Effect of Thymic Extracts on Restoration of Immunologic Competence in Thymectomized Mice (32843)

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It has been established that thymectomy during the neonatal period in the mouse and in other rodents leads to immunologic defects. Failure of normal bodily growth with subsequent death and a striking depletion of small lymphocytes from the blood and lymphoid tissues is also characteristic of thymectomy in certain strains of mice (1–5). The immunologic defects are principally of those reactions mediated by lymphoid cells such as delayed hypersensitivity, Arthus reaction and homograft rejection of normal tissues or tumor cells; there is also known to be a decrease of some but not all humoral antibodies (6, 7).

Restoration of normal immunologic function in thymectomized mice and rats has been achieved by re-equipping the animal with immunologically competent cells or by restoring to the animal the factors necessary for its own immune system to mature to a state of immunologic competence. Thymic grafts (8) or thymic tissue in cell-impermeable Millipore diffusion chambers (9, 10) have been effective. The latter experiments point to the existence of a humoral factor in thymic tissue, designated THF (11), that is capable of restoring immune reactivity and stimulating lymphopoiesis. Results of more recent experiments have indicated that THF is not strain-specific (12) nor species-specific (13). It would appear also that THF is produced by the reticuloepithelial components of the thymus and that it must be released in order to be effective at a critical period in the development of the immune system.

The present report concerns experiments designed to restore the deficits in mice thymectomized at birth through the use of thymic extracts derived from syngeneic mice. Immunologic competence was determined by use of the sensitive graft versus host (GVH) reaction. The GVH is known to result from an immunologic attack by grafted cells against a host incapable of rejecting these cells (14). The effective cells are most likely small lymphocytes. Lymphoid cells obtained from neonatally thymectomized C57BL mice (H-2^b) do not produce GVH in newborn BALB/c recipients (H-2^d) whereas lymphoid cells from intact C57BL mice produce a rapid and severe reaction resulting in early death.

Materials and Methods. Mice. The C57BL/KaLw mice from our inbred colony and BALB/c An mice from the inbred colony of this laboratory or from the NIH production colony were used throughout.

Thymectomy. Surgical removal of the thymus of C57BL mice was accomplished always within 18 hours of birth. Usually, half the litter was thymectomized and half retained as intact mice. The mice were weaned at 5 weeks of age and at this time they were used as donors of spleen cells. Tissue was removed from the upper mediastinum in order to check for the presence of thymic remnants. Donors showing the presence of thymic remnants were not included in the results.

Preparation and inoculation of extracts. Thymuses were removed from C57BL donors, 3–7 days of age using aseptic methods. These were cut finely with scissors, placed in medium 199 (10 thymuses to 1.2 ml of medium), then homogenized in a Servall Omni-Mixer with a microhomogenizer attachment for 5 min. The speed attained by the micro attachment, empty, is known to be approximately 50,000 rpm. Finally, centrifugation was accomplished at 3000 rpm for 5 min and the supernatant used for injections. The procedures were carried out at 4°C.

Splenic extracts were prepared in a similar manner using a volume of splenic tissues, from 3 to 7-day-old syngeneic donors, equal to that of thymic tissue used. Very few viable cells were ever found in smears of the thymus or spleen extracts. The highest concentration of intact cells found in a preparation was 50 cells/0.1 ml.

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TABLE I. GVH Reactions in BALB/c Recipients Inoculated at Birth with C57BL Dissociated Spleen Cells.

Donor group	No. of donors	No. of litters inoculated	No. with GVH /no. inoc.	Age at death (days) mean (range)
I. Intact	4	4	16/18 (89%)	15.8 (13–20)
II. Thymectomized	14	14	0/66	—
III. Thymect. + thymic extract	18	26	48/63 (76%)	14.8 (8–27)
IV. Thymect. + splenic extract	12[a]	15	0/41	—

[a] Residual thymic tissue was found in 2 donors of Group III and 1 donor of Group IV. Dissociated spleen cells from these mice produced GVH reactions. These are not included in the table.

Thymic or splenic extracts were inoculated in a 0.1 ml volume, subcutaneously immediately after preparation into thymectomized C57BL mice, beginning when the mice were 1 week of age and continuing three times a week for 4 weeks or a total of 12 inoculations.

Preparation and inoculation of dissociated cells from spleen. Suspensions of viable cells from the spleens of thymectomized, intact and thymectomized C57BL mice receiving injections of thymic or splenic extracts were prepared by the method described by Billingham (15) except that medium 199 was used throughout as suspending fluid. Donor mice were 5–6 weeks old at time of sacrifice. Dissociated cells were washed twice and finally suspended in a volume of 0.04–0.06-ml range with a concentration of cells of 5×10^6.

Each BALB/c recipient therefore received 5×10^6 dissociated spleen cells administered intravenously by way of the orbital branch of the anterior facial vein. Each recipient was less than 18 hours of age at inoculation. Litter size was standardized, for the most part, using 3–4 inoculated mice and 2 noninoculated mice as controls in each litter.

GVH reaction and fatal runt disease. The GVH reaction is severe in the combination C57BL (H-2^b) → BALB/c (H-2^d) and acute signs of GVH reaction occur with 1×10^6 dissociated spleen cells. First signs of the reaction are observed usually between 1 and 2 weeks at which time normal growth is arrested and there appears in most mice a dermatitis and diarrhea. In the combination used here, GVH was most usually fatal within 2–3 weeks. Only those litters in which the control litter mates did not show signs of poor growth or diarrhea were considered in the results. Body weights were taken once a week beginning at 1 week of age.

Results. The capacity of 5×10^6 dissociated cells obtained from the spleens of the several types of C57BL donors in evoking GVH reactions in newborn BALB/c recipients is shown in Table I. Only the acute form of GVH is recorded here. Several animals showed "runting" without the other signs of the GVH reaction; these were classified as negatives. Fourteen of 18 neonatally thymectomized C57BL mice that received 12 inoculations, 3 ×/week, of thymic extracts, yielded spleen cells capable of producing GVH reactions at a frequency and latent period not too dissimilar from the reactions of BALB/c mice receiving spleen cells from intact C57BL donors or from neonatally thymectomized C57BL mice reconstituted with syngeneic thymic grafts (14/19 = 74%). Splenic extracts, in contrast, did not restore the capacity of lymphoid cells from the thymectomized recipients to evoke a GVH reaction.

In a separate experiment an attempt was made to determine the minimum size of thymic fragments that could be grafted to syngeneic neonatally thymectomized C57BL mice with resulting restoration of competence of the recipient's spleen cells to initiate GVH reaction. When the donor 1- to 3-day thymus was cut into approximately 16–32 pieces and grafted into subcutaneous pocket in 7-day recipients, these mice were then found to be immunologically competent in producing GVH reactions; 21/31 (70%) of BALB/c newborn recipients developed GVH reaction,

TABLE II. Mortality, Body Weights, and WBC in C57BL Mice: Intact, Thymectomized, and Thymic-Extract Treated.

	Mortality at 5 weeks	Body wt. at 5 weeks	Total WBC (lymphocytes) at 5 weeks
Thymectomized	6/20	9.9 (11)[a]	5030 (1960)
Thymectomized + thymic extracts	9/22	10.0 (15)	5060 (1850)
Intact	0/15	13.9 (12)	6400 (4480)

[a] Numbers in parentheses = no. of mice used to determine mean weights. Same numbers of mice in each group used to determine total WBC.

but only if the host had recoverable grafts of thymic tissue. Serial sections taken of such minute grafts showed the presence of medullary reticuloepithelial tissue and cortical lymphoid tissue and were fully reconstituted, resembling intact thymic tissue. These findings are similar to those reported by Metcalf (16). Of interest is the fact that if thymuses were homogenized in a Potter-Elvejem homogenizer, no reconstituted thymic tissue was found at the site of grafting and spleen cells from neonatally thymectomized C57BL mice receiving such preparations did not produce GVH in BALB/c newborns (0/22). Of the 20 mice in Group III, Table I, receiving multiple thymic extracts, none showed evidence of growth of medullary thymic tissue at subcutaneous sites of inoculation of the extracts.

Although thymic extracts given over a 4-week period were quite effective in restoring the capacity of thymectomized recipients to evoke a GVH reaction these extracts failed to lower the frequency of "wasting" and mortality occurring in these mice as a consequence of thymic removal at birth (Table II). In addition there was not found any overt lymphoid regeneration in the thymic-extract treated mice as revealed by comparative lymphocyte populations in the peripheral blood (Table II), or in any histologically detectable regeneration of lymphoid structures in lymph node when examined at 5–6 weeks of age. The yield of viable, dissociated cells obtained from donor mice was nevertheless somewhat higher in the thymic-extract treated mice: 40–48 $\times$ 10^6 cells/spleen and for thymectomized mice, 25–35 $\times$ 10^6 cells/spleen; both values were lower than the yield for intact mice, 60–70 $\times$ 10^6 cells/spleen.

Discussion. Restoration of certain deficiencies in immunocompetence and other defects resulting from neonatal thymectomy have been achieved using thymic grafts, xenogeneic (17) as well as syngeneic (8), and by thymuses placed in diffusion chambers (9, 10). Recently, it has been shown that competence of the spleen to evoke a GVH reaction *in vitro* is dependent specifically upon thymic tissue incubated simultaneously with spleen. Thymic activity was also demonstrated to occur directly, across an intervening Millipore filter barrier (18). These results suggest that the thymus influences recovery through the liberation of a diffusible humoral factor acting within the environment of the thymic tissue and also upon seeded cells within lymphoid organs.

If this is so, extracts from thymic tissue should be expected to restore neonatally thymectomized mice towards normalcy providing proper conditions are met. There is no doubt that thymic extracts, as employed in the present experiment, did restore the immunologic competence to spleen cells of C57BL recipients as detected by the sensitive GVH reaction. This reaction is known to be a homograft reaction on the part of the inoculated allogeneic spleen cells against the transplantation antigens of the host. Extracts of spleens from these syngeneic donors on the other hand were ineffective. Whether this indicates a specific effect of thymus remains to be determined.

The absence of intact viable cells in most thymic extracts and the failure to find macroscopically visible thymic tissue at the site of subcutaneous inoculations indicate the operation of an acellular or subcellular factor in restoration. As high a concentration of 10 $\times$ 10^6 dissociated thymic cells from neo-

natal donors, injected intravenously, is known not to be capable of restoring thymectomized mice (21).

In the present experiments there was no detectable regeneration of lymphoid elements nor was recovery of normal bodily growth attained; yet, immunocompetence as measured by the GVH reaction was restored in a high percentage of mice by thymic extracts. Previous studies of the effect of thymic tissue in diffusion chambers have shown that restored immune reactivity in mice was not always associated with detectable lymphoid regeneration (12, 13). A separation between morphologic recovery and reactivation of the immune response has been reported also using an organ culture system (18). The results reported here suggest that the deficiency of lymphoid cells of the spleen in thymectomized mice is the result not only of a quantitative deficiency but is of a qualitative cellular nature as well (19, 20). The subcellular factor detected therefore appears to be responsible for "differentiation" of lymphoid precursors already present in the spleen.

The relationship of the factor in syngeneic thymic extracts capable of restoring immunocompetence, as described here, to the active extracts obtained from such diverse species as the calf, sheep, and rabbit (22-26) is not known.

Summary. Extracts of thymic tissue from syngeneic donors restored the immunologic capacity of neonatally thymectomized C57BL mice. Dissociated spleen cells of thymic-extract treated mice evoked a GVH reaction in newborn BALB/c mice. In contrast, splenic extracts were ineffective. Thymic extracts, however failed to lower the frequency of "wasting" with consequent death; neither did overt lymphoid regeneration occur in these treated mice thymectomized at birth.

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