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## Role of the Thymus in Anti-Hapten Responses in the Rabbit

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**Abstract** Rabbits thymectomized within 2 months after birth presented a more marked anti-hapten antibody response against DNP-BGG than non-thymectomized control animals. A reduction in anti-hapten response was observed in rabbits injected with allogeneic thymocytes, although it was of short duration. A marked reduction in anti-hapten response was observed in rabbits pre-immunized with the carrier, showing a sharp contrast with an ordinary response observed in the mouse.

The results mentioned above seemed to show the possibility that the immunological function of the rabbit T cells might be qualitatively or quantitatively different from that of the mouse T cells.

Great progress in the study of the cellular basis of immunity has been made in the past decade, since Miller<sup>1)</sup> has first reported on the immunological significance of the thymus in 1961. One of the most important advances in cellular immunology may be the classification of immunocompetent lymphocytes into two categories, T and B, which not only perform distinct roles in immunity but also interact with each other in humoral immune responses. Thus, Mitchison<sup>2,3)</sup> has clarified, using hapten-carrier systems, that T cells recognize carrier portion of the antigen, resulting in the anti-hapten antibody synthesis by B cells which recognize haptenic determinants attached onto the carrier protein.

Studies by several investigators on the T-B cooperation in immune responses<sup>4-8)</sup> have been mostly carried out by the use of mice, and those conducted in rabbits were far less easy to find in literatures except a series of studies by Richter and his co-workers<sup>9-35)</sup>. The latter fact seems to result from two reasons. Firstly, rabbits are the species difficult to obtain an inbred strain in sufficient numbers and secondly, it is doubtful whether rabbit lymphocytes, especially T cells have the same function as those of mice, because the effect of thymectomy is reported to vary greatly with the species of animals used. According to Burnet's observation<sup>36)</sup>, there was no visible effect of neonatal thymectomy in the dog and very little in the rabbit. In addition, Abdou and Richter<sup>37)</sup> have reported that the antigen-reactive cells of the rabbit (i. e., mouse T cell equivalent) are not originated from the thymus but are normally found in the bone-marrow and the organ source of the rabbit antibody-forming cells remains to be clarified. If this were true, it should be said that the immunological significance of the rabbit thymus and thymus-derived cells is beyond comprehension. Thus, our present study was undertaken to clarify the role of the rabbit thymus in immunity and it was demonstrated that thymectomy of the the rabbit within 2 months after birth resulted in a marked enhancement of the humoral response against DNP.

## Materials and Methods

**Animals.** Outbred white albino rabbits of both sexes were used throughout. In order to minimize the deviations due to differences in individualities, choice was made of rabbits having the same allotype of immunoglobulin,  $a^s a^s b^t b^t$ , except where otherwise indicated. When the effect on the anti-hapten antibody responses is to be tested, comparison was made among littermates born in the same day from the same venter, after their being divided into two groups, thymectomized and non-thymectomized.

**Antigen.** 2, 4-dinitrophenyl (DNP) conjugates of bovine  $\gamma$ -globulin (BGG) were prepared according to the method of Eisen et al<sup>38</sup>. Average number of the haptenic determinants was estimated as 38 per carrier molecule.

**Immunization procedures and serum assay.** Two mg of DNP<sub>38</sub>-BGG in Freund's complete adjuvant (FCA) were injected into both footpads by halves (day 0). Three weeks after priming, animals were titrated for their primary anti-hapten antibodies (day 21) followed by second challenges of the same antigen. Secondary antibody responses were assayed on day 28. The concentration of rabbit anti-DNP was titrated by complement-fixation test according to the technique of Stein and Ngu<sup>39</sup> with slight modification of ours, the details of which have been published elsewhere<sup>40</sup>.

**Depletion of T cells.** To delete the T cells in adult thymectomized rabbits, animals received 800 rads at a dose rate of 60 rads/min, using a cobalt<sup>60</sup> source and were repopulated with  $5 \times 10^8$  allogeneic bone-marrow cells within 2 to 3 hrs.

**Statistical analysis.** Arithmetic means and standard errors of the mean were calculated from the reciprocal values of complement-fixation titers. P values were determined by Student's test, values less than 0.05 being chosen as significant in the comparison of any two groups.

## Results

### *Effect of thymectomy on anti-hapten response in rabbits at early ages*

#### **1. Thymectomy one month after birth**

Rabbits were thymectomized one month after birth and their antibody responses against DNP were compared with those of non-thymectomized littermates. Experiments were carried out 2 times; in Experiment I, antigenic challenge was given immediately after thymectomy and in Experiment II, it was given 3 weeks after the operation. Table 1 shows the results of these experiments. As shown in the table, thymectomized rabbits showed an enhanced antibody response against DNP as compared with non-thymectomized controls, difference between these two groups being proved to be statistically significant.

#### **2. Thymectomy two months after birth**

The same experiment as above except that thymectomy was carried out 2 months after birth (in this experiment first antigenic challenges were given 3 weeks thereafter) were undertaken with similar results as shown in the upper 2 columns of Table 2. A point which differs from the data of Table 1 is that the difference of secondary antibody response between thymectomized and non-thymectomized rabbits was not significant when animals were thymectomized two months after birth. When rabbits were thymectomized more than 3 months after birth, the difference of antibody responses in both groups were no longer observed (data not shown).

**Table 1.** Elevation of anti-DNP antibody titers in rabbits thymectomized 1 month after birth

Exp. <sup>a</sup>	Rabbit #	Tx <sup>b</sup>	Anti-DNP response <sup>c</sup>	
			primary	secondary
I	1	+	200	ND <sup>d</sup>
	2	+	500	2000
	3	+	400	1200
	4	+	250	500
			(338 ± 59) <sup>e</sup>	(1233 ± 354)
	5	-	20	20
	6	-	20	20
	7	-	330	1100
II	8	-	120	20
			(123 ± 63)	(290 ± 234)
	9	+	2300	4330
	10	+	1340	2200
	11	+	760	1560
			(1467 ± 366)	(2696 ± 683)
	12	-	120	290
	13	-	350	850
II	14	-	530	1170
	15	-	330	500
			(332 ± 73)	(702 ± 120)

a. In Experiment I, antigenic challenge was given immediately after thymectomy and in Experiment II, it was given 3 weeks after operation. b. Tx: thymectomy. c. titrated by complement-fixation test. d. ND: not determined. e. mean titer ± SEM.

**Table 2.** Anti-hapten responses in rabbits thymectomized 2 months after birth and those in animals received allogeneic thymus cell transfer

Rabbit #	Treatment	Anti-DNP response	
		primary	secondary
16	thymectomized 2 months after birth	1000	1540
17		2010	1720
18		2290	1730
19		1000	ND
20		2100	ND
Mean titer ± SEM		1680 ± 252	1663 ± 54
21	non-thymectomized (control)	1200	1440
22		1200	1390
23		470	1090
24		1010	1780
25		380	910
Mean titer ± SEM	825 ± 159	1322 ± 134	
26	transferred with $5 \times 10^9$ allogeneic thymocytes	300	550
27		400	1000
28		270	1270
29		300	ND
Mean titer ± SEM	318 ± 23	940 ± 171	

*Effects on antihapten responses of thymus cell transfer, pretreatment by carrier protein and T cell depletion*

Since an enhancement by thymectomy of anti-DNP response was observed, we examined whether or not the thymocyte transfer results in a decrease of response in contrast. Results were shown in lower part of Table 2, to which middle part of the table served as a control. In primary response, mean titer of anti-DNP in rabbits being transferred allogeneic thymocytes was less than half the titer of those not transferred thymocytes (318 vs 825), thus showing that the thymocyte transfer would reduce the anti-hapten response to a considerable extent ( $P < 0.05$ ). Such a depression in mean antibody titer, however, is of short duration, recovering to a comparable level with that of non-transferred controls in the secondary response. It was felt that the simplest explanation of this results is that the transferred cells should be impaired rapidly in recipients owing to not being syngeneic. Then, an attempt was made by pre-immunization with carrier protein to have their own T cells proliferated. Thus, rabbits were immunized with BGG followed by challenges with DNP<sub>35</sub>-BGG 3 weeks after pretreatments. Anti-hapten responses in rabbits were completely inhibited when carrier-reactive T cells were previously stimulated by BGG, as shown in Table 3 (group C). Instead of non-thymectomized normal animals, rabbits of group B re-injected intra-venously with autologous thymocytes after thymectomy were used as a control for group C in

**Table 3.** Effects on anti-DNP responses in the rabbit of thymectomy, pretreatment by carrier protein and T cell depletion

Group	Rabbit #	Allotype	Treatment	Anti-DNP response	
				primary	secondary
A*	9	$a^3 a^3 b^4 b^4$	Tx	2330	4330
	10	"		1340	2000
	11	$a^1 a^3 b^4 b^5$		760	1560
	Mean titer $\pm$ SEM			1467 $\pm$ 366	2697 $\pm$ 683
B	33	$a^1 a^3 b^4 b^6$	Tx and re-infused with autologous thymus cells ( $1 \times 10^9$ )	500	700
	34	$a^1 a^3 b^4 b^4$		580	640
	35	"		690	1000
	36	$a^3 a^3 b^4 b^4$		520	520
	37	"		510	540
Mean titer $\pm$ SEM		560 $\pm$ 32	680 $\pm$ 77		
C	38	$a^3 a^3 b^4 b^4$	pre-immunized with carrier protein (BGG)	20	20
	39	"		20	20
	40	$a^3 a^3 b^9 b^9$		20	20
	41	"		20	20
Mean titer $\pm$ SEM		20 $\pm$ 0	20 $\pm$ 0		
D	42	$a^3 a^3 b^4 b^4$	TXBM**	20	20
	43	"		20	640
	44	"		20	640
	45	"		20	20
	46	"		20	20
Mean titer $\pm$ SEM		20 $\pm$ 0	268 $\pm$ 136		

\* data listed in Table 1.

\*\* thymectomized, X irradiated and bone marrow reconstituted.

this table. From the data of group B, it was shown that the re-infusion of the removed thymus cells into self results in the loss of enhancing effect of thymectomy on anti-hapten response in the rabbit. For convenience, the data of thymectomized animals (group A) already listed in Table 1 were added in Table 3 for comparison. Differences between following two groups in both primary and secondary responses were statistically significant, P values of which were given in parentheses: Group A vs Group B ( $<0.05$ ), Group A vs Group C ( $\ll 0.01$ ), Group A vs Group D ( $\ll 0.01$ ), Group B vs Group C ( $\ll 0.01$ ), and Group B vs Group D ( $\ll 0.01$ ).

As evidenced by these results, the magnitude of anti-DNP response in the rabbit seems to have something to do with the quantity of T cells, i. e. T cells larger than normal levels in quantity will induce reduced anti-DNP responses, whereas T cells smaller than normal will do increased ones. However, if T cells were depleted by means of both thymectomy and irradiation, no anti-DNP response occurred as shown in Group D of Table 3, indicating that helper T cells are also necessary for antihapten responses in the rabbit as in the case of the mouse.

### Discussion

The discovery that T cells not only act as helpers in humoral antibody responses to assist B cells, but also act as suppressors to regulate them for antibody production is considered to have ushered in a golden age of so-called T-ology. Generally speaking, antibody formation is influenced by a balance between helpers and suppressors. As to whether these two functions of T cells are carried out by the same T cells or by those belonging to different subsets, data reported by several investigators were quite controversial.

On the one hand, the same population of T cells is considered to be responsible for both enhancement and suppression of humoral responses with the suggestion that suppression is mediated by supraoptimal numbers of helper cells ("too much help")<sup>41-43</sup> while on the other hand, evidence has been accumulated that stimulation and inhibition of antibody response represent the activities of different T cell subpopulations specialized to carry out these distinct roles<sup>46,47,55</sup>. The present study is not designed to clarify these alternatives directly. The most important point to emerge from our findings is that the rabbit thymocytes or thymus-derived cells seem to have much stronger suppressive activities than the mouse T cells as a whole. Our results could be symbolically re-summarized as Table 4, being in sharp contrast to the data of mice appearing in many papers thus far reported. Suppose that normal level of T cells were arbitrarily marked with  $\ddagger$  sign, then the T cell level in animals received allogeneic thymocyte transfer could be given  $\#\#$  sign, that of thymectomized animals  $+$ , and finally, the level in animals deprived of T cells by thymectomy and irradiation could be marked with  $-$  sign. As to a strength of anti-DNP response, let us designate the response of normal animals as  $\ddagger\ddagger$ . In conclusion, reverse relationship was observed

**Table 4.** Relationship between the quantity of T cells and the magnitude of anti-DNP response in the rabbit

Treatment	Quantity of T cells	Response
T cell transfer	$\#\#$	$+$
None (normal)	$\ddagger$	$\ddagger\ddagger$
Thymectomy	$+$	$\#\#$
TXBM*	$-$	$-$

\* thymectomized, lethally irradiated and bone marrow protected.

between the magnitude of anti-DNP response and the quantity of T cells in rabbits except T cell-deprived animals. Elevation of anti-hapten antibody titers by thymectomy is thought to be due to a partial removal of suppressor cells, while lowering of the titers by allogeneic thymocyte transfer might be interpreted as indicating that suppressive activity by transferred cells was added to the normal response. Failure of antibody formation in animals received both thymectomy and irradiation is best explained by an assumption that this treatment removed not only suppressors but also helpers as well. The fact that the anti-hapten response was almost completely inhibited by priming of animals with carrier protein is of special interest. As to the inhibition of anti-hapten response, we can take two possibilities into consideration.

One possibility is that carrier-specific suppressor cells were selectively induced by priming with carrier protein (BGG), which resulted in reduced or abrogated responses. Alternative possibility will be that the carrier protein stimulated overall helpers and thus proliferated excess helpers might bring about the suppression in their net effect. At all events, true mechanism is not known from this experiment. However, judging from the fact that the same or at least apparently similar experiments conducted in mice resulted in the enhancement of antibody response with no or little, if any, exceptions, we want to interpret our data as indicating that the rabbit T cells have something functionally different from the mouse T cells. In addition to the species difference, however, it must be borne in mind the does effect of carrier-priming. In this connection, the results reported by Sarvas et al.<sup>48)</sup> might be suggestive. They observed that the anti-hapten response in chickens was enhanced to fivefold titers if the birds were given a small dose of carrier protein a week before, but the dose of the carrier was larger, it reduced the subsequent anti-hapten response against the hapten-carrier conjugate. They explained their data on the basis that, in the former, carrier-specific helper T cells stimulated by small dose of carrier-priming have increased anti-hapten response, whereas in the latter, a good anti-carrier antibody response was connected with the specific inhibition of anti-hapten response. Apart from whether this explanation is valid for the rabbit or not, the similar results as ours can be found bibliographically. For example, Baum et al.<sup>49)</sup> reported that a marked enhancement of antibody production against KLH was observed in rats when the animals were pre-treated with anti-rat lymphocyte globulin (ALG) by which selective reduction of T cells is considered to occur.

Baker et al.<sup>50)</sup> have observed that mice treated with antilymphocyte serum (ALS) and immunized with type III pneumococcal polysaccharide (SSS-III) showed a ten-to hundredfold increase in the number of direct splenic plaque-forming cells (PFC). Such an enhancing effect on PFC responses of ALS treatment has been also reported by Barthold et al,<sup>51)</sup> Kerbel et al.<sup>52)</sup> and others<sup>53,54)</sup>. Concerning the mechanism of enhanced response by ALS, Baker et al<sup>50)</sup> have interpreted their findings that ALS may have selectively eliminated a population of cells which exerts a suppressive effect on the antibody response to SSS-III.

In recent years, evidence has been accumulated to consider that helper and suppressor T cells should belong to the distinct populations in that they are different from each other at least in the following points. Helper T cells have surface differentiation antigen Ly-1<sup>57,58)</sup>, are radioresistant, relatively insensitive to anti-T cell antisera in the presence of complement, and disappear slowly from adult thymectomized mice but are absent in the spleens of nu/nu mice. By contrast, suppressor T cells have Ly-2, 3, are radiosensitive, are sensitive to anti-T cell antisera and disappear rapidly from adult thymectomized animals. At the same time, these two types of cells are to be considered from the viewpoint of ontogeny. According to Droege<sup>59)</sup>, suppressors were the predominant type of T cells in newborn chickens (0-2 wks of age), whereas no suppressive activity was

found in T cells of 8 month old birds. Mosier et al<sup>60)</sup> have also made the same observation that the marked suppressor activity found in the thymus of newborn mice was progressively decreased with ages. In this respect, the relationship between net immune responses and biological or immunological maturities with age is supposed to remain to be investigated.

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