Response of Lymphocytes of Japanese Quails to Mitogens

Hiroyuki TANIGUCHI, Yasuhiro YOSHIKAWA and Kazuya YAMANOUCHI

Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato, Tokyo 108

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ABSTRACT. Blastogenesis of quail lymphocytes with phytohemagglutinin (PHA) and bacterial lipopolysaccharide (LPS) were examined by incorporation of \(^3\)H-Thymidine as a parameter to evaluate lymphocyte functions. The optimal conditions of PHA-response were \(5 \times 10^8\) spleen cells/ml, 1.0 \(\mu\)g/ml PHA, 7.5\% fetal bovine serum (FBS), and 48 to 72 hr isotope labeling. In LPS-response, the conditions were essentially similar to PHA-response, except for 70 \(\mu\)g/ml LPS and 0.5\% FBS. Among the lymphoid cells, spleen cells were stimulated to proliferate by both PHA and LPS, while thymus cells by only PHA and bursal cells by none of these mitogens. Spleen cells from bursectomized quails responded to PHA similarly to those from sham-operated controls, while they completely lacking the response to LPS. To PHA a few thymectomized cases responded at relatively low level, whereas their response to LPS was normal. Immunofluorescence revealed immunoglobulin on the surface of LPS-induced blastoid cells but not of PHA-induced ones. These results suggest that T and B lymphocytes of quails respond to PHA and LPS, respectively, as observed were chicken lymphocytes and that mature lymphocytes are stimulated by mitogens at higher level than immature ones.

Proliferative response of lymphocytes to polyclonal mitogens is widely used as a parameter of the cell-mediated and humoral immune responses. It is well known in mammalian immune systems that phytohemagglutinin (PHA) and lipopolysaccharide (LPS) stimulate T and B lymphocytes, respectively. In avian immune system, PHA was also reported to induce the proliferation of chicken lymphocytes of the peripheral blood and spleen [1, 2, 3].

Japanese quails are recently paid much attention as an experimental animal for the analysis of host defence mechanisms against virus-induced tumors [4], however, very little is known about the functions of quail lymphocytes in in vitro system. In this study, we examined optimal conditions for mitogen-induced blastogenesis to establish the in vitro method to evaluate lymphocyte functions.

MATERIALS AND METHODS

Animals: Japanese quails (Coturnix coturnix japonica) raised in a closed colony in Nippon Institute for Biological Science, Tokyo, were used at the age of 4 to 8 weeks.

Mitogens: PHA-P (Difco Lab., Detroit, MI) was dissolved in 5 ml of sterile water. LPS from Escherichia coli (Lot 0117; Difco Lab.) was suspended in distilled water and autoclaved for sterilization. Another type of LPS prepared from Serratia marcescens (TCA extract; Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water and passed through a millipore filter. These mitogens were stored at ~80°C until used.

Preparation of lymphocytes: Quails were killed by cardiac puncture. The bursa of Fabricius, spleen and thymus were collected aseptically, minced and passed through a wire screen of 200-mesh with a spatula into a small volume of RPMI 1640 supplemented with 10\% fetal bovine serum (FBS), 60 \(\mu\)g/ml of kanamycin and 10 mM N-2-hydroxy piperazine N'-2-ethane sulfonic acid (HEPES). To remove the adherent cells, cell suspension in 60 mm plastic dish (Falcon 3002) was incubated at 37°C for 30 to 60 min and non-adherent cells were resuspended in the
growth medium under varying conditions.

Assay for PHA and LPS responsiveness: The cell suspensions with various concentrations ranging 1×10^6 to 1×10^7 cells/ml were mixed with various doses of PHA-P (0.25–10 μg/ml) or LPS (1.0–100 μg/ml which is a mixture of two types of LPS) and put in microtest plates (Falcon 3042) at 0.2 ml/well by using quadruplet wells for each sample and incubated at 37°C in CO2 incubator for various time length. Twenty-four hr before the harvest of cells, 1 μCi of 3H-Thymidine (20 Ci/m mol, The Radiochemical Centre Ltd, Amersham, England) was added to each well. The cells were collected on filters with a semi-automatic cell harvester (Labo-Mash; Labo Science, Tokyo), air dried, and placed in glass vials of the liquid scintillator. Radioactivity was counted in a Beckman liquid scintillation counter.

Thymectomy and bursectomy: Since the thymus of quail is too small to conduct thymectomy within 24 hr after hatch, thymectomy was performed at the age of 1 week. Before the thymectomy each 0.2 ml of anti-thymocyte serum (ATS) was injected subcutaneously at the age of 1, 3 and 7 days. Details of the preparation of the ATS were described previously [5].

The bursa was removed surgically with forceps within 24 hr after hatch. Then the bursectomized quails were injected twice intraperitoneally with 0.6 mg of cyclophosphamide (CY; Endoxan, Shionogi Seiyaku, Co. Ltd., Osaka, Japan) at the age of 1 and 3 days. Details of the bursectomy were described previously [6].

Immunofluorescence: Immunofluorescence was performed by indirect method employing rabbit anti-quail Ig serum [7] as first antisera and goat anti-rabbit IgG serum conjugated with fluorescein isothiocyanate (Cappel Lab.) as second one. To minimize non-specific reactions, the rabbit anti-quail Ig serum was absorbed several times with 3×10^6 quail thymus cells before use. Cultured lymphocytes were sedimented by centrifugation at 1,500 rpm for 10 min, fixed in acetone at -20°C for 20 min, and then reacted with first and second antiseraums at 37°C for 30 min each.

RESULTS

Optimal conditions for PHA-response of spleen cells:
The optimal conditions for PHA-induced blastogenesis of quail spleen cells were examined on the following four factors, i.e. PHA dose, serum concentration, spleen cell concentration, and time of isotope labeling.

Optimal dose of PHA was examined by incubating 5×10^6 spleen cells/ml in medium containing 10% FBS and varying doses of PHA, and by labeling with isotope from 48 to 72 hr. As shown in Fig. 1a, range of optimal dose of PHA was narrow, the maximum response being observed at dose of 1.0 μl/ml in which isotope incorporation was 9×10^4 cpm in contrast to 3×10^3 cpm in control one. The concentration higher than 5 μl/ml was cytotoxic to spleen cells. Optimal concentration of FBS was examined by fixing the other conditions as follows; 1.0 μl/ml of PHA, 5×10^6 cells/ml and isotope labeling from 48 to 72 hr. The highest incorporation of isotope was found with 7.5% FBS (Fig. 1b). FBS at higher or lower concentrations than 7.5% was insufficient for stimulation. Varying concentration of spleen cells from 1×10^6 to 1×10^7 cells/ml was examined by fixing the other conditions as follows; 1.0 μl/ml of PHA, 7.5% FBS and isotope labeling from 48 to 72 hr. Isotope incorporation increased in parallel with increasing concentration up to 5×10^6 cells/ml and reached plateau (Fig. 1c). Since it was technically difficult to obtain more than 5×10^7 spleen cells from an individual quail, we determined 5×10^6 cells/ml as an optimal concentration. Optimal starting time of isotope labeling which yields maximum response was found to be 48 to 72 hr of incubation (Fig. 1d).

From these results, the optimal conditions for PHA-response were determined as follows; spleen cells are suspended at a concentration of 5×10^6 cells/ml in RPMI 1640 medium containing 7.5% FBS and 1.0 μl/ml of PHA and pulse labeled with isotope for 48 to 72 hr of incubation.

Optimal conditions for LPS-response of spleen cells:
The optimal conditions for LPS-induced
Fig. 1. Response of spleen cells to PHA at varying conditions, i.e. dose of PHA (a), concentration of FBS (b), cell numbers (c), and time of isotope labeling (d).
- incorporation of isotope (cpm) in the presence of mitogens
- incorporation of isotope (cpm) in the absence of mitogens
Fig. 2. Response of spleen cells to LPS. See legend to Fig. 1.
blastogenesis of spleen cells were examined in an approach similar to PHA-response. Since the mixture of two LPS from *E. coli* and *S. marcescens* was found to induce proliferation of spleen cells more effectively than either LPS alone in preliminary experiments, LPS mixture consisting of 5 parts of *E. coli*-LPS and 2 parts of *S. marcescens*-LPS at concentration of 1 mg/ml each was used.

As shown in Fig. 2a, increase in isotope incorporation was observed in increasing dose of the LPS mixture up to 100 μg/ml, the highest dose tested. Since marked difference in response was not observed at doses between 50 and 100 μg/ml, the dose of 70 μg/ml was determined as a standard LPS dose in the subsequent experiments. The similar level of LPS response was observed for relatively broad range of FBS concentration of 0.5 to 5% (Fig. 2b). The concentration of 0.5% was determined as a standard one, because of low isotope incorporation in the absence of LPS. The cell concentration-dependent increase in isotope incorporation was observed from $1 \times 10^6$ to $1 \times 10^7$ cells/ml similar to PHA response, and the optimal cell concentration was determined to range between $5 \times 10^6$ and $1 \times 10^7$ cells/ml (Fig. 2c). Therefore, the concentration of $5 \times 10^6$ cells/ml was used in the subsequent experiments based on the same consideration as for PHA-response. The labeling time with isotope between 48 to 72 hr was found to induce the highest incorporation of isotope (Fig. 2d).

Thus, the optimal conditions for LPS response of spleen cells were determined as follows; LPS dose of 70 μg/ml, FBS concentration of 0.5%, cells concentration of $5 \times 10^6$ cells/ml and isotope labeling time between 48 and 72 hr of incubation.

**Proliferative response of thymus and bursal cells to PHA and LPS:**

The thymus and bursa of quails are considered to consist mainly of precursors of T cells and those of B cells, respectively. Thymus cells from 4 to 8 weeks old quails suspended in RPMI 1640 supplemented with 10% autologous serum were found to respond consistently to PHA but not to LPS (Fig. 3a). In contrast, thymus cells suspended in RPMI 1640 supplemented with various concentration of FBS or pooled quail serum failed to respond to PHA (data not shown).

Bursal cell suspensions prepared from the quails with the same ages as above-mentioned were incubated with various concentrations of PHA or LPS. They were also incubated in serum-free RPMI 1640 medium, F10 medium or either one of these media supplemented with various concentrations of FBS, pooled quail serum or autologous serum. For the observation period from 1 to 4 days, bursal cells responded neither to PHA nor LPS under any conditions above-mentioned. As shown in Fig. 3a, conditioned media prepared
from the supernatant fluids of spleen cell cultures in the presence of PHA or LPS, or 10% extracts of the thymus or bursa were added to the culture but failed to induce the response of bursal cells to PHA or LPS.

**Effect of thymectomy and bursectomy on proliferative response of spleen cells to mitogens:**

Bursectomized quails were confirmed to be of hypogammaglobulinemia by rocket immunoelectrophoresis, whereas thymectomized quails were found to have normal Ig level (data not shown). To identify the type of spleen lymphocytes responding to PHA or LPS, mitogen response of spleen cells were examined on thymectomized and bursectomized quails.

Spleen cells from many of thymectomized quails responded to PHA and LPS almost at normal level as quails with the intact thymus although major parts of the thymus lobes were removed. However, in a few cases of the thymectomized ones, spleen cells responded to PHA at considerably low level.

Spleen cells from bursectomized quails responded to PHA at low level but not LPS compared with the normal quails as shown in Fig. 3b.

**Detection of surface immunoglobulin:**

Presence of surface immunoglobulin was examined on mitogen-induced blastoid cells of the spleen as well as on thymus and bursal cells. Bursal cells and LPS-induced blastoid cells were found to bear the surface immunoglobulins, whereas thymus cells and PHA-induced blastoid cells were negative (data not shown).

**DISCUSSION**

Spleen lymphocytes of quails were shown to respond to PHA and LPS, both of which are mitogens widely used for chicken lymphocytes as well as for mammalian lymphocytes. Under the optimal conditions for the mitogen-induced blastogenesis determined in the present study, quail spleen cells proliferated consistently at relatively high level by PHA stimulus, but at low level by LPS stimulus. Such lower response of spleen lymphocytes to LPS than that to PHA had been confirmed for chicken lymphocytes [3, 8]. The optimal conditions for the response of spleen lymphocytes to PHA and LPS were essentially similar to those reported for the chicken lymphocytes [1, 2, 3].

Thymus cells responded to PHA consistently but not to LPS. Spleen cells from bursectomized quails also responded to PHA but not to LPS. Thymus cells are considered to consist mainly of precursors of T cells, and bursal cells to consist of precursors of B cells. Surface Ig was demonstrated neither in thymus cells nor in PHA-induced blastoid cells of the spleen. In contrast, surface Ig was present in both bursal cells and LPS-induced blastoid cells of the spleen. These results suggest that the PHA-responding cells consist of T cell populations and the LPS-responding cells consist of B cell populations, similar to the findings on lymphocytes of the human, mouse and chicken.

Bursal cells failed to respond to either LPS or PHA under the conditions tested. The similar result was previously reported for the chicken [3, 9]. We noticed that bursal cells were very fragile in vitro culture in the standard culture medium.

Since bursal cells consisting of immature B cells were speculated not to be viable under the ordinary culture condition, the following condition media were used: supernatant fluids of spleen cell cultures in the presence of PHA or LPS which are expected to contain factors analogous to
T cell replacing factor in mouse [10, 11], or putative B cell differentiation factor, and 10% extracts of the thymus or bursa which are also expected to contain thymic or bursal hormones [12, 13]. However, responses of bursal cells to mitogens were not induced under any of these conditions. Attempt of differentiation of bursal cells into LPS-responding cells in vitro by the bursal hormone remains for the further study.

Thymus cells failed to respond to PHA in the medium supplemented with FBS and pooled quail serum, however the addition of 10% autologous serum into the medium conferred the responsiveness. The result may suggest that stabilizing effect on fragile thymus cells are exerted by autologous serum but not by heterologous or allogeneic sera.

Spleen cells from many of thymectomized quails responded to PHA and LPS almost at normal level as those with the intact thymus, whereas significant suppression of PHA response observed in a few thymectomized quails. Since the thymectomy was performed 1 week after hatch, mature thymus cells are speculated to have migrated into the circulation during this one-week period. Because of small size of the thymus lobes even at 1 week of age, incomplete removal of the thymus may also be responsible for the positive PHA response in thymectomized quails.

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REFERENCES

要約

ウズラ・リンパ球のミトオージェンに対する反応：谷口博之・吉川泰弘・山内一也（東京大学医学部医学研究所実験動物研究部門）——実験用小型鳥類としての有用性が期待されているウズラのリンパ球のミトオージェンによる幼若化反応の至適条件を検討し、反応に関与するリンパ球の同定を試みた。マイクロプレートを用いて RPMI 1640 培地で培養。PHA または LPS を加えて ³H-チミジンのとりこみを測定すると、4～8 週齢のウズラの脾リンパ球は PHA および LPS に対してよく反応し、至適条件は細胞数：5×10⁶ 個/ml、PHA：1 μl/ml、LPS：70 μg/ml。ウシ胎仔血清濃度：7.5%（PHA）、0.5%（LPS）、アイソトープのラベル時間：48～72時間であった。胸腺リンパ球は、ウシ血清に代えて自己ウズラ血清を用いた場合、PHA に対してのみ反応したが、骨髓リンパ球は PHA、LPS のいずれに対しても反応しなかった。胸腺摘出ウズラの脾リンパ球は同ミトオージェンに反応し、骨髓出ウズラのそれは PHA に対してのみ反応した。胸腺・脾の PHA 反応性リンパ球は表面免疫グロブリン（S-Ig）を欠き、骨髓リンパ球および脾の LPS 反応性リンパ球は S-Ig 陽性であった。以上の成績から、ウズラ脾リンパ球の T 細胞に対しては PHA が、B 細胞に対しては LPS が、それぞれ特異的に働かと考えられた。