

# Changes in Peripheral Blood Lymphocyte and Neutrophil Counts and Function Following Long-Term Road Transport in Thoroughbred Horses

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## ABSTRACT

**Problem Statement:** Although there is much information on the effects of long-term road transport on the equine systemic immune status, there is still no consensus regarding these effects.

**Approach:** To better define the effects of long-term road transport on systemic cellular immune function in thoroughbred horses, changes in the counts of peripheral lymphocyte subpopulations, lymphocyte blastogenesis, and nitroblue tetrazolium (NBT), reduction activity of neutrophils were examined in eight thoroughbred horses after 36 hrs of road transport over a distance of 1,676 km. **Results:** Immediately after the transport, the counts of lymphocytes, CD<sup>4+</sup> and CD<sup>8</sup>β<sup>+</sup> and CD<sup>5+</sup> significantly decreased compared with those before the transport. The NBT reduction activity of

neutrophils increased and the mitogen-induced blastogenesis of lymphocytes tended to be enhanced immediately after the transport.

**Conclusions:** The decrease in the counts of peripheral lymphocyte subpopulations may be due to transport-induced stress, and may lead to subsequent compromise of lymphocyte function after the transport. The increased NBT reduction activity of neutrophils and the enhanced lymphocyte blastogenesis may represent a temporary enhancement of cellular immune function in response to bacterial challenge in the respiratory tract during long-term road transport. These findings provide insights into the pathogenesis of respiratory disease in association with long-term road transport.

## INTRODUCTION

The transport of horses exacerbates the incidence of respiratory disease (so-called “shipping fever”).<sup>1,2,3</sup> Transport stress-induced immunosuppression is assumed to be one of the factors for this disease in hors-

es<sup>4,5</sup>. Although there is pathomorphological information that neutrophils might have an important role in the development of equine respiratory disease during long-term road transport,<sup>2,6</sup> studies of the effects of road transport on the peripheral blood neutrophil function in relation to the development of this disease have yielded conflicting results.<sup>3</sup> A study on the effect of road transport on the peripheral blood lymphocyte function showed the transport-associated suppression of lymphocyte blastogenic response in horses.<sup>5</sup> However, Stull et al. reported the difficulty in interpreting results of such a study owing to the high variability of lymphocyte proliferative response between individuals. Thus, there is still no consensus regarding the effects of long-term transport. The lymphocyte subpopulation responses in horses during long-term road transport have been reported,<sup>5</sup> but the functional significance of these responses in transported horses remains obscure.

These findings indicate that the changes and possible effects of long-term transport on peripheral neutrophils and lymphocyte function are poorly understood in horses. The objectives of the present study were to examine the effects of road transport stress on cellular immunological responses by measuring the ratio of the count of peripheral blood neutrophils to that of lymphocytes (N: L) as a good indicator of stress<sup>4</sup> and, in particular, changes in the counts of lymphocyte subpopulations, lymphocyte blastogenesis, and nitroblue tetrazolium (NBT) reduction activity, as an indicator of bactericidal activity of neutrophils.

## **MATERIALS AND METHODS**

### **Animals and Study Design**

A detailed description of the experimental horses used and the methods of transport were reported previously.<sup>6</sup> Briefly, eight thoroughbred horses aged 27 to 29 months, without transport-associated respiratory disease described previously, were used as the test animals to examine physical condition, changes in the count of lymphocyte subpopulations, lymphocyte blastogenesis,

and neutrophil bactericidal activity immediately before and after the transport. The thoroughbred horses were transported 1,708 km by road in trucks, taking approximately 36 hrs on May 2009. The vehicles had internal partitions that divided the lorry into four compartments. The horse in each compartment was untethered and able to lower its head freely (over a front wooden bar height, 1 m). Horses were given free access to hay throughout the trip and were given water during 1 hr rest intervals following each 4-5 hr period of transport. During and after the transport, the horses were treated in accordance with the Humane Use Guideline equivalent to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

### **Hematology**

Heparinized blood was collected by jugular venipuncture immediately before departure and immediately after the road transport. Total leukocyte counts were determined using a Hitachi Automatic Analyzer (Model 747, Hitachi High-Technologies Co, Minatoku, Tokyo, Japan). Differential counts were obtained by examination of May-Grunwald-stained blood smears. The neutrophil to lymphocyte ratio (N:L ratio) was calculated.

### **Lymphocyte preparations**

All the materials and reagents used for the isolation of peripheral blood leukocytes were sterile. Blood samples were collected in vacuum tubes containing a serum-separating agent (6 mL: Terumo Co, Tokyo, Japan), disodium ethylenediaminetetraacetic acid (EDTA-2Na; 5 mL: Terumo Co., Tokyo, Japan), and heparin (10 mL: Terumo Co., Tokyo, Japan) by jugular venipuncture immediately before departure and just after transportation.

### **Flow Cytometry Analysis of Subpopulations of Peripheral Blood**

#### **Leukocytes**

Surface antigens of blood leukocytes were analysed with indirect fluorescence antibodies using a flow cytometer (Cytomics FC500,<sup>®</sup> Beckman Coulter Inc, Brea, CA,

USA). Briefly, leukocytes were incubated for 60 min at 4°C with 10 µL of 100-fold-diluted primary antibody (Anti-CD4 IgG1, cell line HB61A; Anti-CD5 IgG1, cell line HT23A; Anti-CD8βIgG1, cell line HT14A; VMRD, Inc, Pullman, WA, USA). After washing in phosphate-buffered saline (PBS), cells were incubated for 60 min at 4°C with 4,000-fold-diluted phycoerythrin (PE)-labelled anti-mouse immunoglobulin (Ig)G1 (RPE Obmstar81PE,® Cosmo Bio, Tokyo, Japan) or 2,000-fold-diluted fluorescein isothiocyanate (FITC)-labelled anti-mouse IgM (Human Adsorbed; Southern Biotech., Birmingham, AL, USA). The cells were then washed, suspended in PBS, and transferred into a standard round-bottom polypropylene test tube for analysis in the Cytomics FC500 (Beckman Coulter Inc, Brea, CA, USA). The absence of nonspecific reactions was confirmed using PE-labelled anti-mouse IgG1 or FITC-labelled anti-mouse IgM without the primary antibodies as a negative control. The results were analysed using FlowJo® software (Tree Star, Inc, Ashland, OR, USA). Numbers of surface antigen-positive cells were calculated from their percentages and the actual leukocyte count ( $\times 100/\mu\text{L}$ ).

#### **Blastogenic response of lymphocytes:**

Heparinised blood (10 mL) was centrifuged at 1,500 rpm for 3 min. The buffy coat was transferred to a second tube and diluted twofold in PBS. The resulting mixture was diluted twofold in 1/15 M PBS (pH 7.4), layered over 4.2 mL of separating fluid with a density of 1.077 (Lymphosepar I®: Immuno-Biological Laboratories Co, Ltd., Gunma, Japan), and centrifuged at 4°C at 1,500 rpm for 20 min to separate leukocytes. The lymphocytes obtained were washed several times in PBS and suspended in Roswell Park Memorial Institute (RPMI: Invitrogen Co., Tokyo, Japan) 1,640 medium containing 10% (v/v) foetal horse serum (Biowest, Miami, FL, USA) at a concentration of  $1 \times 10^6/\text{mL}$ . The lymphocytes were cultured in the presence of phytohaemagglutinin (PHA; SIGMA Chemical Co, St Louis, MO, USA) for 96 hrs at 37°C under

a humidified atmosphere containing 5% CO<sub>2</sub>. The concentration of PHA was 10 µg/mL. After incubation, the lymphocytes were stained with 5 µg/mL of methylthiazolyldiphenyl-tetrazolium bromide (MTT; Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 2 hrs, then lysed with sodium lauryl sulphate (Bio-Rad Laboratories, Inc, Hercules, CA, USA). After 12 hrs, the samples were analysed spectrophotometrically at 595 nm (Model 3550 microplate reader®; Bio-Rad Laboratories, Inc, Hercules, CA, USA) to determine the stimulation index (SI) of the blastogenic response of the lymphocytes.

The SI was calculated as follows:

- $SI = (I_1 - I_0/I_0) \times 100$
- I<sub>0</sub>: Fluorescent intensity of control
- I<sub>1</sub>: Fluorescent intensity of samples (PHA)

#### **Neutrophil Preparations**

Neutrophils were recovered from the packed erythrocyte layer after the density gradient centrifugation. Briefly, the erythrocytes were lysed by three volumes of cold phosphate buffer with gentle mixing for 60 seconds in a 50 ml round bottom centrifuge tube. Isotonicity was restored by 1.5 volumes of cold phosphate buffered 2.7 % NaCl (pH 7.2) solution. The cells recovered by centrifugation were rinsed and suspended at  $5 \times 10^6$  cells/ml in Eagle's minimal essential medium (MEM). Generally, >95% of the cells were neutrophils.

#### **Nitroblue Tetrazolium (NBT) Reduction Activity of Neutrophils**

The test was undertaken with a modified method described by Murata et al.<sup>7</sup> In brief, 0.5 ml of neutrophil suspension containing  $2.5 \times 10^6$  were mixed with 0.4 ml of 1 mg/ml NBT (Sigma, USA) solution in Hanks' balanced solution, and 0.1 ml of 1 mg/ml opsonized zymosan A (Sigma Chemical Co, St. Louis, MO, USA) solution in Eagle's MEM or 0.1 ml of Eagle's MEM (control). After 30 min, incubation in a 37°C water bath, the reaction was stopped by adding 1 ml of 0.5 N HCL. After centrifugation, the cell precipitates were extracted by 3 ml of dimethyl-

**Table 1.** Changes in counts of peripheral blood leukocytes in 8 thoroughbredhorses after 36 h of road transport

Item evaluated (/μl or Unit)	Time of sampling				
	Pr		Po		
Leukocyte counts	9712	1264	11112	2288	
Neutrophils	4332	762	6048	1542	*
Lymphocytes	4756	632	3789	612	*
N:L ratio (%)	0.86.	0.34	1.56	0.57	**
Eosinophils	218	134	83	67	*
CD4 <sup>+</sup>	1426	118	624	87	**
CD5 <sup>+</sup>	1716	129	876	55	**
CD8β <sup>+</sup>	561	54	238	65	**
Blastogenesis (S.I.)	54.90	1.74	71.79	1.28	(P<0.10)
NBT reduction (OD at 565 nm)	0.27.	0.04	0.39	0.08	*

Pr, prior to transport; Po, post-transport. Values are expressed as mean ± S. E. \* P < 0.05, \*\* P < 0.01. Mean value is significantly different compared with baseline values taken immediately prior to road transport.

sulfoxide (DMSO). The optical density (OD) of the extracts was determined at 565 nm by a spectrophotometer (Hitachi 220-A, Tokyo, Japan).

### STATISTICAL ANALYSIS

The data were analysed by using Microsoft Excell 18 (2003. Microsoft Corp., Seattle, WA, USA). Statistical evaluations were performed by using Student's t-test. P values < 0.01 or < 0.05 were considered statistically significant.

### RESULTS

The rectal temperature of all thoroughbred horses increased compared with that measured before transport (< 38.5°C). Although individual variations in some leukocyte parameters were large, the following results were obtained. Both the counts of neutrophils and NBT reduction activity of neutrophils measured immediately after arrival significantly increased (Table 1). Significant decreases in the counts of total lymphocytes, CD4<sup>+</sup>, CD5<sup>+</sup> and CD8β<sup>+</sup> were observed (Table 1). Lymphocyte blastogenetic activity tended to increase immediately after 36 h of road transport (Table 1). N:L ratio significantly increased immediately after the transport (Table 1). There was a significant

decrease in eosinophil count after the transport in comparison with the count before the transport (Table 1).

### DISCUSSION

We reported previously that road transport increases the concentrations of blood adrenocorticotrophic hormone (ACTH) and 11-hydroxycorticosteroid (11-OHCS) in horses during the transport.<sup>6,8</sup> Road transport has been shown to increase the circulating cortisol concentration in other studies of horses.<sup>4,5,9</sup> Although these indices were not evaluated in the present study, the observations indicated a significant increase in N:L ratio, which may be a more reliable indicator of stress than cortisol concentration,<sup>4</sup> and a significant decrease in eosinophil count, which is as an index of stress. These findings indicate that the majority of horses in the present study experienced transport-induced stress to same extent. Thus, a significant increase in N:L ratio may be due to transport-induced stress, and may lead to transport-associated decreases in the counts of total lymphocytes, the counts of T cells, and B cells (CD4<sup>+</sup> and CD8β<sup>+</sup> and CD5<sup>+</sup>) in the horses. Stull et al also determined that there were transport-associated decreases in the counts of both lymphocyte subpopula-

tions by measuring the T cell surface antigen (CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup>) and B cell surface antigen (CD21<sup>+</sup>) levels in mature horses aged 5 to 15 years 1 day after transportation.<sup>5</sup> The functional significance of the lymphocyte subpopulation responses in transported horses is as yet unclarified. However, in humans, the counts of peripheral blood lymphocyte subpopulations have been used to assess susceptibility to a disease.<sup>5</sup> It is probable that significant decreases in the counts of both lymphocyte subpopulations in horses would lead to the subsequent compromise of lymphocyte function, and thus a risk of an infectious disease after the transport.

Raidal et al. reported that there was a trend for an increased oxidative burst activity of peripheral neutrophils and lymphocytes with suppressed phagocytosis in circulating neutrophils in horses immediately after the transport as an example of dissociation of function.<sup>3</sup> In other animal species, studies on the effects of road transport on the systemic immune system have yielded conflicting results. For example, lymphocytes from calves transported for 48 hrs showed a significantly enhanced blastogenesis, whereas those in sera from these same calves showed a suppressed blastogenesis.<sup>7</sup> A study on transport-induced stress in pigs revealed no significant variation in the proliferative response of lymphocytes.<sup>11</sup> Otherwise, in young pigs transported to a fattening farm, peripheral blood mononuclear cells showed a markedly diminished proliferative response to T-cell mitogen,<sup>12</sup> but the possible effects of such changes on peripheral lymphocyte function are poorly understood in horses.

We have already reported that the concentration of serum granulocyte-colony stimulating factor (G-CSF) correlates positively with peak body temperature and with increased peripheral neutrophil count during the transport, suggesting that a microbial challenge increases neutrophil activity<sup>13</sup> and that the concentration of serum nerve growth factor (NGF) significantly increases immediately after transport, implying that

NGF activity, which has been reported to have chemotactic activity for neutrophils, increases during transport.<sup>13</sup> These findings indicate that the changes in neutrophil count and NBT reduction activity of neutrophils (indicator of intracellular microbial killing capacity) observed in the present study probably reflect a temporary enhancement of cellular functions in response to neutrophilic cytokines such as G-CSF and NGF, possibly owing to bacterial challenge in the respiratory tract during transport.<sup>2,6,13,14,15,16</sup> Stull et al. have reported that the counts of T cells and B cells (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and CD21<sup>+</sup>) decrease in response to the long-term road transport-induced stress.<sup>5</sup> Conversely, the finding obtained from the present study that an enhanced lymphocyte blastogenesis probably reflected a temporary enhancement of these cellular functions in response to bacterial antigens during transport also seems to support this view, ie, enhancement of lymphocyte blastogenesis due to bacterial challenge in the respiratory tract during transport.<sup>2,3,6</sup> Further research is needed to explore the functional implications of alterations in peripheral blood leukocytes.

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