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## Nerve Growth Factor Activity Detected in Equine Peripheral Blood of Horses with Fever after Truck Transportation

Keiko KAWAMOTO<sup>1</sup>, Hiroaki SATO<sup>1</sup>, Masa-aki OIKAWA<sup>2</sup>, Toyohiko YOSHIHARA<sup>2</sup>, Mikihiro KANEKO<sup>2</sup>, and Hiroshi MATSUDA<sup>1</sup>\*

<sup>1</sup>Department of Veterinary Clinic, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183 and <sup>2</sup>Equine Research Institute, Japan Racing Association, 5-27-7 Tsurumaki, Setagaya-ku, Tokyo 154, Japan

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*We examined nerve growth factor (NGF) activity in sera obtained from 5 Anglo-Arab and 6 Thoroughbred horses after long-distance truck transportation. NGF activity was evaluated by neurite outgrowth from rat pheochromocytoma cells that respond to NGF. Five of 11 horses had mild or severe fever after the truck shipping; significant NGF activity was detected in serum samples collected from 4 of the febrile horses. The addition of polyclonal antibodies to murine 2.5S NGF completely abrogated the neurotrophic activity detected in the samples. These results suggested a possible role of NGF in the pathogenesis of equine shipping fever associated with continuous stress by truck transportation.*

**Key words:** horse, nerve growth factor, shipping fever, stress

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Shipping fever is one of the most common diseases in horses loaded onto vehicles for long-distance transportation, and results in severe economic loss and decreased racing performance. Although the etiology of this disease has not been defined yet, physical and physiological stress and microbial infections are speculated to be important factors in the onset and development of this disease [7]. Horses suffering from shipping fever often have other clinical signs such as depression, pyrexia, and cough. In severe cases, infectious respiratory diseases including pneumonia and pleuropneumonia are observed. An increase in the number of neutrophils in the peripheral blood and marked infiltration of neutrophils into the lung are commonly observed in febrile horses with or without the other signs [7, 12], and the involvement of neutrophilia in the pathogenesis of the shipping fever and pneumonia has been unclear.

Nerve growth factor (NGF) is a neurotrophic peptide necessary for the maintenance of sympathetic and embryonic sensory neurons. In addition to the neurotrophic activity, NGF has broader biological effects

on various kinds of immune and inflammatory cells including neutrophils [3-6, 8, 9, 13, 14]. We recently demonstrated that NGF regulates survival [4, 5], differentiation [3], and functional properties of murine neutrophils [5]. NGF also has been reported to have a chemotactic activity for neutrophils [2]. Since NGF is increased in murine sera after fighting stress [1] and in equine sera after running stress [10], there is a possibility that truck transportation stress may induce NGF release into the peripheral blood and lead to fever and neutrophilia. In the present study we examined NGF activity in the sera of horses after experimental transportation and discussed the possible involvement of NGF activity in the pathogenesis of shipping fever.

Five Anglo-Arab (3 male and 2 female) and 6 Thoroughbred (2 male and 4 female) horses, which had no clinical abnormality, were used at 23 to 27 months of age. Two groups of horses were transported by horse trailers for a total of 36 hr (1,676 km) from a Hidaka rearing farm to Rittoh Training Center on April 10, 1993 and on April 23, 1993, respectively. During transportation, clinical signs and rectal temperature were monitored. Blood samples were collected from the jugular vein within 1 hr before and after the transportation by using evacuated blood collection tubes.

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\*corresponding author.

**Table 1.** NGF activity in sera of horses after truck transportation

Horse no.	Breed	Sex	Fever	Neurite-bearing PC12 cells (%) <sup>a)</sup>	
				Before	After
1	Anglo-Arab	Male	Yes	6.5	41.5
3	Anglo-Arab	Male	Yes	6.5	16.0
5	Anglo-Arab	Female	Yes	9.0	23.0
9	Thoroughbred	Male	Yes	5.5	17.5
11	Thoroughbred	Female	Yes	6.3	7.5
2	Anglo-Arab	Male	No	6.5	6.5
4	Anglo-Arab	Female	No	8.5	9.0
6	Thoroughbred	Male	No	7.5	9.8
7	Thoroughbred	Female	No	8.2	6.5
8	Thoroughbred	Female	No	6.4	6.9
10	Thoroughbred	Female	No	0.8	1.8

<sup>a)</sup>PC12 cells ( $10^5$  cells/ml) were incubated with 10% serum collected from horses before and after truck transportation, and 72 hr later the percentage of cells with neurite outgrowth was calculated.

Sera were obtained by centrifugation and were stored at - 20°C until use. NGF activity in sera was examined by neurite outgrowth from a rat pheochromocytoma cell line (PC12) which was obtained from American Type Culture Collection (Rockville, MD, USA) according to the method described previously [15]. Briefly, PC12 cells were suspended at a concentration of  $10^5$  cells/ml in RPMI 1640 supplemented with 10% heat-inactivated horse serum (Hyclone Laboratories, Logan, UT, USA), 5% fetal calf serum (Hyclone), 50 U/ml penicillin, and 50 mg/ml streptomycin, and were incubated with or without 10% collected serum in collagen-coated 24-well culture plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere flushed with 5% CO<sub>2</sub>. The addition of ultrapurified murine 2.5S NGF, which was provided by Drs. A.M. Stanisz and J. Bienenstock (McMaster University, Ontario, Canada), served as a positive control. In other experiments, a 1000-fold dilution of rabbit polyclonal antibody to murine 2.5S NGF (Sigma Chemical Co., St. Louis, MO, USA) was added to the culture medium. Neurite outgrowth from PC12 cells per well was examined 72 hr after the initiation of culture under a phase-contrast microscope. A cell with at least one process exceeding one cell diameter was judged as a neurite-bearing cell, and the percentage of cells showing neurite growth was calculated in 400 cells. Student's *t*-test was performed for statistical analysis of the data obtained in this study, and  $P < 0.05$  was taken as the level of significance.

In a biological assay with PC12 cells, we examined NGF activity in sera collected from horses with or without shipping fever following experimental truck transportation for 36 hr. As shown in Table 1, the

addition of sera collected from all horses before the transportation resulted in a low percentage of PC12 cells with neurite outgrowth (<10% cells). In contrast, significant increases in the percentage of cells with neurite outgrowth were noted in cultures supplemented with sera collected after the transportation from 4 of 5 cases with pyrexia (38.3 to 41.0°C) which were accompanied with coughing and depression (Table 1). In the next experiment, we investigated the NGF specificity of the neurotrophic effect found in serum samples from the fevered horses. The addition of anti-NGF antibodies completely abrogated the neurotrophic activity in sera collected from Nos. 1 and 5 horses (Fig. 1). Since the amino acid sequence of NGF is very similar in mouse, man and other animals [11], this result suggested that, even though the amino acid sequence of horse NGF is not known, NGF activity was detected in the serum of horses with fever.

The present results demonstrated that long-distance truck transportation led to significant NGF activity in sera of horses with fever and other clinical signs. NGF is not detectable in sera of normal adult mice, but aggressive behavior (fighting stress) rapidly increases serum NGF levels up to 300 ng/ml [1]. Our previous studies showed that NGF-like activity in equine sera was detected after running exercise [10]. Neutrophils released from the bone marrow are the main cellular component emigrating from blood to areas of acute inflammation. In addition, it is known that exercise, excitement and many other types of stress lead to rapid neutrophilia. NGF has been reported to have a strong chemotactic activity for neutrophils *in vivo*. We demonstrated that NGF regulates not only the survival of murine neutrophils

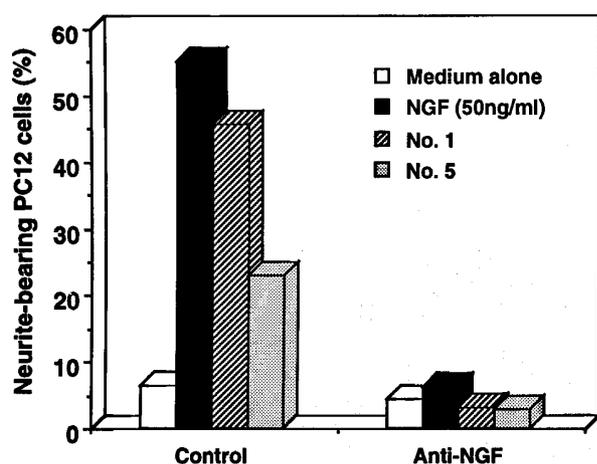


Fig. 1. Neutralization of neurotrophic activity in equine sera by anti-NGF antibodies. PC12 cells ( $10^5$  cells/ml) were incubated with 10% horse serum (horses no. 1 and 5) or with 50 ng/ml 2.5S NGF in the absence or presence of 1:1000-diluted anti-NGF antibodies.

but also their functions, such as enhancement of phagocytosis and superoxide production [3–5]. Since neutrophils are speculated to play a key role in the pathogenesis of shipping fever [7, 12], rapid release of high levels of NGF into the bloodstream may influence a rapid increase in the number of neutrophils and their functions in this disease.

Interleukin 1 which is known as an endogenous pyrogen, is produced by activated macrophages. NGF can induce transcription and secretion of interleukin 1 in murine macrophages [13]. Since the present study demonstrated a good correlation between an outbreak of shipping fever and serum NGF activity, there is a possibility that NGF produced by continuous stress in long-distance truck transportation, may lead indirectly to fever.

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