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STUDIES ON THE IMMUNE STATUS  
DEMONSTRATED BY LYMPHOCYTE FUNCTION  
IN DOMESTIC ANIMALS EXPOSED TO  
ENVIRONMENTAL STRESSORS

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## LITERATURE REVIEW

### Definition of Stress

In general, "stress" is defined in physiology as the following connotation. Namely, any stimulus, as fear or pain, that disturbs or interferes with the normal physiological equilibrium of an organism (38).

### Endocrine Aspect of Stress

Within a number of endocrine responses which occur during stress, two systems have been received much attention. These are the hypothalamic-pituitary-adrenocortical (HPA) system, and the sympathetic-adrenomedullary (SA) system.

Mason (49) and Maickel et al. (47) emphasized that stressors activate the sympathetic system to cause it to release norepinephrine, and the adrenal medulla to cause it to release both epinephrine and norepinephrine. In addition to the peripheral sympathetic response, Stone (81) and Anisman (2) indicated that an activation of cerebral catecholaminergic neurons leads to increased levels of catecholamines in discrete brain regions.

On the other hand, since Selye (73) reported that diverse noxious agents cause an enlargement of the adrenal cortex as a consequence of the "stress syndrome", many studies have been conducted to show that a variety of stressful events cause a

release of adrenocorticotrophic hormone (ACTH) from cells of the anterior pituitary (91). The secreted ACTH then stimulates the synthesis of corticosteroids in the adrenal cortex, which results in an elevated level of blood corticosteroids. As for regulation of ACTH secretion, the release of ACTH from the pituitary is regulated by a corticotropin-releasing factor (CRF), which was isolated, purified, and its structure was characterized as a 41 amino acid peptide (86), from the hypothalamus. In the point of the HPA system, it was shown that  $\beta$ -endorphin and  $\beta$ -lipoprotein are released at the pituitary level along with ACTH during stress (25).

Furthermore, the evidence is accumulating to indicate that these two systems (the HPA and SA) are not independent from each other. Rats exposed to immobilization by using a model that combines emotional stress (escape reaction) and physical stress (muscle work) revealed activation of both the sympathetic-adrenomedullary and the pituitary-adrenocortical systems (42). In this type of stress, the activity of catecholamine biosynthetic enzymes such as tyrosine hydroxylase, dopamine  $\beta$ -hydroxylase, and phenylethanolamine N-methyltransferase (PNMT) are known to be increased (40, 41). Wurtman and Axelrod (89, 90) also examined the effect of hypophysectomy on the activity of PNMT to make sure whether or not glucocorticoids and indirectly ACTH modify the formation of epinephrine in the adrenal medulla. Their results

demonstrated that the activity of PNMT in the medulla showed a marked decrease after hypophysectomy in rats, and the repeated administration of ACTH or the potent glucocorticoid dexamethasone to hypophysectomized rats restored PNMT activity to almost normal values. Similar results were also obtained by Kvetnansky et al. (43), indicating the interrelationship of ACTH in the pituitary and glucocorticoids in the adrenal cortex in affecting the synthesis of epinephrine.

#### Immunological Aspect of Stress

Immune system is one of the most important self-defense mechanisms by which animals can protect themselves from invasion of foreign substances. It is well known that immune function can be altered under several conditions. A typical example is psychological stress which suppresses the immune response (84). A question, here, is raised that how the immune system is regulated in such conditions. One possibility is elevated level of glucocorticoids, which are synthesized in and secreted from the adrenal cortex. Many studies have demonstrated that glucocorticoids suppress mitogen-induced lymphocyte blastogenesis (15, 21, 77). It is generally accepted that this suppressive effect of glucocorticoids is due to an inhibition of interleukin-2 (IL-2) production by T-cells (15, 21). In addition, Snyder and Unanue (79) have found that glucocorticoids inhibit interleukin-1 (IL-1), which used to be

called "lymphocyte activating factor" produced by macrophages. These discoveries have greatly contributed to the underlying of how glucocorticoids suppress primary immune response. Suppressive effect of glucocorticoids on leukocyte population has also been received much attention. Fauci and Dale (18) demonstrated that single intravenous injection of hydrocortisone to humans resulted in profound decreases in absolute numbers of circulating lymphocytes and monocytes. Similar results were also obtained in humans by Thompson et al. (85). That is, lymphocyte levels and plasma cortisol concentrations of healthy adults had circadian variations that were inversely related while the patients with adrenal insufficiency had low plasma cortisol levels and lacked significant lymphocyte variations. And administration of replacement quantities of cortisol to these patients caused a dose-dependent lowering of lymphocyte levels. In addition, Rey et al. (64) have shown that an inverse relation among endogenous levels of glucocorticoids, splenic mass, and cellularity in mice. Namely, decreased endogenous corticosterone blood levels resulted in an increased number of immunoglobulin-secreting cells in the spleen, and high corticosterone blood levels resulted in a reduced number of immunoglobulin-secreting cells, suggesting that endogenous glucocorticoid levels are also able to control the B-cell activity.

#### Lymphocyte Blastogenesis

Lymphocyte blastogenesis is the process which include new deoxyribonucleic acid (DNA) synthesis and cell division after receiving some type of stimulus. The lymphocytes, then, increase in size, the cytoplasm becomes more extensive, the nucleoli are visible in the nucleus, and the lymphocytes resemble blast cells. This process is seen from immunized animals. That is, the lymphocytes proliferate and transform in response to antigens to which they are sensitized. This *in vitro* response correlates with the existence of delayed-type hypersensitivity in the host. Similar series of changes are induced by polyclonal activators which include plant lectins. These plant lectins, including pokeweed mitogen (PWM), phytohemagglutinin (PHA) and concanavalin A (Con A), are called mitogens (52, 65, 66). In 1960, the first study of mitogen-induced lymphocyte blastogenesis was reported by Nowell (55) who demonstrated the proliferation of human lymphocytes in response to PHA. Since then, the analysis of *in vitro* lymphocyte responses to plant lectins has been used to evaluate functional lymphocytes (26, 58, 60). Based on these studies, it is now generally accepted that PHA and Con A preferentially stimulate T-lymphocyte blastogenesis, and PWM stimulates both T- and B-lymphocytes (26, 58, 60).

It has been demonstrated recently that the T-cell blastogenic response after lectin or antigen stimuli requires the production

of interleukin-2 (IL-2) which used to be called T-cell growth factor (54). Components of the signalling process activated during mitogen-induced lymphocyte blastogenesis include IL-2 production by T-cells, IL-2 receptor expression by T-cells, and subsequent T-cell response to IL-2 (14).

Current methods to measure these responses frequently utilize radiolabeled precursor of DNA (usually tritiated thymidine) incorporation which is a quantitative analysis of increased DNA synthesis (66).



CHAPTER I

SUPPRESSED PERIPHERAL BLOOD LYMPHOCYTE BLASTOGENESIS IN PRE- AND  
POST- PARTAL SHEEP BY CHRONIC HEAT-STRESS, AND SUPPRESSIVE  
PROPERTY OF HEAT-STRESSED SHEEP SERUM ON LYMPHOCYTES

## ABSTRACT

Phytohemagglutinin (PHA) and concanavalin A (Con A)-induced blastogenesis of peripheral blood lymphocytes was examined in heat-stressed pre- and post-partal sheep. The peak responses of lymphocytes to PHA and Con A in heat-stressed sheep revealed significant reduction before and after parturition compared with those in the corresponding control animals kept under thermoneutral conditions. Furthermore, the effect of serum from control or heat-stressed sheep on PHA-induced lymphocyte blastogenesis was examined. Supplementation of serum from heat-stressed sheep significantly suppressed the blastogenesis of lymphocytes obtained from healthy sheep, bovine, and human donors. Unlike dexamethasone, heat-stressed sheep serum did not inhibit IL-2 production by PHA-stimulated human peripheral blood lymphocytes. These results indicate that the immunosuppression of heat-stressed sheep is in part mediated by serum factor(s) that can modulate T-cell function in a species non-specific manner.

## INTRODUCTION

Susceptibility of animals to infectious diseases could be

altered under the stressful conditions (33, 37, 48, 61, 72). Although unfavorable environments termed as "stress" are well known to influence the immune response (30, 36, 48), the stressors applied are often of physical nature, such as exposure to repeated electric footshocks or subjection to immobilization. In these experiments, relationship between the effect of stress and the immune system is considered to be unidirectional. That is, if a physical or behavioral stressor of sufficient intensity and length is imposed on an animal, the ability of the animal to initiate or maintain optimum immunological responses will be suppressed via endocrine system, usually defined in terms of the hypothalamic-pituitary-adrenal system (17). The direct effect of glucocorticoids or the indirect effect of stress followed by the stimulated hypothalamic-pituitary-adrenocortical system is well known (17, 20, 67, 74, 75, 88). For instance, reduction in lymphatic tissue mass and a depression in the number of circulating lymphocytes are included. In contrast, although several studies on the suppressive effect of thermal stressors on the immune response have been reported, the underlying cause of suppression of the immune system is still unclear. In mice, Pitkin (61) reported that delayed-type hypersensitivity reactions were smaller in heat-stressed than in control animals. In chickens, Regnier and Kelley (63) noted that both heat and cold exposure for 5 days suppressed expressions of the

cellular immune response. In calves, however, conflicting results have been reported. That is, although chronic heat and cold stressors reduced contact sensitivity and tuberculin dermal reactions (38), neither heat nor cold exposure had a direct effect on mitogen-induced lymphocyte blastogenesis (39).

In animal husbandry, environmental heat is considered to be one of major factors which challenges the ability of animals to maintain energy, thermal, water, hormonal, and mineral balances (31, 46, 72). In addition, environmental heat could also affect the immune status as mentioned above. The purpose of the work presented here, therefore, is to provide additional basic information on the environmental heat effect on an expression of the cell-mediated immune response as measured by mitogen-induced lymphocyte blastogenesis using sheep as experimental animals.

## MATERIALS AND METHODS

### Experimental Animals

Twenty three pregnant cross breed ewes (Dorsett x Finn x Rambouillette), 5-6 years of age, were used in this study. Alfalfa hay supplemented with 114 gm corn/day/sheep and water were available ad libitum. After parturition, newborn lambs were nursed by their

mothers.

#### Thermal Stressor

Animals were divided into two groups. Control (n=12) were maintained in a 20 °C and 65% relative humidity (RH) thermoneutral environment, while heat-stressed animals (n=11) were maintained in a 35 °C and 55% RH environment. The animals were kept under their respective conditions for a period of 16-40 days (depending on date of lambing) before parturition and for 6 weeks after parturition (See Fig.1 for experimental schedule).

#### Rectal Temperature

Rectal temperatures were measured weekly throughout the experiment. Temperatures were obtained by a electronic thermometer (Fischer Scientific, St. Louis, MO).

#### Blood Collection

Ten ml of heparinized venous blood for the assay of lymphocyte blastogenesis were obtained by jugular venipuncture 4-21 days before parturition, and 1 and 4 weeks after parturition. Ten ml of anti-coagulant free blood for serum were obtained by jugular venipuncture 6 weeks after parturition. The blood was then centrifuged at 1,800 x g for 30 min. Sera were harvested and frozen at -20 °C until used.

#### Reagents and Chemicals

Ficoll-Isopaque gradient media (Histopaque®) and

dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). Hepes-buffered RPMI 1640, heat-inactivated fetal bovine serum and human AB serum were obtained from Hazleton Research Products (Lenaxa, KS). Phytohemagglutinin (PHA) and concanavalin A (Con A) were purchased from Difco Laboratories (Detroit, MI) and Pharmacia Co. (Uppsala, Sweden), respectively. Human natural interleukin-2 (IL-2) was purchased from Collaborative Research Inc. (Bedford, MA). (Methyl-<sup>3</sup>H)-thymidine (2 Ci/mmol) was obtained from NEN Research Products (Boston, MA).

#### Peripheral Blood Lymphocytes

Ten ml of sheep heparinized venous blood were mixed with 10 ml of RPMI 1640 supplemented with 2 mM L-glutamine, penicillin G potassium (100 units/ml) and gentamicin (50  $\mu$ g/ml). This supplemented medium will be referred to as RPMI 1640 throughout this chapter. Twenty ml of blood-medium mixture were then carefully layered onto 10 ml of Histopaque®. After centrifugation at 800 x g for 30 min, sheep peripheral blood lymphocytes appeared as a white band between the plasma-medium mixture and Histopaque® at apparent density 1.083 g/ml. Isolated lymphocytes were taken out by a syringe with a spinal needle, transferred to sterile plastic tubes, and centrifuged at 600 x g for 10 min. After the supernatant was decanted, lymphocyte pellet was resuspended in 5 ml of RPMI 1640, and centrifuged at 600 x g for 10 min (washing procedure). After

the lymphocytes were washed three times in RPMI 1640, lymphocyte numbers were estimated by using a Coulter counter (Model ZBI, Coulter Electronics Inc., Hialeah, FL) based on the principle of counting cells suspended in electrolyte and passing them through an aperture with a specific path of current flow for a given length of time. More in detail, the lymphocyte suspension was diluted to 1:500 with 20 ml of Isoton II (Coulter Diagnostics Inc., Hialeah, FL) by Coulter Dual Diluter III (Coulter Diagnostics Inc., Hialeah, FL). To count the lymphocytes, 6 drops of lysing agent (Zapoglobin II, Coulter Diagnostics Inc., Hialeah, FL) were added to 1:500 diluted sample vials to lyse the erythrocytes. All determinations were in triplicate, and the mean value was used. Lymphocyte viability was evaluated by a Trypan blue exclusion test. Namely, a small aliquot of cell suspension was mixed with an equal volume of 0.4% (w/v) Trypan blue solution prepared in 0.9% NaCl. The mixture of cell suspension and Trypan blue solution was placed on a hemocytometer, and under a microscope blue stained cells were regarded as dead cells. Based on the lymphocyte count and viability, lymphocyte concentration was adjusted to  $2 \times 10^6$  viable cells/ml. For further determining if serum from experimental animals had immunomodulative effect on lymphocytes, bovine and human peripheral blood lymphocytes were obtained in the same way as in sheep lymphocytes except that density of Histopaque® used

for isolation of human lymphocytes was 1.077 g/ml.

#### Mitogens, Human IL-2, and Dexamethasone

Five dilutions of PHA (1:2,000 to 1:10) in RPMI 1640 were prepared and kept frozen at -20 °C until used. Five concentrations of Con A (0.02 to 0.31 mg/ml) dissolved in RPMI 1640 were prepared and kept frozen at -20 °C until used. Human IL-2 was dissolved in RPMI 1640 (200 units/ml) immediately before use. Three concentrations of dexamethasone (42 nM to 4.2  $\mu$ M) dissolved in 95% (v/v) ethanol were prepared and kept at 4 °C.

#### Lymphocyte Culture

Peripheral blood lymphocyte suspension ( $1 \times 10^5$  cells/well) were cultured in triplicate or quadruplicate in 96 well flat-bottomed microplates (Falcon 3072, Oxnard, CA). Each well contained 50  $\mu$ l of cell suspension, 50  $\mu$ l of mitogen solution or medium alone (controls), and 20  $\mu$ l of supplemental serum (fetal bovine serum for sheep and bovine lymphocytes, and human AB serum for human lymphocytes). In the experiment in which the effect of serum from the experimental animals was examined, 20  $\mu$ l of serum from the control and heat-stressed animals were added to the wells instead of those supplemental serum. In some experiments with human lymphocytes, 20  $\mu$ l of dexamethasone solution were pipeted into the wells and the ethanol was allowed to evaporate prior to the addition of cell suspension. As a control, 20  $\mu$ l of 95% (v/v)



ethanol without dexamethasone were dispensed in a similar manner to the remaining wells. In addition to dexamethasone, 20  $\mu$ l of human IL-2 or medium alone were added to the wells at the same time as the addition of mitogen and supplemental serum. The cultures were incubated at 37 °C in 5% CO<sub>2</sub>/95% air. After 72 hr of incubation, 1  $\mu$ Ci of <sup>3</sup>H-thymidine dissolved in 20  $\mu$ l of RPMI 1640 was added to the culture and the cells were incubated for an additional 16 hr. The cells were harvested onto glass fiber filter paper by using a semiautomatic cell harvester (Skatron Inc., Sterling, VA) with physiological saline as a washing solution. Filter paper discs were air-dried and <sup>3</sup>H-thymidine incorporation was determined by liquid scintillation counting. Background dpm by unstimulated lymphocyte cultures were subtracted from dpm obtained from stimulated cultures.

To determine if serum from the experimental animals has cytolytic effect on lymphocytes, 2 ml of sheep peripheral blood lymphocyte suspension ( $1 \times 10^6$  cells/ml) were cultured with 0.4 ml of serum from the experimental animals at 37 °C in 5% CO<sub>2</sub>/95% air for 72 hr. Recovery of viable lymphocytes after the incubation was evaluated based on the number of lymphocyte estimated by counting with a Coulter counter (Model ZBI, Coulter Electronics Inc., Hialeah, FL) and cell viability estimated by a Trypan blue exclusion test.

#### Determination of Human IL-2 Concentrations

IL-2 concentrations following culture of human peripheral blood lymphocytes were measured by Interleukin-2 ELISA Assay System (IL-ISA 2, Collaborative Research Inc., Bedford, MA) with a polyclonal human IL-2 antibody. The protocol used for IL-2 assay was the procedure provided by the manufacture. In brief, culture medium from each well was transferred to an Eppendorf tube (1.5 ml) and centrifuged at 150 x g for 4 min to obtain supernatant fractions containing IL-2. These solutions were incubated in the presence of antibody that had been precoated on the surface of plastic microwells that acted as a solid phase support. Anti-IL-2 antibody conjugated with horseradish peroxidase was then added to the wells. After subsequent addition of o-phenylenediamine dihydrochloride, the amount of colored reaction product was measured at 490 nm by using an ELISA reader (MINIREADER II, Dynatech Laboratories Inc., Alexandria, VA).

#### Serum Cortisol, Total Protein, Albumin, and Immunoglobulin G

Serum cortisol was measured by the radioimmunoassay with solid phase technique (Diagnostic Products Co., Los Angeles, CA). Ten  $\mu$ l cortisol standard solution (0-75  $\mu$ g/dl) or serum, and 1,000  $\mu$ l  $^{125}$ I-cortisol were pipeted to polypropylene tubes coated with anti-cortisol serum. Tubes were then incubated for 45 min at 37 °C. After the incubation, the liquid from each tube was decanted, and radioactivity in all tubes was counted in a gamma counter (1277

Gammamaster, Pharmacia Co., Uppsala, Sweden). The cortisol concentrations of the serum samples were determined from the calibration curve prepared using the standards.

Serum total protein was measured according to the method of Lowry et al. (45) by using a commercial assay kit (Sigma Chemical Co., St. Louis, MO). In brief, 1 ml protein standard solution (bovine serum albumin, 0-400  $\mu$ g/ml) or serum diluted with distilled water (1:300) was mixed with 1 ml Lowry reagent solution. After incubation at room temperature for 20 min, 0.5 ml Folin and Ciocalteu's Phenol Reagent Working Solution was added to each tube. After incubation at room temperature for 30 min, the absorbance of the standards and samples versus the blank at 750 nm was measured by using a spectrophotometer (DU Series 60, Beckman Instruments Inc., Fullerton, CA). The protein concentrations of the serum samples were determined from the calibration curve prepared using the standards and multiplication of the result by the appropriate dilution factor.

Serum albumin was measured according to the method of Dumas et al. (16) by using a commercial assay kit (Sigma Chemical Co., St. Louis, MO). In brief, 30  $\mu$ l standard solution (bovine serum albumin, 0-10 g/dl) or serum were added to 1 ml Albumin Reagent containing bromocresol green. After each tube was mixed gently, the absorbance of the standards and samples versus the blank at 628 nm

was measured by using a spectrophotometer (DU Series 60, Beckman Instruments Inc., Fullerton, CA). The albumin concentrations of the serum samples were determined from the calibration curve prepared using the standards.

Sheep serum immunoglobulin G (Ig G) was measured by a single radial immunodiffusion method by using a commercial assay kit (ICN Biomedicals Inc., Irvine, CA). In brief, serum samples were diluted with 5% (w/v) bovine serum albumin solution (1:40). Standards (sheep IgG, 0-1,000 mg/l) or the samples were applied in 5  $\mu$ l volume to each well of an immunodiffusion plate by using a Hamilton micro syringe (701-N, Hamilton Company, Reno NV). After sample application, a lid was tightly closed and the plate was stored flat at room temperature for 50 hr. After the incubation, the precipitate diameters were measured to the nearest 0.1 mm by using a calliper provided by the manufacture, and each (ring diameter)<sup>2</sup> was calculated. The IgG concentrations of the sheep serum samples were determined from the calibration curve prepared using (ring diameter of each standard)<sup>2</sup> and multiplication of the result by the appropriate dilution factor.

Each assay for serum parameters was performed in duplicate, and the mean value was used.

#### Statistical Analysis

When necessary, the difference in the mean value between the

two groups was assessed by two-tailed Student's t-test or the one way analysis of variance.

## RESULTS

### Lymphocyte Response to PHA and Con A in Pre- and Post-Partal Sheep

To determine an expression of the cell-mediated immune response, PHA- or Con A-induced blastogenesis of lymphocytes from the control and heat-stressed sheep was examined. Rectal temperatures, which reflect the magnitude of thermal stress are shown in Table I-1.

<sup>3</sup>H-Thymidine incorporation into unstimulated cultures is shown in Table I-2. In the absence of mitogens, no significant differences were observed between the control and heat-stressed sheep.

Lymphocyte response to PHA and Con A stimulation is summarized in Fig.I-2. In the presence of mitogens, <sup>3</sup>H-thymidine incorporation into the cultures from pre- and post-partal heat-stressed animals was significantly lower than that in the control animals. As shown in Fig.I-2, the peak response to PHA in the heat-stressed animals revealed 60% (P<0.001), 34% (P<0.05) and 43% (P<0.001) reduction before parturition, 1 week and 4 weeks after parturition, respectively, compared with that in the corresponding control animals. In addition, the peak response to Con A in the

heat-stressed animals also revealed 37% ( $P < 0.01$ ), 29% ( $P < 0.05$ ) and 35% ( $P < 0.01$ ) reduction before parturition, 1 week and 4 weeks after parturition, respectively.

#### Effect of Serum from Control and Heat-Stressed Animals on PHA-Induced Blastogenesis of Lymphocytes from Healthy Donors

To determine if serum from heat-stressed sheep has immunomodulative effect on lymphocytes, PHA-induced blastogenesis of lymphocytes from a healthy sheep donor in the presence of serum from the control or heat-stressed sheep was examined (Fig.I-3).

Supplementation of serum from the heat-stressed animals revealed significant suppression ( $P < 0.05$ ) of  $^3\text{H}$ -thymidine incorporation as compared to that from the control animals. Heat-inactivation (56 °C, 30 min) of serum resulted in higher response of lymphocytes in both groups although lymphocyte response with serum from the heat-stressed animals was still significantly suppressed ( $P < 0.05$ ). This suppression was not due to cytolytic effect of heat-stressed sheep serum since no difference was found in recovery of viable lymphocytes incubated with serum from heat-stressed and control sheep (Table I-3).

Furthermore, to determine if this suppressive effect of serum observed in sheep lymphocytes is expressed in a species-specific manner, effect of heat-inactivated serum from healthy bovine and human donors was examined (Fig.I-4). As shown in the figure,

supplementation of heat-stressed sheep serum also suppressed the blastogenesis of both bovine ( $P < 0.01$ ) and human cells ( $P < 0.01$ ).

Effect of Exogenous Human IL-2 on PHA-Induced Blastogenesis of Human Peripheral Blood Lymphocytes Cultured with Serum from Control or Heat-Stressed Sheep

To characterize the suppressive effect of heat-stressed sheep serum on PHA-induced lymphocyte blastogenesis, we evaluated whether the serum interferes with IL-2 pathway by either 1) inhibition of IL-2 production by stimulated T-cells or 2) suppression of T-cell responsiveness to IL-2. Since neither purified sheep IL-2 nor anti-sheep IL-2 antibody was available at the time these experiments were conducted, it was not possible to examine directly whether IL-2 production by sheep T-cells and/or T-cell responsiveness to IL-2 were altered by heat-stressed sheep serum. Therefore, the following experiment was conducted by using human lymphocytes and human IL-2. In Fig.I-4, effect of the control or heat-stressed sheep serum on PHA-induced blastogenesis of human lymphocytes in the presence or absence of human IL-2 was examined. In this experiment, effect of dexamethasone was also examined at the same time since dexamethasone suppresses mitogen-induced blastogenesis by inhibiting IL-2 production by T-cells (15, 21). As shown in Fig.I-5, dexamethasone-induced suppression of  $^3\text{H}$ -thymidine uptake by T-cells was completely overcome by the addition of IL-2. In contrast,

the addition of IL-2 to the cultures supplemented with sheep serum did not reveal any effect.

#### IL-2 Production by PHA-Stimulated Human Peripheral Blood Lymphocytes Supplemented with Control or Heat-Stressed Sheep Serum

The effect of sheep serum on IL-2 production by human T-cells was then evaluated in comparison with that of dexamethasone (Fig.I-6). Dexamethasone clearly inhibited IL-2 release from human lymphocytes into culture media. In contrast, heat-stressed sheep serum did not reduce the amount of IL-2 produced compared with that with control sheep serum.

#### Serum Concentrations of Cortisol and Major Protein Fractions

To determine if there are great differences in the concentrations of serum components between the control and heat-stressed sheep, serum concentrations of cortisol and major protein fractions (total protein, albumin, and immunoglobulin G) were measured (Table I-4). As shown in the table no significant difference in cortisol concentration was found between the control and heat-stressed sheep. In addition, no significant difference was observed in each protein concentration between the control and heat-stressed sheep serum.



## DISCUSSION

In the present paper, we have demonstrated that long-term heat stress suppressed T-cell mitogen (PHA and Con A)-induced peripheral blood lymphocyte blastogenesis in pre- and post-partal sheep. As reported by Pitkin (61) and Regnier et al. (63), heat-stress has been shown to suppress the immune response in some animal species. Unlike other physical stressors such as immobilization and electric footshock (36, 56), little is known about the mechanisms by which heat-stress induces immunosuppression. Since a major population of T-cells are preferentially stimulated by both PHA and Con A, this immunosuppressive effect of heat-stress may be manifested at two levels; 1) a selective decrease in reactive T-cells and/or 2) changes in concentration of serum factor(s) which can modulate T-cell function. The present study here clearly showed that serum from heat-stressed sheep possessed an immunosuppressive property as expressed by its antiproliferative effect on PHA-stimulated lymphocytes. Since this suppression was still observed in the cells with heat-inactivated serum, complements and other heat-labile proteins were probably not involved in the effect. In addition, the suppressive effect induced by heat-stressed sheep serum was not a species-specific manner because the supplementation of the serum also reduced the  $^3\text{H}$ -thymidine uptake by PHA-stimulated

lymphocytes from bovine and human donors.

How heat-stressed sheep serum mediated suppression of T-cell response to PHA was investigated in these experiments. It is generally accepted that once T-cells are exposed to mitogen stimulation, they produce IL-2, which in turn stimulates T-cell clonal proliferation (22, 59). Components of the signalling process activated during mitogen-induced lymphocyte blastogenesis include IL-2 production by T-cells, IL-2 receptor expression by T-cells, and subsequent T-cell response to IL-2 (14). Disruption of IL-2 production at the level of mRNA transcription (as is the case with dexamethasone treatment, 59) results in the cessation of the blastogenic response induced by mitogen. One possibility, therefore, is that heat-stressed sheep serum, like dexamethasone (Fig.7), inhibits the production of IL-2. The studies reported here with human lymphocytes showed that the suppressive effect of heat-stressed sheep serum was not due to inhibition of IL-2 production. Unlike dexamethasone, addition of IL-2 did not overcome the suppressed lymphocyte response by heat-stressed sheep serum, and human IL-2 production by PHA-stimulated lymphocytes did not reveal any difference between supplementation of the control and heat-stressed sheep serum.

Another question was which serum factor(s) was involved in the suppressive property of the serum on lymphocyte response to mitogen.

Serum total protein, albumin, and immunoglobulin G concentrations revealed no significant differences between the two groups. Furthermore, serum cortisol concentrations were measured since serum glucocorticoids have been considered to be responsible for immunosuppression of animals exposed to stressors (9, 20, 87). As no significant differences in serum cortisol concentrations were found between the two groups, the suppressive effect of heat-stressed sheep serum on lymphocytes was probably not due to an increased level of serum glucocorticoids. This is also supported by the evidence that the suppressive property of serum was different from that of dexamethasone, a synthetic glucocorticoid. Some of the circulating hormones, catecholamines and/or opiate peptides have been considered as candidates for mediators of immunomodulation (4, 7, 10, 13, 19, 23, 24, 27, 34, 35). Therefore, further studies for these candidates as immunomodulators will be important in elucidating the mechanisms involved in the immunosuppression by heat-stress.

CHAPTER I

Figures and Tables

Expressed as Mean Squares (M.S.)

Individual Variations

Pre-portal period (25-70, 40-100 M)

Post-portal period (70-100, 100-150 M)

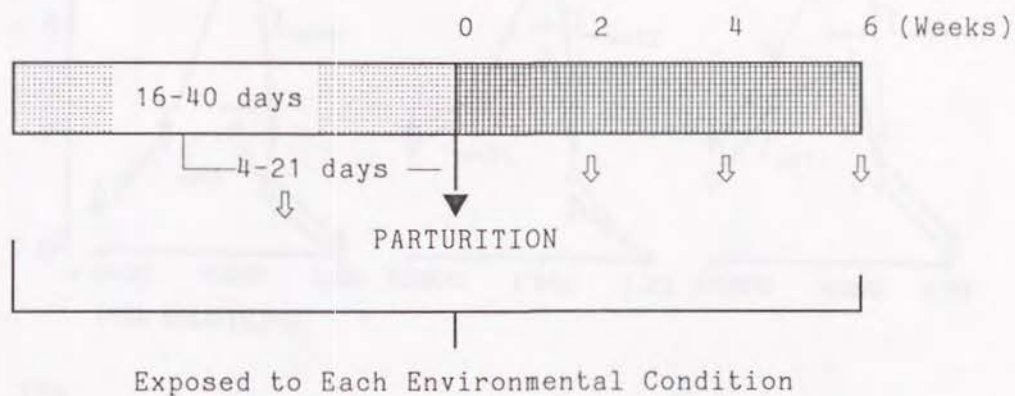
Fig. 1. Experimental results for showing the effect of portal  
blood flow on the metabolism of glucose, lactate, and  
amino acids in the portal vein.



Pre-portal period



Post-portal period



Environmental Conditions:

Thermoneutral Conditions ( 20 °C, 65-75% RH )

Heat-Stressed Conditions ( 35 °C, 55-65% RH )

Fig.I-1 Experimental schedule for studying the effect of chronic heat stress on an expression of cellular immune response in pre- and post-partal sheep

: Pre-partal period     
  : Post-partal period

↓ : Bleeding

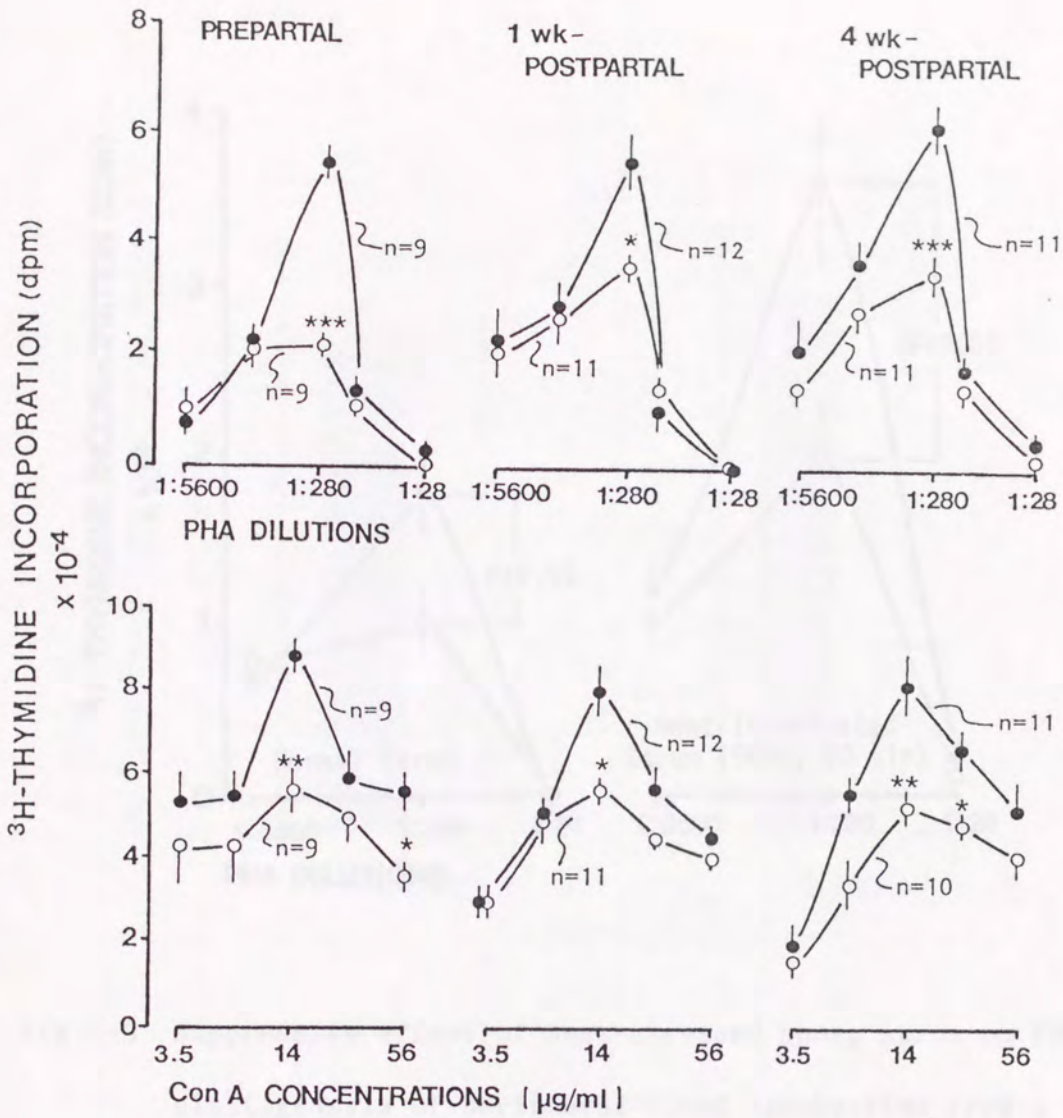


Fig.I-2 Mitogen-induced peripheral blood lymphocyte blastogenesis in the control (TN; ●) or heat-stressed (HS; ○) sheep before and after parturition. Each value represents the mean  $\pm$  standard error and the significance of the difference from the corresponding control group assessed by Student's t-test is shown  $P < 0.05$ ,  $**0.01$ ,  $***0.001$ .

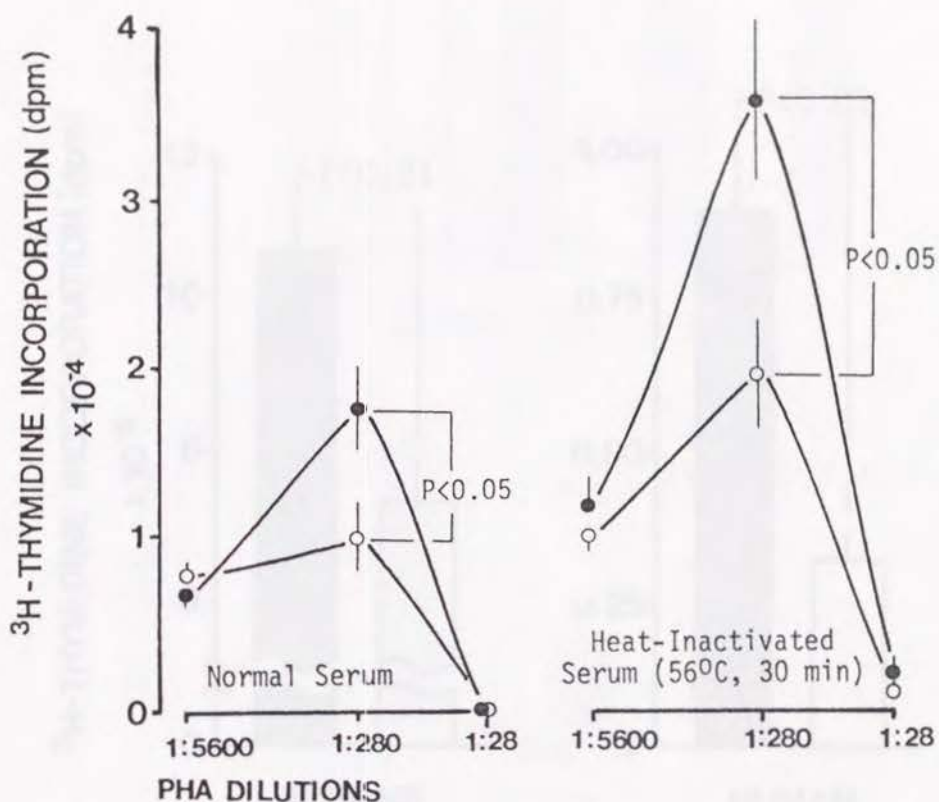


Fig.I-3 Suppressive effect of heat-stressed sheep serum on PHA-induced blastogenesis of peripheral blood lymphocytes from a healthy sheep donor. Left figure shows the effect of normal serum from the experimental animals. Right figure shows the effect of heat-inactivated (56 °C, 30 min) serum from the experimental animals. Each value represents the mean  $\pm$  standard error of the control (TN; ●; n=12) or heat-stressed (HS; ○; n=11). Significance of the difference between the treatments was assessed by Student's t-test.

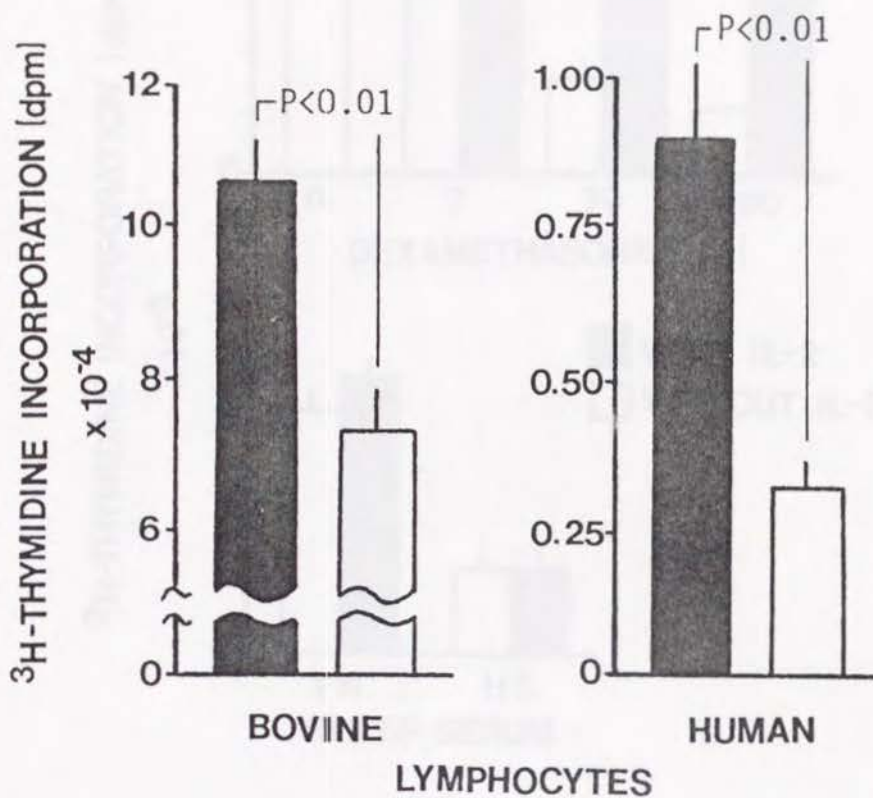


Fig.I-4 Suppressive effect of heat-stressed sheep serum on PHA-induced blastogenesis of peripheral blood lymphocytes from bovine and human lymphocyte cultures were 1:1,400 and 1:140, respectively. Each value represents the mean  $\pm$  standard error of the control (TN; ■; n=12) or heat-stressed (HS; □; n=11) group. Significance of the difference between the treatments was assessed by Student's t-test.



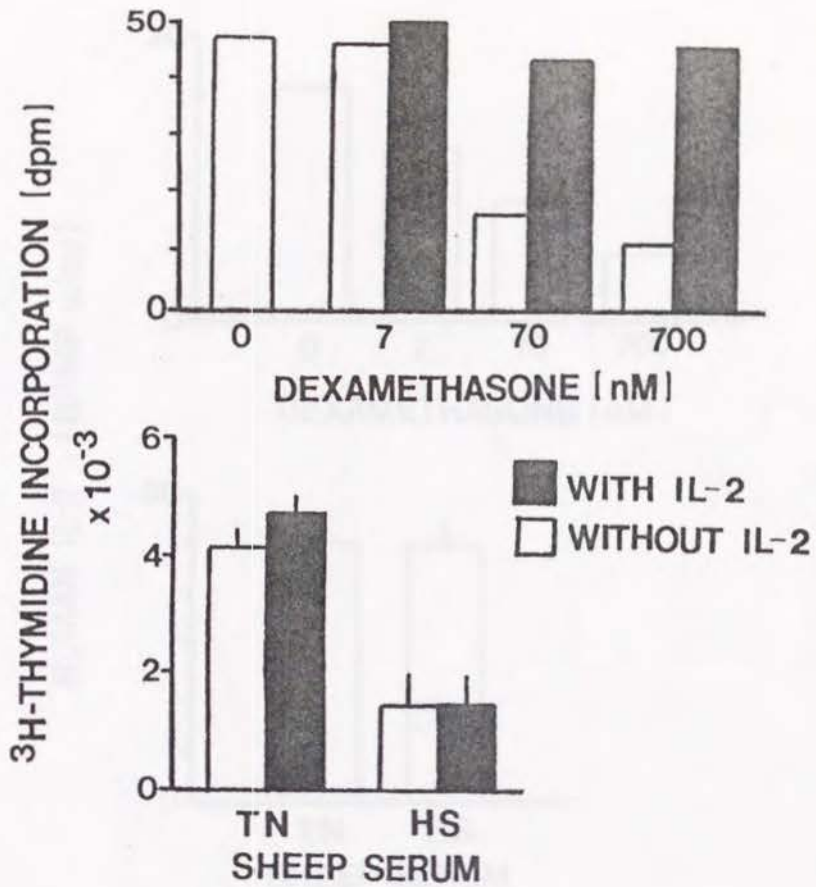


Fig.I-5 PHA-induced human peripheral blood lymphocyte blastogenesis cultured with the control or heat-stressed sheep serum in the presence or absence of human IL-2 (25 units/ml) in comparison with that with varying concentrations of dexamethasone. Final dilution of PHA in the culture was 1:140. Each value represents the mean  $\pm$  standard error of the control (TN; n=12) or heat-stressed (HS; n=11) group. Significance of the difference between the treatments was assessed by Student's t-test.

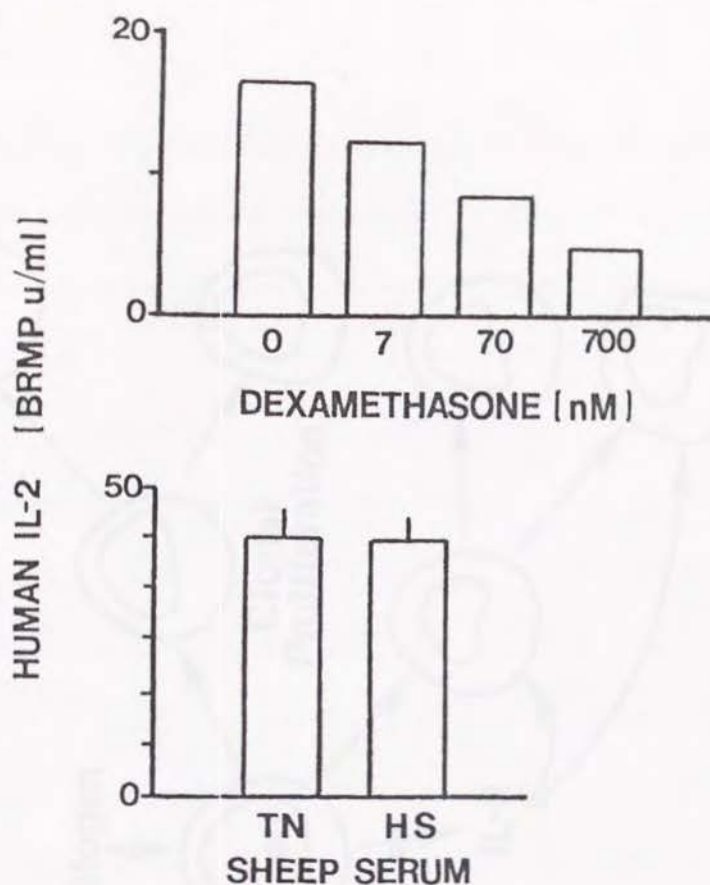


Fig.I-6 Interleukin-2 (IL-2) production by PHA-stimulated human peripheral blood lymphocytes cultured with the control or heat-stressed sheep serum. Final dilution of PHA in the culture was 1:400. Concentrations of IL-2 were defined as BRMP (Biological Response Modifier Program) half-maximal units per milliliter. Each value in the upper figure represents the mean of the duplicate cultures. Each value in the lower figure represents the mean  $\pm$  standard error of the control (TN; n=11) or heat-stressed (HS; n=10) group.

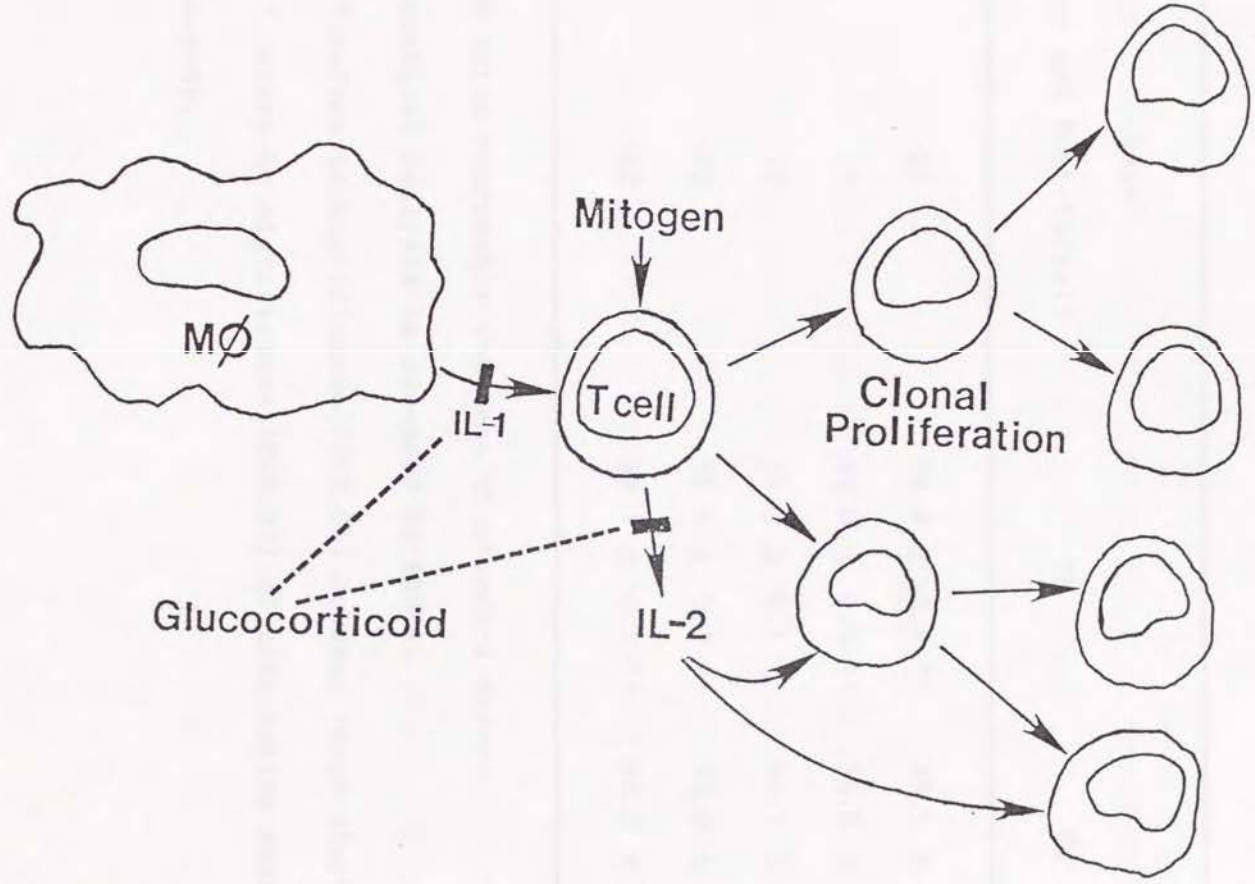


Fig. I-7 Schematic figure of immunosuppressive effects of glucocorticoid on lymphocytes.

Table I-1 Average mean rectal temperatures for pre- and post-partal sheep at thermoneutrality (TN) and during heat stress (HS).

Days (Pre- and Post-Partal)	TN	HS
-21	39.0 ± 0.1 <sup>A a</sup>	39.5 ± 0.1 <sup>B a</sup>
-7	39.0 ± 0.0 <sup>A a</sup>	39.5 ± 0.1 <sup>B a</sup>
+7	39.3 ± 0.1 <sup>A b</sup>	40.1 ± 0.1 <sup>B b</sup>
+28	39.0 ± 0.1 <sup>A a</sup>	40.0 ± 0.1 <sup>B b</sup>
+42	39.1 ± 0.1 <sup>A a</sup>	40.0 ± 0.1 <sup>B b</sup>

Each value represents the mean ± standard error.

Statistical analysis is assessed by ANOVA.

<sup>A</sup>, <sup>B</sup> refers to significance (P<0.01) between temperature treatment.

<sup>a</sup>, <sup>b</sup> refers to significance (P<0.01) of time during same temperature treatment.

Table I-2 <sup>3</sup>H-Thymidine incorporation into unstimulated lymphocyte culture from pre- and post-partal sheep

Treatments	Prepartal	Postpartal	
		1 week	4 weeks
		<u>(dpm/culture)</u>	
TN	1,727 ± 297 (9)	2,795 ± 538 (12)	1,922 ± 370 (11)
HS	1,955 ± 508 (9)	2,432 ± 477 (11)	2,269 ± 586 (10)

TN and HS stand for thermoneutral (control) and heat-stressed, respectively. Each value represents the mean ± standard error. The number in parenthesis represents the number of animals.

Table I-3 Recovery of sheep viable peripheral blood lymphocytes incubated with control (TN ; n=11) or heat-stressed (HS ; n=12) group.

	TN	HS
	75 ± 3 (%)	76 ± 3 (%)

Each value represents the mean ± standard error.

Each value is expressed as % of initial viable cell count.

Table I-4 Serum total protein (TP), albumin (Alb), immunoglobulin G (IgG), and cortisol concentrations in the control (TN; n=12) and heat-stressed (HS; n=11) groups.

Parameters	TN	HS
TP (g/dl)	7.7 ± 0.2	7.2 ± 0.2
Alb (g/dl)	3.0 ± 0.1	2.8 ± 0.1
IgG (g/dl)	2.1 ± 0.2	1.7 ± 0.1
Cortisol (ng/ml)	7.9 ± 1.4	6.9 ± 1.1

Each value represents the mean ± standard error.

CHAPTER II

SUPPRESSED PHYTOHEMAGGLUTININ-STIMULATED LYMPHOCYTE BLASTOGENESIS

OF WHOLE BLOOD CULTURE IN YOUNG PIGS AFTER EXPOSURE

TO MULTI-STRESSORS



## ABSTRACT

A study was conducted to evaluate an expression of the cell-mediated immune response in young feeder pigs after exposure to multi-stressors. Ten pigs were handled, marketed and transported for 36 hr to a new location. Ten pigs (controls) remained in home pens. Blood samples were obtained on d 1, 3, 6, 13, and 21 after relocation. The number of circulating blood lymphocytes and blastogenic response of whole blood (W) and isolated (I) peripheral blood lymphocytes to T-cell mitogen phytohemagglutinin (PHA) were determined. No significant differences in the I-lymphocytes to PHA were found between treatments. In contrast, the response of W-lymphocytes to PHA revealed a 60% and 50% reduction on d 1 and 3, respectively. On d 6 and 13 the degree of suppression gradually lessened and by d 21 no differences were found. Changes in lymphocyte numbers paralleled changes of blastogenic response of W-lymphocytes. The data suggest that the cell-mediated immune response of young pigs is suppressed for several days after exposure to marketing and transporting and such reduction is attributable to decreased number but not function of circulating lymphocytes.

## INTRODUCTION

Major health problems associated with the handling, marketing and transporting of feeder pigs are respiratory distress and diarrhea, with specific pathologic sources varying with reports (29). A survey of midwestern buyers of feeder pigs attributed up to 4% death loss due to pneumonia, scours, and flu. Brumm et al.(12) and Brumm and Peo (11) reported deaths in research trials that ranged from less than 1% to 8.3% and Jesse (28) reported less than 1% death loss with major diseases atrophic rhinitis, pasturella pneumonia and mycoplasma pneumonia. Biehl et al. (8) similarly reported a 5% death loss among commercial pigs and Straw et al. (82) reported a 5.3% death loss among commingled pigs.

A variety of factors may enhance susceptibility to diseases during this cycle of pig production including breed, intensive management, duration and conditions of transport, climatic extremes and commingling of pigs from different disease environments. Knowledge of the physiology and immunological changes occurring in pigs during marketing and transportation would provide a key to understanding the increased susceptibility to and incidence of diseases associated with handling, marketing, and transporting of feeder pigs. Thus, an experiment was conducted to evaluate an expression of the cell-mediated immune response as measured by blastogenic response of lymphocytes in marketed and transported feeder pigs.

## MATERIALS AND METHODS

### Experimental Animals

Twenty crossbred pigs, averaging 22.7 kg and approximately 9 weeks of age, were used in this study.

### Treatment

Ten control pigs remained in home pens with food and water available *ad libitum*. Ten pigs purchased from a commercial salebarn and experienced standard marketing conditions. Pigs were then transported to Columbia, MO. Marketing and transporting occurred over a 36 hr period (See Fig.1 for experimental schedule).

### Blood Collection

Twenty ml blood (10 ml in a heparinized tube, and 10 ml in an anti-coagulant free tube) were collected by jugular venipuncture on days 1, 3, 6, 13, and 21 after relocation. Ten ml of fresh heparinized blood were used for lymphocyte assays and blood cell counting. Ten ml of anti-coagulant free blood were coagulated and then centrifuged at 1,800 x g for 30 min. Sera were harvested and frozen at -20 °C until used.

### Reagents and Chemicals

All of the reagents and chemicals used in this study were the same as those used in Chapter I.

### Isolation of Peripheral Blood Lymphocytes

Procedure applied for isolation of peripheral blood lymphocytes was the same as that for sheep lymphocytes as described in Chapter I except that density of Histopaque® used in this study was 1.077 g/ml.

#### Isolated Lymphocyte and Whole Blood Culture

Protocol for isolated pig lymphocyte culture was identical to that for sheep lymphocyte culture as described in Chapter I. In brief, peripheral blood lymphocyte suspensions ( $1 \times 10^5$  cells/well) were cultured in quadruplicate in 96 well flat-bottomed microplates (Falcon 3027, Oxnard, CA). Each well contained 50  $\mu$ l of cell suspension, 50  $\mu$ l of phytohemagglutinin (PHA) solution or medium (RPMI 1640) alone (controls), and 20  $\mu$ l of fetal bovine serum. In the experiment in which the effect of serum from experimental animals was examined, 20  $\mu$ l of serum from the control and stressed pigs were added to the wells instead of fetal bovine serum. In the experiment with whole blood culture, samples of heparinized whole blood were pipeted into sterile culture tubes and diluted 1:10 in RPMI 1640. Diluted whole blood samples were cultured in the same way as in the isolated lymphocytes. That is, each well contained 50  $\mu$ l of diluted whole blood, 50  $\mu$ l of PHA solution or medium alone, and 20  $\mu$ l of fetal bovine serum. Preliminary experiments in our laboratory indicated that optimal final dilutions of PHA of this lot were 1:1,400 and 1:280 for isolated lymphocyte culture and whole blood culture, respectively. The cultures were incubated at 37 °C in 5% CO<sub>2</sub>/95% air. After 72 hr of incubation,

1  $\mu$ Ci of  $^3\text{H}$ -thymidine dissolved in RPMI 1640 was added to the culture and the cells were incubated for an additional 16 hr. The cells were harvested onto glass fiber filter paper by using a semiautomatic cell harvester (Skartron Inc., Sterling, VA) with physiological saline as a washing solution. Filter paper discs were air-dried and  $^3\text{H}$ -thymidine incorporation was determined by liquid scintillation counting.

Background dpm by unstimulated cultures were subtracted from dpm obtained from PHA-stimulated cultures.

#### Blood Erythrocyte, Leukocyte, and Lymphocyte Count

Total blood erythrocyte and leukocyte counts were determined by using a Coulter counter (Model ZBI, Coulter Electronics Inc., Hialeah, FL) based on the principle of counting cells passing through an aperture with a specific path of current flow for a given length of time. For leukocyte count, heparinized whole blood was diluted to 1:500 with 20 ml of Isoton II (Coulter Diagnostics, Hialeah, FL) by Coulter Dual Diluter III (Coulter Diagnostics, Hialeah, FL). Then 6 drops (0.2 ml) of lysing agent (Zap-oglobin II, Coulter Diagnostics, Hialeah, FL) were added to 1:500 diluted blood sample to lyse the erythrocytes. For erythrocyte counting, 1:50,000 dilution of heparinized whole blood in a 20 ml of Isoton II delivered by Coulter Dual Diluter III was prepared. All determinations were in duplicate, and the mean value was used. Total lymphocyte count was determined based on total leukocyte count and the proportion of

lymphocytes estimated from the differential leukocyte count of more than 200 cells on Giemsa-stained slides of whole blood.

#### Determination of Serum Parameters

Anti-coagulant free whole blood was centrifuged at 1,800 x g for 30 min. Serum was removed and stored at -20 °C until used.

Serum cortisol, total protein, and albumin concentrations were measured according to the same procedures as in Chapter I. Serum globulin concentrations were calculated by subtracting serum albumin concentrations from serum total protein concentrations. Serum albumin/globulin ratio was then calculated based on serum concentrations of albumin and globulin.

Pig serum immunoglobulin G (IgG) concentrations were measured by a single radial immunodiffusion method. Immunodiffusion agarose plates (1 mm thickness) which composed of 1.2% (w/v) agarose, 0.1% NaN<sub>3</sub>, and 10% anti-pig IgG antiserum (ICN Biochemicals Inc., Irvine, CA) were prepared. Serum samples were diluted with 5% (w/v) bovine serum albumin solution (1:40). Standards (Pig IgG, 0-1,000 mg/l, Sigma Chemicals Co., St. Louis, MO) or the samples were applied in 5  $\mu$ l volume to each well (3 mm diameter) of the immunodiffusion plates by using a Hamilton micro syringe (701-N, Hamilton Company, Reno, NV). After sample application, a lid was tightly closed and the plates were stored flat at room temperature for 50 hr. After the incubation, pig serum IgG concentrations were determined in the same way as described in Chapter

I.

All assays were run in duplicate, and the mean value was used.

#### Statistical Analysis

Two-tailed Student's t-test was used to determine the significance of differences between two groups.

### RESULTS

#### Blastogenic Response of Isolated Lymphocytes to PHA

Phytohemagglutinin (PHA)-induced blastogenesis of isolated peripheral blood lymphocytes, as measured by  $^3\text{H}$ -thymidine uptake, revealed no significant differences between the treatments throughout the experiment (Fig.II-2).

#### Blastogenic Response of Lymphocytes in the Whole Blood Culture to PHA

Fig.II-3 shows PHA-induced blastogenesis of lymphocytes in whole blood. Unlike PHA-induced blastogenesis of isolated lymphocytes,  $^3\text{H}$ -thymidine uptake by lymphocytes in whole blood of the stressed animals revealed a 60% ( $P < 0.01$ ) and 50% ( $P < 0.01$ ) reduction on d 1 and 3, respectively, compared to that of the control animals. On d 6 and 13 the degree of suppression gradually lessened and by d 21 no differences were found.

#### The Number of Circulating Blood Erythrocytes, Leukocytes, and

## Lymphocytes

Effect of exposure to multi-stressor on the number of erythrocytes, leukocytes, and lymphocytes in the circulating blood was summarized in Figs.II-4, -5 and -6. The number of erythrocytes per unit volume of blood showed no significant differences between the treatments throughout the experiment, whereas the number of leukocytes of the stressed pigs revealed about 50% reductions ( $P < 0.001$ ) on d 1 and d 3 as compared to that of the corresponding control pigs. On d 6 the degree of reduction in the leukocyte number was lessened to 30% ( $P < 0.001$ ) and after d 13 no differences were found. This reduction in the number of leukocytes was mainly due to a depression in the number of lymphocytes as shown in the figure. Based on these results, the suppression of PHA-induced blastogenesis of lymphocytes in the whole blood culture was probably attributable to decreases in the number of lymphocytes. Indeed, as shown in Fig.II-7, significant correlation between the two parameters of the stressed (marketed/transported) pigs, PHA-induced blastogenesis of lymphocytes in the whole blood culture and the number of circulating lymphocytes ( $P < 0.001$ ) was found.

## PHA-Induced Blastogenesis of Lymphocytes from Healthy Donor in the Presence of Serum from Experimental Animals

To determine if serum from stressed pigs has immunomodulative effect on lymphocytes, PHA-induced blastogenesis of lymphocytes from a



healthy pig donor in the presence of serum from the control or stressed pigs was examined (Table II-1). No significant differences in <sup>3</sup>H-thymidine uptake were observed between the treatments on d 1, 6 and 21.

#### Serum Cortisol Concentrations

To evaluate if such suppression shown in Figs. II-3 and -6 was related to serum glucocorticoid levels, cortisol concentrations were measured (Fig. II-8). On d 1 the stressed pigs showed a higher cortisol level than the control pigs although this difference was not significant. After d 3, cortisol concentrations of both groups were almost the same level (50-70 ng/ml).

#### Serum Concentrations of Albumin, Globulin and Immunoglobulin G, and Albumin/Globulin Ratio

Serum concentrations of albumin and globulin, and albumin/globulin (A/G) ratio are summarized in Tables II-2, -3 and -4. On d 1 the stressed pigs showed significantly higher ( $P < 0.001$ ) serum albumin concentration (3.3 g/dl) than that in the control pigs (2.4 g/dl). The concentration in the stressed pigs then gradually decreased, and after d 13 it returned to the control level. In contrast, on d 1 serum concentration of globulin in the stressed pig (3.1 g/dl) was significantly lower ( $P < 0.001$ ) than that in the control pigs (4.2 g/dl). The concentrations then gradually increased, and on d 21 it reached almost control level. As a result of changes in albumin

and globulin concentration, on d 1 A/G ratio in the stressed pigs was significantly higher ( $P < 0.001$ ) than in the control pigs. The values then gradually declined, and after d 13 no differences were found between the treatments. Furthermore, to determine if such a decrease in serum globulin concentration is due to a lower level of specific globulin fractions, serum immunoglobulin G (IgG) concentrations were measured on d 1, 6, and 21 (Table II-5). The concentration in the stressed pigs was significantly lower than that in the control pigs throughout the experiment although it showed an increasing tendency with time.

#### DISCUSSION

The primary significance of this study was that exposure of young feeder pigs to multi-stressors which mainly consisted of marketing and transporting caused suppression of PHA-induced lymphocyte blastogenesis in whole blood and this immunosuppression lasted for several days after exposure to stressors. This experiment utilized whole blood assay which included both plasma and cells from the animals; therefore, this suppression might be manifested at either of three levels; that is, 1) reduced lymphoid cell population per se., 2) functional impairment of reactive T-cells, and/or 3) changes in plasma factor(s) which is able to

modulate T-cell responsiveness to PHA. When these results were summarized over all experiments, the data indicated that this suppression was probably due to reduced number of circulating blood lymphocytes which paralleled the changes in the lymphocyte blastogenesis of the whole blood culture rather than functional impairment of T-cell responsiveness to PHA since no significant suppression was observed in the PHA-induced blastogenesis of isolated peripheral blood lymphocytes. In addition, plasma factor(s) might not be involved directly in this suppression since supplementation of stressed pig serum caused no significant depression in the PHA-induced blastogenesis of peripheral blood lymphocytes obtained from a healthy pig donor.

Taking the results in which serum IgG concentrations were decreased in stressed pigs, IgG-producing cell population was probably subjected to depression by multi-stressors.

Endocrine regulation of immune events has been discussed for understanding how stress affects the immune response. According to the literatures, a wide variety of hormones is released by stress, such as adrenocorticotropin (ACTH),  $\beta$ -endorphin, glucocorticoids, and all of these hormones have been demonstrated to affect a variety of immune events (23, 27, 32, 34, 50, 78). In these hormones, glucocorticoids have been paid much attention to their immunosuppressive actions which include reduction in lymphatic tissue mass and a depression in the number of circulating lymphocytes (17, 51, 68, 75, 76, 86, 88).

One possibility, therefore, is that such reduction in the number of lymphocytes observed in this study was caused by an elevated level of blood glucocorticoids. To evaluate this point, serum cortisol, which is the major adrenocortical hormone in the pig, was measured. Serum cortisol concentrations in marketed/transported pigs tended to be higher on d 1 after marketing and transportation but after d 3 the concentrations returned to levels similar to those of the control pigs. Unfortunately, serum cortisol concentrations were not measured during exposure of the animals to marketing and transportation; therefore, it seems difficult to explain the involvement of cortisol in the suppression observed in this study. However, since elevated level of cortisol during exposure to transportation stress has been demonstrated elsewhere (6, 80), it is postulated that this type of immunosuppression observed in young feeder pigs marketed and transported during a 36 hr period is mediated by this hormone.

CHAPTER II

Figures and Tables

Fig. II-1. Experimental schedule for studying the effect of sulfur  
dioxide on the growth and development of  
populations of soil-dwelling insects exposed to sulfur  
dioxide.



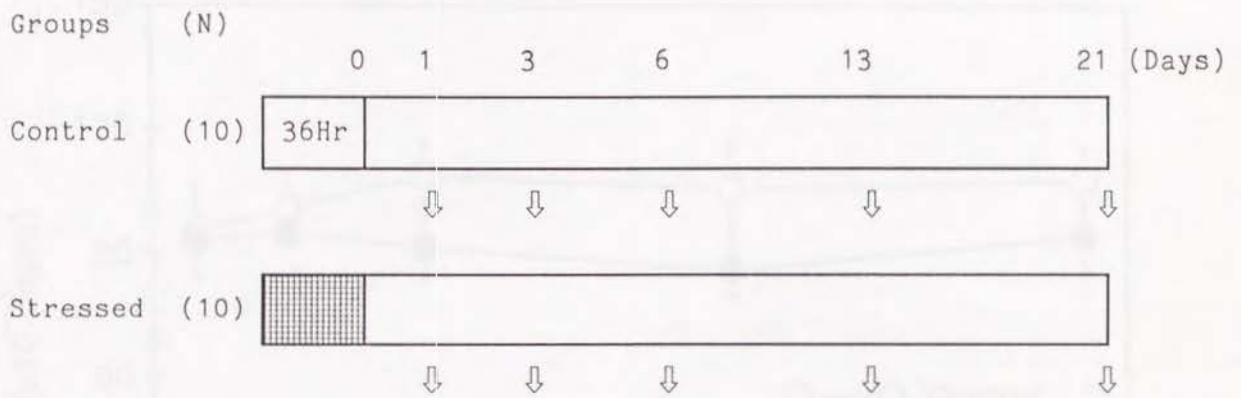



Fig.II-1 Experimental schedule for studying the effect of multi-stressors including marketing and transporting on expressions of cell-mediated immune response in young feeder pigs

 : Marketing and transporting      ↓ : Bleeding

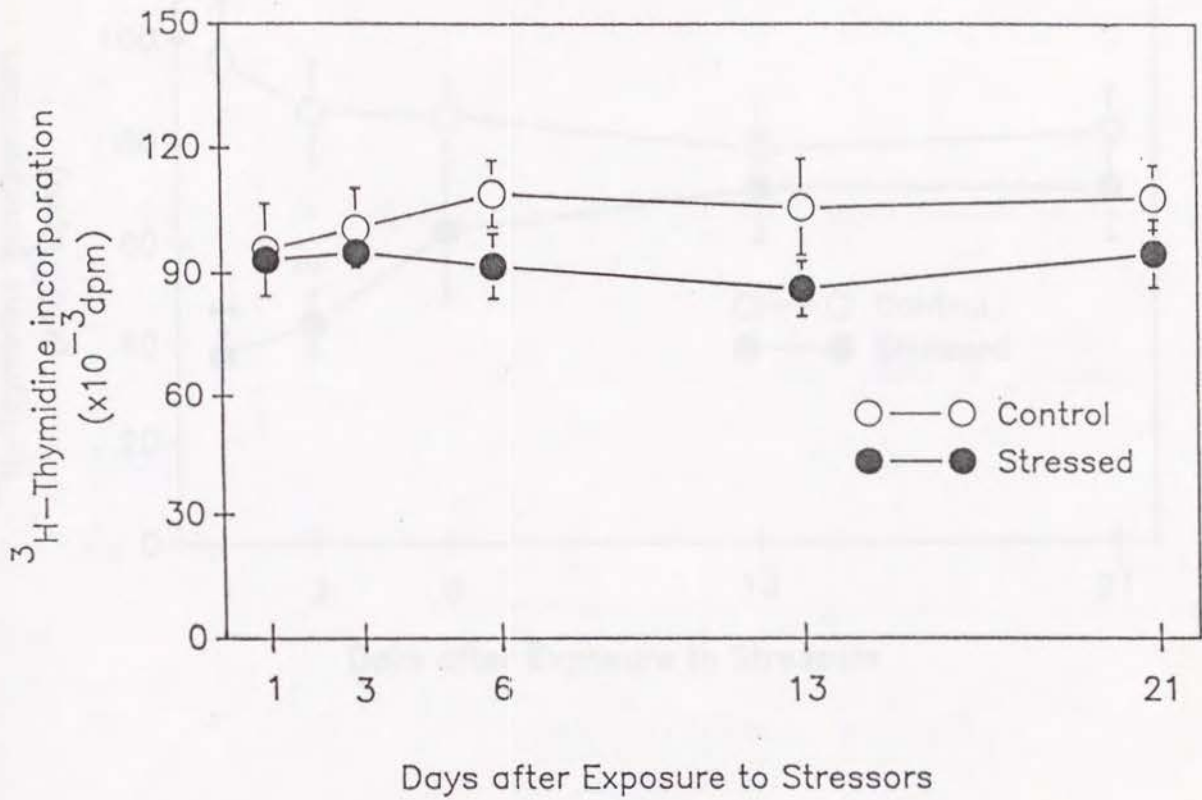


Fig.II-2 Effect of marketing and transporting of feeder pigs on PHA-induced blastogenic response of isolated peripheral blood lymphocytes. Each value represents the mean  $\pm$  standard error.

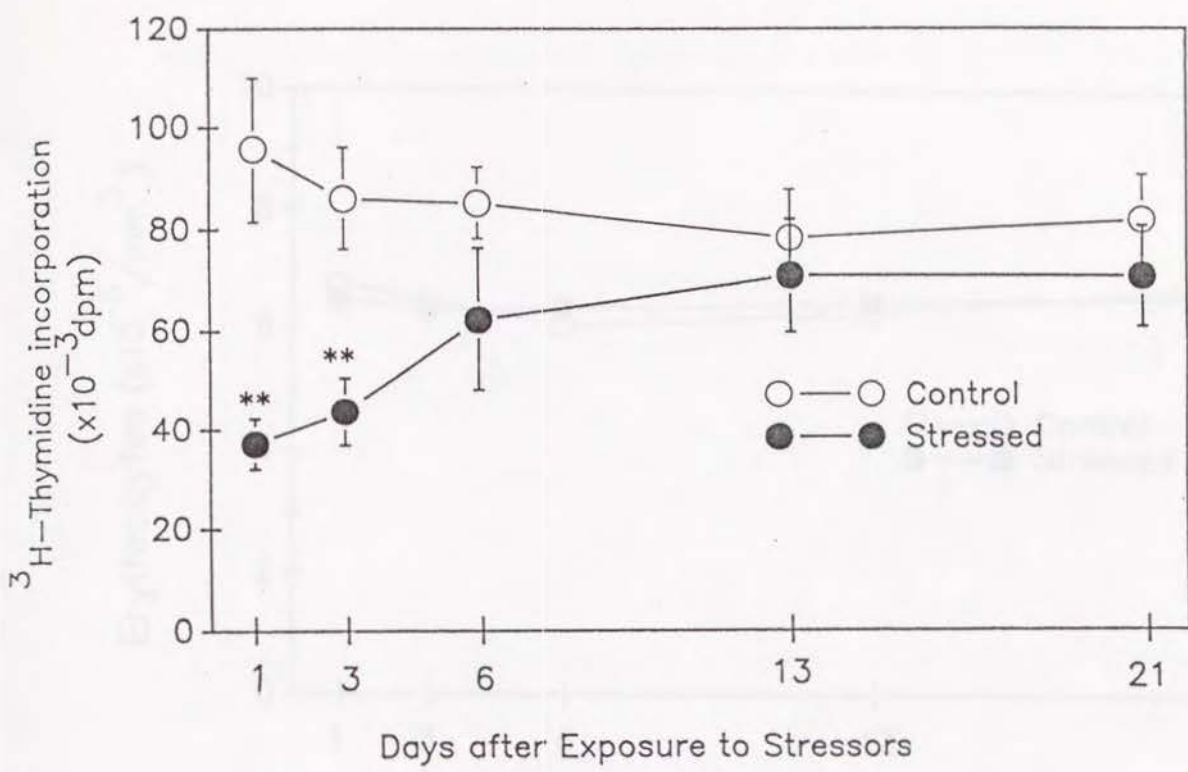


Fig.II-3 Effect of marketing and transporting of feeder pigs on PHA-induced blastogenic response of lymphocytes in the whole blood culture. Each value represents the mean  $\pm$  standard error and the significance of the difference from the corresponding control group is shown  $P < **0.01$ .



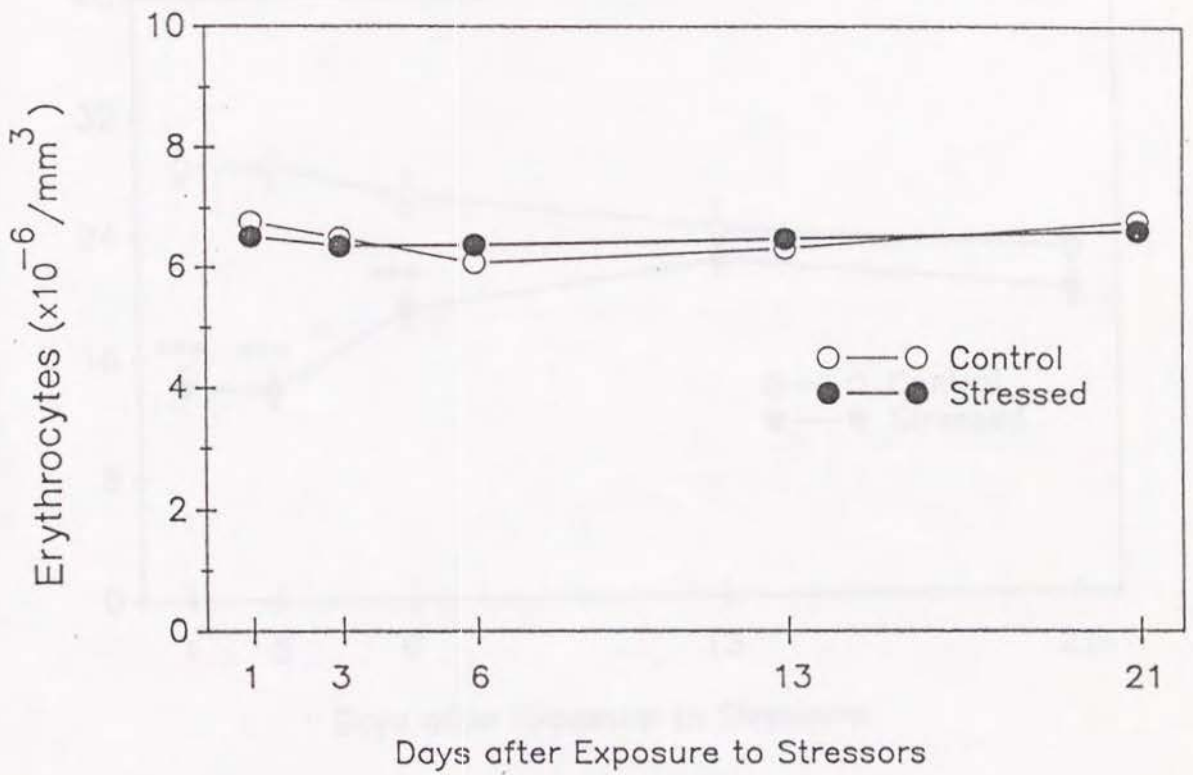


Fig.II-4 Effect of marketing and transporting of feeder pigs on the number of erythrocytes in the circulating blood. Each value represents the mean  $\pm$  standard error.

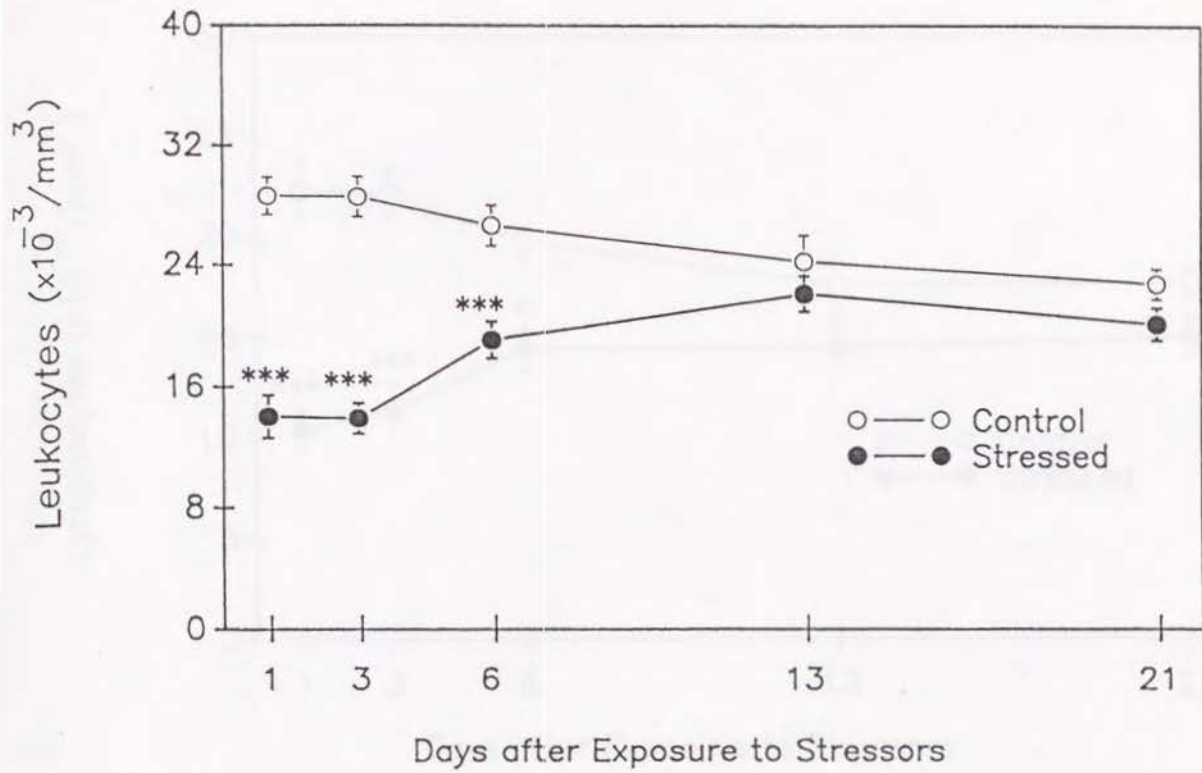


Fig.II-5 Effect of marketing and transporting of feeder pigs on the number of leukocytes in the circulating blood. Each value represents the mean  $\pm$  standard error and the significance of the difference from the corresponding control group is shown  $P < 0.05$ , \*\*\*0.001.

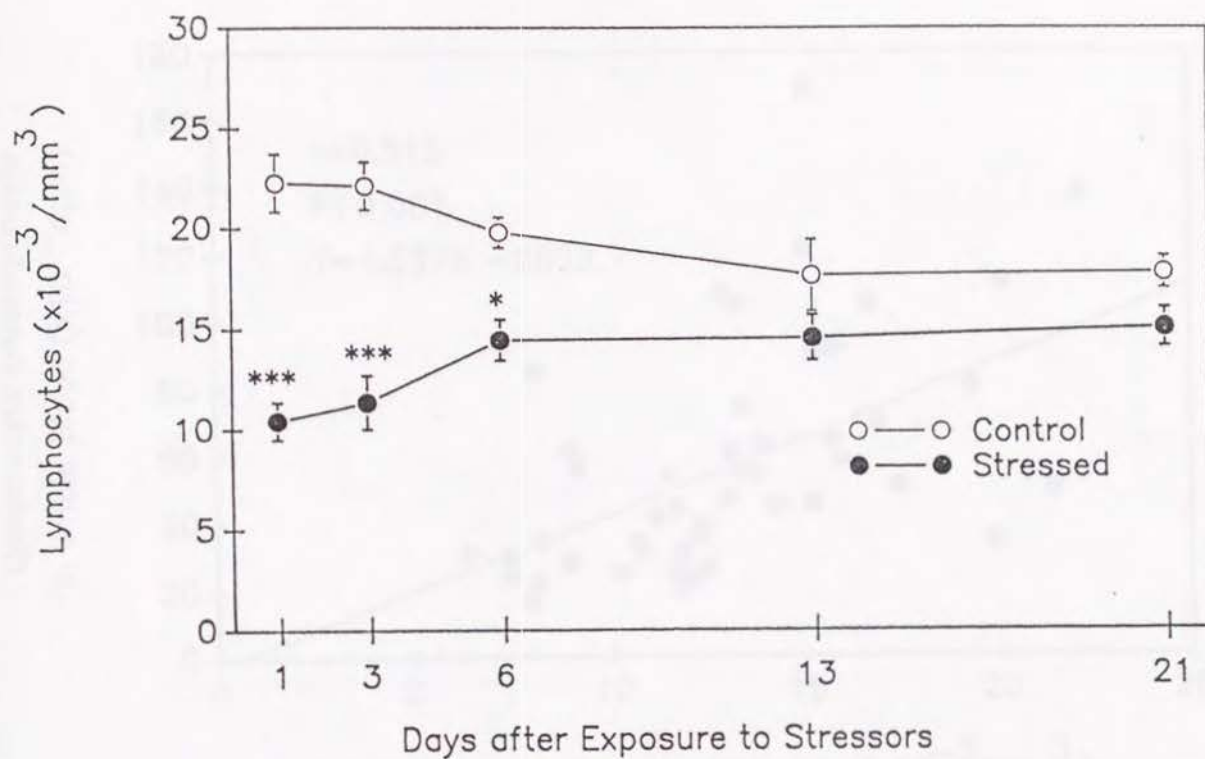


Fig.II-6 Effect of marketing and transporting of feeder pigs on the number of lymphocytes in the circulating blood. Each value represents the mean  $\pm$  standard error and the significance of the difference from the corresponding control group is shown  $P < 0.05$ , \*\*\*0.001.

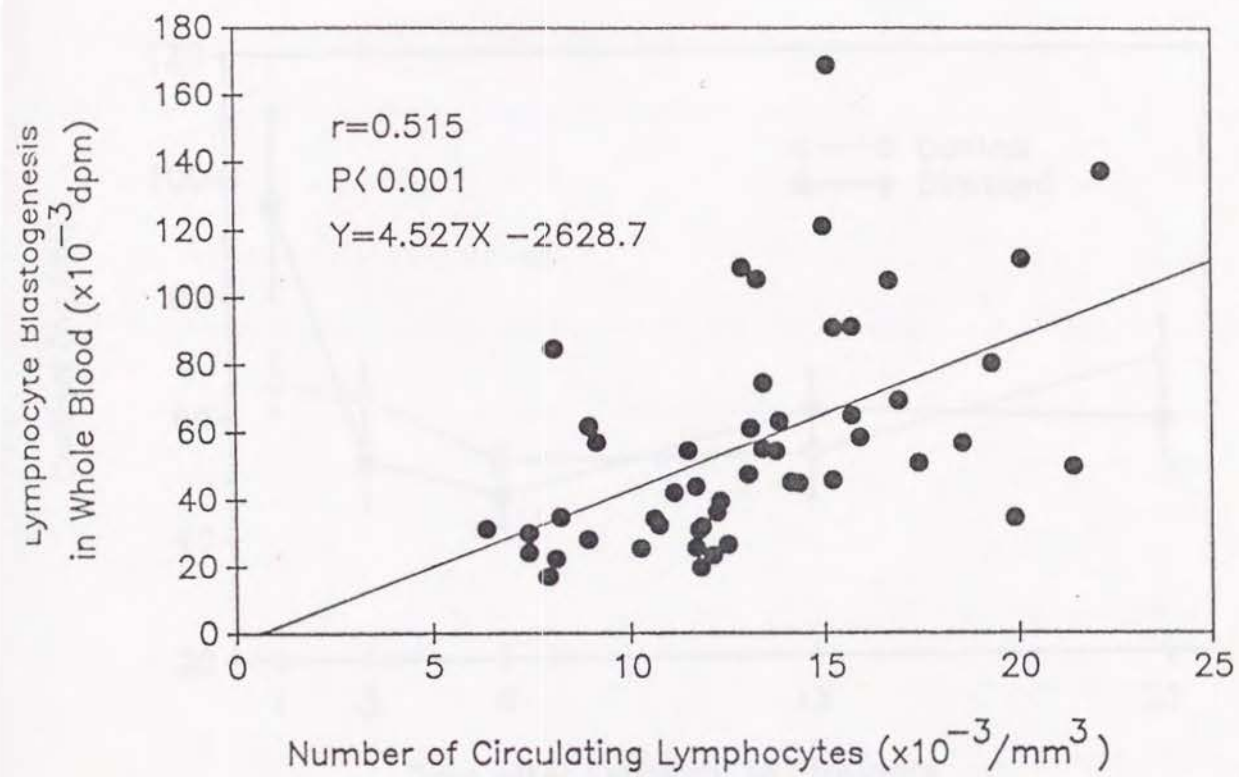


Fig.II-7 Relationship between PHA-induced blastogenesis of lymphocytes in the whole blood culture and the number of circulating lymphocytes of the marketed/transported pigs.

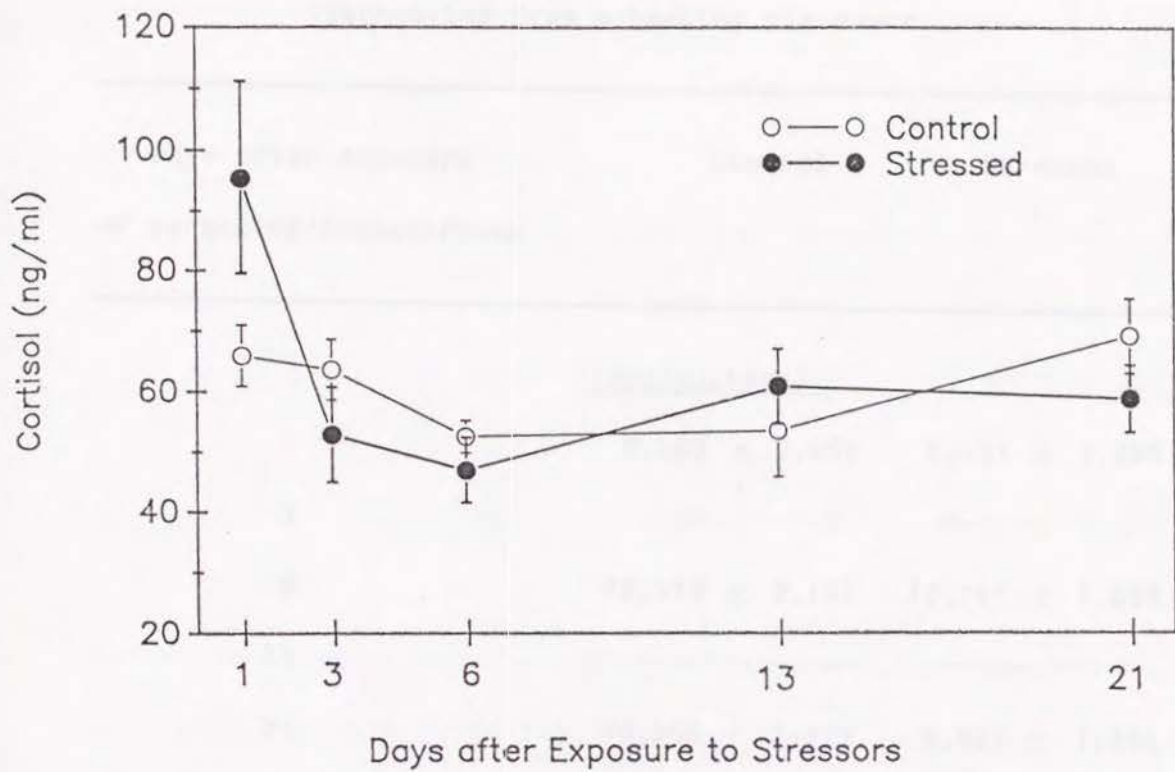


Fig.II-8 Effect of marketing and transporting of feeder pigs on serum concentrations of cortisol. Each value represents the mean  $\pm$  standard error.

Table II-1 Effect of serum from the control and stressed pigs on PHA-induced blastogenesis of isolated peripheral blood lymphocytes from a healthy pig donor.

Days after exposure of marketing/transporting	Control	Stressed
	<u>(dpm/culture)</u>	
1	8,189 ± 2,055	6,191 ± 1,040
3	.....	.....
6	12,519 ± 2,191	10,161 ± 1,699
13	.....	.....
21	10,856 ± 1,973	6,923 ± 1,296

Each value represents the mean ± standard error.

.....: Not determined.

Table II-2 Effect of marketing and transporting of feeder pigs on serum concentrations of albumin (g/dl).

Days after exposure of marketing/transporting	Control	Stressed
1	2.43 ± 0.17	3.31 ± 0.10***
3	2.37 ± 0.17	2.92 ± 0.10*
6	2.36 ± 0.18	2.81 ± 0.10*
13	2.49 ± 0.16	2.38 ± 0.09
21	2.81 ± 0.15	2.34 ± 0.06*

Each value represents the mean ± standard error and the significance of the difference from the corresponding control group is shown P < \*0.05, \*\*\*0.001.

Table II-3 Effect of marketing and transporting of feeder pigs on serum concentrations of globulin (g/dl).

Days after exposure of marketing/transporting	Control	Stressed
1	4.20 ± 0.16	3.06 ± 0.10***
3	4.09 ± 0.17	2.72 ± 0.11***
6	4.14 ± 0.12	3.03 ± 0.11***
13	3.99 ± 0.18	3.25 ± 0.09**
21	4.34 ± 0.19	3.94 ± 0.09*

Each value represents the mean ± standard error and the significance of the difference from the corresponding control group is shown P < \*0.05, \*\*0.01, \*\*\*0.001.



Table II-4 Effect of marketing and transporting of feeder pigs on serum albumin/globulin ratio.

Days after exposure of marketing/transporting	Control	Stressed
1	0.60 ± 0.06	1.09 ± 0.05***
3	0.59 ± 0.05	1.09 ± 0.06***
6	0.58 ± 0.06	0.94 ± 0.05***
13	0.63 ± 0.04	0.74 ± 0.04
21	0.65 ± 0.04	0.60 ± 0.02

Each value represents the mean ± standard error and the significance of the difference from the corresponding control group is shown P<\*\*\*0.001.

Table II-5 Effect of marketing and transporting of feeder pigs on serum concentrations of immunoglobulin G (g/dl).

Days after exposure of marketing/transporting	Control	Stressed
1	1.20 ± 0.13	0.69 ± 0.06 **
3	1.35 ± 0.15	0.53 ± 0.03 ***
6	1.33 ± 0.12	0.74 ± 0.04 ***
13	.....	.....
21	1.47 ± 0.12	0.91 ± 0.10 **

Each value represents the mean ± standard error and the significance of the difference from the corresponding control group is shown P< \*\*0.01, \*\*\*0.001. ....: Not determined.

CHAPTER III

STRESS OF TRANSPORTING PIGS AS MEASURED BY SERUM GLUCOCORTICOIDS  
IN RALATION TO BLASTOGENIC RESPONSE OF LYMPHOCYTES

## ABSTRACT

A study was conducted to evaluate how short-distance and/or short-term period of transportation affect serum glucocorticoids concentration in relation to lymphocyte function. Seven pigs were exposed to transportation by a track for 4 hrs. Seven pigs (controls) remained in home pens. During transportation increased blood neutrophil count with lymphocyte count remained unchanged was observed. Overnight after the exposure of animals to transportation neutrophil count returned to the control level. Phytohemagglutinin (PHA)-induced blastogenic response of whole blood and isolated peripheral blood lymphocytes, as measured for an indicator of lymphocyte function, revealed no significant differences between the control and transported animals. Serum cortisol concentrations prior to transport was  $17.1 \pm 4.0$  ng/ml. A sharp increase to  $55.8 \pm 3.2$  ng/ml occurred 2 hrs after loading, followed by a decrease to  $15.7 \pm 6.6$  ng/ml 18 hrs after unloading. Following several days serum cortisol level in the transported animals lost its circadian rhythm as observed in the control animals.

These results suggest that transportation for several hours is enough to impose stressful stimuli on the pig, but not enough to induce impaired lymphocyte function and/or reduction in the lymphocyte number in the circulating blood.

## INTRODUCTION

Previous study as discussed in Chapter II demonstrated that long-distance transportation including handling and marketing suppressed PHA-induced blastogenic response of whole blood lymphocytes with concomitant reduction in the number of circulating lymphocytes. Of our interests is whether short-distance transportation induce such immunosuppression as observed in the pigs exposed to long-distance transportation.

In human studies, it has been shown that peripheral blood lymphocyte levels have a circadian variation which is due to cyclic changes of plasma levels of adrenal glucocorticoids (18, 83, 85). In animal husbandry stress of transporting animals can be measurable in the serum glucocorticoids (3). An experiment, therefore, was conducted to evaluate if short-distance transportation induce immunosuppression such as reduction in peripheral blood lymphocyte levels in relation to changes in serum cortisol level.

## MATERIALS AND METHODS

### Experimental Animals

Fourteen feeder pigs selected across 14 litters and averaging

32.1 kg were used in this study.

#### Treatment

Indwelling catheters were inserted into the jugular vein of each pig, followed by a 5 to 7 days recovery period. Pigs were then randomly divided into two treatments, transported and control. On d 3 of the experiment, pigs to be transported were loaded into a trailer between 1600-1700 h. The trailer was divided into individual compartments and customized such that catheters would remain intact. Pigs were then transported approximately 402 km between 1700-2100 h. Pigs remained in these pens in an isolated facility. Pens were the same as those housing the animals in the control treatment. Pigs remained in these pens and were provided feed and water *ad libitum* for the following 10 days. Throughout the duration of the experiment, pigs in the control treatment remained in their individual pens with feed and water *ad libitum*.

#### Blood Collection

Blood samples were obtained via indwelling catheters at the following times: d 1, 1500 h; d 2, 0730 h, 1500 h; d 3 (day of transport); d 4, 0730 h (after unloading and moving into individual pens in an isolated facility), 1500 h; d 5-7, 0730 h, 15:00 h; and d 8-9, 0730 h (Fig.III-1).

Ten ml of fresh heparinized blood were used for lymphocyte assays and blood cell counting. The remaining 10 ml were

coagulated and then centrifuged at 1,800 x g for 30 min. Sera were harvested and frozen at -20 °C until used.

#### Reagents and Chemicals

All of the reagents and chemicals used in this study were the same as those used in Chapter I.

#### Isolation of Peripheral Blood Lymphocytes

Procedure applied for isolation of peripheral blood lymphocytes was the same as that described in Chapter II.

#### Isolated Lymphocyte and Whole Blood Culture

Protocols for isolated pig lymphocyte culture and whole blood culture were identical to those described in Chapter II.

#### Leukocyte, Neutrophil, and Lymphocyte Counts

Total leukocyte and lymphocyte counts were determined by the methods described in Chapter II. Total neutrophil count was determined by the similar way to that for total lymphocytes. Namely, total neutrophil count was evaluated based on total leukocyte count and the population of neutrophils estimated from the differential leukocyte count of more than 200 cells on Giemsa-stained slides of whole blood.

#### Serum Cortisol Concentrations

Serum cortisol concentrations were measured by the radioimmunoassay with solid phase technique as described in Chapter I.

All assays were run in duplicate, and the mean value was used.

#### Statistical Analysis

Two-tailed Student's t-test was used to determine the significance of differences between two groups.

## RESULTS

### The number of Circulating Blood Leukocytes, Lymphocytes, and Neutrophils

Effect of exposure of animals to short-distance transportation on the number of leukocytes, lymphocytes, neutrophils are summarized in Figs. III-2, -3 and -4. The number of leukocytes per unit volume of blood showed no significant differences between the treatments throughout the experiment although the number in the transported animals showed a increasing tendency during and immediately after the transportation. The number of lymphocytes also showed no significant differences between the treatments throughout the experiment. In contrast, the number of neutrophils revealed a 40 and 50% increase as compared to that in the corresponding control animals, respectively. As a result of increased number of neutrophils a sharp increase in neutrophil/lymphocyte ratio to  $0.93 \pm 0.15$  and  $0.98 \pm 0.12$  during and immediately after transportation as compared to those ( $0.44 \pm 0.05$  and  $0.57 \pm 0.05$ , respectively) in the corresponding control animals occurred.

### Serum Cortisol Concentrations



Changes in serum cortisol concentration are shown in Fig.III-5. A sharp increase from  $12.7 \pm 3.5$  ng/ml in the control animals to  $55.8 \pm 3.2$  ng/ml in the transported animals during transportation. The higher concentrations of serum cortisol in the transported animals were still observed immediately after and 10.5 hrs after transportation. Then the concentration in the transported animals did not show its cyclic changes as observed in the control animals.

#### Blastogenic Response of Whole Blood and Isolated Peripheral Blood Lymphocytes

Phytohemagglutinin (PHA)-induced blastogenesis of whole blood and isolated lymphocytes, as measured by  $^3\text{H}$ -thymidine uptake, before and immediately after transportation is shown in Table III-1. Neither blastogenesis of whole blood nor isolated lymphocytes showed significant differences between the treatment in both measurement point.

#### DISCUSSION

Stress of movement is measurable in the serum glucocorticoids as previously reported (83). The present study demonstrates that exposure to short-term period of transportation is enough to impose stressful stimuli on young feeder pigs because a sharp increase in serum cortisol concentrations occurred during transportation. However, stress of

transportation such as that applied in this study is not necessarily enough to induce immunosuppression as observed in Chapter II since depressed lymphocyte blastogenesis and/or reduction in the number of circulating blood lymphocytes was not observed in this study. Serum or plasma adrenal glucocorticoids are well known to suppress the cell-mediated immune response which includes depressed lymphocyte blastogenesis and/or reduction in the number of circulating blood lymphocytes (18, 83, 85, 87). Thus it was expected that similar immunosuppression to that observed in the previous study as in Chapter II could occur in this study. One possibility to explain the gap between this study and the previous one is that increased serum glucocorticoid level observed in this study was not enough to regulate the number of lymphocytes. The evidence to support this possibility is that no cyclic variations of the number of lymphocytes was observed although serum cortisol concentration in the control animals showed cyclic changes with the highest concentration of  $52.0 \pm 3.5$  ng/ml which is almost the same level as a sharp increase ( $55.8 \pm 3.2$  ng/ml) obtained in the transported animals. In other words the results of simultaneous measurements of cortisol and lymphocytes in the control animals had no inverse relationship. Thus the question still remains how much cortisol, in amounts small enough to induce changes in serum cortisol within or near the physiological range, is required to alter lymphocyte levels which could also affect blastogenic

response of lymphocyte to mitogen.

CHAPTER III

EXPERIMENTAL DESIGN

Group (1)

Control (1)



CHAPTER III

Experiment (1)

Figures and Tables

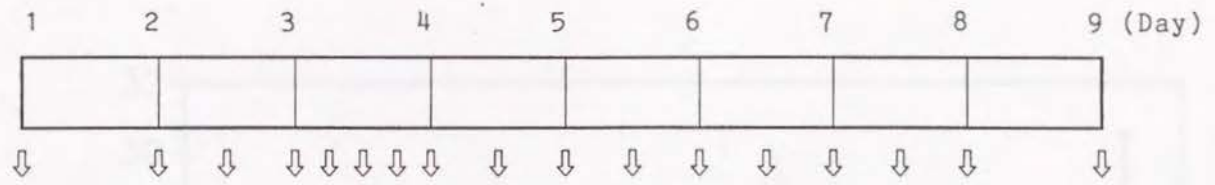


Fig III-1. Experimental results for studying the effect of  
 short light periods of transcription on virus survival.  
 The number of peripheral blood lymphocytes and  
 plating efficiency of lymphocytes is 74%

Legend: 1 - Transcription for 4 hours, 2 - Starving

Groups (N)

Control (7)



Transported (7)

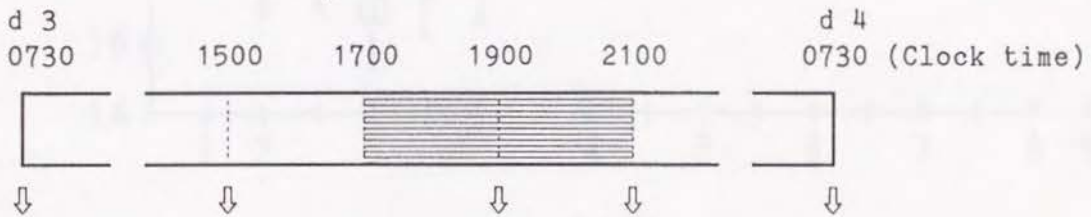
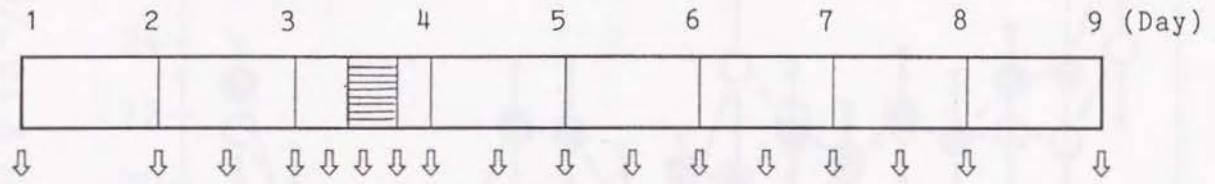



Fig.III-1 Experimental schedule for studying the effect of short-term period of transportation on serum cortisol, the number of peripheral blood lymphocyte, and blastogenic response of lymphocytes to PHA

 : Transportaion for 4 hours    ↓ : Bleeding

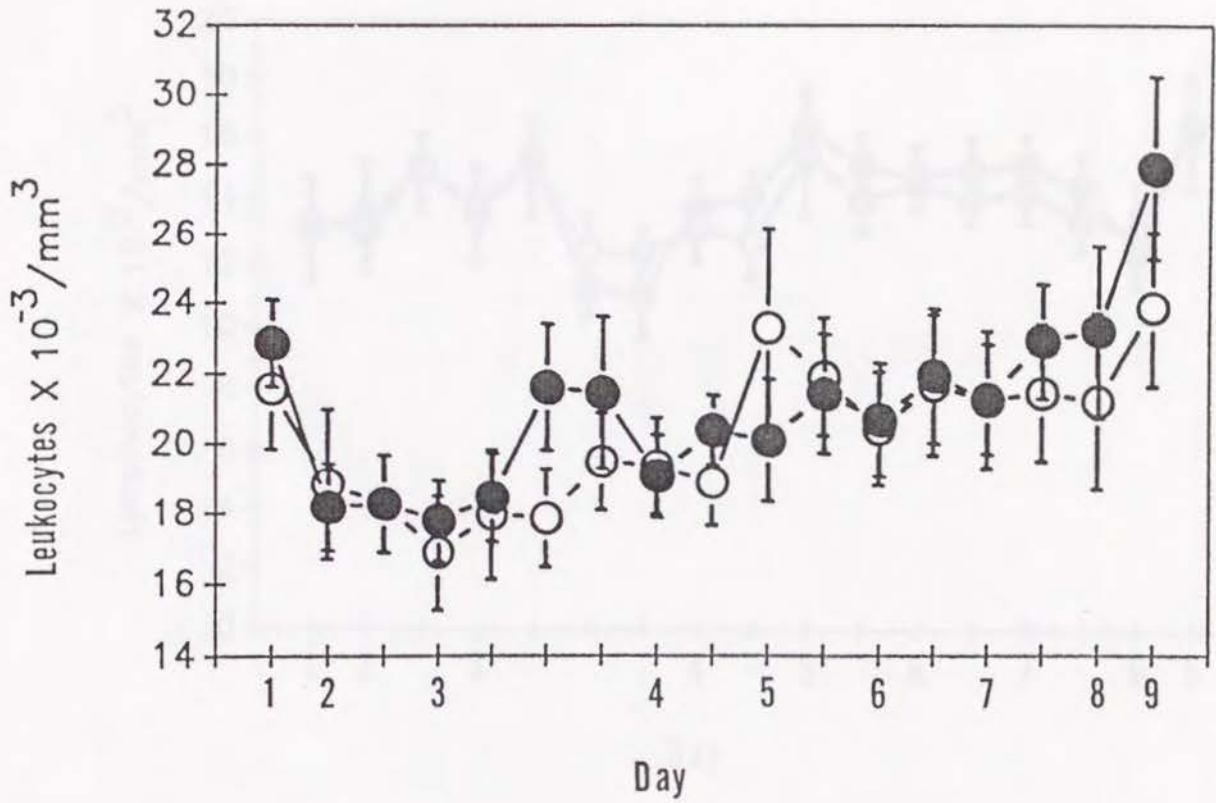


Fig.III-2 Effect of short-term period of transportation on the number of peripheral blood leukocytes in feeder pigs. Each value represents the means and standard error of the control ( O ) or transported ( ● ) group.

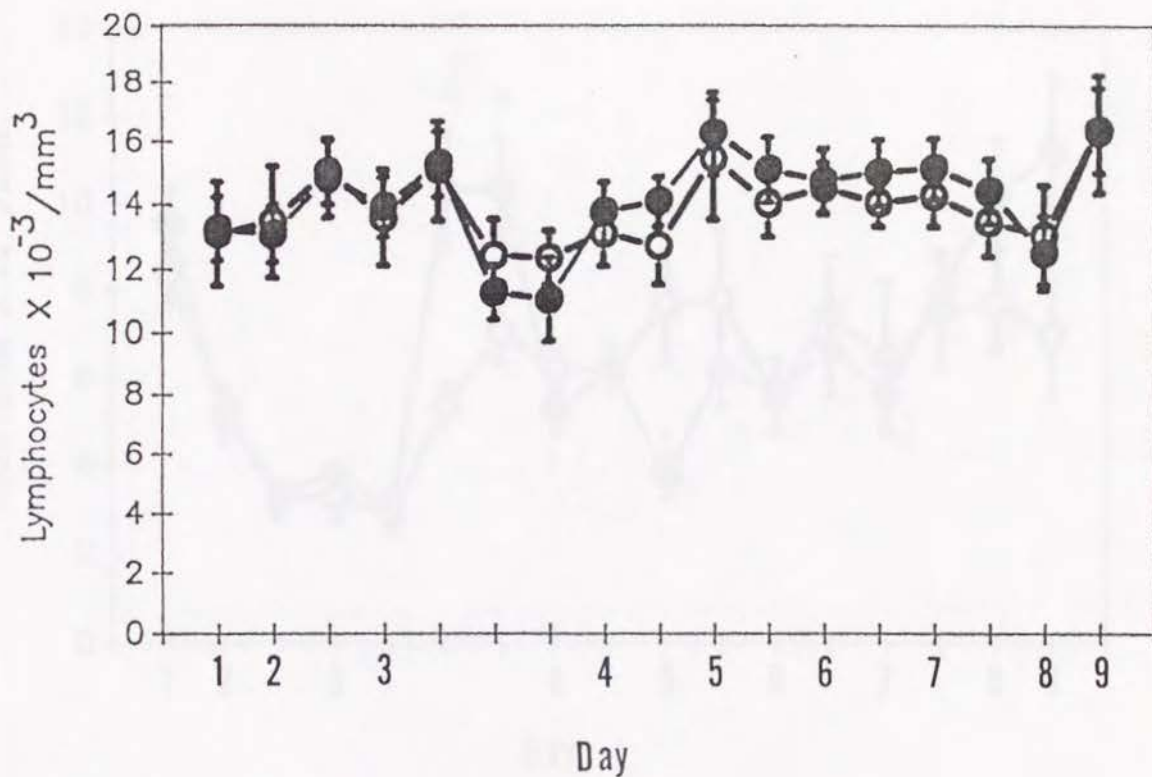


Fig. III-3 Effect of short-term period of transportation on the number of peripheral blood lymphocytes in feeder pigs. Each value represents the means and standard error of the control ( O ) or transported ( ● ) group.

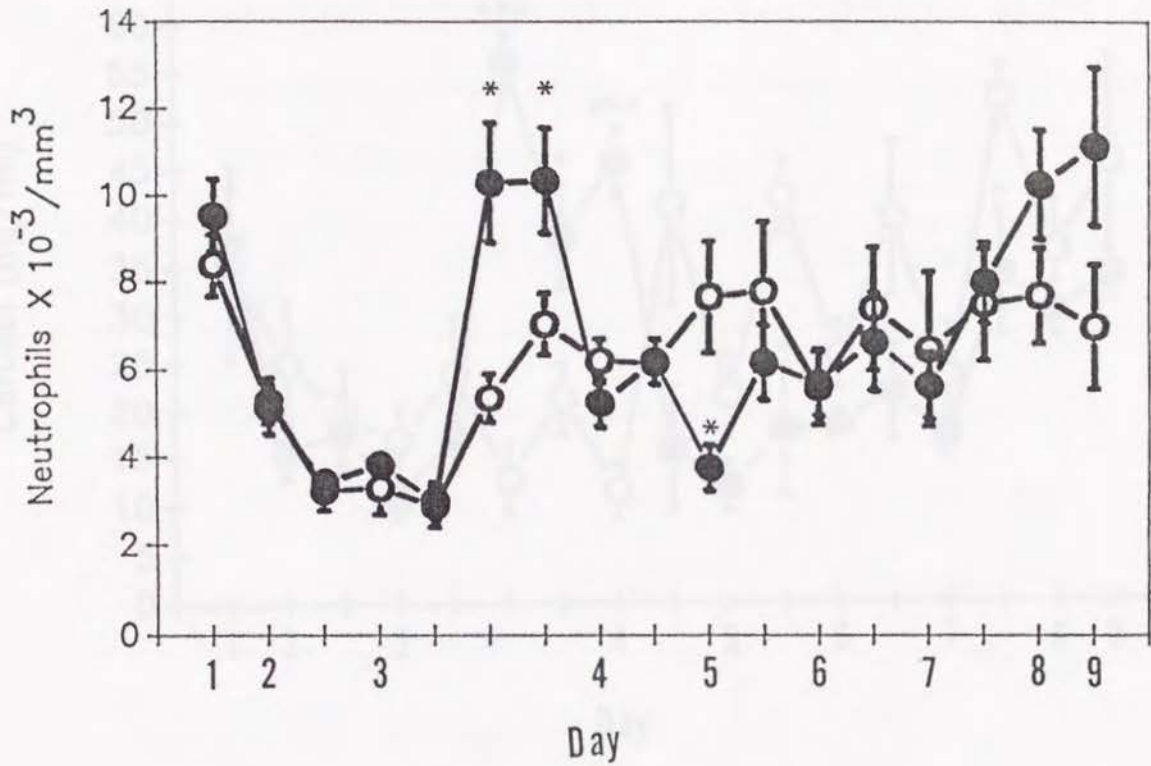


Fig.III-4 Effect of short-term period of transportation on the number of peripheral blood neutrophils in feeder pigs. Each value represents the means and standard error of the control (O) or transported (●) group, and the significance of the difference from the corresponding control group is shown  $P < * 0.05$ .



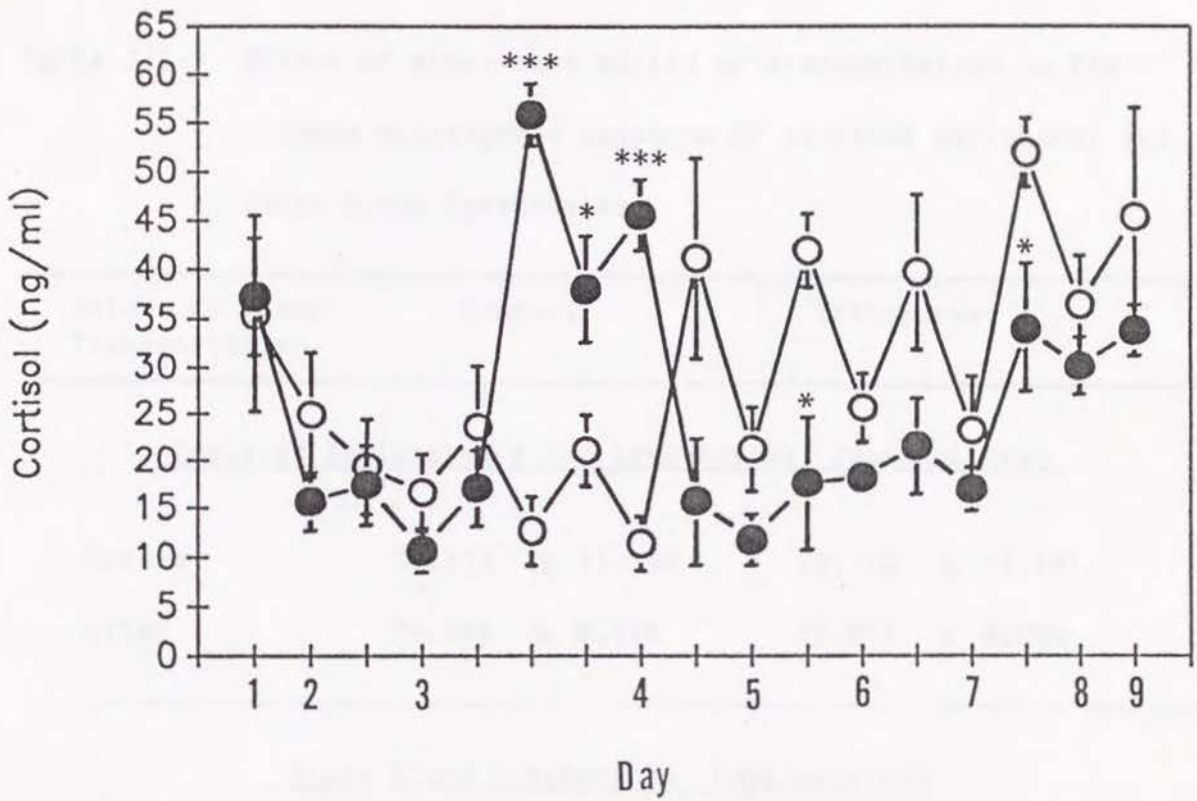


Fig.III-5 Effect of short-term period of transportation on serum concentrations of cortisol in feeder pigs.

Each value represents the means and standard error of the control (○) or transported (●) group, and the significance of the difference from the corresponding control group is shown P < \* 0.05, \*\*\* 0.001.

Table III-1 Effect of short-term period of transportation on PHA-induced blastogenic response of isolated peripheral and whole blood lymphocytes.

Before or After Transportation	Control	Transported
<u>Isolated Peripheral Blood Lymphocytes (dpm/culture)</u>		
Before	77,533 ± 11,590	79,178 ± 11,081
After	74,266 ± 8,726	78,917 ± 8,054
<u>Whole Blood Lymphocytes (dpm/culture)</u>		
Before	71,183 ± 11,509	68,938 ± 8,576
After	67,369 ± 8,364	58,079 ± 14,559

Each value represents the mean ± standard error. Before and after transportation are identical to d2, 7:30 h and d4, 7:30 h as shown in Fig.III-1, respectively.

CHAPTER IV

SUPPRESSED LYMPHOCYTE BLASTOGENESIS IN NEWBORN GOATS SUBJECTED TO  
MATERNAL SEPARATION

## ABSTRACT

Five newborn African Pigmy goats at 1 week of age were used to evaluate if maternal separation affects the cell-mediated immune response of infant ruminants. Animals were isolated from their mothers for 3 days according to the following schedule. First day; 0930-1330, 1730-2130, 0130-0930, Second day; 1230-1530, 1830-2130, 0130-0930, Third day; 1230-1530, 1830-2130, 0130-0830. Blood samples were taken prior to, immediately after, and 4 days after the separation period. Decreased blastogenic responses of peripheral blood lymphocytes to phytohemagglutinin (PHA) and concanavalin A (Con A) were found immediately after the separation period. After 4 days, the responses showed a tendency to restore to pre-separation values. No significant changes were observed in total lymphocyte and neutrophil counts in the circulating blood. The data presented here suggest that maternal separation can produce suppression of the cell-mediated immune response in newborn ruminants.

## INTRODUCTION

Altered immune responses associated with depression, maternal separation and bereavement have been reported in several animal species.

Bartrop et al.(5) and Schleifer et al.(70). found that bereaved spouses had significantly lower lymphocyte blastogenic response to PHA and Con A with no associated changes in absolute T and B cell numbers. Significantly lowered lymphocyte function was also noted in patients hospitalized for major depressive disorder (71). In prematurely separated mice pups, Michaut et al.(53) demonstrated an impaired humoral immune response. In prematurely separated rat pups, Ackerman et al.(1) reported depressed lymphocyte function. Maternal separation in infant monkeys has been used to evaluate if separation and loss affect the immune status. Laudenslager et al.(44) reported that a 14 day separation of infant bonnet monkeys produced suppression of mitogen-induced lymphocyte blastogenesis. Moderately decreased immunoglobulin levels were also reported in rhesus monkey infants subjected to maternal separation (69).

In animal husbandry, "weaning" is the period in the production system when the infant animals are removed from their mothers. Thus transition period involves acute changes in nutrition, and thermal and social environments which possibly produce psychological disturbance for the infant animals. Little information is available on the effect of maternal separation on the immune status of infant ruminants. The objective of this study was to examine if maternal separation alters the immune status of infant ruminants as demonstrated by lymphocyte response to various mitogens.

## MATERIALS AND METHODS

### Experimental Animals

Five newborn African Pigmy goats at 1 week of age were used in this study.

### Treatment

Infant goats were removed from their mothers and placed in different cages from which they could not see their mothers. Isolation was for 3 days during the following time periods; d 1: 0930-1330 h, 1730-2130 h, 0130-0930 h; d 2: 1230-1530 h, 1830-2130 h, 0130-0930 h; d 3: 1230-1530 h, 1830-2130 h, 0130-0830 h (See Fig.IV-1 for experimental schedule). During separation no nutritional supplement was provided for the animals.

### Blood Collection

Heparinized venous blood was obtained by jugular venipuncture prior to, immediately after, and 4 days after the separation, and lymphocyte responses to various mitogens and blood cell counts were determined.

### Reagents and Chemicals

All of the reagents and chemicals used in this study were the same as those used in Chapter I.

### Isolation of Peripheral Blood Lymphocytes

Procedure applied for isolation of peripheral blood lymphocytes

was basically the same as that for sheep lymphocytes as described in Chapter I.

#### Isolated Lymphocyte and Whole Blood Culture

Protocol for separated goat lymphocyte culture was identical to that for sheep lymphocyte culture as described in Chapter I. In brief, peripheral blood lymphocyte suspension ( $1 \times 10^5$  cells/well) was cultured in quadruplicate in 96 well flat-bottomed microplates (Falcon 3027, Oxnard, CA). Each well contained  $50 \mu\text{l}$  of cell suspension,  $50 \mu\text{l}$  of mitogen (phytohemagglutinin ; PHA or concanavalin A ; Con A) solution or medium (RPMI 1640) alone (controls), and  $20 \mu\text{l}$  of fetal bovine serum. Preliminary experiments in our laboratory indicated that optimal dilution of PHA of this lot was 1:1,400, and optimal concentration of Con A of this lot was  $14 \mu\text{g/ml}$ . Protocol for whole blood culture was identical to the pig whole blood culture as described in Chapter II. In brief, diluted heparinized whole blood samples in RPMI 1640 (1:10) were cultured in the same way as in the isolated lymphocytes. That is, each well contained  $50 \mu\text{l}$  of diluted whole blood,  $50 \mu\text{l}$  of mitogen solution or medium alone, and  $20 \mu\text{l}$  of fetal bovine serum. Preliminary experiments in our laboratory indicated that optimal dilution of PHA of this lot was 1:1,400, and optimal concentration of Con A of this lot was  $28 \mu\text{g/ml}$ . Both the isolated lymphocytes and whole blood were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ /95% air. After

72 hr of incubation, 1  $\mu$ Ci of  $^3\text{H}$ -thymidine dissolved in RPMI 1640 was added to the culture and the cells were incubated for an additional 16 hr. The cells were harvested onto glass fiber filter paper by using a semiautomatic cell harvester (Skatron Inc., Sterling, VA) with physiological saline as a washing solution. Filter paper discs were air-dried and  $^3\text{H}$ -thymidine incorporation was determined by liquid scintillation counting. Background dpm by unstimulated cultures were subtracted from dpm obtained from stimulated cultures. The mean values of quadruplicate cultures were used.

#### Erythrocyte, Leukocyte, Lymphocyte, and Neutrophil Count

Total erythrocyte and leukocyte counts were determined by using a Coulter counter (Model ZBI, Coulter Electronics Inc., Hialeah, FL) based on the principle as described in Chapter II. Total lymphocyte and neutrophil counts were determined based on total leukocyte count and the proportion of lymphocytes and neutrophils estimated from the differential leukocyte count of more than 200 cells on Giemsa-stained slides of whole blood.

#### Statistical Analysis

Two-tailed Student's t-test was used to determine the significance of the difference.



## RESULTS

### Isolated lymphocyte Blastogenesis

The effect of maternal separation on blastogenic response of isolated peripheral blood lymphocytes in newborn animals is summarized in Table IV-1. Immediately after the separation period, the lymphocyte response to PHA and Con A revealed a 72% and 57% reduction of pre-separation values, respectively. Four days after the separation period, the response to PHA and Con A restored to 93% and 74% of pre-separation values, respectively.

### Lymphocyte Blastogenesis in Whole Blood

Lymphocyte response to mitogen stimulations in whole blood culture is summarized in Table IV-2. While no significant changes were found in the response to PHA, Con A-induced blastogenesis showed a 69% reduction of pre-separation value immediately after the separation period. Four days after the isolation period, the response showed a tendency to increase although the value was still lower by 46% of pre-separation value.

### Total Erythrocyte, Leukocyte, Lymphocyte, and Neutrophil Count

Total blood cell counts in experimental animals are summarized in Table IV-3. Total erythrocyte, leukocyte, lymphocyte, and neutrophil counts ranged  $5-8 \times 10^6$  cells/mm<sup>3</sup>,  $7-15 \times 10^3$  cells/mm<sup>3</sup>,  $5-11 \times 10^3$  cells/mm<sup>3</sup>,  $1-8 \times 10^3$  cells/mm<sup>3</sup>, respectively, throughout

the experiment. No clear effects of maternal separation on total blood cell counts in newborn goats were observed.

## DISCUSSION

The data presented here demonstrated that in infant ruminants maternal separation can suppress blastogenic response of peripheral blood lymphocytes stimulated by T-cell mitogens with no associated changes in absolute lymphocyte numbers. The mechanisms underlying the suppressive effect of maternal separation on lymphocyte function in newborn goats were not investigated in this study. However, one possibility is that separation causes psychological depression in infants which in turn stimulates the central nervous system to modulate the immune response. For instance, stimulation of the hypothalamic-pituitary-adrenal axis results in increased glucocorticoid secretions from the adrenal cortex (57), which in turn suppresses the immune system (15, 21). Another possibility is that short-term nutritional deprivation may alter the immune response. In this study separated infant Pygmy goats were also subjected to nutritional deprivation during separation although they were allowed to suckle between each separation. The effect of milk deprivation on the immune status

of neonate animals requires further investigation.

In conclusion, in animal husbandry, especially dairy husbandry, maternal separation of the newborns from those lactating mothers is a stressful period of the production cycle. The data from this study, which demonstrated a suppression of immune response in the infant following maternal separation, would suggest that the infants, following maternal separation, would be susceptible to diseases and infection.



CHAPTER IV

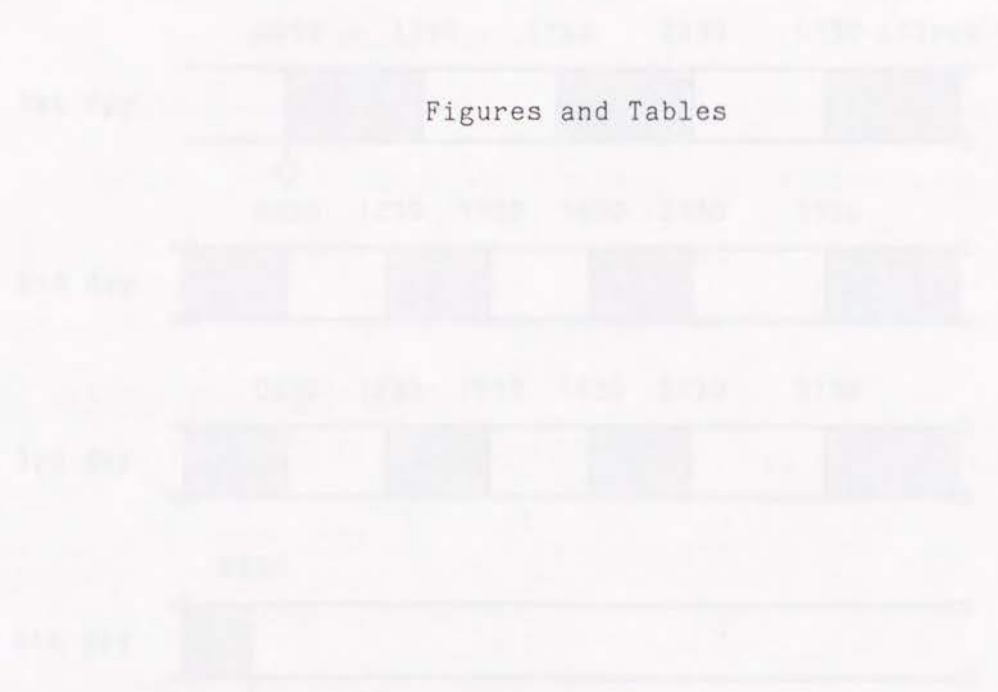


Fig. IV-1. Individual schedule for studying the effects of natural separation on reproduction of collared lemmings during the winter of 1950-1954.

■ Separation from objects      □ No handling

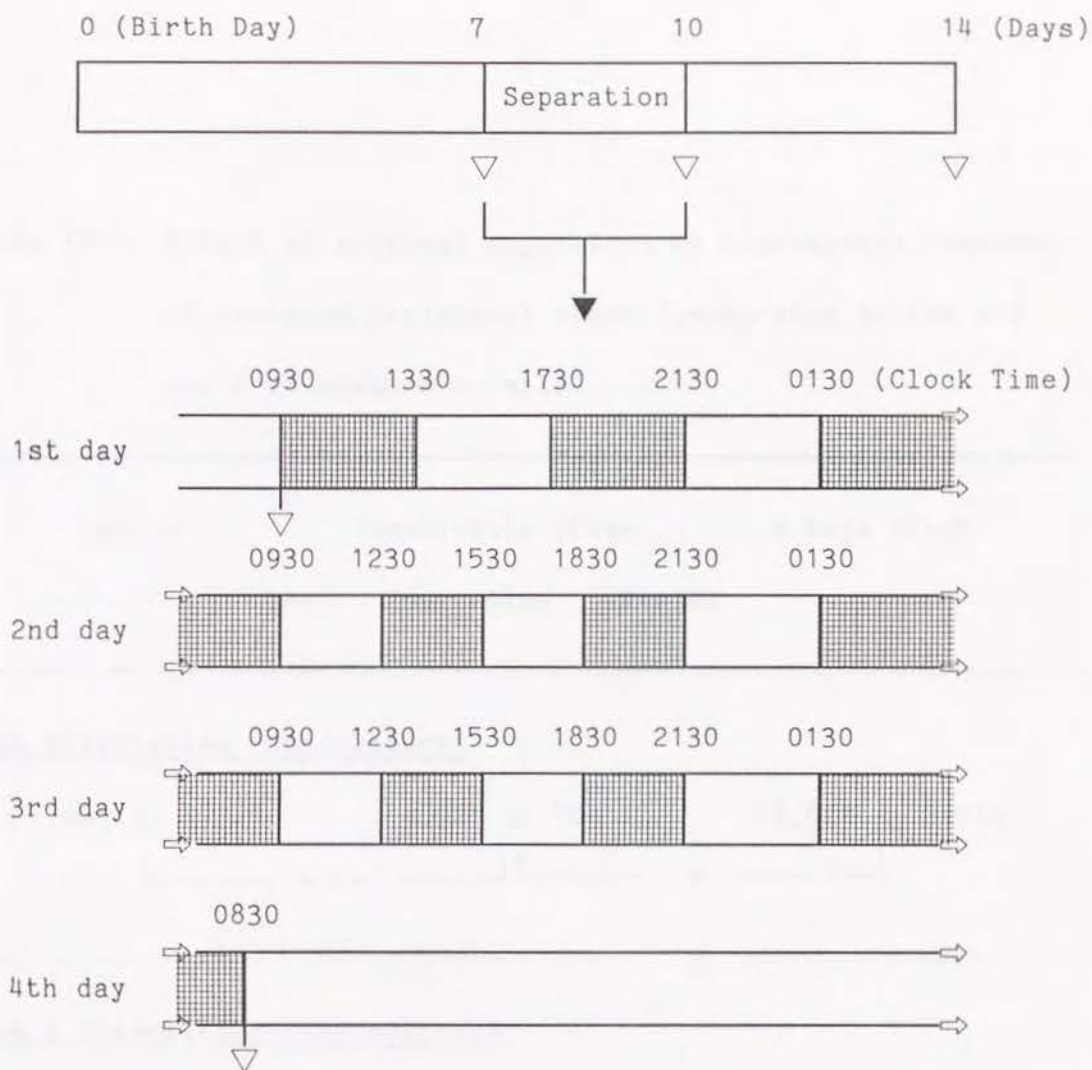


Fig.IV-1 Experimental schedule for studying the effect of maternal separation on expressions of cell-mediated immune response in newborn African Pygmy goats


 : Separation from mothers                      ▽ : Bleeding

Table IV-1 Effect of maternal separation on blastogenic response of isolated peripheral blood lymphocytes to PHA and Con A in newborn animals.

Before	Immediately After the Separation	4 Days After Period
<u>PHA Stimulation (dpm/culture)</u>		
17,605 ± 1,318	4,929 ± 704	15,822 ± 3,540
<u>Con A Stimulation (dpm/culture)</u>		
44,550 ± 2,333	19,166 ± 3,355	32,821 ± 6,559

Each value represents the mean ± standard error, and the significance of the difference between the treatments is shown P < \*0.05, \*\*\*0.001.

Table IV-2 Effect of maternal separation on mitogen-induced lymphocyte blastogenesis of whole blood culture in newborn animals.

Before the	Immediately After the Separation	4 Days After Period
<u>PHA Stimulation (dpm/culture)</u>		
3,176 ± 480	2,432 ± 1,114	2,309 ± 631
<u>Con A Stimulation (dpm/culture)</u>		
20,173 ± 6,303	6,217 ± 1,326	10,812 ± 2,063
<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="border-left: 1px solid black; border-right: 1px solid black; width: 100px; height: 15px;"></div> <span style="margin: 0 10px;">*</span> <div style="border-left: 1px solid black; border-right: 1px solid black; width: 100px; height: 15px;"></div> </div>		

Each Value represents the mean ± standard error.

Table IV-3 Effect of maternal separation on total blood cell counts in newborn animals.

Before	Immediately After	4 Days After
the	Separation	Period
<u>Erythrocyte (x 10<sup>6</sup> cells/mm<sup>3</sup>)</u>		
6.65 ± 0.47	7.17 ± 0.47	6.08 ± 0.53
<u>Leukocyte ( /mm<sup>3</sup>)</u>		
11,749 ± 963	10,381 ± 1,027	10,307 ± 1,544
<u>Lymphocyte ( /mm<sup>3</sup>)</u>		
6,147 ± 516	6,447 ± 278	7,070 ± 1,201
<u>Neutrophil ( /mm<sup>3</sup>)</u>		
4,835 ± 960	3,048 ± 902	2,539 ± 636

Each Value represents the mean ± standard error.



## THESIS SUMMARY

In the present paper, if and how stressful environmental conditions affect the immune status of domestic animals was examined by determining lymphocyte functions. Stressful conditions applied here were 1) Environmental heat, 2) Transportation and/or marketing process, and 3) Maternal separation.

The study in which environmental heat-stress was imposed on pre- and post-partal sheep clearly demonstrated that the cell-mediated immune function was suppressed by chronic high ambient temperature since reduced mitogen-induced blastogenic response of peripheral blood lymphocytes was observed. Further study suggests that this immunosuppression in heat-stressed sheep is in part mediated by altered serum factor(s) that induce suppressed T-cell function. This is supported by the evidence that serum from heat-stressed animals suppressed the blastogenic response of lymphocytes obtained from healthy sheep.

In case of exposure of young pigs to long-term period of transportation, no significant differences in responses of isolated peripheral blood lymphocytes to T-cell mitogen phytohemagglutinin (PHA) were found. In contrast, responses of lymphocytes in whole blood culture to PHA revealed significant reduction after exposure to stressors, and the degree of suppression gradually lessened with day.

Since changes in the number of peripheral blood lymphocytes paralleled changes of the response of lymphocytes in whole blood culture, such reduction is attributable to decreased number but not function of circulating blood lymphocytes. The data, therefore, suggest that the cell-mediated immune function of young pigs is suppressed for several days after exposure of multi-stressors including handling, marketing and long-term period of transportation.

In further determining if short-term period of transportation affect the lymphocyte status in young pigs as is the case with long-term period of transportation, investigations with blastogenic responses of isolated and whole blood lymphocytes to PHA in pigs transported for 6 hrs showed no significant differences from those in control pigs. A sharp increase in serum cortisol concentration was, however, observed in animals during transportation. Therefore, it is suggested that transportation for several hours is stressful to young pigs as measured by serum glucocorticoids, but is not a stressor of sufficient intensity and length enough to impose impaired lymphocyte function and/or reduction in the lymphocyte number on young pigs.

In the study where the effect of maternal separation on the immune status of newborn goats was examined, suppressed blastogenic responses of peripheral blood lymphocytes to T-cell mitogens were found immediately after the isolation period followed by a tendency to restore to preisolation values after 4 days. The data suggest that maternal

separation can produce suppression of the cell-mediated immune response in newborn ruminants.

Taking all the data presented here into consideration, stressful environmental conditions could induce immunosuppression as indicated by impaired lymphocyte function and/or reduction in the lymphocyte population. In other words, stressful environmental stimuli could alter host resistance which results in the increased susceptibility of animals to infectious diseases. The idea that environmental stressors are involved in the etiology of livestock diseases provide a key to understand the physiological basis of disease-environment interaction in animal husbandry.

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