Late Pregnancy Suppresses Relapses in Experimental Autoimmune Encephalomyelitis: Evidence for a Suppressive Pregnancy-Related Serum Factor

Annette Langer-Gould, Hideki Garren, Amy Slansky, Pedro J. Ruiz and Lawrence Steinman

*J Immunol* 2002; 169:1084-1091; 
doi: 10.4049/jimmunol.169.2.1084

http://www.jimmunol.org/content/169/2/1084

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**
This article cites 33 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/169/2/1084.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2002 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Late Pregnancy Suppresses Relapses in Experimental Autoimmune Encephalomyelitis: Evidence for a Suppressive Pregnancy-Related Serum Factor

Annette Langer-Gould,² Hideki Garren, Amy Slansky, Pedro J. Ruiz, and Lawrence Steinman

Women with multiple sclerosis have significantly diminished disease activity during pregnancy. The purpose of our study was to identify the underlying mechanism for the diminished disease activity. We found that during the period of late pregnancy there is protection against paralysis, during both the induction and effector phases of relapsing experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. We did not find any changes in the cytokine secretion profiles or the proliferative activity of autoreactive T cells from mice induced during late pregnancy compared with virgin controls. In mice mated after disease onset, the inflammatory histologic lesions did not clear, despite marked clinical improvement during pregnancy. We found evidence for a serum factor present in late pregnancy that suppresses T cell activation. In the presence of sera taken from mice late in pregnancy, the proliferative response and IL-2 production of proteolipid protein p139–151-specific T cells were significantly diminished as compared with stimulation in the presence of normal mouse sera. In conclusion, serum from late pregnancy has the capacity to down-regulate T cell responses and might be associated with the amelioration of disease activity in experimental autoimmune encephalomyelitis. The Journal of Immunology, 2002, 169: 1084–1091.

Multiple sclerosis (MS)³ is a chronic inflammatory demyelinating illness of the CNS thought to be caused by autoreactive T cells targeting myelin Ags. Women with MS outnumber men with a 2:1 ratio. Remarkably, women with MS have significantly diminished disease activity during late pregnancy (1). The magnitude of this effect far exceeds the benefits from any currently available pharmacological treatments.

Many theories have been proposed to explain the disease-modifying influence of pregnancy on autoimmune and certain infectious diseases. The most popular of these is a generalized Th2 shift in cytokine secretion, perhaps induced by the high circulating levels of estrogens or progesterone (2). Doses of estradiol typical of levels during pregnancy have been shown to significantly enhance IL-10 secretion from Ag-stimulated proteolipid protein (PLP)-specific T cell clones isolated from MS patients (3). Furthermore, PBMCs from normal pregnant women secrete higher amounts of IL-10 when stimulated with PHA as compared with nonpregnant controls (4). IL-10 is thought to ameliorate disease activity in MS (5).

Experimental autoimmune encephalomyelitis (EAE), an animal model of MS, is a prototypic T cell-mediated, organ-specific autoimmune disease. Previously published studies on EAE susceptibility during pregnancy have shown a strain-dependent effect on the induction phase of the disease (6). A decreased incidence of EAE induction in pregnant guinea pigs, rabbits, and Lewis rats has been reported (7–9). No studies have been conducted examining the effect of pregnancy during the relapsing phase of EAE, or in susceptible mice strains.

The purpose of our study was to identify an EAE model in which the disease-modifying effects of pregnancy are similar to that in humans with MS, and to determine the underlying mechanism for diminished disease activity. We studied the effect of pregnancy on both the induction and relapsing phases of EAE in SJL mice. Our data show significantly reduced number of relapses during pregnancy in SJL mice with preexisting EAE and a lower incidence of paralyzed mice, when EAE was induced during late pregnancy. We were unable to identify any change in the proliferative response or profiles of cytokine secretion in autoreactive T cells from mice induced during late pregnancy as compared with virgin controls.

Surprisingly, the CNS inflammatory lesions did not clear during pregnancy in the mice with EAE, despite marked clinical improvement. We found evidence for a serum factor circulating in late pregnancy that suppresses T cell activation. In the presence of late pregnancy sera, the proliferative response to autoantigen and IL-2 production was significantly diminished as compared with stimulation in the presence of normal mouse sera. We conclude that the amelioration of disease activity during late pregnancy seen in EAE of SJL/J mice is due to a transient, circulating immunosuppressive factor, rather than a Th2 shift.

Materials and Methods

Mice and matings

Six- to 8-wk-old female SJL/J mice and PL/J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Two females and one male were housed per cage until a vaginal plug was observed, at which time the pregnant females were housed with other pregnant females. Females were checked daily for the presence of a vaginal plug. The day on which the plug was observed was considered to be day 0 of pregnancy. Pups were euthanized within 48 h of delivery.

Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA 94305

Received for publication January 31, 2002. Accepted for publication May 2, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 H.G. was supported by National Institutes of Health Grant 1K08AI01494-01. P.J.R. was supported by National Research Service Award Grant AI07290-15 from the National Institutes of Health.

2 Address correspondence and reprint requests to Dr. Annette Langer-Gould, Beckman Center for Molecular Medicine B002, Stanford, CA 94305-5316. E-mail address: annettel@stanford.edu

3 Abbreviations used in this paper: MS, multiple sclerosis; E2, 17β-estradiol; E3, estranol; EAE, experimental autoimmune encephalomyelitis; LNC, lymph node cell; PLP, proteolipid protein.

Copyright © 2002 by The American Association of Immunologists, Inc.

0022-1767/02/$02.00
Antigens

Peptides were synthesized on a peptide synthesizer (model 9050; MilliGen, Burlington, MA) by standard 9-fluorenemethoxycarbonyl chemistry. Peptides were purified by HPLC. Structure was confirmed by amino acid analysis and mass spectrometry. Peptides used for the experiments were: PLP139–151 (HSLGKWLGHPDKF) and Vβ5.1 CDR1 (IKGERSILKCIPSGMLSVA).

Immunization and adoptive EAE induction

PLP139–151 was dissolved in PBS to a concentration of 2 mg/ml and emulsified with an equal volume of IFA supplemented with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Mice were injected s.c. with 0.1 ml peptide emulsion. Pregnant mice were immunized during the third trimester (days 14–16). For adoptive transfer of cells, draining lymph nodes were harvested 10 days postimmunization and incubated in enriched RPMI (RPMI 1640 supplemented with 1-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mg/ml), and 2-ME (5 x 10^-5 M)), supplemented with 1% syngeneic, heat-inactivated mouse sera with 10 µg/ml PLP139–151 at 3 days of concentration at 5 x 10^6 cells/ml. Following the 3 days of in vitro stimulation, the cells were injected i.v. (1 x 10^7 cells/0.3 ml sterile PBS per mouse) into naive, virgin SJL mice. Experimental animals were scored as follows: 0, no clinical disease; 1, tail weakness or paralysis; 2, hind limb weakness; 3, monoplegia; 4, paraplegia; 5, moribund or dead.

Cytokine determination

Draining lymph node cells (LNC; 10^7) were taken from experimental animals 10–12 days following immunization and stimulated in vitro with 10 µg/ml PLP139–151. After 24, 72, and 96 h of stimulation, supernatants were collected and tested by ELISA using the following OptEIA kits for IL-2, IL-10, IFN-γ, and IL-4 (BD Pharmingen, San Diego, CA). Total TGFB1 levels in virgin and late pregnant mouse sera (days 15–17) were determined by ELISA kit from Genzyme (Cambridge, MA), according to the manufacturer’s directions.

ELISA for anti-PLP139–151 Ab titers

Polyethylene 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with 0.1 ml PLP139–151 diluted in PBS at a concentration of 10 µg/ml PLP139–151. After 24, 72, and 96 h of incubation, supernatants were collected and tested by ELISA using the following OptEIA kits for IL-2, IL-10, IFN-γ, and IL-4 (BD Pharmingen, San Diego, CA). Mouse sera were incubated for 2 h at room temperature, and Ab binding was tested by the addition of alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Birmingham, AL). After the addition of the enzyme substrate, plates were read at 405 nm in an ELISA reader.

LNC proliferative assays

Draining lymph nodes were removed from mice 10–12 days after immunization and tested in vitro for specific proliferative responses to PLP139–151. Cultures were prepared in flat-bottom 96-well microtiter plates in a volume of 0.2 ml/well at a cell concentration of 2.5 x 10^5/ml in enriched RPMI (see above) and supplemented with either 1–2% of virgin, female SJL mouse sera, or syngeneic sera obtained during late pregnancy. After 72 h of incubation at 37°C, cells were pulsed for 18 h with 1 µCi/well of [3H]thymidine. Plates were harvested, and [3H]thymidine incorporation was measured in a scintillation counter.

Histology

Experimental animals were sacrificed 25–40 days after immunization. Brains were removed and fixed in 5% formalin. Following 1 wk of fixation, paraffin sections were prepared and stained with H&E.

Statistical analysis

Experiments were repeated at least three times. Data are presented as means ± SEM. Student’s t test was used to calculate the significance of the mean proliferative response, mean cytokine levels from triplicate measurements, mean day of onset, and maximum disease score. The significance of incidence of disease was calculated using χ^2 test.

Results

Pregnancy ameliorates disease in mice with preexisting EAE, without abolishing histologic infiltrates

To determine whether pregnancy ameliorates EAE in mice, as seen in humans with MS, SJL mice were immunized with PLP139–151 in CFA, and mated with PL/J males 2 days after the onset of disease. The number of relapses and disease severity were significantly reduced during the latter part of pregnancy in mice challenged for EAE before pregnancy (Table I) as compared with age- and disease duration-matched, virgin controls. The mice that became pregnant all suffered at least one relapse before the second half of pregnancy, and resumed a normal relapsing pattern shortly following delivery (Fig. 1). Infertility and increased spontaneous abortions were not observed in these mice. Most mice were successfully mated during the recovery phase of their first attack, with the average disease severity on the first day of pregnancy being 1.6.

Histologic examination of four mice from each group showed no significant difference in the severity of histologic infiltrate within the brain (Table II), despite marked clinical improvement in the pregnant group. Together these data suggest that the encephalitogenic lymphocytes are locally suppressed within the CNS and that a neuroprotective mechanism may also contribute to improvement in clinical disease scores during late pregnancy.

Mice immunized during pregnancy are less susceptible to EAE

To examine the effect of pregnancy on EAE disease severity and incidence, SJL mice were immunized during early (days 2–7), mid (days 8–13), and late (days 14–16) pregnancy with PLP139–151 in CFA. No disease protection was observed in the early pregnancy group (four of five developed EAE). When immunized during mid- and late pregnancy, there was a significant reduction (p = 0.003) in the incidence of EAE. There was no difference, however, in the mean day of onset or mean peak disease severity as compared with controls (Table III).

Histologic examination of the brains showed evidence of mild inflammatory infiltrates in the mice induced during pregnancy that did not develop EAE. In experimental animals that did develop EAE, no significant difference in inflammatory infiltrates was noted as compared with controls (n = 2 from each group) (Table IV).

Mice immunized during late pregnancy have normal type 1 cytokine and proliferative responses to the PLP peptide

The possibility of a shift in cytokine secretion profile, conferring resistance to EAE induction during late pregnancy, was tested by examining the amount of IL-2, IFN-γ, IL-4, and IL-10 secretion in cultures of draining LNC. Ten days after immunization, mice induced for EAE during late pregnancy and virgin controls were sacrificed, and draining lymph nodes were harvested. The LNC from individual mice were cultured in the presence of PLP139–151 in CFA and mated with PL/J males 2 days after the onset of disease. The number of relapses and disease severity were significantly reduced during the latter part of pregnancy in mice challenged for EAE before pregnancy (Table I) as compared with age- and disease duration-matched, virgin controls. The mice that became pregnant all suffered at least one relapse before the second half of pregnancy, and resumed a normal relapsing pattern shortly following delivery (Fig. 1). Infertility and increased spontaneous abortions were not observed in these mice. Most mice were successfully mated during the recovery phase of their first attack, with the average disease severity on the first day of pregnancy being 1.6.

Histologic examination of four mice from each group showed no significant difference in the severity of histologic infiltrate within the brain (Table II), despite marked clinical improvement in the pregnant group. Together these data suggest that the encephalitogenic lymphocytes are locally suppressed within the CNS and that a neuroprotective mechanism may also contribute to improvement in clinical disease scores during late pregnancy.

| Table I. | Effect of pregnancy on the course of EAE in SJL/J mice |
|---------------------------------|------------------|------------------|------------------|------------------|
| Relapses^a| Maximum Disease Score^a | Relapses^a | Maximum Disease Score^a |
| Virgin | Pregnant | Virgin | Pregnant |
| Prepartum | 2 | 2 | 3.5 ± 0.1 | 3.3 ± 0.3 |
| Early pregnancy | 14 | 10 | 2.8 ± 0.1 | 2.6 ± 0.3 |
| Late pregnancy | 16 | 2^b | 2.8 ± 0.1 | 1.2 ± 0.3^a |

^a Total number of relapses in each group are reported (n = 12 per group). ^ Maximum disease score is given as mean ± SEM. ^ Pregnant: female SJL/J mice immunized with PLP139–151 in CFA age and disease duration matched to pregnancy group. ^ SJL/J female mice were immunized with PLP139–151 in CFA. Two days after the onset of disease, half of the mice were mated with PL/J males. The beginning of pregnancy was determined by the presence of a vaginal plug. ^ Duration of disease prior to beginning of pregnancy varied, from 1 to 16 days (mean = 7 days). ^ Pregnancy days 1 to 10. ^ Pregnancy days 11 to delivery (duration of pregnancy ranged from 18 to 21 days, mean = 19 days). ^ Values of p < 0.001, in comparison with virgin controls by Student’s t test.
Cytokine secretion was determined by ELISA. No significant difference in the amount of IL-2, IFN-γ, IL-4, or IL-10 \((n = 7)\) was seen in the mice induced during late pregnancy as compared with controls (Fig. 2).

**Encephalogenicity of autoreactive cells from mice immunized during late pregnancy can be restored following 3 days of in vitro stimulation with autoantigen**

To test whether induction of EAE during late pregnancy stimulated the production of a suppressive, nontraditional cytokine by lymphocytes or permanently altered their encephalitogenic potential, LNC harvested from mice immunized during late pregnancy were adoptively transferred into naive, virgin recipients. Briefly, mice were immunized during late pregnancy, and LNC were harvested and pooled 10 days postinduction. The pooled LNC were stimulated with PLP139–151 in vitro for 72 h, and \(10^7\) cells were transferred into virgin recipients i.v. The cells adaptively transferred from the pregnancy group were readily able to induce EAE: there was no decrease in disease incidence (three of four mice developed disease); no delay in disease onset (mean day of onset 7.3); and no reduction in severity of disease (peak severity score = 4, in all diseased mice) as compared with control.

### Table II. Histological assessment of brain lesions of mice with EAE during pregnancy

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Clinical score</th>
<th>Lesion score</th>
<th>Clinical score</th>
<th>Lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>++</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>+++</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>++++</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>

* S/JL mice mated after disease onset were sacrificed during late pregnancy (25–40 days after immunization), as were age- and disease duration-matched virgin controls.

* Clinical score at the time of sacrifice is reported.

* Tissue sections from the brains of immunized mice were stained with H&E and assessed for the presence of mononuclear cell infiltrates. Sections were scored as follows: 0, absence of infiltrates; +, small, rare perivascular lesions; ++, small, numerous perivascular lesions and parenchymal infiltration; and ++++, severe, confluent lesions.

### Serum anti-PLP139–151 IgG Abs are reduced in pregnant mice with preexisting EAE as compared with virgin controls

The sera from individual mice with EAE during late pregnancy (25–40 days postimmunization) and age- and disease duration-matched virgin EAE controls were tested by ELISA for the presence of total IgG, and the isotypes IgG1, IgG2a, IgG2b, and IgG3 directed against PLP139–151. A shift from Th1 to Th2 is associated with increases in Ab responses of the IgG1 and IgG2b isotypes. Interestingly, there was a significantly diminished amount of anti-PLP139–151 total IgG in the pregnant mice with preexisting EAE as compared with controls (Fig. 3, \(p = 0.026\)). No statistically significant difference in the amount of anti-PLP139–151 from different IgG isotypes was found (data not shown), as might be expected, given that we did not observe a shift from Th1 to Th2 in T cell responses to PLP139–151.

**Late pregnancy sera significantly diminish proliferation and IL-2 production by autoreactive cells**

Because our data seemed to support only a transient suppression of Th1 encephalitogenic T cells during pregnancy, without any permanent alteration in their pathogenicity, the possibility of a circulating suppressive factor was investigated. Serum obtained from normal S/JL mice during late pregnancy was added to proliferative assays, and cell cultures of LNC were obtained from mice immunized during late pregnancy and virgin controls. The cells were stimulated with PLP139–151, and the proliferative response and amount of IL-2 production were measured and compared with cell cultures stimulated in the presence of equal amounts of virgin mouse sera. The sera obtained from mice during late pregnancy significantly diminished the proliferative response of autoreactive cells in both mice induced during late pregnancy (Fig. 4c, \(p = 0.016\)) and virgin controls (Fig. 4d, \(p = 0.0004\)). IL-2 secretion was also decreased when the autoreactive cells from mice induced during late pregnancy and virgin controls were incubated in the presence of pregnancy sera (Fig. 4a, \(p = 0.036\), and Fig. 4b, \(p = 0.006\), respectively).

**Serum TGF-β levels are unchanged during pregnancy**

Sera obtained from individual S/JL mice during late pregnancy and from age-matched virgin controls were assayed for TGF-β1 content by ELISA. No significant increase in the circulating level of TGF-β1 was observed during pregnancy (Fig. 5, \(p = 0.75\)).

### Discussion

In this study, we have characterized an animal model of MS in which the effect of pregnancy on the clinical course and disease initiation recapitulates that which is seen in women with MS. Late pregnancy improved disability and protected against relapses in S/JL mice with preexisting EAE, and early pregnancy had no effect on disease course. Furthermore, these mice resumed a normal relapsing pattern following delivery when compared with virgin controls. Histologic lesions did not clear during pregnancy, even in those mice that had dramatic improvement in disability during late pregnancy. These results suggest that intralesional suppression of encephalitogenic T cells or a combination of suppression and neuroprotection is responsible for improvement during late pregnancy.

*Consistent with the results of previous studies in Lewis rats, rabbits, and guinea pigs (7–9), S/JL mice that were immunized during late pregnancy showed a significant decrease in the incidence of EAE, but still had mild histological CNS inflammatory infiltrates. Analysis of the proliferative responses and cytokine secretion profiles of the lymphocytes from these mice showed a strong Th1 bias, with no increase in IL-10 production or inhibition.
of proliferation as compared with virgin controls. In fact, when the lymphocytes from these mice induced for EAE during pregnancy, were restimulated, and transferred into naive SJL/J mice, encephalitogenicity was restored and they were able to induce robust disease. In our model, the pregnancy environment did not interfere with development of a Th1 response to an autoantigen and did not lead to the preferential development of Th2-biased autoreactive cells.

Our data concerning the effect of late pregnancy in EAE are consistent with that found in humans with MS. It is well known that late pregnancy affords temporary remission of MS attacks (1). This clinical remission was also associated with lack of new lesion formation on magnetic resonance imaging scans (10). Furthermore, women who first develop symptoms of MS during pregnancy experience less subsequent disability than women who develop the disease at any other time in life (11).

In stark contrast to our results is the commonly held assumption that a generalized Th2 shift during pregnancy is responsible for improvement of disease activity in MS patients. Although it is well established that a Th2 environment dominates the maternal-fetal interface and appears to be essential for survival of the fetoplacental allograft (12), the extent to which this local immune environment influences systemic T cell function is unknown. In normal human pregnancies, decidual leukocytes preferentially express IL-10 (16), an inability to do so appears to play an important role in spontaneous abortions (13). The production of other suppressive cytokines such as IFN-β (14) has also been demonstrated at the maternal-fetal interface. By extrapolation, it has been argued that these local changes also function to suppress Th1-driven immune responses by inducing a systemic Th2 shift (2). Direct evidence supporting such a hypothesis is based mostly on the in vitro effects of isolated pregnancy-related hormones and factors on cytokine secretion. Much effort has been made to identify the pregnancy-related factor that is responsible for this, as it may serve as a potent treatment for MS and other autoimmune disease with similarly affected disease courses.

Previous animal studies addressing the influence of pregnancy on systemic T cell function in disease models have yielded somewhat conflicting results. Krishnan et al. (15) showed that in C57BL/6 mice, which normally recover quickly from infection with Leishmania major by mounting a dominant Th1 response, continual pregnancy both impaired their ability to clear the infection and caused increased production of Th2 cytokines both before and after restimulation with L. major Ag. Using the experimental allergic uveitis model, a T cell-mediated, Th1-driven autoimmune disease similar to EAE, Agarwal et al. (16) showed that induction during late pregnancy in C57BL/6 mice resulted in less severe disease histologically and a dampened Th1 cytokine secretion profile when lymphocytes were restimulated with interphotoreceptor retinoid-binding protein Ag in vitro in the presence of virgin, syngeneic mouse sera. Similar to our results, they did not observe any change in the secretion of IL-10 and IL-4 as compared with virgin controls. However, they did find a significant increase in TGF-β1 serum levels during late pregnancy and postulated that this accounted for the disease amelioration and dampened Th1 cytokine secretion they observed. In contrast, in our SJL mouse model we were not able to demonstrate either a decrease in the Th1 cytokine secretion profile or any increase in serum TGF-β1 levels during pregnancy, raising the possibility of strain-dependent variation in pregnancy-related immune changes.

Our data point to the role of a late pregnancy-related serum factor that inhibits T cell encephalitogenicity. As discussed earlier, when autoreactive T cells were removed from the pregnant animal and hence removed from the pregnancy environment, no differences in proliferative activity or cytokine secretion profile were seen compared with controls. However, when these same T cells were restimulated with Ag in the presence of syngeneic late pregnancy sera, proliferation and IL-2 production were significantly

### Table III. EAE induction during different stages of pregnancy

<table>
<thead>
<tr>
<th>Stage of Pregnancy</th>
<th>Percent Incidence*</th>
<th>Mean Day of Disease Onsetb</th>
<th>Mean Peak Disease Severityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early (days 2–7)</td>
<td>80 (4/5)</td>
<td>13.3 ± 0.3</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Middle (days 8–13)</td>
<td>30 (7/21) (p = 0.003)*</td>
<td>16.4 ± 0.4</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Late (days 14–16)</td>
<td>32 (10/31) (p = 0.003)</td>
<td>16.2 ± 0.3</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Virgin controls</td>
<td>71 (22/31)</td>
<td>14.3 ± 0.2</td>
<td>2.4 ± 0.0</td>
</tr>
</tbody>
</table>

* SJL/J mice were induced with PLPp139–151 in CFA during different stages of pregnancy. The incidence of disease is given as a percentage. Values in parentheses denote sick vs total mice per group.

b Day of onset of clinical disease and peak disease severity scores only include those animals that developed disease. Values are given as mean ± SEM.

### Table IV. Histological assessment of brain lesions of mice immunized during late pregnancy

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Maximum clinical score*</th>
<th>Lesion score*</th>
<th>Mouse</th>
<th>Maximum clinical score</th>
<th>Lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>+</td>
<td>1</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>+</td>
<td>2</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>+++</td>
<td>3</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>+++</td>
<td>4</td>
<td>3</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Mice immunized with PLPp139–151 in CFA during late pregnancy (days 14–16) were sacrificed 25 days later.

* Clinical score at the time of sacrifice is reported.

* Tissue sections from the brains of immunized mice were stained with H&E and assessed for the presence of mononuclear cell infiltrates. Sections were scored as follows: 0, absence of infiltrates; +, small, rare perivascular lesions; ++, small, numerous perivascular lesions; ++++, numerous perivascular lesions and parenchymal infiltration; and ++++++, severe, confluent lesions.
Reduced as compared with incubation in virgin mouse sera. The suppressive action of such a factor acting locally within the CNS lesions could explain why the mice experience significant clinical improvement without clearing of inflammatory infiltrates. Other investigators have encountered this unusual situation of EAE disease amelioration, despite large numbers of inflammatory cells in the CNS. When using an altered peptide ligand of PLPp139–151 to treat SJL mice, Kuchroo et al. (17) and Santambrogio et al. (18) observed sizeable CNS inflammatory infiltrates, yet the mice did not develop any clinical evidence of EAE. They too postulated the presence of a suppressive factor responsible for this intralesional suppression and were able to demonstrate large amounts of TGF-β present in the CNS inflammatory foci of their protected mice as compared with controls. Although we have evidence for the presence of a suppressive factor as well, it does not appear to be TGF-β.

The in vitro immunosuppressive properties of human pregnancy sera have long been recognized. Kaskura (19) showed that human pregnancy serum suppresses the reactivity of mixed leukocyte cultures; the inhibitory effect reached its maximum during late pregnancy and disappeared shortly after delivery. T cell proliferation in response to mitogens is also inhibited in the presence of pregnancy sera (20). This led to the evaluation of inhibitory properties of the many known pregnancy-related hormones and factors. Among these, pregnancy-associated plasma protein A and cortisol are capable of inhibiting T cell proliferation, although neither fully accounts for the suppressive properties of late pregnancy sera (21, 22). In vitro effects of estradiol, estriol (E3), and progesterone on T cell clone stimulation have demonstrated Th2 shifts in cytokine secretion, but no alteration in proliferation (3, 23, 24). Although early pregnancy factor does have immunosuppressive properties, it reaches maximal levels very early in pregnancy and may not even be present during late pregnancy (25). Thus, the possibility of an unidentified serum factor remains.

The presence of an anergy-inducing serum factor that is either first produced or reaches maximal levels during the third trimester, and then is abruptly withdrawn at delivery is also consistent with the clinical observations in MS patients. The protective effect of pregnancy in MS patients is clearly transient, as it is often followed by an increased risk of clinical disease activity in the immediate postpartum period. If the disease amelioration during pregnancy were due to the presence of Th2 clones, one would expect a substantial delay before disease activity would return, similar perhaps to what is seen when treatment with the IFN-β is terminated (26).
Our data do not support the hypotheses that estradiol, E3, progesterone, adrenocorticotrophic hormone, or cortisol, the most commonly recognized hormonal changes of rodent pregnancy, are directly responsible for the disease-ameliorating effects of pregnancy in EAE. All of these hormones have previously been used to treat EAE with varying results. Progesterone has shown no amelioration of disease activity in EAE (27, 28). Treatment of mice with EAE with 17βestradiol (E2), the form of estrogen secreted during the ovulatory cycle, has produced disparate results in different mouse strains. Jansson et al. (29) showed no disease amelioration when given at ovulatory cycle doses and only a delay in

FIGURE 3. Anti-PLPp139–151 IgG Abs. Anti-PLPp139–151 Ab titers in pregnant SJL/J mice 25–40 days after immunization are compared with age- and disease duration-matched virgin controls. Sera were taken during late pregnancy in mice with preexisting EAE and tested by ELISA for the present of anti-PLPp139–151 IgG Ab titers. After incubation with sera at a 1:50 dilution, goat anti-mouse IgG conjugated to alkaline phosphatase was added. Results are expressed as the mean OD of four mice from each group, with bars representing the SEM. Mice with EAE that subsequently become pregnant (EAE + preg; filled bar) have a significant reduction in the amount of anti-PLPp139–151 IgG Abs as compared with their controls (EAE control; open bar) (p = 0.026, unpaired Student’s t test).

FIGURE 4. Pregnancy sera suppress T cell Ag-specific proliferation and IL-2 production. Pooled LNC from mice induced during late pregnancy (preg) and virgin mice (nonpreg) immunized 10 days prior were stimulated with PLPp139–151 in the presence of 1% late pregnancy sera or 1% normal mouse sera. A and B, Levels of IL-2 were tested by ELISA in supernatants. LNC from both groups produced significantly less IL-2 in the presence of pregnancy sera (filled bars) as compared with incubation with normal SJL/J mouse sera (open bars) (A, pregnant, p = 0.036; B, nonpregnant, p = 0.006; paired Student’s t test). C and D, Proliferative response was measured and is expressed as δ cpm = cpm in PLPp139–151 – cpm in medium alone. Values represent mean of four samples ± SEM. Proliferation of PLPp139–151-reactive T cells in the presence of autoantigen is significantly reduced in the presence of late pregnancy sera (C, pregnant group, p = 0.016; D, nonpregnant, p = 0.0004; Student’s unpaired t test).

FIGURE 5. Serum TGF-β1 levels are unchanged during pregnancy. TGF-β1 levels were measured by ELISA in sera obtained from SJL mice during late pregnancy (preg) and virgin controls (nonpreg). Each point represents an individual mouse. The horizontal line represents the mean level of the group. No significant difference was observed (p = 0.75, Student’s unpaired t test).
disease onset (no change in EAE disease incidence or severity) when given at higher doses in B10.RIII female mice. More recently, Bebo et al. (30) reported a reduction in disease severity of EAE in female SJL and B10.Pl mouse strains and in male SJL mice when treated with low and high dose E2 during the induction phase of the disease. This reduction in disease severity was associated with a significant decrease in CNS inflammatory infiltrates (31, 32), unlike the findings in our in vivo pregnancy-EAE model. The treatment of the effector phase of EAE in SJL mice with pregnancy levels of E3, the predominant form of estrogen secreted by the placenta during late pregnancy, did show an amelioration of disease activity. However, inconsistent with the findings in our in vivo model, the disease protection afforded by E3 treatment produced an absence of CNS inflammatory infiltrates, an increase in serum Ag-specific IgG1 Abs, and a significant increase in the production of IL-10 by autoreactive lymphocytes (28).

We cannot exclude that neuroprotection in combination with immune suppression is responsible for disease improvement during late pregnancy. E2 has many reported neuroprotective effects, including the promotion of neurite outgrowth and synaptogenesis, up-regulation of the expression of neurotrophic factors such as nerve growth factor, and up-regulation of acetylcholine synthesis. However, most of the described neuroprotective effects of E2 have been studied in hippocampal and basal forebrain neurons (33), which has unclear relevance to multiple sclerosis, as these are not areas commonly affected by the disease. In addition, as cited above, treatment with E2 at the onset of EAE does not afford any significant disease protection (30), making a predominant E2-induced neuroprotective effect an unlikely explanation for late pregnancy-induced remission of EAE. Although the potential neuroprotective effects of E3 have not been well studied, the results of the work of Kim et al. (28) point to a strong immunomodulatory role for E3 in the EAE model as the main mechanism for disease protection.

The immunomodulatory effects of pregnancy are vast and certainly complicated, many of which are most likely unrelated to the reasons for disease improvement in MS. Identifying exactly which pregnancy-induced change in the immune system results in improvement of EAE and MS has a clear therapeutic significance. In this study, we identified a relapsing mouse model of MS, in which the clinical disease-modifying effects of pregnancy mimic that seen in MS patients. Our data point most strongly to the role of a transient anergy-inducing factor rather than a shift in cytokine secretion profiles as being responsible for the protective effects of pregnancy. These observations will help focus the search for a pregnancy-related therapeutic agent to one with immunosuppressive properties rather than one that induces Th2 cytokines. Further investigation into which factor it may be will lead to the development of a potent disease-alleviating medication for people with MS and other autoimmune diseases. We have recently demonstrated that transcripts for two pregnancy-associated factors, pregnancy-associated plasma protein A and pregnancy-specific β1 glycoprotein, are elevated in MS plaques. We are currently undertaking studies to see whether these factors may contribute to the pregnancy-related decline in disease activity seen in EAE (34).

References
one favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. J. Immunol. 155:128.


