GENETICAL AND DEVELOPMENTAL STUDIES ON THE "Ph" AGGLUTINOGEN IN THE CHICKEN RED CELLS

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The extracts from *Pisum sativum* seeds (PHA) are capable of agglutinating red cells obtained from only egg-laying females in the chicken, but in these egg-laying females some are responsive to agglutination and others fail to be agglutinated. The restriction of red cell agglutinability to egg-laying females is due to the activity of estrogen in egg-laying females which may stimulate the production of the "Ph" agglutinin. This is based on the result of the correlation between the egg production and the red cell agglutinability. The agglutinability become stronger or weaker as the egg production rises and falls. The occurrence of the agglutinin designated "Ph" is restricted to the red cells from egg-laying females of certain strains and the data on the cross of agglutinable strain (BM-C) with non-agglutinable strain (WL-M) suggest that the appearance of "Ph" agglutinin is dependent on genotype as well as hormone (Mizutani and Fujio 1971).

Scheinberg and Reckel (1962) reported on the effects of estrogen and genotype on the production of the "Hi" agglutinin responsive to the extracts from *Lathyrus cicera* and *Pisum arvense* seeds. Salminen (1959, 1962) found that the tick borne encephalitis virus agglutinated red cells from mature male chickens but did not agglutinate those from mature female chickens and that the red cell agglutinability in males was suppressed immediately by an injection of estrogen. Similar phenomenon was found in Japanese quail whose red cells were agglutinated by the extracts from *Glycine max* seeds (Mizutani *et al.* 1973).

In this paper, it is shown that the "Ph" agglutinin was produced in red cells from a certain genotype with an action of estrogen and that the appearance of the agglutinin was age-dependent.

MATERIALS AND METHODS

The seed extracts (PHA) were obtained as follows: the seeds of *Pisum sativum* were soaked in phosphate buffered saline (PBS) and homogenized in equal volume of PBS by a homobrender. The homogenates were passed through a coarse filter to remove debris. The juice was heated at 60°C for 30 minutes and centrifuged at 10,000 rpm for

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30 minutes to obtain clear supernatants. The agglutination test and scoring were performed by the same procedures as those in the previous report (Mizutani and Fujio 1971).

The inbred strains of chickens, Black Minorca C (BM-C) and White Leghorn M (WL-M) were used in the present work. The former is a strain whose red cells are agglutinable and the latter is a strain whose red cells are not agglutinable. Some chicks were given an injection of diethylstilbestrol (10.0 mg) intramuscularly.

RESULTS

1. *Differential appearance of the "Ph" agglutininogen in different ages and sexes.*

The result of tests for the agglutinability of red cells from egg-laying females is shown in Table 1. Red cells from all BM-C egg-laying female chickens were agglutinated but those from all WL-M egg-laying female chickens were not agglutinated. On the other hand, the result of tests for the agglutinability of red cells from mature non-laying females and males showed that red cells from both strains failed to be agglutinated. The red cells from 10 week old chickens were not agglutinated regardless of sex and strain. In these two inbred strains tested through more than 5 generations, there was no segregation of chickens on the basis of the agglutinability of their red cells to PHA in the egg laying state. In the crosses between BM-C and WL-M, all F₁ female progenies showed the presence of agglutininogen in their red cells in the egg laying state. This fact suggests that the agglutinability appears to be hereditary

Table 1. Differential appearance of the "Ph" agglutininogen in red cells from chickens of different ages and sexes

Strain	Sex and age	No. of chickens tested	Response of red cells to PHA	
			Agglutinable	Non-agglutinable
BM-C	male (10-week)	10	0	10
	female (10-week)	10	0	10
	male (adult)	21	0	21
	female (laying)	43	43	0
	female (non-laying)	15	0	15
WL-M	male (10-week)	10	0	10
	female (10-week)	10	0	10
	male (adult)	19	0	19
	female (laying)	37	0	37
	female (non-laying)	12	0	12
(WL-M × BM-C)F ₁	male (10-week)	10	0	10
	female (10-week)	12	0	12
	male (adult)	4	0	4
	female (laying)	12	12	0
	female (non-laying)	5	0	5

Fig. 1. Age-dependent changes in agglutination scores of "Ph" agglutinin in red cells from the BM-C, WL-M, and their hybrid strains.

by a dominant gene.

To test whether red cells from embryos of these strains are agglutinated with seed extracts or not, red cells were obtained from the BM-C, WL-M, and their hybrid embryos at various developmental stages. The results are shown in Fig. 1. It is indicated that red cells from embryos before 11th day in their development were agglutinable regardless of sex and strain. The agglutination score showed a high level in 6th day embryos in all strains, decreased with development of embryos, and reached to zero in the BM-C, WL-M, and their hybrids, at the stages of 3 week old chicks, 14th day embryos, and 13th day embryos, respectively. Thus, the agglutinability of red cells from the WL-M and F₁ hybrid was lost earlier than that from the BM-C. From the curves of decreasing agglutinability, it was indicated that with regard to the agglutinability of red cells the F₁ hybrids were similar to the BM-C strain at the stage of 6-12th day embryos and were similar to the WL-M strain after the stage of 13th day embryos.

Since the time of reappearance of agglutinin was restricted to the egg-laying females in the BM-C strain, it seemed that the production of agglutinin might be associated with female sexual maturity, that is, the egg production. Red cells from mature females were tested for their agglutinability at various times before and after the first egg production (Fig. 2). The agglutinability was not found before the onset of egg laying in both BM-C and WL-M strains. In the BM-C strain, the agglutinability appeared within 1 week after the onset of egg laying but not in the WL-M strain. When the agglutinability of red cells and the egg production in percent in egg-laying females were recorded weekly, an intimate correlation was found between these two properties, that is, the agglutination become stronger or weaker as the egg production

Fig. 2. The relationship between the cumulative egg production and the agglutinability of red cells in the BM-C and WL-M strains. Solid line: agglutination; Broken line: egg production.

risers and falls. This fact suggests that the production of "Ph" agglutinin is affected by two factors, the genotype and the egg production.

2. Genetic analysis

To elucidate the mode of inheritance of the production of agglutinin in red cells, BM-C chickens were crossed to WL-M chickens. In the egg laying state all F_1 female progenies showed the presence of agglutinin in their red cells, and F_2 female progenies were classified into two groups, 24 females which had agglutinin and 10 females which had no agglutinin in their red cells (Table 2). The ratio between the numbers in these two groups was 2.4:1.0 which corresponded significantly to 3:1 in Mendelian ratio ($P > 0.5$). This indicates that the inheritance of agglutinin in red cells was controlled by a single dominant gene. This gene was not sex-linked from the data on the reciprocal crosses of the BM-C and WL-M strains. However, the action of this gene was expressed in red cells from egg-laying females since red cells from early embryos were not dependent on this gene. It is suggested that the red cell agglutinability may be controlled by another structure gene and the gene described above may be a regulatory gene and the function of the latter starts about 11th day of incubation.

Furthermore, the cross experiment was performed to examine the red cell agglutinability from 18th day embryos. The results showed that red cells from agglutinable strain (BM-C) were agglutinated but those from non-agglutinable strain (WL-M) were not regardless of sex. All F_1 progenies from the reciprocal crosses showed the absence

Table 2. Segregation of the "Ph" agglutininogen in adult F_2 progenies from the cross, BM-C $\times$ WL-M

Strain	Sex	No. of chickens tested	Response of red cells of PHA	
			Agglutinable	Non-agglutinable
BM-C	male	21	0	21
	female	43	43	0
WL-M	male	19	0	19
	female	37	0	37
(WL-M $\times$ BM-C) F_1	male	4	0	4
	female	12	12	0
(BM-C $\times$ WL-M) F_1	male	3	0	3
	female	5	5	0
(WL-M $\times$ BM-C) F_2	male	9	0	9
	female	34	24	10

* All female birds were egg-laying.

Supposed that the segregation in F_2 progenies would be 3:1, $\chi^2=0.3529$, D.F.=1, $P>0.50$.

Table 3. Segregation of the "Ph" agglutininogen in 18-day embryonic F_2 progenies from the cross, BM-C $\times$ WL-M

Strain	No. of embryos tested	Response of red cells to PHA	
		Agglutinable	Non-agglutinable
BM-C	10	10	0
WL-M	10	0	10
(WL-M $\times$ BM-C) F_1	10	0	10
(BM-C $\times$ WL-M) F_1	10	0	10
(WL-M $\times$ BM-C) F_2	127	34	93

Supposed that the segregation in F_2 progenies would be 1:3, $\chi^2=0.2064$, D.F.=1, $P>0.50$.

of agglutininogen in their red cells and the F_2 progenies were classified into two groups, 34 embryos which had agglutininogen and 93 embryos which had no agglutininogen in their red cells (Table 3). The ratio between the numbers in these two groups corresponded significantly to 1:3 in Mendelian ratio ($P>0.5$). The result was different from that obtained in egg-laying females. The difference is caused by the difference in phenotypic expression of the red cell agglutinability between egg-laying females and 18th day embryos in heterozygous individuals.

3. Effects of injection of diethylstilbestrol in several immature chicks

The correlation between the agglutinability and the egg production suggests that injection of estrogen into sexually immature chicks of either sex would be effective in the appearance of agglutinability in their red cells. Diethylstilbestrol was injected into 5, 10, and 20 week chicks to determine whether the estrogen is effective in the transformation of the red cells to become agglutinable. The results are presented in Table

Table 4. The time of appearance of agglutination in red cells from chicks injected with diethylstilbestrol

Strain	Sex and age	No. of chicks tested	Weeks after injection					
			0	1	2	3	4	5
BM-C	male (5-week)	5	0.0 (0/5)	0.0 (0/5)	2.6 (4/5)	6.2 (4/5)	2.2 (5/5)	0.0 (0/5)
	female (5-week)	5	0.0 (0/5)	0.0 (0/5)	3.0 (5/5)	6.6 (5/5)	4.0 (5/5)	0.0 (0/5)
	male (10-week)	3	0.0 (0/3)	0.0 (0/3)	2.7 (3/3)	5.7 (3/3)	1.7 (2/3)	0.0 (0/3)
	female (10-week)	3	0.0 (0/3)	0.0 (0/3)	2.3 (3/3)	6.7 (3/3)	3.3 (3/3)	0.0 (0/3)
	male (20-week)	2	0.0 (0/2)	0.0 (0/2)	2.0 (2/2)	5.0 (2/2)	1.0 (1/2)	0.0 (0/2)
	female (20-week)	2	0.0 (0/2)	0.0 (0/2)	2.0 (2/2)	6.0 (2/2)	2.0 (2/2)	0.0 (0/2)
WL-M	male (5-week)	5	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)
	female (5-week)	5	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)	0.0 (0/5)
	male (10-week)	3	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)
	female (10-week)	3	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)
	male (20-week)	2	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)
	female (20-week)	2	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)	0.0 (0/2)
WL-M×BM-C	male (5-week)	4	0.0 (0/4)	0.0 (0/4)	1.0 (2/4)	5.0 (4/4)	1.0 (2/4)	0.0 (0/4)
	female (5-week)	6	0.0 (0/6)	0.0 (0/6)	1.6 (3/6)	6.0 (5/6)	2.0 (4/6)	0.0 (0/6)

All birds received 10.0 mg of diethylstilbestrol intramuscularly.

Number represents the mean agglutination score.

In parentheses the numerator represents the number of chicks positive to PHA and the denominator represents the number of chicks tested.

Table 5. Segregation of the estrogen-stimulated "Ph" agglutininogen in 5-week F_2 progenies from the cross, BM-C $\times$ WL-M

Strain	Sex	No. of chicks tested	Response of red cells to PHA	
			Agglutinable	Non-agglutinable
BM-C	male	5	5	0
	female	5	5	0
WL-M	male	5	0	5
	female	5	0	5
(WL-M $\times$ BM-C) F_1	male	4	4	0
	female	6	6	0
(BM-C $\times$ WL-M) F_1	male	4	4	0
	female	4	4	0
(WL-M $\times$ BM-C) F_2	male	58	43	15
	female	54	38	16

Supposed that segregation of F_2 male and female progenies would be 3:1, $\chi^2=0.4286$, D.F.=1, $P>0.50$.

4. All chicks of the BM-C strain showed the appearance of agglutinability in their red cells 2 weeks after injection and then the agglutination scores rose up to a high level as the time proceeded regardless of sex and ages. After 3 weeks following injection, the red cells exhibited the maximum score of agglutinability, then decreased during another one week and reached to zero 5 weeks after injection. The time required to achieve the maximum level of agglutinability was 3 weeks. On the other hand, all chicks of the WL-M strain showed no agglutinability regardless of sex and ages. Although chicks of the F_1 hybrids showed lower agglutination score than those of the BM-C strain, they were agglutinable. These results suggest that the production of "Ph" agglutininogen began after injection of estrogen and that the genotype of recipient chicks is essential for the responsiveness to estrogen. It was also interesting that the agglutinability is expressed phenotypically in male genotype by the injection of estrogen.

The agglutinability of red cells in the F_2 progenies was examined in 5 week young chicks by injection of diethylstilbestrol as shown in Table 5. The F_2 female and male progenies were classified into two groups by their red cell agglutinability. In female chicks 38 had agglutininogen and 16 had no agglutininogen, and in male chicks 43 had agglutininogen and 15 had no agglutininogen. The ratios between agglutinable and non-agglutinable chicks in these two groups corresponded significantly to 3:1 in Mendelian ratio. The results coincided with those obtained in egg-laying females.

DISCUSSION

The "Ph" agglutininogen in chicken red cells was detected by using a PHA obtained from the seed extract of *Pisum sativum*. The "Ph" agglutininogen was detected only in egg-laying females but not in non-laying females and males, and its appearance was associated with the egg production as shown in the previous report (Mizutani and

Fujio 1971). The injection of diethylstilbestrol revealed that estrogen level was essential for the production of "Ph" agglutinin. The lag between the injection of estrogen and the appearance of agglutinability may be explained by assuming that the agglutinin is produced initially in newly formed red cell stem cells in the bone marrow and that such cells are substituted for the non-agglutinable red cells. Cross experiments between the agglutinable strain and non-agglutinable strain showed that the presence of "Ph" agglutinin was regulated by a single autosomal gene. From the pattern of the agglutinability of red cells with PHA, the "Ph" agglutinin seems to be identical to the "Hi" agglutinin reported by Scheinberg and Reckel (1961a, b, 1962).

In the present study evidence presented that red cells obtained from early embryos were all agglutinable regardless sex and genotype. The previous report indicated that the same agglutinin was present in red cells obtained from embryos and egg-laying females (Mizutani and Fujio 1971). Salminen (1959, 1962) reported that the tick borne encephalitis virus agglutinated red cells from mature males but not those from mature females and that such agglutinin was detected in red cells from newly hatched chicks regardless of sex. In the present work it is interesting that in both agglutinable and non-agglutinable strains red cells obtained from embryos were agglutinable. The agglutination score showed high levels in red cells from 6th day embryos and decreased with the development of embryos. The red cells from the BM-C, WL-M, and their hybrid strains lost their agglutinability 3 weeks after hatching, 14 days of incubation, and 13 days of incubation, respectively. The F_1 hybrids in the reciprocal crosses showed similar decreasing curves of agglutinability to that of the WL-M strain. This fact indicates that the agglutinability was not affected maternally by the cytoplasm from WL-M hens.

Scheinberg and Reckel (1962) reported that the production of "Hi" agglutinin was affected by the genotype and an extrinsic factor (estrogen). Our experiments showed the same result as that of their experiments. The question still remains unanswered how the above interpretation is adapted to explain the fact that red cells from embryos were agglutinable regardless of genotype of their parents. It was reported by several workers that chicken egg yolk contained estrogenic compounds (Altman and Hutt 1938; Marlow and Richert 1940). Thus, it is interpreted that the production of agglutinin in embryonic red cells may be associated with estrogen contained in yolk.

However, it was not interpreted why red cells of embryos were agglutinable in the presence of yolk estrogen in the non-agglutinable strain (WL-M). It seems that the production of "Ph" agglutinin may fit to an operon theory. In other words, the production of agglutinin induced by estrogen may be controlled by a regulatory gene but not by a structure gene and the function of the gene starts from about 11 days of incubation and produce an inhibitor which repress the responsiveness of red cells to the estrogen stimulation. The evidence for the existence of the inhibitor is based on the result of earlier loss of agglutinability in the WL-M and F_1 hybrid embryos than in the BM-C embryos and also no transformation of red cells following the injection of estrogen in the non-agglutinable strain before chickens reach to their sexual maturity. The difference in phenotypic expression of the agglutinability of red cells in the heterozygous condition between egg-laying females and 18th day embryos suggests that there may be an antagonistic competition between the actions of the inhibitor and

estrogen. The lower agglutinability of heterozygous embryonic red cells might be caused by the production of larger amount of the inhibitor than that of heterozygous egg-laying females.

SUMMARY

The "Ph" agglutinin was detected in red cells from egg-laying females but not in those from mature males in the BM-C strain. In the tests for the agglutinability of red cells in egg-laying females, the agglutinable strain (BM-C) and the non-agglutinable strain (WL-M) were found. In regard to the agglutinability of embryonic red cells, "Ph" agglutinin was found in red cells from all embryos tested regardless of sex and strain and it was lost with development of embryos.

Injection experiment of diethylstilbestrol revealed that the estrogen level was essential for the production of agglutinin. Genetic analysis showed that the production of agglutinin was controlled by a single autosomal gene. These results indicate the participation of both estrogen and genotype in the production of "Ph" agglutinin in chick red cells.

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