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Count of T-rosette-forming cells (T-RFC) in the Spleen of Young Rats Born to Azathioprine(Imuran)-administered Pregnant Rats

Wang Jae Lee, Chan Soo Shin, Ka Young Chang and Kwang Ho Lee

Department of Anatomy, College of Medicine, Seoul National University, Seoul 110 Korea

= **Abstract** =This experiment was performed through the rosette formation method to study the inhibition mechanism of the immune response in the spleen of young rats born to azathioprine-administered pregnant rats.

The following results were obtained.

1. In the group I consisting of animals born to normal pregnant rats and immunized with sRBC after birth, there was increase in the number of RFC, compared with that in the group 0 consisting of animals born to normal pregnant rats and not immunized with sRBC.

2. In the group II consisting of animals born to azathioprine-administered pregnant rats and immunized with sRBC, there was decrease in the number of RFC, compared with that in the group I. However the group III, which consists of animals born to azathioprine-administered pregnant rats and inoculated with thymus cells derived from outbred animals and simultaneously immunized with sRBC, showed much more RFC than other groups.

3. Generally the 3rd day post sRBC injection groups showed more RFC than the 7th day post sRBC injection groups.

4. The number of RFC increased with age in the sRBC-injected groups.

Above results suggested that peripheral T lymphocytes were depleted due to retarded fetal development of thymus caused by azathioprine.

Key Words: ***T-rosette-forming cells, Young rats born to azathioprine-administered pregnant rats, Thymus cell inoculation.***

INTRODUCTION

Immunosuppression mechanism of an immunosuppressant, azathioprine has been studied by many investigators. Santos and Owen (1975), Röllinghoff (1973), and Otterness and Chang (1976) reported that azathioprine had an immunosuppressive effect on both cell-mediated and humoral immunities, whereas Camiener and Wechter (1971) and Abdou *et al.* (1973) demonstrated that azathioprine had selective immunosuppressive effect on only the T-cell system. Galanaud *et al.* (1975) and Brown *et al.* (1976) reported the same results as above.

The morphological observation of thymus by Lee *et al.* (1977) showed that the fetal development of the thymus was retarded by azathioprine, and the plaque assay by Lee *et al.* (1978) showed that the number of plaque-forming cells(PFC) to sheep erythrocytes was decreased in the animals, of which the fetal development of thymus was retarded by azathioprine. Thereafter Chang *et al.* (1983) performed an advanced experiment, which showed that in the rats with retarded thymus the number of PFC to sheep erythrocytes was increased after inoculation of thymus cells from outbred rats. However the plaque assay is a method for approaching directly to the B cell system which is responsible for antibody production. Therefore we can't evaluate directly the variations in the number of T helper or T-cells which are involved in the

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T-cell dependent immune response. Needless to say, it is necessary to groove for a method which can approach directly to the T-cell system. The rosette formation method is a representative one for approaching directly to the T-cell system. Specifically human peripheral T lymphocytes have a natural tendency to form rosettes with sheep erythrocytes. Moreover, rosette-forming cells(RFC) are increased in the experimental animals after immunization (Charreire 1973).

In this experiment, only T lymphocytes among all lymphocytes involved in T-cell dependent immune response were observed through the rosette formation method in the experimental animals in which the fetal thymic development was retarded by azathioprine and which were immunized with sRBC after birth.

MATERIALS AND METHOD

1. Animals and grouping

Two hundred young Sprague-Dawley rats were used in this experiment. They were born to 40 female rats, 25 of which had 8 mg/kg of azathioprine orally administered to them on the 7th day of gestation. The animals were divided into 4 groups as follows;

Group 0: young rats born to normal pregnant rats and injected with no sRBC antigen intraperitoneally.

Group I: young rats born to normal pregnant rats and injected with sRBC antigen intraperitoneally after birth.

Group II: young rats born to azathioprine-administered pregnant rats and injected with sRBC antigen intraperitoneally after birth.

Group III: young rats born to azathioprine-administered pregnant rats and injected with sRBC antigen intraperitoneally and with thymus cells intravenously after birth.

2. Antigen(sRBC) preparation

After aseptic sampling of blood from the jugular vein of sheep, the blood was kept in Alsever solution at 4℃ for at least 1 week. The blood was washed 3 times with the aseptic Hank's Balanced Salt Solution(HBSS) at pH 7.2, through centrifugation with 450 g at 4℃ for 10 minutes.

A 30% sRBC suspension was prepared and a 0.5 ml of the suspension was injected into the 4 week old group, 1.0 ml into the 8 week old group, respectively.

3. Thymus cell suspension

Outbred normal Sprague-Dawley rats were killed and their thymuses were resected aseptically. Heterogenic thymus cell suspension was prepared in HBSS. Only thymus cells were collected from that heterogenic thymus cell suspension via centrifugation in Ficoll-Hypaque density gradient solution with 600 g for 30 minutes. The relatively homogenous thymus cell suspension was washed out 3 times with HBSS and diluted with normal saline.

4. Spleen cell suspension

Cell suspensions of the spleens resected 3 days and 7 days after antigen injection were prepared. Only lymphocytes were collected from the suspensions via centrifugation in Ficoll-Hypaque density gradient solution with 600 g for 30 minutes. Splenic lymphocyte suspensions were prepared in HBSS and diluted into 4 x 10/ml with HBSS.

5. Treatment of sRBC with neuraminidase

A 2.0 ml of sRBC(4 x 10/ml) was mixed with 50 u of neuraminidase (General Biochemicals Divisions, Chagrin Falls, Ohio) in 1 ml of phosphate buffered saline (pH 7.4) and the mixture was incubated at 37℃ for 30 minutes.

6. Rosette formation

A 0.5 ml of the splenic lymphocyte suspension was mixed with 0.5 ml of sRBC suspension treated with neuraminidase and the mixture was incubated at 37℃ for 30 minutes. Thereafter it was centrifuged with 100 g for 6 minutes and it was incubated at 0℃ for 1 hour and the mixture was resuspended cautiously with a Pasteur pipette after incubation at room temperature for 30 minutes. A 0.1 ml of 1% sodium azide in phosphate buffered saline was added immediately before resuspending the pellet.

7. T-rosette-forming cells(T-RFC) count

Cautiously resuspended mixture was dropped onto the hemocytometer and the number of T-RFC was counted. The lymphocytes to which 4-10 sRBC were attached were counted as T-RFC in this experiment(Elliott *et al.* 1972).

RESULTS

1. Rosette-forming cells(RFC) in the 4 week old groups

a) 3 days after antigen(sRBC) injection

The group 0(Background Control Group), which consists of the animals born to the normal pregnant rats and not immunized with sRBC showed 0.59×10^5 RFC per 10^6 splenic lymphocytes, whereas the group I(Control Group) consisting of the animals born to the normal pregnant rats and

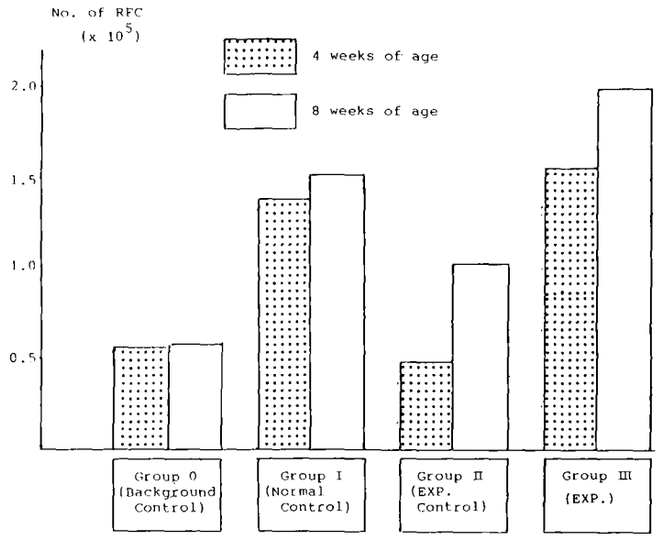

Fig. 1. Changes of the number of RFC with increasing age on the 3rd day after Antigen injection.

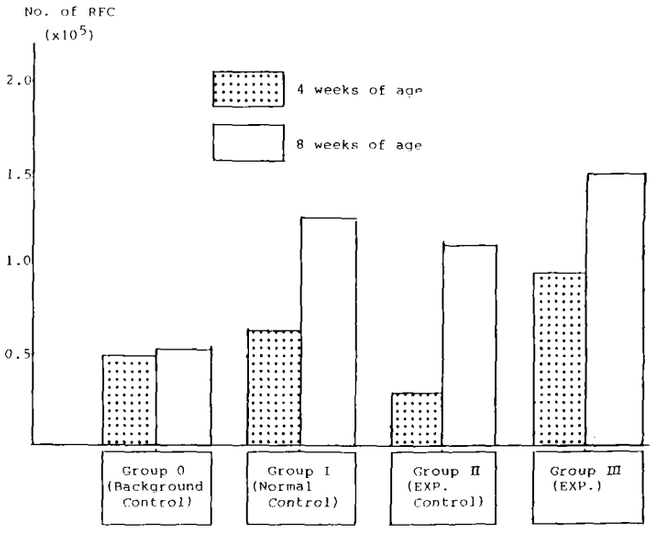

Fig. 2. Changes of the number of RFC with increasing age on the 7th day after Antigen injection.

Table 1. Number of T-RFC per 10^6 spleen cells in each group at the age of 4 weeks.

Groups	Cells inoculated	Days after antigen injection	Average RFC per 10^6 spleen cells	P values*
Group 0		3	$(0.59 \pm 0.05) \times 10^5$	–
(Background Control)	No treatment	7	$(0.51 \pm 0.06) \times 10^5$	–
Group I		3	$(1.36 \pm 0.12) \times 10^5$	$P < 0.01$
(Normal Control)	sRBC	7	$(0.62 \pm 0.05) \times 10^5$	$P < 0.01$
Group II		3	$(0.57 \pm 0.07) \times 10^5$	–
(Experimental Control)	sRBC	7	$(0.30 \pm 0.03) \times 10^5$	–
Group III	sRBC +	3	$(1.68 \pm 0.19) \times 10^5$	$P < 0.01$
(Experimental)	thymus cells	7	$(1.02 \pm 0.09) \times 10^5$	$P < 0.01$

* P values are compared with the average of the group II.

immunized with sRBC after birth, did 1.36 x 10^5. However, 0.57 x 10^5 RFC were observed in the group II (Experimental Control Group) which consists of the animals born to azathioprine-administered pregnant rats and immunized with sRBC after birth, indicating a marked decrease in the number of RFC, compared with that in the group I. Meanwhile 1.68 x 10^5 RFC were counted in the group III consisting of the animals born to azathioprine-administered pregnant rats and inoculated with the outbred thymus cells and immunized with sRBC after birth (Table 1). The ratio of the group I vs. the group II in the number of RFC is 2:1, indicating a relatively marked difference between the two groups (Fig. 1).

b) 7 days after sRBC injection

Generally fewer RFC were counted than in the 3rd day post sRBC injection groups. The group 0 showed 0.51 x 10^5 RFC whereas the group I did 0.62 x 10^5 RFC. In the group II, 0.3 x 10^5 RFC were observed, in the group III, 1.02 x 10^5 RFC were observed (Table 1, Fig. 2).

2. RFC in the 8 week old groups

a) 3 days after sRBC injection

Generally more RFC were counted than in the 4 week old groups. The group 0 showed 0.67 x 10^5 RFC, whereas the group I did 1.54 x 10^5 RFC. The group II showed 1.07 x 10^5 RFC and the ratio of the group I vs. the group II in the number of RFC, 1.5:1, indicating that a relatively greater increase of

Table 2. Number of T-RFC per 10^6 spleen cells in each group at the age of 8 weeks.

Groups	Cells inoculated	Dats after antigen injection	Average RFC per 10^6 spleen cells	P values*
Group 0 (Background Control)	No treatment	3	$(0.67 \pm 0.05) \times 10^5$	–
		7	$(0.63 \pm 0.040 \times 10^5$	–
Group I (Normal control)	sRBC	3	$(1.54 \pm 0.13) \times 10^5$	$P < 0.01$
		7	$(1.45 \pm 0.18) \times 10^5$	NS**
Group II (Experimental Control)	sRBC	3	$(1.07 \pm 0.12) \times 10^5$	–
		7	$(1.37 \pm 0.15) \times 10^5$	–
Group III (Experimental)	sRBC + thymus cells	3	$(2.10 \pm 0.18) \times 10^5$	$P < 0.01$
		7	$(1.78 \pm 0.20) \times 10^5$	$P < 0.01$

* P values are compared with the average of the group II.

** NS; not significant

RFC was in the group II than in the group I, comparing withthe ratio (2.0:1) in the 4 week old groups (Fig.1). The group III showed 2.10×10^5 RFC, and showed a greater number of RFC than the group I as well as the group II (Table 2).

b) 7 days after sRBC injection

Generally fewer RFC were observed in the 3rd day post sRBC injection groups. The general tendency between all groups was similar to that in the 3rd day post sRBC injection groups (Fig. 2, Table 2).

DISCUSSION

Immune responses in mouse to sheep erythrocytes can be observed via a hemolytic plaque assay in a single cell (hemolysin secretion cell) level and a rosette formation method using immunocytoadherence phenomenon.

Zaalberg (1964) described rosette forming cells (RFC) for the first time. The many splenic lymphocytes of the immunized mice formed rosettes with sRBC. Such reactive lymphoid cells involved in the interaction were called as RFC. And the interaction was independent of complement.

The relationship between the functions of RFC and antibody forming cells (AFC) has not been well known. There is a report by Shearer *et al.* (1968) that RFC secrete a agglutinating antibody. However this has not been well accepted due to absence of direct evidence. Zaalberg *et al.* (1968) tried an experiment where they correlated PFC with RFC in one animal. However because the cells used in one experimental method (Plaque assay), good results could not be obtained from the above trial. Thereafter Bach *et al.* (1970; 1972) found in immunized mice that 50% of RFC had a theta(θ) antigen on their surface and that the number of RFC was markedly decreased by treatment of RFC with anti-θ antisera. Wilson *et al.* (1971) and Schlesinger (1971) also found the same results as above in unimmunized mice. Elliott *et al.* (1972) demonstrated that fewer sRBC were attached to T-RFC than to B-RFC. Substantially 4-10 sRBC are attached to T-RFC, whereas more than 10 sRBC are attached to B-RFC. The next year, they suggested that T-RFC were less stable than B-RFC, so the number of sRBC attached to T-RFC were fewer than those to B-RFC.

In this experiment only T-RFC attaching 4-10 sRBC, were counted. And because T-RFC were very friable, a metabolic inhibitor (1% sodium azide in PBS) was used to prevent sRBC from being detached from the surface of T lymphocytes during the experiment.

In general, more RFC were observed in the 3rd day post sRBC injection groups than in the 7th day post sRBC injection groups. This is known to be its own antigenicity of sRBC antigen (Lee *et al.* 1978; Chang *et al.* 1983).

In the group I (Normal control group), there was a statistically significant increase in the number of RFC, compared with that in the group 0 (background control group). This implies that RFC increase by immunization with sRBC (Charreire 1973).

Meanwhile the group II (Experimental control group) showed much fewer RFC than the group I. This is a direct evidence of suggestion by Chang *et*

al. (1983) that the fetal development of thymus is inhibited by azathioprine and consequently peripheral T lymphocytes were depleted.

In the group III (Experimental group) born to azathioprine-administered pregnant rats and inoculated with thymus cells derived from the outbred rats and immunized with sRBC after birth, there were much more RFC than in other groups. As pilot study, we confirmed that 10% of thymus cells used in this experiment formed rosettes with sheep erythrocytes. From comparing the number of inoculated thymus cells to the increased number of RFC it can be suggested that total RFC counted in the group III are the summation of RFC increased by immunization and inoculated RFC with capacity to form rosettes.

The ratio of the group I vs. the group II in the number of RFC is 2:1 at the age of 4 weeks, whereas it is 1.5:1 at the age of 8 weeks. Specifically the number of RFC in the group II, which consists of the animals born to azathioprine-administered pregnant rats, gradually increase with age. It suggests the immunological activity is related to the age, and that depleted peripheral T lymphocytes by inhibited fetal development of thymus can be replenished with age (Lee *et al.* 1978; Chang *et al.* 1983).

In conclusion, it is apparent that the peripheral T lymphocytes are depleted due to retarded fetal development of thymus caused by azathioprine.

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= 국문초록 =

임신흰쥐의 장기 형성시에 투여된 Azathioprine(Imuran)이 신생흰쥐 비장의 T-rosette 형성세포수에 미치는 영향

서울대학교 의과대학 해부학교실

이왕재 · 신찬수 · 장가용 · 이광호

임신중 투여된 Azathioprine이 그 모체에서 태어난 신생흰쥐 림프기관의 기능을 저해시키는 기전을 규명하기 위하여 다음과 같은 실험을 수행하였다.

실험동물로는 Sprague-Dawley 계통의 200 g 내외의 임신한 흰쥐 40마리 (이중 25마리는 임신 확인 후 제 7일에 Azathioprine을 8 mg/kg 경구 투여함)에서 정상분만으로 태어난 신생흰쥐 200마리를 4군으로 나누었고 그 중 3군에는 항원으로 면양으로 면양 적혈구를 투여하고, 이계교배에서 얻은 신생흰쥐 흉선세포를 그 중 1군에 투여하여 항원 투여 후 제 3일과 제 7일에 도살하여 비장을 적출한 다음 비장세포 부유액을 만들어 Rosette 형성방법을 이용하여 Rosette forming ,cells을 조사한 결과 다음과 같은 결론을 얻었다.

1. 임신중 Azathioprine을 투여하지 않은 흰쥐에서 태어난 신생흰쥐에 면양 적혈구를 투여한 군이 임신중 Azathioprine을 투여하지 않은 흰쥐에서 태어난 신생흰쥐에 항원을 투여하지 않은 군에 비해 많은 수의 RFC를 보였다.
2. 면양 적혈구를 투여한 군들에서는 임신중 Azathioprine을 투여한 흰쥐에서 태어난 군이 임신중 Azathioprine을 투여하지 않은 흰쥐에서 태어난 군에 비해 훨씬 적은 수의 RFC를 보였으며 임신중 Aathioprine을 투여한 흰쥐에게 태어났어도 이계교배에서 얻은 신생흰쥐의 흉선세포를 투여한 군에서는 더욱 많은 수의 RFC를 보였다.
3. 면양 적혈구를 항원으로 투여한 군들에서는 항원 투여 후 제 3일에 보다 많은 수의 RFC를 보였다.
4. 면양 적혈구를 항원으로 투여한 군들에서는 흰쥐의 연령이 증가함에 따라 RFC의 수가 증가하는 경향을 보였다.
5. 이상의 결과는 임신중 투여한 Aathioprine에 의해 면역기구의 발육이 억제된 신생흰쥐에서 면양적혈구에 대한 면역반응이 저하되었음을 뜻하며 이것은 말초 T세포의 고갈(depletion)에 의한 결과임을 암시하고 있다.