Nursing Duration and Suckling Intensity: Effects on Plasma Corticosterone, Circulating Leukocytes, and Mammary Nucleic Acids[1] (35822)

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Significant reductions in circulating neutrophils and lymphocytes have been noted after nursing in rats (1) and following machine milking in cows (2). The transitory loss of these leukocytes from the circulation following nursing and milking has been attributed to the passage of these cells into the alveoli and ducts of the mammary gland (1, 2). Neutropenia appears to be due to irritation caused by nursing, whereas lymphopenia depends on corticosteroids since lymphopenia could be abolished by adrenalectomy and restored by administering corticosterone to adrenalectomized rats (3). In view of the apparent dependency of the lymphopenic response on corticosteroids and the fact that suckling stimulates an increase in plasma corticosterone (4) the present study was designed to examine the relationship between circulating leukocytes and plasma corticosterone levels in lactating rats exposed to specific nursing intervals and suckling intensities. Additionally, mammary gland deoxyribonucleic acid levels were determined to assess the influx of leukocytes into the mammary gland. It was anticipated that this approach could provide a more reliable estimate of the leukocytic infiltration than that obtained by examining the mammary gland histologically.

Materials and Methods. Primiparous Sprague-Dawley rats were housed in cages containing five females and one male. At 1–4 days prepartum, females were housed individually and all litters were adjusted to 10 pups on day 1 postpartum. Litter size was maintained at 10 pups throughout the experimental period by replacing dead pups with healthy pups of comparable age.

At 10 a.m. on day 12 of lactation, pups were separated from their mothers and returned at 8 a.m. on the following day. Mothers were allowed to nurse for 2 hr with either 2, 4, 6, and 8 pups, or for 0.25, 0.5, 1, 2, 3, and 4 hr with 10 pups. Some mothers were not reunited with pups and served as nonnursed controls. In addition, pups were placed under their mother's cage to determine whether or not exteroceptive stimuli associated with the presence of young could elicit the leukopenic and adrenocortical response seen after nursing.

Rats were killed by decapitation within 30 sec after being removed from their cages between 8 and 11:30 a.m. Blood was collected in 100-ml beakers containing 8 mg of disodium ethylenediaminetetraacetate. Abdominal-inguinal mammary glands were removed, weighed, and frozen at —20° for nucleic acid analysis (5). Portions of the thoracic mammary gland were removed, fixed, imbedded, sectioned, and stained with Wright's stain for examination of leukocytes in the alveolar lumina.

Immediately after collection, blood smears were prepared for differential leukocyte counts, whereas total leukocyte and erythrocyte counts were determined hemocytometrically as previously described (3). Blood was centrifuged at 5000*g* for 0.25 hr at 5° and the supernatant plasma was removed and stored at —20° until analyzed for corticosterone. Plasma corticosterone was quantitated fluorometrically and corrected for 100% recovery (6). Analysis of variance and

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Duncan's multiple range test were used to determine the significance level of treatment means.

Results and Discussion. Plasma corticosterone concentrations increased 3-fold ($p<0.01$) within 0.25 hr after pups were allowed to nurse rats that had been isolated from their young for 22 hr (Table I). Moreover, plasma corticosterone levels remained significantly higher ($p<0.01$) than nonnursed controls after 0.5 and 1 hr of nursing but decreased significantly ($p<0.01$) after 2, 3, and 4 hr of nursing to control levels (Table I). The striking but transient increase in circulating corticosterone seen after nursing was probably due to the release of adrenocorticotropin (ACTH) and to an increase in the synthesis and secretion of adrenal corticosterone since the amount of corticosterone stored in the adrenal glands (7) could not account for the increase in plasma corticosterone noted in this study. Three- to fourfold increases in plasma corticosterone of mother rats have also been reported by others following nursing durations of either 0.5 or 2 hr (4). However, in the present study plasma corticosterone returned to nonnursed levels after 2 hr of continuous nursing. The present failure to observe elevated plasma corticosterone values after 2 hr or more of nursing may be attributed to a lack of suckling because the mammary glands were virtually devoid of milk after 2 hr. Evidence indicating that the mammary glands were depleted of milk after 2 hr or more of nursing stems from the observation that mammary gland weights (Table II) did not decline significantly ($p>0.20$) after 2 hr of nursing, whereas the average weight of the glands decreased ($p<0.01$) from 9.2 g in nonnursed controls to 5.8 g after 2 hr of nursing. Moreover, at autopsy, milk could only be expressed from glands obtained from rats nursed for 1 hr or less.

The small amount of suckling seen after placing nonnursed pups and mothers together for 2 hr or longer also appeared to be the cause for the failure to show significant changes in plasma corticosterone among mother rats nursing different size litters. Rats nursing 2, 4, or 6 pups for 2 hr had average plasma corticosterone levels that were comparable to nonnursed controls ($p>0.20$); whereas rats nursing 8 pups had significantly higher ($p<0.01$) levels. However, others (8) have observed differences in plasma corticosterone levels for rats nursing 2 or 6 pups for 30 min indicating that subsequent investigations relating plasma corticosterone to suckling intensity should employ short-term nursing periods since long periods of nursing may induce temporary inhibition of the sensory pathways that mediate changes in plasma corticosterone.

In the present study, pups were placed under the mother's cage so that they could be within seeing, hearing, and smelling distance of mother rats for 0.25 and 2 hr (Table I). The average level of corticosterone in mother rats exposed to exteroceptive stimuli associated with placing pups under the mother's cage for 0.25 hr averaged 8.0 μg/100 ml of plasma, whereas nonnursed controls completely isolated from young averaged 3.2 μg/100 ml of plasma. Exposing mothers to pups placed under their cages for 2 hr resulted in an average of 3.4 μg/100 ml of plasma which was not significantly different from control values ($p>0.20$). The observed increase in plasma corticosterone in the absence of suckling suggests that placing pups near the mother for 0.25 hr without allowing them to nurse produced sufficient stimuli to activate the hypothalamo–hypophyseal–adrenal axis. Additionally, others have observed that exteroceptive stimuli associated with nursing triggers the release of prolactin (9), and oxytocin (10) from the pituitary gland.

Precipitous decreases ($p<0.01$) in circulating leukocytes were noted within 0.25 hr after placing pups under their mother's cage or after 0.25 hr of nursing (Table I). Moreover, the number of circulating leukocytes continued to decline up to 2 hr of nursing. After 2, 3, and 4 hr of nursing the number of circulating leukocytes remained relatively constant but significantly lower ($p<0.01$) than that observed after 1 hr of nursing. The reduction in circulating leukocytes noted after 0.25 and 2 hr of nursing represented net losses of 4.0×10^7 and 6.0×10^7 leukocytes, respectively, from the circulatory system. [Calcu-

TABLE I. Influence of Nursing Duration and Suckling Intensity on Plasma Corticosterone and Circulating Leukocytes.[a]

Treatment	Corticosterone (μg/100 ml of plasma)	(per mm^3)					Erythrocytes ($\times 10^4$/mm^3)
		Leukocytes	Neutrophils	Eosinophils	Monocytes	Lymphocytes	
Nonnursed	3.2	8600	2950	280	460	4900	800
Nonnursed with pups under cage for (hr):							
0.25	8.0	6400	2200	220	360	3600	800
2	3.4	6400	2100	340	480	3500	800
Nursed with 10 pups for (hr):							
0.25	9.6	6700	2050	280	480	3920	800
0.50	9.2	6500	1950	260	300	3920	800
1	8.2	6400	1900	200	360	3990	800
2	3.3	5500	1700	200	340	3290	760
3	3.4	5600	1800	220	360	3220	760
4	4.4	5700	1650	160	380	3430	800
Nursed for 2 hr with:							
2 pups	3.5	5300	1700	280	300	3080	760
4 pups	2.2	5800	1600	260	300	3570	800
6 pups	3.7	5700	1750	240	360	3360	800
8 pups	6.4	5400	1650	260	320	3220	800
Overall standard error of treatment mean	$\pm$0.6	$\pm$340	$\pm$170	$\pm$50	$\pm$50	$\pm$215	$\pm$18

[a] Each value represents mean of 14 rats.

TABLE II. Influence of Nursing Duration and Suckling Intensity on Mammary Nucleic Acids.[a]

Treatment	Fresh mammary gland weight (g)	Total (mg)	
		DNA	RNA
Nonnursed	9.2	26.2	83.2
Nonnursed with pups under cage for (hr):			
0.25	9.6	27.6	93.9
2	8.8	25.7	85.7
Nursed with 10 pups for (hr):			
0.25	8.7	27.5	97.9
0.5	8.4	28.5	94.7
1	6.8	24.4	87.9
2	5.8	25.5	84.4
3	5.3	24.0	81.3
4	5.5	24.6	78.3
Nursed for 2 hr with:			
2 pups	7.4	25.1	81.2
4 pups	6.5	24.4	85.6
6 pups	6.0	24.8	81.6
8 pups	5.7	25.2	82.2
Overall standard error of treatment mean	±0.2	±1.2	±4.4

[a] Each value represents the mean of 14 rats.

lated on the basis of av body wt of lactating rats (290 g) and assuming a blood volume, corrected for lactation, of 52 ml/kg of body wt (11)]. These results indicate that the leukopenic response to nursing occurs within 0.25 hr after placing nonnursed pups and mothers together and is dependent upon the duration of the nursing stimulus. This conclusion is consistent with observations in cows which showed that an additional stimulus of 0.25 hr of overmilking depressed the number of circulating leukocytes beyond that associated with complete milk removal (12).

Nursing induced a significant reduction ($p<0.01$) in circulating neutrophils (Table I). The greatest reduction in neutrophils occurred within 0.25 hr after placing pups under the mother's cage. However, the number of circulating neutrophils continued to decline in a somewhat linear manner up to 2 hr of nursing. The number of circulating eosinophils and monocytes tended to be lower than the nonnursed controls particularly for the longer nursing durations. Lymphocytes decreased ($p<0.01$) 0.25 hr after placing pups under the mother's cage, remained relatively constant between 0.25 and 1 hr of nursing, and decreased significantly ($p<0.01$) between the first and second hours. Hence, the leukopenia associated with nursing was primarily caused by a rapidly occurring neutropenia and lymphopenia. The decrease in neutrophils after placement of pups under their mother's cage suggests that the loss of these cells from the circulatory system cannot be solely attributed to tactile stimuli associated with nursing since 60% of the neutrophil decrease was accountable by the exteroceptive stimuli associated with placing pups under their mother's cage for 0.25 hr. Lymphopenia induced by placing pups under their mother's cages or by nursing was probably a response to the high plasma level of corticosterone since this hormone has been shown to cause lymphopenia in rats (3). The reason for the significant decrease in lymphocytes beyond 2 hr of nursing is not readily apparent because plasma corticosterone at this time returned to levels found in nonnursed controls. Even though plasma corticosterone returned to nonnursed levels after 2 hr of nursing, the lymphopenia continued to persist through the fourth hour of nursing. Thus, there is no evidence of a major resupply of lymphocytes or neutrophils to the circulatory system by the hematopoietic system.

Leukocyte counts for rats nursing 2, 4, 6, or 8 pups for 2 hr were less than controls ($p<0.01$), but were not different from each other ($p>0.05$). The failure to observe a relationship between nursing intensity and the number of circulating leukocytes may have been masked by the relatively long nursing period (2 hr) imposed here since the present results indicate that leukopenia was time dependent and did not decline after 2 hr or more of nursing. Thus, the leukopenic response to nursing intensity may become apparent if mother rats were sacrificed at intervals earlier than 2 hr after nursing is initiated. Erythrocyte counts were not measurably different ($p>0.05$) throughout the study, indicating that the observed leukocyte decreases were not due to hemodilution.

For rats nursing 10 pups, total mammary gland weights (Table II) decreased significantly ($p<0.01$) in a somewhat linear manner up to 2 hr of nursing and remained unchanged ($p>0.05$) after 3 and 4 hr of nursing. Thus, the lack of any further decrease in circulating leukocytes at the end of hours 3 and 4 may have been due to a reduction in nursing irritation caused by the absence of milk. Although total mammary gland weight decreased during the first 0.5 hr of nursing, total DNA during this time tended to increase and then decreased significantly ($p<0.05$) at 1 hr of nursing (Table II). Total

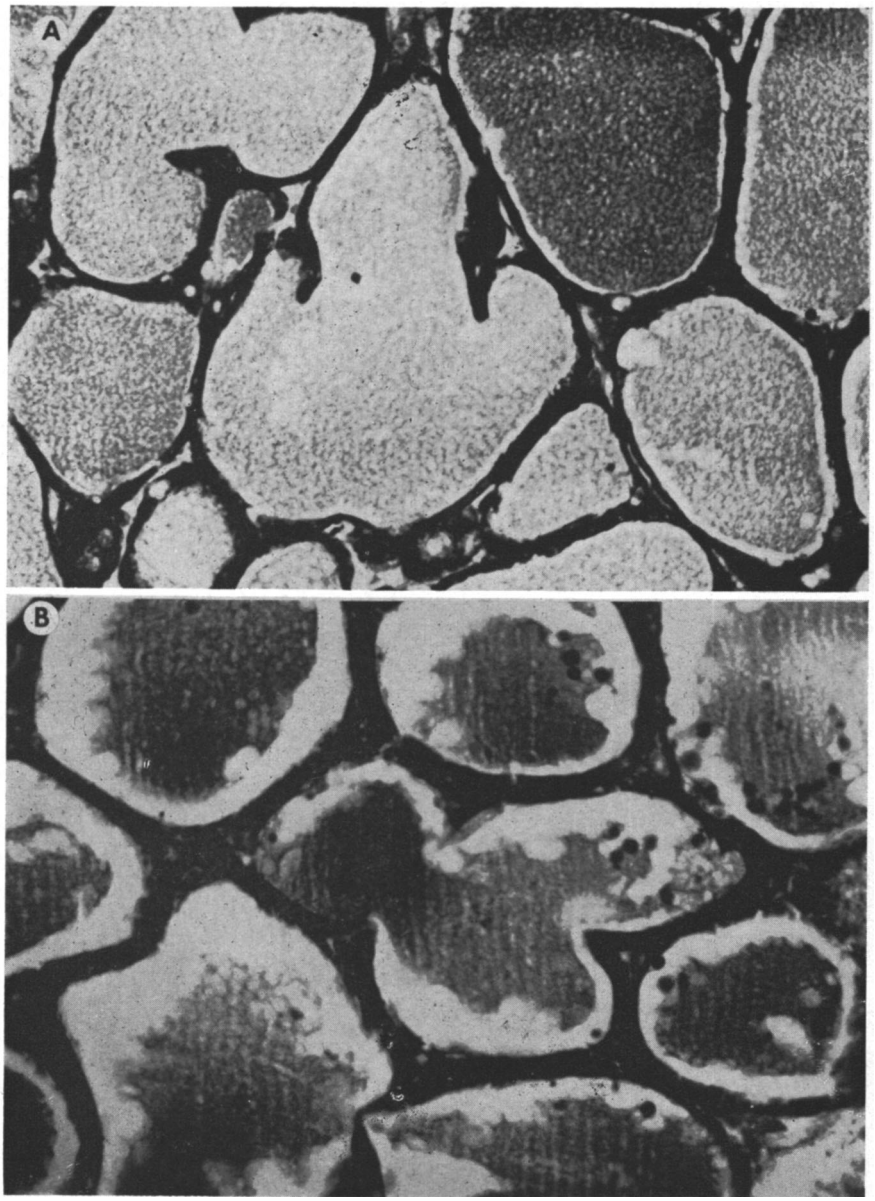

FIG. 1. Leukocytic infiltration into the mammary gland in response to nursing: (A) Lactating rat mammary gland, nonnursed for 22 hr illustrating relative absence of leukocytes in alveolar lumina; Wright's stain, ×250. (B) Mammary gland after 0.5 hr of nursing demonstrating influx of leukocytes in the alveolar lumina; Wright's stain, ×250.

RNA increased significantly ($p<0.01$) 0.25 hr after placing pups under the mother's cage, remained higher ($p<0.01$) than controls after 0.25 and 0.5 hr of nursing, and decreased ($p<0.01$) after 1 hr of nursing (Table II). These changes in mammary nucleic acid content suggest that leukocytes moved into the mammary gland during the first 0.5 hr of nursing, and then were lost in the milk removed during nursing. Moreover, photomicrographs of randomly selected histological sections of mammary glands from nonnursed and nursed (0.5 hr) rats confirmed the presence of appreciably more leukocytes in the alveolar lumina of nursed than nonnursed glands (Fig. 1). Thus, the transitory increase in mammary nucleic acids and the appearance of leukocytes in the alveolar lumina seen here coincide with the results of Emmel *et al.* (1), who reported a movement of leukocytes into the mammary gland in response to nursing.

Rats nursed for 2 hr with either 2, 4, 6, or 8 pups showed no trends in mammary nucleic acids when compared to nonnursed controls. However, mammary weights during these times were well below gland weights of rats nursed 0.25 and 0.5 hr, when the nucleic acid content of the gland reached maximum levels. These observations support the speculation that milk leukocytes were being removed from the mammary gland at a rate faster than the movement of circulating leukocytes into the gland.

Summary. Lactating rats were killed after exposure to specific nursing intensities and suckling intervals to examine the effects of these factors on circulating leukocytes, plasma corticosterone, and mammary nucleic acids. Nursing durations of 0.25, 0.5, and 1 hr caused a 3-fold increase in plasma corticosterone when compared to nonnursed levels. In comparison, plasma corticosterone concentrations were similar to nonnursed control values after hours 2, 3, and 4 of nursing. In addition, exteroceptive stimuli associated with nursing provoked increases in plasma corticosterone that were comparable to values obtained from 0.25 or 0.5 hr of nursing. Circulating leukocytes decreased 24% during the first 0.5 hr of nursing with 10 pups; whereas mammary nucleic acid content increased 9%, suggesting an influx of leukocytes into mammary tissue. Histological examination of mammary glands revealed a striking increase in leukocytes within the alveolar lumina of glands within 0.5 hr after nursing. The results from this study indicate that nursing is a dynamic situation which is capable of provoking striking alterations in leukocytic and adrenocortical function.

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