The Dissertation Committee for Jeffry W. Pennock Certifies that this is the approved version of the following dissertation:

ESTRADIOL AND MDMA AFFECT THE SUSCEPTIBILITY AND IMMUNE RESPONSE TO GENITAL HERPES AND GENITAL HERPES IMMUNIZATION

Committee:

Nigel Bourne, Ph.D., Supervisor

Kathryn A. Cunningham, Ph.D.

D. Mark Estes, Ph.D.

Gregg N. Milligan, Ph.D.

Lawrence R. Stanberry, M.D., Ph.D.

Dean, Graduate School

ESTRADIOL AND MDMA AFFECT THE SUSCEPTIBILITY AND IMMUNE RESPONSE TO GENITAL HERPES AND GENITAL HERPES IMMUNIZATION

by

Jeffry W. Pennock, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

September, 2009

Dedication

This is dedicated to my mother, the Hon. Carol Pennock, for the love and support that put me on the path to this, to Dr. Nigel Bourne & Rachael Stegall, for their wisdom in science and in life that enabled me to do this, to Shelly Wilson, for the fortification of her friendship that sustained and compelled me to complete this, and to Mather Martin, for helping me find where it is that I go from here.

Acknowledgements

I would like to acknowledge Rachael Stegall and Dr. Nigel Bourne, as well as Drs. Jordan Bubar, Milligan, Cunningham, Estes, Vargas, Pyles, and McGowin, in addition to Brent Bell, Sonja Stutz, Chin-Fun Chu, Alison Johnson, Rebecca Johnston, Ron Veselenak, Rae Ann Spagnuolo, Adriane Dela Cruz and Frances Valencia for their instructional and/or technical assistance in the execution of the studies presented here. I would also like to thank GlaxoSmithKline for the generous gift of the vaccine used in these studies and the National Institute on Drug Abuse (NIDA) for supplying us with the (+)-MDMA used in these studies. Lastly, I would like to thank the Sealy Center for Vaccine Development for the funding support provided by their predoctoral fellowship.

Estradiol and MDMA Affect the Susceptibility and Immune Response to Genital Herpes and Genital Herpes Immunization

Publication No._____

Jeffry W. Pennock, Ph.D.

The University of Texas Medical Branch, 2009

Supervisor: Nigel Bourne

Herpes simplex virus type 2 (HSV-2), the most frequent cause of ulcerative genital herpes disease, has infected nearly one-in-five Americans and is even more prevalent in the developing world. The virus is also known to be a synergistic copathogen to HIV and may even facilitate its infection. Nonetheless, the vast majority of HSV-2-infected patients are unaware of their infection and are capable of asymptomatically transmitting the virus during sexual contact, thus a prophylactic vaccine is needed. Therefore, the research presented here examines a factor that could be important in enhancing susceptibility to genital herpes disease as well as one that could be important in enhancing vaccine-afforded protection against it. There is no HSV-2 prophylactic vaccine currently available but there is a subunit vaccine candidate in clinical trials which has shown efficacy in preventing disease in women. Using this vaccine candidate, we were able to demonstrate infection prophylaxis, improved disease prevention and modulated antibody production by complimenting vaccination with estradiol in a murine model. We also showed the effects of estradiol on vaccine efficacy in a guinea pig model. Findings of estradiol-enhanced vaccine efficacy are the first of their kind using a vaccine of this type and have potential clinical relevance to the development of other vaccines, as well as our understanding of gender differences in vaccine efficacy. Meanwhile, 3,4-methylenedioxymethamphetamine

(MDMA, “ecstasy”) was abused by over 1.2 million Americans in 2007 and is popular in the dance club, rave and circuit party scenes. MDMA and other similar drugs are reportedly associated with increased incidence of sexually transmitted infections, like HIV or genital herpes, and may have immunological effects. We were able to demonstrate that MDMA causes increased susceptibility to HSV-2 infection in mice and earlier onset of genital herpes disease. We also demonstrated an MDMA-effect on the cytokines of the innate immune system, both systemically and, for the first time, in the genital tract. These data suggest MDMA may have an important biological role in infection and that estradiol may have an important role in vaccine-elicited protection against it.

Table of Contents

CHAPTER I

Introduction

HERPES SIMPLEX VIRUS TYPE 2 AND HERPES GENITALIS

Human herpes viruses (HHVs) are divided in three groups: α-herpes viruses [herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2) and varicella zoster virus], β-herpes viruses (cytomegalovirus, HHV-6 and HHV-7) and γ-herpes viruses (Epstein-Barr virus and Kaposi's sarcoma-associated herpes virus) (1). The HSV virion consists of four elements: the viral core containing the linear double-stranded DNA genome; an icosahedral capsid surrounding the core; a proteinaceous tegument; and an outer lipid bilayer envelope with spikes on its surface (2). The overall sequence homology of HSV-1 to HSV-2 is approximately 50% (3). The lipid bilayer of the envelope has ten glycoproteins embedded in it: gB, gD, gC, gD, gE, gG, gH, gI, gL and gM (2). HSV replication has both nuclear and cytoplasmic phases. The first step in viral entry is the reversible binding of gC and gB to cell-surface glycosaminoglycans. Mutants lacking surface gB and gC are still viable and removal of their target glycosaminoglycans causes a 10 to 20 fold decrease in plaque formation. The next step in viral entry is the irreversible binding of gD with its cellular receptors and the fusion of the viral envelope with the cell membrane. The gD has three types of cellular receptors: nectins, herpes virus entry mediator and a selected form of 3-O-sulfated heparan sulfate (1, 2). gD acts in concert with gB, gH and gL to enable fusion of the viral envelope with the cell membrane. HSV lacking gD can enter cells, via endocytosis rather than envelope fusion, however this results in an endosomal fusion with lysosomes that destroy the virus particles and induces apoptosis via a characteristic laddering of cellular DNA. At least a very small quantity of gD is required for a productive infection of cells. HSV-1 gD typically interacts with nectin 1 whereas HSV-2 can enter cells via either nectin 1 or 2. Upon fusion of the viral envelope with the cell membrane, several proteins are released from the virion to immediately prepare the cell for virus replication. Transcription of the virus genome, replication of viral DNA and assembly of new capsids take place in the nucleus (4).

The natural history and viral pathogenesis of herpes simplex is well understood, therefore it will only be briefly outlined here (4-6). Herpes is transmitted to neonates predominantly during vaginal delivery and to adults through close contact. The nature of this contact brings virus shed from a peripheral site, mucosal surface, genital secretions or oral secretions into close contact with a skin abrasion, epithelial disruption or susceptible mucosal surface, such as the mucosa of the oronasopharynx, conjunctivae, vagina or rectum. Inoculation of HSV into a susceptible mucosa or abraded skin allows for viral entry and replication in the epidermis, dermis or local epithelium. This is then followed by an infection of sensory and autonomic nerve endings, in which the nucleocapsid is intra-axonally transported to cell bodies in the ganglia. Although replication is usually focused within a given zone of innervation or dermatome, it is not restricted to that dermatome. HSV-1 has historically infected the orolabial region while HSV-2 predominantly infected the anogenital region, however this anatomical separation has diminished in recent decades (4). HSV-2 usually localizes in the dorsal root ganglion (DRG) that corresponds to the inoculated dermatome but it can be found in more than one ganglion, particularly the sacral ganglia 2-5, as there are major communicating branches and nervous plexuses between a ganglion and a nerve terminus in the pelvic region. Replication can continue in the ganglion, contiguous nervous tissue and adjacent dermatomes. Viral replication can spread centrifugally to extend to other mucosal and skin surfaces via peripheral sensory nerves. Therefore, the neurotropism of herpes can sometimes allow it to infect a large surface area, causing ulcerative lesions that are relatively distal from the initial foci of infection. Virus can also spread throughout a given mucosal surface. Histologically, a herpetic lesion is a thin-walled vesicle or ulcer in the basal layer. The ulcer is lined with multinucleated cells and distinctive intranuclear inclusions are often, but not always, observed. The tissue is acutely inflamed and there is some necrosis.

Widely spaced bilateral lesions of the external genitalia are the characteristic symptom of primary genital herpes, the initial presentation of herpes genitalis, usually caused by HSV-2 infection (although genital HSV-1 infection can cause herpes genitalis also) (7). The lesions can

be in various, asynchronous stages including vesicular, pustular or erythematic ulcers. Localized symptoms include pain, itching, dysuria, vaginal and urethral discharge, and tender lymphadenopathy of the inguinal nodes. The localized symptoms of primary genital herpes can be accompanied by fever, headache, malaise and myalgia. A prior oral HSV-1 infection can cause the systemic symptoms resulting from genital HSV-2 infection to be shorter in duration or to not occur at all (4). There have been reports of a reduced attack rate of HSV-2 in an adolescent cohort of girls with previous HSV-1 infections but this is contradicted by adult cohorts, suggesting that the impact of a prior HSV-1 infection in reducing genital herpes disease is more durable than a reduction in HSV-2 seroconversion (4, 8, 9). As the primary genital herpes presentation begins to resolve, the virus becomes latent in the ganglia (4). The virus reactivates from the ganglia frequently to shed infectious virus into the genital tract, often without causing additional symptoms; however, reactivation can episodically cause recurrent genital herpes in some patients, with lesions that are typically localized to a finite mucocutaneous location. Pain is significantly less severe and shorter in duration in recurrent episodes than it was in the primary episode. Usually, lesions are unilateral, the area of involvement is dramatically smaller than that of the primary infection and they last approximately one week (10).

Humans are the only natural reservoir for HSV-1 and -2 and no zoonosis has been reported (however, experimental animal models of infection and disease exist). HSV-1 is more frequently contracted at an earlier age than HSV-2 and more than 90% of adults test HSV-1 seropositive by age 50 (4). Data from the US National Health and Nutrition Examination Surveys (NHANES) suggest that 17% of Americans are seropositive for HSV-2 (11). The epidemiological record of herpes is among the oldest and most interesting in medicine, however, it remains difficult to generate reliable epidemiology about HSV infections to this day, with the best estimations coming from patient pool studies like the NHANES. Herpes prevalence shows a direct correlation with age and an inverse correlation with socioeconomic status (4). In the context of an epidemiological legacy that is centuries old, a distinct shift in pattern has been observed over the last 25-30 years: fewer herpes infections are being acquired in childhood, a

decline that is more than overcome a few years later by increases in sexually contracted herpes infections. Socioeconomic status is now more determinative than age in terms of if and when a person will seroconvert. As with most STIs, women bear a disproportionate burden of HSV-2 infection. The higher female prevalence may be due to a greater efficiency of transmission in the male-to-female direction than female-to-male direction. When examined in terms of race and gender, the cumulative lifetime incidence of HSV-2 infection in the US is 25% among non-hispanic white women, 20% among non-hispanic white men, 80% among non-hispanic black women and 60% among non-hispanic black men (4). Demographically, the greatest incidence of HSV-2 infection is found among patients who self-report to sexually transmitted disease (STD) clinics and among homosexual men. It should be noted that this latter group is a demographical overlap with those most frequently consuming MDMA, as discussed later in this chapter.

It is important to note that between 63% and 87% of HSV-2 infections are entirely asymptomatic, leaving many people unaware of their HSV-2 infection (4, 11). The sensitivity of a self-reported history of genital herpes disease for HSV-2 infection is less than 10% (12, 13). However, it is with seropositivity, rather than disease history, that virus shedding is associated. Infectious HSV-2 is shed into the genital tract in the absence of symptoms, even by people with no history of genital herpes disease as well as by patients between disease episodes (4, 14). While the frequency of detection of asymptomatic shedding varies, based largely on the sensitivity of the assay employed, it is definitively more frequent than recurrent disease and is believed to be the major source of genital herpes transmission to susceptible sex partners (4, 12, 14, 15). It is also important to note that an increasingly large body of data indicates that infection with HSV-2 increases the subsequent risk of HIV infection by at least two- to three-fold (16-22). This is true even when the preceding HSV infection is asymptomatic and the virus is shedding into an anatomically distinct area from that at which the HIV is introduced. At times of active herpetic lesions, the susceptibility to HIV infection is much greater than that of an asymptomatic phase. In patients already infected with both HSV-2 and HIV, the frequency, extent, severity of herpetic outbreaks is significantly increased.

Our knowledge of the synergy between HIV and HSV-2 is new, but our awareness of genital herpes is not; references to genital herpes span several millenia. Nearly a century ago, HSV was confirmed to meet Koch's postulates of an infectious agent (23). In the 17th century, the physician to King Louis VIX', John Astruc, was the first to formally describe herpes genitalis, which he most frequently found in prostitutes (2). The word *herpes* is Greek, and it was used by Hippocrates to describe lesions that "creep" along the skin. The Greek physician Herodotus in 500 BCE noted in a patient's chart that herpetic cutaneous were lesions associated with fever (the derivative English term "fever blister" still persists today), and his contemporary, Galen, was the first to note the lesions recurred at the same anatomic location (23). However, the explanation for why Galen's observation was true, the neurotropic nature of the HSV, would not be understood until the mid 20th Century. There are descriptions of lesions that resemble herpes on the 1500 BCE Ebers Papyrus and on a Sumerian Tablet from the 3rd Millennium BCE. Hebrew also has a word for herpes, shalbeket (24). Herpes (shalbeket), or descriptions consistent with herpes (shekhin), is described in the ancient Torahs as being one of the plagues of Egypt in Exodus, and is enumerated as one of the justifications for divorce in the Babylonian Talmud,. The history of herpes mirrors the history of advances in biomedical technology, even today. While the field of virology developed as a product of The Germ Theory and initiated the advent of molecular biology, studies using herpes viruses were never far behind the cutting edge and were often themselves the viruses used to break new ground in our modern scientific evolution.

An example of one such innovation is acyclovir, which was among the first class of antiviral drugs to be developed; its efficacy in treating herpes was hoped to signal the dawn of a new era in medicine in which the arsenal of antiviral chemotherapy would match that of antibiotics (25). Discovered in 1974 and available by prescription in 1982, acyclovir capsules were taken between three to five times daily at the first sign of symptoms for one or two weeks (longer if it was the first episode of genital herpes) to reduce the severity and duration of a disease episode (25). Newer generations of antiherpetic drugs have since been developed, valacyclovir and famciclovir, both of which are used in clinical practice along with acyclovir.

Acyclovir's benefits include its availability in intravenous form, useful when an HSV infection progresses to neurological complications such as meningitis, as well as the availability of low cost generic forms. Valacyclovir and famciclovir are more expensive but are effective at lower doses and require less frequent dosing. All three antivirals are effective at significantly reducing the number of lesions in an episode, the severity of pain associated with an episode and the duration of an episode (4). The dosing schedule varies depending on which drug is used, but recommendations for the duration of therapy have been revised, shortening the course of treatment across the board; first episodes of genital herpes need only be treated for 10-14 days and recurrent episodes are only treated for three to five days (26). In addition to reducing the gross pathology, studies have shown that these short-course episodic therapies for genital herpes are effective in improving patients' quality of life and that they reduce the psychosexual morbidity. However, short-course therapy does not resolve the infection; the virus persists in the DRG, will continue to cause recurrent disease episodes, will continue to replicate and shed, and will continue to be transmitted to other people.

It has been shown that chronic herpes suppressive therapy, taken daily even in the absence of a recurrent episode, can prophylactically reduce the frequency of recurrent disease episodes as well as reduce the incidence of HSV-2 seroconversion among susceptible sex partners (26-31). However, the use of acyclovir, famciclovir or valacyclovir for chronic suppressive therapy does not completely eliminate virus shedding and transmission to sex partners does still occur in some couples (29). Its efficacy is also limited by patient compliance, specifically the ability of the patient to afford the cost of medication (taken indefinitely, one to three times everyday) and the fidelity with which the patient adheres to the dosing schedule. Moreover, since the vast majority of people infected with HSV-2 are asymptomatic and unaware of their HSV-2 infection, they have no cause to seek suppressive therapy and it is, therefore, unlikely that chronic suppressive therapy will even moderately reduce the transmission of HSV-2 infection from a public health perspective.

GENITAL HERPES VACCINE CANDIDATE

Since the majority of persons seropositive for HSV-2 are unaware of their infection and since chronic suppressive therapies are unlikely to be feasibly employed for widespread chemoprophylaxis against disease or transmission, a prophylactic vaccine would be very beneficial. In recent decades many candidate vaccines, both prophylactic and therapeutic, have been developed and in recent years, multiple vaccines have entered clinical trials *(reviewed by Stanberry* (32) *and Roizman et al* (2)*)*. There is one vaccine that has shown the best safety and efficacy profiles to date in trials and it is undergoing an expanded Phase III clinical evaluation, conducted jointly by manufacturer GlaxoSmithKline (GSK) and the National Institutes of Health (33). The immunogen in this vaccine is a truncated recombinant of the HSV-2 surface glycoprotein D molecule (gD), expressed in plasmid transfected Chinese hamster ovary cells (34). Once purified, the immunogen is added to the propriety adjuvanting system "AS04", which contains 3-O-deacylated monophosphoryl lipid A (MPL) that is adsorbed to aluminum hydroxide (alum) along with the gD. The gD component was selected as the immunogen because it is very stable, well-conserved across various strains, necessary for the infective process (described above, thus escape mutants are unlikely), and elicits high titres of neutralizing antibody, antibody-dependent cellular cytotoxicity (ADCC) and virus-specific CD4 and CD8-mediated responses (35). The exact composition of AS04 is proprietary information but the general effects of alum and MPL are known. Alum serves as depot for the gD antigen of the vaccine, prolonging the duration of time from which gD is released from the vaccine injection site, as well as stimulating T helper 2 (Th2) immune responses (35, 36). The MPL, a lipopolysaccharide (LPS) derivative, stimulates the production of Interleukin-1 (IL-1), IL-6, IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF) from macrophages as well as stimulating interferon-γ (IFN-γ) production and IL-2, thus yielding Th2 helper responses (36). In sum, the vaccine is designed to be able to stimulate both Th1 and Th2 immune responses rather than skewing towards a bias of one at the exclusion of the other.

In 2002, Stanberry *et al*. published the results of two large, double-blind, placebo-controlled, phase III clinical trials conducted in the late 1990s to examine the efficacy of this HSV-2 candidate vaccine from GSK (34). Study 1 contained 847 HSV-1 and HSV-2 double-seronegative subjects aged 18 to 45 years, 268 of which were women, from 57 participating clinical trial sites in the United States, the United Kingdom, Australia and Canada; this study was chiefly designed to quantify the occurance of symptomatic genital herpes disease. All participants in this study were members of HSV-2 serodiscordant couples in which the participant's regular sexual partner (the "source partner") had a clinical history of genital herpes disease who had agreed not to use suppressive antiviral therapy during the study. Study 2 was initially designed as a large safety and tolerability study in 2491 subjects aged at least 18 years at 61 sites in the United States, Italy, Australia and Canada who were seronegative for either, neither or both HSV-1 and HSV-2; among them were 1867 HSV-2 seronegative individuals, 710 of whom were women; an examination of vaccine efficacy at genital herpes disease prevention among women who were HSV-2 seronegative upon entering the study and women who were HSV-1 and -2 double-seronegative upon entering the study were added as primary and secondary endpoints, respectively, based on the results of Study 1 but prior to examining the results of Study 2. All of the subjects Study 2 had a regular sexual source partner who was allowed to take suppressive antiviral therapy (which they may or may not have done). Almost all couples in both studies were oppositely-sexed. Subjects were intramuscularly injected in a deltoid with either vaccine or a control preparation at months 0, 1 and 6 of the study and followed for 19 months. Subjects had three to six scheduled post-immunization follow-up visits at which they were screened for study endpoints. The case definition for genital herpes disease was:

genital symptoms: (at least one)	pain, itching, swelling, papules, vesicles, ulcers, crusts
AND	
laboratory confirmation:	positive HSV culture OR detection of HSV DNA and HSV seroconversion

HSV infection, a secondary endpoint, was defined as having genital herpes disease (as defined above) or asymptomatically seroconverting to HSV antigens other than gD (which was contained in the vaccine). Participants also recorded details of suspected disease-episode onsets and visited a clinic within 48 hrs for examination and blood- and swab-sample collection at those times as well.

The vaccine was well-tolerated. Soreness at the site of injection was frequently experienced but was usually mild to moderate, with only 5% of recipients experiencing pain that interfered with major life functions (compared to 1-3% in the control group), and study drop-outs among the vaccine recipients were similar compared to controls.

The vaccine was effective at preventing genital herpes disease among HSV-1 seronegative women. It did not provide significant protection against HSV infection in either gender. A trend toward protection against infection was observed in HSV-1 & -2 double-seronegative women, with 45% efficacy in Study 1 and 39% efficacy in Study 2, but this fell just short of statistical significance. Nonetheless, these women who entered the study as double-seronegatives did benefit from a significant protection against genital herpes disease, with 73% efficacy in Study 1 and 74% efficacy in Study 2 observed via Cox regression analysis. There were additional surprising results from these trials. Men in either study and of any HSV serological status, including double-seronegative, were not afforded even slight protection against infection, with efficacy values of -7% to -19%. The efficacy of protection against disease among double-seronegative men in Study 1 was a negative number, a positive number in Study 2, but was significant in neither case. The observation that women received better protection

than men was surprising because women are considered to be more susceptible than men to HSV-2; therefore, that a vaccine which initially appeared to be suboptimal would perform more robustly in the population at greater risk is unexpected. Another unexpected finding was the lack of efficacy in women who were HSV-1 seropositive and HSV-2 seronegative at the time of entering the study. There are conflicting studies about whether these women would have naturally been expected to have a reduced HSV-2 infection attack rate because of their preexisting HSV-1 infection; the incidence of infection in this population segment of Study 2 was not reported (4, 8, 9, 12, 34, 37). However, it is reported that these women in the vaccine recipient group experienced a not-statistically-significant 11.6 fold increase in the attack rate of genital herpes disease compared to their cohorts in the control group. It is known that persons infected with HSV-1 are significantly more likely to be asymptomatic if they seroconvert to HSV-2 than people who do not have HSV-1, and it is thought that this is because there is some level of non-sterilizing cross-protective immunity resulting from the HSV-1 infection which has the effect of preventing genital herpes disease (4, 12). Thus, while it is disappointing, it is nonetheless understandable that HSV-1 seropositive women in the vaccine group, already benefitting from a background level of immunity, might not develop a detectably-enhanced resistance resulting from immunization. However, this uptick in disease in HSV-1 seropositive women, combined with the fact that efficacy was demonstrable only in HSV-1 seronegative women, could potentially complicate the future implementation of this vaccine, since HSV-1 infections are more common and typically occur earlier in life than HSV-2 infections (4). If this vaccine needs to precede exposure to HSV-1 to demonstrate efficacy against HSV-2, then the target age would necessarily be much younger than initiation of sexual activity (which might otherwise have been the targeted age group); and this, in turn, would demand that a vaccine regimen administered early in life be able provide a durability of protection that spanned the duration of sexual activity in a recipient's life. This is matter that clinical trials have not yet addressed.

In general, there are three different avenues by which a vaccine can be effective. The most obvious is that of sterilizing immunity, in which vaccination would afford immunity so robust that inoculated HSV-2 would fail to establish infection. The most common type of immunity induced by Advisory Committee on Immunization Practices (ACIP)-recommended vaccines is that of protection against disease, in which an infectious quantity of a pathogen may easily establish a productive infection but does not overwhelm the vaccine-afforded protection against the development of pathological symptoms. The third is transmissibility, in which a non-sterilizing immunity may or may not prevent disease but does function to reduce the quantity of pathogen that an infectious host sheds to susceptible contacts. The latter two avenues are independent of each other, not mutually exclusive and both are desirable when sterilizing immunity is not attainable.

Sterilizing immunity to HSV-2 infection is thought to be an unlikely goal. As discussed in a previous section, subsequent to infection, HSV-2 establishes latency in the innervating DRG from which it periodically reactivates to shed (4, 12, 38). This means that the immune system is exposed to HSV-2 hundreds of times over the natural course of infection and is nonetheless unable to develop immunity robust-enough to impede virus transmission or reduce mucosal viral loads below the level of detection. Therefore, it would be quite challenging to develop a vaccine that elicited a sterilizing immune response to the virus upon the immune system's first encounter with it after only one or even a series of immunizations. The inability to develop sterilizing immunity to HSV-2 when outside of its neurological sequestration, as is the case during symptomatic and asymptomatic reactivation shedding, also suggests that the adjuvanting mechanism employed will be as important as the immunogen the vaccine contains, if an HSV-2 candidate vaccine is to have any efficacy at all. The adjuvant must, by necessity, elicit immune responses both broader and more robust than those elicited over the course of a persistent natural infection. But this too is a difficult challenge because an adjuvant that strongly elicits vigorous, broad-spectrum immune responses may by its very nature also be capable of eliciting adverse events ranging from localized inflammatory reactions to systemic anaphylaxis. Further, reports of

even exceptionally rare serious adverse events during clinical trials can result in trials being halted and/or hesitation by clinicians to recommend the vaccine in the clinical setting; it has been demonstrated by studies within managed care organizations that a physician's acceptance of ACIP recommendations for universal vaccination was the most important factor in determining whether patients were vaccinated (rather than published data on efficacy, need, benefit, etc.) (39-41). The development of adjuvants that stimulate without overstimulating the immune system is being pursued in both academia and industry but is not easy. The efficacy achieved by the GSK candidate HSV-2 vaccine is attributed to their proprietary AS04 adjuvant (32).

The GSK candidate vaccine showed efficacy in the protection against genital herpes disease, although not infection, in a certain population segment of study participants. While reducing the incidence of genital lesions would unquestionably improve the quality of life of vaccinees who may later become HSV-2 seropositive, increasing the size of the asymptomatically infected population as a function of reducing the size of the symptomatic population creates cause for concern as well. In a study of serodiscordant couples in which the source partner was aware of their infection and periodically symptomatic, the annual rate of HSV-2 transmission to their previously uninfected partner was 10%, and in 70% of cases the transmission event took place during periods when source partner was asymptomatic (15). Although unpleasant, periods of symptomatic disease are a signal to the informed-patient that active virus shedding is underway, so that they can inturn impede transmission through barrier protection and/or chemotherapeutic medication. It is hypothesized that an intervention that eliminates this signal of potential transmissibility and increases the size of the population that is unaware that they are infected, and moreover infectious, without reducing the ability to transmit infection could subsequently increase the incidence of transmission, and likewise could increase the disease burden of neonatal herpes and of genital herpes in sex partners who are unimmunized or are not protected by immunization, e.g. men (42). The important decision regarding whether disease prevention in a large population of individuals could justify increased transmission to individuals of a different but also large population was temporarily deferred by data that

suggested it was possible the GSK vaccine candidate might also have an impact on transmissibility.

A vaccine which reduces the transmissibility of an infection that it failed to prevent would nonetheless be an effective vaccine, and this may be an altogether more realistic goal of immunization against pathogens for which sterilizing immunity is more difficult, such as HSV-2. It had previously been reported by *Wald, et al*. that patients with the most frequent recurrent disease also experienced the most frequent symptomatic shedding (43). It was hoped that the converse would be true and that an immunization which reduced the incidence of disease would reduce transmissibility by a default reduction in shedding. This rationale, although untested in the human efficacy trials because no shedding data was collected, was buoyed by data from *Bourne, et al*. who, utilizing an animal model of HSV-2, were the first to demonstrate that this vaccine candidate was effective at significantly reducing the incidence of disease as well as the mean quantity of latent virus in the DRG and the quantity virus being shed into the vaginal mucosa (44, 45). The intuitive explanation is that the immunity afforded by vaccination, non-sterilizing but effective at reducing disease, also reduced the amount of virus ascending to the DRG, which in turn reduced the quantity of virus that was being shed from the DRG and which may have been even further reduced by the memory immune responses elicited by the shedding virus. Interestingly, however, the frequency that this, albeit smaller quantity of virus being shed was not also reduced. Nonetheless, the concept was affirmed by the 2004 report that valacyclovir, taken as chronic, daily suppressive therapy by HSV-2 seropositive study-participants significantly reduced both the frequency and copy number of viral shedding from source partners as well as the frequency of infection transmission to their susceptible sex partner (29). This report was compelling because it was larger than similar studies of this type, containing 1484 participants with an 89-person daily-swabbing substudy, and because it also showed a significant reduction in the incidence of recurrent disease in the source partner and the incidence of primary disease in the susceptible sex partners who became seropositive. The data was important, specifically in a herpes vaccinology context, because it was consistent with the

Bourne animal data that a reduced frequency of recurrent disease would be coupled with a reduction in viral shedding and because it appeared to demonstrate, in a large-scale clinical setting, that the previously-suggested linkage of the endpoints of reduced recurrent disease and asymptomatic shedding with the endpoint of reduced transmission from source partner to susceptible partner existed. Mathematical modelling data indicates a vaccine effective only at disease and shedding reduction, administered only to double-seronegative females, and using the magnitude of efficacy observed in the GSK-vaccine trials, would, in 20-30 years, yield a significant HSV-2 herd immunity that, as a result of reduced transmission, would have protective effects in both genders and HSV-1 seropositive individuals as well (42).

However, data from the same valacyclovir clinical study published three years later contained additional details that were surprising. It was reported that, although the overall study did observe significantly reduced infection transmission from source partners to susceptible partners in suppressive-treatment groups compared to placebo controls, none of the susceptible partners of 89 source partners participating in the shedding substudy became HSV-2 seropositive (27). None of the source partners in the placebo group and none of the participants shedding the greatest quantity of virus on the most frequent number of days actually transmitted the infection over the course of the >65 100-person-years that the substudy participants were followed; this was despite the fact that the study was designed with an anticipated clinically symptomatic transmission rate to susceptible partners to be 5% per 100 person years and with much more frequent asymptomatic transmissions anticipated (27, 29). Moreover, when stratifying source partners within their respective treatment groups based on frequency of recurrent disease, they observed no correlation with viral shedding rate nor with the risk of infection transmission to susceptible sex partners (27). This could call into question previous assertions that either more or less frequent disease is immunologically, rather than coincidentally, correlated with more or less viral shedding, respectively, as well as the relevance of whether shedding rate reduction translates into reduced transmissibility. An explanation of how to reconcile these observations in

this large clinical study with the body of literature that preceded it or a confirmatory reproduction of comparable magnitude remains outstanding.

Meanwhile, four additional phase III clinical trials of the GSK vaccine, currently referred to by the tradename HERPEVAC, have been initiated. A study examining the prophylactic efficacy against genital herpes disease in 8327 subjects, women only, aged 18 to 30 years, over 20 months and who must enter the study seronegative for both HSV-1 and HSV-2, has completed recruitment and is still ongoing (33, 46). Pregnant women in that study were being enrolled in a neonatal substudy to quantify the vertical transmission of vaccine-specific antibody at two and six months postpartum, however this observational substudy was terminated for reasons not stated on the NIH Clinical Trial information sheet (47). A phase III safety and immunogenicity study in 5960 girls of any HSV serological status who are aged 10-17 years, which is a more rational target-age for implementation of this vaccine than ages 18-30, was completed in April, 2009 (48). The manufacturer also completed a phase III lot-to-lot immunogenicity, safety and consistency substudy in November, 2008, from among 671 of these 10-17 year old girls (49).

ESTRADIOL

Androgens, estrogens and their precursor progestins, the three classes of sex hormones, are primarily synthesized by the gonads and the adrenals and have biological functions which extend far beyond the reproductive system or the induction of primary and secondary sex characteristics (50) (Figures 1 & 2). They interface with every system of the body and a full discussion of the scope of their impact is beyond this introduction. The gender differences seen in the efficacy of protection against genital herpes disease in the vaccine clinical trials and the fact that current trials of HERPEVAC recruited female-only cohorts demonstrate the need to better understand the impact of gender and sex hormones on the mucosal immune system, starting with the female sex hormones, which classically encompass the estrogens and progestins.

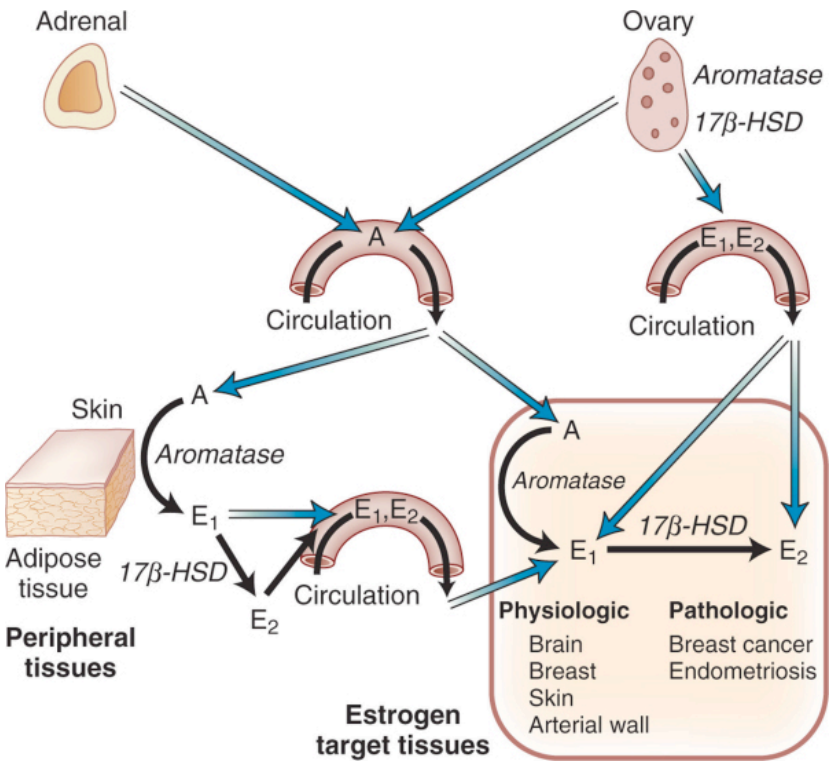

Figure 1. Estrogen secretion in women.

The biologically active estrogen estradiol (E2) is produced in at least three major sites: (1) by direct secretion from the ovary in reproductive-age women; (2) by conversion of circulating androstenedione (A) of adrenal or ovarian origins, or both, to estrone (E1) in peripheral tissues; and (3) by conversion of A to E1 in estrogen target tissues. In the latter two instances, estrogenically weak E1 is further converted to E2 within the same tissue. The presence of the enzyme aromatase and 17β-hydroxysteroid dehydrogenase (17β-HSD) is critical for E2 formation at these sites. E2 formation by peripheral and local conversion is particularly important in postmenopausal women and in estrogen-dependent diseases such as breast cancer, endometriosis, and endometrial cancer. From Kronenberg: Williams Textbook of Endocrinology, 11th ed. (51). Reproduced with permission.

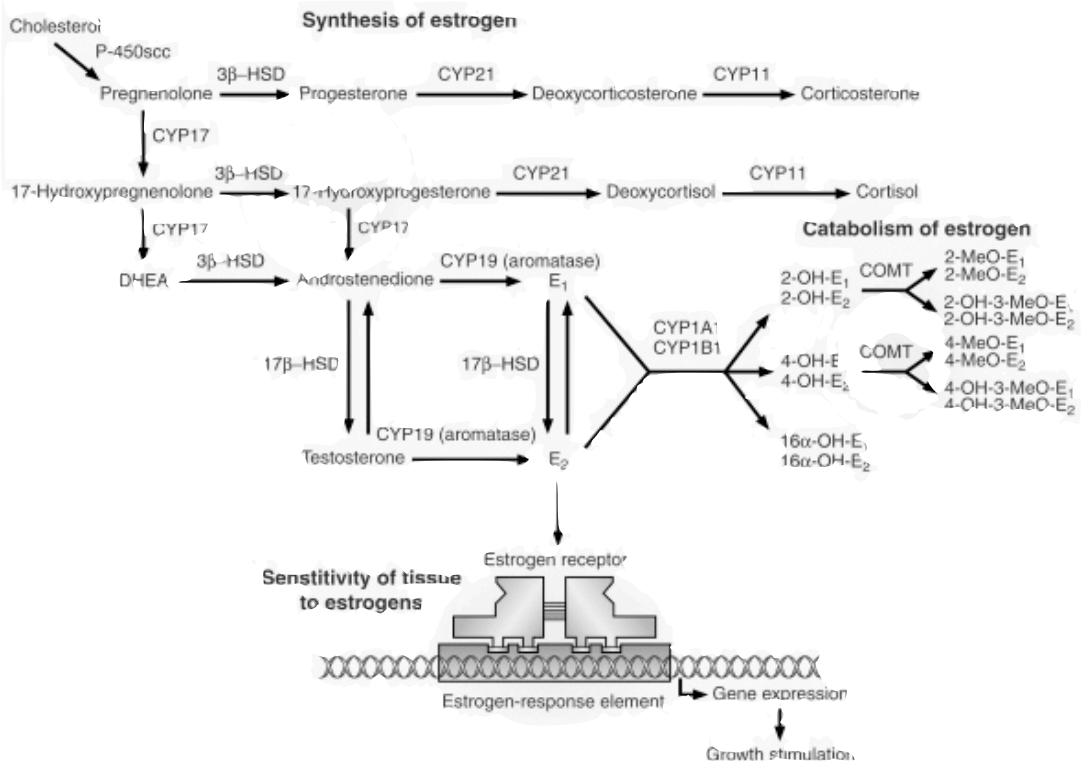

Figure 2. Synthesis and activity of estrogen.

The synthesis of estrogens, progesterone and the androgens and other corticosteroids all begin with cholesterol, although only synthesis and catabolism of estrogen is emphasized here. 2-MeO-E1, 2-methoxyestrone; 2-MeO-E2, 2-methoxyestradiol; 2-OH-3-MeO-E1, 2-hydroxyestrone 3-methyl ether; 2-OH-3-MeO-E2, 2-hydroxyestradiol 3-methyl ether; 2-OH-E1, 2-hydroxyestrone; 2-OH-E2, 2-hydroxyestradiol; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 4-OH-3-MEO-E1, 4-hydroxyestrone 3-methyl ether; 4-OH-3-MeO-E2, 4-hydroxyestradiol 3-methyl ether; 4-OH-E1, 4-hydroxyestrone; 4-OH-E2, 4-hydroxyestradiol; 16α-OH-E1, 16α-hydroxyestrone; 16α-OH-E2, 16α-hydroxyestradiol; 17β-HSD, 17β-hydroxysteroid dehydrogenase; COMT, catechol O-methyltransferase; CYP11, 11β-hydroxylase; CYP1A1, cytochrome P-450 1A1; CYP1B1, cytochrome P-450 1B1; CYP17, 17β-dehydroxylase; CYP19, P-450 aromatase; CYP21, 21-hydroxylase; DHEA, dehydroepiandrosterone; E1, estrone; E2, estradiol; P-450, cytochrome P-450; scc, side-chain-cleavage enzyme. From Clemons M, Goss P: Estrogen and the risk of breast cancer. N Engl J Med 344:276-285, 2001. Reproduced with permission.

The mucosal immune system of female reproductive tract has been shown to be a somewhat-independent immunological region capable of local antibody production, in response to cervically deposited antigens, and participating in the common mucosal immune system, with specific IgA and IgG antibodies to antigens from the oropharynx and gastrointestinal tracts, as well as multisystem immune responses, with antibodies to bloodborne antigens (52-57). The immune system of the female reproductive tract has evolved to balance the necessity of warding off invading pathogens with that of tolerating a fetus that is 50% foreign. The product of this selective pressure is an immune system optimized for both maternal and fetal survival at each stage of menstruation and pregnancy and the instruments used to achieve this optimization are the female sex hormones which enhance and suppress components of the mucosal immune system at each anatomical site of the female reproductive tract (e.g., vagina vs cervix vs uterus) and at each reproductive/endocrine state (58-62). For example, cytotoxic T cell activity in the uterus has been shown to be present during the proliferative phase of menstruation, absent during the secretory phase, and greatest in postmenopausal uterine tissues, whereas it is persistent in the vagina and up to the cervix throughout menstruation and postmenopause (63, 64). It has also been demonstrated in rats that estradiol and progesterone concentrations dramatically impact the quantity of IgG and IgA in the uterus and that their affect is exactly the opposite in the vagina (60, 65, 66). Women with autoimmune disorders such as multiple sclerosis, asthma or systemic lupus erythematosus have disease profiles that shift as they move through the menstrual cycle (67, 68). Bouman *et. al* review the data supporting the rationale that estradiol concentration in women affects the number and function of circulating monocytes and the cytokines produced by them, although the mechanisms have been more difficult to elucidate as the human *in vitro* data often conflicts with the human *in vivo* and animal data (67).

Multiple kinds of estrogen can be synthesized and catabolized by the body; estradiol is the most potent form acting via the classically understood estrogen mechanism of action (Figure 2). Estrogens enter the cell by passive diffusion through the plasma membrane and enter the nucleus to bind one of two types of estrogen receptors, ER α or ER β, in their inactive,

monomeric form (50). Upon binding the hormone, the ER undergoes a conformational change and forms either a homo- or heterodimer with an additional ER, which in turn binds to the promoter region of a target gene, known as the estrogen response element (ERE). The range of consensus sequences that can act as EREs conveys an estrogen specificity to target gene transcriptional activation. The three-dimensional structure of the ER/DNA complex, which is a result of the estrogen binding, the composition of the ER (ER α or β homodimers or a heterodimer), and the sequence of the ERE, determines which co-activators or co-repressors can then be recruited and governs the transcriptional activity of, or lack thereof, the target gene. Variability in any component of this complex will have biological implications for the gene product and it is by this mechanism of action that estrogens, including the most metabolically potent form, 17β-estradiol, affect many systems of the body, interacting with ER α and/or β, which are differentially expressed in various cell types throughout the body.

Among circulating lymphocytes, for example, ERs have been routinely observed in cytotoxic and suppressor T cells and B cells (67). The expression of ERs in monocytic phagocytes has been suggested to be dependent on the phase of differentiation, in which monocytes express ER β and macrophages express ER α, and that it is monocytes which may express ERs on their surface (69, 70). Neutrophils express both kinds of ERs and the expression of adhesion molecules CD11b and CD18 as well as TNF-α have been shown to correlate with menstrual cycle status (71). It has also been shown that the ability of LPS-activated monocytes to recruit neutrophils is impaired if they are estradiol-pretreated, as is their release of the proinflammatory cytokine CXCL8 (72). The female reproductive tract is populated with a distinct, uterine class of natural killer cells (uNK) with estrogen receptors and the tract also has large lymphoid aggregates composed of a central core of B cells surrounded by large numbers of CD8 T cells with antigen presenting cells migrating in and out of this core; the establishment, size and composition of these aggregates appear to be modulated by estradiol fluctuations (64, 73-75). Some researchers have begun even more basic explorations of the mechanisms by which estradiol may modulate the immune system via ERs and selective estrogen receptor modulators;

conducted *in vitro* or in severe combined immunodeficient mice, these postulated mechanisms may or may not prove to have translatable biological relevance but they do provide possible avenues of exploration and, given how varied they are in nature, they provide strong support for the intuitive concept that estradiol's immunomodulating capacity is potentially wide-spread and impacts virtually all cell types of the immune system (76-81). And, as reviewed in Wira *et al*., epithelial cell immunofunctions, which are at the nexus of the innate and adaptive immune response, are also modulated by estradiol in the genital tract (82). Our understanding of the mechanisms by which estradiol affects the immune system is not yet complete, but its importance is demonstrated by the gender dimorphism in the nature of cellular and humeral reactions, the incidence of autoimmune diseases and the efficacy of vaccination (67, 83, 84). As this knowledge gap continues to be filled, other researchers have begun to explore the possibility of translating these observed phenomena into targeted, enhanced vaccine efficacy and immunomodulating therapies.

Researchers have begun to explore the possibility of using estrogen treatment to enhance genital herpes vaccine efficacy in animal models of genital herpes. As is reflected in subsequent chapters, the murine model of genital herpes is frequently used to model human infection and primary disease and the guinea pig model is used to model recurrent disease and virus shedding. The vaginal epithelial cell layers of mice are affected more dramatically by estradiol than humans, such that at times in the estrous cycle when estradiol concentrations are high, the epithelium is virtually impervious to HSV-2 infection. The effect of estradiol in these models was imaged by Pessina *et al*., and it has been well-understood for two decades that mice are unlikely to become vaginally infected with HSV-2 unless they are in diestrus, the low-estrogen phase of the estrous cycle (85-87). No part of the menstrual cycle (Figure 3) renders women physiologically impervious to vaginal herpetic infection, thus this resistance is an artifact of the murine model and is easily corrected by a chemical induction of a diestrous-like state using an injection of progesterone (e.g., medroxyprogesterone acetate, the contraceptive Depo-Provera) or via ovariectomization. However, the affect of the estradiol with respect to HSV-2 in an

immunological context, rather than physiological, is less well understood, although it is an area under active investigation. Using an adenovirus vector vaccine expressing HSV gB in mice, the ratio of gB-specific IgG to IgA in the vagina was observed to be lower during estrus (high estrogen) compared to other phases of estrous (e.g., proestrus and diestrus, when estrogen is not the predominating hormone) (88). This may be explained by the fact that the expression of the polymeric IgA receptor (which translocates multimeric IgA into the mucosa) and the mRNA encoding it has been observed to be suppressed in estradiol-treated ovariectomized animals, when compared to its expression under progesterone- or combined-treatment conditions (89). However, the relevance of these observations is unknown because the affect of the reduced expression of the polymeric IgA receptor and the reduced IgG:IgA cannot be easily correlated infection and/or disease outcomes since the animals are physiologically resistant to HSV infection when under the influence of estradiol. Also, it is believed that IgG is a more important antibody isotype in responding to HSV-2 infection than IgA, although this hypothesis may also be somewhat contaminated by limitations of model systems. Using a system in which ovalbumin-specific T cells and vaginal epithelial cells were incubated in the presence of ovalbumin after hormone treatment, it has been suggested that estradiol regulates antigen presentation by vaginal epithelial cells and that these estradiol-regulated cells are, in turn, influencing antigen presentation as well as B and T cell proliferation (90).

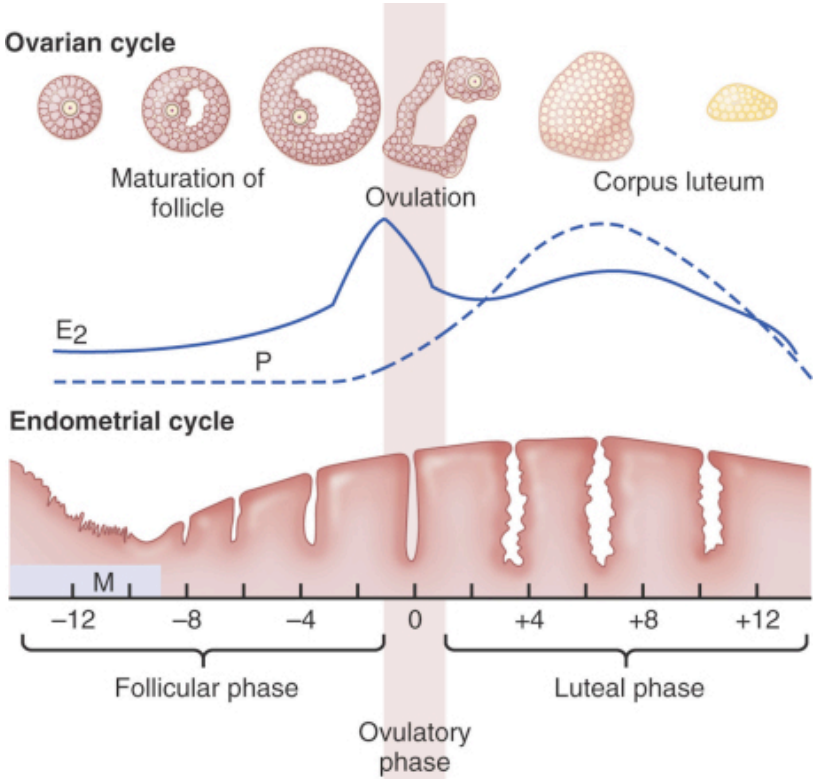

Figure 3. The changes observed during the menstrual cycle.

The idealized cyclic changes observed in estradiol (E_2), progesterone (P), and uterine endometrium during the normal menstrual cycle. The data are centered on the day of ovulation (day 0). Days of menstrual bleeding are indicated by M.
Adapted from Goldman: Cecil Medicine, 23rd ed. (91). Reproduced with permission.

A sequence of published studies using an ovariectomized mouse model first hypothesized that estradiol regulated susceptibility to vaginal HSV-2 infection by demonstrating that estradiol-treated mice rarely become infected whereas saline-, progesterone- or mixed-treated mice readily became infected (92). While this is undoubtedly true, this is mostly likely because the estradiol treatment merely restored the murine vaginal epithelium to the nonpermissive thickness displayed during estrus, rather than an immunological-effect of the estradiol. The authors failed to isolate the physiological effect of estradiol, whose magnitude is an artifact of the murine model, from any potential immunological effects in the naive murine model, which would be of great interest and potential clinical relevance. The authors proceeded to examine the effect of estradiol on vaccine efficacy in ovariectomized mice, using a live, attenuated, replication-restricted HSV-2 vaccine that is administered intravaginally (93). When challenged intravaginally with virulent HSV-2 three weeks later, only the estradiol-treated groups experienced frequent infection, disease and mortality, compared to the other treatment groups. The authors concluded that estradiol abrogated the protection afforded by immunization without ever establishing the mice had actually been vaccinated. For a live, attenuated vaccine to induce protective immunity, the vaccine itself must cause an infection. Therefore, the estradiol-treated mice were as physiologically impervious to the vaccine virus as they were to the wild-type virus in the previous publication and thus, the actual effect of estradiol treatment was not to immunologically abrogate the protection afforded by immunization, but rather to physically render these mice unvaccinated and highly susceptible to virulent challenge once the effects of estradiol lapsed. Incidentally, this vaccine is only moderately effective in animals, initial human trials of an HSV-1 analog of it were unimpressive, and further pursuits of this vaccine type in humans is not anticipated (32). Across this series of studies, these authors administered three 500 ng/day subcutaneous injections of reconstituted 17-β-estradiol, with intravaginal immunization (or virus challenge in the publication using naive mice), occuring 24 hours after the third injection. The pharmacokinetics of this particular preparation are unknown, and while they are clearly potent, their duration would most likely be short, possibly measured in a number

of hours and not exceeding a very small number of days. Therefore, the effect of this design would be that much of the immune response and development of immunological memory probably took place when they were no longer under the influence of estradiol, even though administration of the vaccine occured when the mice possibly were. Alternative study designs were later adopted by some of these authors in which the live, attenuated vaccine was administered either via the intranasal or subcutaneous routes and the estradiol was administered via a sustained release subcutaneous pellet (94). Although this might typically be considered a weakness, the strength of delivering the live, attenuated vaccine via a nonvaginal route is the avoidance of the epithelial-thickening effect of on vaccine permissivity. An additional advantage of intranasal delivery over the subcutaneous route would be the induction of a more robust mucosal immunity (per the common mucosal immune system), but both routes are deprived of the induction of locally generated immunity from vaginal aggregated lymphoid tissues, which is the product of live vaccines delivered intravaginally. The advantage of pelletized hormone is a prolonged delivery of the estradiol, thus whatever immunomodulating effects estradiol has can be exerted on the immune system as it responds to the vaccine and develops a memory response. The weakness of these delivery systems that have not gone through FDA approval is the absence of reliable pharmacokinetic data documenting the quantity, duration and bioavailability of the dose. These authors unfortunately do not independently establish that estradiol dosing has lapsed prior to infectious challenge. Nonetheless their observations are interesting and their improved study design led to conclusions which deviated widely from previous ones. They observed that mice non-vaginally immunized with the live attenuated vaccine virus under the influence of estradiol were seen to have increased rates of survival and decreased incidence of pathology compared to placebo groups, when challenged intravaginally (94).

METHYLENEDIOXYMETHAMPHETAMINE

3,4-Methylenedioxymethamphetamine (MDMA) is a drug of abuse, most commonly known by the street name “ecstasy” (95). MDMA is a sympathomimetic substituted amphetamine and a derivative of methamphetamine (Figure 4). Unlike many other

amphetamines, MDMA is structurally similar to serotonin. MDMA stimulates an efflux of serotonin, norepinephrine and dopamine into the synapse by binding to their respective transporters (with that order of affinity), thus preventing accumulation at uptake sites, and reversing them, causing an efflux of previously accumulated serotonin, norepinephrine and dopamine (96, 97). MDMA is most frequently ingested and acute consumption can cause physiological symptoms which include hypertension, tachycardia, hyperthermia and hyponatremia as well as a serotonin syndrome (95). Autonomic dysfunction, abnormal neuromuscular activity and altered mental status form the triad of symptoms that characterizes serotonin syndrome, which can be fatal (98, 99). The structural similarity of MDMA with serotonin and its ability to inhibit serotonin reuptake is the likely cause for this serotonergic toxicity that is distinctive among more traditional amphetamine toxicities (95, 100, 101). The cardiovascular stimulation induced by MDMA intoxication can cause hypertensive crises, aortic dissection, myocardial infarction, intracranial hemorrhage and dysrhythmia, which can be fatal as well (102-104). The stimulant effect of the amphetamine component of MDMA combined with serotonin build-up can cause life-threatening hyperthermia that is only exacerbated by prolonged physical exertion and hot environmental conditions, the context in which the drug is often consumed (105, 106). MDMA causes a psychogenic polydipsia and some MDMA abusers believe they can overcome the hyperthermic dangers by hyperhydration; this, combined with an MDMA-induced rise in antidiuretic hormone levels, can result in hyponatremia and hypoosmolality, leading to seizures, cerebral edema, cerebral herniation and death (107-109).

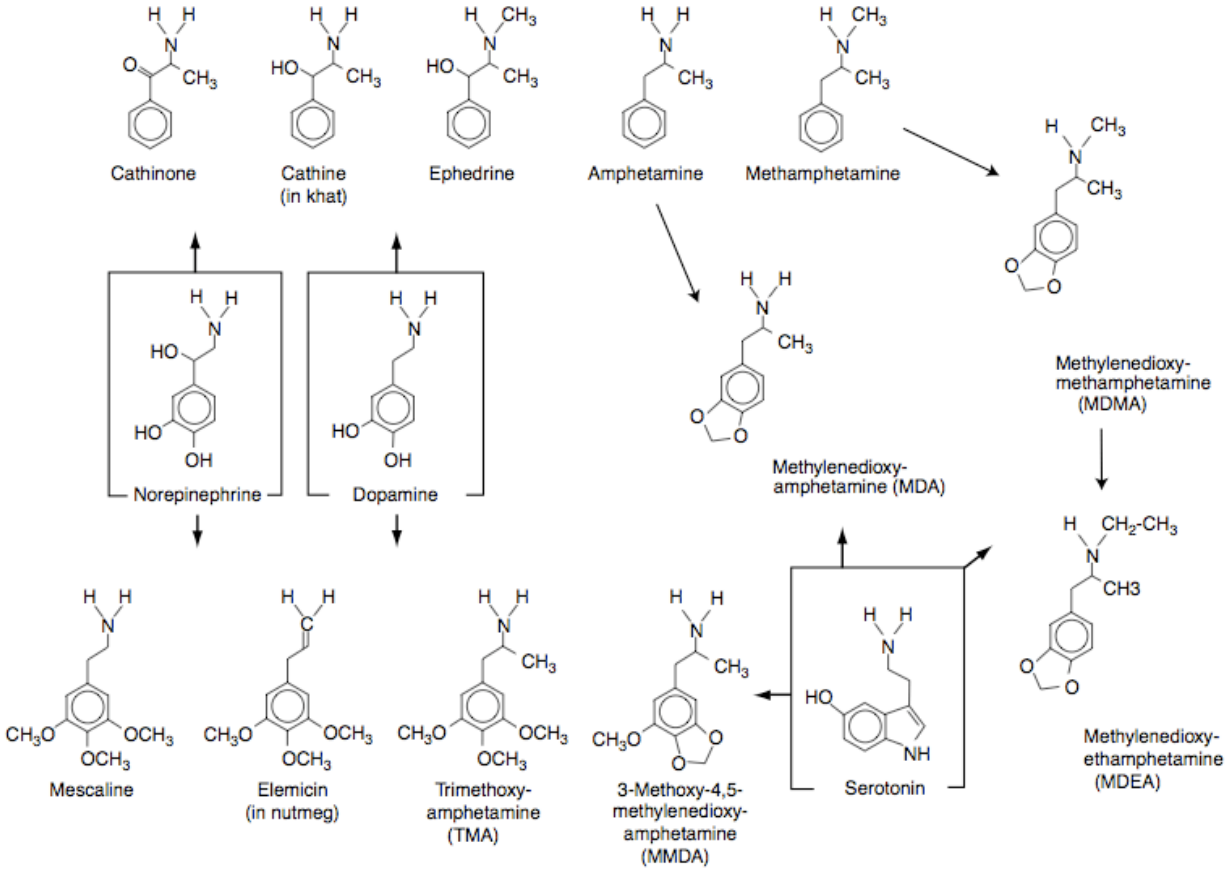

Figure 4: Chemical structure of MDMA, methamphetamine and related drugs compared with those of the monoamine neurotransmitters.

Arrows do not represent pathways of synthesis or metabolism; they merely indicate the closest resemblances of structure. From Kalant, H. CMAJ 2001; 165:917-928. Reproduced with permission.

Despite all the hazards associated with MDMA intoxication, reports from 2007 indicated that over 1.2 million Americans abuse the drug (110). Its popularity stems from the acute subjective effects which are empathogenic and euphoric as well as stimulatory, inducing feelings of wakefulness, intimacy, sexual arousal and behavioral disinhibition (111). Demographically, the drug is most frequently abused by young adults and is popular among homosexuals, particularly those participating in the circuit party scene, as well as being among one of the widely-popular "club drugs", a group of drugs frequently abused by people attending dance clubs and raves; tragically, usage in these population segments is spurred on by the widely held misconception that MDMA is a "safe" drug (110, 112-115).

The empathogenic effects and behavioral disinhibition elicited by MDMA are frequently associated with increased numbers of sex partners and increased frequency of unprotected sex; this in turn has been associated with increased incidence of sexually transmitted infections (112, 116-118). However, the impact of MDMA on infection may not be entirely behavioral. Some studies have shown that, even when controlling for sexual behavior (including number of sex partners and frequency of condom usage), MDMA users still reported an elevated incidence of HIV and other infections when compared with controls that had not consumed MDMA (114-120). MDMA could increase the incidence of infection by compromising the innate immune response. Others have observed an MDMA-effect on proinflammatory cytokines, including TNF-α and IL-1, antiinflammatory cytokines, such as IL-10 and tumor growth factor beta (TGF-β), and immune cell counts, e.g., the number of natural killer (NK) cells in circulation (121-129). MDMA may also have an impact on the adaptive arm of the immune response by suppressing T-cell proliferation, Th1 cytokine production and antibody isotype switching, while stimulating Th2 cytokine production (122, 126, 127). While these reports are interesting, their translational relevance is unclear. The published reports have been performed either *in vitro* or *ex vivo*; studies in which both the drug administration and the observed immunomodulation occur *in vivo* are rare (122). In all but one of the published studies, the immune system was stimulated with antigenic preparations that are artificial, such as high concentration LPS and concanavalin

A. These studies were important in that they served as a proof-of-concept that there could be interaction between MDMA and the immune system. In attempts to replicate the *in vitro* observations in animal models, authors frequently reported a failure to do so or contradictory observations (122, 123). Recently, Nelson *et al*. published findings in which they used gamma herpes virus 68, a murine herpes virus (130). It was the first report to demonstrate that a drug of abuse could alter the immune response to a virus in aspects such as macrophage-directed responses to an infection, including a significantly reduced production of IL-6, TNF-α and IL-10 by MDMA-treated macrophages infected with virus. However, the research was performed *in vitro* and therefore could not report critical endpoints such as outcome of infection or systemic affects on the immune response.

Genital herpes is a pathogen of significant public health importance. The synergy between HSV and HIV makes the need for a protective vaccine and an understanding of the contexts in which the vaccine must maintain efficacy even more urgent. The research presented here explores the possibility of boosting the protection afforded by a suboptimal vaccine candidate using estradiol. We also explore the impact of MDMA on the susceptibility to HSV-2 infection.

CHAPTER II

Estradiol Improves Genital Herpes Vaccine Efficacy in Mice

INTRODUCTION

Herpes Simplex Virus type 2 (HSV-2) is a pathogen of significant public health importance. The US National Health and Nutrition Examination Surveys (NHANES) reported that 17% of Americans are seropositive for HSV-2 and there are published reports that HSV-2 prevalence in parts of the developing world is as high as 80% (11, 131). HSV-2 causes genital herpes and, as with most sexually transmitted infections, women bear a disproportionate burden of HSV-2 infection (11). HSV-2 is commonly transmitted to women via the reproductive tract where it infects and passes through the vaginal mucoepithelial layer to establish a lifelong infection in the dorsal root ganglia (DRG) (4). Between 63% and 87% of HSV-2 infections are asymptomatic, leaving many people unaware of their infection (4, 11). Virus shed in the absence of symptoms is believed to be the major source of genital herpes transmission to susceptible sex partners. Much effort has gone into developing a prophylactic vaccine as is discussed in the previous chapter (32, 34). HSV-2 is also known to be a synergistic co-pathogen to HIV and it is believed that resolution of the HSV-2 pandemic could provide assistance in the fight against the HIV pandemic (17).

Clinical trials indicate that the most effective candidate vaccine thus far is a glycoprotein D vaccine formulated in AS04 which provided HSV-seronegative women significant protection against genital herpes disease, although it failed to provide sterilizing immunity to HSV-2 infection; additional Phase III trials are currently underway (33, 34, 46, 48). Notably, this protection was only seen in women, which is surprising since women are more susceptible to infection.

Many vaccines have shown gender-specific differences in the protection they afford (84). Others have already begun to explore the role that estradiol might have in susceptibility to

HSV-2 infection and vaccine efficacy and they have shown that estradiol impacted vaccine-elicited protection using a live attenuated virus vaccine (88, 92-94, 132). Extending their previous work, here we used the nonreplicating gD-antigen vaccine in the AS04 formulation that is currently in clinical trials, rather than a live attenuated vaccine, in order to completely separate the issues of vaccine-elicited protection from both vaccine- and pathogenic-viral susceptibility and replication. This enabled us to isolate and observe the impact of estradiol on the efficacy of a clinically relevant vaccine in a small animal model of a pathogen of major public health importance. The results of our studies clearly demonstrate that estradiol enhanced vaccine-elicited protection, even in the context of already-robust protection and even in exquisitely sensitive models, using a subunit vaccine in clinical trials.

METHODS

Virus

HSV-2 strain 186 was prepared on Vero cell monolayers and stored frozen (-80˚C) until used, as previously described (133).

Animals and hormone treatments

Ovary-intact and ovariectomized female Swiss Webster mice (Jackson Laboratories, Bar Harbor, ME) approximately eight-weeks-old were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved quarters for these studies and all procedures were approved by the University of Texas Medical Branch Intuitional Animal Use and Care Committee. Animals were allowed to acclimate to the vivarium for seven days prior to use.

Based on published information and personal correspondence with the principal investigator who participated in the animal phases of the research and development of estradiol valerate, we determined that a 0.2 mg/kg injection in small rodents had a half-life of 9 days and that the area under the dose-elimination curve was 3.314 ng/mL/day (134, 135). Hence, the drug clearance was 60.34 mL/$g_a$day (where g_a is the mass of the animal) and that the drug volume of distribution was 783.6 mL/g_a. When chose a target concentration of 75 pg/mL, because we

wanted to deliver a large but not physiologically irrelevant quantity of estradiol. We selected a dosing interval of 38 days, in order ensure that the influence of estradiol would span the immunization regimen and much of the formation of immunological memory; the approximate mean mass of the mice at this stage of the study was 27 g_a. Because of the ramp-up pharmocodynamics of the delivery vehicle and the delivery/diffusion rate out of the tissue, a loading dose can typically be built into the maintenance dose of this drug to ensure that a single dose delivery reaches the target concentration quickly and the that estradiol delivery is sustained for the duration of the dosing interval. Thus, we calculated that each mouse needed to be injected with 6.23 μg of estradiol valerate for the purposes of our study design, using standard pharmacological formulas (136, 137). Because the volume of estradiol valerate containing that quantity is too small of a volume to be reliably delivered, it was added to a mineral oil preparation that would mix well with viscosity of estradiol valerate but would not affect the bioavailability or kinetics of the dose. Mice in estradiol-treated groups were intramuscularly injected with a 50 μL mineral oil solution (heavy paraffin oil, Fisher-Scientific, Fair Lawn, NJ) containing 6.23 μg estradiol valerate (Monarch Pharmaceuticals, Bristol, TN) in the right hind leg 7 days prior to vaccination. Control mice were injected with 50 μL of saline. For challenge studies using ovary-intact mice, the animals were treated 2 mg medroxyprogesterone acetate (The Upjohn Company, Kalamazoo, MI) one-week prior to HSV-2 inoculation to make the epithelium permissive to viral infection, as previously described (87, 138). Ovariectomized mice did not receive progesterone. See Figure 5 for a timeline of the main experimental design.

Cellvizio confocal imaging system

In vivo confocal images of the vaginal surface were collected in situ using a confocal microendoscope imaging system (Cellvizio Lab Mauna Kea Technologies, Paris, France). The system provides 488 nm illumination via an optical fiber bundle to the distal imaging probe and collects fluorescence emission in the 505-700 nm range. The imaging probe was 1.5 mm in diameter and provided a field of view of 600x500 mm with an axial and lateral resolution of 15 and 3.5 μm, respectively. Images provided by this system are single plane *en face* images of the

sample surface. The system was used to collect real time videos at an acquisition rate of 12 frames/sec.

Mouse imaging experiments

Mice were anesthetized with an intraperitoneal injection (50 mg/kg) of Sodium Pentobarbital. The vaginal tract was gently flushed with 1.5 mL saline, and then approximately 0.1 mL of 0.2% W/V Acridine Orange (Product #31,833-7, Aldrich Chemical Company) was administered. Five minutes later, the vaginal tract was again flushed with saline to remove excess dye prior to imaging. The confocal microprobe was then gently inserted into the vaginal tract and a 30-second video was collected of the vaginal tract wall, from which representative still images were selected (134). Animals were then euthanized and the reproductive tract excised and fixed in neutral-buffered formalin fixative for a minimum of 24 hours.

Histological processing and analysis

Samples were submitted for routine histology processing. Several, 4-5 micron transverse sections of the vaginal tract were collected at 100μm intervals. Slides were stained with Hematoxylin and Eosin (H&E) and reviewed under a light microscope (Olympus IX71, Olympus America, Center Valley, PA). For each sample, one histology cross-section near the cervix was chosen for epithelial thickness measurements. From that section 1 to 3 micrographs of the cervicovaginal epithelium were collected using a color digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, MI) at a magnification of 200x. Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI) was used to measure the epithelial thickness from the digital images using a calibrated measuring tool within the software program. Twenty randomly chosen epithelial sites were measured from the microphotographs in order to obtain a mean cervicovaginal epithelial thickness value for each animal.

Vaccine

The gD/AS04 vaccine formulation, described in the previous chapter, was kindly provided by GlaxoSmithKline Biologicals (Rixensart, Belgium). Each mouse was vaccinated

intramuscularly in the left hind leg with 50 μL of the vaccine, which contained 2 μg of HSV-2 surface glycoprotein D. Animals received a second vaccination two weeks later, while estradiol-treated animals were still under the influence of the estradiol dose.

Mouse model of genital herpes

Four weeks after the second vaccination, and one week after progesterone treatment in ovary-intact mice, all vaccinated animals and age-matched naïve control animals were intravaginally inoculated with HSV-2. Depending on the study, the inocula used ranged from 1 x 10^1 to 1 x 10^6 pfu in 15 μL, as previously described (133). On days 1 and 2 postinoculation, vaginal swab samples were collected from all mice. Prior to freezing, a portion of the samples were plated on Vero cell monolayers and incubated for 5 days at 37°C to determine infection. Animals were defined as infected if viral cytopathic effects of HSV-2 were observed from either swab sample. Mice were examined daily for 21 days postinoculation for clinical signs of genital herpes disease and were defined as having such if they showed pathological signs of cutaneous disease (hair loss and erythema on the perineum) or signs of more severe, neurological disease (urinary incontinence and hind-limb paralysis). Mice progressing to severe neurological involvement either quickly succumbed to encephalitis or were euthanized to prevent unnecessary suffering.

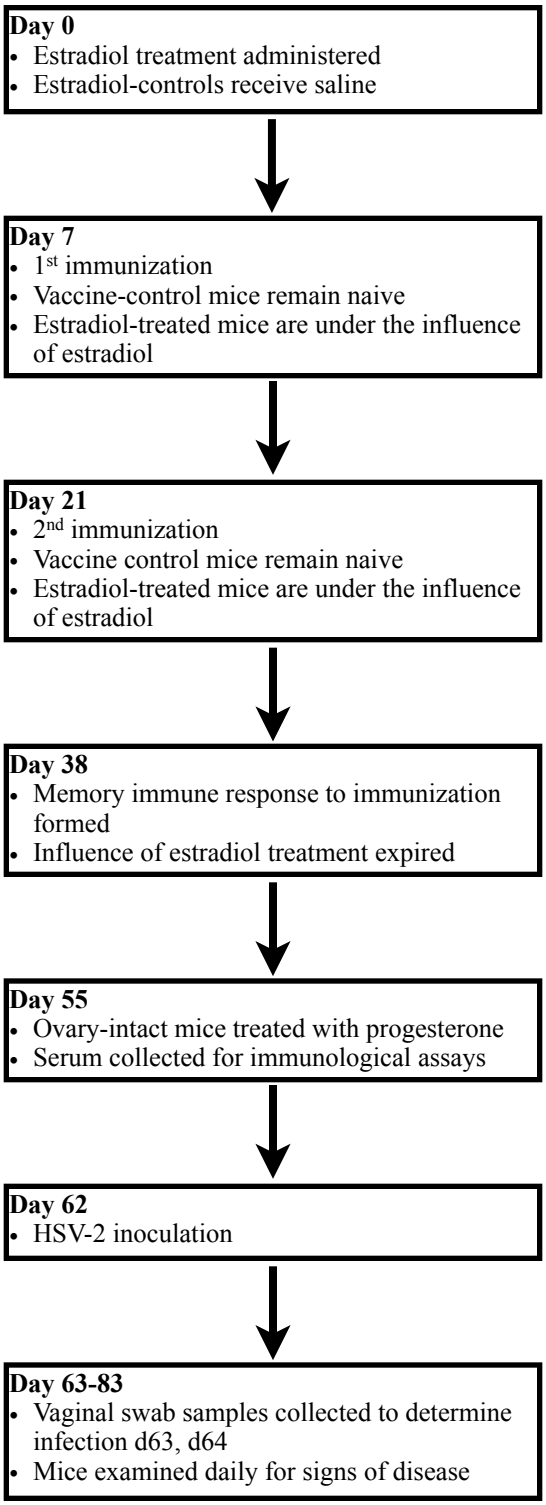

Figure 5. A timeline of the experimental design.

Figure from: Pennock et al. Vaccine 2009 (134).

ELISA for HSV-specific antibodies

One week prior to viral inoculation, blood samples were collected from the retro-orbital plexus of each mouse. ELISA assays were performed as previously described (139, 140). Briefly, serum samples were plated in duplicate wells coated with HSV-2 glycoprotein as the antigen (or glycoprotein from uninfected cells as the control mock antigen). Plates were developed using biotinylated anti-mouse IgG antibody (Southern Biotech, Birmingham, AL), streptavidin peroxidase (Sigma, St. Louis, MO) and o-phenylenediamine dihydrochloride with hydrogen peroxide. The OD490 values were obtained using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA), compared to the linear portion of the standard curve, and HSV-2 gD-specific antibody concentrations were calculated using SoftMax Pro software (Molecular Devices).

Neutralization assays

Neutralizing serum antibody titres were determined by a modification of our previously described technique (141). Briefly, serum from vaccinated and naïve control mice was heat inactivated at 56°C for 15 min. A series of two-fold dilutions was then made in 2% titration media and Low-Tox H Rabbit Complement (Cedarlane Laboratories, Burlington NC) at a final concentration of 1/20480. Approximately 100 pfu HSV-2 strain 186 was added to each tube in the dilution sequence. Following incubation at 37°C for 1 hour, the dilution sequence was plated on Vero cell monolayers for pfu quantification, as above. After incubation for 3 days, the plates were stained with crystal violet and the number of viral plaques were counted. The end-point neutralizing antibody titre was defined as the $\log_{10}$ of the final serum dilution that produced a >50% reduction in the number of viral plaques compared to the number of plaques in control serum wells (45).

Statistics

Fisher's exact tests were used to compare all infection, disease and outcome data. The Student's unpaired t-test was used to compare group mean values. All reported P values are two-tailed and values <0.05 were considered to indicate statistical significance. All statistical analysis was performed using SPSS 16.0 for Mac OS.

RESULTS

Impact of estradiol on the vaginal epithelium of ovary-intact mice

The aim of these studies was to observe the impact of estradiol on vaccine-elicited protection. In order to do so, we wanted to deliver a single, biologically significant dose of estradiol that would span the duration of the vaccination regimen but would have lapsed by the time of viral inoculation. Estradiol is known to cause the vaginal epithelium to thicken; we wanted to be certain the estradiol treatment had lapsed prior to inoculation because otherwise it would be impossible to determine whether a reduced incidence of infection was the result of an estradiol-enhanced vaccine response or a physiological artifact of an estradiol-thickened vaginal epithelium. We selected estradiol valerate as our estrogen treatment because it is available by prescription, therefore its sustained-release kinetics have been established in both rodents and humans (135). We calculated a single dose that we believed would be potent for the duration of the two-injection vaccination regimen and would lapse prior to viral inoculation, as discussed above (135, 142). To confirm this, we used two imaging modalities, Cellvizio and traditional H&E histology, that served two objectives: verify that the dose was in fact biologically potent and verify that its duration of action was short enough to not pose a physiologic barrier to infection. This was done by measuring the thickness of the vaginal epithelium in histological cross-sections and the characteristics of the epithelial surface with Cellvizio. Groups of ovary-intact mice were sacrificed and imaged on days 0, 7 and 62 post-estradiol treatment along with age-matched saline-treated controls (N=5 per group). As shown in Table 1 and as would be expected, somewhat variable results were seen because the ovaries continued to be a source of estrogen during estradiol treatment and thus they were in various phases of their estrous cycles. On day 0, the mean thickness was 62 μm ± 13; on day 7, when the estradiol would be expected to be thickening the vaginal epithelium and the vaccination regimen would begin, the mean had increased to 82 μm ± 13. By day 62, long after the estradiol treatment should have lapsed and

one week after the progesterone, when the viral challenge would occur in the following studies, the mean thickness had decreased to 39 μm ± 4 (Figure 6K). We were able to verify that the impact of estradiol on the epithelium had lapsed and that progesterone had primed (i.e., thinned) the vaginal epithelium for viral challenge because the saline-control mice also had a mean thickness of 39 μm ± 5 (Figure 6I). The Cellvizio images (black & white) showed the brightly stained nuclei of the uppermost cell-layer of the epithelium, which provided supplementary information depicting the nuclear size and density as an overall *in vivo* qualification of epithelial health and an indicator of the influence of estradiol; the crossectional histological images (color) enabled an actual measurement of epithelial thickness, also an indicator of the influence of estradiol as well an indicator of physiological infection permissivity. Progesterone-priming is required for viral challenge in ovary-intact mice (133, 143). Therefore, as an indicator of potency and bioavailability, the estradiol did thicken the vaginal epithelium; and this affect had lapsed, as anticipated, thus posing no physiologic barrier to infection on day 62.

Table 1. Impact of estradiol on epithelial thickness of the vagina of ovary-intact and ovariectomized mice.

Group	Post-estradiol treatment epithelial thickness, mean ± SD			
	Day 0	Day 7	Day 38	Day 62
Ovary-intact	62 µm ± 13 [a]	82 µm ± 13 [b]	--	39 µm ± 4
Ovariectomized	13 µm ± 3	37 µm ± 9 [c]	17 µm ± 4	16 µm ± 3

[a] P<0.05 versus day 62 via Student's unpaired t test
[b] P<0.05 versus day 62 via Student's unpaired t test
[c] P<0.05 versus day 0, day 38 and day 62 via Student's unpaired t test

Data from: *Pennock et al*., *Vaccine 2009* (134).

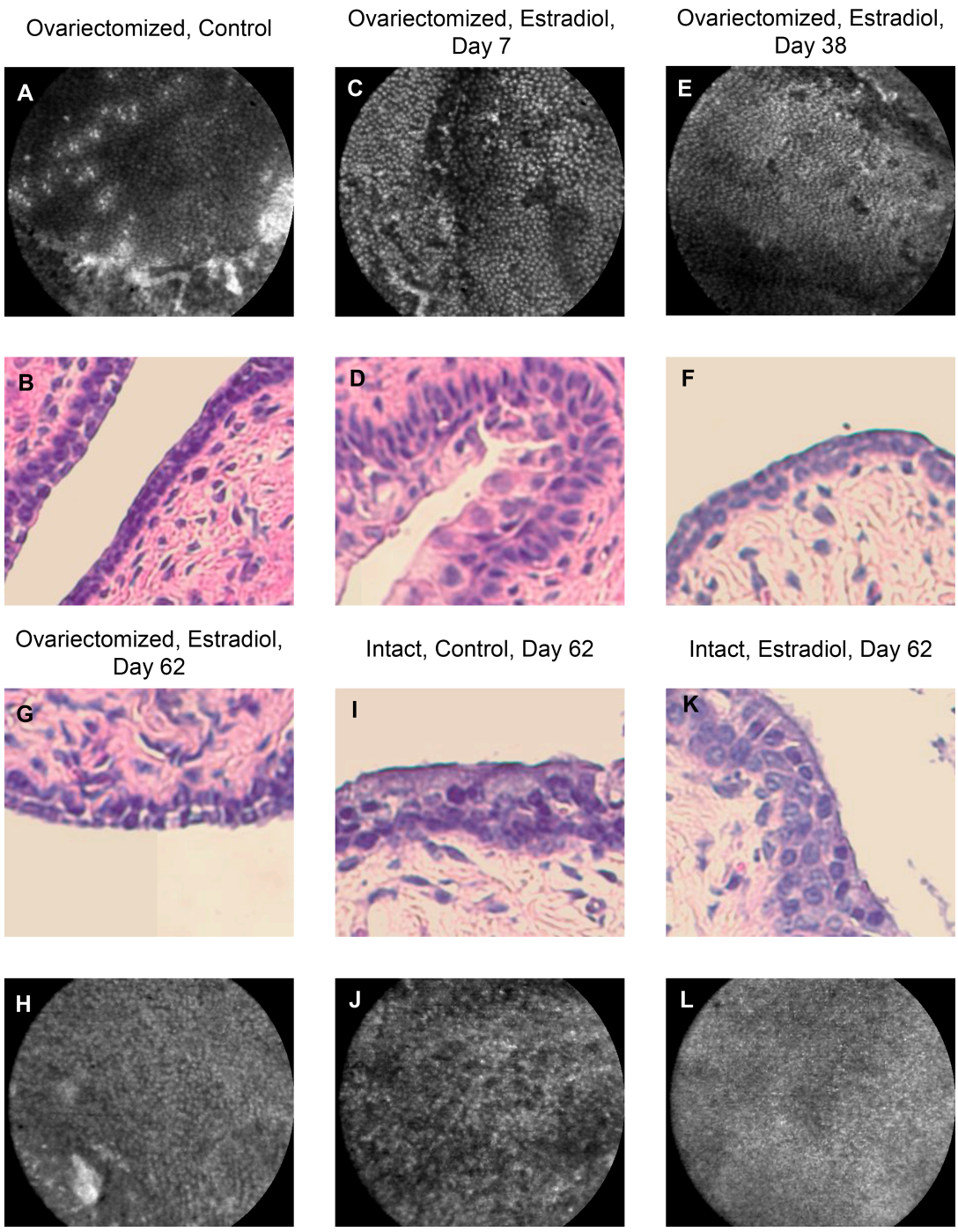

Figure 6. Images of mouse epithelium after estradiol treatment.

Panels A-H are representative images of the vaginal epithelium of ovariectomized mice; A, B were not treated with estradiol; C-H were taken on the indicated number of days after estradiol treatment. Panels I, J are from an ovary-intact mouse 62 days after saline treatment and 7 days after progesterone treatment; K, L are from ovary-intact mice 62 days after estradiol treatment and 7 days after progesterone. Black and white images are Cellvizio confocal micrographs showing the nature of the vaginal epithelial surface; color images are H&E crossectional histology micrographs showing epithelial layers and thickness. Panels A, B (controls) show the

thin, simple, squamous epithelium of the vaginal tract in an ovariectomized mouse; approx 13 µm. Panels C, D show the estradiol-thickened, stratified epithelium with large, proliferating nuclei seven days after estradiol treatment; approx 37 µm. Panels E, F show the epithelium has returned to a thin, simple, squamous layer of cells 38 days after estradiol treatment; approx 16 µm. Panels G, I, K (day 62) show the epithelial thickness to be approx 16 µm, 39 µm and 39 µm, respectively; the effects of estradiol treatment have lapsed. The ovary-intact mice have been progesterone-primed to make them infection-permissive and the ovariectomized mouse's thin vaginal epithelium is already very susceptible to infection. (N=5/group).
From: Pennock et. al., Vaccine 2009 (134).

Impact of estradiol on vaccine-elicited protection in ovary-intact mice

We next examined the effect of estradiol using a high titre inoculum of HSV-2 (1 x 10^5 pfu) in ovary-intact mice. All naïve-control animals in these studies became infected and developed disease, as expected. Vaccination, with and without estradiol treatment, resulted in a significant reduction of both infection and disease in these studies; however, the protection against infection and disease was greater in the estradiol-treated animals (Table 2). Adding estradiol reduced the incidence of infection by 50% and disease by 75%, compared to vaccination alone. Even when only examining infected animals, we observed that both vaccination alone and with estradiol still provided significant reduction in the incidence of disease compared to the naïve controls (raw data not shown, $p<0.01$), but the difference between the two former groups was not significant.

We also examined whether adding estradiol to the vaccination regimen would impact the inoculum required to establish infection at the genital mucosa and/or the inoculum required to cause genital herpes disease in ovary-intact mice. Groups of mice (N=15/group) were challenged with a range of HSV-2 inocula (10 thru 10^6 pfu) to determine if the inoculum required to infect half of all animals in a group (ID_{50}) or cause disease in half of all animals in a group (DD_{50}) would be altered. The ID_{50} for mice in the naïve group was approximately 1.6 x 10^2 pfu; the ID_{50} in the vaccinated groups, both with and without estradiol, were approximately 4 x 10^3 pfu (Table 3). The DD_{50} for the naïve group was 2 x 10^2 pfu; the DD_{50} for the group receiving vaccination alone was approximately 3.16 x 10^5 pfu; and the DD_{50} for the group with estradiol added to the vaccination regimen was $>10^6$ pfu. Both these benchmark inocula were increased by vaccination and disease protection was improved further by the addition of estradiol to vaccination regimen. This, combined with the high dose challenges above, suggested a trend of estradiol-enhanced vaccine-elicited protection, however the robust protection afforded by vaccination alone made quantification of the enhancement difficult. This prompted us to examine the impact of estradiol in ovariectomized mice, which are known to be more susceptible to HSV-2 infection (92).

Table 2. Impact of estradiol on vaccine efficacy in ovary-intact mice.

Group	N	Infection		Clinical Disease		Mortality	
		#	P[a]	#	P[a]	#	P[a]
Naïve	19	19	-	19	-	19	-
Vaccinated	20	12	0.003	4	<<0.001	0	<<0.001
Estradiol + Vaccinated	20	6	<<0.001	1	<<0.001	0	<<0.001

[a] P values were calculated to test for a statistically significant difference in the observed incidence compared to naïve controls.

Table from: Pennock et al., Vaccine 2009 (134)

Table 3. Impact of estradiol on the threshold of vaccine-afforded protection against infection and disease in mice.

Group	Naive	Vaccinated	Estradiol + Vaccinated
ID_{50} [a]	1.6 x 10^2 pfu/mL	4 x 10^3 pfu/mL	4 x 10^3 pfu/mL
DD_{50} [b]	2 x 10^2 pfu/mL	3.16 x 10^5 pfu/mL	>10^6 pfu/mL

[a] ID_{50} is the viral inoculum required to establish infection in 50% of mice in a group, calculated using the Reed-Muench formula.
[b] DD_{50} is the viral inoculum required to cause disease in 50% of mice in a group, calculated using the Reed-Muench formula.

Data from: *Pennock et al., Vaccine 2009* (134).

Impact of estradiol on the vaginal epithelium of ovariectomized mice

Ovariectomized mice also provide a cleaner hormonal background since their primary source of endogenous estradiol has been removed. Therefore, we examined the impact of estradiol on the vaginal epithelium of ovariectomized mice to ensure our dosing schedule was appropriate. Groups of ovariectomized mice were sacrificed and imaged on days 0, 7, 38 and 62 post-estradiol treatment along with age-matched saline-treated controls (N=5 per group). Figure 6 shows representative images from the ovariectomized, estradiol-treated groups. On day 0 (Figure 6, A and B), the mean vaginal epithelial thickness was 13 μm ± 3 in the ovariectomized group. On day 7 (Figure 6, C and D), when the estradiol would be expected to be thickening the vaginal epithelium and the vaccination regimen would begin, the mean thickness was 37 μm ± 9. On day 38 (Figure 6, E and F), when the vaccination regimen was complete and the estradiol was pharmacologically expected to have lapsed, the mean thickness had in fact returned to 17 μm ± 4 (day 38 controls had a mean thickness of 16 μm ± 3, not shown). And on day 62 (Figure 6, G and H), when viral challenge would occur, the mean epithelial thickness was 16 μm ± 3. Therefore, as an indicator of potency and bioavailability, the estradiol did thicken the vaginal epithelium; and this affect had lapsed, as anticipated, 38 days later, thus posing no physiologic barrier to infection on day 62. This was a significant thickening from day 0 to day 7 ($P<0.05$), a significant thinning between day 7 and day 38 ($P<0.05$), and no significant difference at the time points of days 0, 38 and 62, with respect to each other (Table 1). It is also interesting to note that the epithelial thickness observed in the ovariectomized mice never reached reached equivalence with the intact mice, even when the ovariectomized mice were at the height of estradiol treatment.

Impact of estradiol on vaccine-elicited protection in ovariectomized mice

Having shown that the estradiol treatment was appropriate for the ovariectomized mice, we next examined the impact of estradiol on vaccine efficacy in this model and Table 4 shows the results of those studies. All but one of the naïve-control mice challenged with the large 1 x

10^5 pfu inoculum became infected and developed disease. Vaccination alone reduced incidence of infection, but the effect did not reach significance. Overall, this mirrors what was reported from the gD/AS04 vaccine trials in humans, where the vaccine significantly reduced the incidence of genital herpes disease but did not significantly reduce the number of women acquiring HSV-2 infection (34). These results also demonstrated the increased stringency of the ovariectomized mouse model. Estradiol-treated vaccinated animals had a significantly reduced incidence of disease compared to naïve-controls ($P<0.01$). Mice receiving estradiol treatment also experienced significantly reduced incidence of disease when compared to both the naïve controls ($P<0.01$) as well as the vaccine-only group ($P<0.05$). Among infected mice, although both vaccination alone and vaccination with estradiol significantly reduced the incidence of disease compared to naïve controls (raw data not shown, $p<0.05$), the difference between the two former groups was not significant. However, the difference in the severity of the disease was significant. Among the 32 vaccinated mice that became infected, 13 (40%) progressed to severe neurological involvement that resulted in mortality. In contrast, 0 out of 25 infected mice that had also been estradiol-treated developed signs of neurological involvement and all of them survived until the end of the study.

Table 4. Impact of estradiol on vaccine efficacy in ovariectomized mice

Group	N	Infection		Clinical Disease			Mortality		
		#	P[a]	#	P[b]	P[a]	#	P[b]	P[a]
Naïve	30	29	-	29	-	-	29	-	-
Vaccinated	40	32	NS	25	-	<0.01	13	-	<0.01
Estradiol + Vaccinated	40	25	<0.01	14	<0.05	<0.01	0	<0.01	<0.01

[a] P values were calculated to test for a statistically significant difference in the observed incidence compared to naïve controls. NS indicates that the difference was not significant.

[b] P values were calculated to test for a statistically significant difference in the observed incidence compared to mice receiving vaccine only.

From: Pennock et al., Vaccine 2009 (134)

Impact of estradiol on HSV-2-specific antibody production

Serum was collected from mice prior to viral inoculation and the HSV-2 gD-specific IgG antibody was quantified by ELISA. The mean titer of vaccine-specific antibody in the estradiol-treated group (1.62×10^5 ng/mL ± 1.58×10^5 SD; N=21) was higher than that in the group receiving vaccine alone (1.24×10^5 ng/mL ± 9.54×10^4 SD; N=21), however the difference was not statistically significant.

The functionality of the vaccine-elicited antibody was assessed by neutralization assay. The neutralizing antibody titre of the estradiol-treated vaccinated mice was 3.15 $\log_{10}$ (± 0.023 SD), which was significantly greater than that of mice vaccinated alone, 2.99 $\log_{10}$ (± 0.015 SD, $P<0.05$; N=21/group).

DISCUSSION

Our data shows that estradiol improves vaccine-elicited protection against genital herpes infection and disease and results in an enhanced antibody response. Others have previously examined the impact of estradiol on vaccine efficacy against genital herpes in mouse models using a thymidine kinase deficient virus as an attenuated vaccine (92-94, 144). The results presented here are consistent with and extend some of those studies. If estradiol was going to modulate susceptibility to and/or protection against HSV-2 infection, as we and they hypothesized, then the potential existed that it could be modulating susceptibility to the vaccine virus as well as or instead of the pathogenic virus challenge. We avoided this potential problem by using a subunit vaccine, so that replication of the immunogen was not a factor. Further, this vaccine candidate showed efficacy in initial phase III clinical trials, lending additional translational relevance to our studies. Another concern we had was in the dosing schedule of the estradiol. In establishing whether estradiol could enhance vaccine-elicited protection, we wanted to be certain that an estradiol-thickened vaginal epithelium did not confound our ability to detect alterations in vaccine efficacy by leaving the mice physiologically impermeable to virus, especially since the magnitude of this effect is much greater in mice; at no time in the menstrual cycle is a woman's vagina impermeable to HSV-2 infection. We confirmed that our results were

not contaminated by any physiological impact of estradiol by directly imaging it, rather than trying to gauge or time the estrous cycles of the mice or serologically quantify the estradiol. The imaging studies presented here enabled us to visually verify that our administration of a sustained-release estradiol into the mouse model posed no physiological barrier to viral infection. Because we are able to demonstrate in Figure 6 that the vaginal epithelial thickness had returned to pre-estradiol values (or to progesterone-regulated values in the case of intact mice), it is then supportable to accept that the differences observed in the subsequent studies (incidence of infection, disease, circulating antibody, etc.) represented an estradiol-mediated impact on the immune system. Secondarily, the imaging studies confirmed the bioavailability and metabolic potency of the estradiol valerate preparation and dosage used.

It was also interesting to note, from the imaging studies, that the ovariectomized mouse vaginal epithelium, before estradiol treatment (Figure 6B) and after estradiol treatment has lapsed (Figure 6 F&G), is thinner than the progesterone-treated epithelium of an ovary-intact mouse (Figure 6 I&K), which explains why ovariectomized mice are exquisitely sensitive to HSV-2 infection and are thus an ideal model to examine the impact of estradiol on vaccine efficacy. The gD/AS04 vaccine alone was able to reduce the incidence of infection by 20%, a trend towards protection against infection but falling short of statistical significance (Table 4). It should be noted that, in this regard, the ovariectomized mouse model parallels the clinical observations using the same vaccine candidate with which additional expanded phase III clinical trials are currently underway (32) . In ovariectomized mice treated with estradiol valerate one week prior to the first vaccination, an additional gain of 20% vaccine efficacy was observed in comparison to vaccination alone, significantly reducing the incidence of infection by almost 40% compared to naïve controls ($P<0.01$) (Table 4). This is an exciting observation. Sterilizing immunity has not been widely believed to be a feasible goal of an HSV-2 vaccination in clinical application. It is anticipated that an HSV-2 vaccine brought to market would provide immunity sufficient to prevent disease and might reduce the burden of latent virus in the ganglia, which could in turn reduce the shedding into the genital shedding and transmission; modeling indicates

that, in this way, a vaccine that provides nonsterilizing immunity can still significantly reduce the incidence of HSV-2 infection (42, 145). However, adding estradiol to the vaccination regimen in ovariectomized mice directly enhanced protection against infection as well as enhancing the protection against disease. Vaccination alone significantly reduced the incidence of clinical disease overall by 34% ($P<0.01$) and among infected mice by almost 22% ($P<0.01$) (Table 4). The estradiol-treated groups had a reduced incidence of disease by an additional 27.5% overall compared to vaccination (61.7% compared to naïve). Among infected mice, estradiol treated mice had an additional 22% reduction in incidence of disease compared to vaccination alone (not significant) and a 44 % reduction in incidence of disease compared to naïve controls ($P<0.01$). Thus the rigorous ovariectomized mouse model shows that estradiol treatment not only boosts vaccine efficacy to provide enhanced protection against disease but it also boosts it up to a threshold adequate to prevent infection.

Key effector and regulatory cells of the immune system express estrogen receptors and a mechanism involving an interfacing of the endocrine and reproductive systems with the immune system is not a novel idea (67, 85). It has long been understood that the immune system of the female reproductive tract must be modulated to not attack and reject a fetus that is 50% foreign. In contrast, the idea that estradiol could be systemically modulating the formation of adaptive memory immune responses is much newer and is supported by our data (94, 146). While the gD/AS04 vaccine is designed to stimulate both Th1 and Th2 responses, our HSV-2-specific IgG ELISA data and neutralization assay suggest estradiol-treated mice produce additional HSV-2 gD-specific antibody. This may explain, at least in part, why the estradiol-treated vaccinated mice had a better outcome than their vaccine-only counterparts.

The ovary-intact studies were consistent with the principles demonstrated in the ovariectomized mice and the literature. A complicating variable in intact studies is endogenous estrogen. This is particularly true when the studies are designed to test the modulation of vaccine-elicited immunity. In non-hormone studies this is controlled for by the administration of progesterone prior to viral challenge, which synchronizes and halts, at least temporarily, the

estrous cycles of the mice. However, this is too late for the purposes of our studies because the endogenous estrogen may or may not have been significantly contributing the sum estradiol levels in the mouse. Moreover, such contributions, if significant, would have come at unsynchronized times in the development of the memory immune response to the vaccine. Also, progesterone is known to play an antagonistic role to estradiol in many endocrine pathways; some have suggested it does so in immune ones as well and this is an area of ongoing investigation (92). Therefore, we thought it important to examine and report the impact of estradiol on vaccine efficacy in intact animals because it provides an additional perspective on the translational relevance of our data (noting the ovariectomized mice performed more like the human vaccine trials than the intact mice) and because it then enables us to put this data in the context of all the other published HSV-2 mouse model data. Also noteworthy, there was a reduction in the incidence of disease across all viral challenge titres in the estradiol-treated groups compared to the groups receiving vaccination alone (data not shown), again suggesting an enhancement of efficacy against the background of already-robust protection, as is more fully explored in the ovariectomized studies.

While there are still additional questions to be explored regarding the impact of estradiol on infection and immunity, the studies described here present an important demonstration that estradiol's effects go far beyond physiology and can shape the host response to vaccination. The conclusions drawn from these studies have implications that need to be explored using other animal models, other candidate vaccines and other infectious organisms so that, once the mechanism of action of estradiol on the host-response is clearly understood, it may be exploited in a wide range of scenarios to elicit optimal protection from otherwise suboptimal vaccines.

CHAPTER III

Estradiol Affects Genital Herpes Vaccine Efficacy in Guinea Pigs

INTRODUCTION

As discussed in Chapter I, much work has gone into the development of a prophylactic vaccine for genital herpes. Herpes Simplex Virus type 2 (HSV-2) is the predominant cause of genital herpes disease and is reported to have infected 17% of Americans, while prevalence in parts of the developing world is as high as 80% (11, 131). Women are believed to acquire the infection via the reproductive tract where the virus passes through the vaginal mucoepithelial layer before establishing a lifelong infection in the innervating dorsal root ganglia (DRG) (4). Genital herpes disease can be divided into two phases, primary and recurrent. The primary disease develops rapidly as a result of viral infection and is characterized by widely spaced vesiculoulcerative bilateral lesions on the external genitalia and may be accompanied by localized symptoms (e.g., pain, itching, dysuria, vaginal and urethral discharge) as well as systemic symptoms (e.g., fever, headache, malaise and myalgia) (7). After several days, the primary disease presentation begins to resolve in most patients and the infection goes into a period classically referred to as latency. The virus can reactivate episodically, descending from the DRG to cause a recurrent disease that presents with lesions that are typically localized to the finite mucocutaneous dermatomes that are innervated by ganglia which harbor the virus; pain is less severe and shorter in duration during recurrent disease than it was during the primary disease and it may be preceded by prodrome as well as accompanied by localized and systemic symptoms (4, 7). Patients may experience both phases of disease or one phase without the other, and the vast majority of people infected with HSV-2 will not experience any symptoms that they identify as genital herpes (4, 11). However, infectious virus can shed even in the absence of symptoms and asymptomatic shedding is thought to play an import role in the sexual

transmission of the infection. This provides a major impetus for the development of a prophylactic vaccine.

Phase III clinical trails have demonstrated that the glycoprotein D vaccine formulated in AS04, developed by GlaxoSmithKline (GSK), can provide significant protection against genital herpes disease in HSV-1&-2 seronegative women, as described in Chapter I (34). However, the vaccine does not provide significant protection against infection, provides no protection in men or HSV-1 seropositive women and it is not known what affect, if any, the vaccine has on the incidence, frequency or severity of recurrent disease, the frequency or quantity of viral shedding or transmissibility. Further clinical trails are currently underway (33, 48, 49). The guinea pig is an excellent animal model of genital herpes disease because it mirrors the natural history of disease in humans in that infection with the human virus results from intravaginal inoculation, without a prerequisite progesterone priming, and the animal develops a mucocutaneous ulcerative primary disease which then resolves into latency, followed by episodic reactivation from the DRG, viral shedding and recurrent vesicular disease. *Bourne, et al*. have previously published studies using the GSK candidate vaccine in the guinea pig model and found that it does not prevent infection with a high titer virus challenge but does reduce the incidence of primary genital disease, mirroring what was seen in the clinical trials (44, 45) (Table 5). It was also demonstrated that immunized guinea pigs had a significantly lower titer of virus in the vagina during the primary phase of infection, had a significantly reduced incidence and frequency of recurrent disease and were shedding a significantly reduced quantity of PCR-detectable virus during a longitudinal examination of the recurrent phase of infection (44, 45). In Chapter II, we demonstrated that estradiol supplementation during immunization with this vaccine yields enhanced protection in the mouse model of genital HSV-2 (134). We therefore decided to explore the impact of estradiol modulation on vaccine efficacy in the guinea pig model because of the additional endpoints it affords, such as the primary disease severity, frequency of recurrent disease and magnitude of viral shedding.

Table 5. Impact of vaccination on genital herpes disease in guinea pigs

Group	Primary disease			Recurrent disease		Viral shedding	
	Incidence [a]	Severity [b]	Titer, pfu/mL [c]	Incidence [d]	Frequency [e]	Incidence [f]	Quantity [g]
Vaccinated	0/12	0.0	1.00×10^4	2/12	0.2	12/12	3.7
Naive	10/11	5.3	1.26×10^5	9/10	6.6	10/10	5.6

[a] All guinea pigs that were challenged became infected. Data shown are the number of guinea pigs with clinical disease / number of guinea pigs infected. P<0.01
[b] Severity is reported as the mean cumulative primary disease lesion score among infected guinea pigs.
[c] Virus titres were calculated via plaque assay from vaginal swab samples from infected guinea pigs collected two days postinoculation. P<0.01
[d] Number of guinea pigs experiencing at least one episode of recurrent disease on days 15-70 postinoculation / number of infected guinea pigs surviving to day 15. (One naive pig had to be removed from the study after primary disease.) P<0.01
[e] Mean number of recurrent lesion days per infected guinea pig. P<0.01
[f] Number of guinea pigs shedding PCR-detectable viral DNA on day 21-48 postinoculation / number of infected guinea pigs.
[g] Mean $\log_{10}$ of viral equivalents detected by PCR per mL vaginal swab samples. P<0.01
Source: data reformatted from tables in *Bourne et al. JID 2005* [45].

METHODS

Virus

HSV-2 strain MS was prepared on Vero cell monolayers and stored frozen at -80°C until used, as described previously (44).

Animals and hormone treatments

Female Hartley guinea pigs (Charles River Breeding Laboratories) were used in the studies presented here. For some studies, as indicated in the results section, animals underwent ovariectomization prior to arriving at the vivarium. Control animals in studies utilizing ovariectomized animals underwent sham surgery to control for the procedural effects of surgery. All animals were housed under conditions approved by the American Association for the Accreditation of Laboratory Animal Care and all procedures were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Animals were allowed to acclimate to the facility for one week prior to use.

It has been previously established, a 0.2 mg/kg injection of estradiol valerate in rats has a half-life of 9 days and that the area under the dose-elimination curve was 3.314 ng/mL/day (134, 135). Hence, the drug clearance was 60.34 mL/$g_a$day (where g_a is the mass of the animal) and that the drug volume of distribution was 783.6 mL/g_a. We chose a target concentration of 51 pg/mL, which is the peak estradiol concentration during guinea pig estrus and is therefore a large but not physiologically irrelevant quantity of estradiol, and we selected a dosing interval of 25 days, in order ensure that the influence of estradiol would last long enough to impact the memory response to immunization (147). The mean mass of the guinea pigs at the time of estradiol administration was 406 g_a. Based on the ramp-up pharmacodynamics of the delivery vehicle and the delivery/diffusion rate out of the tissue, we calculated that each guinea pig needed to be injected with 47.47 μg of estradiol valerate for the purposes of our study design, using standard pharmacological formulas (136, 137). Because the volume of estradiol valerate containing that

quantity is too small a volume to be reliably delivered, it was added to a mineral oil preparation that would mix well with the viscosity of estradiol valerate but would not affect the bioavailability or kinetics of the dose. Guinea pigs in estradiol-treated groups were intramuscularly injected with a 100 μL mineral oil solution (heavy paraffin oil, Fisher-Scientific, Fair Lawn, NJ) containing 47.47 μg estradiol valerate (Monarch Pharmaceuticals, Bristol, TN) in the right hind leg 7 days prior to vaccination. Even though the dosing interval for the calculated dosing schedule was 25 days, a full 31 days were allowed to pass prior to a second 47.47 μg estradiol valerate injection, followed one week later by the second immunization. A single estradiol dosing interval long enough to span both immunizations was undesirable because guinea pigs naturally have very small quantities of circulating estradiol, measuring approximately 51 pg/mL and remaining relatively unchanged throughout the estrous cycle (147). It was not feasible to deliver a single dose of estradiol valerate at a dosing interval that would span both immunization steps and allow time for a memory immune response to form at a relevant target concentration without the peak concentration being supraphysiological by several fold for a prolonged duration. See Figure 7 for a general timeline of the experimental design in estradiol-supplemented guinea pigs.

Vaccine

The gD/AS04 vaccine formulation, described in Chapter I, was kindly provided by GlaxoSmithKline Biologicals (Rexinsart, Belgium). Each pig was immunized intramuscularly in the left hind leg with either 50 or 125 μL of vaccine (containing either 2 or 5 μg of gD, respectively), as indicated below, depending on the study. Animals received a second immunization 4-weeks later.

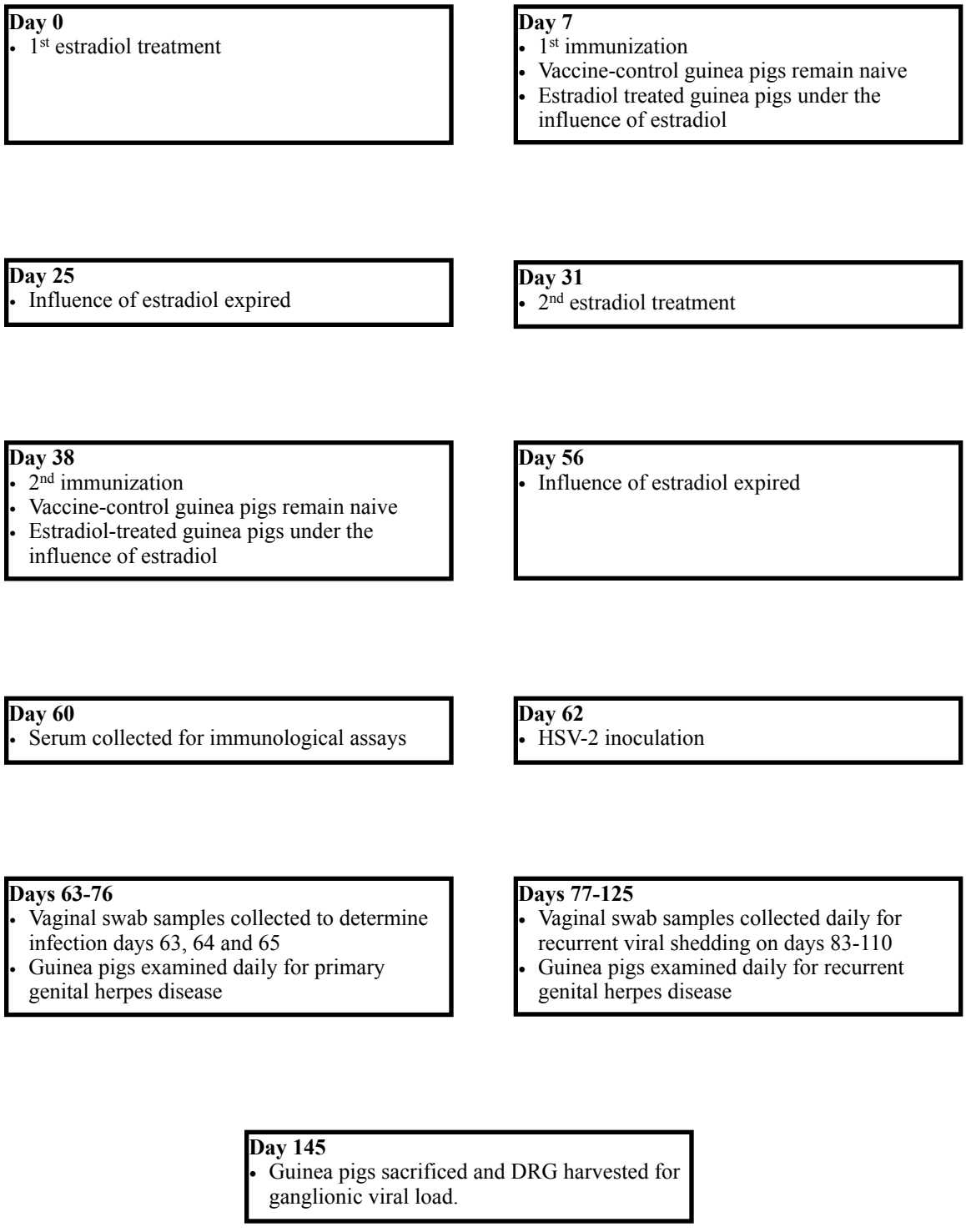

Figure 7. Experimental design timeline for estradiol-treated guinea pigs.

Guinea pig model of genital herpes

Four weeks after the second immunization, serum samples were collected from the animals and they were intravaginally challenged with 1 x 10^6 pfu HSV-2 strain MS, as previously described (44). Vaginal swab samples were collected on days 1, 2 and 3 from all guinea pigs and a portion of these samples were plated without freezing on Vero cell monolayers and incubated for 5 days at 37°C to determine infection; the remainder of the samples were stored at -80°C until they could later be plated on Vero cell monolayers and incubated for 2 days for viral quantification via plaque assay. Animals were defined as being infected if viral cytopathic effects of HSV-2 were observed from one of the swabs. As previously described, guinea pigs were evaluated daily, and primary genital disease was quantified using a 0-4 lesion score scale (44). The mean cumulative lesion score was used as a measurement of disease severity and is the sum of the lesion scores divided by the number of infected guinea pigs in the group. After their recovery from primary disease, infected guinea pigs continued to be evaluated daily for recurrent disease through day 63 postinoculation. The number of recurrent lesions days was used as a measure of the frequency of recurrent disease. Vaginal swab samples were collected from infected guinea pigs in the estradiol supplementation study and stored at -80°C until processing for PCR analysis to quantify the frequency and magnitude of shedding into the vaginal mucosa. Infected guinea pigs surviving to 63 day postinoculation were humanely sacrificed. The DRG were extracted postmortem and frozen at -80°C until processing for PCR analysis for viral DNA quantification.

Neutralization assay

Neutralizing serum antibody titres were measured by a modification of our previously described technique (141). Briefly, serum from vaccinated and naïve control animals was heat inactivated at 56°C for 15 min. A series of two-fold dilutions was then made in 2% titration media and Low-Tox H Rabbit Complement (Cedarlane Laboratories, Burlington NC) at a final concentration of 1/20480. Approximately 100 pfu HSV-2 was added to each tube in the dilution sequence. Following incubation at 37°C for 1 hour, the dilution sequence was plated on Vero cell

monolayers for pfu quantification, as above. After incubation for 3 days, the plates were stained with crystal violet and the number of viral plaques counted. The end-point neutralizing antibody titre was defined as the $\log_{10}$ of the final serum dilution that produced a >50% reduction in the number of viral plaques compared to the number of plaques in control serum wells (45).

HSV-2 DNA quantification

HSV-2 DNA was extracted and quantified by a method adapted from our previous publications (45). DNA was extracted from the samples using the DNeasy 96-well-format extraction (Qiagen). Each plate run included negative controls, to ensure the integrity of the samples. DNA samples were eluted in total organic carbon-free water and were stored at -20° C until analysis.

Quantitative PCR was performed using a CFX96 real-time system and associated reagents (Bio-Rad). HSV-2 DNA was quantified in 96-well plates using primers that targeted the HSV-2 glycoprotein B gene with adaptations for real-time analyses (148). Each reaction, which was completed in duplicate, contained 1x iQ Supermix (Bio-Rad) with TaqMan Probe, 5pmol each of forward and reverse HSV-2 primers, and 5 μL of template DNA (1% of the total sample). The HSV-2 primer sequences were as follows: forward, 5'-CGC ATC AAG ACC ACC TCC TC -3', and reverse, 5' - GCT CGC ACC ACG CGA - 3'. HSV-2 amplimer identity was confirmed by melting temperature analysis. Control reactions, including PCRs that lacked template to serve as negative template contamination controls, were extracted in parallel with the DNA. A 10-fold-dilution series of cloned amplimers was used for quantification standards. Sample integrity and extraction efficiency were assessed by a housekeeping gene *GAPDH*, as previously described (45). The quantity of GAPDH copies served to normalize the HSV-2 copies in each sample for accurate comparisons. PCR efficiencies were between 93% and 107%.

Statistics

Fisher's exact tests were used to compare all infection, disease and outcome data. The Student's unpaired t-test was used to compare pairs of group mean values. And an ANOVA with

the Bonferroni post-hoc test was used to compare more than two group mean values. All tests were two-tailed and all reported P values are two-tailed with values <0.05 being considered to indicate statistical significance. All statistical analysis was performed using SPSS 16.0 for Mac OS.

RESULTS

Impact of ovariectomization on vaccine efficacy against infection and primary genital herpes in guinea pigs

Because the gD/AS04 vaccine provides good protection against disease in the guinea pig model (Table 5), we began the studies presented here using ovariectomized guinea pigs in an attempt to determine if this was a more susceptible model, as it was in mice, and to evaluate the possibility that the absence of ovarian-synthesized estradiol may compromise the protection afforded by immunization. To that end, 20 guinea pigs underwent ovariectomization and 20 guinea pigs underwent a control, sham surgery. Ovariectomization serves as means to reduce estradiol synthesis and thus reduce the amount of circulating estradiol. After recovery from surgery, arrival in our animal facility and acclimation, 10 ovariectomized and 10 sham guinea pigs were immunized with two injections of gD/AS04 vaccine containing 5 μg of gD, given four weeks apart. Four weeks later, all 40 guinea pigs were intravaginally inoculated with 1 x 10^6 pfu HSV-2 and the results are shown in Table 6. All guinea pigs in the vaccinated groups (groups 1 and 2), all ovariectomized naive-control guinea pigs (group 4) and all but three sham surgery naive-control guinea pigs (group 3) became infected. There was no statistical difference in incidence of infection between any group and the vaccine afforded no protection against genital HSV-2 infection in guinea pigs.

Immunization did result in a reduction of HSV-2 replicating in the vaginal tract two days postinoculation (Table 6). Each vaccinated group showed significantly reduced HSV-2 titers compared to their respective naive control groups (group 1 versus 3; group 2 versus 4). However there was no significant difference between the vaccinated groups (group 1 versus 2) or the naive control groups (group 3 versus 4) resulting from ovariectomization.

Table 6. Impact of ovariectomization on genital herpes vaccine efficacy against HSV-2 infection and primary disease in guinea pigs.

Group		Infection		Primary Disease	
#	Intervention	Incidence [a]	Virus titer, mean ± SD, pfu/mL [b]	Incidence [c]	Severity, mean ± SD [d]
1	Sham surgery & vaccinated	10/10	$1.01 \times 10^3 \pm 12.36$ [e]	0/10 [f]	0.00 [g]
2	Ovariectomized & vaccinated	10/10	$8.62 \times 10^2 \pm 4.44$ [h]	1/10 [i]	0.10 ± 0.32 [j]
3	Sham surgery	7/10	$2.12 \times 10^4 \pm 10.09$	6/7	4.79 ± 3.87
4	Ovariectomized	10/10	$1.22 \times 10^4 \pm 2.19$	10/10	5.80 ± 2.38

[a] Data are the number of guinea pigs from which virus was isolated by plaque titration of vaginal swab samples collected on days 1, 2 or 3 postinoculation with Herpes Simplex Virus type 2 (HSV-2) strain MS / number of pigs inoculated.
[b] Virus titres were calculated via plaque assay from vaginal swab samples collected two days postinoculation from infected guinea pigs.
[c] Data are number of guinea pigs experiencing primary genital herpes skin disease / number of guinea pigs infected with HSV-2 strain MS.
[d] Disease severity is reported as the mean of the cumulative primary lesion score among infected guinea pigs.
[e] $P<0.01$ versus group 3, $P>0.05$ versus group 2, via ANOVA with Bonferroni's post-hoc test.
[f] $P<0.01$ versus group 3, $P>0.05$ versus group 2, via Fisher's exact test.
[g] $P<0.01$ versus group 3, $P>0.05$ versus group 2, via ANOVA with Bonferroni's post-hoc test.
[h] $P<0.01$ versus group 4, $P>0.05$ versus group 1, via ANOVA with Bonferroni's post-hoc test.
[i] $P<0.01$ versus group 4 via Fisher's exact test.
[j] $P<0.01$ versus group 4 via ANOVA with Bonferroni's post-hoc test.

Immunization afforded significant protection against primary genital herpes skin disease in HSV-2-infected guinea pigs. As shown in Table 6, none of the sham surgery animals and only one of the ovariectomized animals who were vaccinated developed primary disease; this was a statistically significant protection versus their respective naive controls (group 1 versus 3; group 2 versus 4), since 86% and 100% of them did present with signs of primary disease, respectively. No significant difference in the incidence of primary disease was observed between the immunized groups (group 1 versus 2) nor the naive control groups (group 3 versus 4).

Everyday for 14 days postinoculation, animals were inspected for signs of primary genital herpes disease lesions and those lesions were scored. The group mean of the cumulative primary disease lesion scores is used as a benchmark of the disease severity experienced by the infected guinea pigs in a given group; those values are reported in Table 6. Since none of the sham surgery animals that received immunization presented with primary disease, the mean cumulative primary disease score for group 1 is 0. The disease in the one ovariectomized-immunized animal that manifested primary disease consisted of a single, minor, non-ulcerative erythematic area and lasted two days; the animal's cumulative primary disease score was (0.5 x 2) 1.0 and thus the group's mean score was 0.1. Both of these groups had significantly lower mean severity scores than their respective naive control groups, in which all but one (group 3) or all animals (group 4) experienced primary disease and had lesion scores which were higher and of greater duration (Table 6). However, there was no significant difference in the primary disease severity of the two vaccinated groups (group 1 versus 2) nor in the naive control groups (group 3 versus 4) resulting from ovariectomization. Therefore, comparing the ovariectomized setting to the ovary-intact setting, we see no significant increase in the incidence of infection, virus replication or primary disease severity. Immunization in either setting affords no protection against infection but did significantly reduce the amount of replicating virus as well as the incidence and severity of primary disease. The absence of an affect resulting from ovariectomization deviates from our previous observations of the impact of ovariectomization on vaccine efficacy in mice (134).

Ovariectomization does impact vaccine efficacy in the recurrent phase of genital herpes disease in guinea pigs

Differences in vaccine efficacy were observed during the recurrent phase of genital herpes disease due to ovariectomization of the guinea pigs, as shown in Table 7. Only 10% of guinea pigs in the sham surgery group that were immunized (group 1) had any recurrent disease whereas 83% of the sham surgery naive control guinea pigs (group 3) experienced as least one recurrent disease episode. Therefore, the vaccine was effective at providing significant protection against the incidence of recurrent disease in the sham guinea pig groups ($P<0.01$, Fisher's exact test). However, the significant protection afforded to the sham surgery animals was lost in the ovariectomized animals. Forty percent of the ovariectomized animals that received vaccine (group 2) experienced at least one recurrent disease episode while 89% of the ovariectomized naive control guinea pigs (group 4) experienced recurrent disease ($P>0.05$, Fisher's exact test). There was no significant difference in the incidence of recurrent disease between the two immunized groups (group 1 versus 2, $P>0.05$, Fisher's exact test) nor was there a significant difference in the incidence of recurrent disease between the two naive control groups (group 3 versus 4, $P>0.05$, Fisher's exact test).

The significant reduction in the incidence of recurrent disease among sham surgery animals who were vaccinated (group 1) compared to their naive controls (group 3) was not accompanied by a significant reduction in the number of days that the group experienced recurrent lesions (Table 7). The opposite was observed in the ovariectomized animals. Among ovariectomized guinea pigs, although the number of vaccinated animals (group 2) that developed recurrent disease was not significantly reduced compared to naive controls (group 4), there was a significant reduction in the mean number of days immunized guinea pigs had recurrent lesions compared to ovariectomized naive control animals ($P<0.01$, ANOVA). No significant differences were observed between the vaccinated groups (group 1 versus 2) nor the naive control groups (group 3 versus 4) in the frequency of recurrent disease. Therefore, vaccination afforded significant protection against any recurrent disease only to sham surgery animals and a

significant mitigation of the frequency of recurrent disease only in ovariectomized animals, when compared to their respective naive controls.

Table 7. Impact of ovariectomization on vaccine efficacy during the recurrent phase of genital herpes in guinea pigs

Group		Recurrent Disease	
#	Intervention	Incidence [a]	Frequency, mean ± SD [b]
1	Sham surgery & vaccinated	1/10 [c]	0.10 ± 0.32 [d]
2	Ovariectomized & vaccinated	4/10 [e]	1.00 ± 1.41 [f]
3	Sham surgery	5/6 [g]	3.67 ± 3.83
4	Ovariectomized	8/9 [g]	5.67 ± 3.50

[a] Number of guinea pigs experiencing at least one episode of recurrent genital herpes disease on days 15-63 postinoculation / number of guinea pigs infected with HSV-2.
[b] Frequency is reported as the mean number of days with recurrent lesions per infected guinea pig.
[c] $P<0.01$ versus group 3, $P>0.05$ versus group 2, via Fisher's exact test.
[d] $P>0.05$ versus group 2 and versus group 3 via ANOVA with Bonferroni's post-hot test.
[e] $P>0.05$ versus group 4 via Fisher's exact test.
[f] $P<0.01$ versus group 4 via ANOVA with Bonferroni's post-hot test
[g] One animal had to be removed at the end of the primary phase from both groups 3 & 4 because the severity of their primary disease precluded retaining and scoring them in the recurrent phase.

Impact of estradiol on vaccine efficacy against infection in guinea pigs

Because of the good protection afforded by the vaccine in the guinea pig model, it is difficult to show improvement, much as we initially observed in the murine model. Our study above shows that unlike mice, where we overcame this problem by using ovariectomized animals with increased susceptibility to HSV-2 to provide a more rigorous model, ovariectomization of guinea pigs did not result in increased susceptibility. Therefore, as an alternative approach, we decreased the amount of vaccine used by 2.5 fold in an attempt to decrease the protection afforded. We added estradiol to the immunization regimen in some pigs to examine whether estradiol supplementation would restore the compromised protection afforded by the lower dose of vaccine.

Fifty-four guinea pigs were divided into three groups: estradiol + vaccine, vaccine-only or naive controls, and they were treated and immunized per the schedule outlined in Figure 8, prior to intravaginal inoculation with 1 x 10^6 pfu/mL of HSV-2. The functionality of the vaccine-elicited antibody was assessed by neutralization assay using serum samples collected just prior to viral challenge. The neutralizing antibody titer of the guinea pigs treated with estradiol as part of the immunization regimen was 2.62 $\log_{10}$ (± 0.082 SD), similar to that of guinea pigs receiving vaccine only, 2.76 $\log_{10}$ (± 0.034 SD). Upon viral challenge, all but one or two guinea pigs in each group became infected. Among the naive control groups, 89% became infected with HSV-2 after the intravaginal inoculation; 94% of guinea pigs who were treated with estradiol prior to immunization became infected and 94% of guinea pigs receiving immunization alone also became infected. The titers of the virus quantified from the vaginal swab samples collected two days postinoculation were significantly lower in the two vaccinated groups (Vaccinated and Estradiol + Vaccinated) than the naive control group, but were not significantly different from each other (Table 8). Therefore, neither vaccination alone or in the presence of estradiol provided protection against infection, and immunizing under the influence of estradiol neither improved the neutralizing antibody titer nor lowered the titer of replicating virus two days after infection when compared to immunization alone.

Table 8. Impact of estradiol on vaccine efficacy against infection and primary disease in guinea pigs

Group	Infection		Primary Disease	
	Incidence [a]	Virus titre, mean ± SD, pfu/mL [b]	Incidence [c]	Severity, mean ± SD [d]
Naive	16/18	$3.96 \times 10^4 \pm 31.90$	15/16	5.28 ± 3.02
Vaccinated	17/18	$1.59 \times 10^2 \pm 70.11$ [e]	8/17 [f]	1.26 ± 1.88 [e]
Estradiol + Vaccinated	17/18	$7.40 \times 10^2 \pm 14.84$ [e]	4/17 [f]	0.73 ± 1.23 [e]

[a] Data are the number of guinea pigs from which virus was isolated by plaque titration of vaginal swab samples collected on days 1 and 2 postinoculation with HSV-2 strain MS / number of pigs inoculated.
[b] Virus titres were calculated via plaque assay from from vaginal swab samples collected two days postinoculation.
[c] Data are the number of guinea pigs experiencing genital herpes skin disease / number of infected guinea pigs.
[d] Disease severity is reported as the mean of the cumulative primary lesion score among infected pigs.
[e] $P<0.01$ for each vaccinated group versus naive group, via ANOVA with Bonferroni's post-hoc test; however, $P>0.05$ for vaccinated verus estradiol + vaccinated.
[f] $P<0.01$ for each vaccinated group versus naive group, via Fisher's exact test; however $P>0.05$ for vaccinated verus estradiol + vaccinated.

Estradiol may improve vaccine efficacy in preventing genital herpes disease in guinea pigs

All but one (94%) of the infected naive control guinea pigs developed primary genital herpes disease (Table 8). The number of infected guinea pigs receiving vaccine-only and developing primary disease was 47%, which was half the number of the naive group and a significant level of protection against disease ($P<0.01$). The number of infected guinea pigs developing disease was halved again in the estradiol + vaccine group, with only 23.5% having primary disease; this was a significant improvement in efficacy compared to the naive control group but was not significantly different than the vaccine efficacy in animals receiving vaccine alone. The severity of the primary disease in the naive guinea pigs was significantly worse, as indicated by the mean cumulative disease lesion score, compared to either of the vaccinated groups (Table 8). The severity of the primary disease was improved by more than four-fold in guinea pigs receiving vaccine alone compared to those in the naive group ($P<0.01$). When estradiol was added to immunization, the severity was additionally lessened by almost two-fold compared to vaccine-only, however these two groups were not significantly different from each other. Therefore, vaccination alone provided significant protection against primary genital herpes and reduces its severity, as expected, and there was a trend toward even better protection when immunized under the influence of estradiol.

Impact of estradiol on vaccine efficacy in the recurrent phase of genital herpes in guinea pigs

The examination for the impact of estradiol on vaccine efficacy extended into the recurrent phase of genital herpes disease. All but one of the infected guinea pigs experienced at least one episode of recurrent disease, as is shown in Table 9, while approximately half of the animals in the two vaccinated groups experienced recurrent disease. The mean number recurrent lesion days was reduced in the vaccine only group and was reduced slightly more in the estradiol + vaccine group, when compared to the naive control group, but these differences were not statistically significant.

Table 9. Impact of estradiol on vaccine efficacy in guinea pigs during the recurrent phase of infection.

Group	Recurrent Disease		Viral Shedding		Ganglionic Load
	Incidence [a]	Frequency, mean ± SD [c]	Quantity, mean ± SD [d]	Frequency, mean ± SD [e]	Quantity, mean ± SD [f]
Naive	8/9	2.67 ± 2.12	$1.64 \times 10^{13} \pm 7.7 \times 10^{13}$	11.18 ± 4.1	$6.80 \times 10^{6} \pm 9.7 \times 10^{6}$
Vaccinated	5/11	1.91 ± 2.84	$1.93 \times 10^{12} \pm 6.4 \times 10^{12}$	11 ± 3.7	$2.24 \times 10^{6} \pm 2.0 \times 10^{6}$
Estradiol + Vaccinated	6/11	1.64 ± 2.58	$2.22 \times 10^{12} \pm 9.1 \times 10^{12}$	10.67 ± 3.0	$8.73 \times 10^{6} \pm 1.2 \times 10^{7}$

a Data are number of guinea pigs experiencing at least one episode of recurrent genital herpes disease on days 15-63 postinoculation / number of guinea pigs infected with HSV-2.
b Disease severity is reported as the mean cumulative recurrent lesion score among infected guinea pigs.
c Frequency is reported as the mean number of days with recurrent lesions per infected guinea pig.
d Mean numbers of PCR-detectable genomic copies of HSV-2 /mL from genital swabs taken on days 21-48 postinfection, normalized to quantity of guinea pig DNA per swab.
e Mean number of days on which HSV-2 DNA was detectable per infected guinea pig. All infected guinea pigs experienced at least some viral shedding.
f Mean numbers of PCR-detectable genomic copies of HSV-2 /mL of digested DRG harvested from infected guinea pigs at the termination of the study, normalized to the quantity of guinea pig DNA per DRG sample.
No significant differences existed between any group.

The quantity and frequency of HSV-2 shedding into the vaginal mucosa was assayed via PCR analysis of four weeks from vaginal swab samples collected daily from all infected guinea pigs. The mean quantity of virus being shed into the reproductive tract by the estradiol + vaccine group was similar to that of the vaccine only group and, moreover, the standard deviation was large and thus no significant difference existed between any of the three groups. The frequency of viral shedding may have been lower in the estradiol + vaccine group but, again, the differences in these numbers were small. Therefore, this would indicate that vaccination alone or under the influence of estradiol does little to improve the incidence or frequency of recurrent disease and they do not significantly affect the quantity or frequency that viral shedding occurs. Lastly, the mean viral load in the DRG, harvested from infected guinea pigs at the end of the study and quantified by PCR, also showed no significant differences between the groups (Table 9).

DISCUSSION

We have previously shown in mice that adding estradiol at the time of immunization with this subunit vaccine candidate improved vaccine efficacy (134). Others have observed similar results using a live attenuated vaccine and other routes of administration in mice (94). We also found that if we worked with ovariectomized mice, the most rigorous, susceptible form of the genital herpes murine model, we were better able to demonstrate the improved protection afforded by immunization because the estradiol-enhancement occurs at the upper limits of protection in what is an already fairly robust vaccine. The enhanced vaccine efficacy we observed from the addition of estradiol prompted us to explore this further using a second animal species with which we have had previous success, the guinea pig. While the murine model readily enables studies of infection and incidence of primary disease, the guinea pig model of genital herpes allows us to collect several additional points of data not permitted by the mouse model: the ability to qualify primary and recurrent disease in terms of severity and duration, as well as measure viral shedding; this is desirable because the spectrum and natural history of

genital herpes disease in guinea pigs more closely mirrors that of humans than the mouse model does. However, the guinea pig model also provides additional challenges.

As Bourne *et al*. have published before, vaccination alone affords virtually no protection against infection but significantly reduces the incidence and severity of primary disease, the incidence and frequency of recurrent disease and the quantity of viral shedding (Table 5) (44, 45). Further, the protection against primary and recurrent disease afforded by immunization was so robust that an estradiol-enhancement of protection would not have been detectable for those key endpoints. Therefore, we decided to examine whether we could compromise vaccine efficacy in the guinea pig model via ovariectomization, thus suppressing endogenous levels of estradiol synthesis. This hypothesis was plausible because of the increased susceptibility we observed in ovariectomized mice and because men were afforded no protection by this vaccine in the human clinical trials (34).

The data in Table 6 show that the vaccine performed similarly in ovariectomized guinea pigs as it did in guinea pigs undergoing sham surgery and also very similarly to our previously published guinea pigs results, in which immunization afforded little protection against infection but significant protection against primary and recurrent disease (Table5) (44, 45). Specifically, we saw nearly universal infection in all groups, almost no primary disease in vaccinated groups almost no animals without primary disease in the naive groups, a significantly reduced severity in primary disease and a significantly reduced titer of replicating virus in the genital tract two days postinoculation. It was not until the recurrent phase of disease that we saw a notable effect of ovariectomization. When comparing vaccinated groups to their respective naive control groups, only the sham surgery group benefited from a significant protection against having any recurrent episodes (Table 7). However, the mean severity and number of days with recurrent lesions was not significantly improved in the sham surgery group that was immunized, compared to sham surgery naive controls. Meanwhile, the opposite was observed in ovariectomized groups, in which immunization did not yield significantly greater numbers of guinea pigs experiencing no recurrent disease at all but did afford significantly reduced frequency.

The mixed results in Table 7 complicate an attempt to draw conclusions about the affect of estradiol suppression via ovariectomization on vaccine efficacy. The guinea pig natural hormonal environment is a confounding variable and it differs notably from many other mammals (including humans) in that circulating estradiol concentrations do not vary as the guinea pig cycles through estrous (147, 149). This is not only because guinea pigs naturally have low concentrations of circulating estradiol and because ovarian estradiol makes up a comparatively smaller amount of the total circulating estradiol in guinea pigs, but also because guinea pigs develop large volumes of adipose tissue on their habitus. Fat is a major depot for estrogens in the body, thus a constant source of circulating estradiol exists independent of the ovarian synthesis of estradiol.

Therefore, we employed an alternative approach to examine the impact of estradiol on vaccine efficacy, this time by supplementing with estradiol while using a vaccine dose that was reduced 2.5-fold. The estradiol dosing rationale, relative to the physiological milieu, was equivalent to that which we had successfully used in mice, although the actual dose schedule was modified to be suitable for guinea pigs. We hoped that the reduced vaccine dose would enable us to create the room for improvement in which we would detect any enhancement resulting from estradiol treatment. The primary disease data in Table 8 show that estradiol may in fact improve vaccine efficacy at this phase of the infection. We were effective in compromising the protection afforded by vaccine alone against primary disease (Table 8 versus Tables 5 and 6). Further, against this background of reduced immunization efficacy, we saw a 50% reduction in the incidence of primary disease in the estradiol + vaccine group as well as reduction in the mean severity of primary disease; however, this additional protection fell short of statistical significance. During the recurrent phase of infection we observed a compromised efficacy of immunization alone without a detectable improvement in the recurrent disease or viral shedding as a result of estradiol supplementation. The significant protection afforded by immunization with this low dose of vaccine appeared to have been breached by the recurrent phase of disease, independent of the influence of estradiol; this is plausible since it is believed that the components

of this vaccine which afford efficacy in the primary phase of infection are not necessarily the components which afford protection in the recurrent phase (44, 45).

It is unclear how to assimilate this new data into our understanding of the impact of estradiol on vaccine efficacy and the immune response. It would not be accurate to say that estradiol and ovariectomization had no affect in the studies presented here. It would also not be accurate to characterize estradiol's affects as predominant. This raises the question of whether past observations were a phenomenon unique to the mouse model or whether these results are a guinea pig-specific phenomenon. It also begs the much more important question of which model will better translate to a clinical application. The observations in the mouse model are important because they enable us to replicate what was seen in the clinical trials and, in a broader sense, they mirror the developing picture of a gendered immune system in which some vaccines and autoimmune diseases have a gender-specific profile. The observations in guinea pigs are important because they experience a more clinically relevant disease and because they engage in episodic viral shedding, an endpoint that it potentially important to transmissibility and an area of much focus regarding this vaccine. Answers to these questions will most likely come via two different avenues. In the basic science setting, the question of estradiol's influence on this vaccine's efficacy should probably be translated to at least a third animal model to see which set of previous results can be most commonly translated across species barriers; a cotton rat model of HSV-2 has been used to evaluate an intervention's efficacy in the past (150). And in the clinical setting, further human trials are underway with this vaccine; from these and future trials we will hopefully learn what, if any, affect vaccination has on recurrent disease, viral shedding and transmissibility as well as how strongly guinea pigs data correlates with outcomes in humans.

CHAPTER IV

MDMA Increases Susceptibility to Genital Herpes Infection

INTRODUCTION

Over 1.2 million Americans abused the stimulant 3,4-methylenedioxymethamphetamine (MDMA) in 2007, the majority of whom were young adults (110). MDMA elicits empathogenic, euphoric and stimulant effects, including feelings of wakefulness, intimacy, sexual arousal and disinhibition (113). Its abuse as a "club drug" in the dance club, rave and circuit party scenes is spurred on by the misconception that MDMA, where it is commonly known as "ecstasy", is a "safe" drug. However, acute intoxication with MDMA can cause hypertension, tachycardia, hyperthermia, CNS stimulation, hyponatremia and a serotonin syndrome (95). Further, chronic, heavy usage of MDMA is associated with depression, sleep disorders, as well as increased anxiety, impulsivity and hostility (151). MDMA elicits these effects by stimulating the release of endogenous serotonin, dopamine and norepinephrine into the synapse upon binding to and reversing their respective transporters (96, 97).

The empathogenic effects and behavioral disinhibition elicited by MDMA are frequently associated with increased numbers of sex partners and increased frequency of unprotected sex, which are in turn associated with increased incidence of sexually transmitted infections (STIs) (116). However, the association between MDMA and STIs may not be entirely behavioral. There are an increasing number of reports that MDMA consumption can impact the innate immune response, as mentioned in Chapter 1 and reviewed by *Connor* (122). Animal and human studies, conducted in vitro and ex vivo, suggest that MDMA can modulate key pro- and anti-inflammatory cytokines, including TNF-α, IL-10 and IFN-γ (122-124, 130). However, in vitro observations do not always correspond with the in vivo data and contradicting reports of MDMA's effects on immunomodulation are not uncommon (122, 123).

Herpes Simplex Virus 2 (HSV-2) is the most frequent cause of genital herpes, an STI which presents as vesiculopustular ulcerative lesions. Data from the US National Health and Nutrition Examination Surveys (NHANES) report 17% of Americans are seropositive for HSV-2 (11). As with most STIs, women bear a disproportionate burden of HSV-2 infections, with the virus infecting the vaginal mucoepithelial layer and then spreading to establish a lifelong infection in the innervating dorsal root (4). The virus becomes latent in the ganglia, from which it can reactivate periodically to cause recurrent disease or, more frequently, to shed infectious virus into the genital tract asymptomatically. Virus shed in the absence of symptoms is believed to be the major source of genital herpes transmission to susceptible sex partners (4, 11). Genital herpes lesions can be painful. However, between 63% and 87% of HSV-2 infections are asymptomatic, leaving many people unaware of their HSV-2 infection (4, 11). Importantly, a large body of data indicates that prior infection with HSV-2 increases the risk of HIV infection by at least 2 to 3-fold, even when the HSV infection is asymptomatic and shedding is occurring in an anatomical area distinct from that in which the HIV is introduced; and at times of active lesions, the susceptibility to HIV infection is even greater than during asymptomatic periods (16-21). Thus any impact that MDMA has on HSV-2 infection would have implications for HIV infection as well.

In the studies described here, we tested the hypothesis that MDMA would impact genital herpes infection using a well-established mouse model of genital herpes. We show for the first time that MDMA administration alters both the susceptibility to HSV-2 infection and the course of genital herpes disease. Further, we show that these changes are accompanied by an altered innate immune response to the virus both systemically and at the vaginal mucosal surface.

METHODS

Mice

Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) approximately eight-weeks-old were housed in Association for Assessment and Accreditation of Laboratory Animal

Care-approved quarters and all procedures were approved by the University of Texas Medical Branch Institutional Animal Use and Care Committee. Animals were allowed to acclimate to the vivarium for seven days prior to use.

MDMA and virus

(+)-3,4-Methylenedioxymethamphetamine HCl [(+)-MDMA; National Institute of Drug Abuse, Research Triangle, NC] was dissolved in sterile saline. HSV-2 strain 186 was prepared on Vero cell monolayers and stored frozen (-80°C) until used, as previously described (133).

Mouse model of genital herpes and MDMA

Mice were injected with 2 mg medroxyprogesterone acetate (The Upjohn Company, Kalamazoo, MI) one-week prior to inoculation to make the vaginal epithelium permissive to viral infection, as previously described (133). Five days later, treatment was initiated with mice receiving a 50 μL injection of 10 mg/kg (+)-MDMA subcutaneously once daily for five days. Age-matched control mice received 50 μL of saline. All mice were intravaginally inoculated with HSV-2 on day 3 of drug administration. The inocula used ranged from 1×10^1 to 1×10^4 pfu in 15 μL, depending on the study, as specified in the results section. On days 1 and 2 postinoculation, vaginal swab samples were collected from all mice. Samples were plated on Vero cell monolayers and incubated for 5 days at 37°C to determine infection. Animals were defined as being infected if viral cytopathic effects characteristic of HSV-2 were observed from either swab sample. In addition, mice were examined daily until 21 days postinoculation for clinical signs of genital herpes disease and were defined as having such if they showed pathological signs of cutaneous disease (hair loss and erythema on the perineum) or signs of more severe, neurological disease (urinary inconstance and hind-limb paralysis). Mice progressing to severe neurological involvement either quickly succumbed to encephalitis or were euthanized to prevent suffering.

Cytokine assay

In studies quantifying cytokine and chemokine responses, vaginal lavage and serum samples were collected from mice six hours after HSV-2 challenge on day-3 of (+)-MDMA or saline treatment as well as from (+)-MDMA- and saline-treated mice not challenged with virus. Vaginal mucosal samples were collected by lavage using a positive displacement pipet to instill 25 μL of sterile saline 5 times. Blood samples were collected from the retro-orbital plexus and centrifuged to extract the serum. Recovered lavage fluid and serum was stored at -80°C until cytokine quantification was conducted using cytometric bead arrays with mouse cytokine 23-plex panels (Bio-Rad, Hercules CA).

Calculations and statistics

Fisher's exact test was used to compare all infection, disease and outcome data. The Student's unpaired t-test was used to compare group mean values. All reported p values are two-tailed and values of $p < 0.05$ were considered to indicate statistical significance. The viral inoculum required to infect half of all animals in a group (ID_{50}) was calculated using the Reed-Muench formula. All statistical analysis was performed using SPSS 16.0 for Mac OS.

RESULTS

MDMA impacts the course of genital herpes infection and disease

We first examined the effect of (+)-MDMA on the course of genital herpes disease in mice intravaginally inoculated with 10^4 pfu HSV-2 on day 3 of treatment with (+)-MDMA (10 mg/kg/day x 5 days) or saline (n=25/group). As expected, this high titre inoculum caused infection and produced disease in nearly all animals in both the control and (+)-MDMA-treated groups (152). However, Figure 8 and Table 10 show that the onset of disease was significantly earlier in (+)-MDMA-treated animals (mean day of onset 5.55 ± 0.96 postinoculation) compared to that seen in saline-treated controls (mean day of onset 6.24 ± 1.22; $p<0.05$) (152). Associated with the earlier onset, Table 10 shows the titre of replicating HSV-2 was determined from vaginal

swabs collected two days postinoculation and we observed that (+)-MDMA treatment significantly increased viral titres in the genital tract. A significantly higher viral titre [1.82 x 10^3 pfu/mL (± 4.57 SD, $p<0.05$)] was seen in (+)-MDMA-treated mice compared to saline-treated mice [4.07 x 10^2 pfu/mL (± 7.9 SD)].

MDMA increases susceptibility to HSV-2 infection

We next examined whether (+)-MDMA would change the quantity of virus required to establish infection in the genital tract and impact genital disease. To this end, (+)-MDMA- or saline-treated animals were challenged with a range of HSV-2 inocula (10^1, 10^2 or 10^3 pfu) on the third day of treatment (n=30/group). The results of three independent repeats of the study were used to determine the virus inoculum required to establish infection in 50% of animals, the ID_{50}, a key benchmark of susceptibility. In saline-treated mice, the ID_{50} was 1.91 x 10^2 pfu. However, in mice treated with (+)-MDMA, it was markedly reduced to ID_{50} = 4.56 x 10^1 pfu, indicating an increased susceptibility to infection.

Since not all HSV-2 infections produce genital herpes disease, we monitored these mice to determine whether an increased incidence of infection was accompanied by an increased incidence of disease. Figure 9 shows the incidence of disease across the viral inocula. We observed a greater incidence of disease across all inocula in (+)-MDMA-treated groups compared to saline-controls, including the 10^3 inoculum groups, which had equivalent incidence of infection. Also, the number of (+)-MDMA-treated mice with disease in the 10^2 inoculum group was more than double that of saline-treated group.

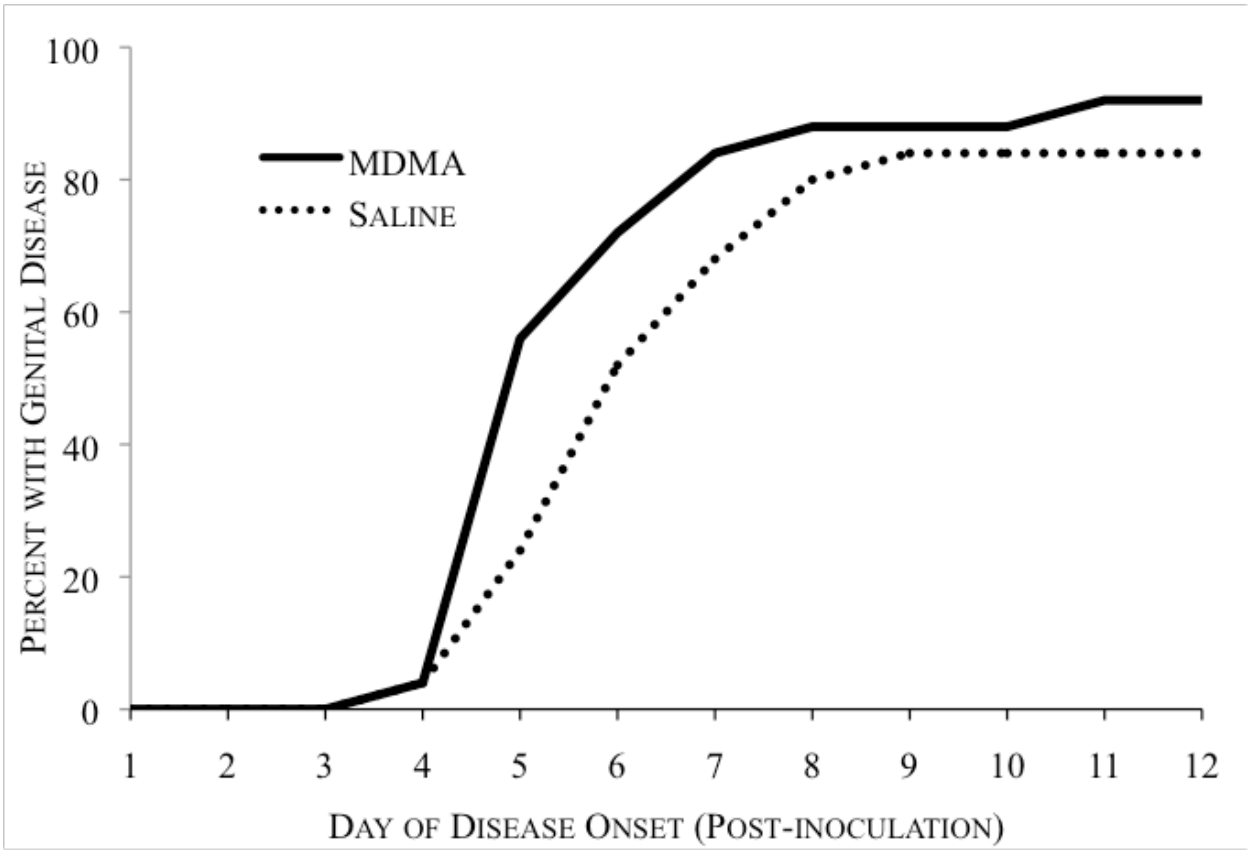

Figure 8. Impact of MDMA on day of genital herpes disease onset.

Mice were administered saline or (+)-MDMA 10 mg/kg/day x 5 days via subcutaneous injection and intravaginally inoculated with 10^4 pfu HSV-2 on day 3 of treatment. Mice were examined daily for the development of genital herpes disease. MDMA-treated mice had a significantly earlier mean day of onset (5.55 ± 0.96 days postinoculation) than saline-treated control mice (6.24 ± 1.22 days postinoculation; $p < 0.05$). n = 25/ group.
Data from: Pennock et al, JID 2009 (152).

Table 10. Impact of MDMA on replicating HSV-2 titers and disease onset.

Group	Virus titer, mean ± SD, pfu/mL [a]	Day of disease onset, mean ± SD [b]
MDMA	$1.82 \times 10^3 \pm 4.57$ [c]	5.55 ± 0.96 [c]
Saline	$4.07 \times 10^2 \pm 7.9$	6.24 ± 1.22

[a] Virus titers were calculated from plaque assay from vaginal swab samples collected two days postinoculation from infected mice.
[b] Data are the mean number of days postinoculation on which the first signs of genital herpes disease was detected in infected mice examined daily.
[c] $P < 0.05$ versus saline-treated controls, via student's unpaired t test. n = 25/group.

Data from: *Pennock et al, JID 2009* (152).

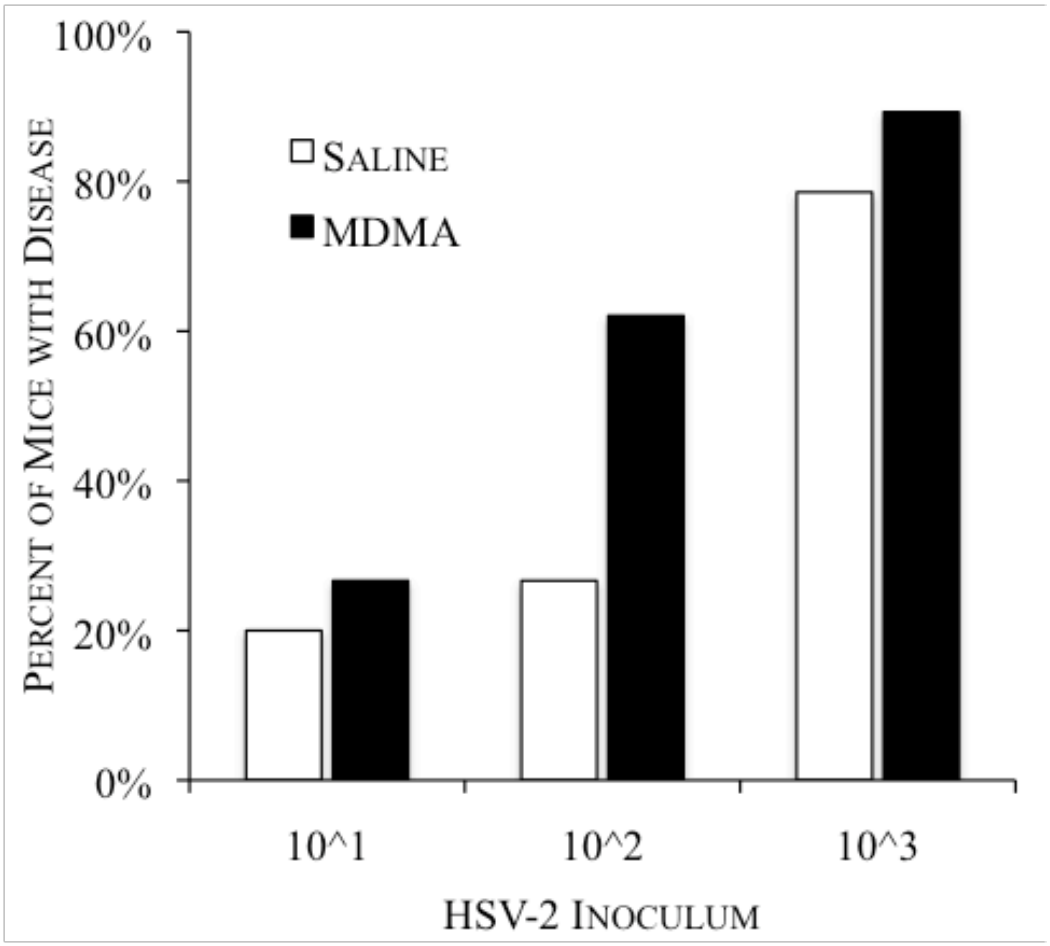

Figure 9. Impact of MDMA on genital herpes disease across various HSV-2 inocula.

Mice were administered saline or (+)-MDMA 10 mg/kg/day x 5 days or saline via subcutaneous injection and intravaginally inoculated with the indicated HSV-2 pfu inoculum on day three of treatment. Mice were examined daily for the development of genital herpes disease. n = 30/group.
Data from: Pennock et al, JID 2009 (152).

MDMA impacts the innate immune response

Our observations that (+)-MDMA treatment altered not only the incidence of infection and disease but also the course of disease and viral replication in the genital tract were suggestive that (+)-MDMA treatment altered the innate immune response to the virus. Therefore, we sought to evaluate the impact of (+)-MDMA on the innate immune system, both in the presence of HSV-2 and without virus challenge. Mice were treated with (+)-MDMA or saline and, on the third day of treatment, inoculated intravaginally with 10^4 pfu HSV-2 or saline. Six hours later, lavage and serum samples were collected from all animals to quantify the impact of (+)-MDMA on the secretion of cytokines and chemokines. Figure 10 shows results for selected cytokines and chemokines in vaginal lavage samples while Figure 11 shows selected systemic results from the serum. Table 11 show the mean values for all the measured cytokines in each group. Our results indicated that (+)-MDMA had a marked impact on a wide range of cytokines assayed, both in infected and uninfected animals. The impact of (+)-MDMA was disregulating and disordering to the innate immune response, rather than skewing either pro- or anti-inflammatory or having a particular Th-1 vs Th-2 bias. While the magnitude of impact on vaginal cytokines varied between the uninfected and infected groups, the direction of the impact (increased in (+)-MDMA-treated mice compared to saline-controls) was usually the same; in contrast, the impact was in opposite directions in the serum (decreased in uninfected (+)-MDMA-treated mice but increased in infected (+)-MDMA-treated mice, compared to controls). Also, the impact of (+)-MDMA-treatment on the cytokines assayed in these two body compartments was not the same in either infected or uninfected mice.

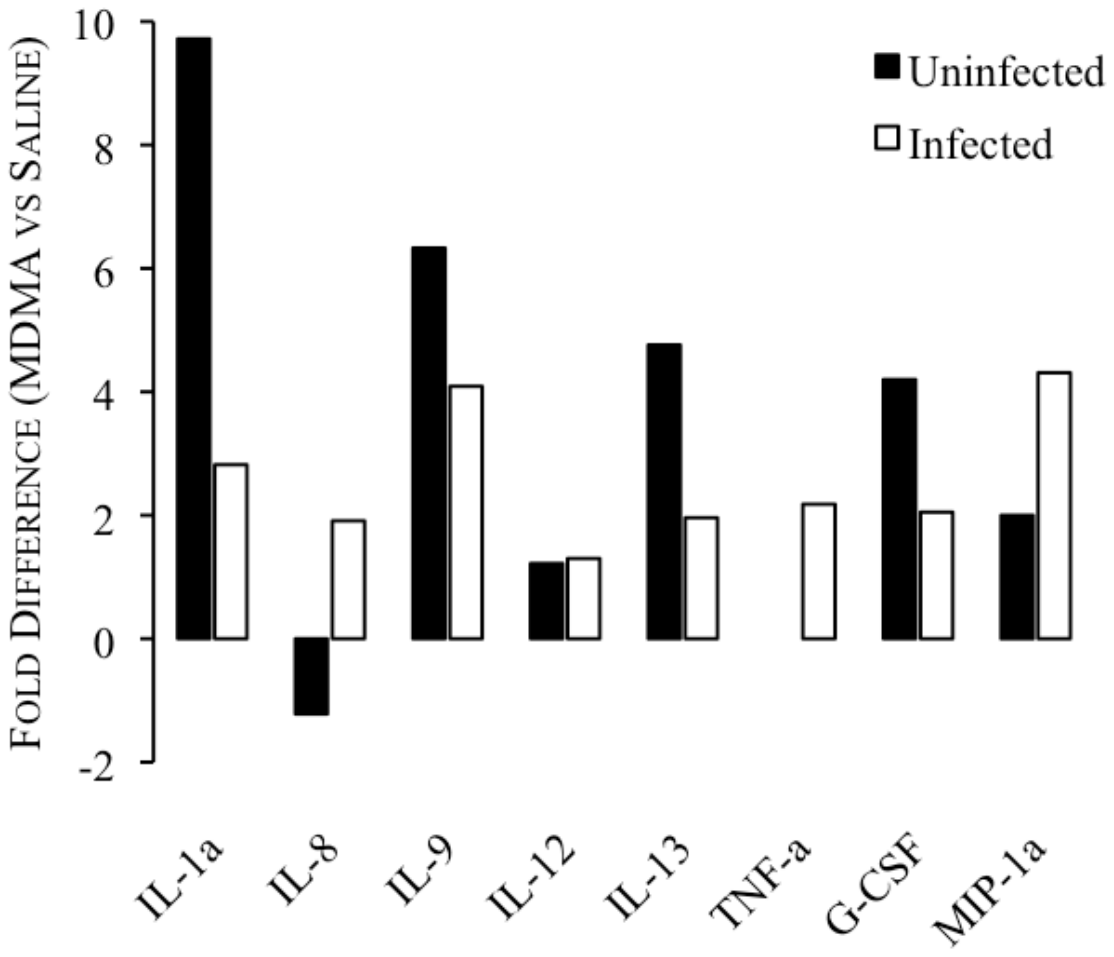

Figure 10. Impact of MDMA-treatment on cytokines in the vaginal mucosa.

Mice were administered (+)-MDMA 10 mg/kg/day x 5 days or saline via subcutaneous injection. Mice were either intravaginally infected with 10^4 pfu HSV-2 (White) or were not inoculated (Black). Samples were collected via vaginal lavage 6 hours after infection on day 3 of treatment. Bar height is an indication of the fold difference of MDMA-treated mice compared to their saline controls. Twenty-three cytokines were assayed, a selection of which are shown here. The value of TNF-α in saline-treated was below the lower limit of detection of the assay; therefore, the value of [the assay's lowest sensitivity - 0.1] was substituted in its place so that the minimum fold difference with respect to the MDMA-treated value could be calculated. A comprehensive index of values can be found in Table 11. n = 15/group
Figure from: Pennock et al, JID 2009 (152).

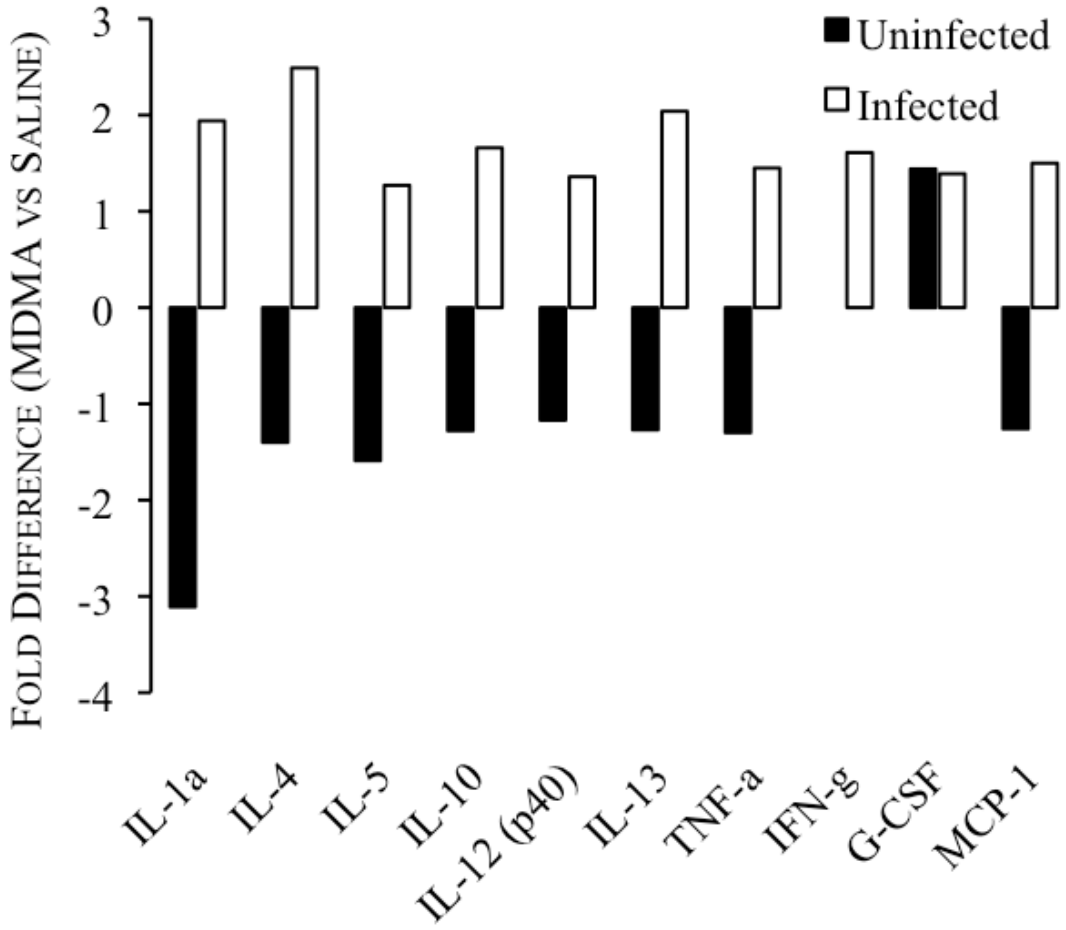

Figure 11. Impact of MDMA-treatment on cytokines in the serum.

Mice were administered (+)-MDMA 10 mg/kg/day x 5 days or saline via subcutaneous injection. Mice were intravaginally infected with 10^4 pfu HSV-2 (White) or were not inoculated (Black). Serum samples were collected 6 hours after infection on day 3 of treatment. Bar height is an indication of the fold difference of MDMA-treated mice compared to their saline controls. Twenty-three cytokines were assay, a selection of which is shown here. A comprehensive index of values can be found in Table 11. n = 15/group.
Figure from: Pennock et al., JID 2009 (152).

Table 11. Impact of MDMA on cytokine secretion in the serum and vaginal mucosa of uninfected and HSV-2 infected mice

Cytokine	Treatment Group [a]	Vaginal Lavage				Serum			
		Uninfected		Infected		Uninfected		Infected	
		Mean (pg/mL)	P [b]	Mean (pg/mL)	P	Mean (pg/mL)	P	Mean (pg/mL)	P
IL-1α	Saline	0.50	<0.01	6.49	<0.05	0.50	--	1.53	--
	MDMA	4.84		18.32		0.16		2.96	
IL-1β	Saline	19.72	--	83.46	--	21.58	--	93.71	<0.01
	MDMA	12.87		96.23		15.53		143.05	
IL-2	Saline	0.85	--	LOD [c]		10.16	--	15.93	<0.01
	MDMA	0.87		LOD		8.65		26.26	
IL-3	Saline	0.18	<0.05	LOD		3.08	<0.01	2.67	<0.05
	MDMA	0.25		LOD		2.10		5.01	
IL-4	Saline	0.40	--	LOD		2.30	<0.05	2.37	0.01
	MDMA	0.49		LOD		1.61		5.91	
IL-5	Saline	0.72	--	3.38	--	8.69	<0.05	22.84	--
	MDMA	0.69		2.25		5.45		28.95	
IL-6	Saline	0.06	--	LOD	<0.01	31.50	--	92.89	0.05
	MDMA	0.09		0.58		25.37		144.99	
IL-8	Saline	20.49	--	12.33	--	18.05	--	40.36	--
	MDMA	16.84		23.50		24.30		43.28	
IL-9	Saline	0.32	--	0.33	--	221.27	<0.05	237.32	<0.01
	MDMA	1.99		1.36		169.78		346.78	
IL-10	Saline	LOD		LOD		59.81	<0.05	441.85	<0.01
	MDMA	LOD		LOD		46.88		734.87	
IL-12 (p40)	Saline	0.64	--	1.60	--	314.12	--	196.55	--
	MDMA	0.78		2.08		266.49		266.96	
IL-12 (p70)	Saline	LOD		LOD	--	32.95	--	128.12	<0.01
	MDMA	LOD		0.32		34.89		221.27	
IL-13	Saline	4.01	--	7.33	--	80.30	--	41.95	<0.01
	MDMA	19.11		14.38		63.26		85.77	
IL-17	Saline	0.61	--	LOD		39.30	--	145.54	0.01
	MDMA	0.76		LOD		40.53		184.15	
IFN-γ	Saline	LOD		LOD		19.62	--	53.20	<0.01
	MDMA	LOD		LOD		19.55		85.91	

Cytokine	Treatment Group [a]	Vaginal Lavage				Serum			
		Uninfected		Infected		Uninfected		Infected	
		Mean (pg/mL)	P [b]	Mean (pg/mL)	P	Mean (pg/mL)	P	Mean (pg/mL)	P
TNF-α	Saline	LOD		LOD		210.09	0.05	1123.25	<0.01
	MDMA	LOD		2.84		161.60		1625.06	
G-CSF	Saline	243.38	<0.01	154.70	--	25.11	0.05	24.76	<0.05
	MDMA	1022.01		316.64		36.22		34.52	
GM-CSF	Saline	LOD		LOD		15.48	<0.01	20.29	<0.05
	MDMA	LOD		LOD		11.27		34.55	
MIP-1α	Saline	LOD	--	3.83	<0.05	26.13	--	28.22	<0.01
	MDMA	2.07		16.52		26.27		43.44	
MIP-1β	Saline	3.76	--	22.48	--	28.76	<0.05	82.35	--
	MDMA	5.79		12.59		18.95		107.43	
RANTES	Saline	0.55	--	20.76	--	15.56	--	46.79	--
	MDMA	0.94		15.03		22.79		44.30	
Eotaxin	Saline	LOD		792.87	--	62.24	--	384.06	--
	MDMA	LOD		412.36		32.55		211.33	
MCP-1	Saline	LOD	--	7.12	--	269.37	--	238.13	<0.01
	MDMA	1.55		8.90		212.16		358.09	

[a] Mice were administered (+)-3,4-methylenedioxymethamphetamine HCL (MDMA) 10 mg/kg/day x 5 days or saline via subcutaneous injection. Animals were either intravaginally infected with 10^4 pfupfupfu herpes simplex virus type 2 (infected) or were not inoculated (uninfected). Vaginal lavage and serum samples were collected 6 hours after infection on day 3 of treatment and the mean cytokine and chemokine production per group was quantified. n=15/group.

[b] Mean values were statistically analyzed by comparing the MDMA-treated group for each cytokine from each sample type to the saline control group using the student's unpaired t-test. Values of $p<0.05$ were considered to indicate significance; cases where $p<0.01$ are also indicated and "—" indicates that the differences were not significantly different.

[c] LOD indicates cases where the quantity of the cytokine is below the lower limit of detection of the assay.

Table from: *Pennock et al., JID 2009* (152).

DISCUSSION

The popularity of MDMA as a drug of abuse is in part due to the widely-held misconception that it is safe (95). However, acute MDMA intoxication poses significant physiological and neurological hazards, including hypertension, tachycardia, hyperthermia and hyponatremia, and there additional complications associated with chronic abuse, including depression, sleep and anxiety disorders, impulsivity and behavioral hostility, some of which persist long after consumption is terminated (95, 151). There is increasing, albeit disparate, evidence that MDMA consumption can also impact the immune response. Researchers have worked with purified bacterial components, mitogens and non-human viruses in studying MDMA drug-effects on the immune system (122-124, 130). Most of this work has been done in vitro and ex vivo. Translating these important observations into animal studies has resulted in mixed and sometimes contradictory results (122, 123). For example, the impact of MDMA on nonreplicating-immunogen-(LPS, concanavalin or phytohaemagglutinin)-induced INF-γ production showed no change in vitro but was suppressed in vivo as a result of IL-12 suppression and IL-10 stimulation (121, 122, 129, 153, 154). We are only aware of one publication reporting an effect of MDMA in regards to an infectious organism; in that study *ex vivo* murine macrophages infected with a mouse gamma herpes virus were observed to produce less IL-6 upon exposure to MDMA (130).

Our studies are the first to examine the impact of (+)-MDMA on a human pathogen, using HSV-2 in an established animal model of genital herpes inoculated via the natural route of infection. We observed significant (+)-MDMA-evoked alterations in the pathological outcomes of virus exposure. The course of HSV-2 infection was altered by (+)-MDMA, with the drug-treated mice experiencing a significantly earlier onset of disease compared to saline-treated mice. Pivotal to both the innate resistance to HSV-2 and the course of HSV-2 infection is the ability of the immune system to control and contain replicating virus in the vaginal tract early in infection. (+)-MDMA-treated mice had significantly higher HSV-2 titres in the genital tract two days after inoculation, compared to saline-treated mice. Also, studies to examine the virus inoculum

required to establish infection at the genital mucosal surface showed that (+)-MDMA treatment was associated with a marked increase in susceptibility to HSV-2 infection, as measured by the four-fold decrease in the ID_{50}. Moreover, more drug-treated animals developed clinical disease over the entire range of inocula, compared to saline controls.

Taken together, these results suggest that the innate immune response of (+)-MDMA-treated mice is less effective in controlling HSV-2 infection and replication. We observe that the impact of (+)-MDMA is to skew and disorder the innate immune system, rather than to functionally shape it, for example, in a pro-inflammatory or anti-inflammatory direction or with a Th1/Th2 bias. Although there is a limited body of literature on the impact of (+)-MDMA on the systemic innate immune response, the results of the present studies, obtained utilizing new, highly sensitive measures, confirm previous reports that (+)-MDMA induces systemic immunological dysregulation (121-124, 130). We are the first to examine the affect of (+)-MDMA on cytokine secretion at a mucosal surface, namely the vaginal mucosa. Interestingly, the impact of (+)-MDMA at this site varied considerably from that seen in the serum, both in the presence and absence of replicating virus. Our data strongly suggest that a measurement of systemic serum immune responses may not accurately reflect that which is seen at a mucosal surface in response to invading pathogens.

The increased incidence of STIs like HIV and HSV-2 among people who consume (+)-MDMA has previously been explained by the behavioral effects of the drug, such as an increased number of sex partners and an increase in high-risk sex practices. Here, we show for the first time that (+)-MDMA can alter the innate immune response and can increase susceptibility to important, human STI pathogens. This observation has direct public health significance. Furthermore, studies in other animal models of genital herpes, such as the guinea pig, in which the impact of (+)-MDMA on recurrent disease and asymptomatic virus shedding into the genital tract can be examined, are necessary as they could have implications for a potential increase in transmissibility to susceptible sex partners (4, 44, 45, 155). In conclusion, from the clinical perspective, knowledge that MDMA abusers are not only more likely to be exposed to STIs but

are also more susceptible to infection and disease is important information for patient education regarding the risks associated with drug abuse, for clinicians performing medical evaluations and screenings of patients who abuse drugs, and for public health officials in anticipating the health needs of at-risk populations.

CHAPTER V

Conclusions

The problem of genital herpes infection and disease is not a small one. It is made larger by the synergy between HSV and HIV infection. Therefore an urgent need exists to better understand the measures by which a vaccine against HSV-2 could be optimized and implemented, as does the need for an understanding of the spectrum of real-world scenarios in which the infection is being acquired and in which the vaccine needs to be effective.

We have shown that estradiol improves vaccine efficacy in ovary-intact and ovariectomized mice. From the images shown in Chapter II, we can see that the intervention with estradiol is biologically potent in ovary-intact mice during the immunization period because we observe a thickened vaginal epithelium. We can also see that the estradiol-thickening effects have lapsed prior to viral challenge, therefore the estradiol treatment and its epithelial effects pose no physical barrier to infection. Therefore, we can be confident that the differences observed between the groups immunized under the influence of estradiol and the groups receiving vaccine only are immunologically mediated, rather than physiologically. In ovary-intact mice, we saw immunization alone affords significant protection against infection and disease and universal protection against mortality. The addition of estradiol to the immunization regimen affords even further protection against infection, nearly universal protection against disease and universal protection against mortality. The estradiol-enhanced vaccine efficacy was significant when compared to naive control mice but was not when compared to vaccine alone, and this was at least partly due to the fact that the vaccine alone is so effective in ovary-intact mice that even large increases in the percentage of mice with improved outcomes are unlikely to be statistically significant, unless the groups sizes are tremendously large. In fact, the robustness of the immune response to the vaccine in the ovary intact mouse model that yielded a significant protection

against infection is an artifact of the murine model since women in the clinical trails of the vaccine did not benefit from significant sterilizing immunity. No such artifact exists when working with ovariectomized mice because they are intrinsically more susceptible to HSV-2 infection than ovary-intact mice. Moreover, our results from using the vaccine in ovariectomized mice mirrored the human clinical trials very well. An additional advantage of the ovariectomized model is that it provides a cleaner hormonal background in which to examine the effects of estradiol.

We observed that the physical effect of estradiol in the ovariectomized mice was the same as it was in the ovary intact mice; estradiol significantly thickened the vaginal epithelium, which subsequently thinned back to the ovariectomized-baseline thickness by the anticipated time and remained at that thickness through viral challenge. Because the baseline thickness of the ovariectomized murine vaginal epithelium is so thin, they are exquisitely susceptible to infection and suffer much more extensive disease than ovary-intact mice. It is interesting to note that although the estradiol-thickening affect of estradiol treatment in ovariectomized mice significantly thickened the epithelium compared to untreated controls, it did not increase the thicknesses to parity with that of the intact mice; this may suggest that our estradiol dosing schedule did not exceed biological relevance for an extended time.

In ovariectomized mice, vaccination alone did not provide significant protection against infection; this mirrors the human clinical trials and deviates from the observations in intact mice. Adding estradiol to the immunization regimen resulted in a sterilizing immunity to infection in a significant number of ovariectomized mice. Vaccination alone provided significant protection against disease, again mirroring the human clinical trials. Immunizing under the influence of estradiol provided protection against disease to a significantly greater number of ovariectomized mice than immunization alone did. Vaccination alone afforded significant but not universal protection against mortality to infected ovariectomized mice. However, adding estradiol resulted in universal protection against mortality in ovariectomized mice. We also began to examine how estradiol treatment may be affecting the immune response to immunization, thus yielding the

improved efficacy. The vaccine-specific IgG antibody titers in estradiol-treated mice were slightly (although not significantly) higher than the antibody titers in mice receiving only the vaccine. We also observed a trend in which the antibody titers correlated well with improved efficacy, i.e., greater numbers of estradiol-treated mice had higher vaccine-specific antibody titers and had reduced rates of infection and disease; however this trend did not reach significance. The difference in the functional, neutralizing antibody titers was significant.

This data is noteworthy because it demonstrates that estradiol has immunological potency than can yield an enhanced immune response rather than just a suppressed one. Our findings contradict the historic majority of published papers on this subject but we were not the first to conclude that estradiol could enhance vaccine efficacy. We were, however, the first to demonstratively isolate the effects of estradiol to that of the immune system such that estradiol did not interfere with the administration of the vaccine, as it may have when others used a live attenuated vaccine, nor did it interfere with the viral challenge itself. We were also the first to demonstrate estradiol-enhanced efficacy using a genital herpes vaccine candidate that was clinically relevant. Notably, one of the estradiol-induced enhancements was the achievement of sterilizing immunity in a significant number of mice where immunization alone could not. As discussed previously, sterilizing immunity to HSV-2 is not widely believed to be a realistic goal of HSV-2 immunization and was not observed in the clinical trials with this vaccine. But given how much more rigorous the ovariectomized mouse model is than the intact model and that the ovariectomized mice receiving vaccination alone mirrored the human clinical trial results, the observation that adding estradiol to the immunization afforded sterilizing immunity suggests that the subject of sterilizing immunity in humans may not have reached cloture yet. The plausibility of this, as well as any other forms of estradiol-enhanced efficacy, needs to be investigated by determining how well these observations translate into various mammalian models. The differences observed between other animal models may also suggest an approach for elucidating the mechanism by which estradiol enhances vaccine efficacy. For this reason, we examined the impact of estradiol on vaccine efficacy in guinea pigs.

Our observations of estradiol-enhanced vaccine efficacy in mice were not entirely reflected in the guinea pig model. Because the vaccine alone is so effective at preventing disease in guinea pigs, it would have been impossible to demonstrate a significant reduction of disease due to estradiol in the normal guinea pig model. And because guinea pigs have very low, constant concentrations of circulating estradiol, it is reasonable to hypothesize that their immune system is less sensitive to and/or modulated by serum estradiol levels than it may be in species that have more pronounced fluctuations in circulating estradiol due to phasic ovarian estradiol synthesis. But the additional endpoints that can be collected using the guinea pig model, such as recurrent disease and viral shedding, made it nonetheless an important system in which to evaluate estradiol's impact on vaccine efficacy. Thus it made sense to see if ovariectomization of the guinea pigs, and the consequential elimination of ovarian contributions to circulating estradiol, would compromise some or all of the vaccine's efficacy.

Ovariectomization of naive animals did little to impact the incidence of infection, primary disease or recurrent disease in the guinea pigs. In immunized animals, ovariectomization had no impact on the efficacy of vaccination in the prevention of infection or primary disease. However, we did observe differences between the groups in the recurrent phase of disease, in which ovariectomized guinea pigs receiving immunization did not benefit the from significant protection against recurrent disease that sham surgery animals did; conversely, ovariectomized guinea pigs did have a significant reduction in the number of days on which they had recurrent disease, which the sham surgery pigs did not.

We also examined the impact of estradiol supplementation on vaccine efficacy in guinea pigs, employing a methodology similar to that used in the murine model. However we used a markedly reduced vaccine dose, compared to what we had previously used in the guinea pig model, to elicit a less-robust immunity from vaccination alone, thus enabling us to demonstrate an estradiol-enhanced efficacy. The protection afforded by this low dose of the vaccine was definitively less robust; the significant protection against disease elicited by the full-dose was not elicited by the low-dose. Further, the compromised vaccine efficacy was not restored to

significance by estradiol supplementation, although there was a trend toward protection against primary disease. Why this trend of estradiol impacting vaccine efficacy was not maintained or magnified in the recurrent phase of disease, as might have been suggested by the ovariectomized studies, is unknown. Moreover, it is unclear if this is suggestive that estradiol's immunologically enhancing effects are transient or if this is because the sub-prime immune response elicited by the low dose of the vaccine had begun wane to an extent that was greater than it could be buoyed by estradiol's persistent immunological effects. It is also possible that the elements of the estradiol-enhanced vaccine-elicited protection against infection and primary disease, as best seen in our mouse data, are not the same elements of the vaccine-elicited immune response which are protective against recurrent disease or viral shedding. This was a possibility we had hoped to examine in the guinea pig model. However, it may be that the guinea pig model is never going to be one in which ovariectomization results in enhanced susceptibility; and it may be that, in attempting to create room for an estradiol-elicited improvement in efficacy, the vaccine's efficacy was so compromised that we left estradiol with no vaccine-elicited-protection to improve upon. To that end, further studies could examine the impact of estradiol supplementation on vaccine efficacy in which a less compromising vaccine dose is administered, although the translational relevance of an additional, large vaccine-dose ranging study in guinea pigs to titrate the optimal vaccine dose for this study design is questionable. The fact that estradiol had some effects on vaccine efficacy, even though they were small effects, is quite remarkable, considering the complicating hormonal backdrop of guinea pigs. Since we saw significant effects in ovariectomized mice and more subdued effects in guinea pigs, further studies should pursue estradiol's impact on vaccine efficacy in the cotton rat model of genital herpes. There is a single report of a cotton rat model which develops human-like genital herpes primary disease and episodic recurrent disease and this model could be very useful since the pharmacokinetics of estradiol valerate and the endogenous estradiol environment are much simpler and well-understood in rats (150).

Among endeavors to prevent genital herpes, an understanding of the conditions in which HSV-2 is transmitted is of equal importance as vaccine development is. Specifically, it is important to understand the biological and situational contexts in which various population segments are acquiring this and other sexually transmitted infections because these contexts likely have implications affecting the innate susceptibility to infection, as well as the potential to abrogate the protection afforded by vaccination. In fact we observed both of these to be the case regarding the impact of MDMA on genital herpes in mice.

Until recently, epidemiologists had not begun to thoroughly examine the impact of the club drug "ecstasy" (MDMA) on the incidence of sexually transmitted infections, like HSV-2; their results seemed a foregone conclusion because the behavioral and sexual disinhibition, compounded by the decreased utilization of condoms, that are known to be associated with MDMA use would naturally be expected to yield an increased incidence of all sexually transmitted infections. A causal linkage between drug-induced behaviors and greater infection seemed epidemiologically obvious. And it undoubtedly is. It is also undoubtable that infection is only one of several very serious health hazards asociated with MDMA consumption. However, these realities are not consistent with how ecstasy is widely perceived in the contexts and population segments in which it is frequently consumed. On the contrary, MDMA is often believed to be a "safe" drug in the night club, rave and circuit party scenes. In these circles, long-term harmful effects of the drug are believed to be few and overdoses are believed to be rare and preventable. Despite the fact that the medical community knows this not to be true, this knowledge that has not permeated the most at-risk populations because they individually observe it being used without any detected health defects far more frequently than they observe it resulting in a fatal overdose or bad outcome. For example, a linkage between MDMA and incidence of infection is easily missed because drug-consumption during the transmission event and the diagnosis are likely be separated by days, if not months.

We observed that MDMA administration to mice who were intravaginally challenged with HSV-2 resulted in a significantly earlier onset of disease, a significantly greater quantity of

virus replicating in the genital tract, a reduced threshold of infection and an increased incidence of disease across a range of inocula. These results suggest two important conclusions. First, the elevated incidence of sexually transmitted infections among people who consume MDMA may not exclusively be a result of sexual behaviors, since our animal studies are conducted in controlled environment. This has implications on how a patient history of drug abuse could necessarily impact the clinical approach and what diagnostic screenings are chosen, even in the absence of the patient reporting higher-risk sexual practices in conjunction with drug use. Further, in the context of a sexually transmitted infection diagnosis, if an exposure history is being collected, our data emphasizes the importance of collecting a drug history that is not limited to intravenous drug use. Meanwhile both an exploration of the duration of MDMA's effects on pathogenesis (which could be conducted in guinea pigs) as well as a more thorough, mechanistic understanding of how MDMA induces increased susceptibility is required. Our cytokine and chemokine analyses in Chapter IV suggest a broad-spectrum dysregulation and disordering of the immune system. An explanation of this impact and how specific parts of this across-the-board dysregulation elicits specific immunological defects pushes the leading edges of our understanding of molecular immunology. However, the need for this level of understanding will continue to be a pressing question well into the future. We have conducted initial pilot studies which show mice immunized against genital herpes lose the significant protection afforded by vaccination if their challenge inoculation occurs during exposure to MDMA. The pursuit of the possibility that MDMA could abrogate the functionality of a pre-existing memory immune response is an important future direction for this research which needs be explored in both mice and guinea pigs, to quantify the extent as well as duration of such an effect. And the impact of what is suggested by this is that the dangers posed by MDMA may not be rendered moot by prophylactic measures, such as vaccination. This only increases the importance of the second conclusion to be drawn from our data.

We have established the foundation for a new rationale of why MDMA is not "safe". The most at-risk patient populations for MDMA abuse have not proven to be very responsive to

patient education pertaining to the dangers of hyponatremia, acute toxicity resulting in serotonin syndrome or the behavioral risks asociated with ecstasy consumption (in fact the later better pertains to the drug's appeal). However, these are also the populations that are the most at-risk for sexually transmitted infections, including HSV-2, syphilis, HIV, etc.. These are dangers they are not entirely unfamiliar with, they are the directed focus of public health education operations and, as a result many individuals in these communities make personal choices about with whom they will have sex, in what venue and what methods of protection they will or will not use. While evidence suggesting that MDMA consumption results in a heightened risk of infection in populations that were already at a high-degree of risk is very unfortunate information, it is nonetheless very important information for them to have and its integration into the public health community's education efforts may finally deliver the message that MDMA is definitively not safe in a manner that will have greater resonance with them than previous attempts.

Our data suggest that one point of commonalty in the differences effected by both estradiol and MDMA is that they are immune mediated. However, the mechanisms by which these immune effects are mediated are well-beyond our capabilities to elucidate them presently because these changes involve a broad spectrum of effector functions. For example, while it is reasonable to speculate, given our efficacy and antibody data from estradiol-treated immunized mice, that estradiol treatment during immunization could have resulted in a modulated transcription of B cell survival and proliferation signals, thus resulting in the establishment and maintenance of a better-educated population of antibody-producing memory cells, even if true, this would almost surely not be a comprehensive explanation of why we observed improved vaccine efficacy in estradiol-treated mice. ERs are observed on effectors cells of almost every type and, during pregnancy and hormone replacement therapy, many of these cell types are modulated in number and localization, therefore the greater leap would be to postulate that only a single or small number of effector pathways are estradiol-modulated than it would to assert that the immunological mechanism of estradiol action exceeds current knowledge of the immune system and our ability to study this question presently. Even more complex is the skewing effect

that MDMA has on the immune system. Every step in the molecular pathways that lead from a resting immune system to the detection of an antigen and then to the launching of a host response are mediated the very cytokine signalling molecules that we observe to be disordered by MDMA. How MDMA mediates this, either by a direct chemical effect of the drug or as a secondary effect of the neurochemical and physiological effects of the drug, is unknown. The net immunological effect of MDMA is clearly deleterious because our drug-treated animals have worse outcomes. However, another unknown is whether we are measuring the cumulative deleterious effect of MDMA or if MDMA impacts some immunological mechanisms even more severely than we are able to detect because MDMA is also rescuing some of the effector pathways that it had disabled upstream. The capability to study the concerted affect of MDMA on so many cytokines at once while examining its impact on immune effector mechanisms in isolation and then adding those isolated effects back together to credibly arrive at the net immunological impact does not exist and probably will not exist in the immediate future.

However, while we wait for the basic science knowledge gaps to be filled which prevent a mechanistic explanation how our immunological data yields our outcome data, we do not have wait for this knowledge to speculate about how our observations are useful. Moreover, what we do know about the epidemiology of today should compel us and our colleagues in the field to continue to study these observations, to translate them through increasingly higher animal models and to examine the adjunct questions our data raises. Poly-drug use is common among people who consume MDMA and the combinatorial effects of these drugs on infection and disease merits exploration, because it is reasonable to speculate that MDMA is not the only commonly-abused substituted amphetamine to disorder the innate immune response in a deleterious manner. And it is unlikely that these detriments are herpes-specific; therefore, studies investigating the impact of MDMA and other drugs on the susceptibility to other pathogens, particularly sexually transmitted agents, should be undertaken. Similarly, it is reasonable to assume that, as an understanding of how estradiol impacts the development of a memory immune response comes to light, this knowledge can be employed to boost other suboptimal

vaccines to improved levels of efficacy. This knowledge has a wide spectrum of clinical applications and it is urgent that these areas of research be pursued in order for discoveries made in the basic science setting to be able to translate into interventions that help patients in the real-world contexts in which they live their lives.

BIBLIOGRAPHY

1. Roizman B and Knipe DM. Herpes simplex viruses and their replication. In: Fields BN, Knipe DM and Howley PM, editors. Fields virology. 4th ed. Philadelphia: Williams & Wilkins; 2001. p. 2399.
2. Roizman B, Knipe DM and Whitley RJ. Herpes simplex viruses. In: Knipe DM and Howley PM, editors. Fields virology. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2503-2601.
3. Gentry GA, Lowe M, Alford G and Nevins R. Sequence analyses of herpesviral enzymes suggest an ancient origin for human sexual behavior. Proc Natl Acad Sci U S A. 1988 Apr; 85 (8):2658-2661.
4. Corey L. Herpes simplex virus. In: Mandell, Bennett and Dolin, editors. Principles and practices of infectious diseases. 6 ed. Philadelphia: Elsevir; 2005. p. 1762-1780.
5. Wald A, Zeh J, Selke S, Ashley RL and Corey L. Virologic characteristics of subclinical and symptomatic genital herpes infections. N Engl J Med. 1995 Sep 21; 333 (12): 770-775.
6. Lafferty WE, Coombs RW, Benedetti J, Critchlow C and Corey L. Recurrences after oral and genital herpes simplex virus infection. Influence of site of infection and viral type. N Engl J Med. 1987 Jun 4; 316 (23):1444-1449.
7. Corey L, Adams HG, Brown ZA and Holmes KK. Genital herpes simplex virus infections: Clinical manifestations, course, and complications. Ann Intern Med. 1983 Jun; 98 (6):958-972.
8. Langenberg AG, Corey L, Ashley RL, Leong WP and Straus SE. A prospective study of new infections with herpes simplex virus type 1 and type 2. Chiron hsv vaccine study group. N Engl J Med. 1999 Nov 4; 341 (19):1432-1438.
9. Stanberry LR, Rosenthal SL, Mills L, Succop PA, Biro FM, Morrow RA and Bernstein DI. Longitudinal risk of herpes simplex virus (hsv) type 1, hsv type 2, and cytomegalovirus infections among young adolescent girls. Clin Infect Dis. 2004 Nov 15; 39 (10):1433-1438.
10. Diaz-Mitoma F, Ruben M, Sacks S, MacPherson P and Caissie G. Detection of viral DNA to evaluate outcome of antiviral treatment of patients with recurrent genital herpes. J Clin Microbiol. 1996 Mar; 34 (3):657-663.
11. Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, Lee FK, Nahmias AJ, Berman SM and Markowitz LE. Trends in herpes simplex virus type 1 and type 2 seroprevalence in the united states. Jama. 2006 Aug 23; 296 (8):964-973.
12. Wald A. Herpes simplex virus type 2 transmission: Risk factors and virus shedding. Herpes : the journal of the IHMF. 2004 Aug 1; 11 Suppl 3:130A-137A.
13. Fleming DT, McQuillan GM, Johnson RE, Nahmias AJ, Aral SO, Lee FK and St Louis ME. Herpes simplex virus type 2 in the united states, 1976 to 1994. N Engl J Med. 1997 Oct 16; 337 (16):1105-1111.

14. Koelle DM and Wald A. Herpes simplex virus: The importance of asymptomatic shedding. J Antimicrob Chemother. 2000 Apr; 45 Suppl T3:1-8.

15. Mertz GJ, Benedetti J, Ashley R, Selke SA and Corey L. Risk factors for the sexual transmission of genital herpes. Ann Intern Med. 1992 Feb 1; 116 (3):197-202.

16. Blower S and Ma L. Calculating the contribution of herpes simplex virus type 2 epidemics to increasing hiv incidence: Treatment implications. Clin Infect Dis. 2004 Nov 1; 39 Suppl 5:S240-247.

17. Corey L. Synergistic copathogens--hiv-1 and hsv-2. N Engl J Med. 2007 Feb 22; 356 (8): 854-856.

18. Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA and Hayes RJ. Herpes simplex virus 2 infection increases hiv acquisition in men and women: Systematic review and meta-analysis of longitudinal studies. Aids. 2006 Jan 2; 20 (1):73-83.

19. Gisselquist D and Potterat JJ. Uncontrolled herpes simplex virus-2 as a cofactor in hiv transmission. J Acquir Immune Defic Syndr. 2003 May 1; 33 (1):119-120.

20. Gray RH, Wawer MJ, Brookmeyer R, Sewankambo NK, Serwadda D, Wabwire-Mangen F, Lutalo T, Li X, vanCott T and Quinn TC. Probability of hiv-1 transmission per coital act in monogamous, heterosexual, hiv-1-discordant couples in rakai, uganda. Lancet. 2001 Apr 14; 357 (9263):1149-1153.

21. Stamm WE, Handsfield HH, Rompalo AM, Ashley RL, Roberts PL and Corey L. The association between genital ulcer disease and acquisition of hiv infection in homosexual men. Jama. 1988 Sep 9; 260 (10):1429-1433.

22. Corey L, Wald A, Celum CL and Quinn TC. The effects of herpes simplex virus-2 on hiv-1 acquisition and transmission: A review of two overlapping epidemics. J Acquir Immune Defic Syndr. 2004 Apr 15; 35 (5):435-445.

23. Roizman B and Whitley RJ. The nine ages of herpes simplex virus. Herpes. 2001 Mar; 8 (1):23-27.

24. Romano A and Gertz S. Herpes (shalbeket) in judaic sources: A synopsis of a book in preparation. Metab Pediatr Syst Ophthalmol. 198811 (1-2):29-30.

25. King DH. History, pharmacokinetics, and pharmacology of acyclovir. J Am Acad Dermatol. 1988 Jan; 18 (1 Pt 2):176-179.

26. Corey L, Bodsworth N, Mindel A, Patel R, Schacker T and Stanberry LR. An update on short-course episodic and prevention therapies for herpes genitalis. Herpes. 2007 Jun 1; 14 Suppl 1:5A-11A.

27. Kim HN, Wald A, Harris J, Almekinder J, Heitman C and Corey L. Does frequency of genital herpes recurrences predict risk of transmission? Further analysis of the valacyclovir transmission study. Sex Transm Dis. 2007 Oct 1.

28. Wald A, Selke S, Warren T, Aoki FY, Sacks S, Diaz-Mitoma F and Corey L. Comparative efficacy of famciclovir and valacyclovir for suppression of recurrent genital herpes and viral shedding. Sex Transm Dis. 2006 Sep; 33 (9):529-533.

29. Corey L, Wald A, Patel R, Sacks SL, Tyring SK, Warren T, Douglas JM, Jr., Paavonen J, Morrow RA, Beutner KR, Stratchounsky LS, Mertz G, Keene ON, Watson HA, Tait D and Vargas-Cortes M. Once-daily valacyclovir to reduce the risk of transmission of genital herpes. N Engl J Med. 2004 Jan 1; 350 (1):11-20.

30. Romanowski B, Marina RB and Roberts JN. Patients' preference of valacyclovir once-daily suppressive therapy versus twice-daily episodic therapy for recurrent genital herpes: A randomized study. Sex Transm Dis. 2003 Mar; 30 (3):226-231.

31. Patel R, Tyring S, Strand A, Price MJ and Grant DM. Impact of suppressive antiviral therapy on the health related quality of life of patients with recurrent genital herpes infection. Sex Transm Infect. 1999 Dec; 75 (6):398-402.

32. Stanberry LR. Clinical trials of prophylactic and therapeutic herpes simplex virus vaccines. Herpes. 2004 Aug; 11 Suppl 3:161A-169A.

33. GlaxoSmithKline and NIAID. A double-blind, randomized, controlled phase iii study to assess the prophylactic efficacy and safety of gd-alum/mpl vaccine in the prevention of genital herpes disease in young women who are hsv-1 and -2 seronegative. NIH; [updated September 25, 2008; cited 2009 June 2]; Available from: http://clinicaltrials.gov/ct2/show/NCT00057330.

34. Stanberry LR, Spruance SL, Cunningham AL, Bernstein DI, Mindel A, Sacks S, Tyring S, Aoki FY, Slaoui M, Denis M, Vandepapeliere P, Dubin G and Group GHVES. Glycoprotein-d-adjuvant vaccine to prevent genital herpes. N Engl J Med. 2002 Nov 21; 347 (21):1652-1661.

35. Langenberg AG, Burke RL, Adair SF, Sekulovich R, Tigges M, Dekker CL and Corey L. A recombinant glycoprotein vaccine for herpes simplex virus type 2: Safety and immunogenicity [corrected]. Ann Intern Med. 1995 Jun 15; 122 (12):889-898.

36. Rudbach JA, Johnson DA and Ulrich JT. Ribi adjuvants: Chemistry, biology and utility in vaccines for human and veterinary medicine. In: Stewart-Tull DES, editor. The theory and practical application of adjuvants. Chichester: John Wiley & Sons; 1995. p. 287-313.

37. Brown ZA, Selke S, Zeh J, Kopelman J, Maslow A, Ashley RL, Watts DH, Berry S, Herd M and Corey L. The acquisition of herpes simplex virus during pregnancy. N Engl J Med. 1997 Aug 21; 337 (8):509-515.

38. Crespi CM, Cumberland WG, Wald A, Corey L and Blower S. Longitudinal study of herpes simplex virus type 2 infection using viral dynamic modelling. Sex Transm Infect. 2007 Aug; 83 (5):359-364.

39. Britto CD, Atherton HD and Dewitt TG. Immunizing adolescents in private practice: Lessons learned from hepatitis b. Prev Med Managed Care. 20012:127-134.

40. Siegel RM, Baker RC, Kotagal UR and Balistreri WF. Hepatitis b vaccine use in cincinnati: A community's response to the aap recommendation of universal hepatitis b immunization. J Natl Med Assoc. 1994 Jun; 86 (6):444-448.

41. Zimet GD, Mays RM, Winston Y, Kee R, Dickes J and Su L. Acceptability of human papillomavirus immunization. J Womens Health Gend Based Med. 2000 Jan-Feb; 9 (1): 47-50.

42. Garnett GP, Dubin G, Slaoui M and Darcis T. The potential epidemiological impact of a genital herpes vaccine for women. Sex Transm Infect. 2004 Feb; 80 (1):24-29.

43. Wald A, Corey L, Cone R, Hobson A, Davis G and Zeh J. Frequent genital herpes simplex virus 2 shedding in immunocompetent women. Effect of acyclovir treatment. J Clin Invest. 1997 Mar 1; 99 (5):1092-1097.

44. Bourne N, Bravo FJ, Francotte M, Bernstein DI, Myers MG, Slaoui M and Stanberry LR. Herpes simplex virus (hsv) type 2 glycoprotein d subunit vaccines and protection against genital hsv-1 or hsv-2 disease in guinea pigs. J Infect Dis. 2003 Feb 15; 187 (4):542-549.

45. Bourne N, Milligan GN, Stanberry LR, Stegall R and Pyles RB. Impact of immunization with glycoprotein d2/as04 on herpes simplex virus type 2 shedding into the genital tract in guinea pigs that become infected. J Infect Dis. 2005 Dec 15; 192 (12):2117-2123.

46. NIAID, NIH and HHS. Herpevac trial for women. [cited 2009 June 2]; Available from: http://www.niaid.nih.gov/dmid/stds/herpevac/default.htm.

47. NIAID. Transfer of herpes simplex virus type 2 gd subunit vaccine-induced antibodies from mothers to neonates. NIH; [updated May 17, 2007; cited 2009 June 2]; Available from: http://clinicaltrials.gov/ct2/show/study/NCT00138320.

48. GlaxoSmithKline. A study to evaluate the immunogenicity and safety of glaxosmithkline biologicals' herpes simplex candidate vaccine (gd2-as04) in healthy hsv seronegative and seropositive female subjects aged 10-17 years. [updated April 30, 2008; cited 2009 June 2]; Available from: http://clinicaltrials.gov/ct2/show/NCT00224484.

49. GlaxoSmithKline. Compare immunogenicity & safety of 3 lots of gsk208141 vaccine in healthy hsv-1 & -2 seronegative (hsv 1-/2-) females of 10-17 y & vaccine immunogenicity in healthy hsv 1-/2- females of 10-17 y with healthy hsv 1 /2- adult females. [updated November 6, 2008; cited 2009 June 2]; Available from: http://clinicaltrials.gov/ct2/show/NCT00224471.

50. Loose DS and Stancel GM. Estrogens and progestins. In: Brunton LL, Lazo JS and Parker KL, editors. Goodman & gilman's the pharmacological basis of therapeutics. 11th ed. New York: McGraw-Hill; 2006. p. 1541-1571.

51. Bulun SE and Adashi EY. The physiology and pathology of the female reproductive axis. In: Kronenberg HM, Melmed S, Polonsky KS and Larsen PR, editors. Williams textbook of endrocrinology. 11th ed. ed. Philadelphia, PA: Saunders Elsevier; 2008. p. Figure16-21.

52. Kerr WR. Vaginal and uterine antibodies in cattle with particular reference to brucella abortus. Br Vet J. 1955111:169-178.

53. Yang SL and Schumacher GF. Immune resposne after vaginal application of antigens in the rhesus monkey. Fertil Steril. 197932:588-598.

54. Parr EL, Parr MB and Thapar M. A comparison of specific antibody responses in mouse vaginal fluid after immunization by several routes. J Reprod Immunol. 198814:165-176.

55. Wira CR and Prabhala R. Sex hormone, glucocorticoid and cytokine regulation of mucosal immunity: Hormonal influences on antibody levels and antigen presentation in

the female genital tract. In: Walker WA, Harmatz PR and Wershil BC, editors. Immunology of the gut. New York: Academic Press; 1993. p. 184-205.

56. Wira CR and Sandoe CP. Specific iga and igg antibodies in the secretions of the female reproductive tract: Effects of immunization and estradiol on expression of this response in vivo. J Immunol. 1987 Jun 15; 138 (12):4159-4164.

57. Wira CR and Sandoe CP. Effect of uterine immunization and oestradiol on specific iga and igg antibodies in uterine, vaginal and salivary secretions. Immunology. 1989 Sep; 68 (1):24-30.

58. Harbour DV and Blalock JE. Lymphocytes and lymphocytic hormones in pregnancy. Prog Neuroendocrinol Immunol. 19892:55-63.

59. Wira CR and Stern JE. Endocrine regulation of the mucosal immune system in the female reproductive tract: Control of iga, igg and secretory component during the during the reproductive cycle, at implantation, and throughout pregnancy. In: Pasqualini JR and Scholler R, editors. Hormones and fetal pathophysiology. New York: Marcel Dekker; 1992. p. 343-367.

60. Wira CR and Sandoe CP. Sex steroid hormone regulation of iga and igg in rat uterine secretions. Nature. 1977 Aug 11; 268 (5620):534-536.

61. Schumacher GF. Humoral immune factors in the female reproductive tract and their changes during the cycle. In: Dindsa D and Schumacher GF, editors. Immunological aspects of infertility and fertility control. New York: North Holland/Elsevier; 1980. p. 93-141.

62. Sullivan DA, Richardson GS, MacLaughlin DT and Wira CR. Variations in the levels of secretory component in human uterine fluid during the menstrual cycle. J Steroid Biochem. 1984 Jan; 20 (1):509-513.

63. White HD, Crassi KM, Givan AL, Stern JE, Gonzalez JL, Memoli VA, Green WR and Wira CR. Cd3+ cd8+ ctl activity within the human female reproductive tract: Influence of stage of the menstrual cycle and menopause. J Immunol. 1997 Mar 15; 158 (6): 3017-3027.

64. White HD, Yeaman GR, Givan AL and Wira CR. Mucosal immunity in the human female reproductive tract: Cytotoxic t lymphocyte function in the cervix and vagina of premenopausal and postmenopausal women. Am J Reprod Immunol. 1997 Jan; 37 (1): 30-38.

65. Wira CR and Sandoe CP. Hormonal regulation of immunoglobulins: Influence of estradiol on immunoglobulins a and g in the rat uterus. Endocrinology. 1980 Mar; 106 (3):1020-1026.

66. Wira CR and Sullivan DA. Estradiol and progesterone regulation of immunoglobulin a and g and secretory component in cervicovaginal secretions of the rat. Biol Reprod. 1985 Feb; 32 (1):90-95.

67. Bouman A, Heineman MJ and Faas MM. Sex hormones and the immune response in humans. Human Reproduction Update. 2005 Jul-Aug; 11 (4):411-423.

68. Whitacre CC. Sex differences in autoimmune disease. Nat Immunol. 2001 Sep; 2 (9): 777-780.

69. Mor G, Sapi E, Abrahams VM, Rutherford T, Song J, Hao XY, Muzaffar S and Kohen F. Interaction of the estrogen receptors with the fas ligand promoter in human monocytes. J Immunol. 2003 Jan 1; 170 (1):114-122.

70. Stefano GB and Peter D. Cell surface estrogen receptors coupled to cnos mediate immune and vascular tissue regulation: Therapeutic implications. Med Sci Monit. 2001 Sep-Oct; 7 (5):1066-1074.

71. Smith JM, Shen Z, Wira CR, Fanger MW and Shen L. Effects of menstrual cycle status and gender on human neutrophil phenotype. Am J Reprod Immunol. 2007 Aug; 58 (2): 111-119.

72. Pioli PA, Jensen AL, Weaver LK, Amiel E, Shen Z, Shen L, Wira CR and Guyre PM. Estradiol attenuates lipopolysaccharide-induced cxc chemokine ligand 8 production by human peripheral blood monocytes. J Immunol. 2007 Nov 1; 179 (9):6284-6290.

73. Henderson TA, Saunders PT, Moffett-King A, Groome NP and Critchley HO. Steroid receptor expression in uterine natural killer cells. J Clin Endocrinol Metab. 2003 Jan; 88 (1):440-449.

74. Yeaman GR, Collins JE, Fanger MW, Wira CR and Lydyard PM. Cd8+ t cells in human uterine endometrial lymphoid aggregates: Evidence for accumulation of cells by trafficking. Immunology. 2001 Apr; 102 (4):434-440.

75. Yeaman GR, Guyre PM, Fanger MW, Collins JE, White HD, Rathbun W, Orndorff KA, Gonzalez J, Stern JE and Wira CR. Unique cd8+ t cell-rich lymphoid aggregates in human uterine endometrium. J Leukoc Biol. 1997 Apr; 61 (4):427-435.

76. Narita S, Goldblum RM, Watson CS, Brooks EG, Estes DM, Curran EM and Midoro-Horiuti T. Environmental estrogens induce mast cell degranulation and enhance ige-mediated release of allergic mediators. Environ Health Perspect. 2007 Jan; 115 (1):48-52.

77. Zaitsu M, Narita S, Lambert KC, Grady JJ, Estes DM, Curran EM, Brooks EG, Watson CS, Goldblum RM and Midoro-Horiuti T. Estradiol activates mast cells via a non-genomic estrogen receptor-alpha and calcium influx. Mol Immunol. 2007 Mar; 44 (8): 1977-1985.

78. Curran EM, Berghaus LJ, Vernetti NJ, Saporita AJ, Lubahn DB and Estes DM. Natural killer cells express estrogen receptor-alpha and estrogen receptor-beta and can respond to estrogen via a non-estrogen receptor-alpha-mediated pathway. Cell Immunol. 2001 Nov 25; 214 (1):12-20.

79. Curran EM, Tassell AH, Judy BM, Nowicki B, Montgomery-Rice V, Estes DM and Nowicki S. Estrogen increases menopausal host susceptibility to experimental ascending urinary-tract infection. J Infect Dis. 2007 Mar 1; 195 (5):680-683.

80. Lambert KC, Curran EM, Judy BM, Lubahn DB and Estes DM. Estrogen receptor-alpha deficiency promotes increased tnf-alpha secretion and bacterial killing by murine macrophages in response to microbial stimuli in vitro. J Leukoc Biol. 2004 Jun; 75 (6): 1166-1172.

81. Lambert KC, Curran EM, Judy BM, Milligan GN, Lubahn DB and Estes DM. Estrogen receptor alpha (eralpha) deficiency in macrophages results in increased stimulation of cd4+ t cells while 17beta-estradiol acts through eralpha to increase il-4 and gata-3 expression in cd4+ t cells independent of antigen presentation. J Immunol. 2005 Nov 1; 175 (9):5716-5723.

82. Wira CR, Fahey JV, Sentman CL, Pioli PA and Shen L. Innate and adaptive immunity in female genital tract: Cellular responses and interactions. Immunol Rev. 2005 Aug; 206:306-335.

83. Ansar Ahmed S, Penhale WJ and Talal N. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. Am J Pathol. 1985 Dec; 121 (3):531-551.

84. Cook IF. Sexual dimorphism of humoral immunity with human vaccines. Vaccine. 2008 Jul 4; 26 (29-30):3551-3555.

85. Pessina MA, Hoyt RF, Jr., Goldstein I and Traish AM. Differential regulation of the expression of estrogen, progesterone, and androgen receptors by sex steroid hormones in the vagina: Immunohistochemical studies. J Sex Med. 2006 Sep; 3 (5):804-814.

86. Pessina MA, Hoyt RF, Jr., Goldstein I and Traish AM. Differential effects of estradiol, progesterone, and testosterone on vaginal structural integrity. Endocrinology. 2006 January 1, 2006; 147 (1):61-69.

87. Teepe AG, Allen LB, Wordinger RJ and Harris EF. Effect of the estrous cycle on susceptibility of female mice to intravaginal inoculation of herpes simplex virus type 2 (hsv-2). Antiviral Res. 1990 Oct-Nov; 14 (4-5):227-235.

88. Gallichan WS and Rosenthal KL. Effects of the estrous cycle on local humoral immune responses and protection of intranasally immunized female mice against herpes simplex virus type 2 infection in the genital tract. Virology. 1996 Oct 15; 224 (2):487-497.

89. Kaushic C, Frauendorf E and Wira CR. Polymeric immunoglobulin a receptor in the rodent female reproductive tract: Influence of estradiol in the vagina and differential expression of messenger ribonucleic acid during estrous cycle. Biol Reprod. 1997 Nov; 57 (5):958-966.

90. Wira CR, Rossoll RM and Kaushic C. Antigen-presenting cells in the female reproductive tract: Influence of estradiol on antigen presentation by vaginal cells. Endocrinology. 2000 Aug; 141 (8):2877-2885.

91. Rebar RW and Erickson GF. Menstrual cycle and fertility. In: Goldman L and Ausiello D, editors. Cecil medicine. 23rd ed. Philadelphia, PA: Saunders Elsevier; 2008. p. Figure256-251.

92. Gillgrass AE, Fernandez SA, Rosenthal KL and Kaushic C. Estradiol regulates susceptibility following primary exposure to genital herpes simplex virus type 2, while progesterone induces inflammation. J Virol. 2005 Mar; 79 (5):3107-3116.

93. Gillgrass AE, Tang VA, Towarnicki KM, Rosenthal KL and Kaushic C. Protection against genital herpes infection in mice immunized under different hormonal conditions

correlates with induction of vagina-associated lymphoid tissue. J Virol. 2005 Mar; 79 (5): 3117-3126.

94. Bhavanam S, Snider DP and Kaushic C. Intranasal and subcutaneous immunization under the effect of estradiol leads to better protection against genital hsv-2 challenge compared to progesterone. Vaccine. 2008 Nov 11; 26 (48):6165-6172.

95. Kalant H. The pharmacology and toxicology of "Ecstasy" (mdma) and related drugs. Cmaj. 2001 Oct 2; 165 (7):917-928.

96. Battaglia G, Brooks BP, Kulsakdinun C and De Souza EB. Pharmacologic profile of mdma (3,4-methylenedioxymethamphetamine) at various brain recognition sites. Eur J Pharmacol. 1988 Apr 27; 149 (1-2):159-163.

97. Rudnick G and Wall SC. The molecular mechanism of "Ecstasy" [3,4-methylenedioxy-methamphetamine (mdma)]: Serotonin transporters are targets for mdma-induced serotonin release. Proc Natl Acad Sci U S A. 1992 Mar 1; 89 (5):1817-1821.

98. Sternbach H. The serotonin syndrome. Am J Psychiatry. 1991 Jun; 148 (6):705-713.

99. Mueller PD and Korey WS. Death by "Ecstasy": The serotonin syndrome? Ann Emerg Med. 1998 Sep; 32 (3 Pt 1):377-380.

100. Rochester JA and Kirchner JT. Ecstasy (3,4-methylenedioxymethamphetamine): History, neurochemistry, and toxicology. J Am Board Fam Pract. 1999 Mar-Apr; 12 (2):137-142.

101. Shannon M. Methylenedioxymethamphetamine (mdma, "Ecstasy"). Pediatr Emerg Care. 2000 Oct; 16 (5):377-380.

102. Mas M, Farre M, de la Torre R, Roset PN, Ortuno J, Segura J and Cami J. Cardiovascular and neuroendocrine effects and pharmacokinetics of 3, 4-methylenedioxymethamphetamine in humans. J Pharmacol Exp Ther. 1999 Jul; 290 (1): 136-145.

103. Madhok A, Boxer R and Chowdhury D. Atrial fibrillation in an adolescent--the agony of ecstasy. Pediatr Emerg Care. 2003 Oct; 19 (5):348-349.

104. Duflou J and Mark A. Aortic dissection after ingestion of "Ecstasy" (mdma). Am J Forensic Med Pathol. 2000 Sep; 21 (3):261-263.

105. Callaway CW and Clark RF. Hyperthermia in psychostimulant overdose. Ann Emerg Med. 1994 Jul; 24 (1):68-76.

106. Williams H, Dratcu L, Taylor R, Roberts M and Oyefeso A. "Saturday night fever": Ecstasy related problems in a london accident and emergency department. J Accid Emerg Med. 1998 Sep; 15 (5):322-326.

107. Budisavljevic MN, Stewart L, Sahn SA and Ploth DW. Hyponatremia associated with 3,4-methylenedioxymethylamphetamine ("Ecstasy") abuse. Am J Med Sci. 2003 Aug; 326 (2):89-93.

108. Hartung TK, Schofield E, Short AI, Parr MJ and Henry JA. Hyponatraemic states following 3,4-methylenedioxymethamphetamine (mdma, 'ecstasy') ingestion. QJM. 2002 Jul; 95 (7):431-437.

109. Holmes SB, Banerjee AK and Alexander WD. Hyponatraemia and seizures after ecstasy use. Postgrad Med J. 1999 Jan; 75 (879):32-33.

110. Substance Abuse and Mental Health Services Administration OoAS. Results from the 2007 national survey on drug use and health: National findings. DHHS: NSDUH Series H-34, DHHS Publication No. SMA 08-4343; 2008. http://www.oas.samhsa.gov/nsduh/2k7nsduh/2k7Results.cfm#TOC.

111. Baylen CA and Rosenberg H. A review of the acute subjective effects of mdma/ecstasy. Addiction. 2006 Jul; 101 (7):933-947.

112. Clatts MC, Goldsamt LA and Yi H. Club drug use among young men who have sex with men in nyc: A preliminary epidemiological profile. Subst Use Misuse. 2005 Jan 1; 40 (9-10):1317-1330.

113. Arria AM, Yacoubian GS, Jr., Fost E and Wish ED. The pediatric forum: Ecstasy use among club rave attendees. Arch Pediatr Adolesc Med. 2002 Mar; 156 (3):295-296.

114. Ross MW, Mattison AM and Franklin DR, Jr. Club drugs and sex on drugs are associated with different motivations for gay circuit party attendance in men. Subst Use Misuse. 2003 Jun; 38 (8):1173-1183.

115. Mattison AM, Ross MW, Wolfson T and Franklin D. Circuit party attendance, club drug use, and unsafe sex in gay men. J Subst Abuse. 200113 (1-2):119-126.

116. Colfax G and Guzman R. Club drugs and hiv infection: A review. Clin Infect Dis. 2006 May 15; 42 (10):1463-1469.

117. Klitzman RL, Greenberg JD, Pollack LM and Dolezal C. Mdma ('ecstasy') use, and its association with high risk behaviors, mental health, and other factors among gay/bisexual men in new york city. Drug Alcohol Depend. 2002 Apr 1; 66 (2):115-125.

118. Klitzman RL, Pope HG, Jr. and Hudson JI. Mdma ("Ecstasy") abuse and high-risk sexual behaviors among 169 gay and bisexual men. Am J Psychiatry. 2000 Jul; 157 (7): 1162-1164.

119. Parrott AC, Buchanan T, Scholey AB, Heffernan T, Ling J and Rodgers J. Ecstasy/mdma attributed problems reported by novice, moderate and heavy recreational users. Hum Psychopharmacol. 2002 Aug; 17 (6):309-312.

120. Purcell DW, Moss S, Remien RH, Woods WJ and Parsons JT. Illicit substance use, sexual risk, and hiv-positive gay and bisexual men: Differences by serostatus of casual partners. Aids. 2005 Apr; 19 Suppl 1:S37-47.

121. Boyle NT and Connor TJ. Mdma ("Ecstasy") suppresses the innate ifn-gamma response in vivo: A critical role for the anti-inflammatory cytokine il-10. Eur J Pharmacol. 2007 Oct 31; 572 (2-3):228-238.

122. Connor TJ. Methylenedioxymethamphetamine (mdma, 'ecstasy'): A stressor on the immune system. Immunology. 2004 Apr; 111 (4):357-367.

123. Connor TJ, Harkin A and Kelly JP. Methylenedioxymethamphetamine suppresses production of the proinflammatory cytokine tumor necrosis factor-alpha independent of a

beta-adrenoceptor-mediated increase in interleukin-10. J Pharmacol Exp Ther. 2005 Jan 1; 312 (1):134-143.

124. Pacifici R, Pichini S, Zuccaro P, Farré M, Segura M, Ortuño J, Di Carlo S, Bacosi A, Roset PN, Segura J and de la Torre R. Paroxetine inhibits acute effects of 3,4-methylenedioxymethamphetamine on the immune system in humans. J Pharmacol Exp Ther. 2004 Apr 1; 309 (1):285-292.

125. Pacifici R, Zuccaro P, Farré M, Pichini S, Di Carlo S, Roset PN, Lopez CH, Ortuño J, Segura J, Camí J and de la Torre R. Immunomodulating activity of mdma. Ann N Y Acad Sci. 2000 Sep 1; 914:215-224.

126. Pacifici R, Zuccaro P, Farré M, Pichini S, Di Carlo S, Roset PN, Ortuño J, Pujadas M, Bacosi A, Menoyo E, Segura J and de la Torre R. Effects of repeated doses of mdma ("Ecstasy") on cell-mediated immune response in humans. Life Sci. 2001 Nov 2; 69 (24): 2931-2941.

127. Pacifici R, Zuccaro P, Farré M, Pichini S, Di Carlo S, Roset PN, Palmi I, Ortuño J, Menoyo E, Segura J and de la Torre R. Cell-mediated immune response in mdma users after repeated dose administration: Studies in controlled versus noncontrolled settings. Ann N Y Acad Sci. 2002 Jun 1; 965:421-433.

128. Pacifici R, Zuccaro P, Farré M, Poudevida S, Abanades S, Pichini S, Langohr K, Segura J and de la Torre R. Combined immunomodulating properties of 3,4-methylenedioxymethamphetamine (mdma) and cannabis in humans. Addiction. 2007 Jun 1; 102 (6):931-936.

129. Pacifici R, Zuccaro P, Hernandez Lopez C, Pichini S, Di Carlo S, Farre M, Roset PN, Ortuno J, Segura J and Torre RL. Acute effects of 3,4-methylenedioxymethamphetamine alone and in combination with ethanol on the immune system in humans. J Pharmacol Exp Ther. 2001 Jan; 296 (1):207-215.

130. Nelson DA, Nirmaier JL, Singh SJ, Tolbert MD and Bost KL. Ecstasy (3,4-methylenedioxymethamphetamine) limits murine gammaherpesvirus-68 induced monokine expression. Brain Behav Immun. 2008 Aug 1; 22 (6):912-922.

131. Weiss H. Epidemiology of herpes simplex virus type 2 infection in the developing world. Herpes. 2004 Apr; 11 Suppl 1:24A-35A.

132. Yao XD, Fernandez S, Kelly MM, Kaushic C and Rosenthal KL. Expression of toll-like receptors in murine vaginal epithelium is affected by the estrous cycle and stromal cells. J Reprod Immunol. 2007 Jun 15.

133. Bourne N, Milligan GN, Schleiss MR, Bernstein DI and Stanberry LR. DNA immunization confers protective immunity on mice challenged intravaginally with herpes simplex virus type 2. Vaccine. 1996 Sep; 14 (13):1230-1234.

134. Pennock JW, Stegall R, Bell B, Vargas G, Motamedi M, Milligan GN and Bourne N. Estradiol improves genital herpes vaccine efficacy in mice. Vaccine. 2009:doi:10.1016/j.vaccine.2009.1007.1052.

135. Dusterberg B and Nishino Y. Pharmacokinetic and pharmacological features of oestradiol valerate. Maturitas. 1982 Dec; 4 (4):315-324.

136. Buxton ILO. Pharmacokinetics and pharmacodynamics: The dynamics of drug absorption, distribution, action and elimination. In: Brunton LL, Lazo JS and Parker KL, editors. Goodman & gilman's the pharmacological basis of therapeutics. Eleventh ed. New York: McGraw-Hill; 2006. p. 1-39.

137. Holford NHG. Pharmacokinetics & pharmacodynamics: Rational dosing & the time course of drug action. In: Katzung BG, editor. Basic & clinical pharmacology. 9 ed. New York: Lange Medical Books McGraw-Hill; 2001. p. 34-50.

138. Bourne N, Stegall R, Montano R, Meador M, Stanberry LR and Milligan GN. Efficacy and toxicity of zinc salts as candidate topical microbicides against vaginal herpes simplex virus type 2 infection. Antimicrob Agents Chemother. 2005 Mar; 49 (3):1181-1183.

139. Milligan GN, Bernstein DI and Bourne N. T lymphocytes are required for protection of the vaginal mucosae and sensory ganglia of immune mice against reinfection with herpes simplex virus type 2. J Immunol. 1998 Jun 15; 160 (12):6093-6100.

140. Bernstein DI, Stanberry LR, Harrison CJ, Shukla R, Kappes JC and Myers MG. Antibody response to herpes simplex virus glycoprotein d: Effects of acyclovir and relation to recurrence. J Infect Dis. 1987 Sep; 156 (3):423-429.

141. Bourne N, Stanberry LR, Bernstein DI and Lew D. DNA immunization against experimental genital herpes simplex virus infection. J Infect Dis. 1996 Apr; 173 (4): 800-807.

142. Kuhnz W and Putz B. Pharmacokinetic interpretation of toxicity tests in rats treated with oestradiol valerate in the diet. Pharmacol Toxicol. 1989 Sep; 65 (3):217-222.

143. Kaushic C, Ashkar AA, Reid LA and Rosenthal KL. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. J Virol. 2003 Apr; 77 (8): 4558-4565.

144. Gillgrass AE, Ashkar AA, Rosenthal KL and Kaushic C. Prolonged exposure to progesterone prevents induction of protective mucosal responses following intravaginal immunization with attenuated herpes simplex virus type 2. J Virol. 2003 Sep; 77 (18): 9845-9851.

145. Garnett GP. Role of herd immunity in determining the effect of vaccines against sexually transmitted disease. Journal of Infectious Diseases. 2005 Feb 1; 191 Suppl 1:S97-106.

146. Parr MB and Parr EL. Vaginal immunity in the hsv-2 mouse model. Int Rev Immunol. 2003 Jan-Feb; 22 (1):43-63.

147. Hutz RJ, Bejvan SM, Durning M and Dierschke DJ. Changes in follicular populations, in serum estrogen and progesterone, and in ovarian steroid secretion in vitro during the guinea pig estrous cycle. Biol Reprod. 1990 Feb 1; 42 (2):266-272.

148. Corey L, Huang ML, Selke S and Wald A. Differentiation of herpes simplex virus types 1 and 2 in clinical samples by a real-time taqman pcr assay. J Med Virol. 2005 Jul; 76 (3): 350-355.

149. Westfahl PK and Vekasy MS. Changes in serum and ovarian steroids during reproductive development in the female guinea pig. Biol Reprod. 1988 Dec; 39 (5):1086-1092.

150. Yim KC, Carroll CJ, Tuyama A, Cheshenko N, Carlucci MJ, Porter DD, Prince GA and Herold BC. The cotton rat provides a novel model to study genital herpes infection and to evaluate preventive strategies. J Virol. 2005 Dec; 79 (23):14632-14639.

151. Morgan MJ. Ecstasy (mdma): A review of its possible persistent psychological effects. Psychopharmacology (Berl). 2000 Oct; 152 (3):230-248.

152. Pennock JW, Stegall R, Bubar MJ, Milligan GN, Cunningham KA and Bourne N. Mdma increases susceptibility to genital herpes infection in mice. Journal of Infectious Diseases. 2009:In Press.

153. Connor TJ, Connelly DB and Kelly JP. Methylenedioxymethamphetamine (mdma; 'ecstasy') suppresses antigen specific igg2a and ifn-gamma production. Immunol Lett. 2001 Sep 3; 78 (2):67-73.

154. Connor TJ, Kelly JP and Leonard BE. An assessment of the acute effects of the serotonin releasers methylenedioxymethamphetamine, methylenedioxyamphetamine and fenfluramine on immunity in rats. Immunopharmacology. 2000 Mar; 46 (3):223-235.

155. Bourne N, Bravo FJ and Bernstein DI. Cyclic hpmpc is safe and effective against systemic guinea pig cytomegalovirus infection in immune compromised animals. Antiviral Res. 2000 Aug; 47 (2):103-109.

VITAE

NAME: Jeffry W. Pennock DATE: 8/5/09

PRESENT POSITION AND ADDRESS:
Graduate Student
Department of Microbiology & Immunology
University of Texas Medical Branch
301 University Boulevard
Galveston, Texas 77555-0436

BIOGRAPHICAL: Born: September 8, 1981 in El Paso, Texas.

EDUCATION: Bachelor of Science, Microbiology and Immunology, University of Miami, Coral Gables, Florida awarded 2004

MD-PhD Combined Degree Program, University of Texas Medical Branch, Galveston, Texas 2004-2005; Withdrew from the School of Medicine 2005

PROFESSIONAL AND TEACHING EXPERIENCE

OTHER RESEARCH ACTIVITIES:

A. Isolation and identification of a novel presumptive alpha-virus from the gill structure of *aplysia californica* via Transmission Electron Microscopy. August 2002 – May 2004, Research Assistant in Electron Microscopy, University of Miami, Coral Gables, Florida
B. *Yersinia pestis* type three secretion system transduction, expression and electron microscopy. June 2003 – May 2004, Research Assistant in Microbiology, Miller School of Medicine, University of Miami, Miami, Florida
C. The role of Fas-Fas Ligand in hepatocellular toxicity in murine model of *ehrlichia*. Summer 2005, Graduate School Rotation Student, University of Texas Medical Branch, Galveston, Texas
D. Efficacy of topical microbicides and vaccine candidates against genital herpes in murine and guinea pig models. January 2005 – August 2005, Research Assistant in Microbiology, University of Texas Medical Branch, Galveston, Texas

TEACHING RESPONSIBILITIES:

Teaching assistant in the graduate school course "Techniques in Electron Microscopy" at the University of Miami, Coral Gables, Florida in 2003.

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

American Society for Microbiology
American Medical Association
Texas Medical Association

HONORS:

Summer Scholars Program, University of Miami, Summer 1999
Academic Merit Scholarship, University of Miami, 2000-2004
Dean's and Provost's Honor Roll 2000-2004
University of Miami Honors Program 2000-2004
Bachelor of Science, Cum Laude, General Honors, 2004
Sealy Center for Vaccine Development Predoctoral Fellow, 2007-2009

BIBLIOGRAPHY:

PUBLISHED:

Torruellas J., Jackson M,W., Pennock J.W., Plano G.V. The *yersinia pestis* type III secretion system plays a role in the regulation of Yop secretion. Molec. Microbiol. 57(6): 1718-33; 2005

IN PRESS:

A. Pennock J.W., Stegall R., Bell B., Vargas G., Motamedi M., Milligan G.N., Bourne N. Estradiol improves genital herpes vaccine efficacy in mice. Vaccine; 2009
B. Pennock J.W., Stegall R., Bubar M.J., Milligan G.N., Cunningham K.A., Bourne N. MDMA increases susceptibility to genital herpes infection in mice. J. Infect. Dis.; 2009