Optimum Concentration of Four Mitogens for the Activation of Peripheral Blood Lymphocytes in Japanese Quail (*Coturnix japonica*)

By

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Summary : The aim of this study was to identify and establish optimal concentrations of four different mitogens : ConcanavalinA (ConA), pokeweed mitogen (PWM), phytohemagglutinin-M (PHA-M), and phytohemagglutinin-L (PHA-L) in stimulating purified peripheral blood lymphocytes (PBL) from Japanese quails. PBL were analyzed on their ability to respond to each of the four mitogens. Our results suggest that the effective dose for PBL activation and proliferation in Japanese quails are 50～1,000 μg/ml for 48 h with PWM, 1.25～2.5 μg/ml for 72 h with PHA-M, and 1～1,000 μg/ml for 48 h with PHA-L. ConA was least effective among 4 mitogens to activate quail PBL and higher concentrations was adversely affect PBL viability. PWM did not promote PBL response at low concentrations (1～12.5 μg/ml), but concentrations greater than 500 μg/ml PWM induced the activation of PBL. PHA-M stimulated PBL in a dose-dependent manner, and the most effective response was observed at the PHA-M concentrations of 50 μg/ml or more and culture time of 72 hours. On the other hand, PHA-L was effective at a wide range of concentrations, making it the most suitable mitogen for Japanese quail PBL activation and proliferation. Further, these findings support the idea that a high dose of ConA can induce apoptosis in quail PBL. Although PWM had relatively little effect on overall quail PBL activation and proliferation, there is reason to believe that certain lymphocyte subsets within the Japanese quail PBL population might be activated with a high dose of PWM.

Key words : Quail PBL, mitogens, ConA, PWM, PHA

Introduction

Currently, it is known that only three mitogens ; concanavalinA (ConA), phytohemagglutinin (PHA) and pokeweed mitogen (PWM), have been successfully used for the stimulation of avian lymphocytes, however only PWM can stimulate both avian B and T cells[1].

ConA is a lectin originally extracted from the jackbean *Canavalia ensiformis*. It was the first lectin to be available on a commercial basis and is widely used in biology and biochemistry to characterize glycoproteins. ConA is also a lymphocyte mitogen[2] and has been used for the detection of chicken macrophages[3] and for the production of interleukin-2 (IL-2)[4,5].

TALEBI *et al.* (1995) established the optimal conditions for measuring the blastogenic effect of ConA as a T cell specific mitogen, on whole blood lymphocytes of chickens, in order to facilitate the quantitative assessment of T-cell function[6]. PHA is a lectin found in plants, especially red kidney beans, which contains potent, cell agglutinating and mitogenic activities[7]. It actually consists of two closely related isolectin subunits, called Leucoagglutinin (PHA-L) and PHA-E. The letters E and L point to the fact that these proteins agglutinate Erythrocytes and Leukocytes, respectively. L has high affinity for Iymphocyte surface receptors but little for those of erythrocytes and is responsible for the mitogenic properties of the isolectins. E is responsible for the erythrocyte agglutinating properties. PHA-P is the protein form and PHA-M is the mucoprotein form

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of these isolectins which is used for the stimulation of cell division in lymphocyte cultures[7,8]. The optimal conditions for PHA stimulation with purified lymphocytes from chicken[9], duck and turkey PBL[10], and for ConA stimulation in turkey PBL[11,12] have already been established and characterized. Lastly, PWM is a lectin mitogen derived from the American Pokeweed plant (Phytolacca americana) and contrary to either ConA or PHA which are T-cell specific, PWM appears to activate B lymphocytes in addition to T-lymphocytes[2]. Vainio *et al.* (1984) showed that PWM is able to induce a large number of B cells producing cytoplasmic immunogloblin, and that the proliferative response of chicken PBL to PWM is macrophage dependent[13].

Although the findings from previous studies provide a number of insights, there are no reports on the effect of mitogens on Japanese quail (*Coturnix japonica*) PBL. The present study aimed at defining the optimum concentrations of four plant mitogens, ConA, PWM, PHA-M, and PHA-L, to stimulate quail PBL.

Material and Methods

Quails : Ten (5 males and 5 females) 10- to 20 week-old Japanese quails (*Coturnix japonica*), which have been maintained at a closed colony in our laboratory, were used for analysis.

Separation of PBL : Heparinized blood was collected from the jugular vein. The collected blood was diluted with the same volume of sterile phosphate buffered saline (PBS, pH7.4, Ca^{2+} and Mg^{2+} free), layered over a Percoll solution (Pharmacia : specific gravity 1.075 ± 0.005) in a 15 ml polystyrene tube, and centrifuged at $450 \times g$ for 30 min at 20℃. The white layer of PBL was separated on the Percoll solution. This layer was collected, and the PBL were pelleted by centrifugation at $150 \times g$ for 10 min at 10℃. Then, PBL were washed twice in RPMI 1640 (GIBCO BRL, USA) with 100 U/ml penicillin and 100 μg/ml streptomycin sulfate without serum. The washed PBL were resuspended in RPMI 1640 medium, counted in a hemacytometer using Natt-Herrick stain[14], and adjusted to 2×10^6/ml.

Cell Culture for Stimulation : To identify the optimum concentrations of the mitogens, the PBL were cultured with concanavalinA (ConA : Sigma, Cat. No. C 5275, USA), pokeweed mitogen (PWM : EY Laboratory, Cat. No. L-1901-5, USA), phytohemagglutinin-M (PHA-M : EY Laboratory, Cat. No. L-1803-10, USA) and phytohemagglutinin-L (PHA-L : EY Laboratory, Cat. No. L-1801-7, USA). Cell suspensions (2×10^6/ml) were dispensed in 50 μl aliquots into wells of 96-well flat bottom microculture plates. Each of the mitogens at a final concentration of 0～1,000 μg/ml in serum-free RPMI

1640, was added to each well. A zero concentration was used as a control for each mitogen. All tests were conducted in triplicate (3 wells/concentration). Cells were stimulated for 24, 48, and 72 hrs, respectively in a humidified 5% CO_2 incubator at 40℃.

Measurement of mitogen response : 4 hours just before the end of the culture period, 10 μl of the 5 mg/ml solution of MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, USA) in RPMI 1640 was added to each well. The plates were gently shaken and incubated for 4 hours under the same culture condition. At the end of the incubation period, the culture plates were centrifuged for 10 min at $450 \times g$ at 4℃. The supernatants were aspirated, and 100 μl of 0.04 N HCl-isopropanol containing 10% dimethylsulfoxide solution was added into each well. Subsequently, the plates were vigorously shaken on a plate-mixer for 30 min at room temperature in order to ensure the solubilization of blue formazan, and the reaction was stopped by adding 100 μl of a 4N H_2SO_4 solution. The optical density of supernatants in each well was measured using an automatic plate reader (Corona, model No. MT-120, Japan) with a 570 nm test wavelength and a 630 nm reference wavelength.

The stimulation index was calculated as follows :

The stimulation index $= (ABS_{570\,nm} - ABS_{630\,nm})$ at the cultivation of 24 hrs or more/$(ABS_{570\,nm} - ABS_{630\,nm})$ at the start of the culture.

Statistical analysis : All data were expressed as mean $\pm$SEM. Significant differences among concentrations were investigated with Sheffer's analysis. Differences were considered statistically significant at $P < 0.05$.

Results

Figures 1 to 4 showed the activity ratios of quail PBL cultured with different concentrations of ConA, PWM, PHA-M, and PHA-L. In the control culture (without mitogen), the formazan production of PBL decreased with culturing time, thus, indicating that cell survival generally decreased in culture medium alone. As shown in Fig. 1, PBL were stimulated with ConA in a range of more than 50 μg/ml for 48 and 72 hrs. A dose-dependent suppression effect, that appeared to induce cell death, was observed at all time points.

On the other hand, stimulation with PWM (Fig. 2) resulted in a dose-response manner when utilized at a dose of more than 50 μg/ml. In the range of 1–25 μg/ml of PWM, the response of PBL was similar to that of unstimulated cells, irrespective of the concentrations. The maximum level for PBL stimulation was reached at a dose of 50–125 μg/ml for 24 hrs after initial culturing. However, PWM induced the highest response

amongst the 4 mitogens under a 24 hr-stimulating culture maintaining the stimulation index at 0.6–0.8, but this response reduced significantly (P<0.05) after 48 hrs of culture.

Two different PHA were tested resulting in two very different trends. PBL stimulated with PHA-M demonstrated a response resembling that of ConA (Fig. 3). Further, PHA-M appeared to suppress PBL response in a dose-response manner at higher dose than 5 μg/ml for 48 and 72 hrs. However, the maximum level for PBL stimulation was reached at a dose of 2.5 μg/ml for 72 hrs after the initial culture. Alternatively, PBL stimulation with PHA-L generated the highest response at 48 hrs in a wide concentration range (Fig. 4). However there was no significant difference in activities between stimulated and un-stimulated cells (0 μg/ml). The responses of PBL after 72 hrs with 12.5 and 25 μg/ml of PHA-L were significantly lower than those after 48 hrs. The activation response induced by PHA-L was very different from any of the other three mitogens and was dose-independent.

Discussion

It is generally known that, under in vitro conditions, differences exist in cellular viability and survival rate between different animal species. Cell survival rates in 24+ hr cultures significantly decrease in serum-free medium. In fact, the activity rate of unstimulated cells after 24 hrs, became less than 1 in this study. As such, usually in the majority of cases with cell culturing, experimental conditions are usually not performed within the first 24 hrs of a primary culture, since this period is considered necessary for cell stabilization time. However, in the present study, knowing cell viability significantly decreases after 24 hrs, we chose not to follow through with a cell stabilization time. Instead, we started collecting data as early as possible, from cell inoculation 0 hour as a reference point for basal stimulation level in order to show that our cultures could be directly used for survival and proliferation experiments with different mitogens within the first 24 hrs. The cellular response using serum-free medium varied according to the kind of the mitogen and the concentration within 24 hrs.

It has been suggested that serum-free condition is especially best for stimulation with low doses of ConA and PHA-P, whereas variable results are produced with higher doses of ConA or PHA-P[15].

Similar to the results of previous reports, our findings indicate that ConA is an efficient lymphocyte activator at low doses in a serum-free condition, but high doses of ConA are toxic rather than mitogenic to quail PBL.

Vainio et al. showed that chicken peripheral blood leukocytes exhibited macrophage-dependent proliferation in response to PWM that induced the production
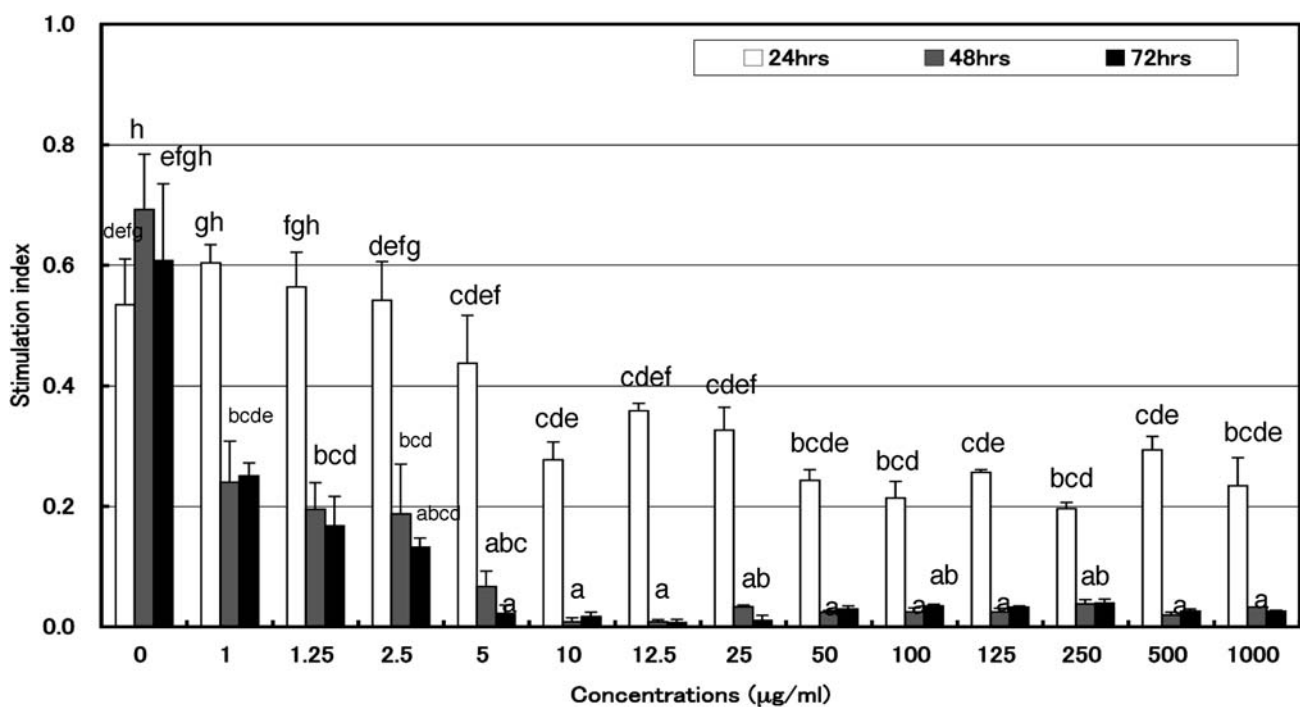

Fig. 1   The activity of quail PBL by stimulation of various concentrations of ConA
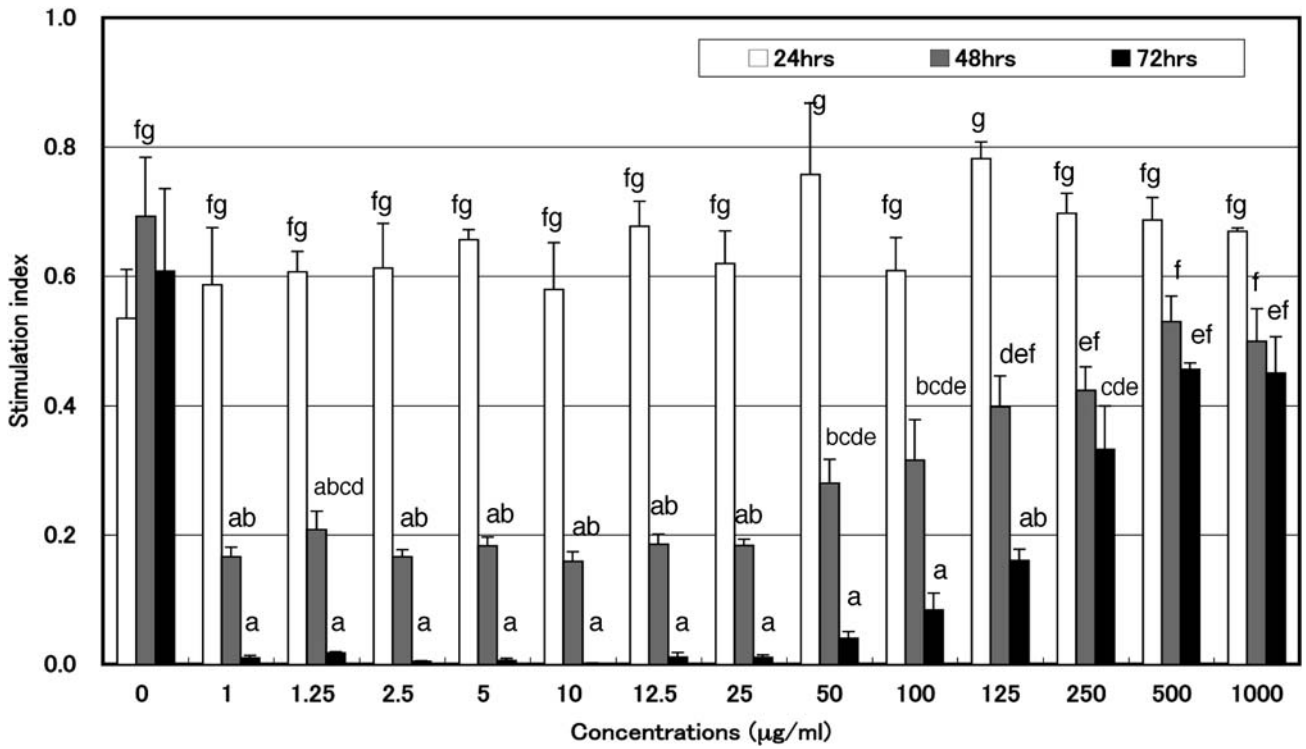(P<0.05 ; a<b<c<d<e<f<g<h)

Fig. 2　The activity of quail PBL by stimulation of various concentrations of PWM
　　　　　(P＜0.05 ; a＜b＜c＜d＜e＜f＜g＜h)
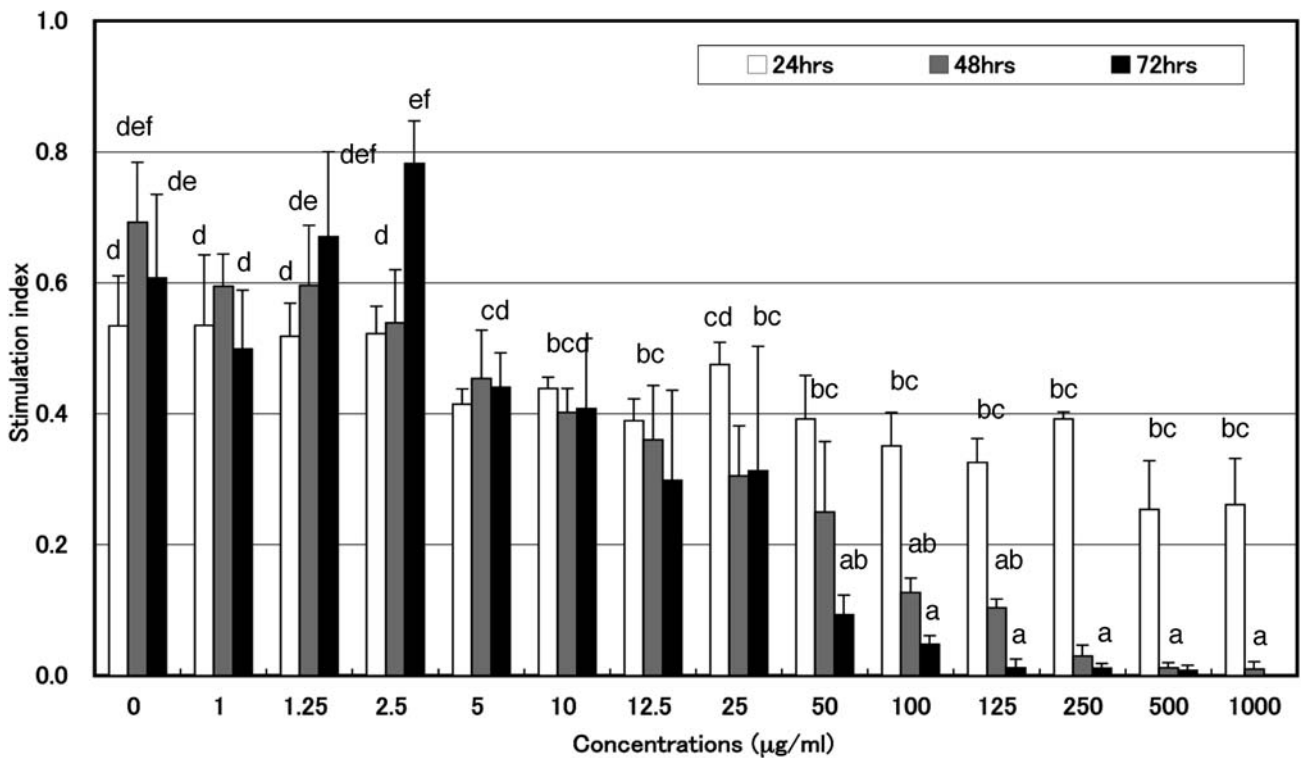
Fig. 3　The activity of quail PBL by stimulation of various concentrations of PHA-M
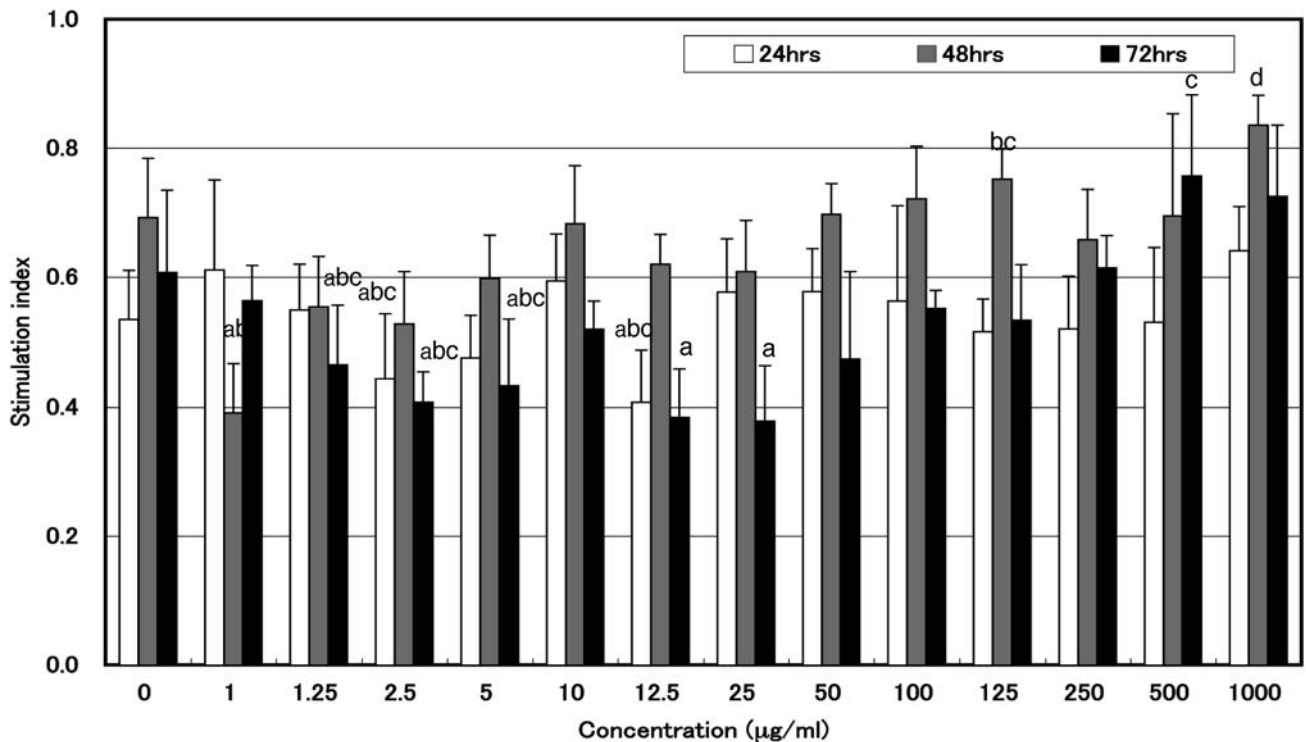　　　　　(P＜0.05 ; a＜b＜c＜d＜e＜f)

Fig. 4 The activity of quail PBL by stimulation of various concentrations of PHA-L
(P＜0.05 ; a＜b＜c＜d, no character : bcd)

of cytoplasmic immunogloblins[13]. T cell percentage in chicken blood as defined by an anti-CD3 monoclonal antibody (T3) is approximately 65–70％[16]. In other words, the percentage of PWM-responsive quail B cells is low, and other cellular interactions would be necessary to induce a response. It is assumed that the proliferation of T cells is markedly suppressed by culturing with low concentration of PWM for 48hr. This indicates that after 48 hrs of culture, the mitogen-induced suppression of T cells might be stronger than the activation of intracellular events.

The PHA-L induced activation response was different from that of the other mitogens and was dose-independent. Hovi *et al.* (1978) reported that the chicken PBL required 100 times more concentrated PHA than that required for human and mouse PBL[17]. However, the mitogens used in their experiment were highly purified and the concentrations were higher than general mitogens. These results suggest, however, that the components in a crude PHA extract, which stimulate chicken lymphocytes, might be different from those inducing DNA-synthesis in mouse or human cells, hence mitogens are not always restricted to a single component.

Barta *et al.* (1992) determined the optimum conditions of lymphocyte transformation tests for chicken (*Gallus gallus*) PBL[15] focusing on 4 factors : Cell densi-

ty, presence of fetal serum in media, incubation temperature, and incubation time. First, chicken buffy-coat cells obtained by slow centrifugation ($40 \times g$ for 10 min.) showed better response to mitogenic stimulation than lymphocytes isolated on separation media containing Ficoll. Maximum PBL responses were obtained using a cell density of 2×10^7 lymphoid cells/m*l*. An average of 10 m*l* in blood volume is available from 2–3 kg chicken. Otherwise, about 2 m*l* is the maximum volume of blood which can be obtained from a 100 g quail and contains 2×10^7 lymphocytes. Therefore, the optimal cell number for a PBL stimulation test in Japanese quails is 2×10^6 cells per well of the 96-well plate that we used in the present study. Second, responses of chicken lymphocytes to mitogens were greatest in a medium containing 5％ fetal bovine, 1.25％ pooled chicken serum or autologous plasma. When the 1.25％ chicken serum was used in the cultures, the greatest responses were generally obtained by final concentrations of 30～50 μg/m*l* either of ConA or PHA-P. The optimum concentration of PWM varied between 1 to 40μg/m*l* among the chickens and it was practically impossible to establish in general. Third, cell culture incubation in humidified air with 5％ CO_2 was significantly better at 40℃ than at 37℃. Lastly, the total culturing time of 40 hours including pulsing with 3H-thymidine during the final 16 hours of incubation was the best for ConA and

PHA-P stimulated cells, whereas a longer incubation of 64 hours made the best result in a PWM stimulation.

In conclusion, our overall results suggest that the optimal conditions for using PHA-L and PHA-M for the activation and proliferation of Japanese quail lymphocyte are under serum-free culturing conditions for up to 48 hrs at a concentration of $1000\,\mu g/ml$ and $1.25\sim2.5$ $\mu g/ml$. Further, our findings support the idea that the high dose of ConA can induce apoptosis in quail PBL. PWM had relatively little effect on the activation and proliferation in quail PBL, and our data indicates that particular lymphocytes subset appears to be highly activated by a high dose of PWM. Therefore, as a future study,we hope to able to separate Japanese quail PBL into several lymphocyte subsets to identify which cell types are responding to 4 different mitogens tested in this current study.

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PHA-M　　$1 \sim 2.5\,\mu\mathrm{g/m}l$　，48　72　　　　　，　　一
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