Journal of NeuroVirology, 13: 233–241, 2007

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280701308467

informa
healthcare

Changes in immunoglobulin levels related to herpes simplex virus type 1 brain infection in pregnant mice

Javier S Burgos, Carlos Ramirez, Anna Brachet, Juan M Alfaro, Isabel Sastre, and Fernando Valdivieso

Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa (C.S.I.C.-U.A.M.), Universidad Autónoma de Madrid, Spain

Disseminated herpes simplex virus type 1 (HSV-1) infection during pregnancy is poorly described even though it is associated with high maternal and fetal morbidity and neonatal mortality in humans. In a previous paper using mice as a model, the authors demonstrated that HSV-1 is transmitted hematogenously from mother to offspring, the virus colonizing the central nervous system and provoking high mortality. In the present study, viral DNA levels in latently infected mothers were investigated during pregnancy and after delivery in mice. Samples from different organs were obtained before gestation (latency), three times during pregnancy (17, 4.5, and 1 day before delivery), and four times after delivery (1 day, 1 week, 1 and 2 months). A dramatic decrease in viral DNA concentration was observed during pregnancy, especially in the nervous system, with postnatal recovery to latent levels. All the brain regions studied showed similar trends. The viral copy numbers detected in mothers at delivery +1 day were independent of viral inoculum size. The spread of the virus to the above organs was examined immunohistochemically and, in general, more intense viral staining was observed after delivery in each. Because immunoglobulin levels can be modified by infections during pregnancy, the authors examined the levels of specific HSV-1 antibodies. Variation in HSV-1 DNA concentration was found to be associated with changes in the full spectrum of immunoglobulins (but especially immunoglobulin M [IgM]) over pregnancy, whereas at delivery −1 day a significant inverse relationship between immunoglobulins and HSV-1 DNA was observed. IgGs provided protection during the postnatal phase. *Journal of NeuroVirology* (2007) **13,** 233–241.

Keywords: central nervous system; herpes simplex virus type 1; pregnancy; mice; immunoglobulins

Introduction

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) can both cause genital infection and be transmitted to the neonate, although HSV-2 is the more important one (>70% of cases) (Annunziato and Gershon, 1996). However, HSV-1 is a ubiquitous virus associated with several human diseases such as fever blisters, severe encephalitis (Meyer *et al*, 1960), and Alzheimer disease (Itzhaki *et al*, 1997). HSV (type 1 and type 2) infections are common during pregnancy, where the manifestations vary from no symptoms to widely disseminated disease (Chiquet *et al*, 2003; Dupuis *et al*, 1999; Pardo *et al*, 2004; Peacock and Sarubbi, 1983; Young *et al*, 1976). Fortunately, severe cases are very rare, and generally HSV infection is mild or symptomless. The most serious complication of HSV infection during pregnancy is transmission of virus to the neonate, and this can have devastating consequences. In fact, the prevalence of infection with HSV during pregnancy has increased during the past three decades (Brown *et al*, 1997).

Address correspondence to Dr. Javier S. Burgos or Prof. Fernando Valdivieso, Lab. CX340, Centro de Biología Molecular, Universidad Autónoma de Madrid, 28049, Cantoblanco, Madrid, Spain. E-mail: jburgos@neuronbp.com; fvaldivieso@cbm.uam.es

This work was supported by a grant from the Obra Social Caja Madrid to the Asociación de Familiares de Enfermos de Alzheimer (AFAL), and by an institutional grant by the Fundación Areces to the Centro de Biología Molecular Severo Ochoa. The authors thank Prof. F. Mayor for continuous encouragement and help, and Dr. L. Carrasco for providing the HSV-1 KOS strain. The authors are grateful to J. Ripoll for excellent technical assistance.

Received 3 November 2006; revised 19 January 2007; accepted 20 February 2007.

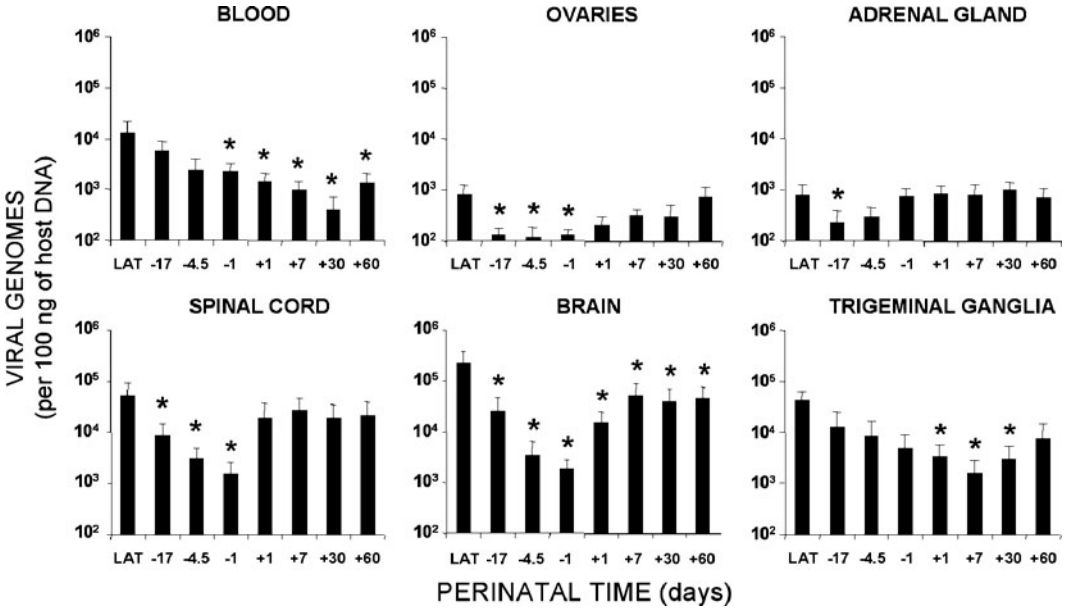

Figure 1 Quantification of HSV-1 DNA concentrations (by organ) in infected mothers at different perinatal time points. A group of female mice ($n = 69$) was infected intraperitoneally with 1×10^6 PFU of HSV-1 (KOS strain), mated at 37 days post-infection with mock-infected male mice, and sacrificed at several times: latency (LAT; previous to cross) and delivery -17, -4.5, -1, $+1$, $+7$, $+30$, and $+60$ days. The bar graph represents the viral copy number detected in each organ expressed on a logarithmic scale. Values are the mean ± SEM of the quantity of viral DNA (expressed as viral genomes and normalized with respect to the quantity of mouse genomes [in 100 nanograms of host DNA by amplifying the β-actin housekeeping gene]). Fisher's exact test was used to compare the values against latency ($^*P < .001$).

Neonatal HSV infection most commonly results from contact between the newborn and HSV that is present in the birth canal of an asymptomatic mother during labor and delivery (Brown *et al*, 1991; Prober *et al*, 1988; Whitley *et al*, 1991). Recently, we demonstrated in mice that HSV-1 is predominantly transmitted by vertical hematogenous infection, the virus specifically colonizing the nervous system of newborns (Burgos *et al*, 2006b). However, there have been few studies of the frequency of HSV dissemination at different times during pregnancy, and variations in HSV-1 DNA in latently infected pregnant mothers have never before been longitudinally analyzed.

The study of the epidemiology of HSV infections in pregnant women has been complicated by the fact that neither mother nor child may exhibit symptoms and due to the lack of appropriate animal models of investigation. For these reasons, the factors influencing transmission of HSV infection from the mother to the fetus are not fully understood. An animal model is required to study the complex epidemiology of HSV-1 during pregnancy. In this sense, the present study describes a model of infection to evaluate the viral shifts during pregnancy in asymptomatic infected mice that will be useful to investigate new therapeutical approaches.

Placental transmission of antibodies from mother-to-fetus forms the basis of passive immunity (Kohler and Farr, 1966). Because maternal antibodies transmitted to the fetus are of great importance during gestation and early life, prenatal and postnatal maternal levels of immunoglobulins were determined to provide insight into the risk of viral reactivation in both mother and offspring. The aim of the present study was to evaluate whether viral shedding in pregnant mice varies during gestation, delivery, and the postnatal phase, and whether this variation might be due to variations in immunoglobulin levels.

Results

Detection and quantification of HSV-1 DNA during the perinatal period

The results show a significant change in viral DNA levels in every tested organ over gestation and the postnatal stage (Figure 1). In general, viral copy numbers decreased consistently over pregnancy, but recovered after delivery. The nervous system had the highest viral DNA level, the brain showing the greatest concentration at every analysis time, followed by the spinal cord and the trigeminal ganglia. The encephalon showed a trend similar to that of the spinal cord. Interestingly, the blood and the trigeminal ganglia showed similar trends in HSV-1 DNA concentration, the levels of both diminished further after delivery. The adrenal glands and ovaries showed the lowest number of viral genomes, as well as similar trends over time.

To determine whether the viral copy number variations detected in brain were restricted to a specific area or whether they were generalized, the encephalon was subdivided into four subregions and HSV-1 DNA levels determined (Figure 2). All

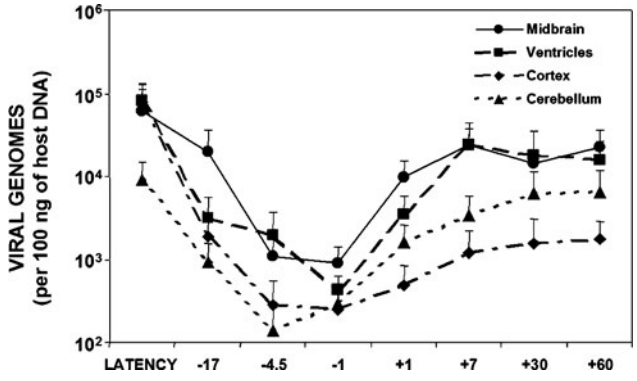

Figure 2 Quantification of HSV-1 DNA concentrations in infected mothers at different perinatal times in the midbrain, ventricles, cortex, and cerebellum. A group of female mice ($n = 69$) was infected intraperitoneally with 1×10^6 PFU of HSV-1 (KOS strain), mated at 37 days post-infection with mock-infected male mice, and sacrificed at latency (previous to cross) and delivery −17, −4.5, −1, +1, +7, +30, and +60 days. The lines represent the viral copy number detected in each brain region expressed on a logarithmic scale. Values are the mean ± SEM for the quantity of viral DNA expressed as viral genomes and normalized with respect to the quantity of mouse genomes (in 100 ng of host DNA by amplifying the β-actin housekeeping gene).

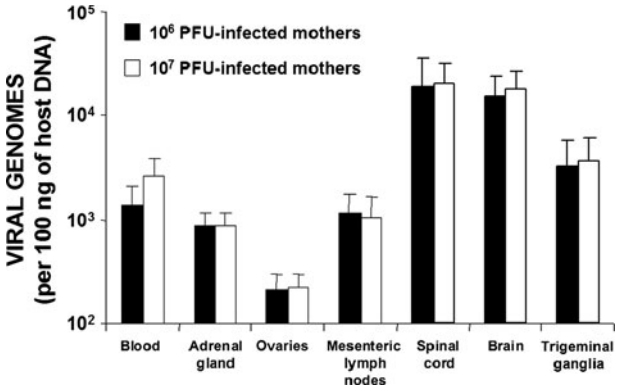

Figure 3 Quantification of HSV-1 DNA concentrations with respect to the viral dose injected in several organs. A group of female mice ($n = 15$) was infected intraperitoneally with two HSV-1 suspensions (KOS strain), 1×10^6 and 1×10^7 PFU, mated at 37 days post-infection with mock-infected male mice, and sacrificed at delivery +1 day; viral DNA concentrations were studied in all mentioned organs. The bar graph represents the viral copy number expressed on a logarithmic scale. The values are the mean ± SEM of the quantity of viral DNA (expressed as viral genomes and normalized with respect to the quantity of mouse genomes expressed in 100 ng of host DNA by amplifying the β-actin housekeeping gene).

encephalic regions showed similar profiles—a constant fall in viral DNA load during gestation and an increase after delivery to recover latency levels.

With respect to the number of viral genomes around the time of delivery (delivery −1 day compared to delivery +1 day), only the spinal cord and the brain showed a significant difference ($P < .001$), with increases of 12.7- and 8.1-fold, respectively. Both organs showed a parallel increment after delivery. The ovaries and adrenal glands also showed a similar (but non-significant) increase. In contrast, the blood and trigeminal ganglia showed a reduction in viral DNA concentration. In brain, the midbrain was the region with the highest increment (10.6-fold; $P < .001$), followed by the ventricles (8.1-fold; $P < .001$) and cerebellum (5.5-fold; $P < .001$). The cerebral cortex showed the smallest increase in viral DNA concentration around delivery time (1.9-fold; $P > .05$). These results indicate that the midbrain to be the region with the greatest variation in viral DNA around delivery.

Effect of viral dose

To evaluate the influence of the injected viral dose on viral DNA variation after delivery, experiments were performed comparing mothers latently infected with 1×10^6 and 1×10^7 plaque-forming units (PFU); the results for these animals at 1 day post delivery were compared. Figure 3 shows the viral DNA concentrations detected in several organs. The concentration in each organ was essentially identical in both groups ($P > .05$), suggesting that after reaching latency the infective dose is irrelevant to subsequent phenomena. Independent of the dose, the organs with the highest viral DNA concentrations were again those of the nervous system (spinal cord, brain, and trigeminal ganglia), followed by the blood. However, although both groups of infected mothers experienced mating problems, all 1×10^6 PFU–infected mothers (36 out of 36) obtained progenies, but 27.7% of them (10 out of 36 mothers) had stillborns or pups that died soon after birth. Five of the 23 (20.8%) mothers infected with 1×10^7 PFU never gave birth. In addition, 55.5% of these mothers (10 out of 18) had some stillborns or pups that died within a few days.

Perinatal viral immunodetection

Tissue sections of the main organs were studied at delivery −1 day and +1 day, and HSV-1 was detected immunohistochemically using anti-VP16 antibody (Figure 4). At −1 day, HSV-1 was observed in the adrenal glands, showing foci of infection (*asterisk*, Figure 4A) with low intensity staining, especially in the zona reticulata. However, at +1 day, the number of foci and the intensity of staining were greater (Figure 4B). Some foci were found in the zona fasciculate (*arrowhead*). In the ovaries, before delivery, the foci of infection were generally seen in the stromal cells (*arrow*) and in some external thecal cells of the graafian follicles (Figure 4C); this pattern was also observed in the ovaries of mothers at delivery +1 day, but the number of infected cells (*arrow*) and the intensity of staining were greater (Figure 4D). HSV-1 was also immunodetected in the central nervous system (CNS). The most infected area was the midbrain (Figure 4E and F), although infection was also clear in the spinal cord (data not shown). In the brains of mothers at delivery −1 day, some ganglionar cells of the mesencephalic trigeminal nucleus were

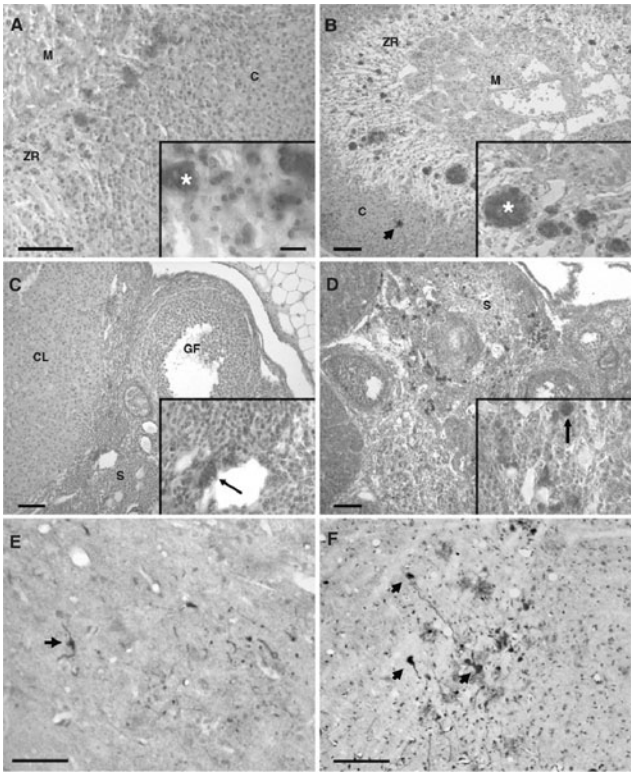

Figure 4 Immunohistochemical detection of HSV-1 antigens in different organs in mothers sacrificed at delivery −1 day compared to delivery +1 day. A group of female mice ($n = 10$) were infected intraperitoneally with 1×10^6 PFU of HSV-1 (KOS strain), mated at 37 days post infection with mock-infected male mice, and sacrificed at delivery −1 or +1 day. The organs were then embedded in paraffin wax and serially sectioned. Immunodetection was performed using the anti-VP16 tegument viral protein and develop as detailed in Materials and Methods. (**A**) Immunohistochemistry of the adrenal gland from a mother at delivery −1 day. In this micrograph, some foci of infection (*asterisk*) can be observed (especially in the zona reticularis of the adrenal cortex) but with a weak expression. (**B**) An adrenal gland from a mother at delivery +1 day. The infection pattern was similar to that found in (**A**). However, the number of infectious foci (*asterisk*) was higher and staining more intense. Further, in this case, a number of infectious foci were detected in the zona fasciculata of the adrenal gland (*arrowhead*). (**C**) HSV-1 labeling in ovary from mother at delivery −1 day, showing VP16 staining in the stroma (*arrow*) and in groups of thecal cells from some follicles. (**D**) The pattern of staining in ovaries from mothers at delivery +1 day was similar to that observed in those at delivery −1 day. However, more infectious foci and stronger immunoreactions (arrow) were seen. (**E**) Sagittal section of brain from mother at delivery −1 day. This micrograph shows some neuronal cells (*arrowhead*) and several nervous fibers infected with HSV-1 in the midbrain. (**F**) As before but with a stronger expression. An immunoreaction to VP16 in the brain of a mother at delivery +1 day; this occurred almost exclusively in neurons of the trigeminal nucleus (*arrowheads*) of the midbrain neural soma and nerve fibers. M, medulla; C, cortex; ZR, zone reticularis; CL, corpus luteum; S, stroma; GF, Graafian follicle. Bar: 100 μm; inset: 30 μm.

immunodetected (Figure 4E, *arrowhead*) with low intensity staining. The same was seen in the midbrain of mothers at delivery +1 day. In these last samples (Figure 4F), the number of neurons stained (*arrowheads*) was higher and the immunoreactions more intense.

Variation in immunoglobulins

The sandwich–enzyme-linked immunosorbent assay (ELISA) technique was used to detect immunoglobulins IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM in the blood of mice at different perinatal time points (Figure 5). Compared to mock-infected mice, IgG1 and IgG2a showed significant increments at every time point in infected mothers, indicating that both are involved in HSV-1 infection (Figure 5, *asterisks*). At delivery −1 day, IgG3 and IgM levels also increased significantly in infected mothers compared to mock-infected mice.

In infected mothers, IgM and IgG2a showed significant increments at delivery −1 day and +60 days, respectively, compared to latently infected mice (Figure 5, *pounds*). However, a general increase in the immunoglobulins was usually observed at delivery −1 day. These results suggest that both immunoglobulins are mainly produced around delivery, IgM playing a role in the prenatal phase and IgG2a becoming the most important during the postnatal stage. Although they showed slight prenatal increments, no significant differences were found for IgA and IgG2b in any comparison.

To determine whether the overall trend for immunoglobulin titers varied over perinatal time, a full immunoglobulin comparison was undertaken. At delivery −1 day significantly higher titers were seen than at all other perinatal sampling times (Figure 6). The immunoglobulin peak (*line*) coincided with the lowest viral copy numbers (*bar graphs*); the other time points showed lower levels of immunoglobulin and higher HSV-1 DNA levels. An inverse correlation was found between increased blood concentrations of immunoglobulin and CNS and blood viral DNA concentrations. This was dependent only on perinatal time in the infected mothers. The mock-infected mothers showed no significant differences in immunoglobulin levels at delivery −1 day and +1 day (data not shown).

Discussion

Little is known about the effectiveness of prenatal immunity against HSV-1 infection in murine mothers and newborns, and there is little direct evidence that maternally derived antibodies actually provide any protection. In earlier work we showed HSV-1 infection to be predominantly restricted to females (Burgos *et al*, 2005) and that pure vertical transmission is essentially due to hematogenous infection (Burgos *et al*, 2006b). Neonatal herpes and its associated disseminated disease have been adequately described in humans (Brown *et al*, 1991), but no information has been reported on clinical outcome in latently infected asymptomatic mothers (candidates for developing herpes-associated diseases after critical stimuli such as delivery). The dissemination of HSV-1 infection can occur even without evident clinical lesions.

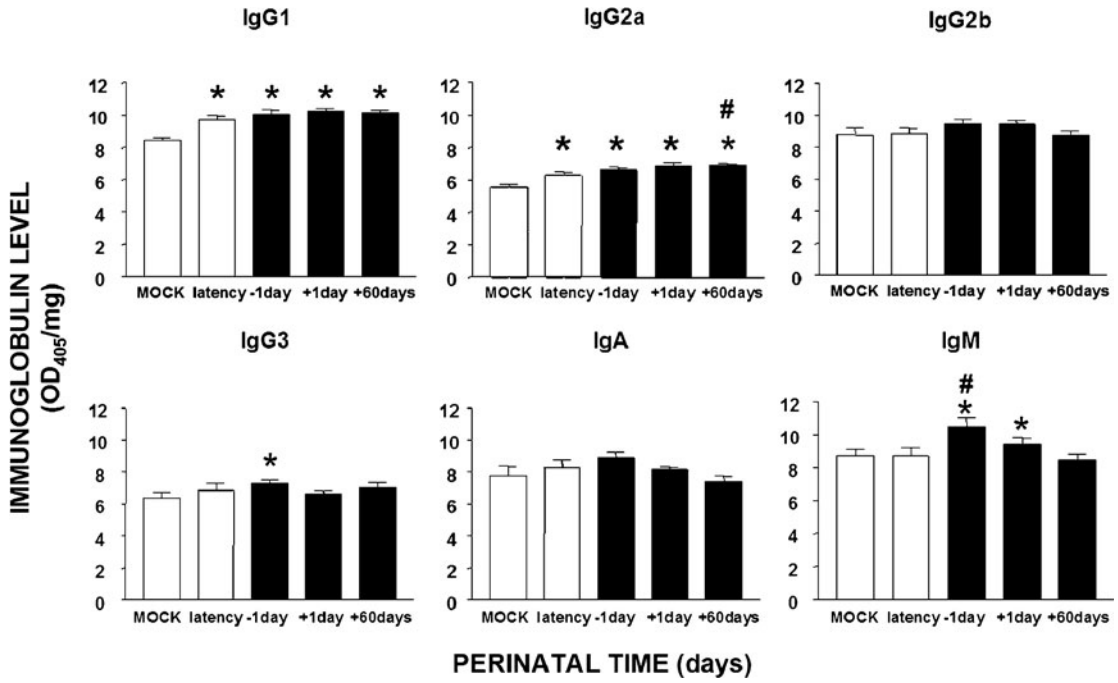

Figure 5 Quantification of immunoglobulin levels in blood. A group of female mice ($n = 34$) was intraperitoneally infected with 1×10^6 PFU of HSV-1 (KOS strain), mated at 37 days post-infection with mock-infected male mice, and serum obtained through retro-orbital bleeding at latency (previous to cross), delivery −1, +1, and +60 days. Immunoglobulins were measured individually by sandwich-ELISA assays as described in Materials and Methods. The mock group was injected with PBS. The bar graphs represents the levels of each immunoglobulin (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) detected over time (expressed as mean ± SD in arbitrary units, OD_{405} per mg of protein). The one-tailed unpaired Student *t* test was used to compare immunoglobulin levels against mock (*$P < .05$) or against latency groups (#$P < .05$).

However, the onset of some diseases caused by HSV-1 such as gingivostomatitis (Pardo *et al*, 2004) or encephalitis (Dupuis *et al*, 1999) (usually associated with seizures or neurological or psychiatric disorders) have been documented in pregnant mothers. Other manifestations of disseminated herpes infection during pregnancy includes hepatitis, pancreatitis, myocarditis, and retinal necrosis (Chiquet *et al*, 2003; Peacock and Sarubbi, 1983; Young *et al*, 1976). Despite this putative association between pregnancy and the dissemination of herpes, the specific predisposing factors remain poorly defined.

The mode of HSV transmission to the neonate is usually from the maternal birth canal to the fetus intrapartum; but occasionally hematogenous transplacental infection can affect the developing fetus months prior to birth (Corey and Flynn, 2000). When HSV is transmitted transplacentally, disseminated hematogenous infection of the fetus can occur, leading to severe systemic consequences (Mansour and Nichols, 1993). Presence of virus in blood can be anticipated by the high percentage of seropositive pregnant women for HSV (Brown *et al*, 1985). Furthermore, viremia with HSV-1 in adults was firstly demonstrated in 1976 (Naraqi *et al*, 1976), and polymerase chain reaction (PCR) techniques have subsequently confirmed that viremia is common in neonatal HSV infection (Diamond *et al*, 1999; Kimura *et al*, 1991; Malm and Forsgren, 1999). These results together with our previous data (Burgos *et al*, 2006b) indicate that the viral shedding occurs from the maternal bloodstream. In the present study, the HSV-1 DNA concentrations of the blood and trigeminal ganglia showed similar profiles during gestation, delivery, and postnatal phases, confirming results previously described (Burgos *et al*, 2005, 2006b, 2006c) and indicating that the trigeminal ganglia are the viral reservoir in latently infected mice that supplies significant quantities of virus to the blood. These results are in concordance with data showing a recovery of HSV-1 from trigeminal ganglia in humans. In fact, observations about the concept that latent infection of sensory ganglia may be the source of virus in recurrent herpetic disease in man is known from the early 1970s (Bastian *et al*, 1972).

Although animal models must be used and interpreted with caution, they are highly desirable to monitor longitudinal and observational phenomena. The present results in mice show a general reduction in the number viral genomes during gestation, followed by their postdelivery recovery, especially in the CNS. The encephalon was the region where the HSV-1 DNA level was highest and where variation in viral DNA concentrations was most dramatic. Spinal cord showed similar trends, confirming the neurotropic character of this virus. After dissecting the brain into four regions and analyzing the variation in viral copy numbers over perinatal time, identical

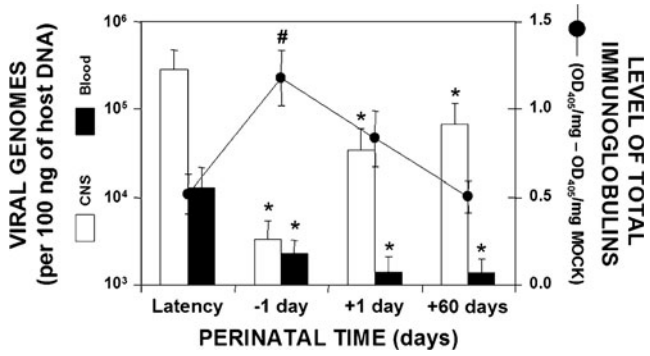

Figure 6 Immunoglobulin levels compared with HSV-1 DNA concentrations in the CNS and blood. A group of female mice ($n = 34$) were intraperitoneally infected with 1×10^6 PFU of HSV-1 (KOS strain), mated at 37 days post-infection with mock-infected male mice, bled, and sacrificed at latency (previous to mating), delivery −1, +1, and +60 days. The solid line represents the total immunoglobulin level (expressed in arbitrary units, OD_{405} per mg of protein) quantified by sandwich-ELISA assays (mock level subtracted). The bar graphs represent the viral copy number detected in blood and CNS (expressed on a logarithmic scale as viral genomes normalized with respect to the quantity of mouse genomes expressed in 100 nanograms of host DNA by amplifying the β-actin housekeeping gene). Values are means and standard deviation for immunoglobulin levels, whereas viral DNA levels are expressed as means and standard errors. Comparisons against latency group were performed using Fisher's exact test for HSV-1 DNA concentrations ($^*P < .001$) and the one-tailed unpaired Student t test for immunoglobulin levels ($\#P < .05$).

behavior was seen in each. This indicates this is a general phenomenon occurring in the whole brain, and is not restricted to any specific region of the encephalon.

The present study shows that asymptomatic infected mothers vary the virus levels around delivery. The immunohistochemical approach of the present work confirmed there to be differences in virus infectivity around the critical date of delivery. In general, the main organs of infected mothers at delivery +1 day showed ever more intense foci of infection and stronger immunoreactions than at delivery −1 day. The present results also show that the inoculum size is irrelevant in the establishment of maternal virus levels; the perinatal time of sacrifice is more important. Although no reactivation was studied, the general behavior of the viral DNA concentrations around delivery suggests that no important viral reactivation occurred. In fact, reactivation from neuronal tissues should be restricted to the nervous system. However, our data show general variation in the whole animal, suggesting a systemic phenomenon involving all organs, especially when reactivation is restricted to specific areas such as the trigeminal ganglia (Cabrera *et al*, 1980; Steiner and Kennedy, 1995). One of the main disadvantages to study the HSV-1 reactivation is that the induction of this phenomenon is notoriously difficult to achieve in experimental animals (Harbour *et al*, 1983; Openshaw *et al*, 1979; Shimeld *et al*, 1990), especially for the KOS strain (Sawtell and Thompson, 1992). However, we have previously showed that HSV-1 reactivates in fetal neurons after hyperthermia (Burgos *et al*, 2006b), and it has been described that transmitting mothers shed virus at delivery but have subclinical HSV-1 infection (Brown *et al*, 1991). Although focal spontaneous molecular reactivations (Feldman *et al*, 2002) cannot be discarded, shifts in specific molecules could explain the two log increase in viral copy number observed after delivery. In this sense, two types of molecule are candidates for regulating virus levels around delivery: hormones and immunoglobulins. Although the potential increase in immunoglobulins should reduce viral loads, hormones should have an opposite effect, especially because it has been shown that an increase in female hormones cause an increase in HSV levels (Bujko *et al*, 1988; Yirrell *et al*, 1987).

It has been described that, in the prenatal stage, an increase in immunoglobulin production occurs to protect the fetus (Hitti *et al*, 1997), and that an imbalance in the ability to produce HSV-specific IgG, IgA, and IgM may be responsible for recurrent herpes lesions (Tokumaru, 1966). On the contrary, other authors have documented a decrease in serum immunoglobulin levels during pregnancy, especially IgG (Benster and Wood, 1970; Studd, 1971). The present serological studies in mice showed a general increase in immunoglobulins during gestation, correlating with a reduction of virus levels in the mother. In fact, the highest immunoglobulin levels (delivery −1 day) coincided with the lowest viral DNA concentration. The present results also show that the increase in antibody levels was across the spectrum, although IgM appears to play a role in this protective response in the mother. Maternal IgG1 and IgG2a increased not only prenatally but also postnatally, whereas IgG3 and IgM showed a significant peak at delivery −1 day, after which they decreased in number. These results in animals agree with the results of clinical studies in which serological tests have found IgM antibodies against HSV-1 (Pardo *et al*, 2004), and with those of experiments in mice where infection with a variety of live viruses led to a preferential increase in IgG2a (Coutelier *et al*, 1987). In agreement with previous results obtained in HSV-2–infected mice, IgA and IgM levels fell during the postnatal phase, whereas IgG levels increased after delivery (Hayashi *et al*, 1983). These results indicate that maternal IgG, acquired prenatally and transmitted to the offspring postnatally via the breast milk, plays an important role in protecting newborn mice against HSV-1 infection. In fact, the mortality in newborn mice intraperitoneally infected with HSV-2 is reduced when such pups are fed the milk of immunized mothers (Hayashi *et al*, 1983). These studies indicate that maternal immunoglobulins, acquired not only postnatally but also prenatally, play an important role in protecting newborns against HSV infection. This agrees with several studies on larger numbers of animals where equivalent maternal

and neonatal antibody levels of HSV-1 were found (Gotlieb-Stematsky *et al*, 1983; Smith and Hanna, 1974).

Altogether, these results in mice suggest that antibody transfer across the placenta is an active and selective process, and that the relative efficiency of immunoglobulin transfer might be responsible (at least in part) for the varied susceptibility of newborns and their mothers to infection. Inefficient maternal production of antibodies may pose a *bona fide* risk to newborns. Immunization of the mother would appear to be a potential therapeutic strategy for interrupting neonatal HSV infection and consequent maternal dissemination. We have previously demonstrated that after reducing maternal viremia with acyclovir, viral infection can be prevented in the offspring (Burgos *et al*, 2006b).

This report in mice provides several new observations concerning maternal changes in HSV-1 load, the anatomical sites of viral shedding, and the involvement of immunoglobulins in the regulation of the virus during the perinatal phase. Clinical and experimental studies are needed, however, if we are to obtain a better understanding of the pathological role of HSV-1 both in mothers and infants, and if the capacity of maternal antibodies to prevent to herpes-related diseases is to be determined.

Materials and methods

Inoculation and dissection

Experiments were performed in accordance with the guidelines of the European Community Animals Act (Scientific Procedures) of 1986. HSV-1 (KOS strain; kindly supplied by Dr. L. Carrasco) was propagated and titrated by plaque assay in confluent monolayers of Vero cells (Burgos *et al*, 2002a). C57BL/6 female mice were inoculated intraperitoneally with 1×10^6 or 1×10^7 plaque-forming units (PFU) as previously described (Burgos *et al*, 2002a, 2003, 2005, 2006a, 2006b, 2006c). Viral and mouse strains were selected in order to compare the results from this study with already published data (Burgos *et al*, 2002a, 2003, 2005, 2006a, 2006b, 2006c) and also because KOS can be considered a prototypic laboratory strain of HSV-1. Doses of inoculation were selected from our previous results (Burgos *et al*, 2006c). Because overt infection subsides 2 weeks after inoculation and all HSV-1 present is a latent state by 4 weeks after inoculation (Miller *et al*, 1998), these animals were separated into two groups at 37 days post infection (when latent infection was assured in these experimental conditions (Burgos *et al*, 2006c)); a small group ($n = 6$) was sacrificed (taken to represent latent infection or time 0 with respect to delivery and defined as *LAT*), while a second group ($n = 96$) was mated with mock-infected male mice. These females were then sacrificed at different time points; delivery −17 days (or 1 day after the appearance of the postvaginal plug [PVP]), delivery −4.5 days (PVP +13.5 days), delivery −1 day (PVP +17 days), and at 1, 7, 30, or 60 days after delivery. Mock-infected animals were used as controls. The mice used ($n = 102$; all survivors of infection) remained free of clinical disease until the end of the experiment. The viral DNA concentrations of the whole blood, adrenal glands, ovaries, spinal cord, trigeminal ganglia, and brain were determined. For more precise analyses, the brain samples were subdivided into four regions as previously described (Burgos *et al*, 2006b): midbrain, ventricles, cerebral cortex, and cerebellum.

HSV-1 DNA quantification from tissue homogenates

DNA from homogenized samples was extracted by conventional methods (High Pure PCR template preparation kit, catolog no. 1 796 828; Roche, Germany). The concentration of HSV-1 DNA in several organs was then quantified using real-time quantitative PCR as previously described (Burgos *et al*, 2002a, 2002b, 2003, 2005, 2006a, 2006b, 2006c). An appropriate concentration range of virus was used for the optimization of the standard curve, and the viral DNA concentration expressed in terms of viral copy numbers. PCR calibration was performed using the β-actin housekeeping gene (results expressed as nanograms of host DNA) (Burgos *et al*, 2002a, 2002b, 2003, 2005, 2006a, 2006b, 2006c). Real-time PCR was performed using a LightCycler rapid thermal cycler (Roche Diagnostics, Lewes, UK) and a LightCycler FastStart DNA Master SYBR Green I kit (catalog no. 3 003 230; Roche, Germany). All experiments were performed in triplicate. Melting curve analyses, agarose and acrylamide gel electrophoresis, restriction analysis and nested-PCR confirmed the specificity of the products (Burgos *et al*, 2002b, 2006b).

ELISA determination of IgG, IgA, and IgM

Total maternal blood was collected and stored at −80°C and tested for immunoglobulins. The total titer for all subclasses of immunoglobulins was determined by sandwich-ELISA using the Mouse MonoAB ID kit (HRP; Zymed Laboratories, San Francisco, CA) with minor modifications. Prior to ELISA, proteins from blood samples were extracted by incubation in 1% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics, Germany) in phosphate-buffered saline (PBS) for 30 min in an ice-water bath, followed by separation by centrifugation at 1870 × *g* for 15 min at 4°C. Protein quantification was performed by the BCA method (Pierce, Illinois, US) (100 to 150 μg protein/well). Immunoplates were coated with 50 μl of diluted goat anti-mouse IgGAM antibody and incubated overnight. Later, the immunoplates were incubated for 1 h at 37°C with 200 μl of blocking solution, and samples were bound to the IgGAM in microtiter plates for 30 min at 37°C. These immunoplates were then washed three times in PBS–Tween 20. Primary antibodies (rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM) were

then applied and incubation allowed to proceed for 1 h at 37°C. After washing, horseradish peroxidase (HRP)-goat anti-rabbit IgG (H+L) was added and incubation allowed to proceed for 45 min at 37°C. Ready-to-use ABTS was added as a substrate for the reaction. The reaction was terminated at 30 min and results taken. At least five animals were evaluated for each time point and comparisons with mock-infected mice were made. Each experiment was performed in triplicate. The quantity of specific immunoglobulins was expressed as arbitrary units of absorbance at 405 nm per mg of protein.

Histological procedures and immunodetection of viral antigens

The tegument viral protein VP16 was used as the target antigen. The ovaries, adrenal glands, and brains belonging to 10 (5 mice at delivery −1 day and 5 at delivery +1 day) latently infected mothers were examined for immunoreactive evidence of HSV-1 infection. The organs were washed with 10 ml of PBS and fixed in formaldehyde 10% in phosphate buffer, dehydrated, and embedded in paraffin wax. Sections (6 μm thick) were processed using the avidin-biotin complex methods. To retrieve the antigen, sections were deparaffinated, hydrated, and incubated with 0.1 M citrate buffer (pH 6) for 1 min in a conventional pressure cooker. After rinsing in Tris-buffered saline (TBS), the sections were incubated with blocking solution (3% normal donkey serum [NDS] in TBS containing 0.05% Triton X-100) for 30 min to prevent nonspecific binding of the primary antibody. They were then incubated overnight at 4°C with rabbit polyclonal anti-VP16 (BD Biosciences Clontech, US) (1:100 dilution) primary antibody diluted in blocking solution. The sections were then washed in TBS and incubated for 1 h with an anti-rabbit biotinylated immunoglobulin (Amersham Biosciences, UK), diluted 1:250 in 1:10 blocking solution. After being washed in TBS, the sections were incubated with avidin-biotin-peroxidase complex and avidin-biotin-phosphatase complex (Dako, Barcelona, Spain) for 45 min, and developed using the DAB PLUS substrate kit and AP-red substrate kit (Zymed), respectively. Counterstaining was performed with hematoxylin to emphasize the absence of non-specific immunostaining. The sections were mounted in DPX (BDH Laboratory Supplies, Poole, UK) for peroxidase, and Acuatex (Merck, Bärmstabt, Germany) for phosphatase. For negative controls, contiguous sections of each sample were incubated as above but without the primary or secondary antibodies. The organs from HSV-1–infected and mock-infected females were included in every experiment to monitor the specificity of viral localization. At least six sections per organ were examined to ensure sufficient observations were made.

Statistical analysis

Fisher's exact test was used to compare viral DNA concentrations. Significance was set at $P < .001$. The one-tail unpaired Student t test was used to compare immunoglobulin levels at different time points. Significance was set at $P < .05$.

References

Annunziato PW, Gershon A (1996). Herpes simplex virus infections. *Pediatr Rev* **17:** 415–423; quiz 424.

Bastian FO, Rabson AS, Yee CL, Tralka TS (1972). Herpesvirus hominis: isolation from human trigeminal ganglion. *Science* **178:** 306–307.

Benster B, Wood EJ (1970). Immunoglobulin levels in normal pregnancy and pregnancy complicated by hypertension. *J Obstet Gynaecol Br Commonw* **77:** 518–522.

Brown ZA, Benedetti J, Ashley R, Burchett S, Selke S, Berry S, Vontver LA, Corey L (1991). Neonatal herpes simplex virus infection in relation to asymptomatic maternal infection at the time of labor. *N Engl J Med* **324:** 1247–1252.

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

Brown ZA, Vontver LA, Benedetti J, Critchlow CW, Hickok DE, Sells CJ, Berry S, Corey L (1985). Genital herpes in pregnancy: risk factors associated with recurrences and asymptomatic viral shedding. *Am J Obstet Gynecol* **153:** 24–30.

Bujko M, Sulovic V, Zivanovic V, Lako B, Dotlic R (1988). Effect of progesterone and pregnancy on the replication of herpes simplex virus type 2 in vivo. *Clin Exp Obstet Gynecol* **15:** 34–37.

Burgos JS, Guzman-Sanchez F, Sastre I, Fillat C, Valdivieso F (2006a). Non-invasive bioluminescence imaging for monitoring herpes simplex virus type 1 hematogenous infection. *Microbes Infect* **8:** 1330–1338.

Burgos JS, Ramirez C, Guzman-Sanchez F, Alfaro JM, Sastre I, Valdivieso F (2006b). Hematogenous vertical transmission of herpes simplex virus type 1 in mice. *J Virol* **80:** 2823–2831.

Burgos JS, Ramirez C, Sastre I, Alfaro JM, Valdivieso F (2005). Herpes simplex virus type 1 infection via the bloodstream with apolipoprotein E dependence in the gonads is influenced by gender. *J Virol* **79:** 1605–1612.

Burgos JS, Ramirez C, Sastre I, Bullido MJ, Valdivieso F (2002a). Involvement of apolipoprotein E in the hematogenous route of herpes simplex virus type 1 to the central nervous system. *J Virol* **76:** 12394–12398.

Burgos JS, Ramirez C, Sastre I, Bullido MJ, Valdivieso F (2003). ApoE4 is more efficient than E3 in brain access by herpes simplex virus type 1. *Neuroreport* **14:** 1825–1827.

Burgos JS, Ramirez C, Sastre I, Valdivieso F (2006c). Effect of apolipoprotein E on the cerebral load of latent herpes simplex virus type 1 DNA. *J Virol* **80:** 5383–5387.

Burgos JS, Ramirez C, Tenorio R, Sastre I, Bullido MJ (2002b). Influence of reagents formulation on real-time PCR parameters. *Mol Cell Probes* **16:** 257–260.

Cabrera CV, Wohlenberg C, Openshaw H, Rey-Mendez M, Puga A, Notkins AL (1980). Herpes simplex virus DNA sequences in the CNS of latently infected mice. *Nature* **288:** 288–290.

Corey RP, Flynn JT (2000). Maternal intrauterine herpes simplex virus infection leading to persistent fetal vasculature. *Arch Ophthalmol* **118:** 837–840.

Coutelier JP, van der Logt JT, Heessen FW, Warnier G, Van Snick J (1987). IgG2a restriction of murine antibodies elicited by viral infections. *J Exp Med* **165:** 64–69.

Chiquet C, Thuret G, Poitevin-Later F, Gain P, Najioullah F, Denis P (2003). Herpes simplex virus acute retinal necrosis during pregnancy. *Eur J Ophthalmol* **13:** 662–665.

Diamond C, Mohan K, Hobson A, Frenkel L, Corey L (1999). Viremia in neonatal herpes simplex virus infections. *Pediatr Infect Dis J* **18:** 487–489.

Dupuis O, Audibert F, Fernandez H, Frydman R (1999). Herpes simplex virus encephalitis in pregnancy. *Obstet Gynecol* **94:** 810–812.

Feldman LT, Ellison AR, Voytek CC, Yang L, Krause P, Margolis TP (2002). Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. *Proc Natl Acad Sci U S A* **99:** 978–983.

Gotlieb-Stematsky T, Meron I, Modan M, Sayar Y, Leventon-Kriss S, Fogel A, Gurewitz R, Insler V (1983). Viral antibodies in maternal and cord sera. *Med Microbiol Immunol (Berl)* **172:** 67–74.

Harbour DA, Hill TJ, Blyth WA (1983). Recurrent herpes simplex in the mouse: inflammation in the skin and activation of virus in the ganglia following peripheral stimulation. *J Gen Virol* **64(Pt 7):** 1491–1498.

Hayashi Y, Wada T, Mori R (1983). Protection of newborn mice against herpes simplex virus infection by prenatal and postnatal transmission of antibody. *J Gen Virol* **64:** 1007–1012.

Hitti J, Watts DH, Burchett SK, Schacker T, Selke S, Brown ZA, Corey L (1997). Herpes simplex virus seropositivity and reactivation at delivery among pregnant women infected with human immunodeficiency virus-1. *Am J Obstet Gynecol* **177:** 450–454.

Itzhaki RF, Lin WR, Shang D, Wilcock GK, Faragher B, Jamieson GA (1997). Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* **349:** 241–244.

Kimura H, Futamura M, Kito H, Ando T, Goto M, Kuzushima K, Shibata M, Morishima T (1991). Detection of viral DNA in neonatal herpes simplex virus infections: frequent and prolonged presence in serum and cerebrospinal fluid. *J Infect Dis* **164:** 289–293.

Kohler PF, Farr RS (1966). Elevation of cord over maternal IgG immunoglobulin: evidence for an active placental IgG transport. *Nature* **210:** 1070–1071.

Malm G, Forsgren M (1999). Neonatal herpes simplex virus infections: HSV DNA in cerebrospinal fluid and serum. *Arch Dis Child Fetal Neonatal Ed* **81:** F24–F29.

Mansour AM, Nichols MM (1993). Congenital diffuse necrotizing herpetic retinitis. *Graefes Arch Clin Exp Ophthalmol* **231:** 95–98.

Meyer HM Jr, Johnson RT, Crawford IP, Dascomb HE, Rogers NG (1960). Central nervous system syndromes of "vital" etiology. A study of 713 cases. *Am J Med* **29:** 334–347.

Miller CS, Danaher RJ, Jacob RJ (1998). Molecular aspects of herpes simplex virus I latency, reactivation, and recurrence. *Crit Rev Oral Biol Med* **9:** 541–562.

Naraqi S, Jackson GG, Jonasson OM (1976). Viremia with herpes simplex type 1 in adults. Four nonfatal cases, one with features of chicken pox. *Ann Intern Med* **85:** 165–169.

Openshaw H, Asher LV, Wohlenberg C, Sekizawa T, Notkins AL (1979). Acute and latent infection of sensory ganglia with herpes simplex virus: immune control and virus reactivation. *J Gen Virol* **44:** 205–215.

Pardo J, Yogev Y, Ben-Haroush A, Hod M, Amir J (2004). Primary herpes simplex virus type 1 gingivostomatitis during the second and third trimester of pregnancy: foetal and pregnancy outcome. *Scand J Infect Dis* **36:** 179–181.

Peacock JE Jr, Sarubbi FA (1983). Disseminated herpes simplex virus infection during pregnancy. *Obstet Gynecol* **61:** 13S–18S.

Prober CG, Hensleigh PA, Boucher FD, Yasukawa LL, Au DS, Arvin AM (1988). Use of routine viral cultures at delivery to identify neonates exposed to herpes simplex virus. *N Engl J Med* **318:** 887–891.

Sawtell NM, Thompson RL (1992). Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *J Virol* **66:** 2150–2156.

Shimeld C, Hill TJ, Blyth WA, Easty DL (1990). Reactivation of latent infection and induction of recurrent herpetic eye disease in mice. *J Gen Virol* **71(Pt 2):** 397–404.

Smith RN, Hanna L (1974). Herpesvirus infections in pregnancy: a comparison of neutralizing antibody titers in mothers and their infants. *Am J Obstet Gynecol* **119:** 314–319.

Steiner I, Kennedy PG (1995). Herpes simplex virus latent infection in the nervous system. *J NeuroVirol* **1:** 19–29.

Studd JW (1971). Immunoglobulins in normal pregnancy, pre-eclampsia and pregnancy complicated by the nephrotic syndrome. *J Obstet Gynaecol Br Commonw* **78:** 786–790.

Tokumaru T (1966). A possible role of gamma-A-immunoglobulin in herpes simplex virus infection in man. *J Immunol* **97:** 248–259.

Whitley R, Arvin A, Prober C, Corey L, Burchett S, Plotkin S, Starr S, Jacobs R, Powell D, Nahmias A, et al. (1991). Predictors of morbidity and mortality in neonates with herpes simplex virus infections. The National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group. *N Engl J Med* **324:** 450–454.

Yirrell DL, Blyth WA, Hill TJ (1987). The influence of androgens on paralysis in mice following intravenous inoculation of herpes simplex virus. *J Gen Virol* **68(Pt 9):** 2461–2464.

Young EJ, Killam AP, Greene JF, Jr. (1976). Disseminated herpesvirus infection. Association with primary genital herpes in pregnancy. *JAMA* **235:** 2731–2733.