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Nerve growth factor-like immunoreactivities in rodent salivary glands and testis

Lars Olson[1], **Christiane Ayer-LeLievre**[1], **Ted Ebendal**[2], **and Åke Seiger**[1]

[1] Department of Histology, Karolinska Institutet, Stockholm, Sweden;
[2] Department of Zoology, Uppsala University, Uppsala, Sweden

Summary. A series of polyclonal affinity-purified antibodies against mouse submandibular-gland nerve growth factor (NGF) are described. Using the submandibular gland of the male mouse and indirect immunofluorescence, the specificity and sensitivity of affinity-purified immunoglobulins and various other fractions from the immunized animals have been tested. It will be shown that affinity-purification schemes, including pre-purification of protein A-fractionated immunoglobulins to remove antibodies that bind to unrelated hydrophilic and hydrophobic proteins, significantly enhance the signal-to-noise ratio and specificity of the antibodies. The antibodies effectively detect NGF-like immunoreactivity in both fresh and fixed glandular tissue. Optimal fixation procedures are described. Fluorescence intensities are linearly correlated to log antibody concentration. By use of the best antibody fractions and optimal fixation protocols, the distribution of NGF-like immunoreactivity is described in eight different salivary glands (rat and mouse, male and female, submandibular and sublingual glands). In addition to the well-known large numbers of immunoreactive cells in the submandibular gland of the male mouse, immunoreactive cells were found in the sublingual gland of male mice and in the submandibular and sublingual glands of female mice. One antibody revealed a weak specific fluorescence also in the submandibular gland of the male mouse. In a survey of genital organs of male mice, one antibody revealed fluorescence in the germ cell line. We conclude that several polyclonal affinity-purified antibodies have been characterized that show a strong NGF-dependent binding to the secretory granules of tubular cells in the submandibular gland of male mice. These antibodies should make it possible to locate endogenous and perturbed NGF levels immunocytochemically, e.g., in the peripheral and central nervous system, where NGF concentrations may be several orders of magnitude lower than in the salivary glands.

Key words: Nerve growth factor – Salivary glands – Antibodies – Immunocytochemistry – Affinity purification – Specificity tests – Testis – Mouse

The submandibular gland of the adult male mouse is the source most used for purification of nerve growth factor (NGF). Antibodies generated against mouse salivary gland β-NGF are widely used to measure NGF levels in other tissues using radio- or enzyme immunoassays, to visualize NGF-like immunoreactivities in tissues using immunohistochemistry, and as a specificity test in bioassays of nerve growth factor activity. Furthermore, antibodies directed against NGF have been administered systemically to animals pre- and postnatally and to adult animals to counteract the development of NGF-sensitive neuronal systems or to induce degeneration of adult nerve fibers that depend on NGF (cf. Levi-Montalcini and Angeletti 1968; Harper and Thoenen 1981, for reviews).

In view of the extensive use of antibodies directed against mouse salivary gland NGF, comparatively few studies have employed immunohistochemistry to study localization of NGF in salivary glands (Schwab et al. 1976; Gresik and Azmitia 1980; Gresik et al. 1980; Hofmann and Drenckhahn 1981; Ayer-LeLievre et al. 1983; Hazen-Martin and Simson 1984; Rush 1984; Watson et al. 1985; Aloe et al. 1985). The present study is a detailed account of the possibility of localizing NGF by the use of antibodies in rodent salivary glands. Optimal fixation procedures will be described; antibodies raised in several (different) rabbits and one sheep were used to localize NGF-like immunoreactivities in eight different salivary glands. Special attention will be paid to the specificity tests available in immunohistochemistry, and to the possibility of relating estimates of fluorescence intensity to the amount of antibodies present. Finally, an interesting localization of NGF-like immunoreactivity in the mouse testis will be reported.

Materials and methods

Sublingual and submandibular salivary glands from adult male and female N.M.R.I. mice and male and female Sprague-Dawley rats were used. In addition, testis, epididymis, the seminal vesicle, the bulbourethral glands (Cowper) and the prostate from adult mice were studied. Animals were killed under deep ether anesthesia.

Tissue preparations

The following different fixation procedures were evaluated using the submandibular gland of male mice: (1) Perfusion fixation: Animals were anesthetized with barbiturate, and perfused through the ascending aorta with Ca-free heparinized Tyrode's solution at 37° C, then at 4° C followed by

Send offprint requests to: Dr. Lars Olson, Department of Histology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

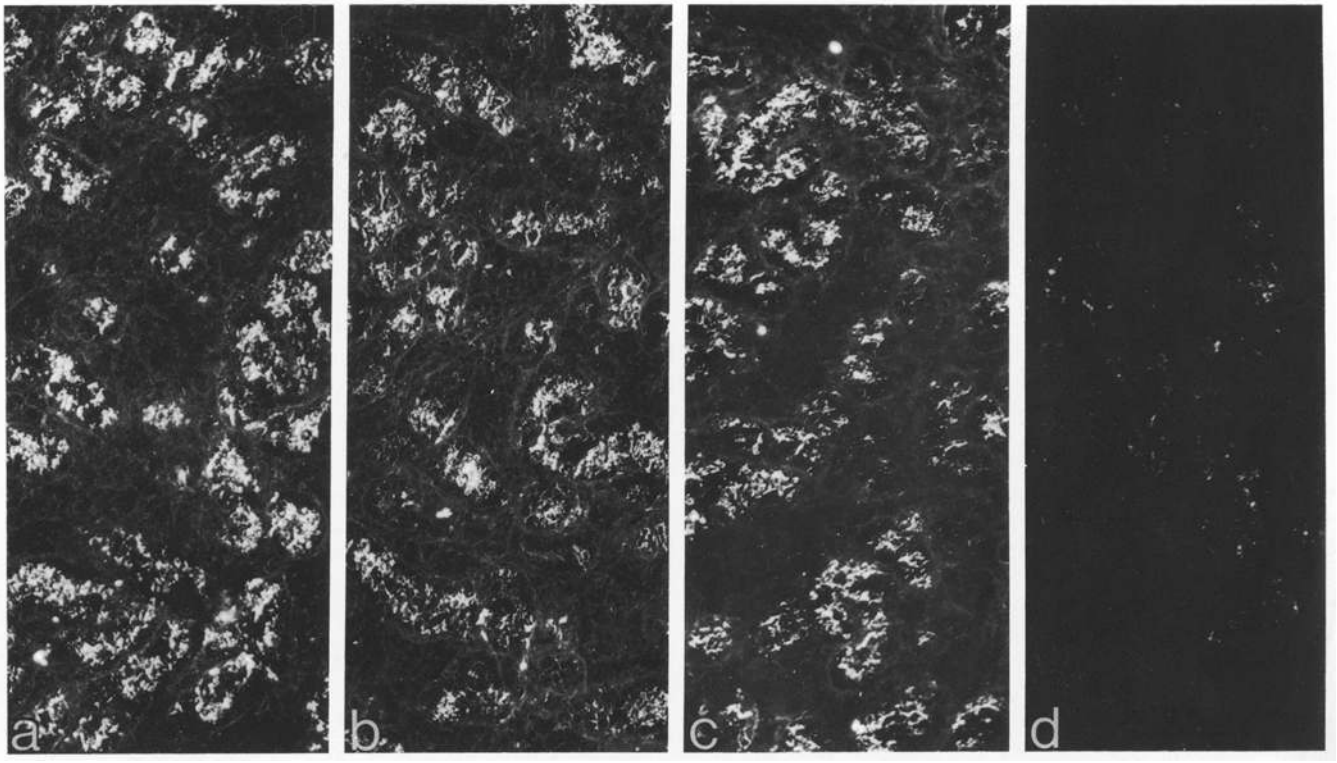

Fig. 1 a–d. Cryostat sections of fresh-frozen submandibular glands from male mice. **a–c** Strongly NGF-immunoreactive granules located to the apical zones of the convoluted secretory ducts. **a** Sheep antibody (F1). **b** Rabbit antibody (K7). **c** Rabbit antibody (K17). In **d** the sheep antibody was preadsorbed, and virtually no immunoreactivity is seen. a–d × 128

4% formaldehyde in phosphate-buffered saline (PBS); (2) immersion fixation in the same fixative; (3) immersion fixation in a mixture of 0.5% formaldehyde and 0.2% picric acid; (4) immersion fixation in a mixture of 2% formaldehyde and 0.2% picric acid; (5) immersion fixation in a mixture of 2% formaldehyde and 0.2% parabenzoquinone; (6) no fixation.

Fixed tissues were rinsed in 10% sucrose in PBS. Time and temperature for fixations and rinsings were varied. All material was frozen on dry ice and sectioned on a cryostat at 14 or 6 μm.

Production of antibodies

The submandibular-sublingual gland complex was rapidly removed from adult male mouse, frozen on dry ice and processed as required for purification of β-NGF (Mobley et al. 1976; Ebendal et al. 1984). This β-NGF preparation was used to immunize rabbits (Ebendal et al. 1983, 1984). In a separate experiment, the sublingual gland, located in the laterocranial quadrant of the submandibular-sublingual salivary gland complex was removed and the submandibular glands alone collected from 200 mice. β-NGF purified from this raw material was used to immunize a sheep. In addition, purified β-NGF was treated with formaldehyde (2%). The formaldehyde-denatured β-NGF thus obtained was used to immunize one rabbit.

Polyclonal antibodies were raised in several rabbits and in one sheep by multiple intradermal and intramuscular injections of β-NGF in Freund's adjuvant. The specificity of the various antisera was evaluated in a bioassay employing chicken sympathetic ganglia as described elsewhere (Ebendal et al. 1984). Ig fractions from the various immunized animals were affinity-purified by passing diluted antiserum over a column of β-NGF coupled to CNBr-activated Sepharose 4B (Stoeckel et al. 1976; Ebendal et al. 1983, 1984). The adsorbed antibodies were displaced by 4.5 M $MgCl_2$ in acetate buffer (pH 5). Normally, 800 μg of anti-β-NGF Ig was recovered per ml antiserum applied. A more detailed fractionation of the sheep antibodies included passing them over a column of hydrophilic chicken proteins coupled to Sepharose followed by a column of hydrophobic chicken proteins coupled to Sepharose followed by the standard column of β-NGF coupled to Sepharose. The rejected antibodies were purified on protein A-Sepharose and used as controls.

Specificity controls. To control for the specificity of the immunoreactivity observed, several control preparations were employed, including "affinity-purified" antibodies from the NGF-Sepharose column derived from the preimmune sheep serum (recovery about 60 μg per ml serum), rejects from the various affinity purifications, antibodies adsorbed to the hydrophilic and hydrophobic chicken proteins (recovery about 20–100 μg per ml antiserum), respectively, normal rabbit serum, and hyperimmune serum from rabbits immunized with chicken embryo proteins. In addition, antibodies against myelin basic protein were tested. Sections where the first antibody in the immunohistochemical procedure was omitted were always included.

The specificity was also tested by preadsorbing the various antibodies with its proper antigen, the mouse β-NGF. Antigen-antibody mixtures were made at least 16 h prior to use. Since NGF was used in excess in these mixtures,

the effects of preincubating sections with NGF followed by incubation with or without the NGF antibodies were also tested.

An increased salt concentration (0.5 M instead of 0.15 M) was tested as a means of avoiding unspecific binding of antibodies. Additional albumin used as a "carrier" of antibody solutions was also tested. Finally, shaking antibody solutions with freeze-dried liver powder was tested as a way of preabsorbing nonspecific components of the polyclonal antibody preparations.

Immunocytochemistry

Using the indirect immunofluorescence technique of Coons (1958), cryostat sections were incubated overnight at 4° C with primary antibodies (sera, Ig fractions, affinity-purified antibodies, rejects, etc.) in various concentrations in PBS containing 0.3% Triton X-100. After thorough rinsing, the second antibody, fluorescein isothiocyanate-labeled swine anti-rabbit or rabbit anti-sheep Ig (Dako-Patts) was applied for 60 min at room temperature in darkness. After a second thorough rinsing, sections were mounted in 90% glycerin in PBS containing 0.1% p-phenylene diamine used as an antifading agent.

Slides were evaluated using dark-field fluorescence microscopy with an oil-immersed dark-field condenser and excitation and barrier filters optimal for FITC-fluorescence. All evaluations were made on coded slides, often by two independent observers. Fluorescence intensities were semiquantitatively estimated on a 0–5 scale. Tri-X (Kodak) was used for photography.

Results

Effects of various fixatives

Fresh-frozen sections not exposed to any fixative showed strong fluorescence with all antibodies tested (F1, K7, K17) in the convoluted secretory ducts of submandibular glands from male mice (Fig. 1). Antibodies preabsorbed with β-NGF gave a weak or very weak signal, as did antibodies from a normal rabbit. When the first antibody was omitted, no specific fluorescence was observed. Treatment of the fresh-frozen cryostat sections with acetone for 3 min caused a slight increase of the specific fluorescence. Both with and without acetone, however, morphological preservation of gland structure was unsatisfactory.

Similarly, fixation by immersion in a mixture of 0.5% formaldehyde and 2% picric acid gave poor results. Although specific fluorescence was observed, morphology and the tensile strengths of the sections were inferior. Increasing the formaldehyde concentration to 2% in this mixture improved morphology and specific fluorescence. Perfusion or immersion fixation in 4% formaldehyde resulted in good morphological preservation and relatively strong specific fluorescence in the convoluted secretory ducts. Optimal results were obtained both with the mixture of 2% formaldehyde and 0.2% parabenzoquinone and with perfusion fixation, which combined good morphological preservation with optimal fluorescence histochemical signals.

Tests of temperature and time parameters, although not complete for all types of fixations, suggest that short fixation in the formalin-parabenzoquinone mixture, in the range of 1–3 h at room temperature with agitation, followed by rinsing in 10% sucrose preferably with two changes at room temperature with agitation overnight gave optimal results. Extended storage in either fixative or rinsing fluid was clearly deleterious.

Table 1. NGF-like immunoreactivity in secretory ducts of the submaxillary gland from male mice, observed with affinity-purified antibodies from rabbits and sheep, and with various control antibodies

Antibodies	Fluorescence intensity[a]	% Fluorescence remaining after preadsorption[b]
A. From animals immunized with NGF:		
Rabbits:		
K4	1.3	60
K7	2.5	4
K10[c]	1.5	83
K16	2.4	2
K17	3	6
K18	1.9	3
Sheep F1	4	16 (3–30)
B. Controls:		
Preimmune sheep	0	
Normal rabbits:		
K3	0	
K19	0.3	
Hyperimmune rabbits:		
K11	0	
K15	0	

[a] Numbers represent means of estimated fluorescence intensities on a 0–5 scale. Each number represents the mean of at least four sections in each of 1–15 different experiments

[b] Percentage values were obtained by dividing mean fluorescence intensities with preadsorption by the mean fluorescence intensities without preadsorption as given in the table

[c] Triple-purification of this antibody, as described for the sheep antibody in Table 2, yielded a much stronger signal (4+) and preadsorption of the triple-purified antibody decreased fluorescence much more (approximately 20% left)

Screening of antibodies and specificity tests

Antibodies from six rabbits and one sheep immunized with native β-NGF were tested in adult male mouse submandibular glands fixed in a mixture of 2% formaldehyde and 0.2% parabenzoquinone (Table 1). Parent sera from the immunized animals were all positive in the submandibular gland of male mice (Fig. 2d). Immunoreactivity was markedly decreased following preabsorbtion with NGF (Fig. 2e, 3c). Affinity-purified antibodies (Table 1) from four rabbits (K7, K16, K17, K18) bound to cells in the convoluted secretory ducts, giving a much better resolution than the antisera. Using optimal concentrations (20 μg of immunoglobulin per ml), this binding resulted in moderate or strong fluorescence intensities and a good signal-to-noise ratio. The affinity-purified antibodies from the sheep generated an extremely strong signal from the salivary duct cells (Fig. 2a, b). Preadsorbing these different antibodies with an excess of the antigen almost completely abolished the specific fluorescence. With two rabbit antibodies giving

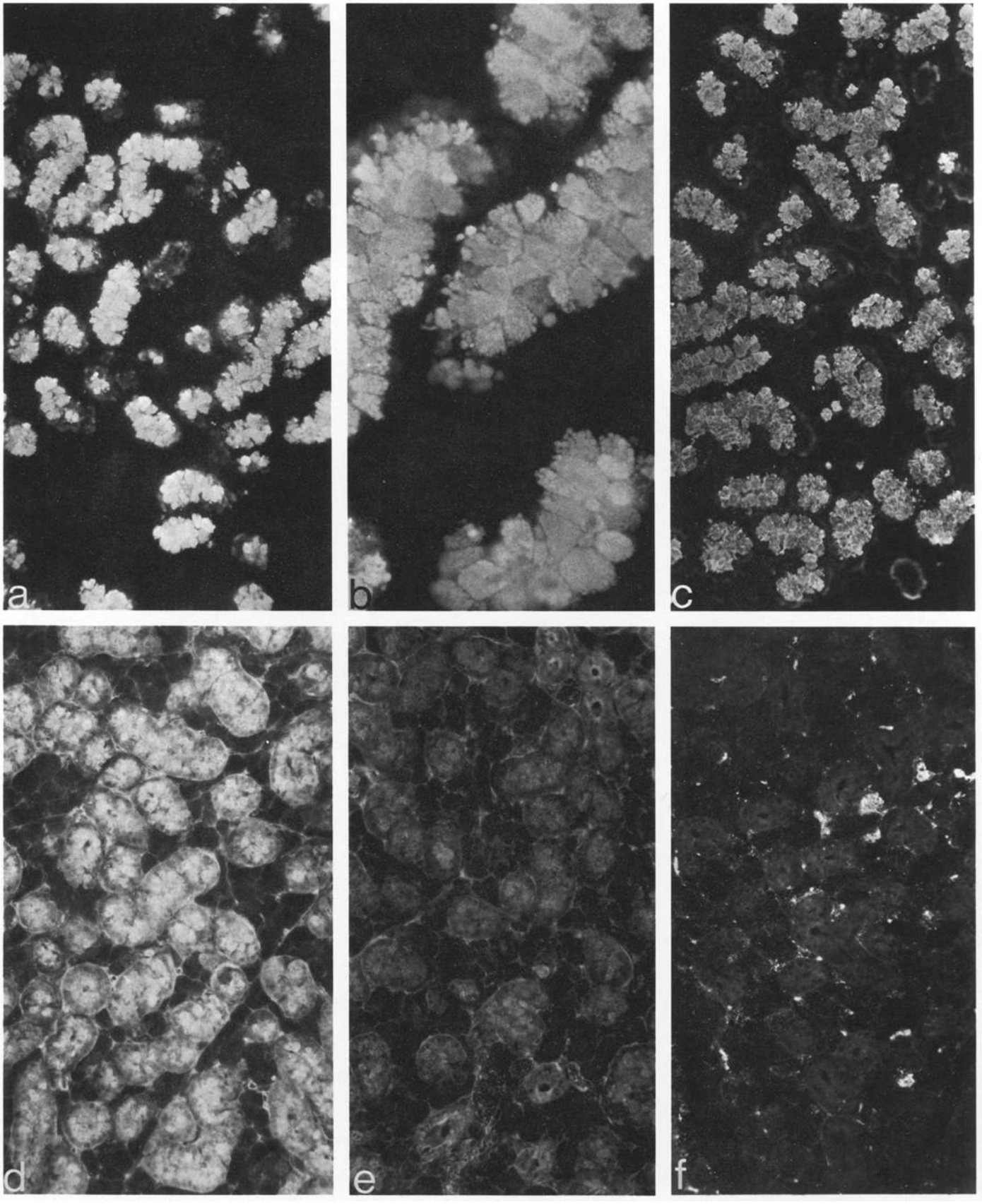

Fig. 2a, b. Perfusion fixation with paraformaldehyde and sheep antibody (F1). Very strong granular NGF-immunoreactivity against a negative background. **c** Paraformaldehyde-denatured NGF was used to raise this rabbit antibody (K30). The signal-to-noise ratio is very good. **d** Parent sheep NGF antiserum shows a strong specific staining, but also some fluorescent membranes. **e** Same as **d**, but preadsorbed; almost no specific staining is seen. **f** Hyperimmune rabbit, immunized to chicken proteins not related to NGF. Only some interstitial cells are positive, leaving all the convoluted secretory ducts negative. a and c–f × 135, b × 300

Table 2. Binding of the various antibody fractions to secretory ducts of submandibular gland from male mice as evaluated by fluorescence immunohistochemistry. Boxes symbolize Sepharose columns with covalently bound proteins as indicated. Standard affinity-purification rejects represent the fraction of an IgG preparation that passes through a β-NGF column without binding to it

Fractionation	Fraction	Fluorescence in % of standard affinity-purified antibody	Preadsorption: Fluorescence in % of non-preabsorbed fraction	Comments
Protein A-fractionated IgG from sheep F1 before immunization				
β-NGF	desorbed preimmune	0		
	preimmune reject	0		
Protein A-fractionated IgG from sheep F1 after immunization				
hydrophilic chicken proteins	desorbed hydrophilic	50	83	High level of unspecific background fluorescence
hydrophobic chicken proteins	desorbed hydrophobic	35	0	Low background
β-NGF	desorbed NGF	133	13	Strong specifically located noise-free signal in all secretory duct cells
	triple reject	67	128	Distinct noise-free signal in apical granules of selected duct cells
Standard affinity-purification rejects:				
sheep F1		54		
rabbit K7		1		
rabbit K17		17		

weak fluorescence intensity in salivary duct cells (K4, K10), preadsorption of the antibodies with β-NGF caused only minor decrease in the fluorescence intensity. As also illustrated in Table 1, three types of control antibodies, generated by "affinity-purifications" of Ig from sheep serum obtained prior to immunization, normal rabbit serum (K3, K19), and serum from the rabbits immunized with a nonrelated antigen (chicken proteins, K11, K15), respectively, generated no or negligible fluorescence in salivary ducts (Fig. 2f).

Immunization of a rabbit with formaldehyde-denatured NGF gave rise to antibodies that recognized the same sites in formalin-fixed salivary glands (Fig. 2c) as antibodies against native NGF. Thus, there seemed to be no advantage in using formaldehyde-denatured NGF for immunization.

Table 2 illustrates the results obtained using those fractions of the various Ig preparations that did not bind to β-NGF-sepharose columns and further fractionation of the sheep antibody. Rejects from the three best antibody producers (F1, K7, K17) differ markedly in that the two rabbit-derived rejected Ig fractions showed little specific fluorescence, while the reject from the sheep-derived Ig fraction gave rise to a fluorescence of the salivary duct that was approximately 50% of that of the affinity-purified fraction (Fig. 3d). This is apparently not unspecific binding caused by antibodies present in the sheep prior to NGF immunization, since neither an "affinity-purified" fraction from the preimmune Ig (20μg/ml) nor a reject from the preimmune Ig caused any fluorescence of the salivary duct at all. To elucidate further the nature of the marked fluorescence found in the postimmunization sheep reject fraction, Ig from the immunized sheep was further fractionated in three steps as illustrated in Table 2. It was found that the crude sheep Ig mixture contained antibodies (0.5–1.5% of Ig recovered) that attach to hydrophilic chicken proteins and that can cause fluorescence of salivary glands both in the

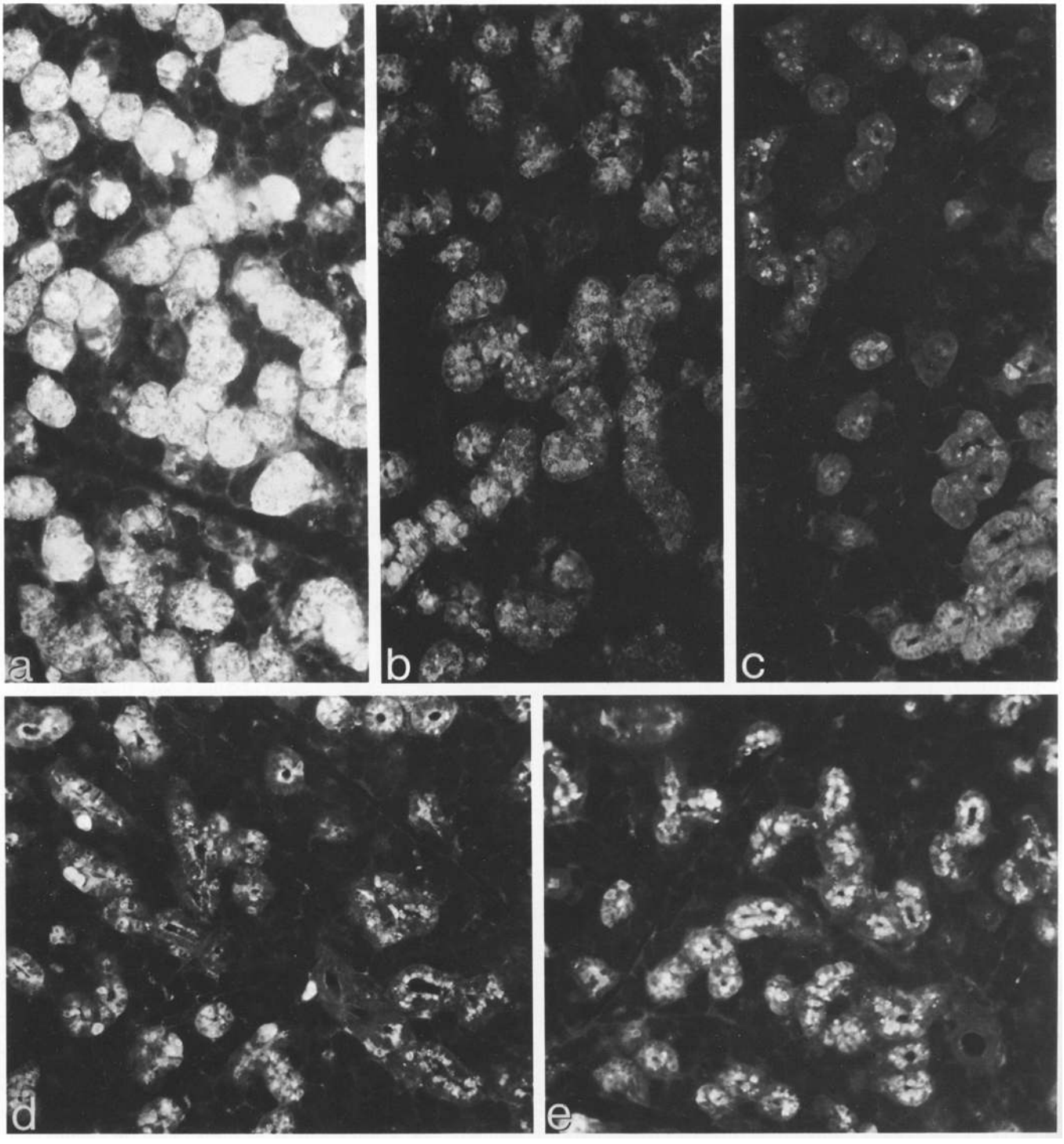

Fig. 3a–d. Triple-purified sheep antibody. **a** Dilution 1:15 with an intense NGF-immunoreactivity. **b** Dilution 1:420 with some remaining NGF-immunoreactivity. **c** The high concentration as in **a**, but preadsorbed with antigen. Almost totally negative. **d** Triple-reject sheep antibody. Several immunoreactive cells in the convoluted secretory duct cells of the submandibular gland from male mouse. **e** Same as **d**, but preadsorbed with β-NGF. The immunoreactivity is not decreased compared with **d**. a–e $\times$ 128

convoluted secretory ducts and elsewhere in the gland in the form of a high unspecific background staining. This fluorescence cannot to any appreciable degree be abolished by preadsorption of the fraction with β-NGF. A second component of the sheep Ig was found to bind to the column of hydrophobic chicken proteins next in sequence (γ 0.1–0.2% of Ig). This component gave rise to a weak to moderate duct fluorescence with a good signal-to-noise ratio and a low background. It could be completely abolished by preadsorption with β-NGF. When material that had passed through the two columns loaded with hydrophilic and hydrophobic chicken proteins respectively, was passed through a β-NGF column, two interesting fractions were obtained: Desorbation of material bound to the column generated antibodies with clearly improved binding characteristics as compared to the standard affinity-purification scheme.

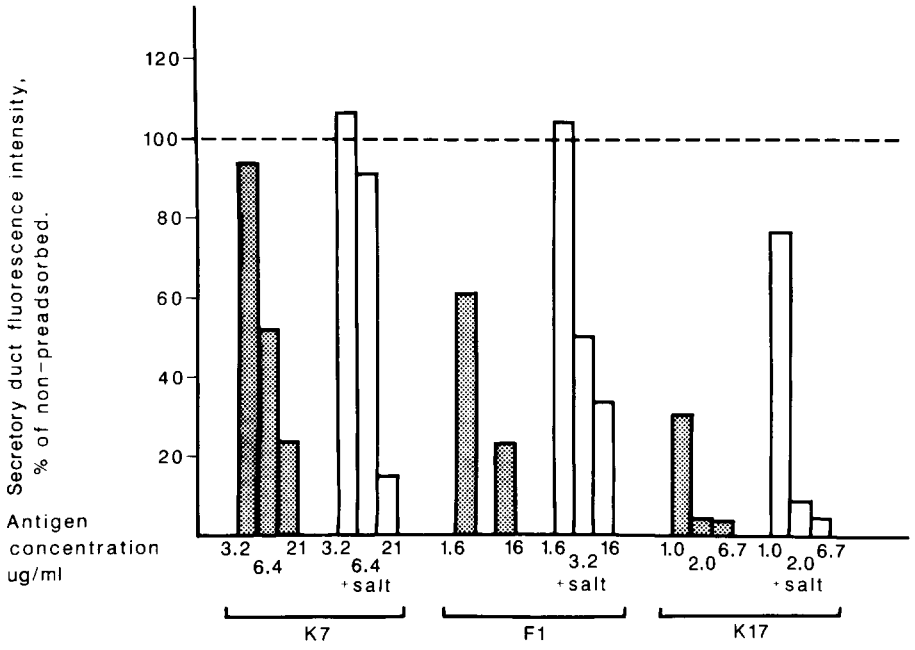

Fig. 4. Effects of graded additions of nerve growth factor to three different affinity-purified antibodies on the fluorescence intensity of secretory tubules in the submandibular gland of male mouse. Each bar represents three independent observations. The intensities of fluorescence were estimated on a blind basis as described in Materials and methods. They have been expressed here as percent of the fluorescence intensity seen with the same affinity-purified antibodies without addition of NGF. Each group of three bars represents a low, a medium and a high amount of NGF present in the antibody solution. For each antibody, the experiment was carried out both with standard salt concentrations and a higher salt concentration as described in Materials and methods. Only two antigen concentrations were used for the standard salt experiments with the sheep (F1) antibody

These antibodies gave a very strong specific signal throughout virtually all cells of the convoluted secretory ducts visualizing both apical granules and a more diffuse cytoplasmic fluorescence (Fig. 3a, b). There was virtually no noise, i.e., no background or membrane staining. Antibodies that passed the β-NGF column without binding to it, the so-called "triple-reject," were found to bind selectively to apical granules in selected cells in the striated ducts (Fig. 3d). This signal was also strong and virtually noise-free. Preadsorption revealed that the signal generated by antibodies bound to the last β-NGF column could be almost completely abolished (Fig. 3c), while the signal generated by the "triple-reject" was, if anything, improved by preadsorption with NGF (Fig. 3e).

In addition to the specific fluorescence in convoluted secretory duct cells, some of the antibodies (e.g., K7) also showed binding to a membranous component of the salivary gland parenchyma. This binding was regarded as unspecific since it was not seen with the majority of the antibodies, and since affinity-purification tended to decrease the amount of such binding as compared to crude Ig or serum preparations. Antibodies against myelin basic protein also stained these membranous components of the gland while leaving the duct cells completely unstained.

Preincubating salivary gland sections with β-NGF was used to test whether NGF would bind to any additional sites in the salivary gland parenchyma or enhance the fluorescence in the ducts. However, NGF-preincubation did not change the distribution or fluorescence intensity observed with any of the three tested affinity-purified antibodies (F1, K7, K17). When sections were incubated overnight with β-NGF instead of with the first antibody and then treated with FITC-marked second antibodies as usual, no fluorescence at all could be detected.

Three different additions to the antibody solutions were also tested: Increasing the salt concentration from 0.15 to 0.5 M in the preadsorption mixtures of antigen and antibody slightly decreased the efficacy of the adsorptions as illustrated in Fig. 4. Addition of albumin as a "carrier" for the immunoglobulins did not cause any observable changes in the distribution or intensity of fluorescent structures. Shaking antibody solutions with freeze-dried liver powder usually did not influence the results, but decreased the amount of unspecific membranous staining when such was present (as with K7). As also illustrated in Fig. 4, graded addition of antigen to the affinity-purified antibody preparations causes a graded disappearance of specific fluorescence.

In a separate experiment three affinity-purified antibodies (F1, K7, K17) were applied in six or seven different concentrations ranging from 0.3 to 20 μg/ml, and the resulting fluorescence intensities in convoluted secretory duct cells of submandibular glands from male mice semiquantitatively estimated on a blind basis. In Fig. 5 the resulting average fluorescence intensities are shown plotted against the ^{10}log of antibody concentration. This relation is almost completely linear within the concentration range studied for all three antibodies, with correlation coefficients ranging from 0.98 to 0.99.

Distribution of NGF-like immunoreactivity in mouse and rat salivary glands using optimal procedures

Specific immunoreactivity, as defined by specificity controls described above, was selectively localized to epithelial cells of the secretory granulated tubules in submandibular glands of male mice. There, duct cells with varying fluorescence intensities were found, some strongly fluorescent throughout the entire cytoplasm, others weakly fluorescent. Thin sections (6 μm) demonstrated the fluorescence to be present in secretory granules located mainly in the apical portions of the cells and more diffusely in the cell cytoplasm (Fig. 6a). Interlobular excretory ducts with single columnar epithelium contained scattered NGF-immunoreactive cells. In larger excretory ducts lined by pseudostratified or stratified epithelium, the most superficial layer was sometimes fluorescent, suggestive of binding of some NGF by the epithelium as it is being excreted. The secretory acini were completely nonfluorescent as were all other parts of the parenchyma and stroma of the gland, including parasympa-

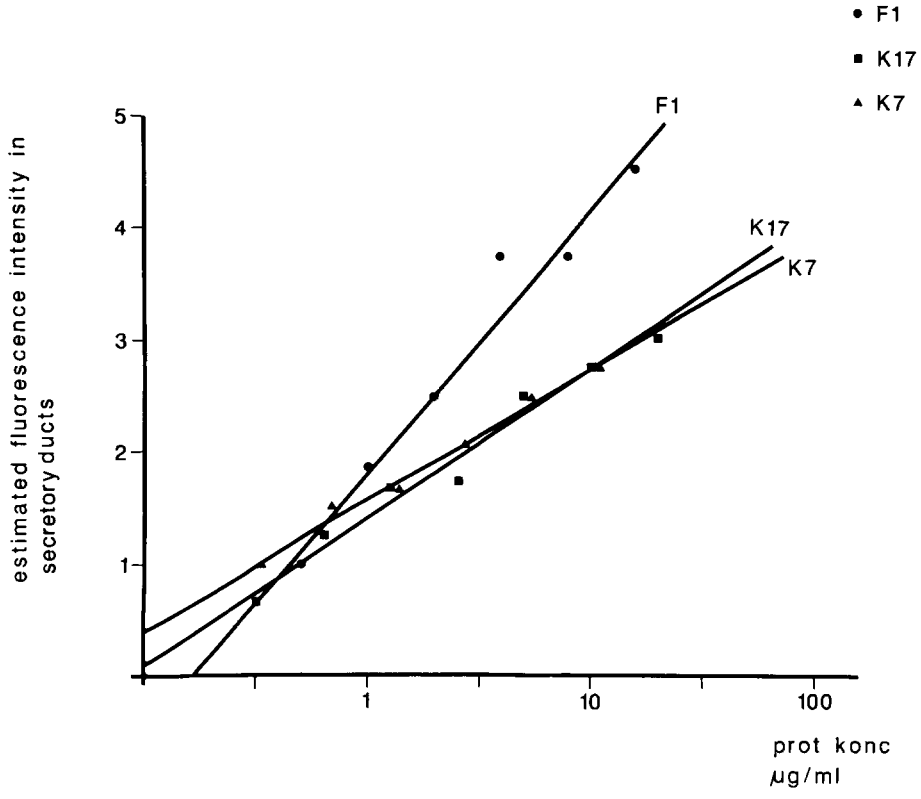

Fig. 5. Correlation between concentration of affinity-purified antibodies and estimated fluorescence intensities in the secretory ducts of submandibular gland from male mouse. The fluorescence intensity was estimated on a blind basis following incubation with 6 (F1, K7) or 7 (K17) different concentrations of antibody. There is a linear correlation better than $r = 0.98$ between ^{10}log concentration of added antibody solution and fluorescence intensity for all three tested antibodies

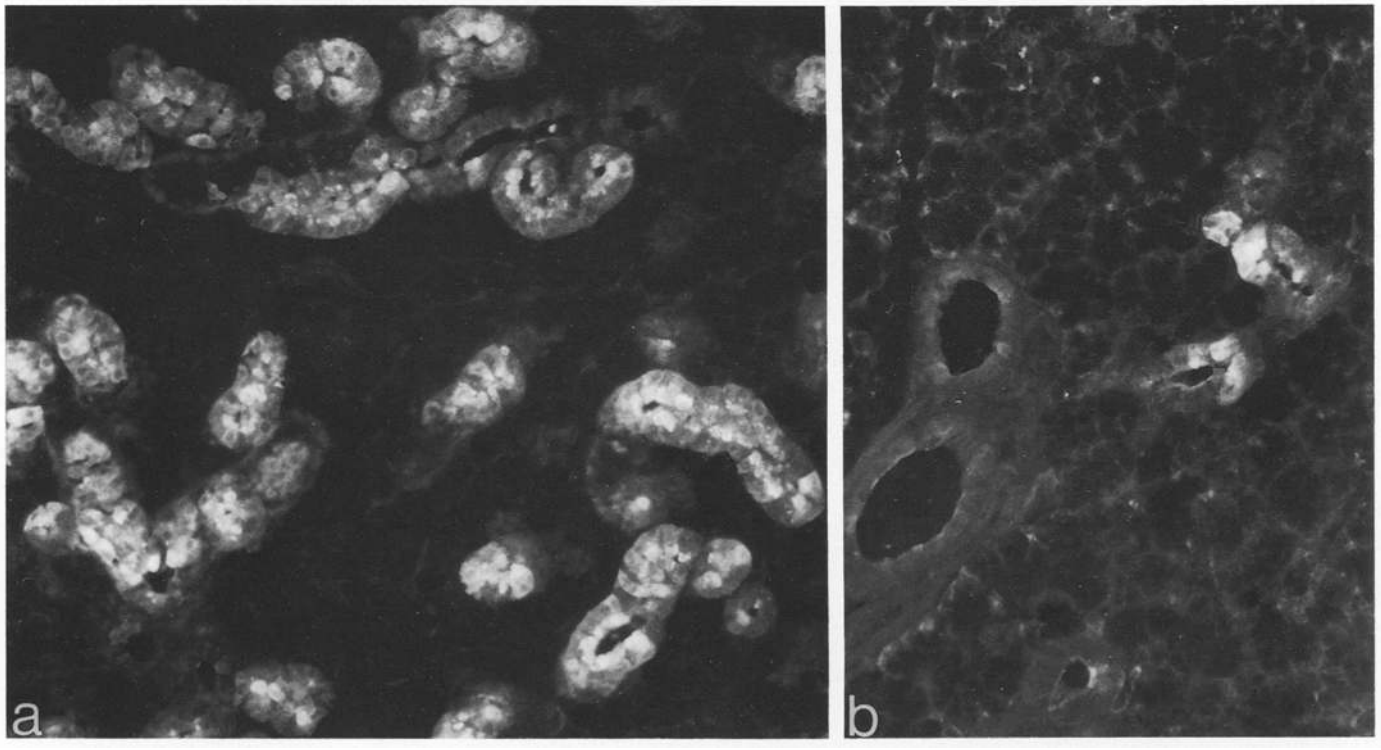

Fig. 6a, b. NGF-immunoreactive cells in secretory ducts of submandibular (**a**) and sublingual (**b**) glands from male mice. Apical parts of the cells in **a** are brightly fluorescent, whereas basal parts of the cells show a diffuse and weaker NGF-immunoreactivity. Scattered cells in ducts of the sublingual gland are also specifically immunoreactive. a, b ×225

thetic ganglia that were occasionally encountered. The sublingual glands of male mice lack convoluted secretory ducts. However, both intra- and interlobular excretory ducts contained a few scattered but strongly immunoreactive cells (Fig. 6b). Their cytoplasm was filled with fine fluorescent granules.

In the female mouse similar scattered fluorescent cells were found in the tubular components of both the subman-

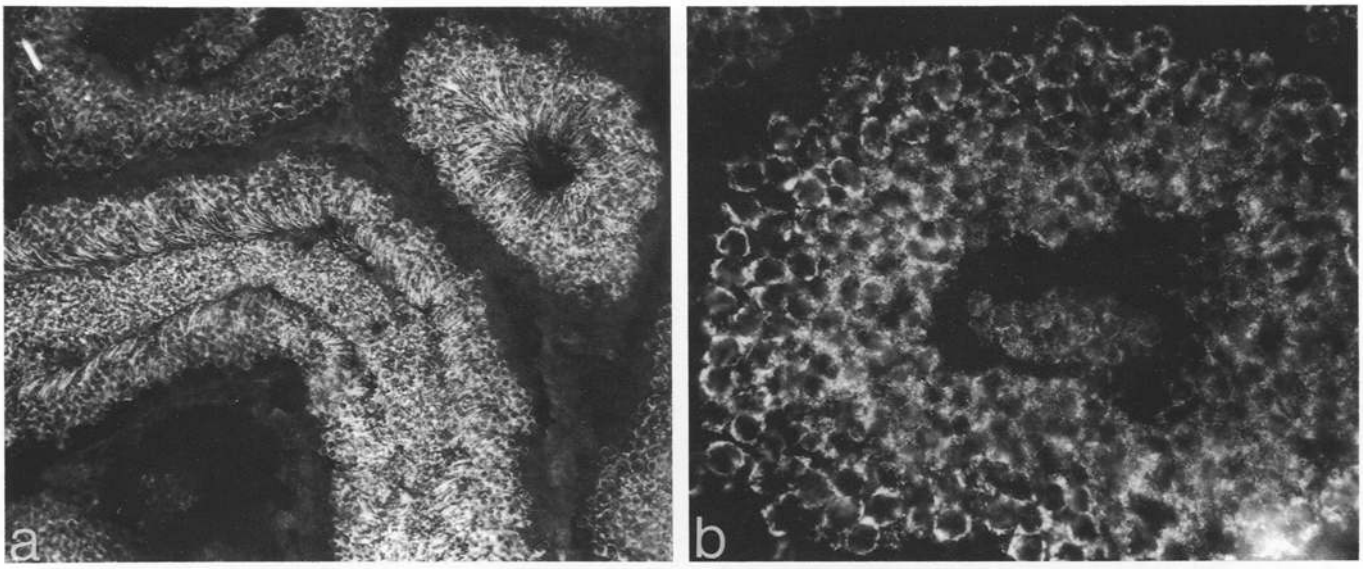

Fig. 7a, b. NGF-like immunoreactivity in mouse testis as shown with affinity-purified sheep antibodies. Most stages of sperm cell development are positive. In **a** large numbers of sperm tails are clearly positive, and in **b** numerous fluorescent spermatogonia are shown. This type of immunoreactivity was not seen with two tested rabbit antibodies (K7, K17). a ×35; b ×300

dibular and the sublingual glands. The number of such cells in both glands was similar to the number of positive cells seen in sublingual glands of male mice, but the fluorescence intensity of the cells found in the female glands was somewhat lower. Specificity tests including preadsorption with β-NGF and the use of reject fractions showed the fluorescence in male sublingual glands and female submandibular and sublingual glands to be of the same nature as that in the submandibular gland of the male mouse.

In the rat, only the submandibular gland of males showed any specific fluorescence. Here, one antibody (K17) showed scattered cells in the ducts to be weakly positive. No other fluorescence was noted in rat submandibular or sublingual glands, either in males or females, except for scattered small interstitial cells possibly of lymphocytic origin, which were sometimes positive, particularly with the sheep antibodies. This interstitial cell fluorescence did not seem to be related to NGF.

Two further observations concerning the submandibular gland of male mice are worth noting: Firstly, considerable variation in granular morphology was noted among animals. Thus, the proportion of large vs. small granules and the amount of nongranular cytoplasmic fluorescence varied. Secondly, and somewhat surprisingly, there was always a gradient in fluorescence intensities with stronger immunoreactivity at the periphery of the gland and less in central parts. This gradient did not appear to be an artifact of fixation and/or rinsing since it was also present in sections of fresh-frozen material.

Genital organs of male mice

A screening of testis, epididymis, the seminal vesicle, the bulbourethral glands of Cowper and the prostate gland with three affinity-purified antibodies (F1, K7, K17) revealed immunoreactivity in only one case, the testis studied by the sheep antibody (F1). In testes, these affinity-purified antibodies apparently bound more or less specifically to the cells of the germ line. Most stages from primary spermatocytes to mature sperms were positive (Fig. 7). More mature forms were found grouped together giving rise to bundles of fluorescent tails. The fluorescence intensity of the germ cells in the convoluted tubules was lower than that seen with corresponding concentrations of antigen in the striated ducts of the submandibular glands. Other components of the mouse testis such as the Sertoli cells and the Leydig cells were nonfluorescent or weakly fluorescent. Controls for the specificity of the fluorescence in the sperm cell line included preadsorption of the sheep antibody with NGF, which completely abolished the specific fluorescence, and treatment with three other Ig fractions from the sheep, "affinity-purified" Ig from preimmune serum, reject from affinity purification of preimmune serum, and reject from affinity-purification of postimmune serum, respectively. The latter three Ig preparations were all negative in the testis.

Discussion

The present study confirms and extends the results from previous reports on the localization of NGF-like immunoreactivity in salivary glands. Early studies yielding partly contradictory results were summarized by Schwab et al. (1976), who showed at the light- and electron-microscopical levels that NGF was localized exclusively to the convoluted secretory tubules in the submandibular gland of the male mouse. By use of anti-NGF antibodies coupled directly to horseradish peroxidase, immunoreactivity was detected only in the apical secretory granules, suggesting that NGF is derived from a precursor molecule that was not immunologically detectable by the antibodies employed. The tubular portion of the male submandibular gland is an exclusive feature of the male gland and its development dependent upon testosterone. NGF-like immunoreactivity increases in the male gland during the first six months of life, remains constant at one year, and apparently begins to decrease in old age (Gresik and Azmitia 1980). The NGF content of the secretory tubules is thus dependent upon testosterone

as is the content of epidermal growth factor, EGF (Steidler and Reade 1982), which is co-localized at both cellular and subcellular levels with NGF in the granulated convoluted tubule cells (Watson et al. 1985). The sexual dimorphism of the submandibular glands is greatly diminished in the testicular feminization syndrome, in which case both EGF and NGF immunoreactivities in male glands are dramatically decreased (Gresik et al. 1980).

NGF-like immunoreactivity in adult mouse salivary glands was also described by Hazen-Martin and Simson (1984) and by Hofmann and Drenckhahn (1981), who in addition reported the presence of NGF-positive cells in the female submandibular gland. Using one of the antibodies employed in the present study (K7), we confirmed the presence of immunoreactivity in the convoluted tubular ducts of submandibular glands of male mice and of scattered positive cells in the female gland (Ayer-LeLievre et al. 1983). Using the K7 antibody, we were unable to locate any immunoreactivity in rat submandibular glands (Ayer-LeLievre et al. 1983). Our report also presented the first evidence for NGF-like immunoreactivities outside the salivary glands in the peripheral and central nervous system. Similar studies of NGF-like immunoreactivity in the innervation apparatus of the iris and in submandibular gland of the male mouse have also been presented by Rush (1984).

In the submandibular gland, β-NGF is stored as a 7S-NGF complex consisting of three distinct proteins including, in addition to β-NGF, α- and γ-subunits (Varon et al. 1968). Antibodies raised against the α-and γ-subunits have recently been used in immunohistochemical studies (Mowry et al. 1984; Murphy et al. 1986) showing that all three components of the 7S-NGF complex exist in the same cells, suggesting that 7S-NGF is formed within these tubular cells and secreted as a complex into the saliva. Indeed, treatment with the secretogogue carbachol, which results in markedly enhanced salivation, is followed by a massive release of secretory granules and the presence of large amounts of NGF in the salivary duct as demonstrated immunocytochemically (Schwab et al. 1976). Our findings of NGF-like immunoreactivity in superficial cell layers of the major excretory duct of the submandibular gland of male mice support this finding and indicate that also under physiological conditions NGF is released in amounts large enough to be observed both in the excretory duct epithelium and in the lumen of these ducts.

Specificity of polyclonal antibodies

Our present study emphasizes problems encountered in using polyclonal antibodies to localize NGF in tissues. Obviously, the first necessary step is to make sure that the antigen is as pure as possible before attempting an immunization protocol. In our case, starting from submandibular glands after specific removal of the sublingual glands, a procedure seldom used, β-NGF was estimated to be better than 92% pure. Injection of this material might nevertheless induce a spectrum of antibodies, most of which directed against various parts of the NGF molecule, but some of which are directed against possible non-β-NGF contaminants of the antigen preparation. To evaluate this risk, it is imperative to analyze antibodies generated from several different animals. In doing so, we found four out of six immunized rabbits to be excellent producers of antibodies that, according to our criteria, seemed to stain NGF sites selectively. Two rabbits, however, contained antibodies that stained secretory cells in the convoluted tubules to a lesser degree, and in addition caused unspecific staining of other components of the salivary gland parenchyma. In these two cases preadsorption with β-NGF was uneffective in abolishing the fluorescence. Moreover, following immunization of a sheep, we obtained antibodies that, after more detailed affinity purifications, contained a major fraction of antibodies highly specific for the secretory granules of cells in the convoluted tubules, and where all criteria for binding to NGF were fulfilled. However, in addition, antibodies were found that, with an almost equally good signal-to-noise ratio, also stained secretory granules, although in only a restricted number of cells in the convoluted tubules. This latter staining was unaffected by preabsorbing the antibody with NGF. Another example of individuality between different polyclonal antibodies is the rabbit K7 antibody, which also after affinity purification carries with it a certain staining of membranous components in the salivary glands.

While these various problems with unspecific, probably non-NGF binding of the antibodies can be detected in the submandibular gland of the male mouse in which there is now a general consensus about the localization of NGF, these constraints are important to bear in mind when new territories are exploited in the search for the presence of NGF in the central nervous system, the peripheral nervous system and its target organs. Thus, we have recently presented evidence for the presence of NGF-related immunoreactivity in both the peripheral and central nervous system (Ayer-LeLievre et al. 1983; Whittemore et al. 1986). By use of thoroughly defined antibodies in combination with cDNA hybridization techniques to measure and localize NGF messenger RNA, both NGF manufacture and storage sites in different areas of the nervous system and periphery can now be located.

One potential problem in immunohistochemistry using formalin-fixed tissues is that the antibodies were raised against native NGF, whereas they must detect formalin-denatured NGF in the sections. Differences in the ability to recognize native versus formalin-denatured NGF might complicate comparisons between, e.g., enzyme immunoassays and immunohistochemistry using the same antibodies. In the present study we have approached this problem two ways: Firstly, we also included fresh-frozen sections of non-fixed salivary glands. Although these sections were of inferior morphological quality, a strong positive granulated fluorescence signal was obtained from the secretory tubules using several different antibodies. Thus, the antibodies detected immunofluorescence in exactly the same locations in fresh tissue as in formalin-fixed tissue. A second approach to this problem is to immunize animals with formalin-denatured antigen with the hope of generating antibodies that would specifically recognize denatured NGF in sectioned material. This approach, however, has so far not improved the results as compared to using antibodies against native NGF. Thus, affinity-purified antibodies from a rabbit immunized with formalin-treated NGF gave the same immunohistochemical signal in the secretory ducts of formalin-fixed material, as did other NGF antibodies.

Quantification of NGF immunoreactivities

As shown by the present results, semiquantitative estimates of fluorescence intensities performed on a blind basis, corre-

late very well with immunoglobulin concentrations used. Within a wide range of antibody concentrations there is a linear relationship between fluorescence intensity and log antibody concentration. Similarly, it is reasonable to expect that varying fluorescence intensities observed in different tissue compartments exposed to the same antibody concentration reflects a varying number of binding sites and thus varying amounts of the antigen. There were marked differences in fluorescence intensities between individual cells in the secretory ducts and clearly lower fluorescence intensities in the salivary glands of the female mouse. Even lower fluorescence intensities, suggesting lower amounts of NGF, were seen in rat submandibular glands.

NGF-like immunoreactivities in male and female rat and mouse submandibular and sublingual glands

Until now the submandibular gland of the male mouse has been the almost exclusive target for NGF immunohistochemistry. As indicated above, a brief mentioning has occurred twice about the submandibular gland of the female mice. The present study clearly shows that in addition to the extensive presence of cells containing NGF-like immunoreactivity in the submandibular gland of the male mouse, all the three related mouse glands, i.e., the female submandibular gland and the sublingual gland of both sexes, contained scattered strongly or moderately NGF-immunoreactive cells in tubular portions of the glands. In the rat, where the sexual dimorphism between the submandibular glands is less pronounced than in the mouse, NGF-like immunoreactivity was detected with only one antibody and only in the submandibular gland of the male rat. Furthermore, the intensity of fluorescence was much weaker than in the corresponding mouse gland. The other three rat glands were negative. These results generally agree well with preliminary studies of NGF activity in the various glands as judged by bioassay. Thus, sublingual glands of female and male N.M.R.I. mice contain approximately 1–3 B.U./animal, while male rat glands are below the level of detection. Submandibular glands of female mice may contain up to 10000 B.U., while male submandibular glands are in the 20000–65000 B.U./animal range (Ebendal et al., unpublished observations).

The mouse testis

Several of the mouse genital organs were analyzed because it had been previously reported that the guinea-pig and rabbit prostate are rich sources of NGF (Harper et al. 1979; Chapman et al. 1979, 1981; Shikata et al. 1984) and that the submandibular gland as well as the prostate in *Suncus murinus* are rich in NGF (Ueyama et al. 1981) as is also semen from the bull. Using three different antibodies, however, we found no appreciable immunoreactivities in the seminal vesicle, the prostate (dorsal part), the bulbourethral gland of Cowper, or the epididymis. Interestingly enough, however, one antibody caused selective fluorescence in the germ cell line of the testis. Thus, both immature cells, intermediate stages and mature sperm were positive with the sheep antibody. This immunoreactivity fulfilled several of our specificity criteria such as not being present in antibodies extracted from the sheep prior to immunization, or in antibodies obtained from the sheep after immunization that passed through the affinity-purification column without binding. Moreover, preadsorption of the affinity-purified antibodies with β-NGF completely abolished this fluorescence. The staining pattern seemed to be selective for the sperm cell line, thus probably excluding the supportive Sertoli cells and probably also excluding the interstitial testosterone-producing Leydig cells. The identity of this immunoreactivity remains to be elucidated, but if it is β-NGF or a closely related substance, the sperm cell line must either have produced it or taken it up from its surroundings. A testiculo-salivary axis has been described involving the dependence of the convoluted tubules in the submandibular gland of the male mouse on testosterone produced by the testis. It has also been reported that removal of the submandibular glands causes testicular atrophy. The presence of NGF-like immunoreactivity in convoluted tubules at both ends of this axis adds yet another aspect to the interesting interdependence between the salivary glands and testes. Whether NGF or an NGF-like substance might be of importance for, e.g., sperm maturation or motility and whether changed levels of such a compound might bear on disturbances of male fertility, are interesting questions generated by the present results.

In conclusion, the present study gives a detailed analysis of fixation parameters and specificity problems involved in localizing NGF immunohistochemically in its richest source, the submandibular gland of male mice and several closely related glands in mice and rats, as well as in male genital organs of the mouse. Several polyclonal affinity-purified antibodies have been characterized that show a strong, NGF-dependent binding to the secretory granules of tubular cells with excellent signal-to-noise ratios that should make it possible to locate endogenous and perturbed NGF levels by means of immunocytochemistry in other parts of the body where concentrations may be three to four orders of magnitude lower than in the salivary glands.

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