«Research Note»

Species Specific Influences of Plasma in Lymphocyte Cytotoxicity Tests on Chickens

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Plasma obtained from 10 different species (swine, rabbit, cattle, mallard, domestic duck, rat, hamster, goose, turkey and guinea fowl) were compared for their suitability as the complement for searching the allo-antigens of chicken. Natural cytotoxicity of plasma against chicken lymphocytes, cytotoxic index (C.I.) in cytotoxicity tests and morphology of lymphocytes were examined.

Rabbit plasma showed the highest cytotoxicity against chicken lymphocytes while those of rat and hamster showed low values.

In cytotoxicity tests, C.I. with avian plasma were lower than those of the mammalian group except for mallard and duck. Swine, rabbit and rat showed high C.I.. On the other hand, hamster, goose, turkey and guinea fowl showed low values.

When plasma of natatorial birds, especially goose, were used in the cytotoxicity tests, the lymphocytes swelled remarkably.

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Key words : lymphocyte cytotoxicity test, plasma.

Introduction

The authors had conducted studies on T lymphocyte specific antigens of chickens by cytotoxicity tests, using fresh plasma of rabbit or guinea pig as the complement¹⁾. However, consistent results were not obtained due to differences in individual or seasonal variations of plasma.

It is not clear if the plasma of these 2 species are the reasonable partner to chicken antibody. Then, this study was planned to find the species of animal which could be used as a good source of complement in cytotoxicity tests of chicken. Plasma obtained from 10 species of animals were compared to determine the suitability as complement.

Materials and Methods

Preparation of chicken lymphocytes

Peripheral blood leukocytes (PBL) were separated by Ficoll-Conray method as described by BÖYME²⁾. Bloods were collected from the wing vein and were diluted 1/2

with phosphate buffered saline (PBS), pH 7.4. Samples were layered over Ficoll-Conray solution (Sp. Gr. 1.077) and centrifuged at 400 *g* for 30 minutes. PBL were then collected from the interface of the gradient. The cells resuspended with PBS were centrifuged at 240 *g* for 10 minutes. Washing was repeated 3 times. Then, the buffer was changed from PBS to veronal buffer (3.8 mM Barbitol sodium, 6.2 mM Barbitol and 0.145 M NaCl, pH 7.4). Viability of the cells were routinely >95%. About 70% of PBL were T lymphocytes. Final concentration of lymphocytes was adjusted to 2.5×10^6 cells/ml for lymphocyte cytotoxicity and to 1×10^7 cells/ml for morphological observation.

Allo-antibodies

Alloantisera were prepared by the immunization of chicken T lymphocytes. These cells were prepared by passing PBL through the nylon wool columns³⁾. Combinations of donor and recipient were designed based on differences in the major histocompatibility complex (B locus). Recipients received intravenous injections of 1×10^7 cells in 1 ml of PBS about 30 times at 7 day intervals. They were bled for serum 7 days after the last injection. The blood was incubated at 37°C for 30 minutes and allowed to stand at 4°C overnight. Sera were separated by centrifugation at 400 *g* for 30 minutes and then heat-inactivated at 56°C for 30 min. The antibodies were extracted from the antisera with saturation of ammonium sulfate (1/3 method). Dialyzed Immunoglobulin was absorbed out with the donor's red blood cells so as to exclude the antibodies against common alloantigen. Antibodies used in this experiment were termed anti Lyt-1 (anti-B⁹), Lyt-2 (anti-B¹¹) and Lyt-3 (anti-B⁹). The dilution used routinely were 1 : 32 for anti Lyt-1 and Lyt-2, 1 : 64 for anti Lyt-3. These dilutions were equivalent to the minimum doses which kill the 100% of the T lymphocytes prepared from the donors by using rabbit plasma.

Complement

Fresh plasma of swine, rabbit, cattle, goose and turkey were collected from each 3 animal, respectively. Similarly, 4 birds were bled for mallard, domestic duck and guinea fowl. Fresh plasma of rat and hamster were gathered from 10 individuals, respectively. Plasma of each species were collected with heparinized syringe and mixed together to exclude the individual effect. And they were divided into small aliquots and were stored at -80°C in a freezer within 3 months. Further dilution of plasma was not made in cytotoxicity tests.

Lymphocyte cytotoxicity test

Cytotoxicity tests were conducted according to the method of CHYOU and NISHIOKA⁴⁾. 2 μ l of a PBL suspension were mixed with the same amount of antibody dilution on a multiplate Terasaki (Sumitomo Bakelite ; MS 31600). As a control, veronal buffer was used in place of the antibodies. Mixture was placed at room temperature for 20 minutes. Then, 2 μ l of plasma was added to the mixture and incubated at 37°C for 30 minutes. The reaction was terminated by placing the plate on an ice bath for 10 minutes. 1 μ l of 0.3% trypan blue solution was then added to the mixture. The percentage of dead cells were determined by observing through an inverted microscope. The cytotoxic index (C.I.) was calculated as following

formula⁵⁾ :

$$\text{C.I.} = \frac{(\% \text{ dead cells in test suspension} - \% \text{ dead cells in the control suspension})}{(100 - \% \text{ dead cells in the control suspension})}$$

Lymphocyte cytotoxicity tests were reiterated 3 times and data were presented by the means of 3 values.

Natural cytotoxicity of plasma

In a multiplate Terasaki, 1 μl of each plasma was reacted with same volume of PBL suspension at 37°C for 30 minutes. For the control, veronal buffer was added to the cell suspension instead of plasma. After placing the plate on an ice bath, the percentage of dead cells were determined by trypan blue exclusion test. Natural cytotoxicity of plasma was also expressed with C.I..

Statistical analysis

The results were evaluated by the analysis of variance, using the least-squares method of HARVEY^{6,7)}.

Results

Natural cytotoxicity of plasma

As shown in Table 1, the plasma of rabbit indicated relatively high cytotoxicity against PBL. However, the coefficient of variation (C.V.) was the lowest among 10 species of animals. The plasma of rat and hamster showed low cytotoxicity. However, C.V. in these two species were higher than in other species.

Effect of plasma on lymphocyte cytotoxicity test

As shown in Table 2, C.I. varied according to the combinations between antibody and the plasma. The plasma of cattle showed high C.I. with anti Lyt-1, although the values with anti Lyt-2 and Lyt-3 were low. Mallard plasma showed low C.I. with anti Lyt-3, but high C.I. was obtained with anti Lyt-2. On the other hand, swine, rabbit and rat plasma showed constant C.I. with each antibody and the value were higher than those of any other plasma. In addition, C.I. of swine plasma always showed low C.V.. C.I. obtained with avian plasma tended to be lower than those of

Table 1. The comparison of natural cytotoxicity of plasma against chicken lymphocytes among 10 species of animals

Rabbit	Swine	Duck	Guinea fowl	Cattle
33.1 $\pm$ 3.4 ^a (23.0)	25.3 $\pm$ 4.0 ^{bc} (35.4)	22.8 $\pm$ 3.4 ^{bcd} (33.3)	17.4 $\pm$ 3.5 ^{bcd} (45.0)	17.0 $\pm$ 3.3 ^{bcd} (43.4)
Mallard	Goose	Hamster	Rat	Turkey
16.6 $\pm$ 3.7 ^{bcd} (49.8)	13.0 $\pm$ 3.7 ^{cd} (63.6)	9.5 $\pm$ 3.2 ^d (74.5)	7.6 $\pm$ 3.7 ^d (108.9)	*

Data are expressed as C.I.: Mean $\pm$ S.E. Values in parentheses indicate the coefficient of variation (C.V.). Means with different superscripts within a row are significantly different ($P < 0.05$).

*; Missing data.

Table 2. The relationships between antibody and plasma on lymphocyte cytotoxicity tests of chicken.

	Lyt-1	Lyt-2	Lyt-3
Swine	*45.9±3.9 ^a (29.4)	30.7±4.1 ^{abc} (26.7)	44.7±5.7 ^a (25.5)
Rabbit	37.4±7.9 ^{ab} (73.2)	45.5±3.4 ^a (14.9)	48.6±3.7 ^a (15.2)
Cattle	48.6±6.6 ^a (47.0)	19.8±7.3 ^{bc} (73.7)	26.1±4.8 ^b (36.8)
Mallard	32.5±5.5 ^{ab} (58.6)	41.2±9.5 ^a (46.1)	23.5±3.5 ^b (29.8)
Duck	34.6±2.8 ^{ab} (28.0)	34.9±4.4 ^{ab} (25.2)	19.2±7.6 ^{bc} (79.2)
Rat	37.4±5.8 ^{ab} (53.4)	41.7±4.3 ^a (20.6)	37.4±5.0 ^a (26.7)
Hamster	20.2±4.6 ^{bc} (78.9)	20.9±2.6 ^{bc} (24.9)	17.2±6.1 ^{bc} (70.9)
Goose	22.5±6.9 ^{bc} (106.2)	20.0±2.3 ^{bc} (23.0)	7.8±5.9 ^c (151.3)
Turkey	22.3±5.8 ^{bc} (90.1)	14.5±7.5 ^c (103.4)	11.2±9.7 ^{bc} (173.2)
Guinea fowl	9.3±4.4 ^{bc} (163.9)	13.4±3.3 ^c (49.3)	22.8±8.8 ^b (77.2)

Data are expressed as C.I. *; Mean ±S.E.

Means with different superscripts within a row are significantly different ($P<0.05$).

The values in parentheses indicates coefficient of variation (C.V.).

mammalian ones except for duck and mallard, and high percentage of C.V. were observed in avian species.

Morphological observations

Lymphocytes were rounded in shape and reflected light in the suspension (Fig. 1). Cells killed with antibodies and plasma were stained with trypan-blue (Fig. 2-5). When the plasma of natatorial birds were used, lymphocytes swelled (Fig. 4, 5). When goose plasma was used, it was quite easy to distinguish the dead cells from viable cells (Fig. 5). When the mammalian plasma was used these phenomena was not observed (Fig. 2, 3).

Discussion

Since a lymphocyte cytotoxicity test is carried out with the treatment of antibody and plasma, low natural cytotoxicity of plasma against chicken lymphocyte should be desirable. So, it was suggested that rabbit plasma is not suitable for the test (Table 1). Although the plasma of rat and hamster showed low natural cytotoxicity, caution must be paid to them by the reason of their high C.V. in their natural cytotoxicity. High C.I. were indicated from the plasma of rabbit, swine and rat on each antibody (Table 2). Accordingly rat and swine plasma were recommended for the test. However, C.I. of rat showed high C.V. with anti Lyt-1. When swine plasma

Fig. 1. is the photomicrograph of chicken lymphocytes of control group which was only incubated at 37°C, for 30 min. The pictures from Fig. 2. to Fig. 5. show chicken lymphocytes treated with anti-Lytl and plasma of different animals.

Fig. 1. Control group. $\times 280$.

Fig. 2. Rabbit plasma was used as complement. Dead cells included trypanblue and some were swelled. $\times 280$.

Fig. 3. Mouse plasma was used. $\times 280$.

Fig. 4. Goose plasma was used. Dead cells included trypan blue and those swelled remarkably. $\times 280$.

Fig. 5. Wild duck plasma was used. Swell of cytoplasm were not so much as goose plasma but Rabbit and Mouse. $\times 280$.

were used low C.V. were indicated on C.I. for each antibody. Natural cytotoxicity of swine plasma was lower than that of rabbit. And swine has good advantage in preparation of homogeneous plasma in large quantities. So swine plasma may reasonable for cytotoxicity test with chicken antibody.

Though the same antibodies and lymphocyte suspensions were used in cytotoxicity tests, high C.V. were indicated by using turkey or guinea fowl plasma. This phenomenon might relate with the fact that chicken antibody is strongly anticomple-

mentary⁵⁾. Perhaps, these two species might differ from the others on their affinity of first component of complement to the chicken antibody.

When the plasma of natatorial birds were used in the tests, lymphocytes swelled remarkably. This phenomenon was not observed when the mammalian plasma were used. The mechanism of the phenomenon is unknown. Further studies are needed to clarify the mechanism.

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ニワトリのリンパ球毒性試験における補体の影響について

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十種類の動物（ウサギ、ブタ、ガチョウ、アヒル、カモ、ラット、ハムスター、ウシ、シチメンチョウ、ホロホロチョウ）より血漿を採取し、補体してニワトリのリンパ球毒性試験に用いた。そして、ニワトリのリンパ球に対する自然細胞傷害性、三種類の同種免疫抗体を用いた際に示される細胞傷害指数（C.I.）ならびに細胞傷害試験におけるニワトリリンパ球の形態変化について、各動物間で比較を行った。

ウサギの血漿は、ニワトリのリンパ球に対して高い細胞傷害性を示した。これに対して、ラットとハムスターの血しょうは細胞傷害性が低く、その他の動物はウサギと、ラットならびにハムスターの中間の値を示した。

C.I. は、3種類の抗体いずれを用いた場合でも、ウサギ、ラットおよびブタの血漿を用いた時高い値が示された。一方、鳥類の血しょうを用いた際は、カモとアヒルを除き C.I. は哺乳類のものを用いた場合より低い値を示した。

抗体と水禽類の血漿で処理を行ったリンパ球は著しく膨化したが、他の動物の血漿を用いた際にはこの現象は認められなかった。

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キーワード：リンパ球毒性試験, 補体