Detection of Mitogen-Induced Lymphocyte Proliferation by Bromodeoxyuridine (BrdU) incorporation in the Chicken

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ABSTRACT. ³H-thymidine (³H-TdR) incorporation assay has been generally used to measure lymphocyte proliferation in the chicken. Disadvantages of this assay are that radioisotope is biological hazard to the person and environment and that it can not measure which subset of lymphocytes proliferates. In this study, bromodeoxyuridine (BrdU) incorporation assay by flow cytometry was compared with ³H-TdR incorporation assay. As a result, BrdU incorporation assay showed a strong correlation with ³H-TdR incorporation assay, and it could be applied simultaneously to detect BrdU incorporation and expression of cell surface marker antigens. These results suggest that the BrdU incorporation assay by flow cytometry is useful to analyze lymphocyte proliferation in detail.

KEY WORDS: bromodeoxyuridine, ³H-thymidine, lymphocyte proliferation.

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Lymphocyte proliferation responses to mitogens are commonly evaluated by a ³H-thymidine (³H-TdR) incorporation assay. However, radioisotope (RI) is biological hazard to the person and environment, therefore, it has been established and utilized various non-RI assays to measure lymphocyte proliferation. Colorimetric assays by using of the tetrazolium salt such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [7] or bromodeoxyuridine (BrdU) [8] have proven to be useful as non-RI assays. A disadvantage of these assays is that they are not available to measure proliferative events in single cells. To solve this problem, flow cytometry was used to measure lymphocyte proliferation. BrdU incorporation assay by flow cytometry allows simultaneously measuring lymphocyte proliferation and expression of cell surface marker antigens [3, 4, 6, 9]. However, there is little information about correlation between ³H-TdR incorporation assay and BrdU incorporation assay by flow cytometry [6]. In this study, to compare ³H-TdR incorporation with BrdU incorporation assay in the chicken, lymphocyte proliferation responses to pokeweed mitogen (PWM) and concanavalin A (Con A) were investigated.

Mononuclear cell (MNC)-rich fraction in the spleen from inbred chickens (genotype B²B²) was prepared as described previously [5]. The final resultant MNC-rich fraction consisted of approximately 90% lymphocytes and 10% monocytes when its Giemsa-stained smear sample was cytologically evaluated. The cells were suspended in Iscove's modified dulbecco's medium (Sigma, U.S.A.) supplemented with 2% normal chicken serum, 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 μ g/ml streptomycin (Meiji Seika, Japan) and 100 U/ml penicillin (Banyu Pharmaceuti-

cal, Japan). The cells (2×10^6 cells/ml) were cultured at 40°C in a humidified atmosphere of 5% CO₂ in the presence or absence of PWM (50 μ g/ml, Sigma) and Con A (5 μ g/ml, Sigma).

A modified method of BrdU incorporation assay by flow cytometry described previously [6] was employed. Cells were dispensed in duplicate (1 ml per well) in a flat-bottom 48 well plate (Nunc, U.S.A.). BrdU (18 μ g/ml, Sigma) was added to cells for the final 20 hr. After culture, cells were washed once in PBS, incubated with anti-Bu-1a, CD4 or CD8 antibody, and then followed by phycoerythrin-conjugated goat anti-mouse immunoglobulins (PE-anti-mouse Ig) (1:100, Southern Biotechnology Associates, Inc., U.S.A.). Following immunofluorescence labeling, cells were washed once in PBS and fixed in PBS containing 1% formaldehyde-0.02% Tween 20 for 24 hr at 4°C. Cells were washed and incubated in PBS supplemented with 0.9 mM CaCl₂, 0.33 mM MgCl₂, and deoxyribonuclease I (50 Kunitz units/ml, DN-25, Sigma) for 30 min at 37°C. Subsequently, after washing, cells were incubated in PBS containing 0.5% Tween 20 with fluorescein isothiocyanate-conjugated mouse anti-BrdU antibody (FITC-anti-BrdU) (Progen, Germany) for 30 min at room temperature. BrdU incorporation rate was measured by a flow cytometer (Epics XL, Beckman Coulter, U.S.A.). Spleen cells cultured without BrdU were used as negative control.

Cells were dispensed in triplicate (200 μ l per well) in flat-bottom 96 well plates (Falcon, U.S.A.). Each well was pulsed with 18.5 kBq of ³H-TdR (740 GBq/mmol, NEN Products, U.S.A.) for 20 hr prior to harvesting cells. Cells were harvested onto glass-fiber filters using a cell harvester (PHD harvester, Cambridge Technology, Inc., U.S.A.). Samples were read on a liquid scintillation counter (LS 6000TA, Beckman Coulter). The association between two assays was determined by calculation of the Pearson correlation coefficient (*r*).

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Fig. 1. PWM- and Con A-induced proliferation of spleen cells in chickens. Time course of ^3H -TdR (A) or BrdU (B) incorporation of spleen cells cultured with medium (○), Con A (△) or PWM (■). (C) Correlation in the proliferative responses of spleen cells cultured with PWM or Con A for 48 (●), 72 (○), 96 (□), and 120 hr (▲). Values are expressed as mean $\pm$ SE from three chickens.

Fig. 2. Correlation of stimulation indexes in cells cultured with PWM or Con A for 72 hr. Stimulation indexes were calculated by values of ^3H -TdR or BrdU incorporated cells cultured with mitogen divided by those of cells cultured without mitogen ($n=12$).

The closely similar pattern of proliferation curve in both assays was observed as shown in Fig. 1, when evaluated by incorporation of ^3H -TdR or BrdU into cellular DNA of mitogen-stimulated cells. The BrdU incorporation rates by flow cytometric analysis showed a positive high correlation with the counts per minute of the ^3H -TdR incorporation in 48- ($r=0.973$) and 72-hr culture ($r=0.910$) (Fig. 1C). This correlation, however, was not shown when cells were cultured for more than 96 hr. The BrdU incorporation rate in the culture without mitogen was significantly increased at 120 hr ($20.0 \pm 4.7\%$) compared to that at 48 hr ($0.6 \pm 0.3\%$) (Fig. 1B). On the contrary, the counts per minute of ^3H -TdR incorporation in the culture without mitogen were moderately increased during culture (342 ± 124 cpm at 48 hr to 671 ± 124 cpm at 120 hr) (Fig. 1A). In flow cytometric analysis, the BrdU incorporation rate represents the percentage of BrdU positive cell number to total lymphocyte number. The BrdU incorporation rate in the culture without mitogen is significantly affected by total cell number which

was decreased with time of culture, indicating that it is important to measure lymphocyte proliferation at the peak of mitogen responses by flow cytometry.

In order to confirm whether correlation between two assays is influenced by different kinds of mitogens, correlation coefficient was determined in the culture with PWM or Con A for 72 hr. Stimulation indexes in proliferative responses to both mitogens by the BrdU incorporation assay positively correlated with those by the ^3H -TdR incorporation assay ($r=0.899$ for PWM; $r=0.934$ for Con A), as shown in Fig. 2. These results suggest that the BrdU incorporation assay by flow cytometry is useful as one of non-RI lymphocyte proliferation assays.

^3H -TdR incorporation assay has been traditionally used as a detection of lymphocyte proliferation, however, this assay does not give information which subset of lymphocytes proliferates. The Alamar blueTM method, a non-RI detection of lymphocyte proliferation assay, is also applied to measure the expression of activated cell surface marker

Fig. 3. The relative proportion of Bu-1a⁺, CD4⁺ and CD8⁺ cells in spleen cells cultured with Con A for 72 hr in the presence (bottom) or absence (upper) of BrdU.

antigens but not to detect simultaneously with cellular proliferation [2]. An advantage of the BrdU incorporation assay by flow cytometry is that it can be applied for double staining of BrdU incorporation and expression of cell surface marker antigens. Figure 3 shows double staining of the cell surface marker antigens and BrdU incorporation in spleen cells cultured with Con A for 72 hr, and indicated that most of BrdU incorporated cells response to Con A were CD8⁺ T cells. This result corresponds to the previous study by Chan *et al.*, who reported that CD8⁺ T cells proliferated response to Con A than CD4⁺ T cells [1].

Taken together, it was confirmed that the BrdU incorporation assay by flow cytometry is more useful to analyze lymphocyte proliferation in detail compared to ³H-TdR incorporation assay in the chicken.

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REFERENCES

1. Chan, M. M., Chen, C. H., Ager, L. L. and Cooper, M. D. 1988. *J. Immunol.* **140**: 2133–2138.
2. Gogal, R. M. Jr., Ansar Ahmed, S. and Larsen, C. T. 1997. *Avian Dis.* **41**: 714–725.
3. Gonchoroff, N. J., Katzmman, J. A., Currie, R. M., Evans, E. L., Houck, D. W., Kline, B. C., Greipp, P. R. and Loken, M. R. 1986. *J. Immunol. Methods* **93**: 97–101.
4. Hirsch, A., Tozzi, C. A., Das, A. K., Thakker-Varia, S., Poiani, G. J., Riley, D. J. and Yurkow, E. J. 1999. *Cytometry* **37**: 81–84.
5. Maslog, F. S., Motobu, M., Hayashida, N., Yoshihara, K., Morozumi, T., Matsumura, M. and Hirota, Y. 1999. *J. Vet. Med. Sci.* **61**: 283–285.
6. Mehta, B. A. and Maino, V. C. 1997. *J. Immunol. Methods* **208**: 49–59.
7. Mosmann, T. 1983. *J. Immunol. Methods* **65**: 55–63.
8. Porstman, T., Ternynck, T. and Avrameas, S. 1985. *J. Immunol. Methods* **82**: 169–179.
9. Rosato, M. T., Jabbour, A. J., Ponce, R. A., Kavanagh, T. J., Takaro, T. K., Hill, J. P., Poot, M., Rabinovitch, P. S. and Faustman, E. M. 2001. *J. Immunol. Methods* **256**: 35–46.