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Modulation of Immunoreactivity to Periodontal Disease-Associated Microorganisms During Pregnancy

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The lymphocyte blastogenic response to a panel of antigens and mitogens was assessed in a group of 20 women throughout their pregnancy. In addition, a group of five nonpregnant women was monitored simultaneously to identify variations in response to the same stimulants. The stimulants included orally associated bacterial antigens (*Streptococcus sanguis*, *Actinomyces viscosus*, *Bacteroides asaccharolyticus*, *Bacteroides melaninogenicus* subsp. *intermedius*, *Bacteroides [Capnocytophaga] ochraceus*, and *Fusobacterium nucleatum*) and non-orally associated stimulants (streptokinase-streptodornase, tetanus toxoid, concanavalin A, phytohemagglutinin, and pokeweed mitogen). Intrinsic (cells cultured in male AB plasma) suppression of the lymphocyte response to these stimulants was observed to occur by the second trimester of pregnancy and was resolved after parturition. Additionally, an extrinsic (cells cultured in autologous plasma) suppression was also suggested to occur in a similar manner. There was no detectable enhancement of the blastogenic response to oral bacteria associated with elevated gingivitis, which is generally reported to occur during nonpregnancy gingivitis. We propose that concomitant immunosuppression occurs during the second trimester, which masks such enhancement.

Recent studies have suggested an impairment of immunocompetency during pregnancy as evidenced by a decreased peripheral blood lymphocyte responsiveness to mitogenic or allogenic stimulation (1, 4, 5, 7-11, 14, 18, 19, 22). However, changes in antigen responsiveness during pregnancy have not been well characterized. Since the antigen-associated lymphocyte transformation response to members of the normal dental plaque flora has been well defined (2, 15), it was of interest to determine whether the lymphocyte responsiveness to these indigenous antigens was altered during pregnancy. This report presents the longitudinal and immunological evaluation of 20 pregnant and five nonpregnant women, characterizing their lymphocyte responsiveness in autologous and male AB(−) plasmas to a panel of orally and non-orally associated antigens. The bacteriological findings from this same group of subjects have been reported separately (K. S. Kornman and W. J. Loesche, J. Periodontal Res.; in press).

MATERIALS AND METHODS

Culture stimulants. Isolates of the following microorganisms were used in this study: *Streptococcus sanguis* ATCC 10558, *Actinomyces viscosus* GA, *Bacteroides asaccharolyticus* W, *Bacteroides melaninogenicus* subsp. *intermedius* 155.6, *Bacteroides* (*Capnocytophaga*) *ochraceus* 374b, and *Fusobacterium nucleatum* 191F. Unless otherwise noted, these isolates were obtained from naturally occurring gingivitis or periodontitis sites during prior clinical studies.

All bacteria were cultured in the basal anaerobic broth of Loesche et al. (13) with esculin eliminated and glucose increased to 1.0%. All incubation was inside an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) maintained at 37°C with an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Screw-cap flasks containing 500 ml of prereduced broth were inoculated with actively growing cells. The cultures were harvested by centrifugation at 12,000 × *g* for 30 min. The cell pellets were washed extensively in sterile phosphate-buffered saline (0.05 M PO_4, 0.15 M NaCl, pH 7.4) and suspended in sterile distilled water to give a concentration of 20 mg (wet weight) per ml. The cell harvest was approximately 100 mg (dry weight) per 1,000 ml of cultured medium.

The washed cells were quick frozen and thawed three times in an ethanol-dry ice bath and then subjected to a total of 40 min of ultrasonic disruption (model W185 D, 85 W; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) delivered in 5-min intervals with alternating periods of cooling in an ice bath. Cell disruption, as evaluated by Gram staining and phase-contrast microscopy, was greater than 95%. The cell wall-whole cell mixture was centrifuged at a low speed (800 × *g* for 5 min), and the supernatant fraction was then centrifuged at a high speed (12,000 × *g* for 30 min) to pellet the cell wall fragments. These centrifugations were repeated six times, followed by a final

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resuspension of the cell walls in phosphate-buffered saline.

The following nonorally associated culture stimulants were obtained from commercial sources: concanavalin A and leucoagglutinin (Pharmacia Fine Chemicals, Inc.); pokeweed mitogen (GIBCO Laboratories); streptokinase-streptodornase (Varidase; Lederle Laboratories, Pearl River, N.Y.); and tetanus toxoid fluid, USP (Eli Lilly & Co., Indianapolis, Ind.).

Subjects. Twenty women, aged 20 to 33 years, whose pregnancies were thought to be less than 13 weeks at the initial examination, were seen monthly until delivery and again postpartum in the University of Michigan Women's Hospital Obstetrics Clinic. Five nonpregnant women in the same age range were seen monthly for 4 consecutive months. Each nonpregnant subject kept a menstrual cycle calendar and was seen each month at approximately the same stage of the cycle. All subjects were part of a study of the bacteriology and immunology associated with the development of pregnancy gingivitis (Kornman and Loesche, J. Periodontal Res., in press).

Thirty milliliters of peripheral blood from the median vein in the antecubital fossa was collected in sterile heparinized vacuum tubes. Blood was sampled at the initial visit (<13 weeks of gestation), at approximately 20 to 24 weeks of gestation, at approximately 32 weeks of gestation, and again postpartum. Blood samples were taken from the five control subjects at each visit.

At each visit the gingival index (12) and plaque index (20) were determined for the mesial of the maxillary right second premolar (site 1) and of the mandibular left cuspid (site 2) in each subject. An evaluation of the tendency toward gingival bleeding throughout the entire mouth was determined by counting the interproximal sites which bled to any extent after gentle probing with a wooden interdental stimulator (Stim-U-Dent, Stim-U-Dent Inc., Detroit, Mich.). A maximum of 22 interproximal sites was available in each patient, mesial to the first molars. No distinction was made between spontaneous and induced bleeding.

Lymphocyte culture. The buffy coat layer from whole blood was centrifuged at 350 × *g* for 30 min, diluted in sterile phosphate-buffered saline, and layered onto Ficoll-Hypaque (3) (Ficoll-paque; Pharmacia Fine Chemicals, Inc.). The tubes were centrifuged at 500 × *g* for 30 min at 23°C. The cells banded at the interface were aspirated, washed three times in phosphate-buffered saline, suspended to a final concentration of 10^6 cells per ml in RPMI 1640 culture medium (GIBCO Laboratories), and supplemented with 40 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml.

Antigen- and mitogen-induced lymphocyte blastogeneses were assessed in a microtiter plate system containing 10^5 mononuclear leukocytes in a final volume of 0.20 ml of RPMI 1640 with either 10% autologous or pooled male AB(−) plasma. Dilutions of each culture stimulant were added to each well and incubated for 3 days (mitogens) or 7 days (antigens). During the last 6 h of culture, 2 μCi of [*methyl*-^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.; TRA 120, 5 Ci/mmol) was added to each well. All cultures were harvested with a multiple automated sample harvester (MASH II; Microbiological Associates, Walkersville, Md.) onto glass fiber filters. The filters were dried, placed in vials (Bio-Vial; Beckman Instruments, Inc., Fullerton, Calif.), filled with 3 ml of scintillation cocktail (OCS; Amersham Corp.), and counted in a Packard model 3320 liquid scintillation spectrometer.

Data analysis. Evaluations of the significant differences between the lymphocyte responses of control (nonpregnant) and pregnant subjects in autologous and male AB(−) plasmas were performed by the Student *t* test. Since the maximum blastogenic response to the same stimulant occurred at different stimulant concentrations for different individuals, comparisons were performed at the maximum counts of [*methyl*-^{3}H]thymidine incorporated per minuute rather than at a predetermined stimulant concentration. Optimal stimulant concentrations for each individual did not vary during this study.

RESULTS

Occurrence of gingivitis during pregnancy. Clinical measurements revealed an increase in gingivitis in the pregnant subjects as indicated by gingival bleeding. The mean number of bleeding sites per patient increased significantly (pairwise *t* test [Table 1]) at 13 to 28 weeks of gestation, from an initial 3.39 bleeding sites per patients to 6.11 bleeding sites per patient at 21 to 24 weeks. The mean gingival index for sites 1 and 2 increased significantly ($P < 0.05$, pairwise *t* test) from 1.16 initially to between 1.44 and 1.61 at 13 to 28 weeks. The mean plaque

TABLE 1. *Mean number of interproximal bleeding sites per pregnant subject*

Trimester	Sample period[a]	Mean	Mean difference[b]	Standard deviation[c]	Significance[d]
First	<13	3.39			
Second	13–16	5.17	1.78	3.28	0.03
	17–20	5.72	2.33	2.93	<0.01
	21–24	6.11	2.72	3.36	<0.01
	25–28	5.50	2.11	2.95	<0.01
Third	29–32	4.39	1.00	3.97	NS[e]
	33–36	3.78	0.39	1.29	NS
	37–40	3.56	0.17	0.38	NS
	Postpartum	3.72	0.33	3.40	NS

[a] Gestational age in weeks estimated from last date of menstruation.

[b] Difference between indicated sample period and initial value.

[c] Standard deviation of the difference.

[d] Significance level determined by pairwise *t* test.

[e] NS, Not significant.

index at both sites (1.13) was essentially unchanged throughout the pregnancy and postpartum. Clinical parameters in the nonpregnant group did not change during the 4-month sampling period, with a mean number of bleeding sites of 3.7 ± 0.10 (standard error), mean gingival index of 1.17 ± 0.18, and mean plaque index of 1.00 ± 0.36.

Blastogenic response of lymphocytes from nonpregnant controls: plasma effects. The average maximum lymphocyte blastogenic response to the stimulant panel by control group lymphocytes is shown in Table 2. The responses of the nonpregnant subjects to orally associated microorganisms were consistent with findings in subjects with gingivitis as reported by others previously (1, 15, 16) and by this laboratory in other studies (unpublished data).

With one exception, a significant stimulation ($P < 0.001$) was observed with all culture stimulants as compared with unstimulated controls, although the magnitude of the blastogenic responses to the stimulant panel varied greatly among individuals. A significant response was not observed to occur in control subjects tested with *B. asaccharolyticus*, a minor component of

TABLE 2. *Blastogenic response of control and pregnant group lymphocytes to immunostimulant panel: plasma effects (extrinsic modulation)*

Stimulant	Plasma[a]	cpm[b] ± standard deviation in following group:				
		Control	Pregnant			
			First trimester[c]	Second trimester	Third trimester	Postpartum
S. sanguis	Autologous	6,957 ± 443	5,475 ± 2,367	3,088 ± 1,563	3,102 ± 2,345	11,013 ± 5,346
	AB	4,401[d] ± 288	6,460 ± 3,452	4,678 ± 2,146[d]	7,195 ± 4,222[e]	7,393 ± 3,664[d]
A. viscosus	Autologous	9,227 ± 2,923	5,490 ± 3,242	2,565 ± 1,444	5,665 ± 3,232	9,725 ± 4,787
	AB	7,091 ± 2,006	6,381 ± 3,745	3,765 ± 2,004[d]	7,268 ± 1,976	5,942 ± 2,543[f]
B. asaccharolyticus	Autologous	1,348 ± 140	1,139 ± 976	1,374 ± 432	3,290 ± 263	1,612 ± 449
	AB	1,685 ± 355	2,963 ± 854[e]	2,102 ± 868[e]	1,888 ± 1,003[e]	1,934 ± 855
B. melaninogenicus	Autologous	9,212 ± 1,879	10,188 ± 6,433	4,836 ± 3,442	5,094 ± 2,972	16,535 ± 8,103
	AB	10,062 ± 3,314	11,561 ± 7,111	6,097 ± 4,310	5,912 ± 3,121	15,877 ± 7,995
B. ochraceus	Autologous	14,788 ± 3,991	13,264 ± 9,559	7,482 ± 5,882	10,810 ± 7,409	17,109 ± 11,242
	AB	12,221 ± 4,248	12,563 ± 5,331	4,802 ± 4,109	8,933 ± 5,342	15,215 ± 9,989
F. nucleatum	Autologous	4,795 ± 1,206	5,288 ± 4,332	2,416 ± 1,900	2,886 ± 1,823	4,334 ± 4,112
	AB	3,569 ± 873	5,824 ± 3,999	3,476 ± 2,664	1,962 ± 893[d]	6,348 ± 3,930
Tetanus toxoid	Autologous	29,254 ± 7,843	18,927 ± 12,849	8,366 ± 5,934	7,050 ± 5,007	21.355 ± 15,456
	AB	25,744 ± 6,746	24,122 ± 16,444	15,240 ± 6,343[e]	10,349 ± 7,993	20,209 ± 12,785
Streptokinase-streptodornase	Autologous	32,451 ± 8,739	43,459 ± 39,881	39,006 ± 21,891	11,228 ± 4,992	40,726 ± 27,349
	AB	29,377 ± 7,234	53,466 ± 47,379	33,959 ± 17,210	15,011 ± 7,112	59,929 ± 32,910
Base line (7 day)	Autologous	1,677 ± 443	2,229 ± 2,641	1,650 ± 1,253	1,420 ± 1,491	1,256 ± 605
	AB	1,419 ± 618	2,971 ± 2,273	1,920 ± 1,498	1,708 ± 1,238	1,952 ± 692
Phytohemagglutinin	Autologous	159,415 ± 44,557	107,605 ± 97,243	83,214 ± 74,967	131,676 ± 64,999	137,415 ± 72,009
	AB	135,085 ± 32,970	146,675 ± 103,444	100,773 ± 89,852	174,799 ± 94,829	186,282 ± 93,011
Concanavalin A	Autologous	89,615 ± 43,829	88,998 ± 69,888	33,777 ± 29,554	85,451 ± 51,332	157,279 ± 133,000
	AB	72,260 ± 43,481	95,166 ± 74,475	38,008 ± 25,586	102,320 ± 69,394	126,599 ± 98,342
Pokeweed mitogen	Autologous	7,402 ± 810	3,020 ± 1,543	1,769 ± 989[d]	2,879 ± 1,332	5,610 ± 3,875
	AB	3,140[f] ± 305	2,179 ± 1,892	1,130 ± 798	2,508 ± 875	3,315 ± 2,965[d]
Base line (3 day)	Autologous	438 ± 214	512 ± 303	536 ± 293	741 ± 586	732 ± 617
	AB	388 ± 196	298 ± 204[d]	348 ± 251[d]	358 ± 245[d]	396 ± 380

[a] Autologous or pooled male AB(−) plasma.

[b] Maximum lymphocyte blastogenic response occurring within stimulant dose ranged tested. Unstimulated control (base line) subtracted.

[c] First trimester, 0 to 13 weeks; second trimester, 14 to 27 weeks; third trimester, 28 to 40 weeks.

[d] $P \leq 0.05$.

[e] $P \leq 0.001$.

[f] $P \leq 0.005$.

dental plaque associated with gingivitis.

Culture in male AB(−) plasma was employed to reveal altered lymphocyte response during pregnancy that was either plasma mediated (extrinsic) or inherent in the cell population (intrinsic). Lymphocytes of the control subjects were cultured in the pooled male AB(−) plasma to evaluate differences in ability to support lymphocyte blastogenesis. In all but two instances, there was not a significant ($P < 0.05$) difference in the ability of the AB(−) plasma to support a blastogenic response (Table 2). The responses to *S. sanguis* and pokeweed mitogen were significantly better when cells were cultured in autologous plasma; however, the responses in AB(−) plasma were still quite strong.

In vitro blastogenic response of the lymphocytes from pregnant subjects to antigen and mitogen stimulations. The in vitro blastogenic response of the lymphocytes from pregnant subjects to the stimulant panel is also shown in Table 2. The responses are indicated as the mean maximum blastogenic response of the lymphocytes to each culture stimulant. As with nonpregnant subjects, culture with *B. asaccharolyticus* did not significantly stimulate the lymphocytes from the pregnant group. Since prepregnancy levels of lymphocyte blastogenesis were unavailable for these individuals, absolute changes in responses relative to prepregnancy values could not be determined. For this reason, all longitudinal comparisons employed the control group for the prepregnancy blastogenic values. This assumed a normal distribution of responsiveness to the stimulant panel, with the only major difference between the two groups being pregnancy.

Modulation of lymphocyte blastogenesis in autologous plasma. The role of pregnancy-associated humoral factors in the modulation of lymphocyte blastogenesis was probed. As shown in Table 2, the blastogenic response to *S. sanguis*, *A. viscosus*, and tetanus toxoid during the second trimester appeared to be suppressed when cells were cultured in autologous plasma as compared with culture in male AB plasma. The response to pokeweed mitogen was minimally, but significantly, enhanced at this time. During the third trimester, the *S. sanguis* response continued to be suppressed, and *F. nucleatum* was minimally, but significantly, enhanced. The postpartum measurements revealed significant enhancement of the *S. sanguis*, *A. viscosus*, and pokeweed mitogen responses.

The ratio of the responses of lymphocytes of pregnant and control subjects when cells were cultured in autologous plasma is shown in Table 3. As shown, a significant depression of blastogenesis occurred during the second and third trimesters, which was resolved after parturition.

Modulation of lymphocyte blastogenesis in male AB plasma. To assess the immunosuppression attributable to nonhumorally intrinsic mediated mechanisms, we made comparisons between the blastogenic responses of the preg-

TABLE 3. *Combined comparison of intrinsic[a] and extrinsic[b] modulations of the lymphocyte blastogenic response: longitudinal comparison between pregnant and control subjects*

Stimulant	cpm (pregnant)/cpm (nonpregnant)[c]							
	First trimester[d]		Second trimester		Third trimester		Postpartum	
	Autologous	AB	Autologous	AB	Autologous	AB	Autologous	AB
S. sanguis	0.90	1.62	0.55[e]	1.13	0.52[e]	1.53	1.42	1.61
A. viscosus	0.71[f]	1.10	0.39[e]	0.67[e]	0.65[f]	1.05	1.01	0.93
B. melaninogenicus	1.15	1.37	0.60[f]	0.75[f]	0.60[e]	0.72[f]	1.63[f]	1.68
B. ochraceus	0.95	1.13	0.55[f]	0.49[e]	0.74	0.78	1.12	1.26
F. nucleatum	1.17	1.76	0.63[f]	1.08	0.67[f]	0.74[e]	0.86	1.66
Tetanus toxoid	0.69	1.00	0.32[e]	0.63[e]	0.27[e]	0.44[e]	0.73	0.82
Streptokinase-streptodornase	1.34	1.83	1.19	1.17	0.37[e]	0.54[e]	1.23	2.01[f]
Phytohemagglutinin	0.68	1.08	0.52[f]	0.75	0.83	1.29	0.86	1.38
Concanavalin A	0.99	1.31	0.38[e]	0.53[e]	0.96	1.41	1.75	1.75
Pokeweed mitogen	0.45	0.70	0.29[e]	0.42[e]	0.46[e]	0.81	0.81	1.05

[a] Lymphocytes were cultured in pooled male AB(−) plasma.

[b] Lymphocytes were cultured in autologous plasma.

[c] Ratio of maximum response of lymphocytes from pregnant subjects divided by maximum response of lymphocytes from control subjects for each stimulant.

[d] First trimester, 0 to 13 weeks; second trimester, 14 to 27 weeks; third trimester, 28 to 40 weeks.

[e] Significant difference between pregnant and control groups, $P < 0.01$ (Student's t test).

[f] $P < 0.05$.

nant and control subjects by culturing cells in AB plasma. As shown in Table 3, with the exception of *S. sanguis* and phytohemagglutinin, there was significant suppression of blastogenesis occuring in either the second or third trimester. After parturition, all values were not significantly different from the control values, with the exception of streptokinase-streptodornase, which appeared to be enhanced.

DISCUSSION

The lymphocyte transformation response to mitogens has been shown previously to decrease during pregnancy (1, 5, 7–11, 14, 18, 19, 22). We observed a significant decrease in both antigen and mitogen responses during the second and third trimesters. This immunosuppression was universal to five of six orally associated antigens, two common nonorally associated antigens, and three mitogens. The response of all subjects to *B. asaccharolyticus* was not significantly different from that of unstimulated cultures; therefore, it is not possible to meaningfully evaluate suppression with that antigen. Depression of the blastogenic response coincided with the development of pregnancy gingivitis (Table 1). In previous studies, enhanced responsiveness to oral bacteria and mitogens has been associated with the gingivitis which accompanies the withdrawal of oral hygiene (15, 16) or which occurs naturally (2). In our study, such enhancement of blastogenesis may have been masked by the coincidental expression of immunosuppressive activity.

A recent report by O'Neil (14) examining changes in lymphocyte response during pregnancy also demonstrated a depression of maternal T-cell responsiveness to mitogens during pregnancy; however, a poor response to *Veillonella alcalescens* ultrasonically treated material precluded evaluation of antigen responses. In our study, intrinsic suppression (Table 3), generally detectable by the second trimester, was evident when lymphocytes were cultured in male AB(−) plasma and occurred with all mitogens and antigens except *S. sanguis* and phytohemagglutinin. Autologous plasma-mediated suppression was not identified in all cases (Table 2). These different types of suppression may actually reflect different lymphocyte populations responding to the various antigens and mitogens. In a study by Fabris et al. (7), humoral and cell-mediated immunity was shown to be differentially suppressed. Baines et al. (1) demonstrated the reduced ability of lymphocytes from pregnant women to respond to thymus-dependent antigens. In addition, recent reports have suggested an alteration in T-cell subpopulations during pregnancy. Strelkauskas et al. (21) evaluated T-cell levels with indirect rosettes, using anti-human T-cell antisera, and found that T-cells dropped from greater than 65% of the lymphocytes to approximately 25% in the early part of the second trimester. Dodson et al. (6), using a different T-cell marker, sheep erythrocyte rosetting, failed to confirm the decrease in T-cells during pregnancy.

At the postpartum evaluation, an elevated response by the pregnant subjects to *B. melaninogenicus* and streptokinase-streptodornase was detected. Such a response would reflect a prior episode of antigen exposure (Kornman and Loesche, J. Periodontal Res., in press) which occurred during the pregnancy. Since intrinsic enhancement of the anti-oral bacteria response did not occur during any trimester, it is not possible to determine whether antigenic sensitization occurred during pregnancy. Reports by Patters et al. (16) indicate that enhanced blastogenesis is transient during experimental gingivitis; thus, the occurrence of immunosuppression concomitant with gingivitis would tend to mask such a response.

A variety of immunosuppressive substances, including progesterone, α-globulin, and β-globulin, increase in the sera of pregnant women (10). Whereas a majority of investigators have reported extrinsic (serum-mediated) suppression of lymphocyte responsiveness, other investigators (17) have reported no difference in responses to mitogens between pregnant and nonpregnant women. The latter study suggests that individual variability, rather than pregnancy-associated factors, is responsible for extrinsic immunosuppression differences. Such an inability to demonstrate immunosuppression during pregnancy may be based in part on several findings in our study. First, not every culture stimulant displayed a reversible extrinsic suppression. Replacement of serum to demonstrate extrinsic suppression assumes that the intrinsic response is not suppressed by nonserum means, for example, a cellular suppression mechanism. Observed suppression (extrinsic or intrinsic) of various stimulants appeared at different stages of pregnancy, suggesting that a variety of mechanisms may have been operating. Detection of humoral suppression relies on cell culture in media containing nonautologous serum or plasma. Such supplements are not necessarily optimal. Therefore, if a suboptimal response in nonautologous serum is compared with a depressed response in autologous serum (which is suppressive), such suppression may not be detected. Finally, humoral (extrinsic) suppression may not be reversible in all instances; therefore,

culture in nonpregnant serum may not uncover a suppressive factor.

In summary, an immunosuppressive activity is mediated by both the plasma and cells of normal pregnant women as determined by their diminished responsiveness to periodontal disease-associated and control stimulants. In contrast to other studies, occurrence of increased gingivitis was not accompanied by a concomitant transient increase in an in vitro blastogenic response to oral bacteria. A possible explanation may be that of masking the response by immunosuppressive mechanisms associated with the pregnancy. Two types of suppression have been described. The first, extrinsic, appears to be mediated by serum factors and can be reversed by culturing the lymphocytes in male plasma. It appears to influence the response to only selected stimulants. The second type, intrinsic, is not reversed by serum supplementation and is probably based on a cellular mechanism. It appears to have a more general suppressive capacity. This suppression is abrogated within 6 weeks after parturition.

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