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Citation	Japanese Journal of Veterinary Research, 40(1), 13-18
Issue Date	1992-05-29
DOI	<a href="https://doi.org/10.14943/jjvr.40.1.13">https://doi.org/10.14943/jjvr.40.1.13</a>
Doc URL	<a href="https://hdl.handle.net/2115/2359">https://hdl.handle.net/2115/2359</a>
Type	departmental bulletin paper
File Information	KJ00002377531.pdf



## DETECTION OF EQUINE IMMUNOGLOBULIN-SECRETING CELLS BY A PLAQUE ASSAY

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(Accepted for publication: May 1, 1992)

A protein A-hemolytic plaque assay was applied to detect immunoglobulin (Ig)-producing cells in horse peripheral blood, using pokeweed mitogen as a B lymphocyte activator. A maximum number of Ig-secreting cells was obtained when horse peripheral blood lymphocytes were cultured in a medium containing horse serum. The number of Ig-secreting cells in young horses (2 years old) was lower than that in adult horses (6 to 23 years old). In addition, the plaque formation was unchanged from blood samples kept at 4 °C for 24 hours, while blood samples kept for 72 hours did not yield plaques. These results indicate that the plaque assay is a reliable and useful method for detecting Ig-secreting cells in the peripheral blood of the horse.

Key Words: hemolytic plaque assay, Protein A, horse, lymphocyte

### INTRODUCTION

Several genetic immunodeficiencies have been reported in horses<sup>7)</sup>. Affected horses have defects of either B- or T-lymphocyte functions and die of infections at an early age. Furthermore, a failure of passive transfer of immunoglobulin (Ig) from mare to foal via colostrum occurs in about 10% of foals of all breeds, resulting in neonatal infections and deaths<sup>5,6)</sup>.

For diagnosis and differentiation of these immunodeficiency disorders of horses, it is essential to evaluate the cell-mediated immune response *in vitro*, in addition to measurement of serum Ig level. The *in vitro* mitogen-induced lymphocyte transformation is generally accepted as a test for evaluating the capacity of lymphocytes in human and animals including horses<sup>1,2,8,9)</sup>. This test however, is not suitable for detecting antibody-forming cells.

The hemolytic plaque assay, which detects single cells secreting Ig, has been used for characterization of B lymphocyte functions in human<sup>3,10)</sup> and swine<sup>11)</sup>. The

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development of plaque techniques has greatly assisted studies on the cellular basis of antibody production. In horses, however, these procedures have not been applied to analysis of the cell-mediated immune response.

In the present paper, the hemolytic plaque assay for detecting single antibody-forming cells in the peripheral blood of horses is described.

#### MATERIALS AND METHODS

##### *Preparation of peripheral blood lymphocytes*

Fifteen Thoroughbred horses (2 to 23 years old), 6 male and 9 female, were used. Heparinized peripheral blood was obtained from the jugular vein. Blood was diluted with an equal volume of PBS (7.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9 mM NaH<sub>2</sub>PO<sub>4</sub>, 154 mM NaCl, pH7.4), and peripheral blood lymphocytes (PBL) were separated over Ficoll-Paque solution (Pharmacia, Sweden) by centrifugation at 2,000 rpm for 30 min at room temperature. Cells obtained were washed once with PBS and twice with RPMI-1640 (Flow Laboratories, U.S.A.). The viability according to the trypan blue dye exclusion test was greater than 98%.

##### *Culture conditions*

Lymphocytes were cultivated in plastic tubes in a final volume of 1 ml of RPMI-1640 supplemented with sodium pyruvate (0.11 mg/ml), glutamine (0.3 mg/ml), sodium bicarbonate (2 mg/ml), penicillin (100 IU/ml), streptomycin (100 µg/ml), 10% heat-inactivated fetal calf serum (Filtron, U.S.A.), and 30% heat-inactivated horse serum (HS). A preliminary experiment employing different doses of pokeweed mitogen (PWM, Gibco, U.S.A.) indicated that six-day culture with 100-fold dilution of PWM solution gave optimal results with regard to the differentiation of peripheral lymphocytes into Ig-producing cells (Fig. 1). The cultures mentioned above were performed at a density of  $1.0 \times 10^6$  cells in 1 ml respectively, and then incubated at 37 °C for six days in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. After that, these cells were used for plaque assay.

##### *Coupling of protein A to the erythrocytes*

Protein A (Pharmacia, Sweden) was coupled to sheep red blood cells (SRBC) using chromium chloride as described previously<sup>4</sup>. Briefly, SRBC stored in Alsever's solution were washed five times in 0.9% NaCl and resuspended in the same solution to yield a packed cell volume (PCV) of 10%. Thereafter 0.5 ml Protein A solution (1 mg/ml) was mixed with 55 µl chromium chloride (50 mM) and 25 ml SRBC suspension. The mixture was incubated at 30 °C for 60 minutes, washed once in 0.9% NaCl, then twice in Hank's balanced salt solution (HBSS), and resuspended in HBSS to yield a PCV of 40%, then kept at 4 °C until use.

##### *Plaque assay*

After cultivation as mentioned above, lymphocytes were washed three times with PBS and resuspended in HBSS. One hundred µl of cell suspension was added together

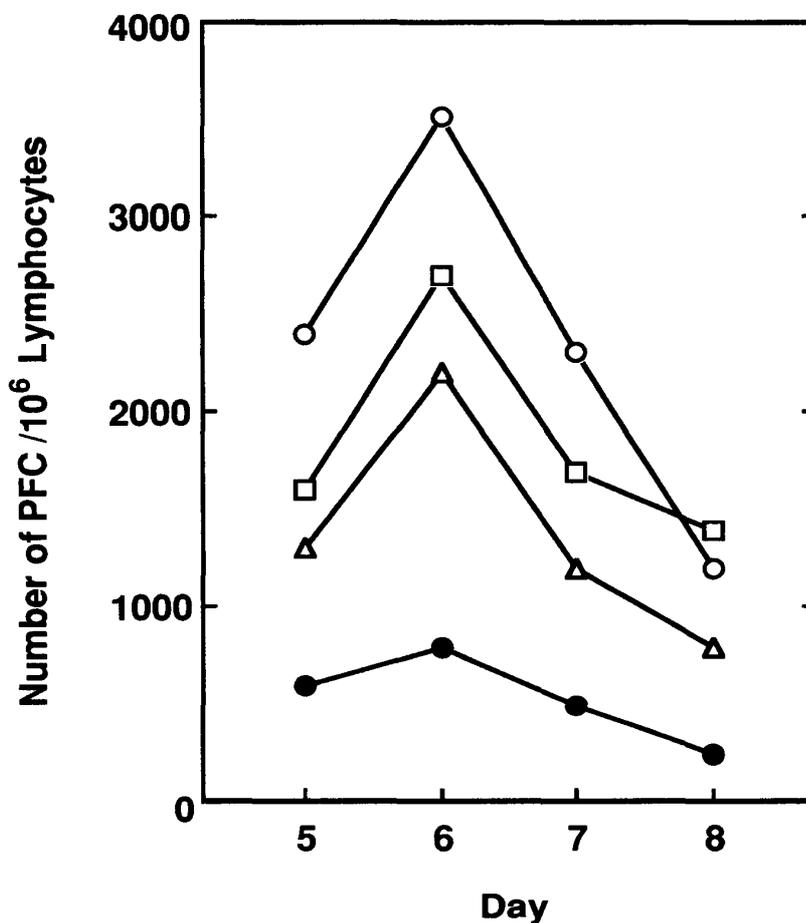


Fig. 1 Effects of incubation period and the concentration of PWM on the generation of plaque forming cells (PFC). Peripheral blood lymphocytes from horses were cultured with different concentrations of PWM (●, none; ○, 1: 100; □, 1: 200; △, 1: 400) for appropriate periods as indicated. After the incubation, plaque assay was performed as described in the Method. Data represent mean of two experiments.

with 20  $\mu$ l of Protein A coupled SRBC suspension, and 20  $\mu$ l of the antiserum diluted 1: 25 [rabbit anti-horse IgG (1.4 mg/ml), Bio-Yeda, Japan] into 300  $\mu$ l of HBSS containing 0.6% agarose (Science, U.S.A.). This mixture was plated on a 9 cm plastic petri dish and incubated at 37 °C for 8 to 10 hours in a humidified incubator under 5% CO<sub>2</sub> and 95% air. After incubation, SRBC-absorbed guinea pig complement (diluted 1: 80) was placed on the gel plate and then incubated for 12 hours under the same conditions. Plaques were counted under indirect light.

## RESULTS AND DISCUSSION

A maximum number of plaque forming cells (PFC), Ig-secreting cells arising after mitogenic stimulation of B cells, was obtained when horse lymphocytes were cultured in a medium containing horse serum (HS). In contrast, only a small number of PFC formed when cultured in a HS-free medium (Fig. 2). This result indicates that equine lymphocyte culture *in vitro* requires supplementation with autologous serum in addition to basic nutrients<sup>8,9</sup>.

In addition, plaque formation varied with different sources of HS (Fig. 2). This difference in number of PFC obtained may be due to different concentrations of essential factors for the optimum growth of lymphocytes in each serum<sup>1</sup>.

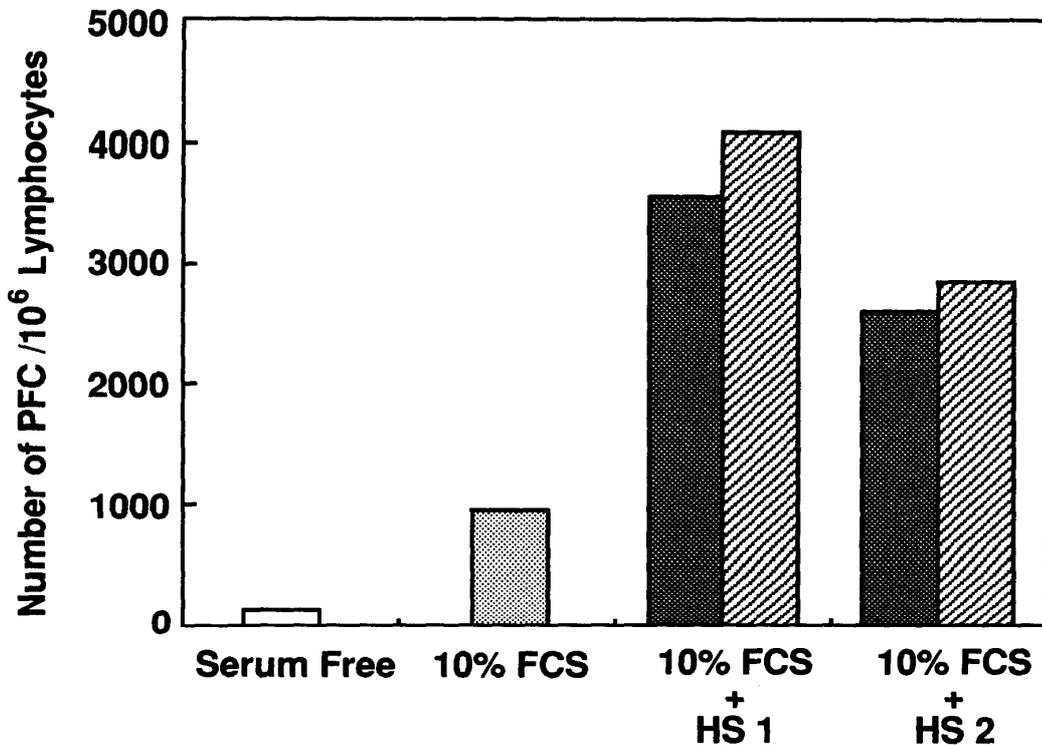


Fig. 2 Effects of serum on generation of PWM-induced PFC in cultures of peripheral blood lymphocytes (PBL) from horses. Plaque assay was performed employing PBL cultured with PWM (1 : 100) for 6 days in the presence of various sera as indicated. HS 1 : a commercial horse serum ; HS 2 : an autologous serum obtained from an experimental horse ; FCS : fetal calf serum. Autologous horse serum was added to a final concentration of 20 % (■) or 30 % (▨). Data represent mean of two experiments.

Table 1 shows the number of PFC in the peripheral blood of horses tested. The number of PFC in young horses (2 years old) was lower than that in adult horses. Since PWM used in the present study has been accepted as a T cell-dependent B cell activator, this result may suggest that the PWM-induced differentiation of B lymphocytes into Ig-producing cells was still suppressed by the T lymphocytes in young horses. Suganuma et al.<sup>11)</sup> reported that the number of PFC in the peripheral blood of suckling piglets was considerably lower than that of adults, and that the suppressor activity of T lymphocytes was consistently demonstrated not only in the newborn period but also throughout the suckling period. However, further study will be needed to elucidate the cause of this interesting finding observed in these young horses.

Table 1. The number of plaque forming cells (PFC) in the peripheral blood

Horses	Number of PFC/10 <sup>6</sup> Lymphocytes
Adult (n=9)	2076±412
Young (n=3)	1512±285

n = number of animals tested

Adult : 6 to 23 years old, Young : 2 years old

Data represent the mean ± SD

Table 2 shows the effect of blood-storage on PFC assay. The number of PFC was unchanged from blood samples kept at 4 °C for 24 hours. Blood samples kept for 72 hours did not yield plaques. From this result, it is clear that the plaque assay should be carried out within 24 hours after the blood collection.

In conclusion, the hemolytic plaque assay using protein A-coated red cells is a

Table 2. The effect of blood-storage on plaque forming cells (PFC) assay

Blood samples	Number of PFC/10 <sup>6</sup> Lymphocytes
Fresh (n=4)	2031±380
24h-blood* (n=5)	2120±426
72h-blood* (n=4)	78±48

\* Blood samples were kept at 4 °C for 24 or 72 hours before use for PFC assay

n = number of animals tested

Data represent the mean ± SD

reliable method for detecting Ig-secreting cells in the peripheral blood of horses. This method, therefore, is useful for clinical studies on equine immunodeficiencies, especially for those disorders caused by a defect of B lymphocyte functions.

#### ACKNOWLEDGEMENTS

We are grateful to Dr. Takuo Hayashi for providing blood from Thoroughbred horses. This study was supported in part by a grant from Equine Research Institute, Japan Racing Association.

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