Mitogenic Responses of Lymphocytes from Two Lines of Chickens Selected for High and Low Competences in Graft-versus-Host Reaction.

Suguru MASHIMA, Jin-ichi OKUMURA¹, Ikuo OKADA²
and Kouzaburoh YAMAMOTO¹

Graduate School of Science and Technology, Niigata University,
Niigata 950-21.

¹Faculty of Agriculture, Niigata University,
Niigata 950-21.

²Faculty of Applied Biological Science, Hiroshima
University, Higashi-Hiroshima 724

Two White Leghorn lines, HA and LA, which were developed by selection for high and low competences in graft-versus-host reaction (GVHR) and found to differ in resistance to Marek's disease, were compared for their responses to the mitogenic lectins *in vitro*. The numbers of peripheral blood lymphocytes were similar in both lines. The HA line showed higher incorporation of ³H-thymidine than the LA line, when they were stimulated with Con A and PHA. These results have suggested that the difference in response to the mitogens between the lines is attributed to their lymphoproliferative ability. Based on these findings, the relationship between resistance to MD and lymphoproliferative ability was discussed.

(*Jpn. Poult. Sci.*, **29** : 98-105, 1992)

Key words : Con A, PHA, mitogenic response, graft-versus-host reaction, chickens.

Introduction

The White Leghorn lines, HA and LA were developed by selection for high and low donor competences in GVHR from the same base population^{1,2)}. The HA line also showed high competence in immune response to diphenyl group and bovine serum albumin³⁾. On the contrary, the LA line showed low immune response and showed strong resistance to Marek's disease (MD) compared with the HA line⁴⁾.

It has been suggested that the resistance to MD correlates with lymphoproliferative ability in chickens^{5,6)}. So, lymphoproliferative ability was compared between the two lines by measuring response to mitogenic lectins *in vitro*.

Materials and Methods

Chickens

Chickens used in the present study were two White Leghorn lines, HA and LA, which were established by selection for high and low GVHR competences. Both lines have the same genotype at the major histocompatibility B complex (MHC) of chickens.

Received Jun. 26. 1991

A group of ten birds was prepared from both lines and used for each experiment. Culture conditions were determined by using outbred male chickens aged 9 to 11 weeks old.

Mitogenic lectins

Phytohaemagglutinin-p (PHA-p, L-9132, Lot No. 58F-9408, Sigma Chemical Co., St. Louis, MO, USA) and Concanavalin A (Con A, Lot No. OD 06496, Pharmacia, Tokyo, Japan) were used as mitogen. Optimum dose of mitogen was examined at the range of 5 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ for Con A and 15 $\mu\text{g/ml}$ to 30 $\mu\text{g/ml}$ for PHA-p as final concentration. The culture was performed as described below.

Mitogen culture

The responsiveness to mitogenic lectins was examined at 3-, 6-, and 9-weeks of age by the method previously described^{7,8)}. Briefly, 0.2 or 0.5 ml of blood was collected with heparinized syringe from a wing vein and diluted to 10 ml with RPMI-1640 medium (Sigma Chemical Co.) supplemented with penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g/ml}$). One ml of the suspension and 50 μl of the mitogen solution were dispensed into a culture tube (No. 2058, Falcon, Oxnard, CA, USA). For the control, the same amount of the medium was dispensed instead of mitogen solutions. The culture was conducted at 40°C in a 5%-CO₂ atmosphere for 72 hr., and pulse-labeling was performed with 18.5 kBq of ³H-thymidine (NET-027, 247.9 GBq/mmol, New England Nuclear Boston, MA, USA) during the last 24 hr. Each experiment was carried out in triplicate.

Determinations of ³H-thymidine incorporation

The culture tubes were centrifuged at 3000 rpm (1660 $\times g$) for 20 min. The sediment was washed twice with ice cold 10% trichloro acetic acid solution. After discarding the supernatant, the sediment was dissolved with 0.5 ml of 10% sodium lauryl sulfate solution and treated with 100 μl of 30% H₂O₂ to avoid color quenching. Radio activity was counted with liquid scintillation counter (Aloka, Tokyo, Japan) by using the Aquasol-2 (New England Nuclear) for the scintillation cocktail. Data was expressed as Δ cpm and stimulation index (S.I.). Those values were expressed by the formula :

Δ cpm = mean cpm of mitogen stimulating cultures - mean cpm of control cultures.

$$\text{Stimulation index (S.I.)} = \frac{\text{mean cpm of mitogen stimulating cultures}}{\text{mean cpm of control cultures}}$$

Statistical analysis

Data was evaluated by analysis of variance using the least squares method of HARVEY⁹⁾.

Result

Optimum culture condition

Suitable dilution of blood was examined for the culture at 40°C. The results showed that 1 : 50 of dilution was more effective than 1 : 20 (Fig. 1). Therefore, a dilution 1 : 50 was chosen for the further experiments.

Next, optimum concentrations of the mitogenic lectins were examined and the

Fig. 1. Effect of different dilutions of blood on incorporation of ^3H -thymidine. C: control cultures. T: 100 $\mu\text{g}/\text{ml}$ of PHA treated cultures.

results are shown in Fig. 2. It was found that suitable concentrations was 20 $\mu\text{g}/\text{ml}$ for Con A and 25 $\mu\text{g}/\text{ml}$ for PHA-p. So, the comparison of mitogenic responses between the lines were conducted at the 1:50 dilution of blood and at the concentration of 20 $\mu\text{g}/\text{ml}$ for Con A and 25 $\mu\text{g}/\text{ml}$ for PHA-p.

Difference in mitogenic responses between the HA and LA lines

As shown in Table 1, Δcpm values of the HA line tended to be higher than those of the LA line for both lectins. Significant differences were observed at 3 and 6 weeks of age for Con A, and at 6-weeks of age for PHA-p.

The differences in stimulation indices (S. I.) between the lines were less clear-cut as compared with Δcpm (Table 2). However, significant differences were obtained at 3 weeks of age for Con A, and at 6 weeks of age for PHA-p. The lower level of significance for the line difference in S.I. as compared with Δcpm was noted to associate with incorporation levels of ^3H -thymidine in each control culture. Indeed HA line showed higher incorporation levels of ^3H -thymidine in the control culture than the LA line (Table 3).

Fig. 2. Dose-response relationship of mitogens and ³H-thymidine incorporation by peripheral blood cells of chickens. (A) Con A, (B) PHA-p. Blood was diluted 1:50. Other conditions of the experiment are given in Materials and Methods.

Table 1. The comparison of Δ cpm between the HA and LA lines in mitogen stimulating cultures

Age (wk)	line	No. of birds	Mitogen	
			Con A	PHA-p
3	HA	10	69,584.0 $\pm$ 9,404.0 ^a ¹⁾	12,808.0 $\pm$ 3,404.0 ^a
	LA	10	20,759.0 $\pm$ 9,404.0 ^b	11,363.0 $\pm$ 3,404.0 ^a
6	HA	10	117,646.5 $\pm$ 11,253.1 ^a	101,965.7 $\pm$ 11,935.5 ^a
	LA	10	45,060.4 $\pm$ 11,253.1 ^b	36,378.7 $\pm$ 11,935.5 ^b
9	HA	10	137,306.1 $\pm$ 24,872.9 ^a	44,365.3 $\pm$ 14,798.9 ^a
	LA	10	116,094.8 $\pm$ 24,872.9 ^a	41,109.1 $\pm$ 14,798.9 ^a

¹⁾ For each group, the means with different superscript within column are significantly different ($P < 0.01$).

In addition, no significant differences were seen for the numbers of peripheral blood lymphocytes between the lines (Table 4). So it was suggested that the difference in Δ cpm between the lines did not depend on the difference in the numbers of peripheral blood lymphocytes.

Fig. 3 A-C show the relationship between responses to Con A and PHA-p. Although no clear-cut relation was observed between the responses to both mitogens, obvious line difference was observed. The responses to both lectins at 6 weeks of age formed the separate clusters for each line (Fig. 3 B).

Table 2. The comparison of stimulation index between the HA and LA lines in mitogen stimulating cultures

Age (wk)	line	No. of birds	Mitogen	
			Con A	PHA-p
3	H A	10	54.63 $\pm$ 7.39 ^a ¹⁾	11.58 $\pm$ 3.26 ^a
	L A	10	22.07 $\pm$ 7.39 ^b	12.95 $\pm$ 3.26 ^a
6	H A	10	101.51 $\pm$ 16.55 ^a	192.60 $\pm$ 25.88 ^a
	L A	10	87.18 $\pm$ 16.55 ^a	32.02 $\pm$ 25.88 ^b
9	H A	10	85.02 $\pm$ 19.13 ^a	28.25 $\pm$ 10.58 ^a
	L A	10	105.00 $\pm$ 19.13 ^a	41.05 $\pm$ 10.58 ^a

¹⁾ For each group, the means with different superscript within column are significantly different ($P < 0.05$).

Table 3. Spontaneous uptake of ³H-thymidine by blood cells of the HA and LA lines¹⁾

line	No. of birds	Age in weeks		
		3	6	9
H A	10	1,357.3 $\pm$ 101.1 ^a ²⁾	1,274.4 $\pm$ 103.7 ^c	1,761.3 $\pm$ 181.5 ^a
L A	10	992.4 $\pm$ 101.1 ^b	667.6 $\pm$ 103.7 ^d	1,250.7 $\pm$ 181.5 ^a

¹⁾ Expressed with cpm.

²⁾ Means with different superscript within a column are significantly different (^{a, b} $P < 0.05$, ^{c, d} $P < 0.01$).

Table 4. The comparison of lymphocyte numbers in peripheral blood between the HA and LA lines¹⁾

line	No. of birds	Age in weeks		
		3	6	9
H A	10	19,700 $\pm$ 924 ^a ¹⁾	21,600 $\pm$ 707 ^a	21,100 $\pm$ 1,141 ^a
L A	10	19,800 $\pm$ 924 ^a	22,200 $\pm$ 707 ^a	21,800 $\pm$ 1,141 ^a

¹⁾ Lymphocytes numbers in 1 μ l of peripheral blood.

²⁾ Means with different superscript within a column are significantly different ($P < 0.05$).

Discussion

Many investigators performed culture of chicken lymphocytes at the temperature 40°C¹⁰⁻¹²⁾. We followed that condition. Previously, LASSILA *et al.* stated that a dilution 1 : 50 was optimum for the whole blood culture system⁸⁾. Contrary to this, KONDO and his colleagues diluted whole blood at 1 : 20 and cultured at the temperature 37°C⁷⁾. The result from present study supported the report of LASSILA and his colleagues⁸⁾. So, it is considered that 1 : 50 of blood dilution is suitable for culturing at 40°C.

Mitogenic responses to Con A and PHA are specific for T lymphocytes in

Fig. 3. Relationship between mitogenic responses to Con A and PHA-p (cpm). Open circles and closed circles represent the individuals from the HA and LA line of chickens, respectively, at the age of (A) : 3-wk., (B) : 6-wk. and (C) : 9-wk. The remainder of legend is the same as in Fig. 2.

chickens^{13,14}. Con A stimulation was shown to be under the control of MHC¹⁵. The PHA response is also under genetic control¹⁶. The present study suggested that responses to these lectins are also under genetic control but the gene(s) are not linked to MHC, since both HA and LA line have same genotype for the MHC complex¹.

The high responder to Con A was not always a high responder to PHA-p (Fig. 3 A-C). The spectra of T lymphocytes subset may be different in the responses to Con A and PHA-p. HOGARTH and MCKENZIE stated that Con A reactive T cells and PHA reactive T cells were distinguished with the surface antigen Ly-7 in mice¹⁷. Such antigen determinants may also exist in T lymphocytes of chickens.

The birds from the HA line showed a strong response to both lectins as compared with the LA line. Con A and PHA-p stimulate T lymphocytes, but do not activate B lymphocytes^{13,14}. So, it is considered that the HA line is higher than the LA line in their competences of either proliferation of T cells or the cooperative effect of antigen presentation cells to T lymphocytes or both. This phenomenon agreed with *in vivo*

response of GVHR^{1,18)}. Since GVHR is thought to be a T lymphocyte dependent function^{19,20)}, the line differences in mitogenic responses to Con A and PHA-p may also reflect the differences in responses of T lymphocytes *in vivo*.

Previous investigations clearly demonstrated that resistance to MD is strongly affected by MHC haplotype^{21,22)}. And it has also been suggested that resistance to MD is associated with lymphoproliferative ability^{5,6)}. In addition, difference in resistance to MD between MHC compatible inbred lines of chickens, RPRL 6₃ and 7₂ was also indicative of the association with lymphoproliferative ability¹⁴⁾. Present investigation agrees with the observation of FREDERICKSEN and GILMOUR¹⁴⁾ and emphasizes the relationship between lymphoproliferative ability and resistance to MD. The inter-line difference in resistance to MD between HA and LA⁴⁾ is thought to be caused by the difference in lymphoproliferative ability of these lines.

References

- 1) OKADA, I and H. MIKAMI (1974) Three generations of selection for high and low donor competences of splenomegaly in chickens. Br. Poult. Sci., **15**: 1-10.
- 2) OKADA, I. (1982) Interaction of *B* locus and GVHR-selected lines in the graft-versus-host reaction in chickens. Anim. Blood Group. Biochem. Genet., **13**: 273-278.
- 3) YANAGIMOTO, T. and I. OKADA (1980) Evidence for major histocompatibility linked and non-linked genetic regulation of immune response in chickens. Jpn. J. Zootech. Sci., **51**: 127-134.
- 4) OKADA, I. and Y. YAMAMOTO (1987) Immunocompetences and Marek's disease resistance in three pairs of chicken lines selected for different immunological characters. Poult. Sci., **66**: 769-773.
- 5) PAZDERKA, F., B.M. LONGENECKER, G.R.J. LAW, H.A. STONE and R.F. RUTH (1975) Histocompatibility of chicken populations selected for resistance to Marek's disease. Immunogenet., **2**: 93-100.
- 6) LEE, F.L. and L.D. BACON (1983) Ontogeny and line differences in the mitogenic response of chicken lymphocytes. Poult. Sci., **62**: 579-584.
- 7) KONDO, Y., Y. TANIGUCHI, Y. SAKOE and A. ABE (1988) Effect of starvation on humoral and cell-mediated immune responses. Jpn. Poult. Sci., **25**: 330-336. *in Jananese*.
- 8) LASSILA, O., J. ESKOLA and P. TOIVANEN (1976) A micro method for stimulation of chicken lymphocytes *in vitro* using whole blood. Clin. Exp. Immunol., **26**: 641-646.
- 9) HARVEY, W.R. (1960) In: Least-squares analysis of data with unequal subclass number. pp. 1-91. Agricultural Research Service. USDA. OH.
- 10) VAINIO, O. and M.J.H. RATCLIFFE (1984) Proliferation of chicken peripheral blood leukocytes in response to Pokeweed mitogen is macrophage dependent. Cell. Immunol., **85**: 235-243.
- 11) HÁLA, K., K. SCHAUENSTEIN, K. NEU, G. KROMER, H. WOLF, B. BOCK and G. WICK (1986) A monoclonal antibodies reacting with a membrane determinant expressed on activated chicken T lymphocytes. Eur. J. Immunol., **16**: 1331-1336.
- 12) KNUDTSON, K.L., M.G. KAISER and S.J. LAMONT (1990) Genetic control of Interleukine-2-like activity is distinct from that of mitogen response in chickens. Poult. Sci., **69**: 65-71.
- 13) WEBER, W.T. (1970) Mixed lymphocytes interaction and PHA response of chicken spleen cells in a chemically defined medium. J. Reticuloendoth. Soc., **8**: 37-54.
- 14) FREDERICKSEN, T.L. and D.G. GILMOUR (1983) Ontogeny of Con A and PHA responses of chicken blood cells in MHC-compatible lines 6₃ and 7₂. J. Immunol., **130**: 2528-2533.
- 15) MIGGIANO, V., M.NOUTH, A. BUDER and J.R.L. PINK (1976) Genetic control of the

- response of chicken leukocytes to T-cell mitogen. *Nature*, **263** : 61-63.
- 16) LASSILA, O., T. NURMI and J. ESKOLA (1979) Genetic differences in the mitogenic responses of peripheral blood lymphocytes in the chicken. *J. Immunogenet.*, **6** : 37-43.
 - 17) HOUGARTH, P.M. and I.F.C. MCKENZIE (1984) lymphocyte antigen. In : *Fundamental Immunology* (PAUL, W.E., ed.) pp. 457-477. Raven Press, New York.
 - 18) OKADA, I., Y. YAMAMOTO and M. MIZUYAMA (1987) Parabiosis between avian embryos selected for high and low competences of the graft-versus-host reaction. *Poult. Sci.*, **66** : 1090-1094.
 - 19) HÁLA, K., R.L. BOYD, H. WOLF, G. BOCK and G. WICK (1984) Functional analysis of B-L (Ia-like) antigen-bearing chicken peripheral blood cells. *Scand. J. Immunol.*, **20** : 15-19.
 - 20) WICK, G., K. HÁLA, H. WOLF, R.L. BOYD and K. SCHAUENSTEIN (1984) Distribution and functional analysis of B-L/Ia antigens on thyroid epithelial cells in spontaneous autoimmune thyroiditis. *Mol. Immunol.*, **21** : 1259-1265.
 - 21) BRILES, W.E., R.W. BRILES, R.E. TAFFTS and H.A. STONE (1983) Resistance to malignant lymphoma in chickens is mapped to subregion of major histocompatibility (*B*) complex. *Science*, **219** : 977-979.
 - 22) SCHIERMAN, L.W and W.M. COLLINS (1987) Influence of the major histocompatibility complex on Tumor regression and immunity in chickens. *Poultry. Sci.*, **66** : 812-818.

移植片対宿主反応によって二方向選抜された HA および LA 鶏における T リンパ球の増殖能について

真島 傑・奥村仁一¹・岡田育穂²・山本興三郎¹

新潟大学自然科学研究科 新潟市 950-21

¹新潟大学農学部, 新潟市 950-21

²広島大学生物生産学部 東広島市 721

HA 系統と LA 系統の鶏は移植片対宿主反応における脾腫能力の違いによって選抜され、マレック病に対して異なった抵抗性を示すことが知られている。本実験では、3, 6, 9 週齢時における T リンパ球のマイトーゲンに対する反応性を両系統間で比較した。

両系統間の値を Δ cpm で示すと、Con A と PHA のいずれのマイトーゲンを用いても、HA 系統で LA 系統よりも高い値が示された。特に 6 週齢では、Con A を用いた際、LA 系統の平均値が 45060.43 ± 11253.08 であったのに対して、HA 系統は 117646.54 ± 11253.08 と 2 倍以上も高い値を示した。また刺激指数は 3, 6 週齢で HA 系統が高く、9 週齢で LA 系統が高い傾向にあった。しかしながら、有意な差が認められたのは、Con A を用いた際の 3 週齢と PHA を用い

た際の 6 週齢だけであった。マイトーゲンで刺激を加えない培養においても、HA 系統は LA 系統に比べ、³H-チミジンの取り込みが高く、3 および 6 週齢で有意な差が認められた。また、末梢血中に含まれていたリンパ球の数は LA 系統で高い傾向にあったが、いずれの週齢でも有意な差が認められなかった。以上の結果より、両系統間で示されるマイトーゲンに対する反応性の違いは、リンパ球の増殖能の違いによってもたらされているものと推察された。そしてさらに、マレック病に対する抵抗性とリンパ球の増殖能の関連について考察が行なわれた。

(家禽会誌, **29** : 98~105, 1992)

キーワード : Con A, PHA, マイトーゲン反応, 移植片対宿主反応