RELATIONSHIP OF ESTROUS CYCLE TO HERPES SIMPLEX VIRUS TYPE 2 SUSCEPTIBILITY IN FEMALE MICE

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In CBA/NJ mice, splenic natural killer (NK) cell activity varies with stages of estrous. Susceptibility of ICR mice to intravaginal inoculation of herpes simplex virus type 2 (HSV-2) decreases with age. Susceptibility of female ICR and CBA/NJ mice to HSV-2 inoculated intravaginally and intraperitoneally was examined during the estrous cycle. In cycling ICR mice, greatest susceptibility to intravaginal inoculation was observed during diestrous and the least during metestrous. CBA/NJ mice were most susceptible to intravaginal inoculation of HSV-2 during diestrous. ICR mice were ovariectomized to mimic diestrous and found to be highly susceptible to intravaginal inoculation at all virus doses. No difference in susceptibility among phases of the estrous cycle was seen following intraperitoneal inoculation.

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INTRODUCTION

The continuing clinical significance and problems associated with infections of herpes simplex virus type two (HSV-2) suggest further investigation because the virus is endemic in the human population and is usually sexually transmitted; it persists latently in neurons and infectious lesions occur at irregular intervals; it is implicated as the cause of cervical cancer (38,131,168); and it causes considerable morbidity in immunosuppressed patients and neonates. HSV-2 and the related virus type one (HSV-1) have been proposed as etiological agents of nervous system disorders such as multiple sclerosis (105), Parkinson's disease (106) and Bell's palsy (180). An etiological link between HSV-1 and peptic ulcers has been proposed (88,137). Although episodes can be managed by antiviral drugs, there is no known cure or means of eliminating the virus from the host body once it is established.

Armerding (9,11) has shown that mice fall into three categories of resistance to HSV-2 inoculated intraperitoneally: resistant, moderately susceptible, and very susceptible. Lopez (95) has demonstrated that two major genetic loci are involved (100) in similar resistance patterns of HSV-1. It is ironic that the genetics of

resistance to HSV-2 inoculated intravaginally have not been studied as this would more accurately resemble natural human infection.

McDermott et al. (109) have reported decreasing susceptibility of BALB/cJ mice to primary intravaginal infection with increased animal age. Resistance rose exponentially from four to ten weeks of age and became constant in older animals. Allen (4) found it necessary to swab vaginas with sodium hydroxide prior to virus inoculation in order to ensure infection and reduce variation of susceptibility. She noted that mucus was often removed during the swabbing (personal communication), which led her to suspect that local factors (mucus, pH, and mucosa) provided a natural mechanism of resistance.

The vagina is a changing environment of mucosal and lumen cell populations (50,174), bacterial flora (26,92) and pH fluctuations (20,35), which varies with phases of estrous or menstrual cycles. Estrogens have been shown to affect vaginal and uterine antibody levels (23), decrease cell-mediated immunity (18), activate natural killer cells (50), enhance antibody production and splenic mitogenesis (90) and activate macrophages (183). The fluctuation of hormone levels during the estrous cycle could, therefore, result in variations of these immune cells. Despite the fact that various natural and immune host factors are known to

vary with estrogen levels or phase of estrous, little is known concerning the effect of ovulatory cycles or estrous phases on susceptibility of animals or humans to HSV-2. Since vaginal infections are the natural route of infection of human females, it is appropriate to investigate the effect of the estrous cycle of mice on susceptibility to intravaginal HSV-2 infection. Also, the estrous cycle provides a system in which variation in immune cells can be examined and compared to resistance to HSV-2 inoculated intraperitoneally.

CHAPTER I

LITERATURE REVIEW

Herpes Simplex Virus and Animal Models

Classification of Herpes Simplex Viruses

Herpes simplex viruses (HSV) belong to the family Herpesviridae, which comprises more than seventy viruses affecting a wide range of eukaryotes from fungi to man (129). This group of viruses has many common characteristics, such as morphology and latency (the capacity to persist within the primary host throughout its lifetime). The viruses are differentiated into three subfamilies and several genera and species (156). Herpes simplex viruses, on the basis of host range, rapid spread of infection in cell culture, and usual establishment of latency in nerve ganglia, have been classified in the alphaherpesvirinae subfamily. By the use of serological, biological, and biochemical means (126); HSV can be differentiated into two distinct types: HSV-1 and HSV-2, which share about one-half of their DNA base sequences. Strains within each of the two types can be differentiated by polypeptide analyses, as well as by restriction endonuclease patterns of their DNAs (28,142). Due to differences in proteins between the two types, immune responses can also be differentiated. Whether the small

differences noted among strains within each type can influence responsiveness in infected hosts is unknown.

Molecular Biology of Herpes Simplex Viruses

The herpesviruses are enveloped, ether-sensitive, and possess capsid icosahedral symmetry of 162 capsomeres. The capsid is approximately 100 nm in diameter and contains the nucleoprotein core, a densely staining area measuring approximately 78 nm in diameter that contains the virus DNA. An envelope, composed of host cell membranes and viral glycoproteins, encompasses and enlarges the complete virion to 150 nm in diameter. Fifty polypeptides present in the envelope have been identified as virus-induced or virus-associated.

Both HSV-1 and HSV-2 DNAs are linear double-stranded molecules, approximately 100 megadaltons in molecular mass (49,82), with base composition of 67 and 69 percent guanine and cytosine, respectively (82). The nucleic acids from the two viruses demonstrate approximately 50 percent base-sequence homology (83,102), which is considerably greater than that shown between any of the nucleic acids of other herpesviruses thus far studied (157).

Cell Types Capable of HSV Infection

While other human herpesviruses tend to be species and even cell specific in host range, HSV-1 and HSV-2 are capable of synthesizing fully infectious virus in a wide range of

species and cell types (149). These viruses can multiply in human, rabbit, guinea pig, mouse and numerous simian cells, in embryonic and adult cell types, in fibroblastoid and epithelioid cells, as well as in cancer lines. It is rare to find a cell that will not accept and replicate HSV to a high titer. The cells most sensitive to HSV infection are primary rabbit kidney cells, the Vero monkey cell line, and a number of human cancer cells such as human epitheloid cells-2 (HEp2) and HeLa cells. Generally, the replicative cycle of HSV-2 is somewhat longer (by a few hours) than that of HSV-1, but this varies depending on the host cell.

Animal Models of Infection

Several different animal models have been developed for study of HSV infections including an ocular model (178), a vaginal model (141), an ear model (65), and an intraperitoneal model (13). Animals that have been used include mice (13), guinea pigs (103), cebus monkeys (46) and rabbits (60). Most studies have utilized HSV-1 and only eight investigations of HSV-2 inoculated intravaginally have been performed.

Herpes Simplex Virus Infections in Mice

Pathogenesis of Infection

Vaginal infection.--Pathogenesis after vaginal HSV-2 infection involves local virus replication in the genital organs, followed by direct spread to the central nervous system (CNS) (25). Clinical vaginitis appears between days four and seven, three days before death by encephalitis. In a detailed study of BALB/c and A/J mice inoculated intravaginally, Walz et al. (185) observed virus antigen in vaginal, uterine, and cervical epithelial cells, as well as cervical and uterine glandular epithelium, myometrium and local autonomic ganglion cells. Viremia does not occur in vaginal infections. Progression to the CNS is often swift with infective virus detected after four to five days (119,153). The liver is only rarely involved and the virus is not usually recovered from kidney homogenates.

Surviving mice are generally resistant to vaginal but not systemic challenge with HSV-2, suggesting that local resistance factors may be involved (118). Cell-mediated immune (CMI) responses, including delayed type hypersensitivity (DTH), proliferative CMI, and spleen cells capable of transferring resistance to recipient mice, are present during acute infection and wane by three to four weeks (24,110). There is essentially no serum antibody response during acute vaginal infection or in surviving mice.

Intraperitoneal infection.--After intraperitoneal (IP) inoculation of HSV-2, virus can be isolated from the liver by day four (153). Kidneys are rarely involved. By day seven, virus is present in the spinal cord. Hepatitis and encephalitis result in death of the animal. Macrophages are the primary barrier (77) and can be overwhelmed by larger virus inoculum, leading to infection of liver, spleen, adrenal cortex and CNS.

It has been shown that IP and intravenous (IV) inoculation of HSV-2 produces a focal necrotic hepatitis in mice, whereas with HSV-1 involvement of the liver is less frequent and less severe (116,153). After HSV-1 infection, virus can be recovered from the liver at twenty-four hours but rapidly clears, and there is infrequent necrosis (153).

Relationship to human infection.--In natural human infections, HSV-1 preferentially infects the perioral region accompanied by latency in the trigeminal ganglia, while HSV-2 exhibits a predilection for the genital mucosa, with latent infection of the lumbosacral ganglia. Experimental herpes infections of mice show a similar anatomical distribution. Inoculated intravaginally, type 2 produces a higher mortality and greater frequency of latent virus in the lumbosacral region than does HSV-1, while type 1 injected intranasally produces a higher incidence of latency in the trigeminal ganglia than does type 2 when injected intranasally (155).

In mice HSV-2 is generally more neurovirulent, yet in humans almost all cases of encephalitis are caused by HSV-1 (130).

In the murine system, the route of infection appears to determine the extent of viral spread and severity of the ensuing disease. For example, in eight week old BALB/c mice the 50 percent lethal dose (LD_{50}) for HSV-1 (strain SC16) injected subcutaneously was 1 to 5 x 10^5 plaque forming units (PFU) compared with an LD_{50} of 10^3 to 10^4 PFU when virus was injected IP or IV.

Role of pregnancy.--In both humans (54,187) and experimental animals (45), pregnant females have been shown to exhibit enhanced susceptibility to several viral infections. In addition, pregnancy has been shown to be associated with alteration of several components of the immune response in humans (37,72,146). These two observations are probably related; for example, the incidence of human genital HSV infections increases during pregnancy (138,143). Enhanced susceptibility of the pregnant mouse to genital herpesvirus infection parallels the clinical observations made in humans (138).

Baley and Schacter (17) have demonstrated decreased natural killer (NK) activity in neonates and in pregnant and postpartum women, compared with healthy, young adults. Dimished activity may be due to different mechanisms in each population. In all three trimesters of pregnancy and in postpartum women natural killing is decreased despite the

presence of normal numbers of large granular lymphocytes (natural killer cells) and normal target cell binding. The inability to lyse bound targets of NK cells of pregnant women remains unexplained. The diminished activity of cord blood cells is more extensive than that of pregnant or postpartum women and involves not only reduced lysis of bound target cells, but, in addition, diminished binding of target cells themselves. In this instance, decreased lysis may be due to an immaturity of the NK cells themselves. No difference was observed between the levels of killing by cells from infant girls and cells from infant boys (57,78).

Several studies have emphasized the role of NK cells in resistance to HSV infection (96,148). In studies with HSV-1, genetically resistant mice generally demonstrated elevated NK activity, while susceptible mice showed decreased lysis of HSV-1 infected cells. Armerding (9,11) has shown that mice resistant to IP HSV-2 inoculation also demonstrated elevated NK cell activity. The increased susceptibility of pregnant females (138,143) and neonates (113) to HSV-2 infections may be due to the depressed ability of NK cells to lyse HSV infected cells.

Genetics of Murine HSV-1 Infection

Susceptibility of various mouse strains to HSV-1.--Lopez (95) has studied the susceptibility of various inbred strains of mice to HSV-1. Twelve inbred strains of mice were

inoculated IP with tenfold dilutions of HSV-1 (strain 2931). Studies were first carried out with one, two, three and four month old BALB/c mice. Three and four month old mice were found to be the most resistant to the virus (99) and this age group was used in subsequent studies with the other inbred strains. Male mice were utilized in most studies although studies of back-crosses failed to show sex-linked resistance (99). The animals appeared to fall into three catagories of resistance: resistant, moderately susceptible and very susceptible. C57BL/6 mice survived initial infection with 10^6 PFU of HSV-1 and were used as the prototype resistant strain. Moderately susceptible strains such as BALB/c demonstrated LD_{50}'s of about 10^3 to 10^4 PFU, and very susceptible strains (A/J strain) were killed by as little as 10^1 to 10^2 PFU (95). Kirchner et al. (87) later confirmed these findings by showing that C57BL/6 mice were far more resistant to HSV-1 (strain WAL) than were DBA/2 mice. Lopez et al. (99) demonstrated the same pattern of resistance as strain 2931 with ten additional strains of virus. C57BL/6 mice were resistant to all virus strains tested and A/J mice were consistently killed by a lower virus concentration than BALB/c mice.

Genes involved in resistance to HSV-1.--To determine the number and characteristics of the genes involved in resistance to HSV-1, a challenge dose of 10^6 PFU of HSV-1 (strain 2931) was used to differentiate between resistant and

susceptible mice. F_1 crosses between C57BL/6 and A/J or BALB/c mice were resistant to the challenge dose of HSV-1, indicating that resistance was a dominant trait (100). A total of 25 percent of the progeny of crosses between the F_1 (C57BL/6 x A/J) and A/J parent were resistant to HSV-1, indicating that resistance was governed by two loci, both of which were required for resistance. Further back-crosses of these survivors with A/J mice resulted in less than 25 percent survivors, indicating that other loci on the A/J background also influenced susceptibility. In contrast, similar back-cross experiments with BALB/c mice continued to yield progeny which were about 25 percent resistant, indicating the influence of only two major loci (100).

Earlier studies by Lopez (95) had indicated that HSV-1 resistance was probably not H-2 (major histocompatability locus) linked since different mice strains of the $H\text{-}2^k$ phenotype were both resistant and susceptible. In a more recent study, congenic strains of mice were challenged with HSV-1 and the results showed that background (non-H-2) loci were important in determining resistance and susceptibility, but that H-2 genes were not (100). The H-2 phenotype of back-crossed mice which survived challenge with HSV-1 were identified and resistance clearly segregated independently of H-2 (99).

Schneweiss and Saftig (164) have evaluated inbred strains of mice for resistance to vaginal infections with

HSV-1. Strain resistance was similar to IP inoculation. Resistance could be diminished by x-irradiation, cyclophosphamide and ^{89}Sr, agents which destroy bone marrow precursors of macrophages and lymphocytes. Macrophage poisons did not affect resistance to intravaginal infections, but increased susceptibility to IP infections. Resistant strains of mice had a slightly decreased replication of virus in the sacral ganglia after intravaginal infection.

Strain Resistance to HSV-2.--In similar studies with HSV-2, Armerding et al. (9,11) found the same spectrum of resistance. Inbred strains of mice resistant to HSV-1 were also resistant to equivalent lethal doses of IP infection with HSV-2. Resistant strains inoculated with HSV-2 demonstrated elevated natural killer (NK) cell activity and augmented capacity of macrophages to restrict HSV-2 replication in vivo (10). In addition, transfer of HSV-2 infection-activated NK cells into irradiated syngeneic mice protected these mice from an otherwise lethal infection. Therefore, NK cells and macrophages, perhaps working in concert, appear to play dominant roles in natural resistance to intraperitoneal infection with HSV-2.

Data concerning genes controlling intravaginal infections with HSV-2 were not found in the literature. It is somewhat ironic that the genetics of the natural vaginal infection have not been studied, while IP infections of HSV-1 (which is an artificial model) have been studied in detail.

Role of Macrophages in Resistance

Age of host.--One of the earliest observations of murine herpesvirus infection was that newborn mice were much more susceptible to parenteral injection with HSV-1 than were adult mice (8). Later Johnson (76,77) showed that age-dependent susceptibility of newborn mice to IP infection could be correlated with the inability of cultures of neonatal peritoneal macrophages to restrict replication of the virus. Furthermore, in vitro studies by Stevens and Cook (172) showed that HSV-1 infection of adult macrophages was aborted. In contrast, macrophages from newborn mice replicated HSV-1 to high titers. Viral antigens were expressed in cultures of macrophages from adult mice, but progeny virus appeared to be incomplete. The restrictive effect of peritoneal macrophages can be explained in part by defective virus DNA formation and in part by incomplete virus assembly and envelopment (172).

Experimental inhibition of macrophages.--A number of studies have used various treatments to inhibit macrophage function in order to determine whether they affected resistance to HSV-1. Macrophage "poisons" such as silica particles (6,39,97) or solutions of kappa carragheenin (97), have been used to show that macrophage function was required for resistance to IP infection with HSV-1. Silica and carragheenin are taken up by the macrophages and eventually

result in death of these cells (6,161). Abrogation of this cellular function in inoculated mice causes widespread dissemination and death of the mice.

Experimental enhancement of macrophages.--Another approach to the study of the role of macrophages in resistance to HSV-1 has been the attempt to specifically enhance macrophage function and determine the effect on resistance to HSV-1 infection. Treatment of mice with *C. parvum*, which has been shown to activate macrophages (59), has resulted in mice with greater resistance to IP infection with HSV-1 than untreated mice (99).

Experimental transfer of macrophages.--Transfer experiments have also been used to identify the cells responsible for resistance to HSV-1. Since immature macrophages appeared to be responsible for HSV-1 susceptibility of newborn mice, Hirsh et al. (71) transferred adherent cells from adult mice to newborns and showed increased resistance to IP infection. A very high percentage of the transferred cells had the morphology of macrophages, suggesting that these were the cells responsible for IP resistance.

Interpretation of experimental data.--Lopez (84) suggests the experimental methods supporting macrophage involvement in natural resistance must be reconsidered. First, neonates have a broad array of cellular immuno-

deficiencies (115,173) any or all of which might contribute to their susceptibility to HSV-1. Deficiencies in NK cell function (14), generation of immune interferon (27) or functions of neutrophils (115) might be as responsible as deficiencies of macrophage functions for susceptibility to HSV-1. Transfer experiments also must be reconsidered; if even a few interferon-producing cells or a small percentage of NK cells were transferred with the macrophages, the beneficial effect of the transferred cells could have been due to the contaminating cells rather than to macrophages. Cells could be activated by separation procedures and other manipulations, leading to observations which might not reflect the role of resident macrophages. Furthermore, an interaction of transferred and resident cells could result in resistance. Finally, depression and augmentation of macrophages by these methods is not entirely selective. For example, silica and carragheenin diminish and *C. parvum* augments NK cell function (68). Therefore, these results could be as easily attributed to NK cell function as macrophage function.

Macrophages and HSV-2 infections.--As with HSV-1, macrophages seemingly play an important role in resistance to IP infection with HSV-2 (52,111,122). There have been two additional observations made with HSV-2 infections of mice. First, McGeorge and Morahan (111) found that treatment of mice with macrophage poisons (silica, trypan blue or dextran

sulfate) increased susceptibility to intravenous infection with HSV-2, yet failed to augment susceptibility to an intravaginal infection. Second, Armerding et al. (11) have shown that HSV-2 infection initiated by IP inoculation is an excellent inducer of murine macrophage phagocytic activity. Thus, macrophages, possibly activated by locally generated interferon (194) or by virus infection (31), might be required for resistance to an IP infection. Intravaginal inoculation is thought to parallel the natural route of infection for HSV-2 in humans and these results would suggest that peritoneal macrophages are not required for defense against intravaginal infection.

Effect of Interferon Production (HSV-1)

There are at least three mechanisms by which interferon (IFN) might play a role in resistance to herpesvirus infections (55). Interferon inhibits the replication of herpesviruses (151) and could thus act by limiting the quantities of infectious virus produced at the focus of infection. A second possible mechanism is augmentation of the lytic capacity of NK cells (179) or by recruitment of pre-NK cells which differentiate into mature, functional effectors (158,176). In addition, IFN could function by activating macrophages so that they sequester virus (73) or lyse virus-infected targets (171). In general, interferon's role has been studied in animals made IFN deficient or in mice or humans with inherited deficiencies of this response.

Although deficiencies of IFN generation have been associated with susceptibility to infection, little information has been derived on which functions of interferons are required for resistance. All are probably involved. Kirchner's group (42) has developed evidence which suggests that the early (four hours after infection) production of IFN correlates with genetic resistance to IP HSV-1 infection, which could result from IFN activation of macrophages and pre-NK cells.

Strong evidence that IFN plays a role in resistance to HSV-1 inoculated IP derives from the studies of Gresser et al. (56). These investigators produced a potent heterologous anti-mouse IFN serum which was inoculated into mice to determine its effects on the pathogenesis of virus infections including HSV-1. The IFN deficiency produced by this treatment significantly increased the susceptibility of the mice to IP infection with HSV-1. The incubation period was shortened, and the amount of virus necessary to produce the LD_{50} was decreased by three logs compared with virus-infected control mice. Treatment of previously HSV-infected mice with anti-interferon globulin did not result in reactivation of virus.

Involvement of Natural Killer (NK) Cells

Several studies emphasize the role of NK cells in early resistance to HSV infection (96,99,148). Neonates and patients suffering from Wiscott-Aldrich syndrome share low NK

cell number and a concomitant high mortality rate from HSV infections (113).

Treatment of mice with radioactive strontium (^{89}Sr), which abrogates NK cell function (58), has been shown to make genetically resistant mice as susceptible to HSV-1 as the genetically most susceptible strains (98). Natural killer function in resistant and susceptible strains of mice (99) has been evaluated. Genetically resistant mice have good NK responses, while the most susceptible strains lack significant lysis of HSV-1 infected cells compared to uninfected cells. Interestingly, the CBA strain has normal to high NK, but is moderately susceptible to HSV-1 infection, indicating that this function is not, by itself, enough to mediate resistance.

Natural killing can be stimulated in vivo by interferon or by agents that stimulate endogenous interferon, such as Newcastle disease virus, tilorone, or poly I:C (51,159). With any of these agents, the stimulation of natural killing is blocked by the concomitant administration of antiserum to interferon, demonstrating the role of interferon as mediator of the stimulus to NK cells (51,67). Interferon may also play a role in regulating normal levels of natural killer cells, since strains of mice that produce high levels of interferon tend to have high resting levels of natural killer cell activity (84), and NK cells of such mice respond better

to interferon activation than cells of strains with low baseline natural killer cell activity (66).

Murine natural killer cell activity can be reduced by sustained high levels of 17 beta-estradiol (165,166). Estrogen-treated mice produce interferon but fail to respond to it. It is suspected (167) that high dose estrogens either inactivate NK precursors or reduce their numbers. Estrogens are not directly toxic to mature NK cells, do not affect target lysis in vitro, and they have no effect in vivo before two weeks, even though high levels of 17 beta-estradiol are rapidly established (165).

Natural killer cells have been shown to act in resistance to HSV-1 infection (98) and be affected by levels of estradiol (165). Studies of the susceptibility of mice to IP HSV-2 at various stages of the estrous cycle would show if susceptibility varies with changing activity of NK cells.

Summary of Natural Resistance

Natural resistance to HSV has been extensively studied in the murine system. Involvement of macrophages, IFN and NK cells has been examined. Contradictory data has been presented for each system studied and much more work remains to be done to clarify the role of nonspecific natural factors in herpes resistance, both murine and human.

Armerding (9,11) has noted an elevated NK response in strains of mice which are resistant to IP inoculation of

HSV-2. Transfer of HSV-2 infection-activated NK cells has been shown to protect irradiated syngeneic mice from an otherwise lethal infection (10). Splenic NK cells have been demonstrated to fluctuate in activity during phases of the estrous cycle (50), thus, susceptibility to IP infection with HSV-2 may alter throughout the cycle. In humans (54,187) and experimental animals (45), the NK response is low during pregnancy which might account for the increased susceptibility to vaginal HSV-2 infection (138). Studies of IP susceptibility to HSV-2 during various stages of the estrous cycle would show if virus susceptibility varies with cycle related changes of NK cells.

Experimental inhibition and enhancement of macrophages has produced strong evidence for their involvement in HSV infection; however, as Lopez (99) has suggested, it is necessary to re-examine the experimental methods to exclude the possibility that NK cells might be causing the results which are attributed to macrophages. In mice, cyclic increases in endogenous estrogen during estrous correlate with increased macrophage activity and numbers (183). Thus, susceptibility of mice to an IP infection with HSV-2 may vary during the different phases of estrous.

Early production of IFN correlates with genetic resistance to HSV-1 (42), anti-mouse IFN increases susceptibility to IP HSV-2 infection (56) and IFN also acts indirectly by activating NK cells or macrophages (195).

Production and/or physiologic response to regulatory IFN may be the main mediator of the observed genetic spectrum of susceptibility-resistance.

Involvement of Cell-Mediated Immunity

It has been generally believed for a number of years that the formation of specific antiviral antibody is not important in promoting recovery from primary or recurrent HSV infection, and that the major host defense against this virus is some aspect of cell-mediated immunity (5,127,139,147,181). Behavior of T cells in acute experimental herpes simplex infections (reviews 133,134,135) has been explored extensively. Patients suffering from T cell deficiencies manifest severe recurrent disease; whereas, patients deficient in B cells are not more susceptible to HSV than normal individuals (113). Similar results have been obtained in experimental murine models. Congenitally athymic nude mice infected with HSV types 1 and 2 are prone to an inexorable infection whether inoculated intradermally, subcutaneously or intraocularly (81,114,124). The nude mouse infection may be controlled by transfer of syngeneic T cells seven days after HSV infection (81). The transfer of antibodies to infected nude mice restricted the spread of virus to and even within the CNS, but failed to clear local infection, whereas adoptive transfer of immune cells from draining lymph nodes conferred complete protection (81). Neonatally thymectomized mice behave in the same fashion

(120) as athymic nude mice. Other forms of immunosuppression such as x-irradiation and treatment with anti-thymocyte serum (ATS) (139) or cyclophosphamide (147) rendered mice highly susceptible to peripherally inoculated virus, and protection was conferred by the transfer of immune spleen cells. Depletion of T lymphocytes by adult thymectomy or lethal irradiation and bone marrow reconstitution produced a tenfold increase in susceptibility of mice to systemic or vaginal infection (111).

Overall, for most viruses that have been carefully investigated, at least in murine systems, viral specific cytotoxic T lymphocyte (CTL) responses have been demonstrated (1,22,195). Typically, such responses first become detectable three to four days postinfection, peak at six to eight days, and decline to background levels by about twenty-one days. Such responses can be characterized as T cell mediated by their viral specificity, major histocompatability locus (MHC) restriction, and alloantigenic markers expressed by the cytotoxic cell types. Specifically, the cells are thy 1^+ and Lyt 2^+ or Lyt 1^+2^+. It is important to establish this identification since along with the induction of CTL there may be an enhanced NK cell response as well as other forms of cytotoxicity such as antibody dependent cell cytotoxicity (ADCC) (13) and direct cytotoxicity of B cells (133). For reasons yet to be fully elucidated, mice do not generate a strong primary or secondary CTL response in vivo

to herpes virus antigens. Weak positive responses of spleen cells have been reported by some (93,169), but not all investigators (13). Armerding has failed to demonstrate splenic anti-HSV CTL induction and has emphasized NK cell responses in systemic infection with HSV-1 (13). Such NK cells lyse virus-infected cells much better than uninfected cells and yet are virus nonspecific.

In a further study of the effects of ATS, Schlabach et al. (163) found that macrophage functions are suppressed during the first few days after treatment. The relative importance of macrophages and lack of importance of T cells in the resistance of C58 mice (a high lymphatic leukemia strain) to primary systemic herpetic infection has been effectively established by this study. If mice were inoculated IP with HSV-1 during this early period, the mice appeared to be very susceptible to HSV-1; but, if inoculated after macrophage function returned to normal and when T cells were still suppressed, the treated mice were no more susceptible than controls. Resistance to HSV-1 was recovered within one week after ATS treatment, whether or not the mice were thymectomized.

McDermott et al. (108) have recently shown that resistance to HSV-2 vaginal infection can be transferred via genital lymph node (GLN) cells. Treatment of GLN cells with anti-Thy 1.2 serum and complement prior to immunization abrogates resistance transfer. Since immunity can be

transferred by the IV injection of single cell suspensions of GLN, these cells apparently have a homing affinity for genital mucosa.

In humans, circulating T cells have been shown to be affected by hormonal levels and are depressed during menstruation (150) and pregnancy. Interestingly, HSV-2 infections recur during menstruation (125) and increase in incidence during pregnancy (138,143). It is possible that GLN cells respond to hormonal fluctuation resulting in varying degrees of susceptibility to HSV-2 throughout the estrous cycle. This hypothesis can be tested in the mouse intravaginal HSV-2 model.

Effects of Humoral Immunity (HSV-1)

A substantial number of investigations have been made in the past concerning the role of antibodies in protection against HSV infection. Nearly all have been based on passive transfer experiments. Conflicting results obtained in various experimental systems led to continued confusion over the role of antibodies at the various stages of natural infection. In 1946, Evans et al. (43) showed that when antibody was given to mice soon after infection, access to the nervous system was reduced. More recent studies in a variety of experimental models confirm this observation (16,85,112,178). However, such has not been the finding in all systems (34,188). The presence of a high level of antibodies does not apply to latency which develops during

primary infection because virus reaches ganglia early (twenty-four to forty-eight hours postinfection) (34,85), long before a detectable antibody response can occur.

Neutralizing antibody is unlikely to moderate acute infection, although it might well be important in preventing recurrent infection and thus recrudescence. Supportive evidence comes from the use of mice treated since infancy with anti-IgM (53). Such mice failed to make anti-herpes antibody; but, provided that their delayed hypersensitivity was intact, they cleared virus as efficiently as did untreated mice (80). However, although the treated mice recovered from infection, the centripetal extension of virus and the establishment of ganglionic latency were more pronounced. The above account suggests that mice deprived of humoral immunity are not much disadvantaged with respect to acute herpes infections, a situation similar to that found in man (113).

More recent observations have suggested that antibody may play a role in recovery from HSV infection in a normal host. First, it has been demonstrated that the formation of antibody to HSV in mice is thymus dependent (5,29,192). Thus, any method that suppressed thymus-dependent cell-mediated immunity would also suppress the formation of thymus-dependent antibody. In addition, administration of anti-HSV antibody protected neonatal mice from fatal HSV

infection when administered several hours after virus (19,104).

Mice immunosuppressed by three different methods (x-irradiation, administration of cytoxan, or anti-thymocyte serum), were markedly more susceptible to primary systemic HSV infection (191). These immunosuppressed mice also did not form neutralizing antibody to HSV. Passive transfer of physiologic amounts of antibody as late as day six after infection exerted a protective effect in these immunosuppressed HSV-infected mice. Immune spleen cells were less effective when transferred after infection, and mice which received these cells appeared to make sufficient antibody to explain the protective effect of the transferred cells (191). Taken together, these results help to reopen the possibility that antibody may play one of the essential roles in recovery from primary HSV infection in the mouse. They also demonstrate that antibody can be effective in the treatment of HSV infection in the severely immunosuppressed mouse. Antibody alone is not sufficient to cause virus clearance in the absence of T cell mechanisms either in vitro (140) or in vivo (85), although clinical disease can be delayed (85).

Vaginal Responses to Hormonal Fluctuation

Cyclical Variation in Vaginal Epithelium

The estrous cycle of the mouse.--Stockard and Papanicolaou (174) and Dierks were the first to demonstrate

in the guinea pig and the human, respectively, that the vaginal epithelium undergoes cyclic morphologic changes that are correlated with phases of the ovarian cycle. Diagnosis of the phases can be ascertained by the microscopical examination of vaginal smears.

The epithelium of the vagina undergoes striking histological changes during the estrous cycle (170). During proestrous, the epithelium consists of three layers: (1) an outer layer of nucleated epithelial cells; four to five cell layers thick; (2) the stratum granulosum which, in estrus, becomes the stratum corneum and; (3) the stratum germinativum, some seven layers in thickness. During proestrous when plasma estrogen levels are low, the thin vaginal mucosa permits leukocytes to enter the vaginal lumen and the cells of the outer layer are delaminated into the vagina. A smear taken at this time contains many leukocytes and numerous small, rounded nucleated epithelial cells. Estrogen levels slowly rise, promoting cell proliferation: the stratum granulosum gradually thickens and the superficial layer becomes cornified. The vaginal mucosa is multilayered, and blocks leukocyte migration. Throughout estrus, cells are delaminated from the cornified layer resulting in a smear consisting predominantly of these cells. The onset of metestrous is characterized by the peeling off of the entire cornified layer. During late metestrous there is a rise in the nucleated cell count indicating that in the last stages

of the delamination process some of the superficial layers of the stratum germinativum becomes heavily infiltrated with leukocytes which also appear abundantly in the smear at this time. As a result of the delamination of the superficial layers, the vaginal epithelium at diestrous contains only one layer, the stratum germinativum, some three to seven cell layers in thickness. A smear taken at this time consists almost exclusively of leukocytes. Late in diestrous, active growth begins in the stratum germinativum, and by proestrous a stratum granulosum has formed several cell layers below the surface, thus completing the cycle.

Vaginal changes in human female.--In the mouse, cyclic histologic changes in the vagina during each phase are obvious, ranging from an almost completely desquamated epithelium in the proestrous phase to a thick cornified structure in the full estrus period. In the human being, however, sufficient estrogen is present at any phase of the normal menstrual cycle to maintain the basic integrity of the epithelial layers. The epithelium is a typical stratified squamous epithelium consisting of forty-five layers of cells during the follicular phase of the cycle and thirty in the luteal phase. The morphology and staining reaction of the vaginal cells are used to obtain a correlation with ovarian function (21).

During the early follicular phase, the vaginal smear shows large numbers of basophilic, squamous epithelial cells,

a moderate number of leukocytes and mucus. In the presence of high estrogen near ovulation, the nuclei may disappear entirely from cells which are acidophilic and cornified. Leukocytes and bacteria are reduced in number and mucus in the preovulatory phase becomes thinner and more watery. During the luteal phase, progesterone opposes estrogen and there is a sharp influx of leukocytes, mucus staining increases and basophilic cells predominate. If pregnancy does not occur, there is a sharp drop in the production of both estrogen and progesterone about four or five days before the next menses. Leukocytes and mucus are abundant and basophilic cells become mucified and folded.

Fluctuation in Vaginal pH

Vaginal pH varies with estrogenic function and marked changes in pH are observed in various stages of the ovulatory cycle. In the human neonate, Cruickshank and Sharman (35) found a pH range from 4.9 to 5.7 during the first nine days of life probably due to the high maternal estrogen. By three to six weeks of age, the pH is neutral and remains so until the onset of puberty when pH ranges between 4.0 and 5.0. During the menstrual cycle, there is a slight fluctuation in vaginal pH which is lowest (about 0.6 lower) at ovulation. Maximum acidity (pH 3.8 to 4.0) of vaginal secretions is during pregnancy when estrogen levels peak.

The presence of glycogen in human vaginal mucosa has been thought to cause the fluctuation in pH since the acidity is

due to racemic lactic acid. Lactic acid is the result of glycogenic fermentation and the glycogen content of the mucosa approximates the estrogen curve. However, in the absence of appreciable amounts of glycogen, the pH may be slightly acidic. In the rat, which has no perceptible glycogen in the vaginal mucosa, the pH undergoes cyclic fluctuations. The lowest pH is seen during estrus, 4.5, and the highest during diestrous, 7.0. If these data reflect that of the mouse, a greater susceptibility to intravaginal HSV-2 infection would be expected during diestrous due to preference of the virus for a neutral pH.

Response of Microbial Colonization to Hormonal Fluctuation

A study by Larsen et al. (92) has shown by plate counts performed on material lavaged daily from the vaginal tracts of several rats, that bacterial counts are elevated in proestrous and estrus phases of the cycle to values several orders of magnitude greater than those observed during metestrous or diestrous phases. Increases in vaginal bacterial counts were associated with the presence of predominantly nonviable cornified epithelial cells in the vagina. Greater than 90 percent were shown to be accessible and elutable by vaginal lavage when compared with electron micrographs of bacteria adhered to surface epithelium. Decreases in the vaginal bacterial content were related to the influx of leukocytes into the vagina after estrus. Ovariectomy results in low

vaginal bacterial counts (91) and estrogen replacement results in an increase in vaginal bacterial counts.

Changes in microbial flora have been studied during the different stages of life in the human female (26). Most relate to the changes associated with pH fluctuation. No literature was found on the fluctuation of numbers or types of vaginal microbes during phases of the menstrual cycle. It is likely that variation occurs during estrogen fluctuation.

Effect of Estrogen on the Immune Response

Variations in the Female Immune Response

There is an increasing amount of data suggesting that estrogens function as immunoregulators. Regulation by estrogens is suggested by studies contrasting male and female immune responses. Males generally produce fewer antibodies following antigenic stimulation compared to females (154,177), and in mice this deficiency can be negated by castration (41). In humans, serum IgM concentrations are consistently higher in females as opposed to males (154). Cell-mediated immunity, however, is relatively depressed in the female (74).

Variations in physiological levels of estrogenic hormones due to the ovulatory cycle, pregnancy and age also affect the immune system. Cyclic fluctuations in estrogen during the estrous cycle correlate with increased macrophage activity and number (183). Lymphocyte counts in women at

ovulation reach a minimum that coincides with the maximum level of estradiol in the blood (107). Pregnancy results in a depression of CMI (30,44) which correlates with increased serum estrogen levels (61).

Clinical observations have shown that therapeutic use of estrogens can alter immunocompetence. Women taking oral contraceptives demonstrate an enhanced antibody production (23) and depressed CMI (18). A depressed CMI also occurs in prostate cancer patients receiving estrogen therapy (2).

Fluctuation of NK Cell During Estrous Cycle and Pregnancy

Natural killer cells, considered to be involved in surveillance of tumors (66), defense mechanisms against viral infections (7), and control of hematopoietic stem cells (62), show different degrees of activity under various physiological conditions. In mice, for example, NK activity depends on age (70). Strain-related differences in mice (69) and diurnal or yearly rhythms in human NK activity have also been demonstrated (144).

Furukawa et al. (50) studied changes in NK activity of various tissues involved in immunity during the estrous cycle and pregnancy in C3H/He mice. Spleens and peripheral blood mononuclear cells (PBMC) are known to exhibit strong NK activity, while lymph nodes and other lymphoid tissues have little NK activity in male mice (75). These distributions were also observed during the estrous cycle and pregnancy.

Studies of splenic NK activity during the estrous cycle revealed relatively constant activity except for a rise during metestrous. The levels decreased considerably during early to mid-pregnancy, rose rapidly on day eighteen, and declined thereafter. Natural killer activity of PBMC demonstrated a similar but more marked fluctuation with its peak also at metestrous. Unlike the splenic NK activity, PBMC showed decreased NK cell levels throughout pregnancy, followed by a rapid increase on day one post-partum.

Previous data obtained in our laboratory demonstrated that human females exhibit greater fluctuation in their natural killer cell activity as compared to males. Activity was found to correlate with serum estrogen levels (64).

Immunoglobulins (Ig) Present in Vagina and Uterus

Female rats.--Studies by Wira (189) have shown that mean Ig levels in rat uterine and vaginal flushings are affected by the estrous cycle. In uterine flushings, maximal values of both IgA and IgG were measured at proestrous. IgG then dropped to just detectable levels at estrus and remained low at diestrous. IgA, however, remained somewhat elevated at estrus and then declined to its lowest point at diestrous. Rats ovariectomized two to three weeks prior to assay also had very low levels of both Ig's. However, serum Ig levels did not change significantly during the estrous cycle or following ovariectomy.

To determine whether similar changes occur in the vagina, Ig levels were also measured in vaginal flushings. The levels of both IgA and IgG were highest at diestrous and lowest at proestrous. These observations suggest that IgA and IgG levels are inversely related to those of blood estradiol. Also that Ig's in the uterus and vagina vary independently of each other.

Changes in Ig levels appear to respond to level of blood estradiol, which is known to increase at proestrous (136,162). Estradiol, when given to ovariectomized rats, both stimulates IgA and IgG in the uterus and inhibits them in the vagina. When vaginal Ig levels are compared with bacterial studies of Larsen et al. (91), Ig's are lowest at proestrous when the number of bacteria in the vagina is highest.

The movement of IgA positive lymphocytes (IgA cells) to the uterus is regulated by estradiol (189). Estradiol has a marked effect since: (1) IgA cells are not present in the uteri of ovariectomized rats; and (2) IgA cells accumulate in both the endometrium and myometrium in response to estradiol administration.

The increase in antibody titers is the result of a direct action by estradiol on B cells which increase synthesis of IgM (123). Estradiol has also been found to increase serum IgM production in male rats (123).

Effects of Estradiol on Cellular Responses in Mice

Females appear to have a less responsive cellular immune system (74,79,160) than males. Decreased cellular immune responses may be partly due to estradiol (74,160), which prolongs graft survival time (184), reduces delayed type hypersensitivity responses (186), causes thymic atrophy (36), and decreases number and function of thymocytes (3).

In a study by Krzych et al. (90), the mitogenic response of spleen cells from young adult female mice were higher than those of males in all comparative tests. Newborn mice did not demonstrate sex-associated differences; and among the weanling mice, slight differences between male and female spleen cells responsiveness were observed. The blastogenic responsiveness of spleen cells from mature female BALB/c mice was greater at proestrous and metestrous, as compared to estrus and diestrous. The peaks of responsiveness correspond to reported elevated levels of estrogen and pregnenolone during these phases of the cycle (193). These results demonstrate a modulation of both splenic T and B cells by endogenous sex hormones. In vivo plaque-forming responses to antigens of sheep red blood cells (SRBC) showed a similar cyclic pattern. Lymphocytes from male controls did not display any cyclicity for either mitogenic or plaque-forming responses; responses of male spleen cells were similar to those of females in estrus and diestrous.

Susceptibility to HSV-2 in Human Female Neonates

Nahmias et al. (132) have reported that HSV-2 infection of newborns presents a wide spectrum of clinical conditions, from severe generalized disease to subclinical infection. The occurrence of severe HSV-2 infections in premature infants supports the concept that host immune deficiency or immaturity results in lack of resistance to HSV-2 (32). Wolontis and Jeansson (190) reported that of five newborn patients, the two surviving infants were females; whereas, the fatal cases occurred among males. In their survey of the literature, fifteen of twenty-three boys and one of nine girls had fatal HSV-2 infection. Although these data cannot give proof of a sex difference in mortality rate, Wolontis and Jeansson suggested that sex-related differences in herpes susceptibility and prognosis may be a profitable field for future study.

CHAPTER II

MATERIALS AND METHODS

Animal Studies

Animals

Female ICR mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) at four to five weeks of age. Female CBA/NJ mice were obtained from Jackson Laboratories (Bar Harbor, ME) at four to six weeks of age. The mice were housed in a biocontainment facility on a twelve hour light/dark cycle and given food and water ad libitum. All animals were rested for one week after arrival in the facility before being utilized in experiments.

Staging of Estrous

Stages of the estrous cycle were determined by microscopically observing vaginal smears. In this method, the mouse vagina was flushed with a few drops of normal saline (0.9 percent) using a Pastuer pipette. The retracted fluid was transferred to a slide and viewed as a wet mount. The smear was examined for the presence of leukocytes, cornified and noncornified epithelial cells (170). Proestrous consists of epithelial cells and leukocytes in approximately equal number, estrus of predominantly

epithelial cells, metestrous of cornified epithelial cells and leukocytes in approximately equal numbers and diestrous of predominately leukocytes.

Establishment of Estrogen Deficient Ovariectomized Mice

Mice were anesthetized by exposure to Metofane (Methoxyflurane) in a chamber, placed ventral side down and metofane exposure continued by using a nose cone containing Metofane and cotton. The skin was retracted using forceps and a one-half inch cut was made perpendicular to the midline. A small incision was made in the abdominal wall above each ovary. The ovary and associated fat were pulled through the incision and the ovary with part of the oviduct was then excised. The remaining tissue was then teased back into the abdominal cavity of the animal and the incision sutured with sterile, nonabsorbable, silk surgical suture (Ethicon). The skin was pulled together and fastened using wound clips. The animals were allowed at least one week recovery time before being utilized in experiments.

In Vitro Virologic Studies

Medium for Growth of Cells and Virus Propagation

The medium used for growth of cells and virus propagation was Eagle's minimum essential medium (MEM) containing Hank's balanced salt, 10 percent fetal bovine

serum (FBS), 1 mM n-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid (HEPES) (Sigma), 50 µg/mL gentamicin (Sigma), 100 units/mL penicillin (Whittaker), 100 µg/mL streptomycin (Whittaker) and sufficient sodium hydroxide (NaOH) (Baker Chemical Company) and/or sodium bicarbonate ($NaHCO_3$) (Irvine Scientific) to adjust the pH to 7.2.

Medium for Virus Titration

The media used for viral titration was MEM, 2 percent FBS, 1 mM HEPES, 50 µg/mL gentamycin, 100 units/mL penicillin, 100 µg/mL streptomycin and sufficient NaOH and/or $NaHCO_3$ to adjust the pH to 7.5. This medium was also used as diluent for in vivo inoculation.

Cell Culture

Baby Hamster Kidney-21 (BHK) cells were grown in MEM plus 10 percent FBS and passaged twice weekly. Low passages were stored in liquid nitrogen and occasionally new starts were initiated in order to keep the passage level low.

Virus Propagation

Twelve 150 cm^2 flasks of BHK cells (passage 63) at a concentration of approximately 3.3×10^5 cells per flask were inoculated with 2 mL of 10^{-3} dilution of stock herpes simplex type 2 (strain 186) (Donald Bone, Celtek) of unknown titer. To facilitate virus adsorbtion, flasks were rocked at fifteen minute intervals during one and one-quarter hours at 37C.

After adsorbtion, 30 mL of sorbitol medium (MEM plus 10 percent FBS plus 1 percent sorbitol) was added to each flask. The flasks were incubated until the cell sheet exhibited 75-100 percent virus-induced cytopathic effect (CPE), which was three days. The cells were removed from the flasks by scraping with a rubber policeman. Cells and medium were placed in 50 mL centrifuge tubes and centrifuged (2200 RPM at 4C for 15 minutes without brake). The supernatant was then poured into a new sterile centrifuge tube. Cells were resuspended in 10 percent of the original volume of medium.

Cells were disrupted and virus released by homogenation in a homogenizer (Vernitron Medical Products, Inc.) while being kept cold. The cell debris and supernatant were transferred to 50 mL centrifuge tubes and centrifuged at 2200 RPM at 4C for 15 minutes. The supernatant was dispensed into ampules in 0.6 mL aliquots which were flame sealed and stored at -70C.

The original stock virus was depleted in studies with ICR mice, therefore, it was necessary to prepare a new pool for use in CBA/NJ mice. The new pool was prepared in approximately the same manner as the original pool. Three mL of $10^{-3.5}$ dilution of stock virus was inoculated into each flask of BHK cells. Only two days were required for the CPE to reach the desired degree.

In Vitro Viral Titration

The potency of virus pools was determined by titration of infection in indicator cells grown in microtiter plates. BHK cells at a concentration of 0.6×10^5 cells/mL were seeded in 0.2 mL volumes into each well of a 96 well microtiter plate, covered, wrapped in Saran Wrap and incubated for 18-24 hours before use. Medium was decanted and 0.2 mL of ten-fold virus dilutions in MEM with 2 percent FBS was added (four wells per dilution). Medium without virus was placed in six wells and served as an uninfected cell control. The plate was covered, wrapped and incubated for three days at 37C. Virus induced cytopathic effect (CPE) was then observed microscopically and recorded. The titer in 50 percent tissue culture infectious doses ($TCID_{50}$) was calculated by the techniques of Reed and Muench (152). The titer of the original virus pool was $10^{7.4}$ $TCID_{50}$/mL and the new pool $10^{6.7}$ $TCID_{50}$/mL.

In Vivo Virological Studies

Intravaginal Inoculation

Three half log virus dilutions were instilled intravaginally in a volume of approximately 0.0125 mL by an automated syringe equipped with an oral feeding needle. Animals were observed daily for deaths. After twenty-one days, any surviving animals were sacrificed.

Intraperitoneal Inoculation

Mice were inoculated intraperitoneally with 0.2 mL virus using a tuberculin syringe and a twenty-five gauge needle. Animals were observed daily for deaths. After twenty-one days any surviving animals were sacrificed.

Immunologic Studies

Detection of Natural Killer (NK) Cell Activity

Medium for natural killer assay.--RPMI 1640 (Flow Laboratories) with 10 percent FBS (Hyclone defined), 10 units per mL penicillin, 10 µg/mL streptomycin and 0.25 M HEPES (Flow Laboratories) was used for NK assays and designated complete medium. FBS was omitted from the medium for preparation of effector cells until the last wash. Medium was prepared by dissolving 10.4 grams powdered RPMI 1640 with L-glutamine and without sodium bicarbonate in approximately 900 mL distilled water. Two grams of powdered sodium bicarbonate ($NaHCO_3$) were added separately with additional water to bring medium to final volume. The pH was adjusted to 7.15 with hydrochloric acid. The medium was filter sterilized through a 0.2 µm membrane using positive pressure. Sterile heat-inactivated fetal bovine serum, penicillin, streptomycin and HEPES were added after filtration. Medium was stored at 4C.

Preparation of effector (NK) cells.--Spleens were removed from CBA/NJ mice after cervical dislocation and placed on ice in a petri dish with a small amount (5 mL) of cold RPMI 1640 without serum. Spleens were mashed with the plunger end of a 5 mL syringe and aspirated through a twenty-one gauge needle to disperse the cells. The spleen cells were transferred to a centrifuge tube via the syringe, thus eliminating many of the large particles. The petri dish was rinsed with ten additional mL of cold RPMI without serum and the transfer of cells to the centrifuge tube repeated. To minimize the presence of large pieces of tissue, the spleen debris/cell suspension was allowed to sit five minutes and the supernatant decanted. Cells were centrifuged (400 g for 10 min), supernatant decanted and cells resuspended in the remaining liquid. One mL of water was added and mixed with the cells for no longer than twenty seconds in order to lyse the red blood cells. The tube was then filled completely with cold RPMI with serum and centrifuged for another ten minutes. The supernatant was decanted and the cells resuspended in one mL in complete RPMI (with serum). The cells were counted using trypan blue dye exclusion and the concentration adjusted to 2×10^7 cells/mL.

Natural killer (NK) assay procedures.--A ^{51}CR release assay was used to measure NK activity. YAC-1 cells derived from a Moloney virus-induced lymphoma in A/Sn mice were used

as target cells. Target cells at a concentration of 1×10^4 cells/mL were labeled for 1 hour with Na_2CrO_4 (ICN Biomedicals). The targets were incubated for 4 hours at 37C under 5 percent CO_2 with varying concentrations of effector cells (200:1, 100:1, 50:1, 25:1, 12.5:1 and 6.25:1) in 96-well round-bottom microtiter plates. Total chromium was established by incubating target cells with 10 percent Triton-X, spontaneous release by incubation of targets with unlabeled target cells (autologous cells) and specific lysis by incubation of targets with effector cells. Each of the ratios, total ^{51}Cr, total lysis and spontaneous release was performed in triplicate. After incubation the plate was centrifuged 10 minutes (400 g) and 100 µL aliquots of the supernatant were removed for counting in the gamma counter (Auto-gamma 5210, Packard). Prior to removal of supernatant in the total chromium wells, the cells were resuspended; this was not the case for the rest of the wells. Using these data, the percent specific ^{51}Cr release was calculated with this equation:

$$\frac{\text{(mean cpm effector wells)} - \text{(mean cpm autologous wells)}}{\text{(mean cpm total lysis wells)} - \text{(mean cpm autologous wells)}} \times 100\%$$

Percent lysis was calculated for each effector:target ratio of each donor.

Calculation of lytic units.--`Lytic units (LU) afford a method for reporting results of radioactive chromium release

assays that includes a complete set of data (all effector to target ratios tested) for an individual effector cell source. The lytic unit is generally defined as the number of effector cells required to produce lysis of a selected percentage of target cells. Lytic units are normalized as the number of lytic units in a designated number of effector cells, thus presenting lytic unit values that increase with increasing lytic activity. Several methods for calculating lytic units have been reported. Our LYTIC computer program (63) uses a method for determining lytic units that is similar to the exponential fit method of Pross et al. (145). In this procedure, LU represents the number of lytic units in 1×10^7 effector cells at 15 percent specific lysis with 1×10^4 target cells per well.

Detection of Peritoneal Macrophages

Retrieval of peritoneal cells.--One 3 mL syringe containing 2 mL cold tissue culture medium with heparin (5 units/mL) was placed on ice for each mouse to be tested. Mice were sacrificed individually by cervical dislocation, and the abdomen washed with 70 percent ethanol. The abdominal skin was retracted with forceps and a 5 mm cut made perpendicular to the medline. A twenty-three gauge needle attached to a 3 mL syringe was carefully inserted through the abdominal wall along the midline so that the bevel was away

from the muscle layer. The medium was then injected into the peritoneum. The needle was removed from the abdomen and the abdomen gently agitated for about thirty seconds. The needle was carefully reinserted through the abdominal wall along the lateral or flank region so that the bevel was away from the muscle layer. One to one-half mL of the medium was aspirated and the needle withdrawn. If the fluid was bloody or cloudy, it was discarded. The needle was then removed from the syringe and the washings placed in a sterile 50 mL centrifuge tube which was kept on ice. This procedure was repeated for each mouse to be tested. Cells were washed once and resuspended in medium with serum to a concentration of 2×10^6 cells/mL. A small amount of the cells were then transferred to a slide and allowed to air dry and stained to determine the percent of macrophages present in the peritoneal cavity.

Staining for macrophages.--Macrophages were stained using the reagents described below, following the method of Koski and colleagues (89). Smears were prepared in serum for preservation of morphology. The cells were dried for thirty seconds at 4C then rinsed with three changes of distilled water and allowed to air dry. Meanwhile, 0.1 gram $NaNO_2$ was dissolved in 2.5 mL of water. One fifth mL of this solution was then added to 0.2 mL pararosaniline stock to hexazotize for one minute. The following compounds were mixed together

in sequence and filtered before use: 47.5 mL of 0.15 M phosphate buffer, 0.25 mL of hexazotized pararosaniline and 2.5 mL of alpha-naphthyl butyrate substrate. The slides were then incubated in this solution for 45 minutes at room temperature. After this incubation period, the slides were rinsed in distilled water and counterstained with 0.5 percent methyl green for 1-2 minutes. The slides were then rinsed well, allowed to air dry and mounted. Two hundred cells were counted for each smear and the percent macrophages was calculated.

Reagents for macrophage staining.--Fixative (pH 6.6) contains 0.0074 M KH_2PO_4, 0.00075 M Na_2HPO_4, 45 percent acetone, and 7.5 percent formaldehyde in aqueous solution, stored at 4C and reused two to three times.

Pararosaniline stock is 4 percent pararosaniline hydrochloride in 2N hydrochloric acid (warm), filtered and stored at 4C.

Alpha-naphthyl butyrate substrate is 2 percent alpha-naphthyl butyrate in ethylene glycol monoethyl ether, mixed in a glass bottle using glass pipettes, stored below zero centigrade (will not freeze) and protected from light.

CHAPTER III

RESULTS

Effect of Estrous Stage on Susceptibility to HSV-2 Infection in Mice

Vaginal Inoculation of HSV-2

ICR mice.--Ninety-four, five-week-old female ICR mice in two experiments were identified for stages of estrous by vaginal smears and inoculated intravaginally with $10^{4.5}$, $10^{4.0}$, or $10^{3.5}$ $TCID_{50}$ HSV-2 strain 186 per mouse. Eight mice were used for each estrous stage and virus concentration except for metestrous which had only six mice available at the $10^{3.5}$ $TCID_{50}$ concentration.

Deaths were the most frequent (75 percent) in mice which were in diestrous at the time of virus inoculation (Table I). The lowest number of deaths was seen in the group which was in metestrous. As illustrated in Figure 1, leukocytes are the predominate cell type in diestrous, with the other phases having epithelial cells only or in addition to leukocytes.

Table II illustrates infection of older ICR mice and CBA/NJ inbred mice. To study the possible effects of age on susceptibility, ten-week-old ICR mice were inoculated intravaginally with HSV-2 in an identical manner. Only those

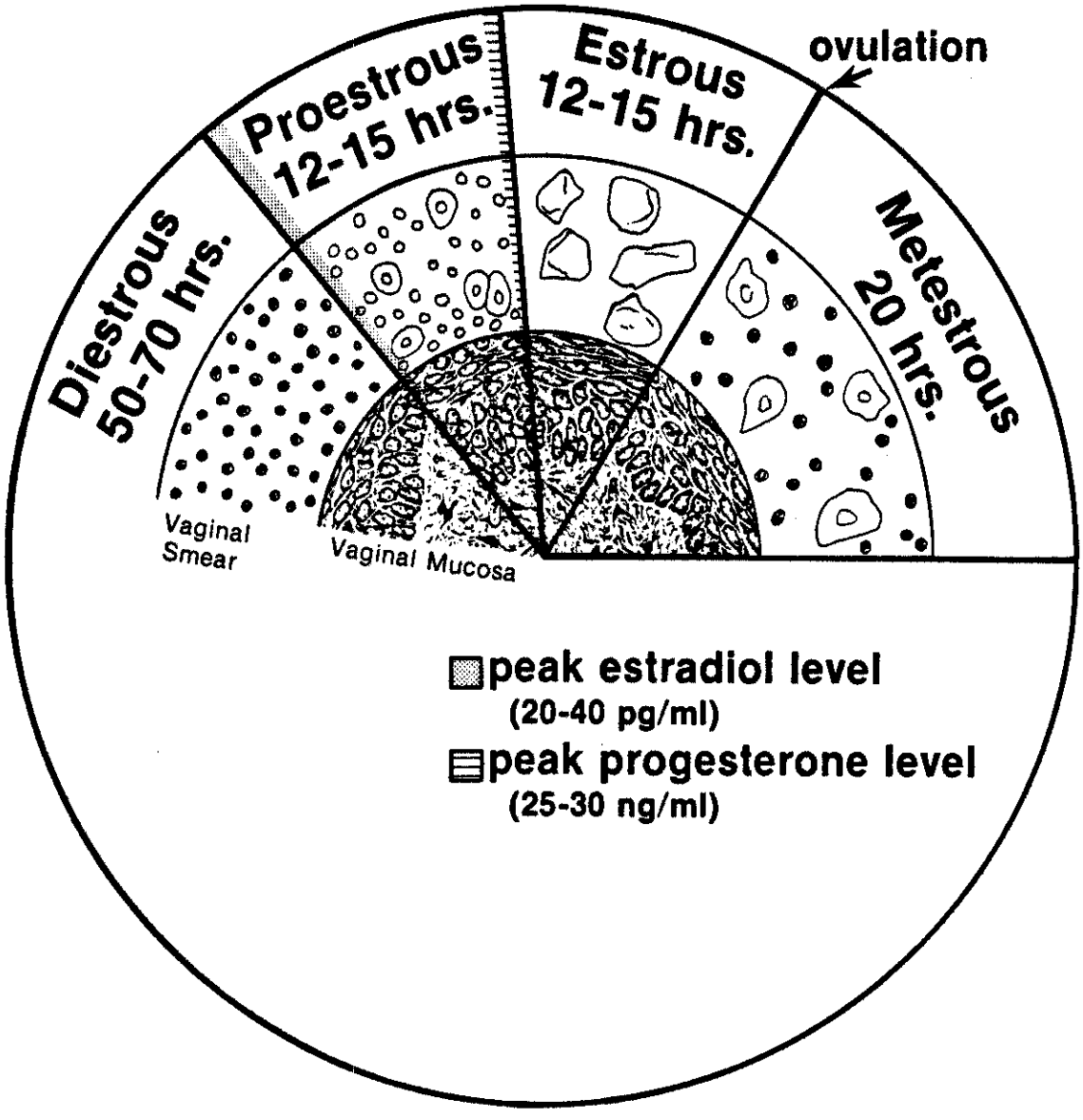

Figure 1. Murine estrous cycle (4-5 days)

mice in diestrous at the time of inoculation were susceptible to virus infection (Table II).

TABLE I

EFFECT OF ESTROUS CYCLE ON SUSCEPTIBILITY OF ICR MICE TO HERPES SIMPLEX VIRUS TYPE 2 INOCULATED INTRAVAGINALLY

	Estrous Stages			
Dosage	Proestrous d/t*	Estrus d/t	Metestrous d/t	Diestrous d/t
$10^{4.5}$ $TCID_{50}$	2/8	3/8	1/8	6/8
$10^{4.0}$ $TCID_{50}$	4/8	1/8	0/8	6/8
$10^{3.5}$ $TCID_{50}$	2/8	0/8	1/6	6/8
Total	8/24	4/24	2/22	18/24
Percent	33%	16%	9%	75%

*deaths/total inoculated.

CBA/NJ mice.--Female CBA/NJ mice were inoculated intravaginally using the same procedures as ICR mice. The new pool of virus, which has a titer of $10^{6.7}$/mL $TCID_{50}$, was used for CBA/NJ mice. This pool was also titered in ICR mice to determine its relative virulence to the original virus stock. No deaths were observed in CBA/NJ mice inoculated with virus concentrations between $10^{3.8}$ and $10^{0.8}$ $TCID_{50}$ per mouse. Therefore, an additional experiment was performed in twenty mice using undilute virus ($10^{4.8}$ $TCID_{50}$ per mouse).

At this virus dose, mice in diestrous were the most susceptible to infection (Table II).

TABLE II

EFFECT OF ESTROUS CYCLE ON SUSCEPTIBILITY OF FEMALE MICE (ICR AND CBA/NJ) TO INTRAVAGINAL HSV-2 INFECTION

Mice	Estrous Stages							
	Proestrous		Estrus		Metestrous		Diestrous	
	d/t*	%dead	d/t	%dead	d/t	%dead	d/t	%dead
5 wk ICR**	8/24	33	4/24	16	2/22	9	18/24	75
10 wk ICR**	0/2	0	0/3	0	0/2	0	9/11	82
7-8 wk CBA/NJ***	0/5	0	0/3	0	1/8	12.5	4/4	100

*deaths/total inoculated.
**$10^{4.5}$, $10^{4.0}$, or $10^{3.5}$ $TCID_{50}$ per mouse.
***$10^{4.8}$ $TCID_{50}$ per mouse.

Intraperitoneal Inoculation of HSV-2

ICR mice.--Ninety-three, six-week-old female ICR mice were inoculated IP with HSV-2 strain 186 using between $10^{5.7}$ and $10.^{1.7}$ $TCID_{50}$ serial log dilutions per mouse. No appreciable difference in death rates was observed between the groups at different estrous stages (Table III). The mice were found to be highly susceptible to all doses of virus during all phases of the estrous cycle.

TABLE III

EFFECT OF ESTROUS CYCLE ON SUSCEPTIBILITY OF FEMALE MICE (ICR AND CBA/NJ) TO INTRAPERITONEAL HSV-2 INFECTION

Mice	Estrous Stages							
	Proestrous		Estrus		Metestrous		Diestrous	
	d/t*	%dead	d/t	%dead	d/t	%dead	d/t	%dead
5-6 wk ICR**	18/24	75	20/23	87	20/22	91	19/24	79
10 wk CBA/NJ***	4/4	100	4/4	100	4/4	100	4/4	100

*deaths/total inoculated.

**$10^{5.7}$-$10^{1.7}$, $TCID_{50}$ serial log dilutions per mouse.

***10^{6} $TCID_{50}$ per mouse.

CBA/NJ mice.--Sixteen, ten-week-old female CBA/NJ mice were inoculated IP with $10^{4.8}$ $TCID_{50}$ per mouse HSV-2 strain 186 ($10^{6.7}$ $TCID_{50}$/mL). All animals at each phase of estrous died (Table III).

Ovariectomized Mice

To confirm the susceptibility of animals in diestrous, thirty-seven fourteen-week-old ICR mice were ovariectomized in order to simulate diestrous. The mice were inoculated intravaginally with $10^{4.5}$, $10^{4.0}$, or $10^{3.5}$ $TCID_{50}$ concentrations of HSV-2 strain 186 ($10^{7.4}$/mL $TCID_{50}$).

As shown in Table IV, this procedure resulted in death of 95 percent of the animals.

TABLE IV

COMPARISON OF SUSCEPTIBILITY OF OVARIECTOMIZED AND OVULATING MICE TO INTRAVAGINAL HSV-2 INFECTION

	Percent Deaths		
Mice	Average all stages	Diestrous	Ovariectomized (pseudodiestrous)
5 wk ICR*	34	75	--
10 wk ICR*	50	82	--
7-8 wk CBA/NJ**	25	100	--
14 wk ICR ovariectomized*	--	--	95

*$10^{4.5}$, $10^{4.0}$, $10^{3.5}$ $TCID_{50}$ per mouse.
**$10^{4.8}$ $TCID_{50}$ per mouse.

Fluctuation of NK Cell Activity During Estrous Cycle

Splenic NK cell activity was measured in twenty-seven naturally cycling five to eight-week-old CBA/NJ female mice which were identified for estrous phases by examination of vaginal smears. Natural killer cell activity was measured using the standard four hour ^{51}Cr release assay. The percent specific lysis and lytic units were calculated as previously described. Percent lysis at effector:target ratio 100:1

(Figure 2) peaks at metestrous, the stage where the greatest range of activity was seen. These data were also calculated in lytic units and the same peak in activity observed. Lytic units were zero for proestrous, estrus 14, metestrous 36, and diestrous 19. Our data for CBA/NJ mice corresponds with the data obtained by Furukawa et al. (50) in C3H/He mice.

Peritoneal Macrophages and the Estrous Cycle

Preliminary studies on the numbers of peritoneal macrophages during each estrous phase were performed with ICR mice. No appreciable difference was observed in numbers of peritoneal macrophages present during the stages of estrous. During proestrous the percent macrophages observed was 73 percent, in estrus 80 percent, in metestrous 86 percent and in diestrous 80 percent. The small increase in peritoneal macrophages during metestrous parallels our findings concerning NK cells; however, only a limited number of mice were studied (2 to 3 per stage). Since an outbred strain was examined, we would have expected greater variation simply due to genetic heterogenicity. Future studies should include inbred animals as well as measuring differences in activated macrophages during each estrous stage.

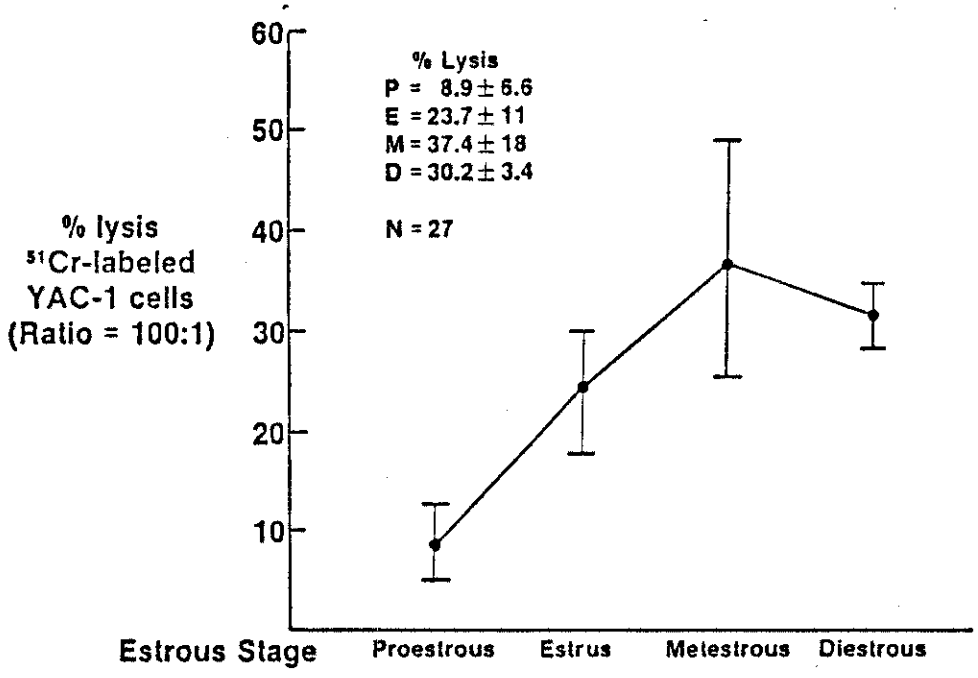

Figure 2. Variation in Percent Lysis of ^{51}Cr Labeled YAC-1 Targets By Natural Killer Cells During Estrous Stages of CBA/N Mice

CHAPTER IV

DISCUSSION

This study shows variations among cycling female mice in susceptibility to herpes. In this case, each phase of the estrous cycle representing a distinct variation in sex hormones was examined for effect on susceptibility to vaginal and IP HSV-2 infection. A significant difference in susceptibility to vaginal, but not IP, inoculation of HSV-2 was observed.

Reports implicate macrophages (9,11), NK cells (9,11) and T cells (12) in resistance to systemic (IP) HSV-2 infection. Each of these cell types has been shown to be affected by changing hormonal levels (18,50,183). Consequently, an effect of estrous stage on susceptibility to IP HSV-2 was suspected. No evidence was obtained in our study to substantiate this hypothesis (Table III); however, the virus concentration used may have been too high (although the lowest dose was $10^{1.7}$ $TCID_{50}$/mouse) and might have masked any effect of changing hormonal levels during estrous stages. All mice were found to be susceptible to the doses used. It may also be that even though the immune factors present in the peritoneum are affected by estrogen, the effects could be subtle and not sufficient to override virulence of HSV-2 in

murine IP infection. The pH of the peritoneum is physiological (7.4) and would provide an ideal environment for growth of herpes virus by aiding in rapid replication of virus and overwhelming the immune system. The IP route of infection is not usually a natural portal of entry for pathogens. Hence, immune defenses may not be as prepared for direct invasion as would the vaginal mucosa, which is often subjected to invasion by pathogens from the external environment.

In intravaginal studies, mice inoculated during diestrous were found to be the most susceptible to HSV-2, while those inoculated during metestrous were the least susceptible (Table I). Further supporting this observation, ovariectomized mice whose vaginal epithelium and mucosa resembles that of diestrous mice, were found to be highly susceptible to intravaginal infection (Table IV).

Although much work has been done regarding systemic HSV-2 infection, little is known concerning the induction and expression of immunity of the genital mucosa. In studies to be discussed later in this chapter, cytotoxic T lymphocytes (CTL) emerge as the main factor in HSV-2 vaginal resistance. The possible involvement of CTL in vaginal HSV-2 resistance does not exclude other physiologic protection factors. In previous studies, mice were not distinguished by stage of estrous and in many cases were swabbed with NaOH

pre-inoculation in order to produce a more consistant infection. It is possible that the epithelium, pH, and mucus are involved as natural resistance factors and are bypassed by the abrasive effects of the swabbing technique.

Those stages we found to be less susceptible (proestrous, estrus, metestrous) all had epithelial cells present in the lumen. Virus could attach and then be sloughed or expelled from the vagina prior to active viral proliferation and stimulation of immune cells present in the genital lymph node cells (GLN). In diestrous, the vaginal mucosa is thinner and no epithelial cells are present in the lumen; consequently, virus may penetrate the outer epithelial layer, overwhelm the cells present in the GLN and more easily initiate a fatal infection.

Vaginal pH may also influence the susceptibility of mice to intravaginal inoculation with HSV-2. Although there are no mouse pH studies, in the rat vaginal pH is lowest during estrus, pH 4.5, and highest during diestrous, pH 7.0 (20). If these data reflect that of the mouse, one would expect greater susceptibility to occur during diestrous since this pH is favorable for the growth of HSV-2. Our results substantiate this theory as 75 percent of the mice in diestrous died as compared to only 16 percent in estrus. Studies by Allen (4) further implicate involvement of pH in susceptibility of mice to vaginal infection. In comparison

tests of swabbing agents, dimethylsulfoxide (DMSO) (pH of 6.5) and DMSO with acetic acid (pH 5), mortality was 87 percent and 33 percent respectively. Vaginal pH appears not to be the only factor involved, since in our study metestrous shows the lowest percent death, 9 percent.

Mucus may play a role in susceptibility to HSV-2 vaginal infection. Mucus was found to be present in the greatest quantity during diestrous, which is the most susceptible stage. This substance has a sticky quality to it which could result in prolonged contact of the viral suspension with the thin vaginal mucosa thereby facilitating infection.

The role of natural resistance factors, which appear with sexual maturity, is further substantiated by the reported increased resistance with age. McDermott et al. (109) have reported resistance of BALB/CJ mice increases exponentially from four to ten weeks of age and becomes constant in older mice. This is in agreement with the observations of Allen who found young mice to be more easily infected; while a great degree of variation was seen in older, sexually mature mice if they were not first swabbed with NaOH (4). From a physiological standpoint, natural as well as immune resistance would be expected to be greatest near estrus. This is when mating occurs and the likelihood of invading pathogens being introduced to the vaginal mucosa is greatest.

To examine the role of these natural resistance factors in resistance, an experiment could be performed in which the mice are inoculated in each phase of estrous and subsequently swabbed to record virus titer. If the virus is being lost from the vagina by adherence to epithelial cells prior to penetration of the epithelium and interaction with the immune system, low amounts of virus would be detected in proestrous, estrus, and metestrous. During diestrous, virus titer would approximate the inoculum amount, provided virus is not yet fully adsorbed by vaginal epithelium.

Hormonal levels have previously been shown to be involved in susceptibility to HSV-2. For example, Baker and Plotkin (15) have shown that the enhanced susceptibility to intravaginal HSV-2 during pregnancy can be mimiced by the administration of progesterone to nonpregnant female mice. The depressed cell mediated immunity (CMI) observed in pregnancy (146) may be due to progesterone (121) and could result in decreased resistance to HSV-2. However, the authors did not exclude the possibility that effects on the vaginal wall play a role in enhanced susceptibility.

Other genital diseases have also been shown to be affected by hormonal levels, although the major effect is on the pathogen rather than the host. In studies of *Neisseria gonorrhoeae* (48) and *Coccidioides immitis* (40), growth of the organisms is affected by direct action of gonadal hormones.

Kinsman and Collard (86) have shown *Candida albicans* to be altered in ability to vaginally infect rats during different phases of the estrous cycle. Both cellular composition of the vaginal wall and direct action of hormones on *C. albicans* appear to be involved.

It will be necessary to further examine the role of natural resistance factors during the estrous cycle. One could thymectomize adult animals and inoculate HSV-2 during the different stages of estrous without swabbing (provided thymectomy does not alter genital mucosa). The same pattern of resistance as our results would suggest that the physiology of the vagina is protective. Morahan and McCord (119) showed that depletion of T cells by adult thymectomy, lethal irradiation, and bone-marow reconstitution produced a ten fold increase in susceptibility of mice to both systemic or vaginal HSV-2 infection. Morahan's work would seem to rule out natural immunity factors, however, her results might also derive from virus concentration too high to observe subtle estrous cycle controls of natural immunity barriers.

Regarding genital mucosal immunity, Morahan (34,110,118) also showed anti-HSV-2 CMI responses are present during acute vaginal infection, including delayed type hypersensitivity, proliferative CMI, and spleen cells capable of transferring vaginal resistance to recipient mice. These wane by three to four weeks. There is essentially no serum antibody response

during acute vaginal infection or in surviving mice. Surviving mice are generally resistant to intravaginal but not systemic challenge with HSV-2. Since spleen cells of mice surviving intravaginal infection may transfer resistance to intravaginal infection, but not protect against systemic challenge, sub-lethal intravaginal infection must produce local resistance factors, which do not recirculate to the peritoneum or spleen. These results present an enigma. Why can spleen cells of mice surviving vaginal infection confer resistance to challenge vaginal infection, but not to IP challenge infection? This may be due to splenic T cells which Schlabach et al. (163) showed were not involved in IP infections, but which appear to be involved in intravaginal infections (108).

Schneweiss and Saftig (164) evaluated inbred strains of mice for resistance to vaginal infections with HSV-1. The degree of strain resistance was similar to the IP inoculation data of Lopez (95) and could be diminished by x-irradiation, cyclophosphamide and ^{89}Sr, agents which destroy bone marrow precursors of macrophages, T and B lymphocytes and the latter also obliterates NK cells. On the other hand, McGeorge and Morahan (113) have shown that IP pretreatment of mice with silica, trypan blue, or dextran sulfate (macrophqage inhibitors) had no effect on HSV-2 vaginal infection. These experiments taken together would seem to exclude macrophages

from development of resistance to vaginal infection with HSV-2, but not clarify whether local T cells or splenic T cells or both are required for local resistance. Ludmila et al. (101) have shown that mice immunized IP with an avirulent strain of HSV-2 demonstrate resistance to vaginal infection with a virulent strain of HSV-2. The survival rate was greatly increased; however, latency was also increased. This experiment is another example that local resistance can be produced by IP inoculation, although it does not differentiate whether the effect is mediated by spleen or mucosal lymphocytes or both.

In further development of the hypothesis that T system immunity accounts for resistance to vaginal infection, McDermott et al. (108) have shown resistance to vaginal infection is mediated by GLN cells. The exact identity of the immune effector cells is not yet known, but HSV-2 specific CTL (94) are known to be present in the GLN after intravaginal immunization. Vaginal immunity can be transferred to normal mice by IV inoculation of HSV-2 stimulated GLN. Injection of serum, splenocytes, mesenteric lymph node cells or peripheral lymph node cells from immune donors did not confer resistance to vaginal inoculation. These results conflict with Morahan's evidence that spleen cells can transfer vaginal resistance. The fact that HSV-2 was eliminated from genital mucosa following IV transfer of

GLN cells suggests these cells have a homing affinity for the genital mucosa. Experiments in progress indicate that injection of GLN cells results in their colonization within the genital mucosa (108).

Exposure of the genital mucosa to HSV-2 (129) results in an antigen-specific antibody response, particularly secretory IgA, in cervicovaginal secretions but not necessarily in the circulation. This correlates with an increase in the numbers of IgA and IgG containing plasmacytes in the cervicovaginal mucosa (33).

Immunoglobulins may play a role in the pattern of resistance we have observed during the estrous cycle. IgA and IgG are at their lowest levels in the uterus during diestrous, which corresponds to the phase most susceptible to intravaginal inoculation of HSV-2. Access to the sacral ganglia, which results in spread of the virus to the CNS and death, may be via the uterus. Further implicating involvement of Igs, IgA is not present in the uteri of ovariectomized rats (189) which are highly susceptible to intravaginal inoculation of HSV-2.

In contrast, the vaginal levels of IgG and IgA are at their highest during diestrous. High levels of antibody in the vagina contradict their involvement in the pattern of resistance we observed; however, since we examined primary HSV-2 infections, presence of specific antibodies would not

be expected. The possible involvement of antibodies in resistance to intravaginal HSV-2 challenge during estrous cycle phases will provide an interesting subject for further study.

IP inoculation of macrophage poisons was shown to have no effect on vaginal HSV-2 infection (113). Such inhibitors may alter circulating macrophages; but perhaps not local fixed macrophages, so macrophages present in the vaginal mucosa may also aid in defense against HSV-2. Peritoneal macrophages have been shown to be altered in number and activity by estrogen levels (183) and any macrophages present in the vaginal mucosa might be expected to act similarly. Estrous related macrophage activity may be lowest in diestrous. We saw no change in macrophage numbers, but did not examine macrophage activity. Further studies are necessary to examine the existence and possible involvement of macrophages in the genital mucosa in resistance to HSV-2 infection during other estrous stages.

NK cells have been implicated in resistance to IP infection with HSV-2 (9,11) and these cells may also be present in the vaginal mucosa. We have shown CBA/NJ splenic NK cells are affected by estrous cycle changes (Figure 2) with highest NK activity occurring during metestrous. If the same influence occurs on lymphocytes in the vaginal mucosa,

it would correspond with the estrous phase we observed to have greatest resistance to HSV-2.

It will be necessary to demonstrate whether the immune system or the physiology of the vagina account for the pattern of resistance we observed in vaginal infection. To accomplish this, animals in all stages of the estrous cycle, excluding diestrous, will be infected and survivors challenged during diestrous. If the mice are as susceptible as in the present study, support for the natural resistance hypothesis is obtained. If the mice are resistant to challenge during diestrous, support for a local immune response during the other estrous phases will be obtained.

Immune and natural resistance factors may work together in defense of vaginal infection with HSV-2. All have been shown to vary with changes in hormonal levels during ovulatory cycles. Vaginal pH, quantity of mucus, local Ab level, and possibly local macrophages and NK cells provide a natural barrier to infection which is not constant during reproductive years, but varies with cyclic hormone fluctuations. Cytotoxic T cells in the GLN may be stimulated if the natural resistance barriers are penetrated. During diestrous, local viral propagation may outrace the GLN response. Virus could then spread through the uterus, which is poorly protected (i.e. low Ab and possibly low T cell

immunity), thus resulting in more rapid CNS infection and death as we have observed.

Greater susceptibility to herpes and other genital infections during pregnancy is well known, as are pregnancy related changes in vaginal pH and suppression of many natural and immune cellular effectors. Pregnancy changes may not parallel those seen during cycles of ovulation, because hormonal factors are at least ten fold higher during pregnancy. Dose dependent bimodal modulation of cell function is a well known, though not entirely understood, phenomenon. At any rate, hormonal changes in local natural and/or immune factors (GLN cells) are probably the best explanation for our observed greater susceptibility to herpes genital infection and encephalitic death during diestrous. Our results also substantiate many other clinical observations of differences in the male and female immune systems.

Talal, the major authority on sex hormones and the immune system, proposes that sex hormones, among their other functions, play a very important role in biological survival by manipulating immune responses to infectious agents. Regulatory T cells appear to be the primary targets of sex-hormone action (175). This hypothesis is clearly supported by the evidence that murine susceptibility to HSV-2 varies with estrous stage. The sex hormones, through natural

or immune mechanisms or both, appear to regulate the immune system in relationship to the vulnerability to pathogens which accompany biological functions during the ovulatory cycle.

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