

EFFECT OF NEUROTROPIN ON THE OXYGEN CONSUMPTION AND ADHERENCE CAPACITY OF POLYMORPHONUCLEAR LEUKOCYTES OF PERIPHERAL BLOOD*

By

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ABSTRACT

Effect of neurotrophin (NSP) was studied on the oxygen consumption and adherence capacity of polymorphonuclear leukocytes of peripheral blood. Its therapeutic doses were found not to affect the phagocytic and adherent ability of leukocytes of healthy adults. In vitro experiments demonstrated that the respiration of leukocytes ceases much earlier in the presence of NSP than in its absence in a dose-dependent fashion, although the initial velocity of oxygen consumption associated with phagocytosis was unchanged. The fact that respiration tended to become normal when dialyzed neurotrophin was used suggested that some inhibitory factors, which are dialyzable, are present in this preparation.

INTRODUCTION

"Neurotrophin Special 3cc" (NSP)¹⁾ is a unique preparation isolated from the inflamed skin of rabbits inoculated with vaccinia virus. It is currently used therapeutically in neuralgia, bronchial asthma and other allergic diseases. Although a favorable clinical response has been documented in a number of patients, its precise mode of action has not been fully elucidated, nor has its effect on cellular functions been studied in detail.

The present communication evaluates the effect of NSP on leukocyte functions both in vivo and in vitro, particularly on the oxygen consumption and adherence capacity of the polymorphonuclear leukocytes of human peripheral blood.

MATERIALS AND METHODS

NSP, donated by Nippon Zoki Pharmaceutical Co. LTD, has a pH of 7.4-7.8. Doses of 1.2 mg/ml were prepared for in vivo use and of 1.56 mg/ml for in vitro experiments. For the latter, NSP was diluted in HEPES buffer (17 mM N-hydroxyethylpiperazine-N'-ethanesulfonic acid, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄ and 5 mM glucose) and saline or heparinized venous blood to the specified concentration. In order to evaluate its effect in vivo, three healthy adult volunteers received a subcutaneous injection of 3 ml of NSP daily for one week, and their leukocytes were examined before, during and after this one-week period until the end of the second week. Polymorphonuclear leukocytes (PMNL)

*¹⁾ 小林陽之助, 白井朋包: 末梢血多核白血球の酸素消費能および付着能におよぼすノイロトロピンの影響

were prepared after removal of lymphocytes in a Ficoll-sodium metrizoate gradient, sedimentation of erythrocytes in 3% dextran saline and removal of contaminating erythrocytes by hypotonic lysis.

The oxygen consumption capacity of PMNL was determined principally by the method of Nakamura et al.²⁾ with a Clark type oxygen electrode, which has been described elsewhere³⁾. Phagocytizable particles were zymosan A (Sigma Chem. Co.) opsonized with fresh human serum (OPZ) and heat-killed *Staphylococcus aureus* 209P. OPZ was prepared by incubating 25 mg zymosan A in 1.0 ml of fresh serum at 37°C for 30 min with shaking. Bacteria were cultured in brain-heart infusion broth for 18 hours, washed with saline, adjusted to 4×10^9 /ml and autoclaved. They were opsonized with an equal volume of fresh serum at 37°C for 30 min, washed and suspended in HEPES buffer to the original concentration and used at a bacteria-to-PMNL ratio of 100:1. Phorbol myristate acetate (PMA, Sigma Chem. Co.), a metabolic stimulator of PMNL, was dissolved in dimethyl sulfoxide (DMSO, 1 mg/ml) and used at a concentration of 100 ng/10⁶ PMNL. DMSO itself had no effect on the determination. N-ethyl maleimide, an inhibitor of glycolysis, was diluted in saline and used at 0.1 mg/ml. NSP was dialyzed in cellulose tubing (8/32, Visking Co.) against 1,000 volumes of physiological saline in the cold for 44 hours with a change of saline at 24 hours. The level-off time (LOT) was defined as the point at which the oxygen consumption of the phagocytizing phase becomes equal to that of the resting phase, and was expressed in minutes from the addition of phagocytizable particles or metabolic stimulator.

Granulocyte adherence was performed accord-

ing to the method of MacGregor et al.⁴⁾ with modifications. Briefly, a glass tuberculin syringe was loaded with 100 (± 1) mg of tetron fiber (Terumo Co.) to 0.5 ml, and was attached to a 21-gauge needle. The above column was placed in a plastic tube and preincubated at 37°C until use. Heparinized venous blood was incubated with either NSP or saline for 15 min at 37°C with constant agitation, after which each 1.0-ml portion of the specimen was applied to the column and allowed to flow through at room temperature. PMNL adherence was calculated by the following equation:

$$\left(1 - \frac{\text{PMNL/ml in effluent}}{\text{PMNL/ml in original}}\right) \times 100\%$$

The values were expressed as a mean of three determinations.

RESULTS

The addition of NSP to an air-equilibrated HEPES buffer at 37°C did not affect the dissolved oxygen. A maximal rate, i.e., a linear portion, of oxygen consumption was essentially similar in either the absence or the presence of NSP irrespective of its concentration. With higher concentrations of NSP, however, PMNL apparently ceased to consume oxygen much earlier than the controls, i.e., at a shorter level-off time (Table 1 and Fig. 1). After the level-off point, the oxygen consumption became less than that of the resting phase. This finding was most prominent with OPZ and *S. aureus* 209P as phagocytizable particles and was not distinctly discernible when PMA was used as a metabolic stimulator. The results were grossly similar with cells which had been preincubated with NSP (100 or 1 μ g/ml) and washed. In an attempt to explain this premature cessation of oxygen consumption in the presence

Table 1. Oxygen consumption capacity of PMNL treated with NSP in vitro

NSP (μ g/ml)	OPZ		<i>S. aureus</i> 209P		PMA	
	OC*	LOT**	OC	LOT	OC	LOT
0	5.3	11'30''	4.9	>15'	3.0	>20'
0.01	5.5	11'30''	—	—	2.8	>20'
0.1	—	—	4.5	>15'	2.8	>20'
1.0	—	—	4.8	7'	2.6	>20'
10	5.5	6'30''	—	—	2.5	>20'
100	5.3	5'00''	4.9	5'30''	2.8	15'

OC*: Oxygen consumption (n moles O₂/min/10⁶ PMNL); LOT: Level-off time.

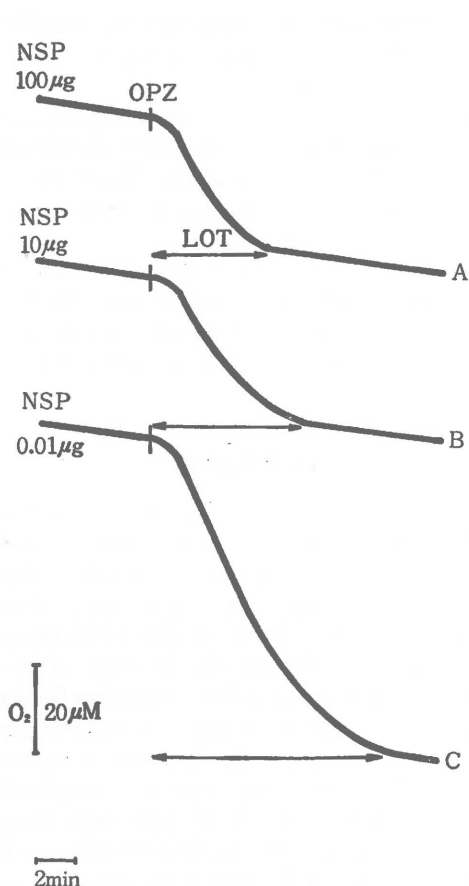


Fig. 1. Effect of NSP on oxygen consumption by polymorphonuclear leukocytes (PMNL) after stimulation with opsonized zymosan (OPZ). A, NSP 100 μg; B, 10 μg; C, 0.01 μg. LOT: Level-off time.

of NSP, the following experiments were performed. Subsequent addition of PMNL at the level-off point induced further oxygen consumption, as shown in Fig. 2C. This observation was interpreted to indicate that newly introduced cells started to phagocytize OPZ which had remained uningested by the cells initially present with NSP. After oxygen consumption reached the level-off point, a second addition of OPZ did not result in any further increase in oxygen consumption. Following the initiation of reaction with OPZ, a second stimulation with PMA, and vice versa, was attempted but failed to induce more oxygen consumption (Fig. 2A and B).

NEM, when added to PMNL at least 30 seconds before OPZ, completely inhibited oxygen consumption (Fig. 3A). When added after

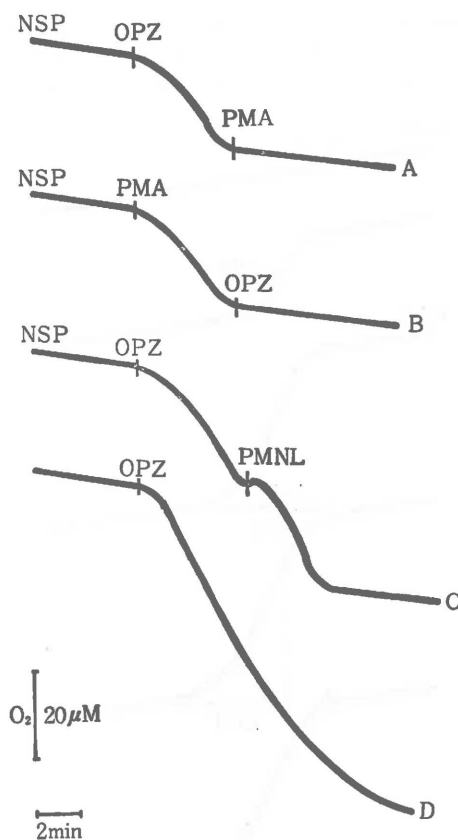


Fig. 2. Effect of second stimulation with phorbol myristate (PMA) or OPZ at the level-off point on oxygen consumption initiated by OPZ or PMA (A, B). Further increase of oxygen consumption induced by PMNL added at the level-off point (C) and control (D).

oxygen began to be consumed, inhibition followed a lag period of about one minute and subsequent respiration was almost terminated (Fig. 3B). In order to define whether NSP has a similar inhibitory action on leukocyte respiration, 100 μg of NSP was added 2 minutes after the oxygen consumption started; it stopped respiration in about 5 to 6 minutes, comparable to LOT with the same amount of NSP present in the mixture from the beginning (Fig. 3C and D). The following treatments of NSP did not alter the characteristic pattern of oxygen consumption when the treated NSP was present in the reaction mixture from the beginning; heating (56°C, 30 min), autoclaving, freezing and thawing, and exposure to sunlight for 6 hours, which caused a slight brownish discoloration of the solution. Like saline-treated OPZ, no

change in oxygen consumption was noted with OPZ which had been treated with NSP for 30 min at 37°C with subsequent washing. Di-

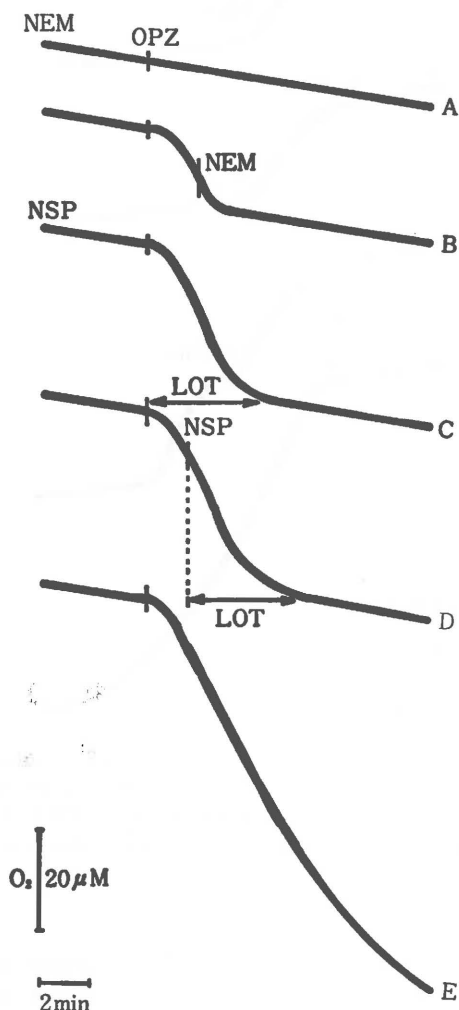


Fig. 3. Effect of N-ethyl maleimide (NEM) and NSP added before (A, C) or after (B, D) the initiation of phagocytosis. LOTs in C and D are comparable. Control (E).

alyzed NSP partially eliminated the inhibitory effect, prolonging the LOT to about 1.5 times that of the control.

The response to OPZ of PMNL from subjects who received NSP did not differ significantly from that of the control adults (Table 2).

Adherence was assessed at a single concentration of NSP (10 $\mu\text{g}/\text{ml}$): 78.3% (range: 70–87%) for controls and 76.3% (range: 73–79%) for NSP, not a significant difference. With NEM-treated (0.1 mg/ml) venous blood (37°C, 10 min), the degree of adherence rate was similar.

COMMENTS

Our studies have shown that therapeutic doses of NSP do not affect the phagocytic and respiratory ability of leukocytes of healthy adults. In vitro experiments, however, have clearly demonstrated that the respiration of leukocytes ceases much earlier in the presence of NSP than in its absence in a dose-dependent fashion, although the initial velocity of oxygen consumption associated with phagocytosis was unchanged by NSP. The fact that respiration was less after the level-off point than during the resting phase and tended to become normal when dialyzed NSP was used suggests that some inhibitory factors, which are dialyzable, are present in NSP.

Two insoluble stimuli, OPZ and *S. aureus* 209P, had a similar effect, but soluble PMA inhibited respiration less. The mode of action of soluble and insoluble materials was expected to differ, but once the respiration initiated by the first stimulus ended, it could be resumed only by the introduction of new PMNL and not by a second stimulus of any type. These findings suggest that the capacity of PMNL to

Table 2. Oxygen consumption capacity of PMNL from subjects who received NSP daily for a week

Subject	Before NSP	After 1 dose of NSP	After 4 doses of NSP	After 7 doses of NSP	A week after the last NSP	Mean \pm 1SD
N.S.	8.6	5.7	7.0	8.3	7.3	7.4 \pm 1.2
R.M.	7.3	6.9	7.3	7.0	10.0	7.7 \pm 1.3
Y.K.	5.9	8.4	7.5	7.3	9.2	7.7 \pm 1.2
Mean \pm 1 SD	7.3 \pm 1.4	7.0 \pm 1.4	7.3 \pm 0.3	7.5 \pm 0.7	8.8 \pm 1.4	
Control 1	7.9	7.5	6.3	7.0	10.0	7.7 \pm 1.4
Control 2	8.0	8.1	7.3	8.0	10.8	8.4 \pm 1.4

respond to stimuli is prematurely aborted in the presence of NSP and that termination of oxygen consumption in its presence is probably not due to the exhaustion of phagocytizable particles in the reaction mixture.

As an analogy of the effect of NEM on respiration, a similar inhibitory mechanism was initially postulated to be operative with NSP. There were differences, however; NEM, when present in the reaction mixture from the beginning, completely inhibited oxygen consumption, whereas NSP under identical conditions, inhibited it only after a lag period of 5 to 6 min after the start of phagocytosis. Therefore, the suppressive effect of NSP appears to be triggered only by phagocytosis and to be greatly facilitated by its internalization with particulate materials. The failure of NSP-treated OPZ to inhibit respiration was probably due to a lack of adsorption of NSP on OPZ or its removal from OPZ by thorough washing.

In the present method, PMNL adherence was not affected at all by NSP. No reports have been published on adherence to tetron fiber. Although further experiences are certainly needed,

the conclusion that PMNL were specifically retained in the tetron column may be partly substantiated by the fact that the erythrocyte count in the effluent was equal to that in the original.

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