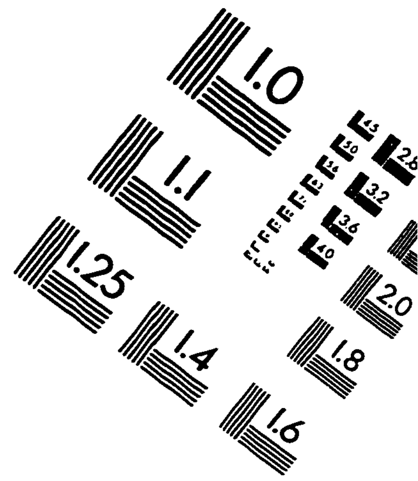

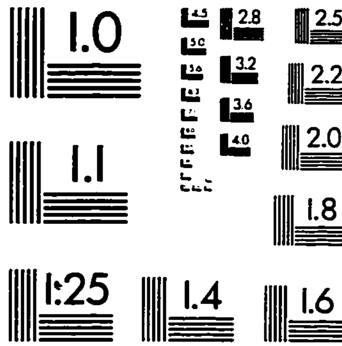

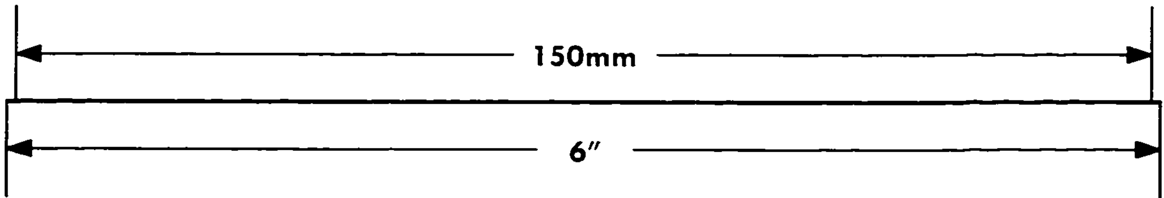

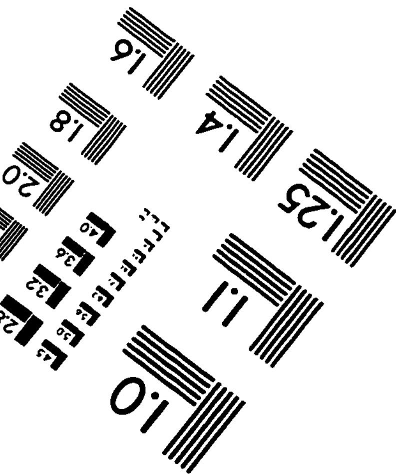

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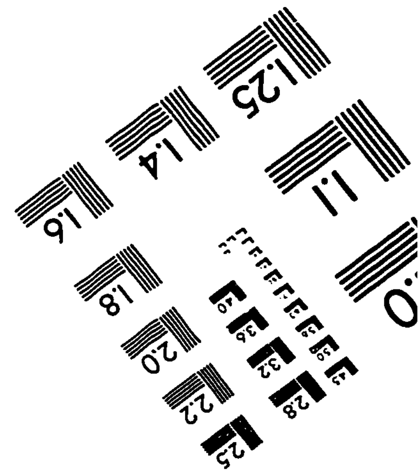

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