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Growth of transplanted tails of infant rats in adolescent allogeneic recipients

(alkaline phosphatase/^{45}Ca/zebra-stripe effect/obligatory growth/sucrose ration)

CHARLES B. HUGGINS AND KENNETH PAUL ANDERSON

The Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, Illinois 60637

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ABSTRACT **The tails of newborn rats, consisting of slender continuous cones of avascular cartilage, were transplanted in subcutaneous spaces of allogeneic adolescents; colloidal carbon was injected in a vein before the transplants were harvested on days 7–28. Between 97 and 100% of the transplants were accepted, underwent differentiation into bone with bone marrow, and grew at a brisk rate.**

Acceptance was recognized by (*i*) a zebra-stripe effect, visible in the gross, resulting from accumulation of carbon in reticuloendothelial cells; (*ii*) increase of alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] and of incorporated ^{45}Ca in the transplant; and (*iii*) the presence of dense cortical bone with lacunae populated with osteocytes demonstrable by histology. Differentiation of the cartilaginous transplant into bone with subsequent growth of the transplant occurred in recipients fed sucrose as their sole ration.

In the present experiments the tails of newborn rats of a noninbred strain were transplanted to other rats from the same colony as the donors. It was found out that the age of the recipient was a factor of high significance in determining acceptance or rejection of the allografts. Nearly all of the transplants were accepted by nonrelated rats, 21–26 days old, whereas many grafts were rejected by the parents of the donors of the transplants.

Differentiation of the rat tail (RT) allografts of cartilage into bony vertebrae, each with two and, later, four growth centers, occurred in the accepted transplants and brisk growth followed. It was found that RT allografts are useful as a simple and reproducible assay system for measuring skeletal growth in addition to indicating immunologic tolerance. In this work it was found out that growth of RT allografts is obligatory in adolescent recipients fed sucrose as their exclusive ration.

Bert (1) observed that the tail of a rat, 8 days old, transplanted subcutaneously in another young rat grew to double its length in 2 months. Huggins and Blocksom (2) found out that a warm physiologic environment is mandatory for hematopoiesis in mammalian bone marrow, including allografts of RT. Loeb (3) observed that lymphocytes were attracted by allografts of rat xiphoid cartilage, but on the whole chondrocytes are well preserved in allogeneic transplants. Zeiss *et al.* (4) found that transplants of tubular pieces of tibia in young adult rats are vascularized by ingrowth of host blood vessels; these are destroyed in allografts 7–12 days after transplantation. Langer *et al.* (5) observed a marked inflammatory reaction in allografts of femur in adult rats commencing 1 week after transplantation. When tubular pieces of tibia were transplanted to young adult rats (6), osteogenesis was slight or absent at 250 days. Marchand (7) discovered that rejected bone or cartilage is replaced by a creeping substitution of newly formed osteoblasts.

Abbreviation: RT, rat tail.

MATERIALS AND METHODS

The experimental animals were rats of the Long-Evans strain that have been maintained as a closed colony for 16 years; the animals were random-bred *inter se* and not by brother × sister matings. The animals are remarkably sturdy and free from intercurrent infections. The rats were kept in air-conditioned rooms at 25° and fed a commercial ration of compressed pellets, which has been the sole food for our colony for 10+ years. In certain experiments, groups of rats were fed a ration consisting exclusively of sucrose; a salt lick was suspended in the cage. All rats were given tap water ad lib.

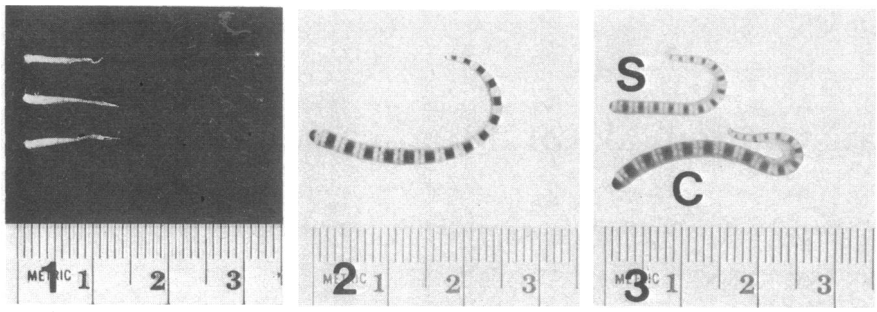
All of the transplants were obtained from newborn rats, 0.5–2 days old. A rat tail was amputated with scissors at its junction with the torso; hemostasis was unnecessary and the wound was not closed; the infant was returned to its mother. The amputated tail denuded of skin was weighed on a torsion balance. To obtain long bones for transplantation, a newborn infant rat was decapitated; humerus, femur, and tibia were excised and cleansed of soft tissue.

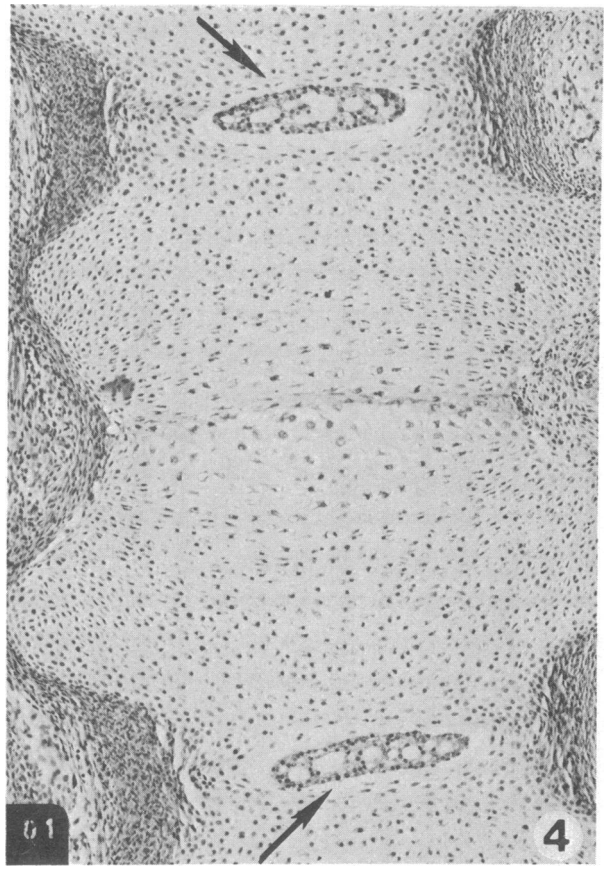
The recipients of the grafts were of two sorts: (*a*) nonrelated Long-Evans males 21–26 days old, and (*b*) the mother and father of the infants on the day of birth. All of the grafts were transplanted subcutaneously in the thoracic region. The recipient was anesthetized with ether. A 0.5 cm incision was made in the skin of the lower chest and a subcutaneous pocket was prepared by blunt dissection. To protect the delicate graft during transplantation it was placed inside a cannula to insert and deposit it in the surgically prepared pocket; the incision was closed with a metallic skin clip. Each animal received four transplants in the thoracic region. The day of transplantation is denoted day 0.

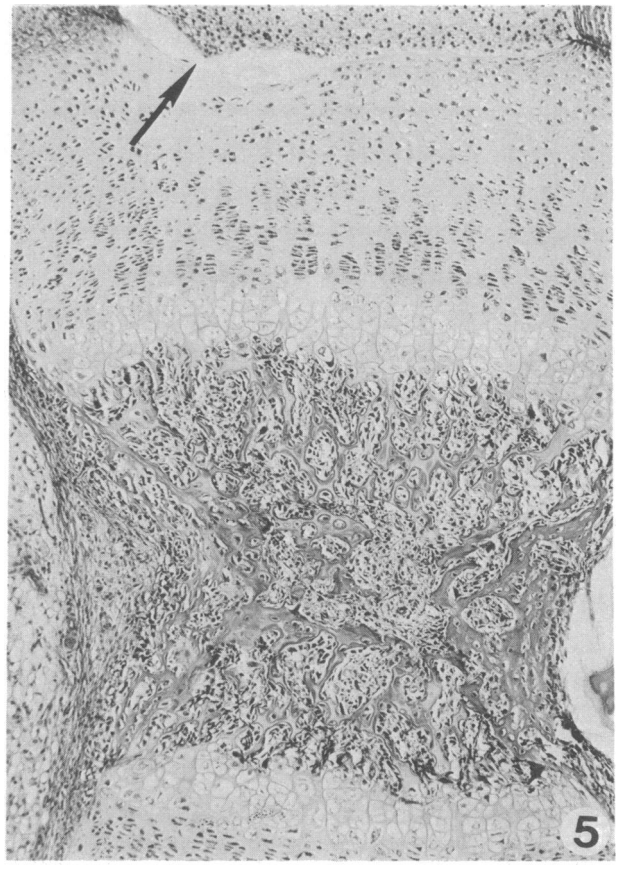
The grafts were harvested on days 7–36. *Twenty-four hours prior to harvest every recipient was injected intravenously with a suspension of colloidal carbon.* The carbon suspension (Pelikan C11/1431A, Günther Wagner, Hanover) contained 10% carbon black. It was diluted 1:3 with saline and injected intravenously in a caudal vein at a dosage of 0.01 ml/g of body weight; the injection time was *ca* 0.5 min.

Radioactivity. $^{45}CaCl_2$ (specific activity about 25 mCi/mg) was obtained from New England Nuclear Corporation. Two hours prior to harvest each rat received an intravenous injection of 1 μCi of ^{45}Ca per g of body weight. Preparation of tissues and measurement of radioactivity have been described (8). The results are expressed as cpm of ^{45}Ca per mg of tissue incorporated in an acid-soluble fraction. Ca^{2+} in the grafts was measured by atomic absorption flame spectrophotometry.

Harvest. At 0 hr the rats were anesthetized with ether and exsanguinated by cardiac puncture followed by decapitation. One graft in each set was preserved in Bouin's fluid for histological examination; paraffin sections were stained with







FIGS. 1–5. Rat tails before and after transplantation. *Fig. 1.* Tails of three rats, 1 day old. *Fig. 2.* Allogeneic transplant of RT in host, 24 days old; the recipient was injected with colloidal carbon on day 8 and the graft was harvested on day 9. The zebra-stripe effect is evident, denoting acceptance of the transplant. *Fig. 3.* Allogeneic transplants of RT in hosts fed respectively: sucrose ration (S); complete diet (C). The recipients were injected with colloidal carbon on day 7 and the grafts were harvested on day 8. The zebra-stripe effect is evident in both specimens. *Figs. 4 and 5.* Photomicrographs of paraffin sections stained with hematoxylin and eosin. ×90. Arrows point to intervertebral discs. *Fig. 4.* Tail of rat, 1 day old. *Fig. 5.* Allogeneic transplant of tail of rat 1 day old; the graft was harvested on day 8.

hematoxylin–eosin. The site of alkaline phosphatase was determined by a histochemical method of Gomori (9).

Most of the grafts were subjected to enzyme assays and determination of radioactivity. The graft, cleansed of adherent tissues, was cut into 3 mm cubes and homogenized in cold 0.15 M NaCl–3 mM $NaHCO_3$ in a Polytron homogenizer for three 10 sec bursts at the maximum setting. The homogenates were centrifuged at 12,000 × *g* for 15 min at 2°; the supernatant was removed for enzyme assays and the washed precipitate was used to determine radioactivity.

Enzyme Assays. Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] was determined in barbital buffer at pH 9.3; acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] was measured in acetate buffer at pH 5.1. One unit is defined as the enzyme activity that liberated 1 μmol of *p*-nitrophenol in 0.5 hr under stated conditions (10). Statistical differences were evaluated by Student's *t* test and *P* values of 0.01 or less were considered significant.

RESULTS

RT transplants: Day 0

At 1 day, rat tail (Fig. 1) used as a transplant was a pearl-gray, serrated, avascular column of cartilage (Fig. 4) surrounded by primitive mesodermal cells; bone and bone marrow were absent. The intervertebral discs were evident in the cartilage rod at periodic intervals; each segment between discs was the site of a developing caudal vertebra. The vertebra at this age consisted of small-cell cartilage with a band (Fig. 4) of hypertrophic chondrocytes at its equator creating a single growth center. In histochemical preparations (9) it was evident that alkaline

Table 1. Acid and alkaline phosphatase activities and incorporation of ^{45}Ca in allografts of infant tails in adolescent recipients

Harvest day	n	Weight (mg)	Phosphatases (units/g)		^{45}Ca incorporation (cpm/mg) $\times 10^{-3}$
			Acid	Alkaline	
0*	10	15.1 ± 1	16.4 ± 2	11.3 ± 3	0.39
7	7	55.5 ± 22	25.3 ± 4	52.2 ± 12	1.70 ± 0.36
8	17	95.9 ± 21	23.1 ± 4	56.8 ± 7	1.78 ± 0.39
10	14	106.5 ± 23	28.7 ± 9	81.9 ± 10	3.46 ± 0.26
14	15	189.0 ± 26	30.7 ± 4	67.1 ± 21	3.10 ± 0.49
26	20	382.5 ± 63	30.8 ± 5	108 ± 20	—

n = number of transplants. Data are given ± standard deviation of mean. Age: donors 1 day; recipients 21–26 days.
* Controls on day of transplantation.

phosphatase was present in two locations: (*a*) the cytoplasm of the hypertrophic chondrocytes, and (*b*) as a narrow line on the top and bottom of the developing vertebra near the intervertebral discs.

At the time of transplantation (Table 1) the weight of the grafts was *ca* 15 mg; acid phosphatase exceeded the activity of alkaline phosphatase; there was a low level of incorporation of ^{45}Ca. In eight grafts the content of Ca^{2+} was 1.1 ± 0.2 mg/g.

RT transplants: Days 8–9

The transplanted tail that had been accepted by its host consisted of 12–24 vertebrae connected to form a firmly united structure. The transplant had alternate black and white bands easily visible in the gross, which formed a striking zebra-stripe effect; the black stripes were alternately thin and thick (Figs. 2 and 3).

The skeletal elements consisted of a heavily ossified vertebral body with plates of hypertrophic cartilage at the metaphyses forming two growth plates (Fig. 5); epiphyses were not present. The vertebral body contained many trabeculae of bone surrounding unabsorbed cartilage. The intertrabecular spaces contained dilated capillaries and hemopoietic bone marrow; many carbon-containing reticuloendothelial cells were visible.

With reference to values obtained on day 0, the following increases were found in the grafts on day 8 (Table 1): weight *ca* 6-fold, alkaline phosphatase *ca* 5-fold, and incorporation of ^{45}Ca *ca* 4-fold. Moreover, on day 8 alkaline phosphatase exceeded acid phosphatase activity.

RT transplants: Day 26

Epiphyses were present. Each of the vertebrae in the accepted grafts contained four growth centers denoted by bands of hypertrophic cartilage in both epiphyses and both metaphyses. With reference to values found in the grafts on day 0, the following increases were found (Table 1): weight *ca* 25-fold, alkaline phosphatase *ca* 10-fold, and acid phosphatase *ca* 2-fold.

Criteria of acceptance

Infant bone transplants which had been accepted or rejected had distinctive characteristics. In the gross, accepted transplants were firm, black, and relatively large; tail transplants displayed the zebra-stripe effect. Microscopic examination disclosed that the bone cortex was thick and unbroken and the lacunae of cartilage and bone were populated with cells. Hematopoietic and fatty bone marrows were present. Many reticuloendothelial cells with ingested carbon particles were observed in the marrow, and lymphocytic infiltration was slight or absent.

Rejected transplants were white, short, and stubby; some were firm, others were flabby. The zebra-stripe effect was absent in rejected tail transplants. Microscopic examination disclosed that bone was devoid of cells in rejected grafts. In the transplants that were white and firm the cortex of the bone was continuous and unbroken but depopulated of cells. In the white flabby transplants, the cortical bone was fractured and fragmented, and osteocytes were absent. Small-cell cartilage survived; hypertrophic cartilage cells were disrupted and acellular. The bone marrow comprised a diffuse protein framework with a rather sparse population of lymphocytes and fibroblasts; hematopoiesis and lipopoiesis were absent. Necrotic blood vessels surrounded by massive clumps of carbon-containing macrophages were abundant in the bone marrow. There were periosteal collections of lymphocytes.

Acceptance of transplants from an infant to its parents

Eight experiments were carried out in which one member of a pair of the long bones of an infant was transplanted to each parent. On the day of birth a newborn rat was sacrificed and the bones of its extremities and its tail were dissected. Each parent received four skeletal elements from the infant rat. The right humerus, femur, and tibia were transplanted subcutaneously in its mother, while the father received the contralateral bones as transplants; half of the tail was transplanted in each parent. The mother continued to nurse the surviving members of the litter until the animals were weaned; the grafts were harvested on days 23 and 36.

Acceptance or rejection of the transplants of infant bones in a parent was comprehensive; the recipient accepted all of the transplants or none of them. Eight mothers (Table 2) accepted transplants from six infants; eight fathers accepted grafts from five infants. It is noteworthy that the bones of one rat (Table 2, infant no. 1) were rejected by mother and father.

A rather similar experiment was performed in which two infant siblings were sacrificed and used as donors of transplants; two femurs and two tibias of an infant daughter were transplanted to its mother; the father received similar bones from his infant son. The transplants were harvested on days 28–31. Acceptance or rejection of multiple bones from a single donor was comprehensive; the parent accepted all or none of the bones of its progeny. Thirty-nine sets were studied: 39 mothers accepted the transplants of 31 daughters; 39 fathers accepted grafts from 26 sons.

Alkaline phosphatase activity, expressed in units per g, in 40 transplants was: 27 accepted grafts, 170 ± 25; 13 rejected grafts, 40 ± 11.

Table 2. Acceptance of transplants of paired bones of an infant by its mother and father

Infant no.	Acceptance of transplants	
	Mother	Father
1	−	−
2	−	+
3	+	−
4	+	−
5	+	+
6	+	+
7	+	+
8	+	+

On the birthday of an infant rat its right humerus, femur, and tibia were transplanted subcutaneously to its mother, while the father received transplants of the contralateral bones; in addition each parent received as transplant half of its infant's tail. The transplants were harvested on days 23–36. +, Acceptance; −, rejection.

Acceptance of allografts by adolescent recipients

Short Term. Thirty recipients, 21–26 days old, received whole tail transplants in the subcutaneous tissue from 94 donors and the transplants were harvested on days 8–10. Ninety-one of the transplants (97%) were accepted and the zebra-stripe effect was visible in every one of the grafts; three transplants were rejected.

Long Term. Ten recipients, 26 days old, received 38 whole tail transplants in the subcutaneous tissue and the grafts were harvested on days 26–28; all of the transplants were accepted by their hosts and all of them displayed the zebra-stripe effect.

Obligatory growth of allografts in recipients fed sucrose ration

Each of six rats, 22 days old, received subcutaneous transplants of whole tails from four infants, 1 day old. Beginning on day 0, half of the rats, designated the sucrose ration group, received sucrose as their sole food; the remainder, as controls, were maintained on the complete diet that is customary in our laboratory. The transplants were harvested on day 8.

In body weight on average sucrose ration rats declined 1 g per day, whereas controls gained 2.4 g. The transplants in animals in the sucrose ration group were smaller (Fig. 3) than those of the controls, but the zebra-stripe effect was evident in all of the transplants in both groups. With respect to the grafts in controls, the transplants in the sucrose ration groups were characterized by significant decreases (Table 3): in weight, in acid phosphatase activity, and in the incorporation of ^{45}Ca. In alkaline phosphatase activity the differences in the two groups in observed values (Table 3) were not significant.

Table 3. Acid and alkaline phosphatase activities and incorporation of ^{45}Ca in tail allografts in recipients fed complete or sucrose rations

Weight of grafts (mg)	Phosphatases (units/g)		^{45}Ca incorporation (cpm/mg) × 10^{-3}
	Acid	Alkaline	
	Sucrose ration		
33.4 ± 6	18.3 ± 5	63.2 ± 12	2.40 ± 0.32
	Complete ration		
90.9 ± 11*	28.1 ± 7*	74.3 ± 14†	3.45 ± 0.39*

n = 12. Data are ± standard deviation of mean. Age of recipients: 22 days. The grafts were harvested on day 8. Differences from respective values for sucrose-fed animals: * $P < 0.01$; † $0.1 < P < 0.05$.

DISCUSSION

Acceptance of the allografts usually was detected with ease by visual methods made possible by phagocytosis of carbon particles by reticuloendothelial cells in the bone marrow and its sinusoids, resulting in the zebra-stripe effect. A high percentage of allogeneic transplants in adolescent rats were accepted, but many grafts were rejected by parents of the donors.

The transplantation of infant tail was advantageous in the study of skeletal growth and immunologic tolerance for many reasons. (*i*). RT at birth is a rod of cartilage devoid of blood vessels, bone, and marrow; Ca^{2+} was present in small amounts. (*ii*) RT was obtained without difficulty and amputation of the tail is not perilous for the donor. (*iii*) Growth is easily assayed by measuring the weight of the transplant, its content of alkaline phosphatase, and the incorporation of ^{45}Ca. (*iv*) Because of the development of 50–90 individual growth centers in the tail transplants, the growth of the column of caudal cartilage after differentiation exceeds that of any other rat bone. In adult rats of the Long-Evans strain, tail length exceeds crown–rump body length. (*v*) Rejection of bone allografts was never total and remnants of the transplant were found after 4 months. The reasons for survival and perpetuation of living cells in rejected grafts are 2-fold: (*a*) small-cell cartilage is not rejected in allogeneic transplants (3), and (*b*) mineral-free bone matrix transforms fibroblasts (8, 11) into chondroblasts and osteoblasts so that dead bone in demineralized rejected transplants is replaced with living bone through a creeping substitution (7). (*vi*) The infant bones bear no load.

The mean survival of rats on a ration consisting solely of sucrose was 26 ± 3.6 days (12). We define obligatory growth as cellular increment in animals on a nitrogen-free ration. Obligatory growth is a remarkable effect since areal growth, often purposeless, takes place despite the catastrophic breakdown of the generality of tissues that results in severe emaciation.

Obligatory growth has been observed in two previous experiments: (*i*) growth of the prostate is vigorous (13) and selective in infantile dogs injected with testosterone while deprived of all food for 3 weeks, and (*ii*) implantation of mineral-free bone matrix induced the transformation of fibroblasts into cartilage and bone in rats fed a sucrose ration (12). In related observations, Rous (14) found that the growth of a transplanted carcinoma in the rat was unaffected by drastic underfeeding of the host. In conclusion, obligatory growth occurs where cell division is intense and preferential because of factors, e.g., testosterone, which urge on the selective multiplication of several sorts of cells.

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