Impact of maternal stress on the transmammary transfer and protective capacity of herpes simplex virus-specific immunity

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MAMMalian NEONATES are born with an immature immune system and are therefore more susceptible to pathogens encountered early in life. Being unable to generate their own protective immune responses, neonates rely heavily on maternally derived immunity acquired both prenatally and postnatally for protection from infection. Mice receive a small portion of maternally derived IgG across the placenta; however, a much larger portion of protective IgG is acquired from the maternal milk (6). This milk-derived IgG is bound by Fc receptors present on the neonatal intestinal mucosa and is subsequently transferred into the serum (18). Hence, milk-derived antibodies represent a large proportion of the circulating IgG in newborn mice and offer protection against pathogens, such as herpes simplex virus (HSV), to which the mother was previously exposed.

It is estimated that 1/3,000 to 1/7,000 human neonates are infected with HSV per year in the United States (23). In stark contrast to adults, in which HSV infection is typically asymptomatic, 95% of neonatal HSV infections are symptomatic (23). Despite antiviral therapy, neonates who develop disseminated infection experience a mortality rate of 50–60% (45). The capacity to resist severe HSV infection is dependent on the passive transfer of HSV-specific immunity from the mother to the offspring. Indeed, a recent report demonstrated that the highest incidence of infection occurred in neonates born to women who were seronegative for HSV, whereas women with a previous history of HSV-2 infection were at a reduced risk for transmitting HSV-2 to their neonate (7). These findings suggested that maternal derived, HSV-specific antibody offers some degree of protection to the neonate. Studies in mice demonstrated that the postnatal transfer of HSV-specific antibody alone is sufficient to protect neonatal mice from HSV-associated mortality (21, 28). This antibody, primarily of the IgG isotype (21, 26), provides protection by both viral neutralization and natural killer cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC; 2, 24).

It is well established that stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and its end product, corticosterone modulate the immune response during both healthy and disease states (reviewed in Ref. 32). Studies in animals have shown that prenatal maternal stress affects the transplacental transfer of immunity and immunocompetence of the offspring (10, 11, 41, 42). However, the effects of maternal stress on the transfer of antibody through the milk have not been investigated. Hence our studies are the first to determine the impact of stress and the associated increases in corticosterone in lactating mice on the passive transfer of immunity and subsequent neonate susceptibility to infection and mortality.

To elicit a stress response in lactating mice, a widely used restraint/light stress model was employed (22, 33, 43, 46, 49). This model has been used in numerous studies to administer stress to rodents and results in elevated serum corticosterone levels in both adult mice and rats. During the maternal restraint/light stress procedure, neonates are subjected to 45-min periods of maternal deprivation. Although neonates typically exhibit a stress hyporesponsive period (37, 44), an age-dependent phenomenon during which potential stressors fail to elevate corticosterone levels, maternal deprivation was shown to increase corticosterone levels in neonates of the CD1 mouse
strains (9, 38). Because our goal was to study the impact of solely maternally derived corticosterone on the transmammary transfer of antibody, we first demonstrated that C57BL/6 neonates exhibit only basal levels of corticosterone when subjected to maternal deprivation. We subsequently used the maternal restraint/light stress procedure to investigate the impact of postnatal stress on the transfer of total and HSV-specific IgG and neonate susceptibility to HSV-2 infection. Restraint/light stress elevated serum corticosterone levels in lactating mice and resulted in an increased transfer of corticosterone to the neonate through the milk. Although previous studies demonstrated decreased transplacental transfer of antibody during prenatal stress (10, 41), postnatal stress did not alter the transmammary transfer of either total or HSV-specific IgG. Interestingly, postnatal stress significantly increased the survival of neonates infected with HSV-2. This increased survival adds to the current literature that indicates that acute exposure to corticosterone may be immunoenhancing (13). Overall, these studies continue to define the role of corticosterone in modulating the passive transfer and development of immunity in the neonate.

METHODS

Mice. Female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 5–6 wk of age and were housed at five mice per cage until breeding pairs were established. Mice were maintained on a 12:12-h light/dark cycle (lights off 1900–0700) and were allowed at least 1 wk to acclimate to these conditions before any experimental manipulation. Food and water were provided ad libitum except during the restraint/light stress procedure. Mice were handled in accordance with American Association for Laboratory Animal Care and National Institutes of Health guidelines.

Virus. HSV-2 strain 186 virus was propagated on Vero cells by infection at a multiplicity of infection (MOI) of 0.01, and virus titer was determined by standard plaque assay on Vero cells. Virus stocks were stored at −70°C.

Maternal immunization and neonatal infection with HSV-2. Similar to previous studies (21), female mice 7–8 wk of age were immunized three times at 1-wk intervals subcutaneously in both of the rear footpads with 1 × 10^5 (first 2 immunizations) and 1 × 10^6 (3rd immunization) plaque-forming units (PFU) of HSV-2 in a volume of 50 μl [PBS/1% (vol/vol) FBS]/footpad. Two-day-old mice were infected intraperitoneally with 1 × 10^5 PFU of HSV-2 in a volume of 10 μl.

Maternal and fostering. At 10–11 wk of age, primiparous HSV-immunized and non-HSV-immunized (naïve) female mice were housed singly with one male. Male and female mice were separated on day 12 postcoitus. At birth, entire litters were randomly assigned to either control or stress groups. Litters were typically comprised of five to eight neonates, and litters of greater than eight were all culled to eight. In a subset of studies, neonates were transferred on day 0 from their nonimmunized mother to an HSV-immunized mother who had delivered less than 24 h before the transfer.

Maternal and neonatal stress. Individual adult female mice were placed into a 4-oz, wide-mouth polypropylene bottle containing ~90 0.5-cm diameter holes for ventilation. The bottle was placed 30 cm from two 150-W lights. The postnatal restraint/light stress procedure was administered twice (1230–1315 and 1630–1715) on the day of delivery (day 0 postnatal) and three times daily (0830–0915, 1230–1315, and 1630–1715) thereafter, continuing through postnatal day 6. Neonatal stress was administered by removing the mother for a period of 45 min, during which time neonatal mice remained within the cotton fiber nest in their home cage. There was no maternal or neonatal mortality associated with these stress models.

Serum collection. Adult mice were euthanized by cervical dislocation, and cardiac puncture was performed. Neonates were anesthetized in a saturated atmosphere of isoflurane and decapitated, and trunk blood was collected. All samples were centrifuged at 16,000 g for 2 min, and serum was collected and stored at −70°C.

Milk collection from postpartum mothers and neonates. Postpartum mice were anesthetized intraperitoneally with ~100 mg/kg pentobarbital sodium (Nembutal, Abbott Laboratories, Abbott Park, IL). At least 5 min before being milked, mice received 1 unit of oxytocin (Sigma) intraperitoneally in a volume of 100 μl PBS/1% (vol/vol) FBS to facilitate milk ejection. Milk was obtained by using an adaptation to a previously described suction apparatus (19). Briefly, the nipples were moistened with water and placed over an 18-gauge, 1-ml syringe that emptied into a weighed 1.5-ml microfuge tube. Milk was obtained by a manually controlled pulsation suction from an Erlenmeyer vacuum flask. Neonatal mice were anesthetized in a saturated atmosphere of isofluran and decapitated, and milk curd was dissected from the stomach. Both maternal and neonatal milk samples were diluted 1:1 (based on weight) with unsupplemented Dulbecco’s modified Eagle’s medium. The diluted milk was mixed for 2 min in a 40-W sonicator and centrifuged for 10 min at 16,000 g (21). The supernatant was collected from below the lipid layer and stored at −70°C.

Quantification of corticosterone. Levels of corticosterone were determined using an RIA kit (ICN; Orangeburg, NY), which measures both free corticosterone and corticosterone bound to corticosterone binding globulin (CBG). The levels of corticosterone were determined using a standard curve generated from standards containing 0–1,000 ng/ml of corticosterone. The mean squared error of the standard curves ranged from 1.1 and 1.5 and had an average r^2 value of 0.99.

Quantification of total and HSV-specific IgG. Total and HSV-specific IgG levels were determined by ELISA as described previously (49). Briefly, plates were coated overnight either with HSV-2 antigen prepared from HSV-infected Vero cells or with anti-mouse IgG for detection of HSV-specific and total IgG, respectively. For determination of HSV-specific IgG, serum samples from adult mice were diluted 1:1,000 and from neonates diluted 1:800 in PBS/10% (vol/vol) FBS. For determination of HSV-specific IgG in the milk, samples from adult mice were diluted 1:100 in PBS/10% FBS. Serial dilutions [in PBS/10% (vol/vol) FBS] of a pooled serum sample from adult mice immunized three times with HSV-2 were used in each assay to generate an HSV-specific IgG standard curve. For determination of total IgG levels, serum samples from adult mice were diluted 1:200,000 and from neonates diluted 1:30,000 in PBS/10% FBS. For determination of total IgG in the milk, samples from adult mice were diluted 1:48,000 in PBS/10% FBS. Serial dilutions of an unlabeled mouse IgG (Fisher Scientific, Pittsburgh, PA) were used in each assay to generate an IgG standard curve. One hundred microliters of the diluted serum samples or standards was assayed in triplicate, whereas the diluted milk samples were assayed in duplicate. Biotinylated anti-mouse IgG (eBioscience, San Diego, CA) or anti-mouse Ig (Pharmingen, San Diego, CA) was used as the detecting antibody for quantification of total and HSV-specific IgG, respectively. In all cases, the specified dilution of serum or milk was chosen such that the resulting optical density values were within the linear portion of the standard curve. The mean squared error of the standard curves ranged from 0.001 to 0.009, and the r^2 values ranged from 0.97 to 0.99. The level of Vero cell antigen-specific antibody in the maternal and neonatal serum and milk was consistently 10–15% of the total optical density.

Detection of HSV-2 in tissues. On days 3, 5, and 7 postinfection, neonates were anesthetized in a saturated atmosphere of isoflurane, decapitated, and briefly frozen at −20°C to facilitate dissection. The brain, spleen, lungs, heart, and kidneys were dissected and frozen at −70°C in 0.5 ml of unsupplemented Iscove’s-modified Dulbecco’s medium. Organs were subsequently thawed and homogenized with an Omni TH homogenizer (Omni International, Warrenton, VA).
mogenates were assayed for infectious virus by standard plaque assay on Vero cells.

Statistical analysis. Statistical significance was assessed by ANOVA using Statview 5.0.1 software (SAS Institute; Cary, NC). Post hoc comparisons between control and stress groups on the indicated days were performed using the Fisher's protected least significance difference test. The Kaplan-Meier method was used to generate survival curves for each group of mice, and the logrank test was used to test for differences between the groups. *P values > 0.05 are considered not significant.

RESULTS

Neonatal mice exhibit a stress hyporesponsive period during maternal deprivation and/or HSV infection. Although the hypothalamic-pituitary-adrenal (HPA) axis is generally hyporesponsive to stressors during the neonatal period (36, 37, 44), previous studies demonstrated that maternal deprivation may result in increased circulating levels of corticosterone in the neonate (9, 38). Because 45-min periods of maternal deprivation are inherent to our stress model, it was important to first demonstrate that maternal deprivation alone does not result in elevated levels of corticosterone in the neonate.

Neonates exposed to a single session of maternal deprivation were killed, and blood from maternally deprived and nondeprived control neonates was assessed for levels of serum corticosterone. Mice 1–7 days of age subjected to maternal deprivation showed no increase in serum corticosterone above that of nondeprived controls (Fig. 1A). With the exception of day 1, when neonatal corticosterone levels are typically at their highest (3), the levels of serum corticosterone in all groups of mice 7 days of age and younger were very low compared with the corticosterone levels typically present in nonstressed adult mice. Mice that were subjected to the same period of maternal deprivation at 21 days of age exhibited a stress response as indicated by significantly increased levels of corticosterone relative to that of nondeprived controls (P = 0.007), whose corticosterone levels were within a range similar to that of nonstressed adult mice. Even when exposed to a somewhat extreme physical stressor (physical shaking), neonates still exhibited no increase in the levels of serum corticosterone relative to nondisturbed controls (data not shown). These maternal deprivation studies suggest that neonatal mice indeed exhibit a stress hyporesponsive period after exposure to maternal deprivation.

Viral infection in adult mice has previously been shown to elevate serum corticosterone levels (15). Because our model requires that maternally deprived neonates also be infected with HSV, it was necessary to ensure that infection does not itself result in increased corticosterone levels. Two-day-old mice born to either HSV-immunized or nonimmunized mothers were infected intraperitoneally with 1 × 10² PFU of HSV-2. At 1, 3, and, if possible, 5 days postinfection, neonates were either exposed to maternal deprivation or remained undisturbed with their mothers. The levels of serum corticosterone were determined after the 45-min period of maternal deprivation. No significant changes in serum corticosterone levels were observed when HSV-infected neonates were exposed to maternal deprivation on any of the days assessed (Fig. 1B). These findings indicate that neither HSV infection alone nor HSV infection coupled with maternal deprivation increases the levels of corticosterone in mice of this age. This demonstra

![Graph A](http://ajpregu.physiology.org/)

**Fig. 1.** Neonatal mice exhibit a stress hyporesponsive period during maternal deprivation. A: mice 1–7 and 21 days of age remained in their nest in the home cage for 45 min after the mother was removed. Levels of serum corticosterone were determined in both these mice and control groups of mice that had remained with their mothers for the entire 45-min period. B: mice born to herpes simplex virus (HSV)-immunized or nonimmunized mothers were infected at 2 days of age with 1 × 10² plaque-forming units (PFU) of HSV-2. On days 1, 3, and, if possible, day 5 postinfection, mice were exposed to 45 min of maternal deprivation. Levels of serum corticosterone were determined in both these mice and control mice that remained with their mother for the entire 45-min period. Values represent means ± SE. *P ≤ 0.05.

Postnatal restraint/light stress increases the levels of corticosterone in lactating mice and results in the transfer of corticosterone to the neonate through the milk. To our knowledge, restraint/light stress has not been used to increase corticosterone levels in lactating mothers. Therefore, it was important to demonstrate that the postnatal restraint/light stress procedure would indeed elevate serum corticosterone levels in the lactating mice used in our model. Lactating mice were subjected to restraint/light stress beginning on day 0 postnatal and continuing through postnatal day 6. Serum corticosterone levels were measured in both stressed and nonstressed control mice on postnatal days 2, 4, and 6 immediately after the first session of stress (0830–0915), a time when basal levels of corticosterone are typically at their lowest of the day. The data
Maternal postnatal restraint/light stress increases the levels of corticosterone in lactating mice and results in the transfer of corticosterone to the neonate. Lactating mice were exposed to postnatal restraint/light stress beginning on the day of delivery. A: serum corticosterone levels were determined in stressed and nonstressed control mice on postnatal days 2, 4, and 6 immediately after the first daily session of maternal stress (0830–0915). Data are expressed as the combined average percentage of control from 2 independent experiments. B: corticosterone levels were determined in milk obtained from the stomachs of neonates nursing on stressed and nonstressed mothers on postnatal days 2, 4, and 6 1 h after the first daily session of maternal stress. Values represent means ± SE. *P < 0.05.

are expressed as the combined average percentage of control (nonstressed) from two independent experiments. Postnatal stress resulted in significantly increased levels of serum corticosterone on each of the days (day 2, P = 0.0005; day 4, P = 0.01; day 6, P = 0.02) compared with nonstressed control mice (Fig. 2A). The serum corticosterone levels in the stressed and nonstressed control mice ranged from 250 to 500 ng/ml and 50 to 100 ng/ml, respectively. Because the increased levels of maternal corticosterone could possibly be transferred to the neonate through the milk, both milk curd and serum were obtained from neonates on postnatal days 2, 4, and 6, 1 h after the first session of maternal stress and were assessed for levels of corticosterone. Neonates nursed by stressed mothers exhibited significantly elevated levels of corticosterone in the milk relative to neonates nursed by nonstressed control mothers (days 2 and 4, P < 0.0001; day 6, P = 0.005; Fig. 2B). Thus postnatal restraint/light stress resulted in the increased transfer of corticosterone to the neonate. No significant increase in serum corticosterone levels was detected in neonates nursed by postnatally stressed females (data not shown), most likely because the corticosterone was not transferred from the milk into the serum within 1 h of nursing. However, previous studies suggest that milk-derived corticosterone can indeed cross the epithelial barrier of the intestine (4). Recent studies from our laboratory have demonstrated that milk-derived corticosterone does eventually result in significantly increased levels of corticosterone in neonatal serum (50). In addition to receiving increased levels of corticosterone, neonates nursed by stressed mothers exhibited a reduction in weight at both 4 (P = 0.0001) and 6 (P = 0.03) days of age (Table 1).

Transmammary transfer of total and HSV-specific IgG is unaffected by postnatal restraint/light stress. We were interested in whether postnatal stress would affect the transmammary transfer of both total and HSV-specific IgG to the neonate. However, to determine the amount of maternal antibody transferred to the neonate, it was important to first determine whether postnatal stress would alter the total and HSV-specific IgG levels present in the mother. A subset of HSV-immunized mothers was exposed to restraint/light stress on days 0–6 postnatal. Because there is a positive correlation between the levels of IgG in maternal serum and milk (1), both serum and milk samples were obtained from the stressed and nonstressed mothers on days 2, 7, and 14 postnatal and were analyzed for total and HSV-specific IgG levels by ELISA. The levels of total and HSV-specific IgG remained unchanged in the mothers over each of the days tested; therefore, the data are represented as an average of all days analyzed. Postnatal stress did not alter the total IgG levels in either the maternal serum (Fig. 3A; P = 0.22) or milk (Fig. 3B; P = 0.14). Similarly, the HSV-specific IgG levels in the maternal serum (Fig. 3C; P = 0.26) and milk (Fig. 3D; P = 0.91) were unaffected by stress. Together, these findings indicate that postnatal maternal stress and the associated increases in corticosterone do not affect the amount of antibody present in maternal serum or milk. Having demonstrated no change in the mother’s antibody levels, we determined whether postnatal maternal stress would affect the kinetics with which the total and HSV-specific IgG accumulated in the neonate’s serum. A subset of mice born to nonimmunized mothers was fostered immediately after delivery onto lactating HSV-immunized mothers. In this manner, the neonates could only obtain the HSV-specific antibody by the transmammary route. Serum samples were obtained from neonates nursing on postnatally stressed and nonstressed HSV-immunized foster mothers on days 2, 7, and 14 postnatal and analyzed for total and HSV-specific IgG by ELISA. There was a significant increase in the total IgG levels between days 2 and 7 in neonates nursing on either stressed (P = 0.03) or nonstressed control mothers (P < 0.0001; Fig. 4A). Postnatal maternal stress did not affect the total IgG levels present in neonate serum on either day 2, 7, or 14. After only 2 days of nursing on HSV-immunized mothers, neonates had acquired ~15% of the mother’s HSV-specific IgG levels (Fig. 4B). From days 2 to 7 and from days 7 to 14, the levels of HSV-specific antibody increased markedly (P < 0.001 for all

Table 1. Weight of neonates nursing on either postnatally stressed or nonstressed control mothers

<table>
<thead>
<tr>
<th>Days Postnatal</th>
<th>Nonstressed mother</th>
<th>Postnatally stressed mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.27±0.03 (13)</td>
<td>1.34±0.02 (11)</td>
</tr>
<tr>
<td>2</td>
<td>1.38±0.04 (12)</td>
<td>1.32±0.03 (11)</td>
</tr>
<tr>
<td>4</td>
<td>2.53±0.10 (12)</td>
<td>1.86±0.03*(16)</td>
</tr>
<tr>
<td>6</td>
<td>2.76±0.05 (13)</td>
<td>2.41±0.15*(14)</td>
</tr>
</tbody>
</table>

Values are means ± SE (no. of mice). *P < 0.05.
comparisons) in neonate serum, such that by 2 wk of age, neonates had acquired ~90% of the mother’s HSV-specific antibody levels. Similar to those findings for total IgG, postnatal stress did not alter the accumulation of HSV-specific antibody in the serum. These findings indicate that the transmammary transfer and accumulation of total and HSV-specific IgG is resistant to postnatal maternal stress.

Postnatal restraint/light stress increases neonate survival after HSV-2 infection despite no apparent protection from viral spread. Although the transmammary transfer of total and HSV-specific antibody was not affected by postnatal maternal stress, we knew that elevated levels of corticosterone were reaching the neonate through the milk (Fig. 2). We hypothesized that this increased corticosterone may affect the ability of the neonate to use the maternally derived antibody and thus alter the susceptibility of the neonate to HSV infection. Hence we examined whether postnatal maternal stress would affect viral spread and the overall susceptibility of the neonate to HSV-associated mortality.

Neonates either remained with their nonimmunized or HSV-immunized mothers or were fostered immediately after delivery from nonimmunized mothers onto HSV-immunized foster mothers. A subset of these HSV-immunized lactating mothers was exposed to postnatal restraint/light stress beginning on the day of delivery. Neonates born to nonimmunized mothers were fostered onto HSV-immunized mothers immediately after delivery. A subset of the HSV-immunized mothers was subjected to postnatal restraint/light stress beginning on the day of delivery. Serum samples were obtained on days 2, 7, and 14 postnatal from neonates nursing on stressed and nonstressed control mothers and were analyzed for total (A) and HSV-specific (B) IgG by ELISA. Values represent means ± SE. *P < 0.05.
of maternally derived, HSV-specific antibody, neonatal mice are indeed protected from extensive viral spread. Those neonates fostered from their nonimmunized mothers onto nonstressed HSV-immunized mothers and therefore receiving the HSV-specific antibody by only the transmammary route were protected from extensive viral spread but had detectable levels of virus on each of the days assessed. This finding indicates that the transmammary transfer of HSV-specific antibody alone is not nearly as protective against infection compared with antibody received by both transplacental and transmammary means. Neonates born to nonimmunized mothers and fostered onto postnatally stressed or nonstressed HSV-immunized mothers had similar scores on all days examined. Therefore, postnatal maternal stress and the associated increases in maternal and neonatal corticosterone did not significantly affect either the rate or extent of viral spread in the neonate.

To determine whether postnatal maternal stress would alter the susceptibility of neonates to HSV-associated mortality, neonates either remained with their nonimmunized or HSV-immunized mothers or were fostered immediately after delivery from nonimmunized mothers onto HSV-immunized mothers. A subset of these HSV-immunized lactating mice was exposed to postnatal restraint/light stress beginning on the day of delivery. In accordance with the rapid viral spread, all but one neonate remaining with their nonimmunized mother succumbed to HSV infection by day 10 postinfection (Fig. 5A). In the absence of fostering, the majority of those neonates nursing on either postnatally stressed or nonstressed, HSV-immunized mothers and receiving the HSV-specific antibody by both transplacental and transmammary means was protected against HSV-associated mortality (Fig. 5A). To determine whether postnatal stress would affect the survival of neonates who could only receive the HSV-specific antibody by transmammary means, neonates were fostered from nonimmunized mothers onto HSV-immunized mothers and infected at 2 days of age. Neonates nursing on nonstressed HSV-immunized mothers and only receiving the HSV-specific antibody by the transmammary route exhibited ~50% survival (Fig. 5B), indicating once again that the transmammary transfer of HSV-specific antibody alone is not as protective as the combination of transplacental and transmammary antibody transfer. Interestingly, neonates fostered onto HSV-immunized, postnatally stressed mothers exhibited a significant increase in survival ($P = 0.0007$) compared with those neonates who nursed on HSV-immunized nonstressed mothers. Thus,

Table 2. Detection of infectious HSV in neonates remaining with their nonimmunized or HSV-immunized mothers or fostered onto nonstressed or postnatally stressed HSV-immunized mothers

<table>
<thead>
<tr>
<th>Days Postinfection</th>
<th>Nonimmunized mother</th>
<th>Immunized mother</th>
<th>Nonimmunized mother</th>
<th>Immunized mother</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Fostering</td>
<td>Fostering</td>
<td>Average Score</td>
<td>Fostering</td>
</tr>
<tr>
<td>3</td>
<td>2.38±0.46 (8)</td>
<td>0.57±0.30 (7)</td>
<td>0.36±0.13 (14)</td>
<td>0.10±0.42 (8)</td>
</tr>
<tr>
<td>5</td>
<td>3.00±0.71 (4)</td>
<td>0±0.00 (6)</td>
<td>1.00±0.42 (8)</td>
<td>0.54±0.27 (8)</td>
</tr>
<tr>
<td>7</td>
<td>NT</td>
<td>0±0.00 (4)</td>
<td>0.88±0.40 (8)</td>
<td>1.20±0.20 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE (no. of mice). Mice were scored based on the presence of infectious herpes simplex virus (HSV) in the brain, spleen, lungs, kidneys, and heart (e.g., 1 = 1/5 organs infected, 5 = 5/5 organs infected). NT, not tested; all neonates died by day 7 postinfection.

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Fig. 5. Maternal postnatal restraint/light stress increases the protective capacity of HSV-specific immunity. A: neonates remained with their nonimmunized or HSV-immunized mothers. A subset of the HSV-immunized mothers was subjected to postnatal restraint/light stress beginning on the day of delivery. B: neonates born to nonimmunized mothers were fostered immediately after delivery onto HSV-immunized mothers. A subset of the HSV-immunized mothers was subjected to postnatal restraint/light stress beginning on the day of delivery. In A and B, all neonates were infected intraperitoneally at 2 days of age with $1 \times 10^2$ PFU of HSV-2 in a volume of 10 μL. Neonatal mice were monitored daily for mortality until 21 days postinfection. Data are representative of 2 independent experiments. For the survival data presented in A, 8 litters of mice comprised both the HSV-immunized, nonstress (8 litters, $n = 41$), and HSV-immunized, postnatal stress (8 litters, $n = 40$) groups. SE for these nonstress and stressed groups were only 4.5% and 7.6%, respectively. For the data presented in B, the nonstress group was comprised of 7 litters ($n = 41$) and the stressed group was comprised of 9 litters ($n = 58$). SE for these groups were only 10.7% and 5.1%, respectively.
Although maternal stress did not affect the rate and extent of virus spread in the neonate, it did increase neonate survival by an as of yet undetermined mechanism.

**DISCUSSION**

It is widely accepted that the development of an immune response to infectious pathogens in the adult animal is partially regulated by HPA axis activation and the associated increases in corticosterone (reviewed in Ref. 5). However, recent studies indicate that maternal stress-induced increases in corticosterone can be transferred to the fetus and neonate and negatively affect the developing nervous, endocrine, and immune systems (reviewed in Refs. 39, 40). Although previous studies have determined the effects of prenatal maternal stress on the transplacental transfer of antibody (10, 41, 42), to our knowledge, no one has investigated the effects of postnatal maternal stress on the transfer of antibody through the milk. These studies are important because human and murine neonates acquire substantial amounts of milk-derived IgA and IgG, respectively, which protects them from a variety of infections early in life.

The HPA axis is hyporesponsive to stressors during the neonatal period in rats and mice (37, 44), resulting in very low circulating levels of corticosterone. This stress hyporesponsive period was recently defined in neonatal mice (37) and partially results from the failure of the pituitary to secrete adenocorticotropic hormone (36). However, previous studies demonstrated increased serum corticosterone levels in 9- and 12-day-old CD1 mice exposed to 24 h of maternal deprivation (9, 38). Therefore, before attempting to examine the effects of increased maternal corticosterone on neonatal susceptibility to infection, it was important for us to demonstrate that neonatal C57BL/6 mice exhibit a stress hyporesponsive period during the relatively short 45-min period of maternal deprivation that is inherent to the restraint/light stress model. Corticosterone levels were not increased in either uninfected (Fig. 1A) or HSV-infected (Fig. 1B) mice younger than 7 days of age that were exposed to a single 45-min period of maternal deprivation. These studies correlate with previous findings that demonstrated that 4- and 8 day-old CD1 mice do not exhibit elevated levels of corticosterone in response to 24 h of maternal deprivation (9). Combined, our studies and previous studies suggest that neonatal mice do not exhibit elevated corticosterone levels in response to maternal deprivation until after the first week of life. In our model, neonates are only subjected to maternal deprivation on days 0 through 6 postnatal. Hence we were unconcerned with determining the neonate’s response to maternal deprivation beyond 7 days of age. However, to verify that maternal deprivation can indeed be perceived as a stressful event in C57BL/6 mice, we subjected 21-day-old mice to maternal deprivation. These mice responded to the maternal deprivation with increased levels of serum corticosterone (Fig. 1A), indicating that 45 min of maternal deprivation can indeed be perceived as a stressful event in mice whose HPA axis is fully functional.

Previous studies from our laboratory have used the restraint/light stress procedure to elevate serum corticosterone levels in pregnant mice (49). However, to our knowledge, the restraint/light stress procedure has not been used to elevate serum corticosterone levels in lactating mice. Lactating mice exposed to restraint/light stress had markedly increased levels of serum corticosterone (Fig. 2A), which resulted in increased levels of corticosterone being transferred to the neonate through the milk (Fig. 2B). Although the levels of corticosterone in the milk obtained from neonates nursing on stressed mothers was higher than in neonates nursing on nonstressed mothers on each of the 3 days examined, there was a steady decline in these levels of corticosterone over time. This decline in corticosterone indicates that the amount of corticosterone present in maternal milk may decrease as the mothers adapt to the restraint/light stress procedure. Although increased levels of corticosterone were detected in milk curd obtained from neonates nursing on postnatally stressed mothers, elevated levels of serum corticosterone were not detected in the neonates. This is most likely a result of the relatively short nursing period (1 h) allowed after maternal stress and before sample collection. Importantly, previous studies from our laboratory demonstrated significantly increased serum corticosterone levels in neonates nursing on mothers with elevated corticosterone levels (50). Although we observed no changes in maternal feeding behavior, neonates nursed by postnatally stressed mothers weighed less at 4 and 6 days of age (Table 1). This failure to gain weight as rapidly as neonates nursed by nonstressed mothers may result from stress-induced suppression of maternal lactation efficiency (29, 30). In addition, the neonates may weigh less due to the decreased amount of time the neonates are able to nurse on their mothers as a result of the restraint/light stress procedure.

Both human and murine neonates receive a significant portion of their protective antibody from maternal milk. Therefore we were interested in determining the impact of maternal stress on the kinetics with which both total and HSV-specific IgG accumulate in the serum of neonates. Both total (Fig. 4A) and HSV-specific IgG (Fig. 4B) were detected in neonate serum on day 2 postnatal. Peak IgG levels were reached by day 7 postnatal, whereas HSV-specific IgG levels continued to increase through day 14 postnatal. These results are similar to studies in neonatal rats in which the absorption of orally administered antibody was shown to be slow shortly after birth but reached peak levels by 14 days of age (31). Absorption of maternal IgG terminates abruptly in mice around postnatal day 16 (18). Treatment of young rats with high doses of cortisol acetate, a corticosterone derivative, resulted in an early termination of antibody absorption (20), indicating that corticosterone may affect the accumulation of antibody in neonate serum. However, our model of maternal stress failed to alter the accumulation of either total or HSV-specific IgG in the serum (Fig. 4). This discrepancy is likely due to the presence of lower concentrations of corticosterone in the neonate due to the acute nature of the stressor used in the current study. We consider our restraint/light stress procedure to be acute because maternal corticosterone levels return to baseline within 4 h after the termination of the stressor (33).

In the absence of maternally derived, HSV-specific antibody, neonatal and young C57BL/6 mice are extremely susceptible to HSV-associated mortality (49). The combined transplacental and transmammary transfer of HSV-specific antibody confers nearly complete protection of neonates from HSV-associated mortality (Fig. 5A). The transmammary transfer of HSV-specific IgG alone also provides neonatal mice with
Some protection from HSV-associated mortality; however, the level of protection achieved is typically only 50% (Fig. 5B). Importantly, this level of protection allowed us to determine whether postnatal maternal stress could either increase or decrease neonatal susceptibility to HSV-associated mortality. Interestingly, postnatal maternal stress significantly increased the survival of neonates who were infected with HSV at 2 days of age. However, there was no apparent alteration in the extent of virus spread (Table 2). One explanation for this finding may be the relatively low sensitivity of the plaque assay, which may prevent us from detecting small difference in viral titer. In addition, there may be differences in the titer of virus in regions not examined, such as the spinal cord.

Previous studies documented enhancement of both innate and adaptive immunity after exposure of animals to an acute stressor (reviewed in Ref. 13). Such enhancement of immunity may be mediated by corticosterone or several other immunomodulatory factors such as corticotrophin-releasing hormone, norepinephrine, and substance P. Potential alterations to an ensuing immune response include increased lymphocyte trafficking to the site of antigen deposition (14), heightened macrophage and complement activity (12, 17), and increased proinflammatory cytokine gene expression (34, 51). In our model, the precise mechanism(s) by which stress is conferring enhanced protection is unknown. Although studies have demonstrated that corticosterone (16) and norepinephrine (35) increase antigen-specific antibody production, the inability of neonates to produce their own HSV-specific antibody suggests that the maternal stress is likely enhancing some aspect of innate immunity rather than adaptive immunity. Indeed, a recent study indicates that acute stress induces extracellular heat shock protein 72, which, in turn, potentiates the production of nitric oxide and proinflammatory cytokines leading to faster resolution of bacterial infection (8). It is possible that maternal stress-induced hormones may be altering the proinflammatory environment within the neonate and therefore altering the trafficking of neonatal and milk-derived lymphoid cells into infected organs (47, 48). Because neonatally derived HSV-specific antibody provides protection through both viral neutralization and antibody-dependent cell-mediated cytotoxicity (2, 24), an increased trafficking of natural killer cells could allow for more efficient utilization of the neonatally derived antibody in the lysis of HSV-infected cells (25). Increased antibody-dependent cell-mediated cytotoxic activity has been correlated with decreased clinical presentation of human HSV infection (27) and may result in more rapid resolution of the HSV infection and enhanced survival.

The current studies add to the growing literature that suggests a potential role for corticosterone in the regulation of the passive transfer of immunity and overall neonatal immunocompetence. Whereas previous studies demonstrated a decreased transplacental transfer of antibody during prenatal stress (10, 41), our results demonstrate that acute postnatal maternal stress does not alter the transmammary transfer of antibody. Rather, postnatal maternal stress increased the survival of neonates infected with HSV-2. Future studies will determine whether the increased survival of the neonate is mediated solely by the presence of increased levels of corticosterone or if other stress-induced neuroendocrine-mediated mechanisms are responsible for these observations. In addition, we will also determine whether this increased survival is the result of an alteration in the innate and/or adaptive immune response after HSV infection.

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