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Nerve growth factor: Activation of the classical complement pathway by specific substitution for component $C\bar{1}$

(wound healing/saliva/zymogen activation)

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ABSTRACT **The interaction of homogeneous preparations of mouse submandibular gland nerve growth factor (NGF) with the classical complement pathway was studied. NGF was found to be capable of carrying out the enzyme activities of the first component ($C\bar{1}$) of the classical complement pathway (i.e., the cleavage of zymogen C4 and C2). NGF would not substitute for any other classical pathway component, C2–C9. The $C\bar{1}$-like activity of NGF was inhibited by human $C\bar{1}$ inactivator. This interaction of NGF with the complement system may account for the previously described ability of NGF to accelerate the rate of contraction of experimentally induced wounds.**

Nerve growth factor (NGF) is a M_r 116,000 protein (1) that is present in high concentrations in mouse submandibular glands (2) and also is secreted in high concentrations in mouse saliva (3–5). Thus, present evidence indicates that the mouse submandibular gland is an exocrine organ with respect to NGF secretion; yet the physiologic reason for this is not known.

Earlier studies by Greene *et al.* (6) first demonstrated that a high M_r form of mouse salivary gland NGF [termed 7S-NGF (6)] displayed enzymic activity toward certain synthetic *N*-substituted arginine and lysine esters. The M_r 116,000 protein secreted in mouse saliva shares these enzymic properties. Enzymic activity is fully inhibited by treatment of the protein with diisopropyl fluorophosphate (iPr_2P-F) (7) and N^{α}-tosyllysine chloromethyl ketone (8); thus, NGF is a member of the general class of serine proteases with highly restricted enzymic specificity (7). Although the physiologically important substrate(s) for the proteolytic activity of NGF is not known, recent studies have shown that it can activate plasminogen with concomitant lysis of a fibrin clot (7). This reaction is strictly plasminogen dependent.

The preceding information on the restricted enzymic activity of NGF may be pertinent to at least four observations on the biologic properties (and biologic sources) of NGF which originated from the early work of Levi-Montalcini. First, Levi-Montalcini and her colleagues demonstrated that experimentally induced mouse granulation tissue displayed NGF-like activity when examined in the neurite-outgrowth sensory ganglion assay system (see ref. 9 for a review). Second, studies from our laboratory have shown that a variety of both transformed and untransformed cells in culture have the capacity to secrete NGF (10, 11). Among these are cultures of primary fibroblasts (12), and these cells are known to be a prominent component of wound-induced granulation tissue. Third, the studies of Hutson *et al.* (13) have revealed (*i*) that mouse submandibular glands contain a substance(s) that can be applied by means of saliva to experimentally induced wounds by the communal licking process and (*ii*) that this process accelerates wound healing. Fourth, we have confirmed the results of Hutson *et al.* (13), but additionally we have shown that topical applications of the M_r 116,000 form of NGF to superficial skin wounds of sialoadenectomized mice significantly and consistently accelerated the rate of wound contraction (14). Thus, it may be that promotion of wound healing is one of the functions of both the NGF present in saliva as well as that associated with fibroblasts and granulation tissue. Inactivation of the NGF enzyme with iPr_2P-F abolished its ability both to activate plasminogen (7) and to promote early wound contraction (14); yet urokinase (a potent plasminogen activator) is inactive in our experimental wound-healing system. Thus, although the enzyme activity of NGF is necessary for its effect upon the rate of wound contraction, this property appears not to arise from plasminogen-mediated fibrinolysis (14).

In the study presented here, we turned to another physiologic system that has been shown to be an important participant in inflammatory processes—the complement (C) cascade (15). Results demonstrate that NGF can specifically substitute for $C\bar{1}$ of the classical C pathway and for no other component (C2–C9). Moreover, the $C\bar{1}$ activity of mouse NGF can be completely inhibited by human serum $C\bar{1}$ inactivator.

MATERIALS AND METHODS

Reagents. NGF was prepared from adult male mouse submandibular glands as described (1). Preparations were shown to be electrophoretically homogeneous (1, 7). All buffer salts were reagent grade. Isotonic Veronal-buffered saline (pH 7.4) contained 1 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (referred to hereafter as Veronal/saline). Veronal/saline with sucrose and Veronal/saline with 0.01 M EDTA were prepared as described by Rapp and Borsos (16). All buffers contained 1 mg of gelatin (Difco) per ml. Anti-Forsmann antiserum was prepared in New Zealand albino rabbits as described (17). The IgM fraction was separated on diethylaminoethylcellulose as described by Boyle and Langone (18).

Fresh guinea pig serum (JEM Research, Kensington, MD) was diluted 1:40 (vol/vol) with Veronal/saline/EDTA to prepare CEDTA (C diluted with Veronal/saline/EDTA). CEDTA provides the functionally active components C3–C9 as a single reagent (16). Functionally purified guinea pig C components and human $C\bar{1}$ inactivator were obtained from Cordis Labora-

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Abbreviations: NGF, nerve growth factor purified as in ref. 1; iPr_2P-F, diisopropyl fluorophosphate; EA, antibody-sensitized sheep erythrocytes; C, complement; EAC4b, EA with the activated fourth component of C bound to erythrocyte membranes; CEDTA, C diluted with Veronal/saline/EDTA. Terminology for the C system is that recommended in the *Bulletin of the World Health Organization* (1968) **39**, 935–938.

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tories (Miami, FL). C4-deficient guinea pig serum was obtained from the National Institutes of Health.

Cells and Cellular Intermediates. Sheep erythrocytes were collected and washed as described (16). IgM antibody-coated erythrocytes (EA) with C4b bound to their surfaces (EAC4b) were prepared by the method of Borsos and Rapp (19). This procedure yields EAC4b indicator cells free of contaminating components C1, C2, or C3–C9.

Measurements of $C\bar{1}$ Activity. $C\bar{1}$ activity was measured by modifications of the methods of Rapp and Borsos (16). Cells ($EAC4bMg^{2+}C2$) were prepared by incubation of 5.0 ml of EAC4b (1.5×10^8 cells per ml) with excess guinea pig C2 (1 ml; 1,000 50% hemolysis units/ml) for 10 min at 37°C, with Veronal/saline/sucrose (ionic strength, 0.065) as solvent. Cell intermediates were washed twice with Veronal/saline/sucrose and were suspended at a final concentration of 1.5×10^8 cells per ml; 0.1 ml of this suspension was then incubated with an appropriate dilution of $C\bar{1}$ for 15 min at 37°C, and the quantity of C3 convertase ($EAC4bMg^{2+}2a$) generated was measured by completing the hemolytic reaction with an excess of components C3–C9 (CEDTA). The reaction mixture was incubated for 1 hr at 37°C and centrifuged to remove intact lysed cells; then the degree of hemoglobin released into the supernatant solution was measured. Extent of lysis was determined by comparison of the absorbance ($\lambda = 412$ nm) of the supernatant solutions of experimental samples with the absorbance obtained after dilution and lysis of an aliquot of the cell sample in water (16). Controls, which lacked $C\bar{1}$, CEDTA, or both, were also included.

Measurement of Hemolytic Activities of C2–C9. These components were measured by the procedures of Gee *et al.* (20). $EAC\overline{14}$ cells were prepared by incubation of EAC4b cells with excess guinea pig $C\bar{1}$ in Veronal/saline/sucrose for 10 min at 37°C. The $EAC\overline{14}$ cells formed were washed twice with Veronal/saline/sucrose and resuspended at a concentration of 1.5×10^8 cells per ml. This solution (0.1 ml) was added to 0.1 ml of a dilution of the component to be tested together with an excess of the purified components necessary to complete the hemolytic sequence. Reaction was carried out in a total volume of 1 ml of Veronal/saline/sucrose, and the extent of lysis was measured after 1.5 hr at 37°C. Control solutions minus each individual component were also included. In each assay, an excess of the purified component to be measured was added to insure that complete cell lysis could be achieved.

C4 activity was determined by measuring the degree of cell lysis after incubation of 1.5×10^7 EA (sensitized with IgM antibody) in the presence of guinea pig C4-deficient serum in a total volume of 1 ml.

RESULTS

The results presented in Table 1 show that NGF can substitute specifically for the first component of the classical C pathway under the conditions described in *Materials and Methods*. No significant hemolytic activity was detected when NGF was substituted for any other C component (C2–C9). Moreover, as shown in Table 1, the degree of hemolytic activity observed was strictly dependent upon the concentration of NGF used to substitute for $C\bar{1}$. A single NGF preparation was used to generate the data in Table 1. However, three different NGF preparations were tested, and the results were virtually identical to those in Table 1.

In light of the fact that NGF is a member of the serine protease class (7) and that bovine pancreatic trypsin can substitute for $C\bar{1}$ in generating EAC4b2a (21), we wished to minimize the possibility that our NGF preparations might be contaminated with $C\bar{1}$ or with another protease having $C\bar{1}$-like activity. For this purpose, 40 μg of NGF was examined by polyacrylamide gel electrophoresis under nondenaturing conditions (7). One gel was stained and another was sliced (1-mm intervals). Individual gel slices were eluted with 0.1 M Tris·HCl (pH 7.5), and the eluates were examined for $C\bar{1}$ activity. The electrophoretic mobilities of NGF and of $C\bar{1}$-like activity were identical (Fig. 1).

Table 1. Ability of NGF to substitute for the first component of the classical C pathway

Component replaced by NGF	Hemolysis from NGF addition, %			
	0 μg	5 μg	2.5 μg	1.25 μg
C1	0	49	35	15
C4	0	0	0	0
C2	2	2	2	2
C3	2	0	0	1
C5	2	0	2	2
C6	8	8	8	7
C7	9	12	10	7
C8	7	6	7	7
C9	1	0	1	1

Hemolytic assays for individual components were performed under pseudo-first-order conditions by the standard assay procedures as described (20).

Studies on the $C\bar{1}$-like activity of NGF that have been described so far have centered upon the ability of NGF to cleave zymogen C2. There is a second enzyme activity of $C\bar{1}$—the ability to cleave zymogen C4. In the next series of experiments, NGF was tested for its ability to cleave zymogen C4 (Table 2). In these experiments, 1.5×10^7 EA were incubated with 5 μg of NGF and excess zymogen C4 (1,000 50% hemolysis units per

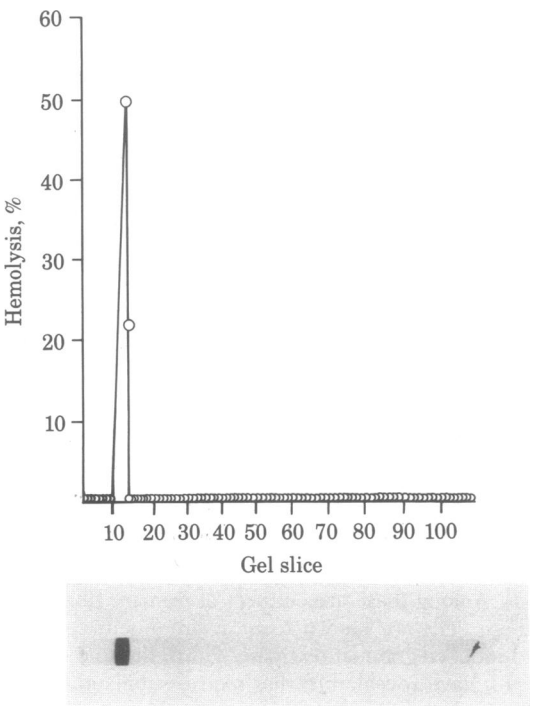

FIG. 1. Forty micrograms of NGF was applied to each of two polyacrylamide gels under nondenaturing conditions as described (1, 7). One gel was stained with Coomassie blue. The other was sliced at 1-mm intervals; the slices were eluted with 0.1 M Tris·HCl (pH 7.5), and the resulting solutions were assayed for $C\bar{1}$ activity.

Table 2. Ability of NGF to activate C4*

Reactants†	Hemolysis,‡ %
EA + buffer + buffer; wash	4
EA + buffer + C4; wash	2
EA + $C\bar{1}$ + C4; wash	100
EA + $C\bar{1}$ + buffer; wash	3
EA + NGF + C4; wash	41
EA + NGF + buffer; wash	3
EA + NGF; wash, C4; wash	3
EA + $C\bar{1}$; wash, C4; wash	100
EA + buffer; wash, C4; wash	4

* These studies were carried out with 5 μg of NGF or 100 50% hemolysis units of guinea pig $C\bar{1}$.
† The designation "wash" means that cells were washed three times with Veronal/saline/sucrose and collected by centrifugation, after they had been treated with the reactants indicated.
‡ After 1-hr incubation at 37°C with guinea pig C4-deficient serum.

ml) in Veronal/saline/sucrose (final volume, 0.2 ml) for 15 min at 37°C. One milliliter of Veronal/saline/sucrose was added, and the cells were collected by centrifugation. This procedure was repeated twice to remove unbound reactants and is designated by the term "wash" in Table 2. The washed cell pellet was resuspended in 1 ml of a 1:40 dilution of guinea pig C4-deficient serum. The extent of lysis was measured after a 1-hr incubation at 37°C. Under these conditions, NGF was capable of depositing C4b on EA (see Table 2). When NGF was incubated with EA for 15 min at 37°C, and the cells were washed prior to the addition of C4 for a further 15 min at 37°C, no C4b could be detected (Table 2). These findings indicate that NGF either does not bind to antigen–antibody complexes or does so with much lower affinity than $C\bar{1}$. $C\bar{1}$ binds firmly to antigen–antibody complexes on the cell surface and is not dissociated by washing the cells. Thus, the results in Tables 1 and 2, taken together, show that NGF can activate both C2 and C4. These proteins are the natural physiologic substrates of $C\bar{1}$.

Inhibition of NGF $C\bar{1}$-like Activity by $C\bar{1}$ Inactivator. $C\bar{1}$ inactivator is a serum protein that is believed to be the physiological regulator of $C\bar{1}$ activity (22). Consequently, we have examined the ability of $C\bar{1}$ inactivator to inhibit the $C\bar{1}$-like properties of NGF as follows. A concentration of NGF (5 μg per ml) that will convert ≈50% of EAC4bMg^{2+} C2 to EAC4bMg^{2+}2a in 15 min at 37°C was chosen, and the effect of increasing concentrations of human $C\bar{1}$ inactivator was measured. The $C\bar{1}$ inactivator inhibits the activity of NGF, and it does so at concentrations similar to those required to inactivate $C\bar{1}$ (Table 3).

Table 3. Effect of human $C\bar{1}$ inhibitor on the $C\bar{1}$ activity of NGF*

Units of $C\bar{1}$ inhibitor†	Hemolysis, %	Inhibition of NGF activity, %
0	51	0
1	50	2
2	25	51
5	5	91
10	1	98

* Cells (1.5×10^7 of EAC4bMg^{2+}C2) were treated with 5 μg of NGF for 15 min at 37°C in Veronal/saline/sucrose (total volume, 1 ml). The extent of conversion to EAC4bMg^{2+}C2a was measured by completing the reaction with CEDTA. Degrees of hemolysis were determined after a 1-hr incubation at 37°C.
† Under identical experimental conditions, 1 unit of $C\bar{1}$-inhibitor will inactivate by 50% the conversion of EAC4bMg^{2+}C2 to EAC4bMg^{2+}C2a when this reaction is mediated by $C\bar{1}$.

DISCUSSION

Taken together, the results presented above indicate that homogeneous preparations of mouse submandibular gland NGF (M_r 116,000) are able to mimic the known enzymic activities of $C\bar{1}$ as follows. (*i*) NGF will cleave zymogen C4 and will deposit C4b on the erythrocyte surface. (*ii*) It also will hydrolyze zymogen C2 that is bound to C4b and, thus, will generate a C3 convertase. (*iii*) These $C\bar{1}$-like activities of NGF can be regulated by human $C\bar{1}$ inactivator. (*iv*) Like $C\bar{1}$, NGF will not, under the experimental conditions used, substitute for C4, C2, C3, or any of the terminal components, C5–C9.

Two lines of evidence indicate that the results presented here do not stem from $C\bar{1}$ or a contaminating $C\bar{1}$-like serine esterase. First, the method of NGF purification should clearly isolate it from an enzyme-like trypsin, from $C\bar{1}$, or from its subunits. Second, the electrophoretic results in Fig. 1 reveal only a single protein component, and all of the $C\bar{1}$-like activity comigrates with this component.

The physiological relevance of the ability of NGF to cleave plasminogen (7), promote wound healing (14), and to initiate the classical C pathway is unclear. Previous studies have indicated that the accelerated rate of wound healing mediated by NGF is not due directly to its capacity to activate plasminogen because other plasminogen activators (e.g., urokinase) were ineffective (14). The observation that NGF can interact with the C system might provide an explanation for the *in vivo* action of NGF in wound healing.

The host response to tissue damage is extremely complex and poorly understood. The consequences of tissue injury depend on the nature of the damage-causing agent and the extent of tissue damage. In man and higher animals, there are a series of cascade systems that function in the host response to tissue damage (23–25). These include the C system, the clotting system, the fibrinolytic system, and the kinin-generating system. These systems are interrelated and are regulated by a single protein, the $C\bar{1}$ inactivator (23, 24). The ability of NGF to promote nerve growth, accelerate wound healing, cleave plasminogen, and initiate the classical C pathway in the absence of $C\bar{1}$ would suggest that it may represent, or be part of, another host defense system with a primary role in the response to, and repair of, tissue damage.

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