Application of Glucose Consumption Test for Evaluating Blastogenesis in Bovine Lymphocytes

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ABSTRACT. The transformation of mitogen stimulated bovine lymphocytes was studied using the glucose consumption test. After four days of incubation of lymphocytes, the glucose concentration in the culture medium was determined. The differences of glucose concentration between control and mitogen stimulated cultures indicated the blastogenetic activity of the lymphocytes. Of four mitogens used, phytohaemagglutinin and concanavalin A responded well whereas responses with lipopolysaccharide and pokeweed mitogen were weak. This simple method was useful in evaluating the blastogenesis of bovine lymphocytes for routine use.—**KEY WORDS:** blastogenesis, bovine lymphocytes, glucose consumption test.

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The measurement of incorporated ³H-thymidine has been widely used for evaluating lymphocyte blastogenesis in humans. However, this method has some limitations for routine use; it requires special instruments and reagents. Furthermore, there are very strict regulations on the use of isotopes.

DeCock *et al.* [3] reported a simple method of evaluating human lymphocyte blastogenesis by measuring the glucose concentrations of terminal incubation medium. This method is based on the increase in glycolysis of lymphocytes by mitogenesis through an energy consumption process [6]. With this method, special instruments are not needed and it is possible to treat many samples at the same time using the microculture plate method.

MATERIALS AND METHODS

Samples: Blood samples were obtained by jugular venepuncture from nine healthy Holstein cows in the university farm. They were four to six years old and all in no milking period. The heparinized blood (20 units per

one milliliter of blood) was diluted 1:1 with phosphate-buffered saline (PBS, pH 7.2) and layered over Ficoll Paque solution (Pharmacia, Sweden). The blood was centrifuged at 400 g for 30 minutes after which the mononuclear cell layer was aspirated. The cells were washed three times with PBS and twice with the culture medium (RPMI 1640 contained 10% fetal calf serum). The cells were resuspended in the medium and were examined for lymphocyte recovery, purity and viability. In this experiment, the mean recovery rate was about 78%, the lymphocyte population was more than 97% pure lymphocytes and they were more than 95% viable by trypan blue exclusion.

Mitogens: Phytohaemagglutinin-P (PHA, Difco, USA) and concanavalin A (Con A, Gibco, USA) were used for T cell mitogen, lipopolysaccharide (LPS, Difco, USA) was used for B cell mitogen and pokeweed mitogen (PWM, Gibco, USA) as both T and B cell mitogen. Each mitogen was reconstituted with sterile PBS and divided into small aliquots and stored at –30°C until used.

Glucose consumption test: Two hundred

microliters of lymphocyte suspension and 20 μ l of mitogen were poured into each well of a disposable microplate. Each culture was triplicated and mitogen free culture was set up as a control. This plate was incubated at 37°C in 5% humidified carbon dioxide (CO₂) in the air for varying periods. After incubation, each culture medium was transferred in a microtube and centrifuged at 400 g for 10 minutes. The supernatant was collected and the glucose concentration was determined with a RaBA-super system (Chugai Pharmaceutical, Japan). The culture medium contained 200 mg/100 ml of glucose.

The lymphocyte stimulation was estimated as the quantity of glucose consumed (minus the concentration of stimulated culture from the control culture).

Thymidine incorporation method: The cells of mitogen stimulated culture and control culture were prepared as described above. The cell concentration was 5×10^6 /ml and the mitogen concentration was adjusted as follows: PHA, 15 μ g/ml, Con A, LPS and PWM, 10 μ g/ml.

These conditions were determined by preliminary experiment. The cell cultures were incubated in 5% CO₂ for 72 hours and then 1 μ Ci 6-³H-thymidine (³H-TdR, specific activity of 26 Ci/mmol, amersham, England) was added to each well and incubated for 24 hours. Then the cells were harvested on the glass fiber filter with automatic cell harvester (Micro cell harvester; Bellco Glass Inc., USA). The filter was transferred in a 10 ml counting vial and dried at 60°C for 60 minutes. Five milliliters of scintillator (Scintizol EX-H, Dotite, Dojindo Laboratory, Japan) was poured into a vial and incorporated counts of ³H-TdR were measured with a liquid scintillation spectrometer (Tracor Analytic 006881) for one minute.

The results were expressed as counts per minute (cpm) and stimulation index (S.I. = cpm of mitogen stimulated culture per cpm of control culture).

RESULTS

Effects of cell concentration on glucose consumption test: The lymphocytes were diluted in the medium to cell concentrations varying from 5×10^5 to 1×10^7 cells/ml. The glucose consumption of mitogen stimulated and control cultures increased with the concentration, especially above 5×10^6 /ml, but the LPS stimulated cultures showed poorer consumption (Fig. 1). The glucose consumption of mitogen stimulated culture was highest at 1×10^7 /ml, but the consumption of the control culture also increased at the same time. In the present study, a cell concentration of 5×10^6 /ml was employed for good stimulation and cell economy.

Effects of mitogen concentration: Each mitogen was diluted with sterile PBS in vari-

Fig. 1. Effects of cell concentration on the response of the cultures to PHA, Con A, LPS and PWM. Stimulation is expressed by the glucose concentration of terminal incubation medium.

Fig. 2. Effects of various concentrations of PHA, Con A, LPS and PWM on lymphocyte stimulation. Each value is expressed as mean of triplicated culture.

ous concentrations; PHA from 1.0 to 25.0 $\mu\text{g/ml}$, Con A from 1.0 to 50.0 $\mu\text{g/ml}$, LPS from 1.0 to 50.0 $\mu\text{g/ml}$ and PWM from 1.0 to 40.0 $\mu\text{g/ml}$.

Dose response curves of these mitogens are presented in Fig. 2. Con A and PHA showed good stimulation with increasing dosage. In contrast the responses of LPS and PWM were rather weak. The optimal dosages were determined as follows: PHA 15 $\mu\text{g/ml}$, Con A 25 $\mu\text{g/ml}$, LPS 10 $\mu\text{g/ml}$ and PWM 5 μl per culture, respectively.

Effects of incubation period: The influence of incubation time were examined daily from two to five days. The results indicated that there were no significant differences between control and stimulated cultures for two days although the glucose consumption increased in parallel with the incubation time. At four days of incubation, PHA, LPS and PWM showed the maximum consumption, whereas Con A indicated an increased stimulation up to five days (Fig. 3). Four days incubation produced good stimulation in all four mitogens and this culture condition was used in the subsequent experiment.

Fig. 3. Influences of incubation period on glucose consumption.

Results of glucose consumption test and ^3H -thymidine incorporation method: The results of the glucose consumption test and ^3H -TdR method in nine cows are given in Table 1. The results of the glucose consumption test were expressed by the glucose concentration of terminal incubation medium and the ^3H -TdR method was shown as S.I..

Significant differences were observed between the glucose consumption of each mitogen stimulated and control cultures ($P > 0.01$) although there were individual variations be-

Table 1. Results of the glucose consumption test from nine adult cows

Cattle No.	No mitogen	Mitogen		
		PHA	Con A	LPS
1	162.2 ^{a)}	144.5 ^{a)} (15.3) ^{b)}	135.4 ^{a)} (32.5) ^{b)}	140.6 ^{a)} (1.2) ^{b)}
2	151.1	140.8 (4.0)	135.5 (3.8)	136.9 (1.0)
3	144.5	123.6 (5.4)	116.2 (6.7)	131.9 (1.7)
4	145.8	119.2 (13.2)	121.3 (10.7)	135.3 (0.9)
5	155.4	137.5 (4.7)	132.9 (8.5)	146.5 (1.1)
6	165.4	144.6 (4.2)	152.1 (3.8)	146.4 (1.3)
7	148.1	138.6 (3.8)	138.7 (8.7)	136.3 (1.2)
8	155.0	120.0 (11.1)	98.0 (15.7)	125.0 (1.4)
9	151.0	102.0 (9.5)	81.5 (14.9)	110.0 (1.5)

a) Glucose concentration (mg/100 ml).

b) Stimulation index (³H-TdR).

tween the cows. In general, the glucose consumption of PHA and Con A stimulated cultures were higher than the LPS.

There were no significant correlations between the results of the glucose consumption test and ³H-TdR method in this experiment which contributed to the increased incorporation of ³H-TdR in the control culture.

DISCUSSION

The results of this study demonstrated that the optimal conditions for blastogenesis of bovine peripheral lymphocytes as measured by the glucose consumption test were: (a) a 96 hours incubation period, (b) 5×10^6 cells per culture in RPMI 1640 containing 10% fetal calf serum and (c) PHA (15 μ g/ml), Con A (25 μ g/ml), LPS (10 μ g/ml) and PWM (5 μ l) added to each culture. Under these conditions, the fluctuations of the data obtained were kept to a minimum. But for mitogen concentration, it requires the preliminary examination before use according to the lot of each mitogen.

The advantages of this method are that it is simple and convenient. Furthermore, it registers the entire event of lymphocyte transformation whereas other methods evaluate only viable cells at the end of the culture.

The application of the glucose consump-

tion test for human lymphocyte transformation was first described by DeCock *et al.* [3] but there is no available information on the glucose consumption test of bovine lymphocytes.

In this preliminary study, there were considerable differences in the glucose consumption of mitogen stimulated cultures compared with the controls, which expressed the degree of mitogenetic ability of lymphocytes to each mitogen. Above all, PHA and Con A stimulated cultures indicated more stimulation but LPS stimulated cultures showed less. These may be due to the differences in response of the lymphocyte subpopulation to each mitogen as PHA and Con A were considered as T cell mitogens [2, 4, 5, 9] and LPS was considered as a B cell mitogen [1, 7-9].

DeCock *et al.* [3] reported that the results of the glucose consumption test of human lymphocytes correlated well with the results of morphological evaluation and ³H-TdR uptake. However, in this experiment, a significant correlation was not observed between the results of the glucose consumption test and the ³H-TdR method, when compared with the quantity of glucose consumption and S.I.. Because, the increased uptake of ³H-TdR in control culture depressed the S.I., relatively. The reasons of this was not made clear enough in this experiment.

The present study suggested that the application of the glucose consumption test for bovine peripheral lymphocyte transformation may provide one of the simple and useful method to evaluate immune status of cattle. It is important to avoid contamination by bacteria or fungi which consume large amounts of glucose and lymphocyte purity should be maintained at more than 95%.

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要 約

グルコース消費試験を応用したウシリンパ球の幼若化反応の測定について：石川 潤・白幡敏一¹⁾（帯広畜産大学附属家畜病院，¹⁾家畜微生物学教室）——ウシリンパ球の幼若化反応の測定にグルコース消費試験を応用するため，基礎的条件の検討を行った。培養条件の検討の結果，細胞数 $5 \times 10^6/\text{ml}$ ，マイトジェン濃度，PHA 15 $\mu\text{g}/\text{ml}$ ，Con A 25 $\mu\text{g}/\text{ml}$ ，LPS 10 $\mu\text{g}/\text{ml}$ ，PWM 5 μl ，培養時間96時間が至適条件と考えられた。この条件下で，成牛9頭の末梢血リンパ球の幼若化反応を測定した結果，本法は操作が簡易で，特殊な器具，試薬を必要とせず再現性もよいことなどから，ウシの免疫機能検査法の一つとして臨床応用も可能と考えられた。