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Nerve growth factor in mouse serum and saliva: Role of the submandibular gland

(sialoadenectomy/exocrine gland)

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ABSTRACT **The concept that the salivary gland of the mouse is an endocrine organ for nerve growth factor (NGF) has been reexamined. Serum concentrations of the protein have been measured by radioimmunoassay in male and female mice and in mice from which the submandibular glands were removed. In spite of the fact that the submandibular glands of male mice contained more NGF than did those of female mice, no sex differences in circulating concentrations of the factor were detected. Furthermore, serum concentrations of NGF did not change after submandibular gland removal or after administration of several autonomic agonists. These results indicate that the submandibular glands are not endocrine organs with respect to NGF. On the other hand, extremely high concentrations of the factor are normally secreted in mouse saliva at levels that reflect the sex differences in the amount of NGF present in the glands. This finding suggests that the salivary gland is an exocrine organ for NGF and that the protein may play a biological role in saliva.**

For unknown reasons, nerve growth factor (NGF) is present in high concentrations in the submandibular glands of mice (1, 2) and in the venom of certain snakes (1, 3). The concentration of NGF is much higher in glands of male mice than in glands of female mice or prepubescent mice of either sex (1, 2). Biologically active NGF can also be detected in mouse blood (1). By using a sensitive radioimmunoassay, Hendry (2) found that the plasma of adult male mice contains 6 times as much NGF as does plasma of adult female mice, and Hendry and Iversen (4) found that sialoadenectomy caused a decrease in circulating levels of the factor. After submandibular gland removal, plasma concentrations of NGF dropped to 15% of control values over a period of 33 days and then returned to normal after an additional 27 days, at which time the sex difference in serum levels was reestablished (4). These findings have given rise to the widely held view that circulating levels of NGF are controlled (at least in part) by the submandibular glands (4, 5). In earlier papers (e.g., see ref. 6), Levi-Montalcini and her colleagues also favored this concept because they observed that sialoadenectomy resulted in the total disappearance of biologically active NGF from the blood. However, more recent papers from this group (e.g., see ref. 7) express the view that the submandibular glands are not primarily endocrine organs that secrete NGF into the blood, because removal of these glands had no apparent deleterious effects on the mouse.

Recent studies in this laboratory have shown that several different kinds of cells secrete NGF in culture (for a review, see ref. 8); in principle, these observations could be made to fit conceptually with the finding (4) that serum levels of NGF are only temporarily decreased by sialoadenectomy. However, the results of Hendry and Iversen (4) still present some difficulties. For example, the half-life of ^{125}I-labeled NGF (^{125}I-NGF) in mouse serum has been found to be on the order of 10–30 min (5). Consequently, if the submandibular glands are a major source of circulating NGF, we would expect that removal of the glands would cause a rapid decrease in serum levels of the protein. However, Hendry and Iversen (4) found that this did not occur.

In the present study, we reexamined the role that the submandibular glands might play in secreting NGF into the circulation. In contrast to the results of Hendry and Iversen (4), we found that removal of the submandibular glands has no effect on circulating levels of NGF over a period of 1–5 weeks after sialoadenectomy. Furthermore, serum levels of the factor in adult male and female mice were indistinguishable and did not reflect the sex differences found in salivary gland levels of the protein. Consequently, we conclude that the submandibular gland in the mouse does not function as an endocrine organ for NGF.

These results then led to the idea that the salivary gland might be an exocrine organ for NGF, and we found that submandibular gland saliva from both males and females contained extraordinarily high concentrations of the protein. Wallace and Partlow (9) have reported that α-adrenergic stimulation of male mice preferentially produces saliva rich in NGF. In contrast, we found that high levels of NGF are continously secreted in saliva in the absence of any exogenously administered agent. Taken together, these findings raise some questions concerning the physiological role that the submandibular glands play in the biological function of NGF. It could be that the submandibular glands dispose of NGF by secreting it into the digestive tract, or, alternatively, that the protein in saliva has some heretofore unsuspected biological role in the alimentary tract.

METHODS AND MATERIALS

Reagents. Doubly glass distilled H_2O was used for all solutions, and buffer salts were reagent grade. NGF was isolated from male mouse submandibular glands by the method of Bocchini and Angeletti (10), and all preparations were shown to be electrophoretically homogeneous as previously described (11). Pilocarpine was obtained from Cooper Laboratories, isoproterenol *d*-bitartrate and bovine serum albumin (three-times recrystallized) were from Sigma, phenylephrine was from Winthrop Laboratories, tribromoethanol was from Aldrich, and *t*-amyl alcohol was from Pfaltz and Bauer (Stamford, CT). Na-^{125}I was purchased from New England Nuclear.

Animals. Swiss mice CD-1 (outbred albino) were obtained from Charles River Breeding Laboratories, Inc., and were fed Purina laboratory chow and water. For general anesthesia, tribromoethanol dissolved in *t*-amyl alcohol (1.6 g/ml) was given intraperitoneally at a dose of 0.4 mg/g of body weight. Pilocarpine, isoproterenol, and phenylephrine were adminis-

Abbreviations: NGF, nerve growth factor; ^{125}I-NGF, ^{125}I-labeled NGF.

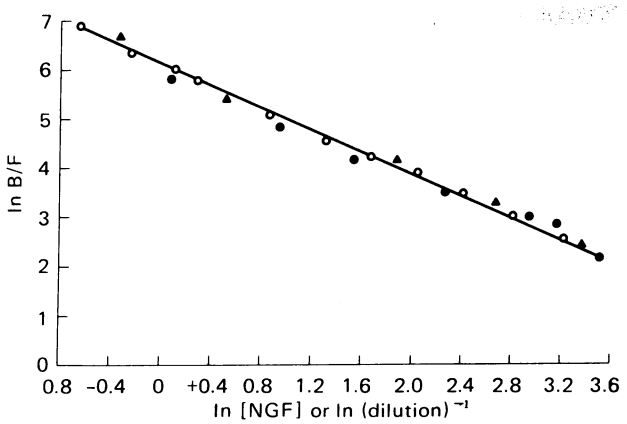

FIG. 1. Quantitative immunologic comparison of submandibular gland NGF (○), submandibular salivary NGF (●), and serum NGF (▲). Values of antibody-bound and free ^{125}I-NGF were measured by radioimmunoassay.

tered by intraperitoneal injection at doses of 30, 6, and 5 mg/kg, respectively. For sialoadenectomy, a midline incision was made in the neck of the anesthetized animal. Glands were dissected free from surrounding structures and removed after ducts and blood vessels were ligated. The skin was closed with 5-0 nylon sutures. Sham-operated animals were prepared by freeing the glands from surrounding structures and then closing the incision. No obvious infections occurred in any of the animals studied. To collect pure submandibular gland salivary secretions, the submandibular ducts were carefully dissected bilaterally from supporting tissue and cut transversely. Saliva was allowed to flow into the pretracheal space. Capillary tubes (10 μl) were used for collection, and any blood-contaminated samples were discarded. Saliva samples were immediately diluted with 0.1 M potassium phosphate containing bovine serum albumin (1 mg/ml) at pH 7.0, and these solutions were frozen. Blood was obtained from anesthetized animals by cardiac puncture and sera were frozen until assayed. Sera were used in all studies after it was observed (8) that NGF immunoassays of serum and plasma yielded identical results.

Radioimmunoassays. Several rabbit antisera to NGF were used in this study. All have been demonstrated to be monospecific (11). Preparation of ^{125}I-NGF and the details of radioimmunoassay have been given elsewhere (11). Several preparations of ^{125}I-NGF were used in this study and all displayed specific activities close to 0.6 gatom I/mol NGF.

Biological Assays. Sensory ganglion bioassays of NGF were performed with 8-day chick embryo dorsal root ganglia as previously described (12).

RESULTS

To compare the immunologic behavior of serum and salivary NGF with that of pure gland NGF, by radioimmunoassay, ^{125}I-NGF displacement curves were constructed. Fig. 1 illustrates plots of ln B/F (B and F are the concentrations of antibody-bound and free ^{125}I-NGF, respectively) versus ln[NGF]. The lines for both submandibular gland saliva and serum are parallel to and superimposable on the line obtained with pure gland NGF. Thus, although the results of Fig. 1 do not prove that salivary and serum NGF are immunologically identical to gland NGF, they do indicate that all three molecules are indistinguishable by the immunoassay. These findings are supported by earlier studies in which mouse serum NGF levels were measured by radioimmunoassay and by a bacteriophage immunoassay. Both methods gave virtually identical results (8).

Table 1. NGF concentrations (ng/ml) in sera from adult mice*

Male mice	Female mice
13.6	9.9
11.8	9.1
7.4	9.1
10.9	8.6
7.0	6.8
11.8	7.9
8.5	8.6
Mean 10.1	9.6
SD ± 2.5	9.0
	7.5
	Mean 8.6
	SD ± 1.0

* Each value is from one animal. Animals (>56 days old) were housed and treated identically. All animals weighed approximately 35 g.

Table 1 presents representative results of radioimmunoassays of NGF in serum from adult male and female mice. No significant difference between the sexes was detected. In these experiments, all animals were housed identically and blood samples were drawn at the same time in order to minimize changes in serum levels that might have resulted from diurnal variation or some other unknown cause. In this regard, it should be noted that normal serum levels in our laboratory routinely range from 10 to 40 ng/ml, and these numbers are in good agreement with those found by Hendry (2) and Hendry and Iversen (4). Moreover, these concentration differences do not stem from variations in the assay, the antibody used, or the preparation of ^{125}I-NGF.

To study the effect of submandibular gland removal on serum concentrations of NGF, control, sham-operated, and sialoadenectomized groups, each containing five adult male mice, were examined at various time intervals after sialoadenectomy. The results (Table 2), from studies of 135 animals, indicate that over a period of 1–5 weeks no significant changes in serum NGF concentrations were detected following submandibular gland removal. However, the NGF concentration increased sigificantly in the serum of sham-operated (but not

Table 2. Effect of sialoadenectomy on NGF levels in serum of adult male mice*

Time after operation	Serum NGF, % of control	
	Sham	Operated
30 min	428 ± 26	104 ± 6
30 min†	98 ± 7	112 ± 9
1 day	103 ± 3	93 ± 7
7	58 ± 5	73 ± 5
11	93 ± 6	93 ± 6
14	75 ± 4	103 ± 6
21	109 ± 3	74 ± 4
28	144 ± 3	100 ± 3
35	100 ± 6	86 ± 7

* Animals were sialoadenectomized and sham-operated as described in the *text*. NGF was measured by radioimmunoassay. Each group (control, sham, operated) contained five animals at each time interval. Values are mean ± SEM.

† In this group of animals, the sham operation consisted of opening and closing the neck incision without gland manipulation.

Table 3. Effects of adrenergic and cholinergic agonists on serum NGF levels in adult male mice*

Drug	Time, min	NGF, % of control
Isoproterenol	5	100 ± 8
	15	85 ± 7
	30	93 ± 3
	60	100 ± 9
Phenylephrine	5	133 ± 29
	15	116 ± 21
	30	123 ± 2
	60	176 ± 38
Pilocarpine	5	107 ± 7
	15	126 ± 11
	30	119 ± 9
	60	128 ± 13

* Anesthetized animals were given intraperitoneal injections of the drugs as described in the *text*. At the indicated times, blood was withdrawn and serum NGF was measured by radioimmunoassay. Values at each time interval represent the means (± SEM) from at least four animals. Statistical analyses (*t* test) showed no significant differences within any of the above series, except that in the phenylephrine group the small changes shown were significant ($P < 0.01$) at 30 min period but not at 5, 15, and 60 min.

sialoadenectomized) animals 30 min after operation. This result suggested that perhaps the surgical sham procedure itself could have resulted in cellular or tubular damage within the gland and concomitant release of NGF into the circulation. To study this possibility, a group of animals was examined in which the sham operation consisted only of opening and closing the neck incision without any gland manipulation. Under these conditions, there were no changes in serum NGF concentrations relative to control or sialoadenectomized animals.

It has been reported that the serum concentration of epidermal growth factor (also found in high levels in the mouse submandibular gland) increases 100-fold within 1 hr after administration of phenylephrine (13). Consequently, mouse serum NGF levels were measured during 5–60 min after administration of phenylephrine, isoproterenol, or pilocarpine. No

Table 4. NGF concentrations (μg/ml) in submandibular saliva from male and female mice*

Adult male mice	Adult female mice	Young male mice
71	0.56	6.0
504	0.38	5.7
37	0.60	6.5
216	0.10	Mean 6.1
113	1.29	SD ± 0.4
45	3.32	
14	Mean 1.04	
22	SD ± 1.2	
180		
241		
214		
229		
24		
Mean 147		
SD ± 139		

* Immunoassays were performed in quadruplicate with saliva diluted with 0.1 M potassium phosphate containing bovine serum albumin (1 mg/ml) at pH 7.0. Each value is from a single animal. Adult animals were at least 56 days old; young males were 30–35 days old.

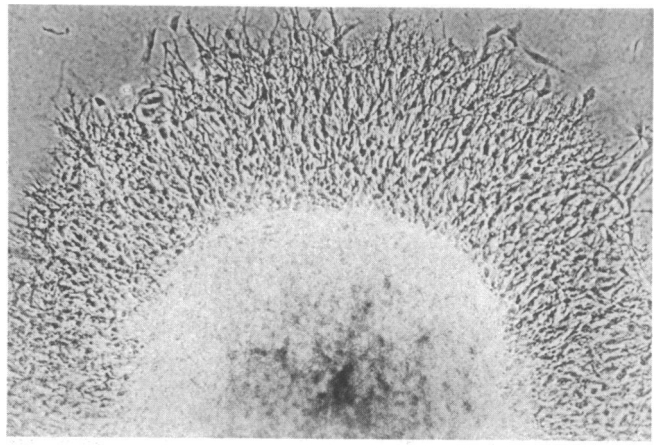

FIG. 2. Sensory ganglion bioassay of salivary NGF. The NGF concentration was 50 ng/ml as measured by radioimmunoassay. (Phase contrast photomicrograph, 18-hr incubation at 37°; ×96.)

consistently significant changes were detected (Table 3). Taken together, all of the preceding results led us to infer that serum NGF does not arise from the submandibular gland and that, in contrast to the prevailing view, the gland is not an important endocrine organ for this growth factor. We then turned to saliva.

Pure submandibular gland saliva, collected from unstimulated animals, contained high concentrations of NGF (Table 4). For example, male saliva contained more than 100,000 times the concentration required to display biologic activity in the sensory ganglion assay system, and female saliva contained 1000 times the biologically active concentration. There was considerable variation in concentration from animal to animal. Yet these values are real and completely reproducible from assay to assay. All of the saliva samples used for the data in Table 4 were serially diluted as described in Fig. 1 and all dilution plots were virtually identical to the one shown in Fig. 1 (which was obtained with the 504-μg/ml sample of saliva noted in Table 4). Moreover, as shown elsewhere (14), when purified mouse gland NGF is added to saliva it remains stable and can be accurately measured by radioimmunoassay. Fig. 2 reveals that highly diluted saliva also yields a strongly positive response in the sensory ganglion bioassay system.

Wallace and Partlow (9) concluded that α-adrenergic stimulation preferentially caused an increase in the concentration of NGF in saliva. That conclusion was based on studies in which NGF in saliva from adrenergically or cholinergically stimulated animals was measured by semiquantitative biological assay and a radial immunodiffusion assay. But no control animals (untreated) were examined by these authors. Further, careful examination of their results (table 1 in ref. 9) reveals that saliva obtained after epinephrine administration (both α- and β-adrenergic) actually contained a 4-times higher NGF concentration than that following norepinephrine administration (predominantly α-adrenergic). Thus, it is difficult to understand how their conclusion was reached. Our results cannot be compared quantitatively with the data of Wallace and Partlow because they examined mixed, not submandibular gland, saliva, and the antibody used in their immunoassay was stated to be heterogeneous and not specific only for biologically active NGF (9).

It has been appreciated for a long time that the submandibular glands of newborn mice contain very low levels of NGF (1, 2). Yet, if NGF has an important role to play in saliva, then it is curious that glands of newborns contain such low levels. This

reasoning led us to measure the NGF concentration in mouse milk. For this purpose, two 10-day postpartum female mice were removed from their young for 24 hr and then injected with pituitrin (Parke-Davis; 0.4 IU/kg of body weight, intraperitoneally). A milking device, similar to that described by McBurney *et al.* (15), was used to obtain between 30 and 80 μl of milk. The milk was then diluted (1:100) with 0.1 M potassium phosphate, pH 7.0, for radioimmunoassay. One sample of milk contained 0.3 μg of immunoreactive material per ml and the other, 1.1 μg/ml. Again it should be noted that these concentrations are high compared to the concentrations required to demonstrate biologic activity in the ganglion system. Moreover, when examined in the immunoassay as described in Fig. 1 for saliva and serum, the milk samples could not be distinguished from pure NGF. However, sensory ganglion assays of both milk samples have given negative results.

DISCUSSION

The results of this study indicate that the submandibular glands of mice are not endocrine glands for NGF, that sera of male and female mice contain comparable concentrations of the factor, and that very high concentrations are normally secreted in submandibular gland saliva.

The present observation that sialoadenectomy does not alter circulating levels of NGF is not consistent with the results of Hendry and Iversen (4), who observed a slow decrease in NGF levels, followed by a slow rise several weeks later. We cannot offer any explanation for the differences between our results and theirs. Yet, if the submandibular glands were actively secreting NGF into the bloodstream, we would expect an almost immediate decrease in circulating levels—particularly in light of the rapid elimination of ^{125}I-NGF from the blood (5). Moreover, the concentration of NGF in saliva is more than 10^4 times that in serum, and we are unaware of any physiologic situation in which a gland is both endocrine and exocrine for the same substance. Consequently, serum NGF must arise elsewhere in the animal and, because many different kinds of cells are known to secrete NGF in culture (8), it seems reasonable to infer that the serum factor arises from multifocal cellular secretion.

Until the present study [and (9)], the fact that mouse saliva contains such high concentrations of NGF seems to have been overlooked. On the other hand, in 1967 Levi-Montalcini and coworkers examined very high dilutions of saliva by bioassay; but no mention was made of the fact that the data would have pointed to high concentrations of the factor in undiluted saliva (16). From a more recent study, Schwab *et al.* (17) reported that carbachol stimulation caused a massive release of submandibular gland tubular cell vesicles into the duct, but they could not detect NGF in saliva (17).

The central question is what is NGF doing in saliva, particularly at such high concentrations. As shown in Table 4, male saliva contains about 100 times the concentration of female saliva—although it will be appreciated that female saliva also contains high concentrations of the factor, based upon its known biological activity in the ng/ml range of concentration. Not surprisingly, this male–female saliva difference parallels that found in the submandibular glands of the two sexes. Several possibilities could account for NGF in saliva. 1. The submandibular gland could be an excretory organ for NGF, removing the factor from the circulation and excreting it in saliva. However, if this were the case, serum NGF levels would be expected to increase after sialoadenectomy, and they do not. Furthermore, it has been reported that intravenously administered ^{125}I-NGF is not accumulated by the gland (5). 2. NGF is synthesized by the gland, utilized for some biological purpose within the gland, and then excreted in saliva. 3. NGF plays a heretofore unsuspected biological role in saliva, perhaps upon some component of the alimentary tract. In this regard, it will be shown in a companion paper (14) that the molecular properties of saliva NGF are different from those of any form of the protein that has been studied so far. The idea that NGF may have a role in the digestive system is reminiscent of the finding that epidermal growth factor (also present in saliva) is a strong inhibitor of gastric acid secretion and that it is probably identical to urogastrone which has been shown to promote healing of experimentally induced gastric ulcers (18).

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