

## THE THYMUS AND RECOVERY FROM CYCLOPHOSPHAMIDE-INDUCED TOLERANCE TO SHEEP ERYTHROCYTES\*

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The technique introduced by Jerne for enumerating antibody-forming cells in agar (1, 2) permits precise analysis of the cellular kinetics of the immune response to sheep erythrocytes. In an earlier paper the kinetic events and the alterations in nucleic acid metabolism observed during the induction of tolerance to this antigen with the immunosuppressive drug cyclophosphamide were analyzed (3). The present manuscript describes the recovery from this type of tolerance and defines the role of the thymus.

### *Methods*

As in the earlier experiments (3), tolerance to sheep erythrocytes was induced in 10-12 wk old female CBA mice (Jackson Laboratories, Bar Harbor, Me.) by the intraperitoneal injection of 160 or 320 mg/kg of cyclophosphamide (Cytosan, Mead Johnson Laboratories, Evansville, Ind.) in four daily divided doses. The tolerance-inducing injection of antigen, 0.8 ml of a 30% suspension ( $6.4 \times 10^8$  cells) of sheep erythrocytes, was injected intravenously immediately before the first drug injection.

The method of Jerne (1, 2), as previously modified (3), was employed to determine the number of hemolysin-producing spleen cells. Animals were challenged with 0.2 ml of 10% sheep cells ( $5 \times 10^8$  cells) 4 days before sacrifice. An appropriate aliquot of sieved spleen was incubated with sheep erythrocytes in Gey's solution which was gelled by the addition of Agarose (SeaKem brand obtained from Bausch and Lomb Incorporated, Rochester, N. Y.). The gelled suspension was incubated for 2 hr at 37°C, 1 ml of 1:10 guinea pig serum was added, and the suspension incubated for an additional hour to develop the direct (19S) plaques. All the data presented except for Table I concern 19S plaques, but representative experiments of each type were analyzed for indirect (7S) plaques by the addition of rabbit anti-mouse gamma globulin serum before the complement addition (3, 4). Unless specified to the contrary, experimental points are arithmetic means of the spleens of four animals individually plaqued.

All animals were subject to postmortem examination, and the data from any thymectomized mice with residual thymus tissue were discarded. Data from operated or nonoperated adult animals weighing less than 20 g or with a spleen cell count of less than 100 million were also discarded. Such animals usually have pneumonia and respond poorly to sheep cell injection.

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## RESULTS

*Decline of Plaque-Forming Cell Response and Thymus Weight with Age.*—Because recovery from drug-induced tolerance occupies a considerable fraction of

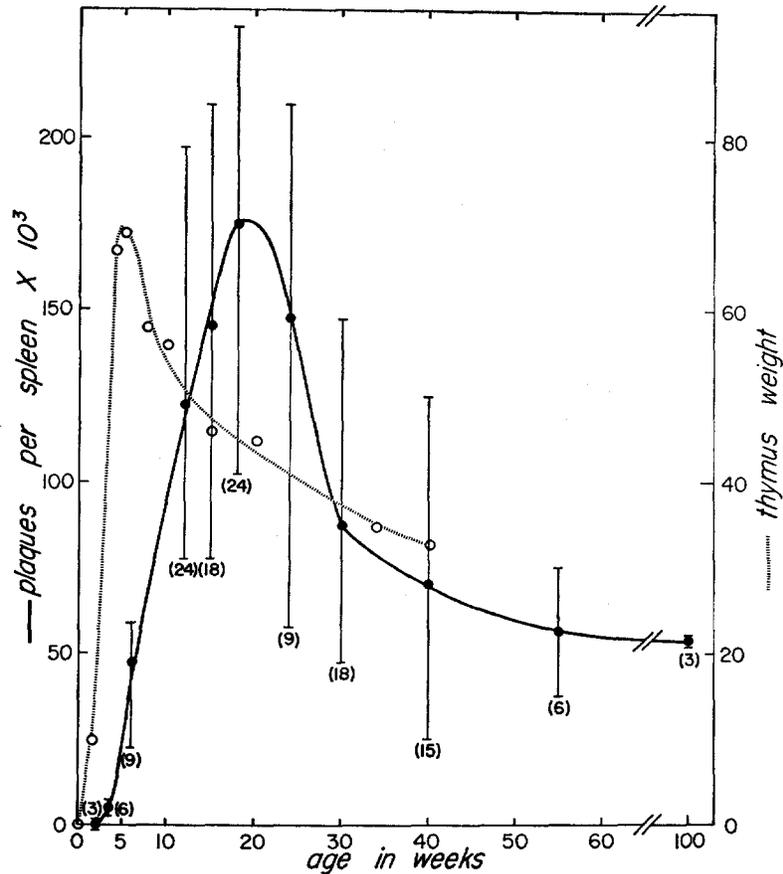


FIG. 1. Direct hemolytic plaques after challenge and thymus weights of female CBA mice of various ages.

The solid line connects the arithmetic means of hemolytic plaques present in the entire spleen 4 days after sheep cell injection. All normal animals of the entire series of experiments have been included, the extreme values are represented by vertical bars, and the number of animals of each age is indicated in parentheses.

The broken line connects arithmetic means of thymus weights (mg) of groups of three animals.

the life span of the CBA mouse, it was necessary first to establish the variation with age of the plaque response to sheep cell injection. Fig. 1 contains a compilation of the plaque response for all normal animals of various ages in the entire series of experiments plotted as plaques per spleen against age. Although there

is wide variation among animals of a given age, the arithmetic means for the various ages fall along a smooth curve (the unbroken line in Fig. 1). This variation is decreased, but not eliminated, when animals of different ages are challenged and plaqued at the same time (Table I), suggesting that the variability is in part due to minor differences in the times between challenge and sacrifice. Appreciable numbers of hemolysin-forming cells are not seen after antigenic challenge in the 1st wk of life (Fig. 1), appear in the 2nd and 3rd week, rapidly rise to a maximum in the 15th to 20th wk, and undergo a substantial decline over the remainder of the life of the animal. The spleens of the oldest animals studied form only one-third as many plaques in response to antigen as do those of mice in their immunological prime. Table I indicates that the indirect (7S)

TABLE I  
*Direct (19S) and Indirect (7S) Plaques in Mice of Various Ages*

Expt. No.	Age* in weeks	19S Plaques per spleen	7S Plaques per spleen
1	2	256	224
	3	8,740	5,760
	16	137,000	65,000
	35	101,000	73,000
2	2	1,400	
	7	51,900	
	18	218,000	169,000
	30	81,800	
	40	83,800	
3	5	54,100	
	14	157,200	
	28	82,700	
	42	54,800	

\* Age at time of plaquing.

plaques, which are not plotted in Fig. 1, closely parallel the direct (19S) results in animals of various ages. In young animals (2 and 3 wk old), animals in their immunological prime (16 and 18 wk old), and in older animals (35 wk old), indirect plaques were between one-half the number and a number equal to the direct plaques 4 days after challenge.

Fig. 1 also indicates the thymus weights of female CBA mice of various ages (broken line). Thymus weight rapidly rises to a maximum in animals 5 wk old and then declines.

*Decline of Immune Responsiveness with Age in Thymectomized Mice.*—In Table II the age-related decline of immunity in normal and thymectomized mice is compared. The sheep cell response declines more rapidly in aging thymectomized mice and eventually reaches a level 25–30% that of non-

operated animals. In these experiments this ratio was largely reached 20 wk after thymectomy, and there was little further disproportionate fall, with subsequent aging, in the plaque response of thymectomized mice.

TABLE II  
*Decline of Immune Responsiveness with Age in Thymectomized Mice\**

Expt. No.	Time after thymectomy	Normal (N)	Thymect. (T)	Ratio N/T
1	5 wk	210,000	167,000	0.80
	10 wk	94,700	63,200	0.67
	20 wk	94,700	30,800	0.30
	30 wk	143,400	43,200	0.30
2	42 wk	126,000	36,000	0.29
	60 wk	73,000	21,000	0.29
	100 wk	54,000	13,700	0.25

\* Thymectomized at 8 wk of age.

TABLE III  
*Plaque-Forming Spleen Cells and Total Spleen Cells at Various Times after Cyclophosphamide and Cyclophosphamide Sheep Cell Administration with and without Challenge*

Time after drug	Antigenic challenge	Normal		Cyclophosphamide		Cyclophosphamide sheep cells	
		Plaques per spleen	Cells per spleen $\times 10^6$	Plaques per spleen	Cells per spleen $\times 10^6$	Plaques per spleen	Cells per spleen $\times 10^6$
18 days	—	34	135	54	137	2	151
	+	93,000	189	30,300	183	10	141
5 wk	—	44	157	47	124	2	128
	+	64,000	222	12,800	162	1,190	113
9 wk	—	67	224	62	114	16	115
	+	290,000	373	70,000	271	41,100	203
14 wk	—	44	141	42	149	48	163
	+	191,000	253	140,000	264	49,000	188

\* Cyclophosphamide 80 mg/kg was administered on days 1, 2, 3, and 4.

*Recovery of Plaque-Forming Cells of the Unstimulated Spleen and of the Proliferative Response during Recovery from Tolerance.*—Table III lists the number of plaques in the spleen 18 days, 5 wk, 9 and 14 wk after cyclophosphamide in an experiment in which parallel groups of animals were plaqued without (base line plaques) and with antigenic challenge. As previously reported (5), base line

plaques are absent 18 days after animals were made completely tolerant to sheep cells by cyclophosphamide and antigen. These plaques are still absent at 5 wk, begin to return at 9 wk (when mice show partial recovery from tolerance), and have completely returned at 14 wk (though recovery from tolerance is not complete at this time). This table also includes the total number of spleen cells (counted in a hemocytometer as a conventional white cell count)

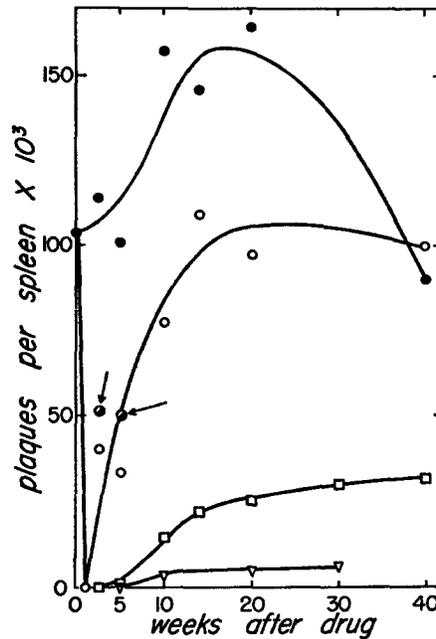


FIG. 2. Recovery from tolerance to sheep erythrocytes induced with cyclophosphamide in high dosage (80 mg/kg on days 1, 2, 3, and 4).

●—●, Normal; ○—○, normal, cyclophosphamide; ◐, thymectomized, cyclophosphamide; ◑—◑, normal, cyclophosphamide and sheep cells on day 1; ▽—▽, thymectomized, cyclophosphamide sheep cells. The 18 day and 5 and 10 wk points are arithmetic means of 18 animals, the 20 wk point of 9 animals, and the 0, 30, 40 wk points of 6 animals. All values are in direct hemolytic plaques per spleen 4 days after challenge with sheep cells.

before and after antigenic challenge, and it can be noted that specific tolerance is accompanied by a loss of the proliferative response to antigen.

*Recovery from Cyclophosphamide-Induced Tolerance.*—In Fig. 2 data are presented on the disappearance of tolerance induced in normal and thymectomized mice with high dosage cyclophosphamide. This drug dosage results in a protracted period of nonspecific immune injury, as indicated by the curve defined by the open circles. In nonthymectomized, drug-tolerant animals (open squares), some recovery from tolerance is noted at 5 wk, recovery is rapid between the 5th and the 14th wk, and much slower improvement continues to the

40th wk when the experiment was terminated. Thymectomized, tolerant animals (open triangles) display almost no recovery at 5 wk, a modest recovery between 5 and 10 wk, and no subsequent improvement.

In Fig. 3, identical data (the normal control curve has been omitted) for an experiment with low dosage cyclophosphamide are plotted. The results appear quite similar to those obtained with the higher drug level, with the exception that recovery from nonspecific drug injury is essentially complete at the end of

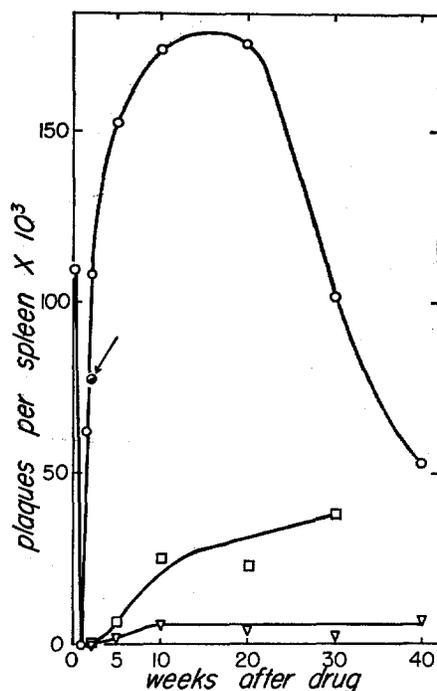


FIG. 3. Recovery from tolerance to sheep erythrocytes induced with cyclophosphamide in low dosage (40 mg/kg on days 1, 2, 3, and 4). Legends the same as Fig. 2, except that the points are arithmetic means of 4 animals.

18 days. Thereafter, the hemolysin-producing cell response in animals treated with the drug alone passes through the age-related maximum and decline that was described in untreated animals. From the plotted data, it appears that the slowly rising curve for animals made tolerant to sheep cells with cyclophosphamide will cross the falling cyclophosphamide control curve about 40 wk after recovery from tolerance is first observed. As at the higher drug dosage, some recovery of specific responsiveness occurs in thymectomized animals in the first 10 wk, but none thereafter. It is of considerable interest that thymec-

tomy does not influence the recovery from the nonspecific immunological depression induced with either high or low dosage cyclophosphamide (points indicated by arrows in Figs. 2 and 3), but only recovery from specific immune tolerance.

*The Influence of Delayed Thymectomy on the Recovery from Tolerance.*—It is important to determine whether the thymus is required only during the period of cellular repopulation after drug administration, or whether the organ must be continually present for recovery from tolerance to proceed. Thus the experiment represented in Table IV contains an additional group of animals in whom tolerance was induced with drug and antigen, and who were thymectomized 18 days later at a time when tolerance was still complete (experimentally verified). The recovery of the late thymectomy group can then be compared with non-thymectomized animals and animals thymectomized before the induction of

TABLE IV  
*Effect of Delayed Thymectomy on Recovery from Tolerance to Sheep Cells Induced with Cyclophosphamide*

Experimental group*	Plaques per spleen†
Normal control	180,700 ± 101,000
Tolerant, nonthymectomized	22,800 ± 10,200
Tolerant, thymectomized before tolerance is induced	4,160 ± 2,030
Tolerant, thymectomized 18 days after tolerance is induced	2,570 ± 1,130

\* All animals plaqued at 22 wk of age, 10 wk after tolerance was induced with 160 mg/kg of cyclophosphamide.

† Groups of 6 animals, ± standard deviation.

tolerance. The results indicate that late thymectomy is equally effective in preventing subsequent immune recovery.

*Indirect Plaques*—Representative experiments of each of the previous sections were analyzed for indirect plaques with findings essentially similar to the direct results reported.

#### DISCUSSION

The decline in the immunological responsiveness of intact mice with advancing age seen in these experiments has been observed before (6, 7), and the more rapid decrease of this responsiveness seen in thymectomized adult animals has been reported by several investigators (7–9). Since it is known (10) that thymus weight goes through a maximum in mice 4–6 wk of age (in CBA mice the maximum is at 5 wk) after which it diminishes, it would be important to establish whether the decline in this organ is related to the subsequent decline in immunological reactivity. (The observation that the peak of the sheep cell-

plaque response occurs about 10–15 wk after the decrease in thymus weight has begun makes it unlikely that the thymus decline is a manifestation of senescence of the lymphoid system in general.) In our own work, during the first 20 wk after thymectomy, operated mice show a disproportionate fall in immune responsiveness reaching a level of 30% that of intact animals of the same age. Thereafter, the fall of responsiveness of thymectomized animals parallels or slightly exceeds the fall in nonoperated mice. Thus, our data in thymectomized animals could be explained as a decline which is a composite of that caused by thymectomy and that caused by aging. Our studies neither support nor contradict Metcalf's view (6), based on his failure to restore the immune response of old animals with newborn thymus grafts, that senescence of immune responsiveness in mice is not caused solely by a decline of thymus function.

Our experiments connect two events with the induction of specific immunological tolerance: the disappearance of hemolysin-producing cells of the unstimulated spleen (base line plaques), and the loss of the proliferative response to antigen. Disappearance with induction, and return with recovery from tolerance are strong evidence that the base line plaque-forming cell is related to the cell that responds to antigen. This conclusion does not contradict the findings of Hege and Cole (11) that such cells are present in immunologically incompetent, neonatally thymectomized mice, or our own data<sup>1</sup> that such cells are also present in similarly incompetent thymectomized, irradiated adult CBA animals. Although possession of 19S hemolysin-producing cells before antigen stimulation is not a sufficient condition for immunological responsiveness, it may be a necessary one. It appears that the number of such base line plaques is not directly related to the final number of antibody-forming cells produced, either during recovery from tolerance in our experiments, or during manipulations made by others (11), to alter the number of such base line cells. Finally, the experiments of Playfair, Papermaster, and Cole (12), which place the number of precursor cells one to two orders of magnitude greater than the number of base line Jerne plaques, should be cited. From the available evidence, the Jerne plaque present before antigenic stimulation is related to or derived from the cell that responds to antigen but is not that cell itself.

It is also of interest that the proliferative response after the injection of sheep erythrocytes is lost with the induction of tolerance and returns with recovery. This increase in spleen cells seen after antigenic stimulation ( $0.5$  to  $1 \times 10^6$  cells) is far greater than the observed increase in plaque-formers ( $1$  to  $3 \times 10^6$  cells). Thus, the proliferative response includes cells either with other types of immunological function or without immunological function. However, whatever the function of these cells, under the present experimental conditions their proliferation is antigen specific.

The curve of waning cyclophosphamide tolerance obtained with the plaque

<sup>1</sup> Aisenberg, A. C., and C. Davis. Unpublished observations.

technique is complex. The initial 18 days during which tolerance is complete is followed by 10 wk of rapid recovery, followed by a much slower restoration over the remaining 20–30 wk of observation. The entire recovery period evidently takes from 40–50 wk. The period of rapid recovery from tolerance corresponds to the time of increase in the plaque response of normal and cyclophosphamide control animals, and this rapid restitution may be related to the age-dependent increase of immune responsiveness. There is a parallel here with the work of Mitchison, who observed that the rate of recovery from erythrocyte tolerance in chickens declined with advancing age (13). Whereas Mitchison observed that tolerance declined more slowly in groups of older animals, we find that the incremental recovery from tolerance is less as animals age. If one views recovery from tolerance as the repopulation of the lymphoid system with new cells that have not been rendered tolerant (14), then it is plausible that more rapid cell proliferation results in more rapid recruitment of nontolerant cells in younger animals. Mitchison has also studied tolerance in mice and finds that it requires over 3 months (his data extrapolate to 20 wk) to induce complete tolerance with low doses of bovine serum albumin (13, 15). The longer time required for recovery from drug-induced tolerance (more than 40 wk in our experiments as compared with 20 wk to induce tolerance in Mitchison's work) may reflect impaired reequipment after drug-induced injury or other differences in mouse strain or antigen.

The present experiments further clarify the role of the adult thymus. First, there can be little doubt that the 19S hemolysin-producing cell of the adult CBA mouse is thymus dependent. Recovery from tolerance proceeds minimally in the first 10 wk (the thymectomized animal always lagging behind the non-operated one in the degree of recovery), and then stops. Thus the ability to generate new cells which will respond to this antigen is markedly diminished, though neither the actual cellular reconstitution of the drug-depleted spleen nor the immune responsiveness of the animal that has received drug *without* antigen is impaired by thymectomy. In this connection our experiments confirm and extend the work of Clamen and Talmage (16) and of Taylor (17) on the contribution of the thymus in recovery from tolerance to protein antigens.

It had not been expected that removal of the thymus 18 days after inducing drug tolerance would be as effective as immediate thymectomy in preventing the recovery from tolerance, since restoration of the spleen cell population is essentially complete at 18 days. Our work is also at variance with the conclusion of Miller et al. (18) that the thymus functions only for the first 7 to 15 days in recovery from the immune impairment of lethal irradiation. If current views of lymphocyte migration are invoked (19–21), marrow cells begin their transit through the thymus and acquire immune competence only some 18 days after drug injection. Our results are equally compatible with a humoral mechanism of thymus function (22, 23).

Unlike lethal irradiation with bone marrow, cyclophosphamide without

antigen cannot eliminate immunocompetent cells. While there is a transient nonspecific depression immediately after drug injection, this probably represents depletion of effector lymphoid cells and is restored in a week or 10 days in animals either with or without their thymus. This unreactivity of drug without antigen contrasts sharply with that seen after drug plus antigen, which is specific and requires the thymus for restoration. Treatment with drug (without antigen) also contrasts with lethal irradiation (with bone marrow) which eliminates cells competent to respond to a variety of antigens (including sheep cells [footnote 1 and reference 24]) and in which restoration is thymus-dependent. Thus drug plus antigen accomplishes the same thing for the specific antigenic response that lethal irradiation (bone marrow) without antigen accomplishes for the responses to a variety of antigens. The conclusion that the target cell is the same in the two latter instances appears reasonable.

#### SUMMARY

Recovery from specific immunological tolerance to sheep erythrocytes induced with the drug cyclophosphamide was studied with the hemolytic plaque technique of Jerne. The base line plaque (19S antibody-forming cell of the unstimulated spleen) and the proliferative response to antigen, both of which had disappeared during tolerance induction, returned with the recovery of specific immunological reactivity.

When cyclophosphamide is injected without sheep cells there is temporary immunological unreactivity and lymphoid depletion of the spleen, but specific tolerance is not induced. Recovery is largely complete at the end of 2 wk and does not require the participation of the thymus.

When cyclophosphamide is injected together with sheep cells, 18 days after drug injection, tolerance is still complete. In nonthymectomized mice there is rapid recovery during the next 10 wk, followed by much slower restoration over the remaining 20–30 wk of observation. The entire recovery process evidently takes 40–50 wk. In thymectomized CBA mice only minimal recovery takes place in the first 10 wk and no further restoration occurs thereafter. Thymectomy performed 18 days after tolerance is induced, when tolerance is complete, is equally effective in preventing this recovery.

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