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ONTOGENY OF T CELL FUNCTION

I. Acquisition of Helper Cell Activity by the Thymus[1]

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The ontogeny of the ability of thymus cells to "help" the response to a T-dependent antigen was studied in a cell transfer system. Lethally irradiated, thymectomized mice were reconstituted with thymus cells from donors of various ages together with rabbit anti-mouse brain antiserum and complement- (C) treated bone marrow cells. Mice were immunized with TNP-BGG and the magnitude and affinity distributions of their splenic plaque-forming cell (PFC) responses were assayed 3 weeks later. The results indicate that neonatal thymic cells are capable of helping a direct PFC response but cannot mediate the shift to indirect plaque formation. The ability to mediate the switch to an indirect PFC response is a separate maturation event that occurs between birth and 2 to 4 days of age. The thymus cell population from 2-day-old donors is already capable of mediating selection of high affinity PFC. Neonatal cells residing in an irradiated, thymectomized or nonthymectomized adult recipient for 7 days, even in the presence of adult rabbit anti-mouse brain and C-treated syngeneic spleen cells, did not mature to be capable of mediating an indirect PFC response.

The maturation of the immune system has been shown to occur as an ordered sequence of events (1–17). The culmination of these events results in the ability to produce an adult-like immune response, which, in the mouse, occurs some time after birth. By using cell surface markers to identify the cells known to be involved in the immune response, it has been found that these cells appear considerably before the animal is able to mount a normal adult-like antibody response (3–13).

Using an adoptive cell transfer system, we (1) have previously shown that the ability of the B cell population to produce a heterogeneous, high affinity response to the T-dependent antigen, dinitrophenylated bovine γ-globulin (DNP-BGG),[3] matures between 7 and 10 days of age. In the present study we have adapted this cell transfer system to investigate the ontogeny of the T cell population with respect to its ability to provide help for a T-dependent antibody response. Lethally irradiated, thymectomized, adult mice were reconstituted with rabbit anti-mouse brain (RAMB) antibody plus complement- (C) treated adult syngeneic bone marrow together with thymus cells from donors of various ages. Mice were immunized 1 day after cell transfer with the T-dependent antigen, trinitrophenylated bovine γ-globulin (TNP-BGG) and their anti-TNP plaque-forming cell (PFC) response was assayed. This experimental design permitted us to evaluate the ability of thymus cells from immature mice to help a T-dependent antibody response by adult B cells in an adult environment. It was found that neonatal thymus cells are already able to provide help for a direct PFC response. The acquisition of the ability of the thymus cell population to bring about a switch to the production of indirect PFC (or to be capable of differentiating to mediate the switch to the production of indirect PFC) occurred between birth and 2 to 4 days of age. However, neonatal thymus cells that resided in an irradiated host (thymectomized or nonthymectomized) together with adult bone marrow cells did not acquire the ability to mediate the switch to indirect plaque formation over a 7-day period of residence.

MATERIALS AND METHODS

Animals. LAF_1 mice obtained from Jackson Laboratories (Bar Harbor, Maine) were used. Thymus cells were used as the source of helper T lymphocytes. The day of birth was designated as day 0 when specifying the age of cell donors. Neonatal thymuses were taken from animals sacrificed less than 24 hr after birth. Adult thymus cells were from 6- to 12-week-old donors. Bone marrow from 6- to 12-week-old mice was used as the source of B cells in all cell transfers.

Cell transfers. In all experiments, LAF_1 adult mice were lethally irradiated 2 to 8 hr before cell transfer by exposure to 800 rads from a gamma source. Where indicated, recipients were thymectomized 1 to 8 weeks before irradiation. Thymus cells were teased through a 200-mesh wire screen, were passed through a thin layer of cotton gauze, were washed once, and were injected i.v. Thymus cells from 5 to 30 animals, of the same age, were pooled. Bone marrow cells were flushed out of femurs and tibias with Hanks' balanced salt solution (HBSS) and were filtered through cotton gauze. The cells were washed once and were treated with RAMB antiserum plus C to remove any T lymphocytes. Spleen cells were teased through an 80-

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[3] Abbreviations used in this paper: BGG, bovine γ-globulin; DNP-BGG, dinitrophenylated bovine γ-globulin; EACA, ε-amino-*n*-caproic acid; HBSS, Hanks' balanced salt solution; RAMB, rabbit anti-mouse brain antiserum; $TNBSO_3$, 2,4,6-trinitrobenzene sulfonic acid, sodium salt; TNP, 2,4,6-trinitrophenyl group; TNP-BGG, trinitrophenylated bovine γ-globulin; TNP-EACA, trinitrophenylated-ε-amino-*n*-caproic acid.

mesh screen, were passed through a thin layer of gauze, were washed once, and were treated with RAMB antiserum and C to remove T lymphocytes. The RAMB antiserum was prepared by the method of Golub (18). The antiserum was absorbed with mouse bone marrow and mouse kidney powder. After absorption it was cytotoxic for over 95% of thymus cells, less than 5% of bone marrow cells, and approximately 50% of spleen cells. Fresh-frozen guinea pig serum, absorbed with agarose was used as the C source. Viability was assayed by trypan blue exclusion. In most experiments the recipients were given 3 to 5 $\times$ 10^7 viable, nucleated bone marrow cells and 3 $\times$ 10^7 thymus cells from donors of known age. Where indicated, recipients were given 2 to 3 $\times$ 10^7 spleen cells. The mice were immunized 1 day after cell transfer. In some experiments, the irradiated mice were initially given only thymus cells or thymus and RAMB-treated spleen cells. Adult bone marrow was then administered 2 or 6 days later and the mice were immunized 1 day thereafter.

Antigens and haptens. TNP-BGG was prepared by the reaction of BGG (Miles Laboratories, Inc., Miles Research Products, Kankakee, Ill.) with 2,4,6-trinitrobenzene sulfonic acid ($TNBSO_3$; Sigma Chemical Co., St. Louis, Mo.) under alkaline conditions essentially as described by Eisen *et al.* (19). The concentration of the conjugated protein was determined by dry weight analysis and its degree of derivatization was estimated spectrophotometrically from its absorbance at 348 nm (ϵ for TNP-lysine was taken as 15,400). The TNP-BGG preparations used in these experiments had 45 to 55 TNP groups per BGG molecule. TNP-ϵ-amino-caproic acid (TNP-EACA) was prepared by the reaction of $TNBSO_3$ with EACA (Sigma Chemical Co.) as described previously (20).

Immunization. Mice were immunized by the i.p. injection of 500 μg TNP-BGG, emulsified in complete Freund's adjuvant (CFA; containing 1.5 mg/ml *Mycobacterium butyricum*), in a final volume of 0.2 ml. Mice were sacrificed by cervical dislocation 20 to 22 days after immunization for assay of their splenic anti-TNP PFC.

Assay of number and affinity of PFC. Anti-TNP PFC were assayed by the Dresser and Greaves (21) modification of the Jerne plaque assay (22) with TNP-conjugated sheep erythrocytes (SRBC) prepared by the reaction of $TNBSO_3$ (Sigma Chemical Co.) with washed SRBC as described by Rittenberg and Pratt (23). Slides were incubated for 1 hr at 37°C. Freshly frozen guinea pig serum (absorbed with 50% SRBC) was added, at a final dilution of 1/30, as a source of C and the slides were incubated for an additional 45 min. Indirect PFC are developed by the addition of 1/300 dilution of rabbit anti-mouse γ-globulin to the assay system. The developing antiserum at this concentration, inhibited roughly half of the direct plaques. Indirect plaques are reported as total plaques in the presence of developing antiserum. The number of direct plaques was not subtracted. The affinity distribution of the anti-TNP PFC was determined by hapten inhibition of plaque formation, as described by Andersson (24) and validated by previous work (25, 26). Concentrations of TNP-EACA ranging from 1 $\times$ 10^{-9} M to 1 $\times$ 10^{-5} M, in half-log increments, were used for inhibition. Plaque formation around high affinity antibody-secreting cells is inhibited by low concentrations of hapten whereas plaque formation around low affinity antibody-secreting cells requires high concentrations of hapten for inhibition.

Thymopoietin treatment. The pharmacologically active pentapeptide from thymopoietin (Lot GOB 3) was synthesized and provided by Dr. Gideon Goldstein (Ortho Pharmaceuticals). Animals were injected with 1 μg thymopoietin i.p. daily for 5 days.

RESULTS

Ontogeny of the capacity of the thymus cell population to provide T cell "help" for the response of adult B cells to a T-dependent antigen. Lethally irradiated, thymectomized, adult LAF_1 mice were reconstituted with 3 to 5 $\times$ 10^7 RAMB antiserum plus C-treated adult bone marrow cells together with, or without, 3 $\times$ 10^7 thymus cells from donors of various ages. The recipients were immunized with TNP-BGG in CFA 1 day after cell transfer and were assayed for splenic anti-TNP PFC 20 to 22 days thereafter. Mice reconstituted with only RAMB plus C-treated bone marrow produced a very low response consisting predominantly of direct PFC (Table I). In contrast, mice reconstituted with adult thymus cells plus T cell-depleted bone marrow give a markedly greater response of high affinity (Fig. 1, Table I).

Mice reconstituted with neonatal thymus cells plus T cell-depleted adult bone marrow produced a response that is significantly greater than that of mice that received no thymus cells. However, this response consisted mainly of direct PFC (Table I). Increasing the number of neonatal thymus cells to 6 $\times$ 10^7 per recipient had no effect on the magnitude or class distribution of the PFC response (Table I). Thus, although neonatal thymus cells were capable of providing some T cell help for the response to a T-dependent antigen they did not behave in a

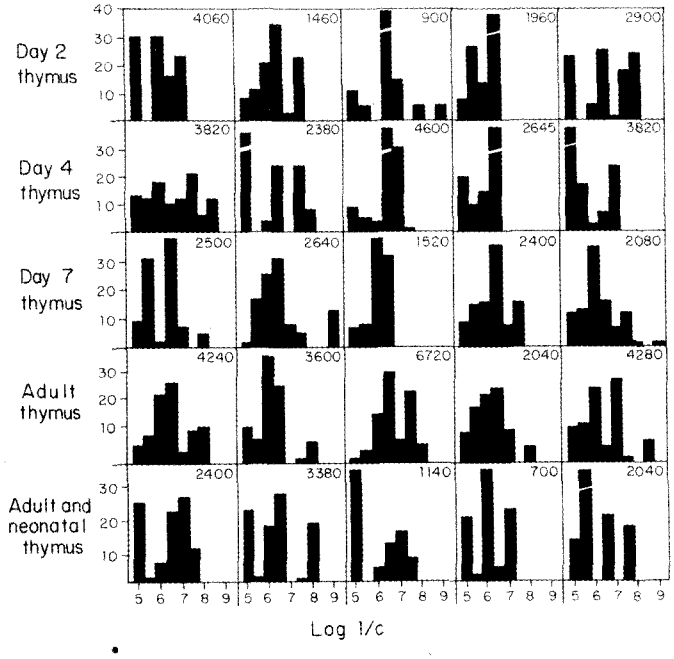

Figure 1. Each histogram illustrates the distribution of indirect PFC with respect to affinity in the spleen of a lethally irradiated, thymectomized mouse reconstituted with 3 $\times$ 10^7 thymus cells from donors of the indicated age (to the left of each row) and 3 to 5 $\times$ 10^7 RAMB and C-treated adult bone marrow cells. Mice were immunized with TNP-BGG in CFA 1 day later and anti-TNP PFC were assayed 20 to 22 days thereafter. The abscissa represents the log of the inverse of the free hapten (TNP-EACA) concentration used in the plaque inhibition assay. The ordinate represents the percentage of the total population of PFC present in each affinity subpopulation. Affinity increases to the right. Total indirect PFC per spleen is given in the upper right corner of each histogram. Five representative animals from groups of 7 to 47 mice are presented. Bars drawn with a diagonal break indicate greater than 40% of the PFC population in that affinity subpopulation.

TABLE I

Anti-TNP response to TNP-BGG by mice reconstituted with T cell depleted adult bone marrow plus thymus cells from donors of various ages[a]

Age of Thymus Cell Donor	No. of Thymus Cells Transferred × 10^{-7}	No. of Recipients Assayed	Anti-TNP PFC/Spleen		Ratio (I/D)
			Direct	Indirect	
days					
None	None	36	258 ± 0.40	133 ± 0.52	0.5
Neonate	3	6	911 ± 0.68	502 ± 0.62	0.6
Neonate	6	10	1090 ± 0.69	852 ± 0.77	0.8
2	3	10	1450 ± 0.68	1810 ± 0.69	1.3
4	3	7	1350 ± 0.66	2150 ± 0.83	1.6
7	3	8	978 ± 0.52	1650 ± 0.57	1.7
Adult	3	47	1300 ± 0.33	2610 ± 0.36	2.0

[a] Lethally irradiated, thymectomized mice were reconstituted with 3 to 5 × 10^7 RAMB and C-treated syngeneic adult bone marrow cells plus the indicated number of thymus cells from donors of the designated age. Recipients were immunized with TNP-BGG, in CFA, 1 day after cell transfer and assayed for anti-TNP PFC 20 to 22 days thereafter. Results are presented as geometric means ± standard error of the mean.

mature manner in that they failed to induce a switch to indirect plaque formation.

When thymus cells from 2-day-old donors were used as the T cell source, the number of indirect PFC increased markedly and, in the majority of recipients, a predominantly indirect PFC response occurred (Table I). Those animals that produced an indirect PFC response had affinity distributions of their PFC that were indistinguishable from those of mice that received adult thymus cells (Fig. 1). If 4- or 7-day-old mice were used as thymus cell donors the anti-TNP response by the recipients was the same as that of mice reconstituted with adult thymus cells. Thus, the thymus cell population acquires the capacity to provide normal, adult-like, T cell help between the day of birth and 2 to 4 days of age.

Evidence that suppressor cells are not responsible for limiting the capacity of the neonatal thymus cell population to provide help. Lethally irradiated, thymectomized, adult LAF_1 mice were reconstituted with RAMB antiserum and C-treated adult bone marrow plus 3 × 10^7 adult thymus cells together with or without 3 × 10^7 neonatal thymus cells. The recipients were immunized with TNP-BGG in CFA 1 day later and were assayed for splenic anti-TNP PFC 20 to 22 days thereafter. The responses of mice that received the mixture of neonatal and adult thymus cells were indistinguishable from those of mice that received only adult thymus cells (Table II). Thus, the inability of neonatal thymus cells to provide normal, adult-like, helper activity does not appear to be due to the presence of excessive suppressor activity.

Lack of maturation of neonatal thymus cell population within the cell transfer recipient. Lethally irradiated, thymectomized, adult LAF_1 mice were given either 3 × 10^7 neonatal thymus cells or 3 × 10^7 adult thymus cells. Two or 6 days later they received 3 to 5 × 10^7 RAMB antiserum and C-treated adult bone marrow cells and were immunized with TNP-BGG in CFA 1 day thereafter. The response of the animals reconstituted with neonatal thymus cells was immature in character (in that it lacked indirect PFC) despite the thymus cells having resided in the adult thymectomized animal for up to 7 days before immunization (Table III). Lethally irradiated, nonthymectomized, adult LAF_1 mice were reconstituted in the same manner. Again the response of the animals reconstituted with neonatal thymus cells remained immature in character despite 7 days of residence in the host (Table III). It is interesting to

TABLE II

Anti-TNP PFC response to TNP-BGG by mice reconstituted with T cell depleted adult bone marrow plus mixtures of adult and neonatal thymus cells[a]

Age of Thymus Cell Donor	No. of Recipients Assayed	Anti-TNP PFC/Spleen	
		Direct	Indirect
Neonate	6	911 ± 0.68	502 ± 0.62
Adult + neonate	12	1452 ± 0.86	2024 ± 0.77
Adult	12	743 ± 0.80	1618 ± 0.85

[a] Lethally irradiated, thymectomized mice were reconstituted with 3 to 5 × 10^7 RAMB and C-treated bone marrow cells plus 3 × 10^7 thymus cells from neonatal donors, from adult donors, or from both neonatal and adult donors (3 × 10^7 cells from each). The animals were immunized with TNP-BGG in CFA 1 day later and were assayed for anti-TNP PFC 20 to 22 days thereafter. Results are presented as geometric means ± standard error of the mean.

TABLE III

Effect of residence of neonatal thymus cells in lethally irradiated adult mice on their capacity to mediate an indirect PFC response[a]

Recipient	Age of Thymus Cell Donor	Additional Treatment	Days of Residence before Immunization	Anti-TNP PFC/Spleen	
				Direct	Indirect
Thymectomized					
	Neonate	None	1	911 ± 0.68	502 ± 0.62
	Neonate	None	3	935 ± 0.65	675 ± 0.81
	Neonate	None	7	928 ± 0.83	917 ± 0.73
	Adult	None	1	1300 ± 0.33	2610 ± 0.36
	Adult	None	3	1840 ± 0.92	3530 ± 0.87
	Adult	None	7	1360 ± 0.66	5180 ± 0.67
	Neonate	Thymopoietin[b]	6	842 ± 0.26	511 ± 1.21
	Adult	None	6	1340 ± 0.60	3120 ± 0.87
	None	RAMB-treated spleen[c]	7	849 ± 0.91	643 ± 0.93
	Neonate	RAMB-treated spleen[c]	7	1570 ± 0.55	919 ± 0.52
	Adult	RAMB-treated spleen[c]	7	3320 ± 1.17	3590 ± 1.14
Nonthymectomized					
	Neonate	None	7	374 ± 0.80	395 ± 0.79
	Adult	None	7	616 ± 0.57	2250 ± 0.69

[a] Lethally irradiated, thymectomized or nonthymectomized (as indicated) mice were reconstituted with 3 × 10^7 adult or neonatal thymus cells. Where indicated, they also received 2 × 10^7 RAMB and C-treated adult spleen cells. Recipients were immunized 1, 3, 6, or 7 days later with TNP-BGG, in CFA, and were assayed for anti-TNP PFC 20 to 22 days thereafter. All mice received 3 to 5 × 10^7 RAMB and C-treated adult bone marrow cells 1 day before immunization. The data are presented as geometric means ± standard error of the mean for groups of 5 to 47 mice.

[b] Recipients were injected with 1 μg of thymopoietin daily for 5 days.

[c] Received 2 × 10^7 RAMB plus C-treated adult spleen cells at the time of the initial cell transfer.

note that the magnitude of the response by nonthymectomized mice is lower than that of thymectomized animals. The explanation for this difference is not known.

Lethally irradiated, thymectomized mice were given either 3 $\times$ 10^7 neonatal thymus cells or 3 $\times$ 10^7 adult thymus cells. Mice receiving neonatal thymus cells were injected with 1 μg thymopoietin i.p. daily for 5 days. A similar dose schedule with this synthetic pentapeptide has previously been shown to reverse the effect of aging on antibody affinity (27). They were then given 5 $\times$ 10^7 RAMB plus C-treated bone marrow cells and were immunized with 500 μg TNP-BGG in CFA. Their PFC response was immature in character in that it lacked indirect plaques. Thus, thymopoietin failed to induce maturation of the immature thymus cell population within the cell transfer recipient (Table III).

There has been some evidence that adult thymocytes require post-thymic maturation before they respond in a completely mature manner (28). In order to see whether a splenic non-θ-bearing cell would induce maturation in the neonatal thymus cell, the following experiment was performed.

Lethally irradiated, thymectomized mice were given 3 $\times$ 10^7 adult thymus cells, 3 $\times$ 10^7 neonatal thymus cells, or no thymus cells plus 2 $\times$ 10^7 RAMB and C-treated spleen cells 6 days before receiving 5 $\times$ 10^7 RAMB treated bone marrow cells. They were immunized 1 day later with TNP-BGG in CFA. As shown in Table III, the response remained immature with respect to their ability to promote an indirect PFC response.

DISCUSSION

It is well known that the membrane surface antigens of the thymus are present before birth (12). However, some functional properties of the mouse thymus cell population do not mature for several weeks (13, 14).

The data presented here indicate that the ability of the thymus cell population to "help" adult B cells to produce a response to a T-dependent antigen is immature at birth and matures by 2 to 4 days of age. At birth, the thymus cell population can provide some augmentation of the direct PFC response but cannot mediate a switch to the production of indirect PFC. It appears that the acquisition of the capacity to mediate a switch to indirect PFC represents a true differentiation event since increasing the number of neonatal thymus cells transferred did not lead to the generation of indirect PFC. Thus, there is probably a qualitative and not just quantitative difference between the neonatal and the 2-day-old thymus cell population. It appears that, in the mouse, T cell helper function (with respect to the helper T cells present in the thymus) matures in at least two steps: first, the ability to "turn on" an antibody response in the form of direct (IgM) plaques; second, the ability to mediate the switch to an indirect (IgG) PFC response. Using similar adoptive transfer systems, both MacGillivray *et al.* (29) and Chiscon and Golub (30) found the neonatal antibody response to be immature when compared to the adult. In terms of the magnitude of the PFC response, Chiscon and Golub (30) found T cells to mature by 2 days of age, and B cells by day 18 of gestation. Parallel results were seen by Wu (31) who, using the cell-mediated lympholysis technique found thymus cells of the B.10 strain mouse developed the ability to respond to allogeneic cells by 2 days of age.

The data presented here clearly indicate that a differentiation event occurs in the T cell population between birth and 2 days of age in LAF_1 mice. This differentiation event is detected by the ability of the thymus cell population to mediate a switch to indirect PFC in a cell transfer recipient. It cannot be formally distinguished whether this differentiation event involves: a) the acquisition of the capacity to mediate the shift to IgG production or b) the acquisition of the capacity to undergo a subsequent differentiation event, in the cell transfer recipient, that then permits the T cells to mediate the switch to IgG production. *A priori* the former possibility appears the simpler and more likely hypothesis.

It is interesting to note that the indirect PFC response by mice reconstituted with adult B cells and thymus cells from 2-day-old donors is indistinguishable from that of adult mice with respect to affinity and heterogeneity of affinity. We have previously reported that the B cell population matures to be able to produce a high affinity, heterogeneous, adult-like response between 7 and 10 days of age in LAF_1 mice (1). This antigen-independent maturation event was shown to require adult thymus cells for its induction (23). The thymus cell population matured, with regard to its capacity to induce this differentiation of the B cell population, between 7 and 10 days of age (32). Thus, the acquisition, by the thymus, of the ability to mediate the switch to IgG production and the ability to induce an antigen-independent differentiation step in the development of the B cell population takes place at different ages, and hence these appear to represent distinct functions of the thymus cell population.

Three distinct stages can therefore be defined in the functional maturation of the thymus cell population of LAF_1 mice: a) the ability to act as helper cells in augmenting the direct PFC response to T-dependent antigens is present at birth; b) the ability to mediate the switch to indirect plaque formation develops by 2 to 4 days of age; c) the ability to induce an antigen-independent differentiation event in the maturation of the B cell population to produce a high affinity response develops between days 7 and 10 of age (32).

The possibility that the immature behavior of the neonatal thymus cell population is due to the presence of suppressor cells was rendered unlikely by the results of mixed cell transfer experiments that showed that neonatal thymus cells failed to suppress the ability of adult thymus cells to cooperate with adult bone marrow cells in a response to a T-dependent antigen, TNP-BGG. The increase in direct PFC seen in the mixed cell recipient group (with a concurrent slight decrease in the ratio of indirect to direct PFC) is almost identical to the expected response calculated by adding the averages of the responses of mice that received only neonatal thymus cells and mice that received only adult thymus cells. These numbers of adult thymus cells previously have been shown to give a linear dose-response in similar experiments (33). These results appear superficially inconsistent with the data of Mosier and Johnson (15) who, using a cell culture system, found significant suppressor activity in the thymus of BALB/c mice up to 7 days of age. Several differences do exist between the experimental system employed by Mosier and Johnson and that used here, which might account for this apparent discrepancy. The Mosier and Johnson studies were carried out *in vitro* and employed mice of a different strain from those used here. Of perhaps greater significance is the fact that the responding population in the Mosier and Johnson system was adult spleen cells whereas in the studies reported here T cell-depleted bone marrow was used. The spleen, of course, contains mature T lymphocytes as well as B lymphocytes. The possiblity exists that the adult splenic T cells participate in the suppression observed by Mosier and Johnson either as inducers of suppressor activity or perhaps as the targets of inducers of suppressor activity. The absence of such interactions with adult thymus cells that we

used as the source of helper activity might account for the difference in our results. It is of course also possible that the splenic B cell population and the adult bone marrow B cell population are affected differently by the neonatal suppressor cells.

Allowing neonatal thymus cells to reside in lethally irradiated, thymectomized or nonthymectomized adult recipients for up to 7 days before immunization did not result in the development of mature helper cell function. Injection of thymopoietin did not induce detectable maturation under these conditions. Since 7 days is well beyond the time required for the thymus cell population to acquire mature function in the intact animal (2 to 4 days of age) it would appear that this maturation of function might involve an induced differentiation event that requires a radiation-sensitive cell. Despite the fact that the thymus has been regarded as relatively radiation resistant, some of its properties have been previously shown by Stutman (28) to be sensitive to high-dose (750 rads) irradiation.

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