Changes in Lymphocyte Blastogenic Response of Mares during the Perinatal Period

Yasuyuki SANADA, Hiroshi NODA, and Hajime NAGAHATA

Department of Animal Health, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan

(Received 3 April 1989/Accepted 27 December 1989)

ABSTRACT. A fluorometric assay was applied to evaluate blastogenesis of equine lymphocytes. Optimal culture conditions were as follows; concentrations of phytohaemagglutinin-P (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) were 1 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, respectively, when 5×10^5 lymphocytes were incubated with culture medium containing 20% pooled horse serum (PHS) for 120 hours. The relative mean stimulation index of healthy non-pregnant mares were 5.107 ± 0.323 ($M \pm SE$) with PHA, 4.019 ± 0.183 with Con A and 3.610 ± 0.131 with PWM. Sequentially the blastogenic responses of lymphocytes from twenty mares were observed during various stages of the perinatal period. Response decreased gradually before parturition was lowest at the time of parturition (PHA: 1.923 ± 0.174 , Con A: 1.698 ± 0.206 and PWM: 1.706 ± 0.177), and then increased gradually after parturition towards non-pregnant levels.—**KEY WORDS:** blastogenesis, equine lymphocyte, ethidium bromide, fluorometric assay.

Jpn. J. Vet. Sci. 52(3): 455–460, 1990

Lymphocyte blastogenic assays have been widely used to determine lymphocyte responses to mitogens. Fluorometric assays using ethidium bromide have been applied to determine blastogenic responses of lymphocytes from cows [10] and pigs [5]. But there are only a few reports concerning equine lymphocyte blastogenesis evaluated by fluorometric assay [11, 14]. Further studies are necessary to standardize culture conditions for blastogenesis.

The purpose of this study was to apply fluorometric assay for the evaluation of equine lymphocyte blastogenesis, and to determine the blastogenic responses of lymphocytes from mares in the perinatal period.

MATERIALS AND METHODS

Animals: Ten clinically healthy mature horses (thoroughbred) were used to determine optimal blastogenic assay conditions. Twenty mares (thoroughbred) were used to observe the pattern of blastogenic response

during the perinatal period (Seven mares were observed continuously, 13 mares were observed on 1–3 points during the perinatal period).

Separation fluid: Ficoll- a sucrose polymer (Ficoll 400, Pharmacia) and Conray- sodium iothalamate (Conray 400, Daiichi Pharmaceutical Co.) were used. The separation fluid was prepared by mixing 9% Ficoll with 33.4% Conray, to make gradients with a density of 1.079.

Separation of lymphocytes: Peripheral blood was collected from the jugular vein and placed into 10 ml syringes containing heparin (20 units per ml of blood). Leucocyte-rich plasma was removed after the blood had been allowed to settle under gravity for 40 min, and used for the separation of lymphocytes [3]. Six milliliters of diluted plasma (1:1) was layered carefully over 3 ml of the Ficoll-Conray gradient and centrifuged at 300 G for 20 min at 20°C. After centrifugation, the mononuclear cells layered in the interface were carefully col-

lected with a Pasteur pipette and transferred to a 10 ml centrifuge tube. The cells were suspended in phosphate buffered saline (PBS) and washed twice. After the final washing, the cells were resuspended in culture medium to give 1×10^6 cells/ml.

Culture medium: RPMI 1640 culture medium supplemented with 25mM Hepes buffer, penicillin (200 IU/ml), streptomycin (200 μ g/ml) (Gibco laboratories) and heat-inactivated fetal calf serum (FCS) (Gibco laboratories) or pooled horse serum (PHS) was used.

Mitogens: Phytohaemagglutinin-P (PHA) (Difco laboratories), Concanavalin A (Con A) (Miles laboratories) and pokeweed mitogen (PWM) (P-L Biochemicals) were used.

Lymphocyte stimulation assay: Five hundred microliters of the lymphocyte suspension was placed into each well of a 24 flat bottomed tissue culture plate (Falcon 3047) and 500 μ l of mitogen was added. The culture were set up in triplicate and incubated at 37°C in humidified 5% CO₂.

Fluorometry of the cultured lymphocytes: Fluorometric microassay of DNA in the cultured lymphocytes was performed according to the method described previously [10]. The cultured lymphocytes were harvested from culture wells using a Pasteur pipette and transferred into centrifuge tubes. After centrifuging at 400 g for 10 min, the supernatant was carefully discarded. Two milliliters of 0.015% sodium laurylsulfate (SDS) (Nakarai Chemical) was added to solubilize the lymphocytes and mixed thoroughly. After 30 min at ambient temperature, 2 ml of 8 μ g/ml ethidium bromide (EB) (Aldrich) was added and mixed. Fluorescence intensity (FI) was measured at an excitation wavelength of 538 nm and an emission wavelength of 586 nm using a spectro fluorophotometer (Shimadzu RF540, Shimadzu).

The results were expressed by a stimula-

tion index (SI). SI was calculated using the following formula: $SI = A - C / B - C$, where as: A is mean FI with mitogen; B is mean FI without mitogen; C is background FI.

RESULTS

The effects of horse serum, mitogen concentration and incubation time on equine lymphocyte blastogenesis were determined. When lymphocytes were stimulated with PHA, Con A and PWM, SIs were higher with PHS compared to with FCS, and higher with 20% PHS compared to with 10% PHS (Fig. 1).

Optimal stimulation with PHA, Con A and PWM occurred at dose levels of 1 μ g/ml, 40 μ g/ml and 10 μ g/ml, respectively (Fig. 2). Optimal incubation time was determined comparing incubation times of 48, 72, 96, 120, and 144 hours after incubation of the culture mixtures with optimal concentrations of each mitogen. The optimal time for incubation which discriminate each blastogenic response was found to be 120 hours (Fig. 1).

The relative mean SIs of stimulated lymphocytes from healthy non-pregnant mares were: 5.107 ± 0.323 (mean $\pm$ SE) with PHA, 4.019 ± 0.183 with Con A and 3.610 ± 0.131 with PWM. The blastogenic responses of lymphocytes collected from mares right before to after parturition, were significantly lower with these three mitogens ($p < 0.01$) compared with non-pregnant mares (Table 1). In order to evaluate immunological responses during the perinatal period, the blastogenic response of lymphocytes collected from twenty mares 28 days before parturition to 53 days after parturition were evaluated. Generally, the response decreased gradually before parturition, was lowest at the time of parturition (PHA: 1.923 ± 0.174 , Con A: 1.698 ± 0.206 and PWM: 1.706 ± 0.177), and then increased gradually after parturition towards non-

Fig. 1. Relationship between stimulation index and incubation time, using different sera and different concentrations of sera.

Mean $\pm$ S.E.

PHS: Pooled horse serum —●— 10% —○— 20%

FCS: Fetal calf serum ---●--- 10% ---○--- 20%

Fig. 2. Stimulation of peripheral lymphocytes from 4 healthy mature horses with various concentrations of PHA, Con A and PWM.

Table 1. A comparison of blastogenic response SI of lymphocytes from mares right before to after parturition and non-pregnant mares

Mares	PHA	Con A	PWM
Mares 1 day before to 1 day after parturition	1.923 $\pm$ 0.174 ^{a)} (n=9) ^{b)}	1.698 $\pm$ 0.206 (n=6)	1.706 $\pm$ 0.177 (n=6)
Non-pregnant mares	5.107 $\pm$ 0.323 (n=10)	4.019 $\pm$ 0.183 (n=7)	3.610 $\pm$ 0.131 (n=10)

* Significant difference $P < 0.01$

a) Mean $\pm$ S.E. of each group.

b) Number of animals used.

Fig. 3. Lymphocyte blastogenic response of mares during the perinatal period.

PHA —●—
 Con A ---●---
 PWM ···●···
 n=4-16 Mean±S.E.

pregnant levels (Fig. 3). Furthermore, the patterns of change in lymphocyte blastogenic response were compared among seven of those mares, which investigated sequentially during perinatal period. Although sequential changes in the response differed with the individual horses, they could be divided into three groups and they showed almost the same pattern to the three mitogens in each horse (Figs. 4-6).

DISCUSSION

Assay of blastogenic response of lymphocytes using ethidium bromide is safer, more practical and simpler than the tritiated thymidine up take assay, as confirmed in previous studies [5, 9, 14]. In this study, the assay of lymphocyte blastogenic response using ethidium bromide was applied to equine lymphocytes.

FCS or pooled homologous serum is commonly used for blastogenesis of lymphocytes [7]. FCS or PHS, of concentrations of 10 or 20%, is used extensively [12, 13, 15]. In our study, PHS was used at a 20%

Fig. 4. The SIs of three groups, grouped according to their pattern.

Group 1: SI decreases early before parturition and increases late after parturition.

Group 2: SI decreases late before parturition and increases late after parturition.

Group 3: SI decreases early before parturition and increases early after parturition.

Letters A-G represent the blastogenic response of lymphocytes from individual mares.

Fig. 5. The SIs of three groups, grouped according to their pattern.

Group 1: SI decreases early before parturition and increases late after parturition.

Group 2: SI decreases late before parturition and increases late after parturition.

Group 3: SI decreases early before parturition and increases early after parturition.

Letters A-G represent the blastogenic response of lymphocytes from individual mares.

Fig. 6. The SIs of three groups, grouped according to their pattern.

Group 1: SI decreases early before parturition and increases late after parturition.

Group 2: SI decreases late before parturition and increases late after parturition.

Group 3: SI decreases early before parturition and increases early after parturition.

Letters A-G represent the blastogenic response of lymphocytes from individual mares.

concentration (Fig. 1). The optimal time for incubation which discriminate each blastogenic response for the horse was found to be 120 hours (Fig. 1), but optimal incubation time for the cow is 72 hours. Optimal concentrations of PHA, Con A and PWM were 1 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, respectively (Fig. 2), but optimal concentration of Con A for the blastogenic response of equine lymphocytes was 4 to 8 times higher than that of the cow [10]. The reason for this phenomenon is unclear, but it may be related to the theory that the sensitivity of the receptors to mitogens on lymphocyte membranes are different depending on the species, as suggested by Tajima *et al.* [14]. In several studies, the blastogenic response of equine lymphocytes to PHA and Con A was higher than that to PWM [1, 7, 8], and the present study showed also similar responses.

The results suggested that the immunoreaction of horses was depressed by the stress which occur during the perinatal period. Blastogenic response of lymphocytes to PHA, Con A and PWM has been demonstrated to decrease in pregnant women and cows [2, 4, 6]. The present study confirmed that the blastogenic response of equine lymphocytes decreases during the perinatal period too.

It is well known that steroid hormones such as progesterone, estrogen and cortisol suppress blastogenic response. We reported that high concentration of cortisol was observed in peripheral blood during perinatal period using sera collected from the same mares with the present study (Nakazawa *et al.* Unpublished data). On the basis of the fact mentioned above, it is suggested that cortisol is one of the major factors which suppress blastogenic response of lympho-

cytes [7].

Evaluation of the blastogenic response of equine lymphocytes by fluorometric assay was considered to be an useful method to obtain information on immunological response.

REFERENCES

1. Banks, K. L. and Henson, J. B. 1973. Quantitation of immunoglobulin-bearing lymphocyte and lymphocyte response to mitogen in horses persistently infected by equine infectious anemia virus. *Infect. Immun.* 8: 679-682.
2. Brunham, R. C., Martin, D. H., Hubbard, T. D., Chochou, Kuo, Critchlow, C. W., Cles, L. D., Eschenbach, D. A., and Holmes, K. K. 1983. Depression of the lymphocyte transformation response to microbial antigens and phytohaemagglutinin during pregnancy. *J. Clin. Invest.* 72: 1629-1638.
3. Dixon, J. B. and Allan, D. 1978. The 'normal range' and precision of phytohaemagglutinin-induced equine lymphocyte transformation in vitro. *Res. Vet. Sci.* 24: 87-91.
4. Fujisaki, S., Mori, N., Sasaki, T., and Maeyama, M. 1979. Cell-mediated immunity in human pregnancy. Changes in lymphocyte reactivity during pregnancy and post-partum. *Microbiol. Immunol.* 23: 899-907.
5. Inoue, T., Tsukamoto, H., Goto, N., and Matsuura, K. 1987. Application of ethidium bromide fluorescence assay to measurement of blastogenesis of swine lymphocytes. *Jpn. J. Vet. Sci.* 49: 681-686.
6. Ishikawa, H. 1987. Observation of lymphocyte function in perinatal cows and neonatal calves. *Jpn. J. Vet. Sci.* 49: 469-475.
7. Kristensen, F., Kristensen, B., and Lazary, S. 1982. The lymphocyte stimulation test in veterinary immunology. *Vet. Immuno. Immunopathol.* 3: 203-277.
8. Lazary, S., de Weck, A. L., Gerber, H., Schatzmann, U., and Straub, R. 1973. Characteristics of the in vitro stimulation of horse leucocytes by phytohemagglutinin and antigen. *Z. Immun. Forsch.* 145: 364-375.
9. Nagahata, H., Noda, H., and Abe, T. 1985. Evaluation of bovine lymphocyte blastogenic response by fluorometric DNA synthesis assay. *Jpn. J. Vet. Sci.* 48: 23-28.
10. Nagahata, H., Yokota, H., and Noda, H. 1984. A simple fluorometric microassay of DNA in mononuclear cell preparations using ethidium bromide. *Jpn. J. Clin. Chem.* 13: 109-113.
11. Oikawa, M., Ohishi, H., Yoshihara, T., Kaneko, M., and Urana, K. 1988. Equine lymphocyte blastogenic assay using the ethidium bromide fluorescence technique. *Bull. Equine Res. Inst.* 25: 20-24.
12. Peters, G. J. and Veerkamp, J. H. 1982. Stimulation by phytohaemagglutinin of peripheral blood lymphocytes from horses, pig, sheep and man. *Vet. Immuno. Immunopathol.* 3: 295-300.
13. Prescott, J. F., Ogilvie, T. H., and Markham, R. J. F. 1980. Lymphocyte immunostimulation in the diagnosis of corynebacterium equi pneumonia of foals. *Am. J. Vet. Res.* 41: 2073-2075.
14. Tajima, M., Fujinaga, T., Koike, T., Okamoto, Y., and Otomo, K. 1987. Assay for equine peripheral blood lymphocytes blastogenic response using ethidium bromide. *Jpn. J. Vet. Sci.* 49: 567-570.
15. Valpotic, Jr., Gerencer, M., Kastelan, M., Kastelan, A., and Basic, I. 1982. Equine lymphocyte transformation induced by mitogens and specific antigens. *Vet. Arh.* 52: 201-207.

要 約

周産期雌馬のリンパ球幼若化能の動態：真田靖幸・野田 寛・永幡 肇(酪農学園大学家畜衛生学教室)——周産期雌馬のリンパ球機能の動態を知ることを目的に、馬リンパ球幼若化試験における至適条件の検討を含め本実験を行った。至適条件は Mitogen 濃度 PHA : 1 $\mu\text{g}/\text{mL}$, Con A:40 $\mu\text{g}/\text{mL}$, PWM:10 $\mu\text{g}/\text{mL}$, 馬プール血清濃度 20%, 120時間培養であった。上記至適条件下での健康成雌馬(非妊馬)のリンパ球幼若化試験の刺激指数は、PHA : 5.107 ± 0.323 , Con A : 4.019 ± 0.183 , PWM : 3.610 ± 0.131 であった。周産期の雌馬の幼若化能は分娩日に近づくに従って低下し、分娩前後1日に刺激指数は最低値[PHA : 1.923 ± 0.174 , Con A : 1.698 ± 0.206 , PWM : 1.706 ± 0.177]を示し、以後漸次上昇した。