The Colorimetric Assay for Swine Lymphocyte Blastogenesis

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ABSTRACT. The colorimetric assay used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was compared with the ^3H -thymidine incorporation assay in swine lymphocyte blastogenesis induced by phytohemagglutinin-P (PHA) and concanavalin A (ConA). The correlation coefficients were 0.70 ($n=18$, $p<0.01$) in PHA and 0.82 ($n=18$, $p<0.01$) in ConA. The maximal responses were observed in cultures of 2.0×10^6 cells/ml with the final concentration of 2.5–10.0 $\mu\text{g/ml}$ in PHA or 2.5–5.0 $\mu\text{g/ml}$ in ConA for 72 hr. The stimulation index of peripheral blood lymphocytes from clinically healthy pigs aged 6 months were 1.77 ± 0.10 ($n=10$) in PHA and 1.96 ± 0.18 ($n=10$) in ConA, respectively.—**KEY WORDS:** blastogenesis, colorimetric assay, swine lymphocyte.

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The colorimetric assay used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was widely used on cell function test in human and other animals in place of ^3H -thymidine incorporation assay [1, 4–6, 8]. This assay is based on reduction of MTT into formazan salt by mitochondrial dehydrogenase of viable cells and the degradation rate of MTT was closely related with the proliferative activity of cells [2]. The purpose of this report is to evaluate the application of this MTT colorimetric assay for the measurement of swine lymphocyte blastogenesis.

All pigs used in this study were clinically healthy, two- or three-way crossbreeds raised on commercial and conventional farms, which were aged 6 months and about 100 kg in weight. Swine blood lymphocytes were obtained from heparinized blood by the gradient differential centrifugation with Ficoll-Conray solution (S.G. 1.077–1.078). Swine lymphocytes were suspended in RPMI1640 (Flow Laboratories Inc., U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (MAB, U.S.A.), 0.1% sodium bicarbonate, 100,000 U/l penicillin (Meiji, Tokyo) and 100 mg/l streptomycin (Meiji, Tokyo). Aliquots (100 μl) were cultured in 96-well flat-bottomed microplates (FALCON® 3072, Becton Dickinson, U.S.A.) with phytohemagglutinin-P (PHA, Difco, U.S.A.) or concanavalin A (ConA, Sigma, U.S.A.) at 37°C in a humidified 5% CO_2 atmosphere. Cultures were pulsed with 10 μl of 5 mg/ml filter-sterilized MTT (Sigma, U.S.A.) in phosphate buffered saline for the final 4 hr of incubation. At the end of incubation, the medium was gently aspirate and then 100 μl of dimethyl sulfoxide (DMSO, Wako, Osaka) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The plates were left at room temperature for a few minutes to ensure that all crystals were dissolved and were read on a ELISA reader (CORONA, Ibaraki), using a test wavelength at 570 nm and a reference wavelength at 630 nm. ^3H -thymidine incorporation assay was carried out according to the method of Inoue *et al.* [3]. The results are expressed as the stimulation index on MTT assay (SI-MTT) or radioisotope assay (SI-RI) calculated according to the following formula from the results of triplicate assays:

$$\text{SI-MTT or SI-RI} = \frac{\text{O.D. (570/630 nm) or cpm of stimulated cells}}{\text{O.D. (570/630 nm) or cpm of unstimulated cells}}$$

Fig. 1. MTT cleavage activity of PHA- and non-stimulated swine blood lymphocytes in relation to cell number. Each point represents the mean and standard deviation of triplicate assays from 3 different pigs.

MTT cleavage activity was examined on swine lymphocytes stimulated with or without PHA for 48 hr. The viable cells were adjusted to various cell number indicated in Fig. 1. The result shows that the absorbance is directly proportional to the cell number between 4×10^5 and 1×10^7 cells/ml. The amount of formazan generated by activated cells was approximately 4-fold higher than that by non-stimulated cells.

MTT colorimetric assay was compared with ^3H -thymidine incorporation assay on blastogenic responses with various concentration of PHA or ConA for 72 hr. The both assays showed an agreement in ConA or PHA stimulation (Fig.2). The maximal responses were obtained from lymphocytes stimulated with 2.5 $\mu\text{g/ml}$ of PHA and with 2.5 to 5.0 $\mu\text{g/ml}$ of ConA. The correlation coefficients were 0.70 ($n=18$, $p<0.01$) in PHA and 0.82 ($n=18$, $p<0.01$) in ConA. However, SI values in MTT assay was less than that in ^3H -thymidine incorporation assay. The MTT assay measures the number and activity of living cells, whereas ^3H -thymidine incorporation assay measures the number of cells synthesizing DNA. Therefore, the differences between SI-MTT and SI-RI values were caused by the amounts of background as resting cells.

Fig. 2. Mitogenic response of swine blood lymphocytes in the MTT assay compared with ^3H -thymidine incorporation assay after stimulation with various concentration of PHA and ConA for 72 hr. Each point represents the mean of triplicate assays from 3 different pigs.

The optimal incubation time on the MTT assay was determined after the incubation of the culture mixtures with the optimal concentration of $2.5\text{ }\mu\text{g/ml}$ of PHA or $5.0\text{ }\mu\text{g/ml}$ of ConA for 24, 48, 72, 96, or 120 hr. The maximal responses were obtained in culture for 72 hr in both mitogens (Fig. 3). The SI-MTT values were 1.77 ± 0.10 ($n=10$) in PHA and 1.96 ± 0.18 ($n=10$) in ConA. These optimal conditions were similar to those in other methods such as ethidium bromide fluorescence or ^3H -thymidine incorporation assay [3]. However, no significant increase of formazan formation was observed in pokeweed mitogen or lipopolysaccharides-stimulated cells (data not shown). Stimulated cell population in these mitogens might be much smaller than those in ConA or PHA, as reported that MTT assay could not evaluate the mitogenic response of swine lymphocytes at 48 hr [7].

As mentioned above, MTT colorimetric assay may be useful for determining mitogenic response of swine lymphocytes to ConA or PHA. The optimal mitogenic response of swine lymphocytes in MTT colorimetric assay can be obtained at the stimulation of $2.5\text{ }\mu\text{g/ml}$ PHA or 2.5 to $5\text{ }\mu\text{g/ml}$ ConA for 72 hr.

Fig. 3. Mitogenic response of swine blood lymphocytes in MTT assay at various incubation time. Each point represents the mean of triplicate assays from 4 different pigs.

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