

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 $\mu\text{g/ml}$ for 48 h with PWM, 1.25~2.5 $\mu\text{g/ml}$ for 72 h with PHA-M, and 1~1,000 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$), but concentrations greater than 500 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ 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 cells¹⁾.

ConA is a lectin originally extracted from the jack-bean *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 mitogen²⁾ and has been used for the detection of chicken macrophages³⁾ 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 function⁶⁾. PHA is a lectin found in plants, especially red kidney beans, which contains potent, cell agglutinating and mitogenic activities⁷⁾. 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 lymphocyte 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 chicken⁹, duck and turkey PBL¹⁰, 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-lymphocytes². VAINIO *et al.* (1984) showed that PWM is able to induce a large number of B cells producing cytoplasmic immunoglobulin, and that the proliferative response of chicken PBL to PWM is macrophage dependent¹³.

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, Ca²⁺ and Mg²⁺ free), layered over a Percoll solution (Pharmacia : specific gravity 1.075 ± 0.005) in a 15 ml polystyrene tube, and centrifuged at 450 × g for 30 min at 20°C. The white layer of PBL was separated on the Percoll solution. This layer was collected, and the PBL were pelleted by centrifugation at 150 × g for 10 min at 10°C. 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 stain¹⁴, and adjusted to 2 × 10⁶/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 × 10⁶/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% CO₂ incubator at 40°C.

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 × g at 4°C. 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 H₂SO₄ 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 ± 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 $5 \mu\text{g/ml}$ for 48 and 72 hrs. However, the maximum level for PBL stimulation was reached at a dose of $2.5 \mu\text{g/ml}$ 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 ($0 \mu\text{g/ml}$). The responses of PBL after 72 hrs with 12.5 and $25 \mu\text{g/ml}$ 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-P¹⁵.

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

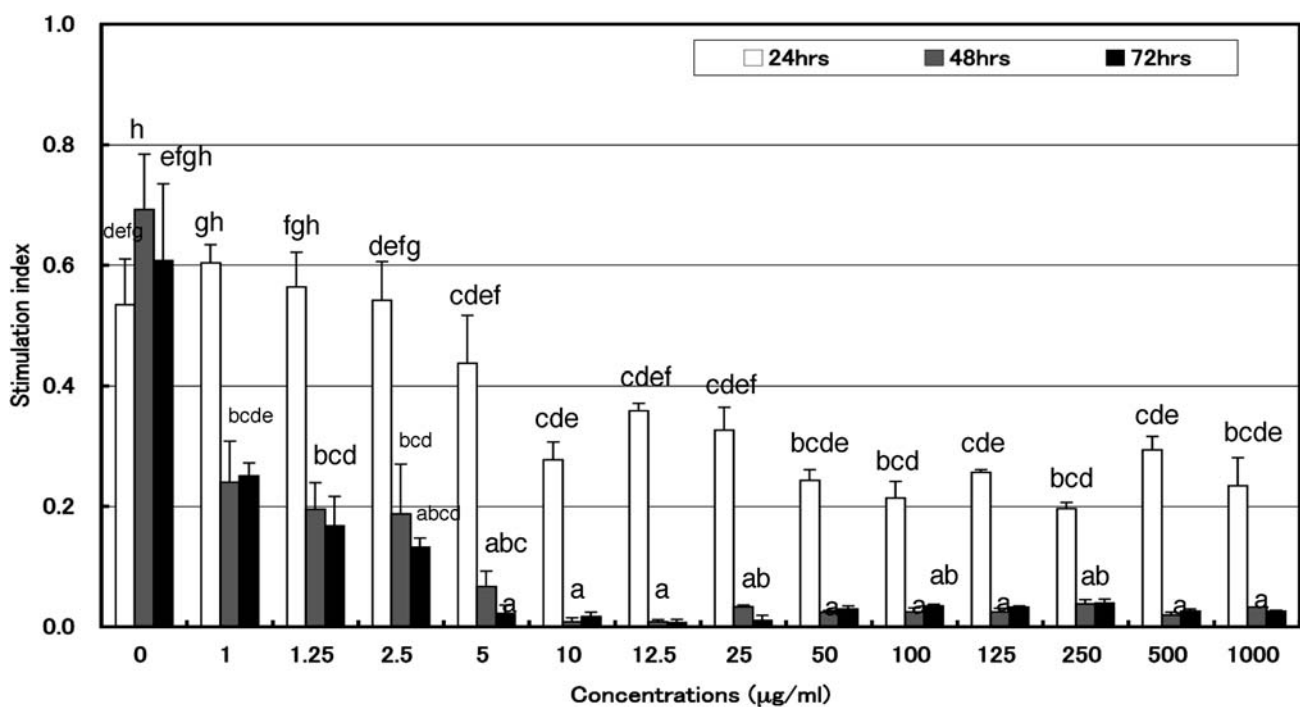


Fig. 1 The activity of quail PBL by stimulation of various concentrations of ConA ($P < 0.05$; $a < b < c < d < e < f < g < h$)

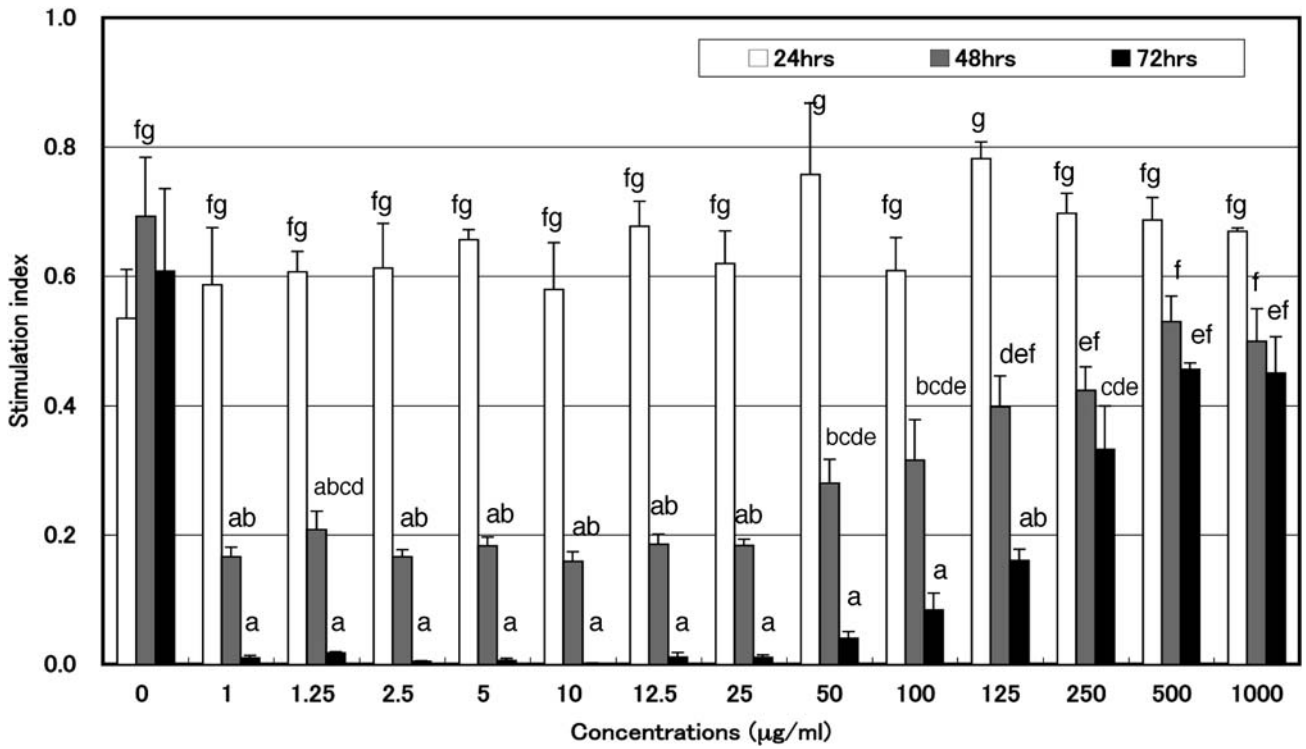


Fig. 2 The activity of quail PBL by stimulation of various concentrations of PWM ($P < 0.05$; $a < b < c < d < e < f < g < h$)

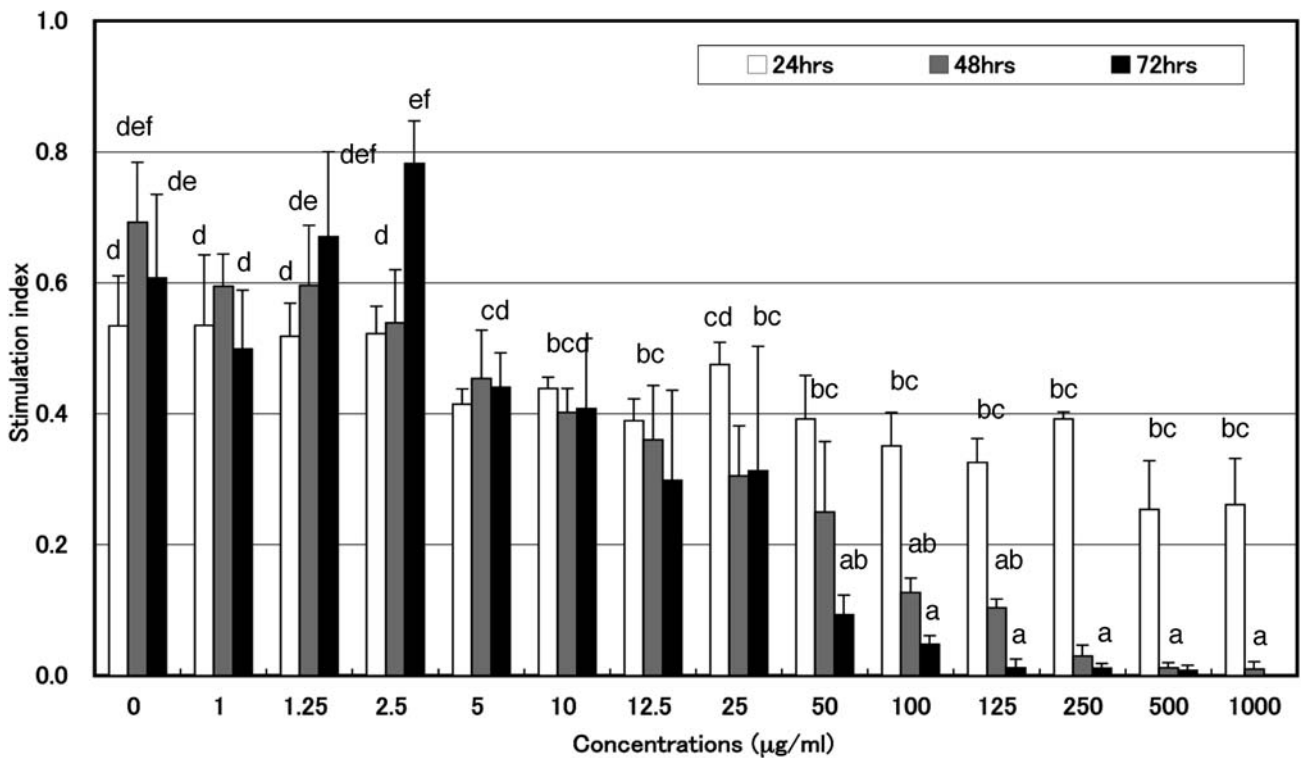


Fig. 3 The activity of quail PBL by stimulation of various concentrations of PHA-M ($P < 0.05$; $a < b < c < d < e < f$)

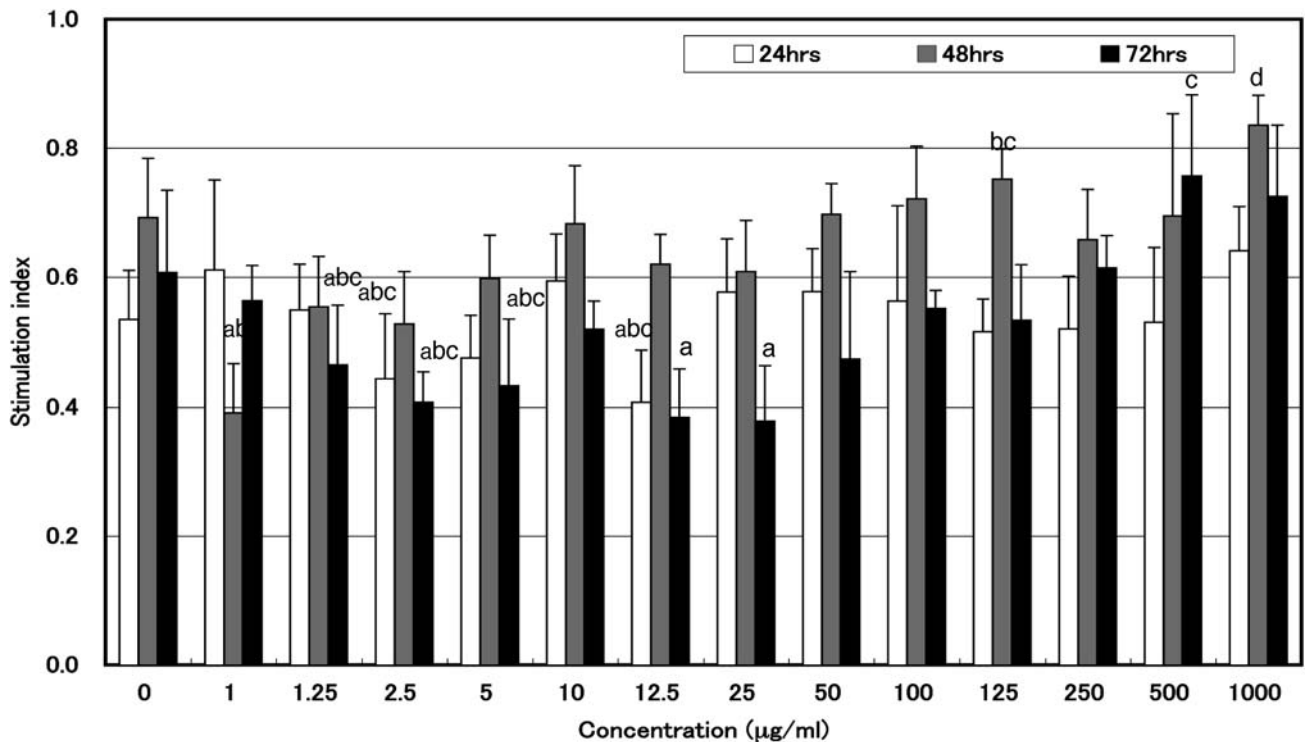


Fig. 4 The activity of quail PBL by stimulation of various concentrations of PHA-L ($P < 0.05$; $a < b < c < d$, no character : bcd)

of cytoplasmic immunoglobulins¹³). 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 PBL¹⁷). 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*) PBL¹⁵) 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/ml. An average of 10 ml in blood volume is available from 2–3 kg chicken. Otherwise, about 2 ml 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 $\mu g/ml$ either of ConA or PHA-P. The optimum concentration of PWM varied between 1 to 40 $\mu g/ml$ 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°C than at 37°C. 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 $\mu\text{g/ml}$ and 1.25~2.5 $\mu\text{g/ml}$. 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 be 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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ニホンウズラ (*Coturnix japonica*) 末梢血リンパ球 活性化における 4 種のマイトージェンの 培養至適濃度

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要約：ニホンウズラの末梢血リンパ球活性化における ConA, PHA-L, PHA-M と PWM のマイトージェンの至適培養濃度を検討した。末梢血よりリンパ球を Percoll 密度勾配遠心法により分離し、 $2 \times 10^6/ml$ 浮遊液に調製し、各マイトージェンを $0 \sim 1,000 \mu g/ml$ 添加した RPMI1640 培地（血清不含有）で 0, 24, 48, 72 時間培養した。各培養時間後に MTT アッセイにより細胞活性を測定し、培養 0 時間の測定値を 1 とし各培養条件での細胞活性率を算出した。その結果、ConA および PHA-M 刺激では濃度依存的に細胞活性率は低下した。一方、PWM 刺激時の細胞活性率は PWM 濃度依存的に上昇した。また、PHA-L 刺激時の細胞活性率は濃度によって異なる反応を呈した。すなわち PHA-L $1.0 \mu g/ml$ では、培養 48 時間後に低下した細胞活性率が 72 時間培養後に高くなり、濃度 $2.5 \mu g/ml$ 以上、培養 48 時間後の細胞活性率は他の 3 つのマイトージェンに比べて常に高く、さらに濃度 12.5 および $125 \mu g/ml$ では、48 時間培養後に最も高くなり、 $500 \mu g/ml$ では 72 時間培養後に最も高くなった。

マイトージェンとして最も利用されている ConA はニホンウズラの末梢リンパ球に対しては生存阻害的に働くことが示唆された。PWM は 4 つのマイトージェンの中で 24 時間培養後に全濃度において最も細胞活性率が高くなり、その活性化された細胞の作用によって 48 時間培養後の $1 \sim 25 \mu g/ml$ の低濃度で低下し、強い抑制細胞間相互作用が働いたことが示唆された。以上のことから、ウズラリンパ球に対し ConA は細胞死誘導に有効であり、PWM は一部の細胞に対してのみ高濃度の $500 \mu g/ml$ 以上で培養時間とその濃度を変えることにより特定なリンパ球サブセットを効率的に活性化しうることが示唆された。ウズラリンパ球に対して PHA-M は低濃度の $1 \sim 2.5 \mu g/ml$ 濃度、48 と 72 時間培養で活性化効果があり、最もマイトージェンとして適しているのは他のマイトージェンと顕著に異なり濃度による活性化抑制・阻害効果を示さない PHA-L であり、48 時間培養で $1 \sim 1000 \mu g/ml$ の広範囲な濃度で活性化効果が得られることが明確となった。

キーワード：ニホンウズラリンパ球、マイトージェン、ConA, PWM, PHA

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