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Nerve Growth Factor in Mouse Milk during Early Lactation: Lack of Dependency on Submandibular Salivary Glands

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ABSTRACT. Using a specific and sensitive nerve growth factor radioimmunoassay we show measurable quantities of β nerve growth factor in mouse milk during the period of early lactation. Partial purification by cationic exchange resin yielded a preparation which exhibited biological activity in a PC-12 cell bioassay system. Submandibular-sublingual sialoadenectomy had no influence on the breast milk NGF concentrations. These results support the presence of bioactive NGF in mouse milk during early lactation, but do not clarify the source. (*Pediatr Res* 19: 934–937, 1985)

Abbreviations

NGF, nerve growth factor
βNGF-RIA, βNGF-radioimmunoassay
SMG, submandibular gland

NGF refers to a family of proteins that play a key role in the survival and differentiation of selected neurons of the peripheral nervous system (1, 2). Despite the potential *in vivo* requirement for NGF during specific stages of development the biological source(s) and routes of NGF supply are not clearly understood. Selective retrograde axonal transport for NGF has been described in target neurons of mature animals (3, 4), suggesting that end organs may supply NGF to neurons through an axonal transport system. Studies with iris muscle partially support the view that end organs may indeed synthesize NGF (5, 6). However, in neonatal animals innervation of end organs by target neurons is not complete (7, 8). This process requires several weeks and during this time NGF must reach target neurons through routes other than specific retrograde axonal transport systems.

The observation that systemically administered NGF in neonatal animals elicits biological responses (9, 10) suggests that NGF may reach target neurons through the general circulation. Orally administered NGF also is effective, suggesting that NGF can be absorbed and transported in bioactive form from the gastrointestinal tract (11). Thus in neonatal animals one of the potential sources for NGF could be milk which is known to contain several hormones and growth factors (12–16). Evidence for the presence of NGF in milk has been inconclusive (16, 17). The present study therefore examines the concentrations of NGF in milk during the period of early lactation in the mouse using both a specific βNGF-RIA and a neurite outgrowth bioassay system. The results suggest that mouse milk contains measurable concentrations of NGF which is present in a biologically active form. Further, studies with sialoadenectomized adult female animals indicate that the bulk of milk βNGF originates from sources other than the submandiblar salivary glands.

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MATERIALS AND METHODS

Animals. Adult female Swiss Webster mice which have undergone at least three to four pregnancies were purchased from Simonsen Laboratories (Gilroy, CA). On arrival to the Harbor-UCLA vivarium, they were placed in individual cages and kept under controlled temperature and lighting conditions. They were given water and pelleted standard mouse food *ad libitum*. In 30 animals the submandibular sublingual salivary glands were removed under general anesthesia with pentobarbital sodium (60 μg/g body weight). Sham operations were performed in another 30 animals. All animals were allowed to recover from surgery for a 3-wk period. Sialoadenectomized animals exhibited disorderly and ungroomed appearances with regard to their body hair. Both sham-operated and sialoadenectomized female mice were housed with males (three females + one male) for mating. Males were separated from females 72 h after their caging with females and pregnant females were separated from each other on day 16 of their pregnancy to be housed in individual cages. In the sialoadenectomized animals, 80% of the female mice became pregnant while all animals sham-operated underwent pregnancy successfully.

Pups were reduced to eight per dam on the day of delivery. The day of birth was considered as day 0. No differences were observed in litter size or nursing capacity of sialoadenectomized mothers and sham-operated animals.

Milk collection. Pups were separated from the mothers at least 4 h prior to breast milk collection. The lactating mothers were anesthetized with pentobarbital sodium. Fifteen minutes before milk collection, oxytocin (160 mU) was injected intraperitoneally. Breasts were gently pressed between two fingers and the milk ejected was collected through a pipetman fitted with polyethylene pipet tips. Milk samples were transferred to polystyrene tubes and stored at −70° C.

Milk samples also were collected from the stomach of the pups. For this purpose, pups were sacrificed on specified days; abdomens were opened with a scissor, the stomach exposed, and the contents were emptied after cutting the stomach wall with a razor blade. Both breast milk and stomach milk were processed similarly for βNGF quantification. βNGF content (expressed per milligram protein) in milk samples collected from the stomach of the pups was not different from that in milk collected from the breast of the respective mothers during the first 4 days after parturition. For partial purification of milk NGF, both breast milk and stomach milk were pooled.

Preparation of milk homogenate supernatants. Milk collected from the breast had variable consistency and the milk collected from the stomach of the pups was semisolid in nature. Thus breast milk samples and the milk collected from the stomachs of the pups were homogenized in a glass-Teflon homogenizer (1:9 w/v) in 0.05 M phosphate buffered saline, pH 7.2, containing 0.1% sodium azide. The homogenates were centrifuged at 145,000 × *g* for 60 min and the supernatants used for βNGF quantification by RIA. Protein content of the supernatants was quantified by the method of Lowry *et al.* (18), using bovine serum albumin as standard.

Partial purification of milk NGF. Milk samples collected from the breast and the stomachs of the pups were pooled, homogenized in phosphate buffered saline, were centrifuged at 145,000 × *g* and the supernatants collected as described above. Aliquots were taken both for protein estimation and βNGF quantification and lyophilized and stored at −70° C until further analysis. A typical purification procedure was as follows: 60–70 mg of lyophilized protein was reconstituted in 2 ml of 0.02 M phosphate buffer and dialyzed against the same buffer for 24 h with two changes. The dialyzed sample was made up to a known volume with 0.02 M phosphate buffer (3–5 ml) and aliquots were taken for protein estimation and NGF quantification. Three milliliters of the sample were loaded on a CM-cellulose column (0.5 × 9 mm) packed in a 13-ml disposable plastic syringe, equilibrated with 0.02 M phosphate buffer as originally described by Mobley *et al.* (19). The column was washed with the same buffer until the absorbancy of the eluate fell below 0.5 OD at 280 nm. The unabsorbed material was pooled and dialyzed against 2 liters of 0.25 mM phosphate buffer for 24 h.

After dialysis sufficient amounts of 0.5 M sodium acetate buffer (pH 4.0) and solid NaCl were added such that the final concentration of the buffer was 0.05 M and NaCl concentration 0.04 M. Samples were centrifuged and the supernatant was loaded on a second CM-cellulose column (0.5 × 6 cm) packed in a 10 ml disposable plastic syringe. The column was washed with 50 ml of 0.05 M acetate buffer containing 0.04 M NaCl. The protein remaining in the column was eluted in 1-ml fractions with 0.05 M Tris-HCl buffer, pH 9.0, containing 0.4 M NaCl. Aliquots of the fractions were tested for βNGF by RIA. The fractions containing βNGF immunoreactive material were pooled and pressure dialyzed against RPMI-1460 buffer using a 10 YM ultrafiltration membrane (Amicon Coporation, Lexington, MA). The volume of the sample was finally concentrated to 1 ml and filter sterilized prior to bioassay.

PC-12 cell bioassay. PC-12 cells were maintained in a culture medium containing 85% RPMI-1640 medium supplemented with 10% horse serum and 5% fetal calf serum, 100 μg/ml streptomycin, and 150 U/ml penicillin. PC-12 cells primed with βNGF (50 ng/ml) were used for neurite outgrowth responsiveness (20). Prior to bioassay, cells were washed three times with culture medium containing no NGF. Finally cells were resuspended in RPMI buffer containing 5% horse serum. One hundred microliters of cell suspension (1×10^4 cell/ml) were added to each well (24 well coaster culture plate), followed by 800 μl of culture medium. Aliquots of the sample to be tested were added in 100 μl volume. Purified βNGF (1–20 ng/ml) was added in parallel to other wells containing PC-12 cells. The culture dish was incubated at 37° C in a CO_2 incubator. The neurite outgrowth response was scored at the end of a 36-h incubation period.

βNGF-RIA. βNGF was quantified by RIA using an antiserum containing at least two high affinity antibodies (kd 7.6×10^{-11} and 7.0×10^{-10} mol/liter) generated to a purified submandibular gland βNGF preparation. βNGF was isolated by the method of Mobley *et al.* (19) with the modification of Chapman *et al.* (21). This preparation was used for immunization, iodination, and as reference standard. The antiserum bound 50% ^{125}I-βNGF in a final dilution of 1:250,000. The assay is sensitive to 16-21 pg βNGF/tube. The antiserum reacts equally with purified βNGF and 7S NGF from SMG tissue. No cross-reaction was observed with purified SMG renin or EGF. Nonspecific binding was consistently less than 6%. The inter- and intraassay variations were less than 10%.

The βNGF RIA was performed in polystyrene (12 × 25) test tubes. The buffer and incubation system were as described by Walker *et al.* (22). The procedure of Walker *et al.* (22) was modified as follows: 24 h after incubation with labeled NGF, 40 μl of goat antirabbit γ globulin were added; 1 h later 60 μl of 25% polyethylene glycol 8000 (J. T. Baker Chemical Co, Phillipsburg, NJ) were added. One hour later the tubes were centrifuged at 2500 × *g* for 30 min. The supernatants were aspirated and the precipitates counted. Results were calculated after log logit transformation. ^{125}I-βNGF used in this study was iodinated using the chloramine T method of Greenwood *et al.* (23); labeled βNGF was purified on a CM cellulose column using a buffer system similar to that described by Mobley *et al.* (19) for the isolation of βNGF. The specific radioactivity of ^{125}I-βNGF, determined by self displacement assay, approximated 5.3×10^6 Ci/mol.

Parallelism of milk homogenate supernatants and standard βNGF was assessed by measuring serial dilutions of milk in the RIA system. The slopes of the regression lines (standard *versus* milk homogenate supernatants) were calculated after log logit transformation and tested for significant difference using Students' *t* test.

Recovery studies were performed by adding varying amounts of standard NGF (range 0.25–0.025 ng) to 100 μl of milk homogenate supernatant. The measured NGF per tube was compared to the amount expected (sum of endogenous level assayed plus known amounts of standard βNGF added). The percentage recovery was calculated from triplicate specimens.

RESULTS

Figure 1 shows log-logit plots of βNGF immunoreactivity of purified mouse SMG-βNGF and breast milk homogenate supernatant over the range of 20–200 μl/tube. The slope of the line for 10-fold dilutions of milk homogenate supernatant was statistically not different from the slope of the line for purified βNGF standard. The mean (±SEM) recovery of unlabeled βNGF added to milk homogenate supernatant was 92 ± 5% (data not shown).

Purification of βNGF immunoreactivity from milk homogenate supernatant by the procedure described yielded only 40% recovery. Figure 2 illustrates the biological response to 5 ng (as measured by RIA) of partially purified milk βNGF. This neurite outgrowth response was comparable to that observed with PC-

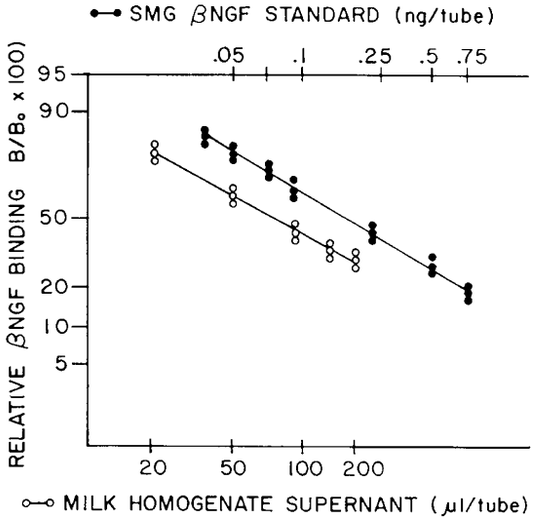

Fig. 1. Representative displacement curves of ^{125}I-βNGF by unlabeled standard NGF and by breast milk homogenate supernatant.

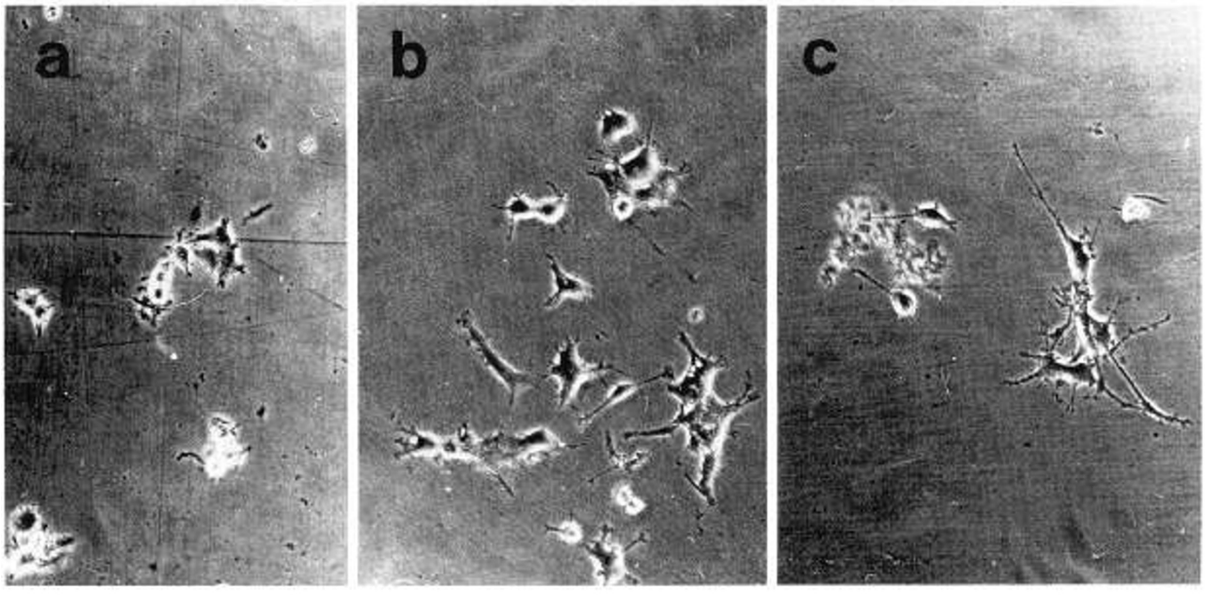

Fig. 2. PC-12 bioassay. Phase contrast micrographs (magnification × 200) of PC-12 cells cultured in RPMI-1640 buffer supplemented with 3% horse serum for 36 h. Additions: none (*a*); 5 ng of purified submandibular gland-βNGF preparation (*b*); and partially purified milk βNGF of 5 ng concentration as measured by RIA (*c*).

12 cells cultured in the presence of similar amounts of purified SMG-βNGF and could be blocked with βNGF antiserum.

Figure 3 compares the relative breast milk βNGF concentrations in sham-operated and sialoadenectomized lactating mothers. No significant differences were observed between the two groups during the first 12 days of lactation. In both groups the contents were low on day 0.

DISCUSSION

The data of the present study confirm the presence of βNGF in mouse milk both by RIA and bioassay. Milk βNGF concentrations (expressed per milligram protein) were consistently less than 400 pg during the first 12 days of lactation. No significant differences were observed between sham-operated and sialoadenectomized lactating mothers. Milk collected from the breast was sticky in nature and there was much variation in consistency among samples. Thus we were unable to express our results per milliliter milk. However, the mean protein concentration of breast milk collected from lactating animals on days 0–12 postpartum ranged from 80–90 mg/ml. Thus, an approximation of the βNGF content per milliliter of milk would be 32–36 ng. This concentration was sufficient to demonstrate the presence of βNGF in the PC-12 bioassay (20).

In the present study milk samples directly added to PC-12 cell cultures were found to be highly toxic. A toxic effect of milk also was observed in the dorsal root ganglion bioassay system by Pantazis *et al.* (17). We did not attempt to assess the nature of factors involved in the toxic effects of milk. Milk does contain several hormones, glycoproteins, and lipids (12). Steroid hormones in excess are reported to inhibit neurite outgrowth elicited by NGF (24). We did not quantify the concentrations of steroid hormones. The characteristics of milk NGF partially purified by cation exchange resin suggests that milk NGF may be a basic protein similar to mouse SMG-βNGF. However, the site of production appears not to be the salivary gland since milk concentrations do not change after sialoadenectomy. The recent identification of βNGF mRNA in organs other than SMG (25) suggests that this growth factor may be synthesized at multiple sites. Whether it is synthesized in the mammary gland itself remains to be determined.

The present observation of a lack of significant difference in milk NGF content in sham-operated and sialoadenectomized

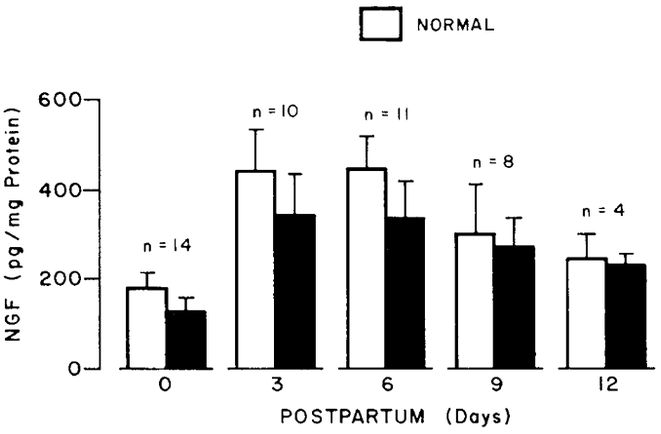

Fig. 3. Concentrations of breast milk NGF in normal (sham-operated) (*open bars*) and submandibular gland-sublingual gland sialoadenectomized animals (*solid bars*): βNGF was quantified by specific RIA. Student's *t* test was used for the comparison of mean values. See text for other details.

mothers is similar to our earlier report of similar milk epidermal growth factor concentrations in sialoadenectomized and sham-operated mice (26). Thus, it appears that both βNGF and epidermal growth factor in breast milk probably originate from non-SMG tissues. The physiological significance of SMG during pregnancy and lactation is not clear.

In our studies no differences were observed in litter size or nursing capacity of sialoadenectomized mothers and sham-operated animals. However, these observations are in contrast to results reported by Okamoto and Oka (27). These authors suggested an endocrine role for the SMG during pregnancy and lactation. According to these investigators, pregestational sialoadenectomy decreases the growth of the mammary gland and its capacity to synthesize milk, leading to an increase in offspring mortality. Our different results could be due to methodological variations. In our study we used experienced adult female mice, >200 days of age and weighing 32–35 g at the time of sialoadenectomy, whereas Okamoto and Oka (27) performed sialoadenectomy in virgin female mice (50–60 days old). Our animals all had undergone three to four successful pregnancies and were

well nourished prior to study. Experience in our laboratory indicates that animals during the first two pregnancies are less suitable than experienced animals for physiological studies involving pregnancy, lactation, nursing, and neonatal and infant growth. Wide variations often are observed in nesting behavior, litter size, and nursing ability in mice during the first one or two pregnancies. Both maternal neglect and maternal cannibalism are greater in young mothers. In addition, young mothers are very sensitive to frequent handling of pups during routine cage changes. Moreover, studies in our laboratory indicate, at least in adult female mice, that removal of the SMG does not influence the epidermal growth factor concentration in tissue and body fluids (28, 29).

The extent to which milk NGF contributes to the growth and development of NGF target cells in suckling animals is not yet clear. Several investigations indirectly suggest that milk NGF may play a significant role in target neuronal growth and maturation. Original studies by Levi-Montalcini and coworkers (30, 31) and by Cohen (32) demonstrate that administration of antisera to mouse NGF to newborns of several species results in the destruction of the peripheral nervous system. Aloe *et al.* (11) demonstrated that oral administration of NGF (pharmacological doses) elicited hypertrophy of sympathetic superior cervical ganglia. This study possibly mimics a normal physiological process. In addition the offspring born to normal rats but suckled by an anti-NGF antibody-producing rat were shown to have relatively small sympathetic superior cervical ganglia with decreased tyrosine hydroxylase activity (33). The irreversible damage elicited by NGF antiserum injected into newborn animals during the first 12 days of life indicates a requirement for this growth factor during this critical period (34). NGF antiserum may exert its effect by neutralization circulating NGF (35). Whether sensitive tissues produce NGF locally and whether circulating NGF is important remain unanswered questions.

In suckling animals milk represents the only source of food. The possibility that milk NGF may participate in development, at least of the sympathetic ganglion, is suggested by studies of Gaetani *et al.* (36). These authors reported changes in protein content and specific neurotransmitter synthesizing enzyme levels as a consequence of alteration in litter size. Their results indicate the maternal nutrition plays a significant role in the growth and maturation of autonomic ganglia at it does in the brain (37–39). Further studies are necessary to delineate the role of milk NGF in maturation of the autonomic nervous system in mice and other species. The increasing list of growth factors in milk of rodents (14) and in humans (15, 16) confirms the importance of these factors for target organ growth and maturation during the perinatal period.

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