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Restoration of Immunologic Competence of Neonatally Thymectomized Mice by Isogeneic and Xenogeneic Thymic Grafts (33738)

GEORGE A. HALLENBECK, THEODORE P. KUBISTA[1] AND ROY G. SHORTER
Mayo Clinic and Mayo Foundation: Section of Organ and Tissue Transplantation, Rochester, Minnesota 55901

The importance of the thymus in the development of immunologic competence has been established in experiments on animals of many species (1). Neonatally thymectomized mice of many strains fail to develop normal immune responses, and the capacity of these animals to form humoral antibody, to reject allogeneic skin grafts, and to repopulate their lymphoid systems can be restored by grafts of isogeneic or allogeneic thymus transplanted as free grafts or in "Millipore" diffusion chambers (2–6). Attempts to restore the immunologic capacity of neonatally thymectomized mice by use of zenogeneic grafts of rat thymus have given varied results. Yunis *et al.* (7) reported that, although subcutaneous grafts of thymus from newborn Holtzman rats survived in neonatally thymectomized C3H mice, the grafts did not alter the wasting disease, the depletion of lymphoid tissue, or the inability of splenic cells to mount a graft-against-host reaction. Law (8), however, found that subcutaneous grafts of thymic tissue from Osborn-Mendel rats or from a Syrian hamster restored immunologic competence in neonatally thymectomized F_1 hybrid mice (C57BL/KA × C3HF/Lw).

Having shown previously that the Walker 256 carcinosarcoma of rats grows only briefly, if at all, in intact C57BL/6 mice but usually flourishes in neonatally thymectomized mice (9, 10), we performed experiments to determine whether grafts of isogeneic thymus or grafts or rat thymus could restore the capacity of such thymectomized mice to reject the tumor.

Methods and Procedure. Mice of the C57BL/6 strain were maintained in an air-conditioned environment at 70 to 72°F, with free access to a commercial chow[2] and to water. Thymectomy was performed in each mouse less than 24 hr after birth by the method of Sjodin and his associates (11) using hypothermia for anesthesia. Intact mice and sham-thymectomized mice served as controls. When 3 weeks of age each mouse was injected subcutaneously in the lumbodorsal region with approximately 5×10^6 Walker tumor cells suspended in Tyrode's solution and prepared as described previously (9). Aliquots of each batch of tumor cells were injected into two Sprague-Dawley rats to be certain that the cells were viable.

Groups of neonatally thymectomized mice received grafts of sliced thymic or other tissues intraperitoneally when 2 weeks of age, 1 week before being inoculated with tumor cells. Grafted tissue included thymus from mice or rats, spleen, skeletal muscle, liver, duodenum, lung, kidney, submaxillary glands, and thyroid glands from mice 2 weeks of age and spleen from mice 2 weeks old that had undergone thymectomy when less than 24 hr of age. Thymus and spleen were implanted both free and in Millipore chambers prepared as described by Amos (12) and using filters of 0.1-μpore size. In one series of tests, thymic tissue was killed before transplantation by freezing five times

[1] Mayo Graduate School of Medicine, University of Minnesota, Rochester: Resident in Surgery.

[2] Rock Island Rat and Mouse Food, Monmouth, Ill.

TABLE I. Effect of Intraperitoneal (i.p.) Transplantation of Grafts of Thymus on Rejection of Walker 256 Carcinosarcoma by Neonatally Thymectomized C57BL/6 Mice.

Grafts			No. of mice	Tumors regressed within 28 days		Significance (neonatal thymectomy alone versus grafted mice)
Source	Av wt. (mg)	Type of i.p. graft		No.	(%)	
None	—	—	124	15	12	—
None	—	Empty millipore chamber	8	1	13	n.s.
Thymus						
Neonatal mice	13	Free	12	4	33	n.s.
14-day-old mice	55	Free	27	14	52	$p < 0.001$
14-day-old mice	165	Free	12	11	92	$p < 0.001$
14-day-old mice (thymus killed by freezing and thawing)	165	Free	6	1	17	n.s.
Neonatal mice	13	Millipore chamber	8	0	0	—
14-day-old mice	55	Millipore chamber	18	7	39	$p < 0.02$
Neonatal rats	10	Free	14	3	21	n.s.
Neonatal rats	30	Free	12	5	42	$p < 0.05$
14-day-old rats	340	Free	11	9	82	$p < 0.001$
Spleen						
14-day-old mice	50	Free	18	14	78	$p < 0.001$
14-day-old mice	50	Millipore chamber	12	2	17	n.s.
14-day-old neonatally thymectomized mice	57	Free	24	4	17	n.s.

in a mixture of acetone and dry ice, with alternate thawing at room temperature, after which aliquots of thymic cells failed to exclude trypan blue.

Each animal was examined daily, and the diameter of any subcutaneous tumor found by palpation was measured in millimeters. After 28 days, mice that were still living were killed and examined. Mice that developed large tumors and appeared about to die before 28 days has passed were killed and examined, and some animals died spontaneously before the experiments ended. Autopsy examinations were performed in all animals found dead or killed, and the presence or absence of Walker tumor was confirmed grossly and microscopically. The completeness of thymectomy was assessed, and only the findings in mice in which thymectomy was adjudged complete were included in the analysis of results. Histologic examination was made of all grafted tissues.

Results. Controls. Aliquots of suspensions of tumor cells that were injected into mice were given to rats in each experiment and produced tumors in all cases, demonstrating that the tumor cells were viable. A total of 160 intact mice received Walker tumor cells. Small subcutaneous nodules were palpable in 142 mice during the first week or two after injection, but all nodules disappeared within the 28-day period of observation. And in none of these mice could tumor tissue be identified grossly or microscopically at postmortem examination. Eight mice that underwent sham thymectomy on the day of birth behaved as did intact controls.

Strikingly different was the growth of tumors in 124 neonatally thymectomized mice. All developed large subcutaneous tumors and in only 15 (12%) did the tumors disappear within 28 days (Table I). The presence of Walker tumor tissue was confirmed microscopically postmortem in the mice that had gross nodules at the time of death.

These data, obtained concurrently with

other data presented in this paper, confirm the findings previously reported from this laboratory (9, 10), and the observations on neonatally thymectomized mice provide a base line to which results of attempts at restoring the capacity of neonatally thymectomized mice to reject the Walker tumor by transplanting thymus and other tissues can be compared.

Effects of intraperitoneal transplantation of isogeneic and xenogeneic thymus on rejection of the Walker tumor by neonatally thymectomized C57BL mice. Neonatally thymectomized mice given free intraperitoneal grafts of three isogeneic thymuses (av total wt. 13 mg) from mice less than 1 day old and subsequently injected with Walker tumor cells rejected their tumors within 28 days in 4 of 12 cases (33%). This incidence of rejection is greater than the 12% observed in untreated neonatally thymectomized mice, but the difference was not significant statistically (Table I).

When free thymic grafts consisted of one thymus, averaging 55 mg in weight, from mice 14 days of age, neonatally thymectomized mice rejected their tumors in 52% of 27 cases, a result that differed significantly from the control value. When the mass of thymic tissue grafted was increased to an average of 165 mg by use of thymus from three mice, each 14 days of age, the capacity of neonatally thymectomized mice to reject the Walker tumor was restored in 11 of 12 animals (92%). In six other animals, thymic tissue was frozen and thawed five times before being used for grafting, and only one of the six thymectomized recipients rejected the Walker tumor subsequently, an incidence not significantly different from that of control mice.

In another group of experiments, isogeneic thymic tissue was transplanted intraperitoneally in Millipore chambers. Preliminary tests showed that implantation of empty Millipore chambers intraperitoneally into neonatally thymectomized mice did not restore their ability to reject the Walker tumor (Table I). Two sets of experiments were then performed in which thymus within Millipore chambers was placed intraperitoneally. When thymic tissue averaging 13 mg in weight and taken from three neonatal mice was used, none of eight neonatally thymectomized mice subsequently rejected their tumors, When a single thymus (av. wt. 55 mg) from a 14-day-old mouse was used, 7 (39%) of 18 thymectomized mice rejected their tumors, an incidence significantly greater than that of control mice.

Xenografts of Sprague-Dawley rat thymus, transplanted as free grafts intraperitoneally, were also tested. Grafts of neonatal rat thymus with an average weight of 10 mg were followed by rejection of the Walker tumor in 3 of 14 cases, an incidence greater than, but not significantly different from, that of the controls. When the dose of neonatal rat thymus was increased to an average of 30 mg, rejection of the tumor occurred in 5 of 12 cases (42%); and when three thymuses from rats 14 days of age were used, an average weight of 340 mg, the incidence of rejection of the tumor increased to the highly significant figure of 82% of 11 animals.

Effects of intraperitoneal transplantation of isogeneic spleen on rejection of the Walker tumor by neonatally thymectomized C57BL mice. Spleens (av. wt. 50 mg) from intact mice 14 days old, implanted intraperitoneally, restored the ability of 14 of 18 neonatally thymectomized mice to reject the Walker tumor. Neither spleens of neonatally thymectomized mice nor spleens from intact mice transplanted in Millipore chambers were effective (Table I).

Results with grafts of other tissues. Free intraperitoneal implantation of isogeneic duodenum, lung, kidney, liver, skeletal muscle, and submaxillary, and thyroid glands failed to restore the capability of neonatally thymectomized C57BL mice to reject the Walker tumor.

Histologic studies. Grafted thymic tissue was sought at postmortem examinations. Generally, the amount of tissue identifiable grossly was always less than the amount that had been transplanted. Some remaining histologically normal thymic tissue after isogeneic grafting was found more often when larger grafts consisting of 165 mg of thymic tissue

had been used (7 of 12) than when 55 mg of tissue from 14-day-old mice or 13 mg of neonatal thymus had been used (2 of 25 and 3 of 12, respectively). Histologically, necrotic tissue was found more often and was present in many mice in which immunologic capacity had been restored to the point that the Walker tumors had been rejected, and when necrosis was present, no epithelial elements could be identified.

Only necrotic thymus was found in the Millipore chambers, and all remaining thymic tissue of the rat appeared histologically to be undergoing necrosis. Grafts of isogeneic splenic tissue behaved similarly, and histologically, normal spleen again could not be demonstrated in all of the animals that rejected the Walker tumor.

Discussion. The data indicate that intraperitoneal grafting of isogeneic thymic tissue, either free or in Millipore chambers, can restore the ability of neonatally thymectomized C57BL/6 mice to reject a xenogeneic tumor, thus adding to the results of others who have demonstrated that thymic grafts can restore various manifestations of immunologic competence. The effectiveness of thymic grafts in Millipore chambers is taken as evidence that a cell-free thymic humoral factor is involved. Grafts of isogeneic splenic tissue from neonatally thymectomized mice were ineffective, presumably because the splenic lymphoid cells had not yet been acted on by the thymic humoral factor, and grafts of spleens from 14-day-old mice were effective when placed free in the peritoneal cavity and when lymphoid cells could migrate freely from the grafts, but not when splenic tissue was in Millipore chambers that confined the cells. The last finding confirms data of Osoba (6) and parenthetically serves as a control of the adequacy of our technique of making cell-tight chambers.

The effectiveness of thymic grafts increased with the weight of tissue transplanted, an observation reminiscent of the findings of Stutman and associates (13), who obtained better reversal of post thymectomy wasting disease in mice after grafts of five thymus glands than after grafts of only one gland. In the present study, residual grafts could not be found in several mice that had rejected their Walker tumors and perhaps grafts that did not survive provided a depot of thymic humoral factor that was released as the grafts disintegrated.

The data indicate clearly that grafts of rat thymic tissue can restore the capacity of neonatally thymectomized C57BL mice to reject the Walker tumor and thus confirm, with a different experimental model, the findings of Law (8). Lack of species specificity is not surprising, especially in view of the recent reports of Trainin and Linker-Israeli (14) and of Law *et al.* (15) that extracts of thymus of the calf can restore some immunologic capability in neonatally thymectomized mice.

Conclusions. The effectiveness of intraperitoneal grafts of thymus and other tissues was tested by observing their ability to restore to neonatally thymectomized C57BL/6 mice their ability to reject the Walker 256 carcinosarcoma of rats. Grafts of isogeneic thymic tissue, both free and in cell-impermeable Millipore chambers, were effective. Grafts of isogeneic splenic tissue from 14-day-old mice were effective if transplanted free but not if enclosed in Millipore chambers. Free grafts of splenic tissue from neonatally thymectomized mice were ineffective. Free grafts of isogeneic duodenum, lung, liver, kidney, skeletal muscle, and submaxillary and thyroid glands were ineffective. Free grafts of xenogeneic thymus tissue from Sprague-Dawley rats, either neonatal or 14 days of age, were effective. The effectiveness of thymic grafts in this model increased as the mass of tissue transplanted was increased and was independent of the finding of histologically normal thymic tissue at the end of the period of observation.

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Feeding Norethynodrel and Mestranol to Immature and Adult Female Rats Cross-Section and Length of Long Bones* (33739)

M. G. YANG, V. L. SANGER, AND O. MICKELSEN

Foods and Nutrition and Pathology Departments, Michigan State University, East Lansing, Michigan 48823

During the last several years, certain estrogenic compounds have been used experimentally to stop the skeletal growth rates of young girls by hastening bone maturation (1–5). The reports indicate that for girls who were treated with estradiol valerate before the onset of puberty, their bone age increased as much as 44 months after 1 year. 17-Hydroxyprogesterone caproate was used to start menstrual bleeding each month of the therapy. Progestational compounds when given to pregnant women have also been found to increase the bone age of their infants (6). Whether this was a direct action of the progestational compounds on the fetal bone is not known.

The preceding studies suggest that oral contraceptive steroid preparations containing a combination of estrogenic and progestational agents may produce comparable effects as those produced in the young girls. For this reason, norethynodrel and mestranol, the progestational and estrogenic compounds commonly used in formulating oral contraceptive pills were fed to female rats to determine whether they had any effect on the long bones. Furthermore, since prepubertal girls appeared to be more susceptible to bone growth inhibition than older girls, 5- and 12-week-old rats were used in the present experiments.

Methods. In the first of two trials, 28 virgin adult female rats, 83 days of age were paired according to body weights. Seven rats, one from each of 7 pairs were fed *ad libitum* a basal diet[1] containing 0.0225 mg of mestranol and 1.562 mg of norethynodrel/kg of diet while each pair-mate was fed a quantity of the basal diet equaling that consumed by the treated rats. The remaining 7 pairs were also pair-fed but the steroid levels were increased to twice that of the first 7 pairs. The

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[1] Percentage composition of the basal diet was: ground corn, 60.7; soybean meal (50% protein) 28.0; alfalfa meal (17% protein), 2.0; fish meal (12.5% protein), 2.5 ; dried whey (67% lactose), 2.5; limestone (38% Ca), 1.6; dicalcium phosphate (18.5% P, 22–25% Ca), 1.75; iodized salt, 0.5. Supplementary minerals and vitamins were added to provide per kg of diet: (in mg) Mn, 121; Fe, 95; Cu, 7; Zn, 4; I_2, 4; Co, 2; choline chloride, 400; Ca pantothenate, 6; riboflavin, 3; niacin, 33; menadione, 2; DL-methionine, 500; (in μg) vitamin B_{12}, 7; (in IU) vitamin A, 8010; vitamin D_2, 750; vitamin E, 5.