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Quantitative Study of Thymocytes Injected into Young Rats born to Azathioprine-administered Pregnant Rats*

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= Abstract= The plaque assay was performed to determine the optimal dosage of thymus cells to be inoculated into the neonatally T-cell depleted rats by azathioprine for the recovery of immune function. The experimental animals were two hundred young rats which were born to sixty normal Sprague-Dawley pregnant rats, of which 45 rats had 8 mg/kg of azathioprine orally administered to them on the 7th day of the gestation. Sheep erythrocytes (sRBC) antigen was intraperitioneally injected into the animals on the 4th & 8th weeks after birth and simultaneously the thymus cells derived from outbred Sprague-Dawley rats were inoculated into 4 experimental groups with the dosages of 10^5, 10^6, 10^7 and 10^8 thymus cells, respectively. The spleens were resected on the 3rd & 7th days after sRBC injection for the plaque assay.

The following results were obtained.

1. In the neonatally T-cell depleted group, there was statistically significant decrease in the number of PFC, compared with that in the control group.
2. In the 4 week old groups, the normal level of PFC could be reached by the inoculation of between 10^6 and 10^7 thymus cells, in 8 week old groups, by the inoculation of between 10^5 & 10^6 thymus cells.
3. In both 4 week old and 8 week old groups, the peak numbers of PFC were obtained by the inoculation of 10^7 thymus cells.

Key Words: ***Plaque assay, Neonatal T-cell depletion by azathioprine, Thymocyte inoculation***

INTRODUCTION

Azathioprine (Imuran^R) is a synthetic purine analogue as one of the derivatives of 6-mercaptopurine (6-MP). Originally it was developed as a chemotherapeutic agent for cancer, but recently it has been frequently used as a immunosuppressant for the treatment of autoimmune diseases, or organ transplantation.

Röllinghoff et al. (1973) and Galanaud et al. (1975) reported that T-cell dependent immune response was suppressed more easily by azathioprine than T-cell independent immune response. According to the research of Chen et al. (1976), azathioprine had nearly no effect on T-cell independent system, or B-cell system.

Lee et al. (1977) and Lee et al. (1978) reported the results of *in vivo* experiments to investigate the immuno-suppression mechanism of azathioprine. Specifically, when azathioprine was administered into the pregnant rats on the 7th day of the gestation, the neonatal rats from them showed decreased hemolysin-forming cells to sheep erythrocytes (sRBC) in the plaque assay. In serial *in vivo* experiments in which outbred thymus cells were inoculated intravenously into T-cell azathioprine suppressed rats, Chang et al. (1983) observed increased hemolysin-forming cells to sRBC, suggesting that the immunosuppression mechanism of azathioprine inhibits the fetal development of thy-

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mus and depletes the peripheral T lymphocytes. Above experiments showed that the collaboration between T and B lymphocytes was necessary for T-cell dependent immune response. As a rule, the optimal number of T & B lymphocytes are involved in the collaboration between them. For that reason, it is important to determine the optimal dosage of thymus cells to be inoculated for the generation of the immune response in the neonatally T-cell depleted rats. There have been, however, almost no reports on the optimal dosage of thymus cells to be inoculated for the recovery of the immune function.

Therfore, the following experiment was performed to determine the optimal dosage of thymus cells to be inoculated into the neonatally azathioprine T-cell depleted rats.

MATERIALS & METHODS

1. Animals and Grouping

Two hundred young rats born to 60 normal Sprague-Dawley pregnant rats, 45 rats of which 8 mg/kg of azathioprine were orally administered to on the 7th gestational day were used. The animals were divided into 6 groups as follows;

Control group:young rats born to normal pregnant rats and injected with sRBC antigen intraperitoneally.

Experimental control group:young rats born to azathioprine administered rats during pregnancy and injected with sRBC antigen intraperitoneally.

Experimental group I:young rats born to azathiprine administered pregnant rats and injected with sRBC antigen intraperitoneally and with 10^5 thymus cells intravenously.

Experimental group II; young rats born to azathioprine administered rats during pregnancy and injected with sRBC antigen intraperitoneally and with 10^6 thymus cells intravenously.

Experimental group III: young rats born to azathioprine administered rats during pregnancy and injected with sRBC antigen intraperitoneally and with 10^7 thymus cells intravenously.

Experimental group IV:young rats born to azathioprine administered rats during pregnancy and injected with sRBC antigen intraperitoneally and with 10^8 thymus cells intravenously.

2. Antigen (sRBC) Preparation

After aseptic sampling of blood from the jugular vein of sheep, the blood was kept in Alsever solution at 4°C for at least 1 weeks. The blood was washed out 3 times with the aseptic Hank's Balanced Salt Solution (HBSS) of pH 7.2, through centrifugation with 1,000 rpm at 4°C for 10 minutes.

30% sRBC suspension was prepared and 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 Spraque-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 2,000 rpm for 30 minutes. This relatively homogenous thymus cell suspension was washed out 3 times with HBSS and diluted with normal saline.

4. Plaque Assay

The method used in this experiment was a slide method of plaque assay, modified by Jerne et al. (1963).

a) Cell suspension

Cell suspension of the spleens resected on the 3rd day and 7th day after injection of antigen was prepared in HBSS. The cells were counted and suitable dilutions were made so that the amounts used in this assay would yield approximately 40-100 plaques per slide. The suspended cells were kept in an ice bath until used

b) Assay procedure

0.5 ml of 1:15 diluted sRBC in HBSS were pipetted into 0.5 ml of 0.5% agarose solution contained in glass tubes in a 45°C water bath. 0.1 ml of spleen cells were added. The contents of the tube were mixed by flicking with the fingers and the agarose solution-cell suspension was then poured onto the clear area of the coated microscope slide. The agarose must be allowed to gel completely. A 10% solution of guinea pig serum in HBSS was prepared. The slides were individually turned over on the slide rack so that the agar ends rested on the slide supports. Then the complement solution was let into the well under the agarose surface with a Pasteur pipette. After all of the slides were so treated, the racks were placed in a humidifying chamber at 37°C for 3 hours.

RESULTS

1. Plaque-forming cells (PFC) in the 4 week old groups

a) 3 days after antingen (sRBC) injection

While 20.2 PFC were counted in the group which the peripheral T lymphocytes were neonatally depleted by azathioprine, 56.0 PFC were observed in control group. Meanwhile much more PFC were generally observed in remaining four groups into which outbred thymus cells were inoculated. Specifically as the number of thymus cells inoculated increased from 10^5 to 10^7 by 10 times, the number of PFC increased 27.7, 37.5 and 87.4, respectively. However, 68.5 PFC were observed in the group into which 10^8 thymus cells were inoculated, so there was decreasing tendency of the number of PFC in spite of its maximal dosage of thymus cells. It should be noticed that the normal level of PFC(56.0) could be reached by inoculation of between 10^6 and 10^7 thymus cells (Table 1).

b) 7 days after sRBC injection

Generally the less PFC were observed than in the groups of 3rd day after sRBC injection.

The comparative relationships among all groups, especially between the normal group and the neonatally T-cell depleted group, between the neonatally T-cell depleted group and the outbred thymus cell inoculated groups were similar to those of groups of 3rd day after sRBC injection (Table 1).

2. PFC in the 8 week old groups

a) 3 days after sRBC injection

Generally, much more PFC were observed than in the 4 week old groups (Fig. 1).

While 52.4 PFC were observed in the neonatally T-cell depleted group, 96.8 PFC were observed in the normal group and were approximately 2 times as those of the former group. In those groups into which outbred thymus cells were inoculated, as the number of thymus cells increased from 10^5 to 10^7 by 10 times gradient, the number of PFC increased 58.7, 125.4 and 212.5, respectively. However, 142.8 PFC were observed in the group into which 10^8 thymus cells were inoculated. In comparision with those in the group into which 10^7 thymus cells were inoculated, there was marked decrease of PFC in the fomer group notwithstanding its maximal dosage of the inoculated thymus cells.

It should be noticed that the normal level of PFC (96.8) could be reached by inoculation of between 10^5 & 10^6 thymus cells (Table 2).

b) 7 days after sRBC injection

In general, much less PFC were observed than in the groups of 3 rd day after sRBC injection (Fig. 2). General tendecy among all 6 groups was similar to that in the groups of 3rd day after sRBC injection (Table 2).

Table 1. Number of PFC per 10^6 spleen cells of neonatally azathioprine T-cell depleted rats and normal rats after injection of sRBC and outbred thymus cells at the age of 4 weeks

Groups	Cells incoulated	Days after antigen injection	Average PFC per 10^6 spleen cells	P values*
Control	sRBC	3	56.0±7.1	P<0.01
		7	18.9±2.2	P<0.01
Experimental control	sRBC	3	20.2±2.2	—
		7	12.1±1.5	—
Experimental I	sRBC+ 10^5 thymus cells	3	27.7±3.2	P<0.05
		7	14.5±1.5	P<0.05
Experimental II	sRBC+ 10^6 thymus cells	3	37.5±3.1	P<0.01
		7	17.1-2.0	P<0.01
Experimental III	sRBC+ 10^7 thymus cells	3	87.4±9.2	P<0.01
		7	23.6±2.1	P<0.01
Experimental IV	sRBC+ 10^8 thymus cells	3	68.5±6.3	P<0.01
		7	28.4±3.0	P<0.01

* P values are compared with the average of experimental control group

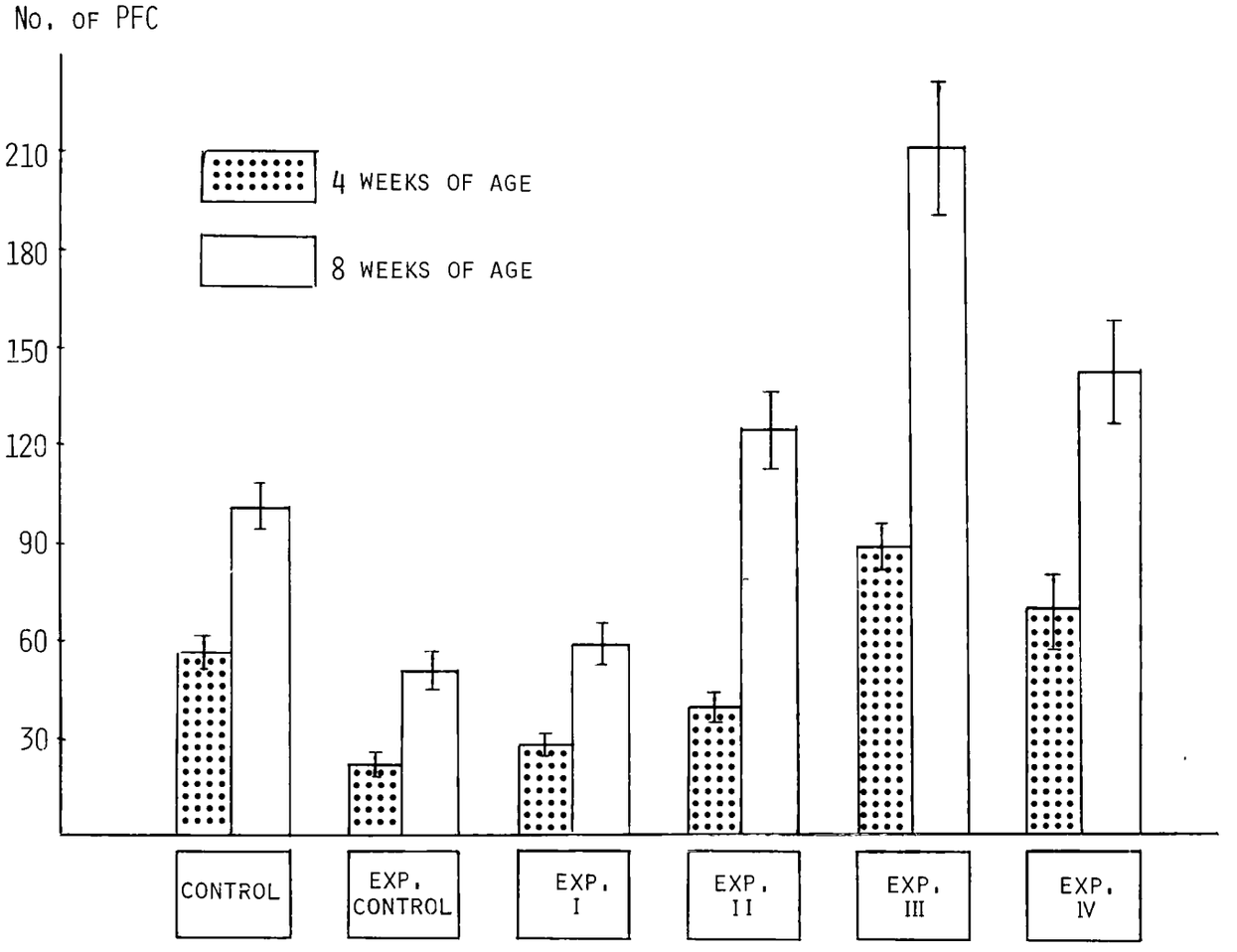

Fig. 1. Comparison of No. of PFC in the 4 week old and 8 week old groups, respectively on the 3 rd day after antigen(sRBC) injection.

Table 2. Number of PFC per 10^6 spleen cells of neonatally azathioprine T-cell depleted rats and normal rats after injection of sRBC and outbred thymus cells at the age of 8 weeks

Groups	Cells incoulated	Days after antigen injection	Average PFC per 10^6 spleen cells	P values*
Control	sRBC	3	96.8±7.5	P<0.01
		7	30.0±2.8	P<0.01
Experimental control	sRBC	3	52.4±6.4	—
		7	20.5± 2.5	—
Experimental I	sRBC+ 10^5 thymus cells	3	58.7± 6.2	NS**
		7	21.4± 1.9	NS
Experimental II	sRBC+ 10^6 thymus cells	3	125.4± 9.8	P<0.01
		7	33.5± 3.7	P<0.01
Experimental III	sRBC+ 10^7 thymus cells	3	212.5±19.7	P<0.01
		7	63.8± 5.9	P<0.01
Experimental IV	sRBC+ 10^8 thymus cells	3	142.8± 6.2	P<0.01
		7	57.6± 6.2	P<0.01

* P values are compared with the average of experimental control group

** NS; not significant

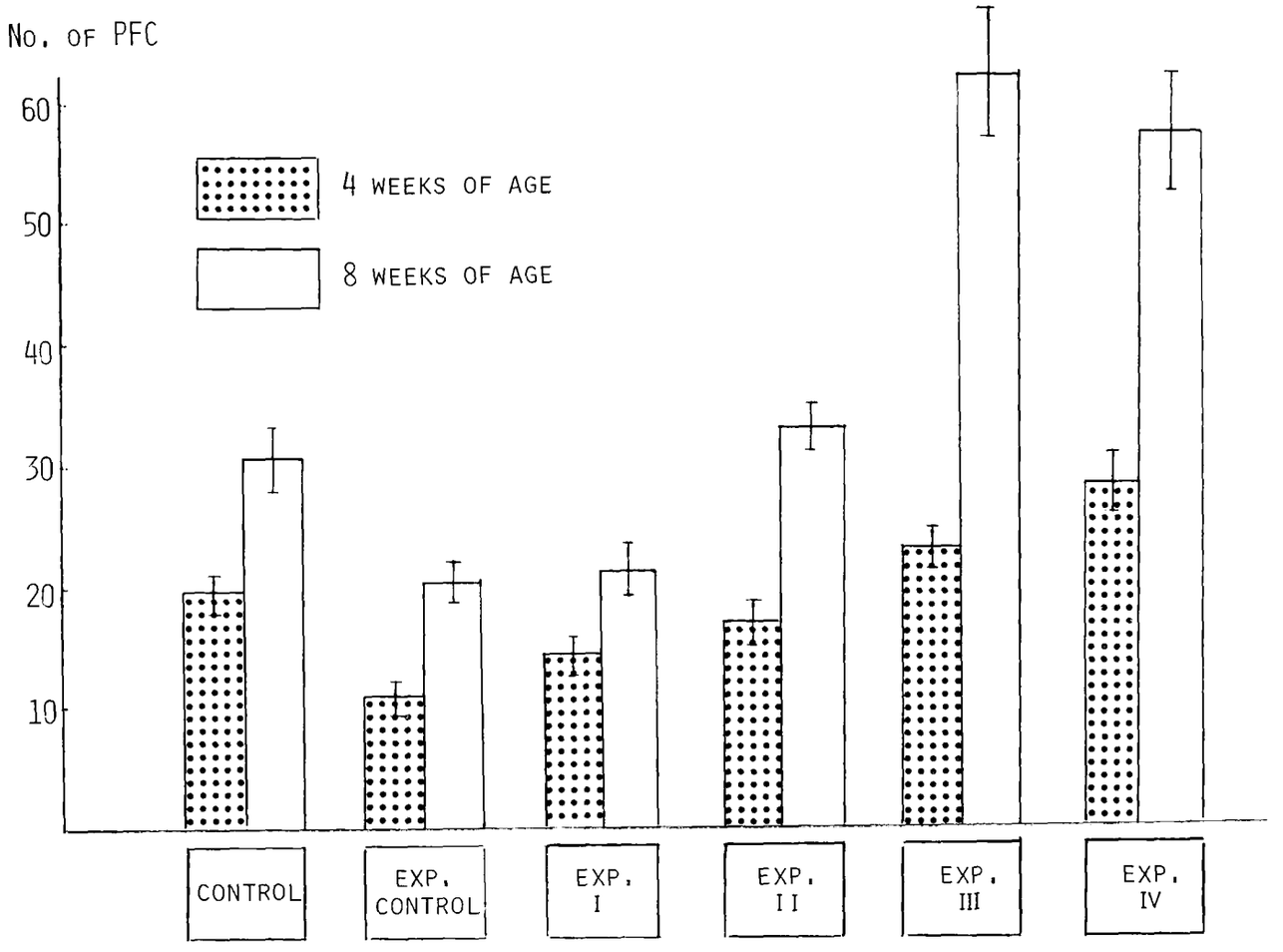

Fig. 2. Comparison of No. of PFC in the 4 week old and 8 week old groups, respectively on the 7th day after antigen(sRBC) injection.

DISCUSSION

The collaboration between T and B lymphocytes in T-cell dependent immune response has been studied by several investigators. The first demonstration that the collaboration between them was required for the generation of the immune responses came from the seminal experiments of Claman et al. (1963). Claman and Co-workers demonstrated that the antibody response to sRBC antigen by lethally irradiated mice could be fully reconstituted by the adoptive transfer of spleen cells. Miller and Mitchell (1968) also demonstrated that the antibody forming capacity to sRBC antigen in the neonatally thymectomized mice could be fully recovered by the inoculation of syngeneic or allogeneic thymus cells. Thereafter, Hamaoka et al. (1973) and Katz et al. (1973) reported that histoincompatible T helper (T_H) and B cells could cooperate with each other. And they demonstrated that the mechanism by which histoincompatible T_H and B cells collaborated was distinct from the mechanism by which histocompatible T_H and B cell collaborated. All these studies are qualitative ones about the collaboration of T & B lymphocytes. However, quantitative study is also necessary for the investigation of more correct mechanism about it.

Therefore it is important to determine the optimal dosage of the inoculated thymus cells at the time of the inoculation of outbred thymus cells for the recovery of the immune function into the neonatally azathioprine T-cell depleted rats.

The optimal dose of antigen, the peripheral T lymphocytes which recognize that antigen, and B lymphocytes which receive the signal from the antigen & T lymphocytes are involved in the serial processes of T-cell dependent immune response from the exposure to antigen to the activation of B lymphocytes and secetion of antibody. In this experiment, only the peripheral T lymphocytes among above three requisites for T-cell dependent immune response were modulated by azathioprine.

That is, because sRBC antigen is a T-cell dependent antigen and the intensity of its antigenicity is dose-dependent, the dosage of sRBC antigen was determined by the body weights of experiment animals. So, by intraperitoneal injection of 0.5 ml of 30% sRBC suspension into the 4 week old groups, 1.0 ml into the 8 week old groups, respectively (Chang et al. 1983), the effects of the changes of antigen dose on the immune response were tried to abolish. And according to the reports by Chen et al. (1976) and Chang et al. (1983), azathioprine has no effect on B-cell system.

In this experiment, by the dosage of thymus cells inoculated, which was approximately determined by the total number of thymus cells in a thymus, four experimental groups were established. These four groups were inoculated with 10^5, 10^6, 10^7 and 10^8 thymus cells respectively.

Generally much more PFC in the 8 week old groups were observed than that in the 4 week old groups. It implies that the immunological activity is related to the age, and that depleted peripheral T lymphocytes by inhibited fetal dovelopment of thymus can be replenished with age (Lee et al. 1978; Chang et al. 1983).

In general, much more PFC were observed in the groups of 3 rd day after sRBC injection than in the groups of 7th day after sRBC injection and it is known to be its own antigenicity of sRBC antigen (Chang et al. 1983). In the neonatally T-cell depleted groups, there was statisticaly significant decrease in the number of PFC, compared with that in the control groups. In the groups into which the thymus cells were inoculated, there was, in general, marked increase in the number of PFC. In the 4 week old groups, the normal level of PFC(56.0 PFC) could be reached by the inoculation of between 10^6 and 10^7 thymus cells. The group into which 10^7 thymus cells were inoculated showed the peak number of PFC(87.4 PFC). In the 8 week old groups, the normal level of PFC(96.8 PFC) could reached by the inoculation of between 10^5 and 10^6 thymus cells and the group into which 10^7 thymus cells were inoculated showed the peak number of PFC (212.5 PFC). Above results also showed that the depleted peripheral T lymphocytes by inhibited fetal development of thymus were replenished with age. There was, however, a decrease rather than an increase in the number of PFC as the number of inoculated thymus cells increased from 10^7 to 10^8. Several considerations could be taken into account as the reasons for the above phenomenon, but further study would seem indicated before firm conclusions can be made.

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= 국문초록 =

임신중 투여된 Azathioprine (Imuran)에 의해 면역기능이 억제된 신생흰쥐에 정주할 흉선세포의 정량에 관한 연구

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임신중 투여된 Azathioprine에 의해서 면역기능이 억제된 신생흰쥐에게 이계교배된 흰쥐의 흉선세포를 정맥주사함으로써 면역기능의 회복 여부를 관찰하고 정주할 흉선세포의 적정용량을 결정하기 위해 다음과 같은 실험을 수행하였다.

실험동물로는 Sprague-Dawley 계통의 200g 내외의 임신한 흰쥐 60마리(이중 45마리에게는 임신 제7일에 Azathioprine을 8mg/kg 경구투여함)에서 정상분만으로 태어난 신생흰쥐 200마리를 6군으로 나누어 사용하였다. 항원으로 면양적혈구를 생후 4주 8주 후에 복강내 투여하였고 Azathioprine에 의해 말초 T임파구가 고갈된 일부의 군에는 이계교배된 흉선세포를 10^5, 10^6, 10^7 10^8개의 순으로 각각 투여하였다. 각 군을 항원 투여 후 제3일과 7일에 도살하고 비장을 적출하여 세포부유액을 만들고 Plaque Assay법을 이용하여 항체 분비세포의 수를 계수하여 다음과 같은 결론을 얻었다.

1. Azathioprine에 의해서 말초T임파구가 고갈된 군에서는 Azathioprine을 투여하지 않은 정상군에 비해서 통계적으로 유의한 정도로 항체 형성세포의 수가 적게 관찰되었다.
2. 생후 4주군들에서는, 흉선세포를 10^6개에서 10^7개 사이의 용량을 투여함으로써 항체 형성세포가 정상 수준으로 회복되었고, 생후 8주군에서는 10^5개와 10^6개 사이의 용량에 의해서 정상 수준으로 회복되었다.
3. 생후 4주와 8주의 모든 군들에서 10^7개의 흉선세포를 투여했을 때 가장 많은 수의 항체형성세포가 관찰되었다.