Chapter 23

INNATE AND INDUCED REGENERATIVE RESPONSE OF THE FORELIMB OF THE RAT*

RICHARD M. LIBBIN AND PHILIP PERSON

Introduction

IN 1972 BECKER (1) described significant partial limb regeneration in the forelimb-amputated twenty-one-day-old white rat in response to minute electrical currents applied directly to the amputation stump by a silver-platinum (Ag-Pt) bimetallic couple joined through a 10-Mohm resistor. Becker's report, studies of Mizell (10) conducted upon the marsupialized opossum fetus into whose limbs exogenous nerve tissue had been implanted prior to amputation, studies of Umanskii and Kudokotsev (16) employing hormonal stimulation, and earlier investigations by Selye (15) and by Nunnemacher (11) describe the most extensive mammalian limb regenerations of which we are aware and have stimulated the endeavors we presently describe.

In setting forth our experiences our intention has been to describe recently published as well as unpublished observations including research currently in progress. While emphasis is placed upon skeletal tissue regeneration phenomena, we also indicate responses in skeletal muscle and connective and other tissues.

Experiments and results to be described below have devolved about three types of general approach. In the first experiments, responses to electrical and mechanical stimulation provided by indwelling bimetallic couples and control devices delivering no current, but duplicating the mechanical aspects of device implantation, have been assessed and recently reported by us (8). In the second group of experiments, we have attempted to examine the innate limb regeneration capacity of young rats. In this context we have used rats only several hours old (100 gm animals), rats intermediate in body weight and age, as well as those somewhat older. As Polezhaev has noted (13), while regeneration capacity is thought to be greatest in younger animals, it may be retained throughout life and may in theory at least be elicited at any time by resort to appropriate stimuli. Thus, this second approach, the study of innate limb regenerative capacity,

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constitutes not only a baseline analysis for future experiments in mammalian limb regeneration research but also an effort directed towards a fuller examination of Polezhaev's perspective. In the third approach, we have recently attempted to duplicate the experiments of Umaskii and Kudokotsev (16) examining the capacity of parathyroid extract (PTE) to enhance the limb regenerative potential of young, forelimb-amputated rats. These workers claimed to have produced the most advanced regenerates observed to date in a eutherian mammal by this hormonal regimen, and described the regeneration of distal humerus, radius, ulna, and several carpal-metacarpal and digital structures in response to administration of PTE. Of particular interest is their premise that PTE might induce destruction of skeletal tissues and thus canalize the limb stump toward processes of dedifferentiation prior to amputation.

We have also directed attention to the question of the existence of epimorphic processes in both the PTE-treated limb and in our most advanced regenerates obtained from experiments in which nonhormonal stimuli were applied, seeking to identify processes of dedifferentiation and redifferentiation.

Studies of mammalian regeneration phenomena in the preceding twenty-five years have been greatly expanded, and it is now recognized that these processes may occur with heretofore unsuspected vigor in humans as well as in lower vertebrate organisms. In this context, several reports have described spontaneous digital regeneration of impressive fidelity in children (4, 5, 6, 14). In view of the presence of many tissues in the digital terminus (bone, cartilage, tendon, nerve, blood vessels as well as integumentary specializations such as nail, cuticle, and dermataglypic pattern) and the full recovery of sensory and motor function in these instances, credence is strongly lent to the view that mammalian regenerative capacity is far greater than had been imagined, and that its study offers opportunities not only for laboratory animal investigations but also for direct application to the clinical setting.

Methods and Procedures

General

In previously unpublished and current research described in this communication, methods have been developed to accurately assess the level of amputation of each forelimb, and to determine whether or not distal humeral growth plate cartilage might be present in the transection plane. Were such the case, this could confer capacities for regeneration upon the postamputation limb that it might otherwise lack had this cartilage been surgically excluded from the amputation plane via a more proximal transection (11).

Additional simple methods bave been employed in later experiments to

initially separate limb specimens with substantial regeneration responses from those in which regeneration responses were not observed. In this way, considerable time and effort were spared and unnecessary routine serial sectioning reduced. Finally, in those instances where forelimb amputation was performed upon rat neonates several hours to one day old, methods were devised to prevent maternal cannibalism, which have been described by us in a recent communication (7). In the latter experiments, litter size and sex distribution were recorded and considered in the context of possible genetic and nutritional factors that might affect regenerative capacity. In all experiments, commercially obtained male Sprague-Dawley-derived rats were used. In those instances where experiments were conducted upon neonates, maternal parents were obtained at thirteen days of gestation and maintained in the laboratory rather than the animal facility through the interval of gestation and weaning.

Surgical

POST-WEANLINGS: Rats that had reached the age of weaning or were older were anesthetized with ether. Under semisterile conditions the skin and superficial fascia of the limb were transected circumferentially in the amputation plane and the neurovascular bundle transmitting the brachial artery was ligated several mm above the proposed level of amputation. The simplest amputation procedure used required only that the muscle and bone of the upper limb be transected by a guillotine-type maneuver followed by closure of the skin with sutures. Prior to amputation the humerus was denuded of soft tissues in such a manner that its periosteum was left undisturbed. The final level of amputation was then determined visually. Subsequent methyl green staining of the amputated portion of the limb followed by clearing in methyl salicylate (9) made possible the determination of the level of amputation of the limb, and the presence or absence of cartilage in the plane of section, with considerably accuracy.

In a second series of experiments, where electrical device implantation was carried out, following the transection described above, the end of the Pt wire was inserted into the humeral medullary cavity for a distance of 1 to 2 mm and the remainder of the device (resistor plus Ag wire) was placed outside the humerus but parallel to it beneath the brachial fascia and skin. The skin was then closed with continuous sutures. Electrical devices were removed either four or seven days later under light ether anesthesia. In several additional experimental groups, muscle tissue of the limb was transected more proximally on the flexor surface of the limb than on the extensor surface. By this means the triceps brachii could be pulled over the cut surface of the humerus, thus forming a muscle covering or myoplasty preparation and then attached to the distal remainder of the biceps brachii by a single suture. The skin was then closed as above.

Other rats two weeks (25 gm), three to four weeks (50 gm), four to five weeks (75 gm), and seven to eight weeks (200 gm) of age were also studied to assess the interplay of age in the elicitation of either spontaneous or muscle covering-induced regenerative capacity. No variations in surgical procedure from those described above were employed.

NEONATES: Rats ranging in age from several hours to twenty-four hours were anesthetized by hypothermia. Male members of a litter were placed in the cold room for twenty-five to thirty-five minutes and then removed singly just prior to surgery. Amputations were performed in two segments, the first consisting of single circumferential incision through all soft tissues of the upper arm exposing the surface of the humerus over several mm of its length. By this method the level of amputation (generally the junction of the lower and middle thirds of the humerus) could be determined visually with fair precision. In the second segment the humerus was transected with a single movement. This skin was left unsutured and the pups were returned immediately to their parents. In a single experiment involving forelimb amputation upon the first day of life, an attempt was made to direct the transection either through the cartilage of the distal humeral growth plate or through the adjacent metaphysis proximal to it. This experiment was undertaken to test the hypothesis of Nunnemacher (11) that the substance of the growth plate possesses unique potencies, leading to the substantial regeneration of the extirpated portion of the long bone. Thirty-six male rats of either fifteen or twenty-five days of age (PTE-and vehicle-treated groups) underwent amputations as described above following administration of PTE at a dose of 10 U per rat neonate per day for ten consecutive days. Two similar groups of rats received injections of the vehicle in which the PTE was suspended (0.1*M* L-cysteine in isotonic saline). After humeral transection had been completed, the skin was either sutured closed directly or closed after a muscle covering had been established.

Rats were killed at various intervals after forelimb amputation. In some experiments these were predetermined, while in others (principally studies involving neonatal animals) rats displaying limbs with bulbous termini, as determined by gentle palpation, were permitted to survive for up to one year. Experimental protocols summarizing surgical, hormonal, electrical, and other study categories are presented in Table 23-I.

Methods for Preliminary Assessment of Level of Amputation and Regenerative Responses

In earlier experiments some difficulty was experienced in precisely assessing the level of amputation. The following procedure was therefore employed. As each amputation was completed, the portion of the limb removed was fixed immediately in Bouin's solution, bleached in dilute

TABLE 23-I
SUMMARY OF EXPERIMENTS

Experimental Group	*Age at Time of Amputation*	*Surgical Procedure*	*Postsurgical Interval*	*Substance Administered*	*Number of Rats in Study*
I. Electrical Stimulation					
a. Effective Device	4-5 wk (100 gm)	simple amputation	4, 7, 14, 28 day	—	4-5 per study interval
b. Ineffective (Null) Device	4-5 wk (100 gm)	simple amputation	14	—	9
c. No Device	4-5 wk (100 gm)	simple amputation	4, 7, 14, 28 day	—	4-5 per study interval
II. Muscle covering	4-5 wk (100 gm)	muscle covering	4, 7, 14, 28 day	—	4-5 per study interval
III. Age-related Assessment of Regenerative Capacity					
a. Neonates	1 day	simple amputation	5-271 day*	—	45
b. Juveniles	2 wk (25 gm)	simple amputation	35 day	—	7
			37 day	—	10
			67 day	—	7
	3-4 wk (50 gm)	simple amputation	59 day	—	12
	4 wk (75 gm)	simple amputation	31 day	—	7
			64 day	—	8
	4-5 wk† (100 gm)				
	7-8 wk (200 gm)	simple amputation	36 day	—	12
IV. Parathyroid Extract Administration‡	15 day (25 gm)	simple amputation	92 day	PTE‡	3
		muscle covering	92 day	PTE	5
		simple amputation	92 day	vehicle§	5
		muscle covering	92 day	vehicle	4
	25 day (50 gm)	simple amputation	92 day	PTE	4
		muscle covering	92 day	PTE	6
		simple amputation	92 day	vehicle	4
		muscle covering	92 day	vehicle	5

* Individual post-surgical healing intervals provided in text.
† Animals comprising this group are those from experimental group Ic.
‡ 10 PTE U per rat daily in a single dose for twelve consecutive days.
§ 0.1*M* L-cysteine in isotonic saline.

hydrogen peroxide, and immersed in methyl green solution, which selectively stains cartilage (9). Following removal of excess stain and transfer of the specimen to methyl salicylate, it was rendered transparent so that the bone and distal growth plate apparatus could be directly visualized. In this way it was possible to determine the plane of transection with accuracy.

The same methyl green staining-clearing procedure was also applied in later experiments to the experimental and control limb specimens themselves. Upon sacrifice the operated as well as the contralateral intact humeri were measured in their longitudinal axes and the limbs were then fixed in Bouin's solution. Following cartilage staining and clearing, only those limbs displaying some evidence of regenerative responses were decalcified and sectioned. In this way considerable histological preparation time was saved. Specimens were frequently x-rayed prior to decalcification. This further assisted in the process of specimen selection and also made it possible to orient the specimens in the most favorable plane during paraffin embedding.

In a single instance the postsurgical limb was roughly trimmed and then placed in a dish containing mealworms for several days for removal of soft tissues. Following the photographing of this specimen it was fixed, cleared, rephotographed, decalcified, and then sectioned.

Histological Procedures: All postsurgical specimens selected for histological examination were immersed in Evans-Krajian formic acid-citrate solution for several days. Upon completion of this decalcification process, the specimens were either bisected in a standard longitudinal plane and embedded in paraffin or were embedded directly. Serial sections through the long axis of the entire forelimb were prepared at 6 μm and stained routinely with hematoxylin and eosin. Occasional slides were stained instead with either 0.1% aqueous toluidine blue O or with Alcian blue and chlorantine fast red.

Assessment Criteria: Examination of stained sections emphasized regenerative responses occurring in muscle, connective tissue, and nerve, as well as in bone and cartilage. We have also, in more recent experiments, measured the lengths of both postsurgical and contralateral unoperated humeri and expressed the longitudinal growth component of the operated humerus as a percentage of the growth in length achieved by the unoperated humerus.

In an attempt to identify cell and tissue changes indicative of epimorphic regeneration processes, we have examined histological preparations for evidence of dedifferentiation, for the possible presence and activation of embryonal cells and for the presence of mesenchyme or mesenchyme-like tissue components, as well as for the appearance of a structure within the limb bearing similarity to the regeneration blastema of amphibians.

Results

Postsurgical Healing and Behavioral Responses

NEONATES: Following forelimb amputation, neonatal rats were returned to their mothers and commenced suckling immediately. Maternal cannibalism was not observed. Several days after surgery the amputation wound covered with a circular scab. Subsequently, neonates developed pelage and opened their eyes at ages identical to those of their unoperated littermates, but in early experiments it was apparent ten days after surgery that those animals that had undergone amputations were smaller than their unoperated siblings. It is unclear whether loss of a limb interfered with nursing, resulting in diminished stature, or whether loss of blood during surgery had brought about this early retardation of growth. In an attempt to improve the nutritional status of the operated pups in later experiments, some unoperated pups were removed from the nest in order to reduce competition for milk. Generally, litter sizes were adjusted in this way with the result that each parent nursed only five to seven pups. In several instances, exceptionally small litters (two to five pups) were used. Nursing litter sizes were recorded and did not correlate with regeneration responses observed.

Surgical wounds appeared uninfected throughout the nursing interval, but thereafter became markedly infected in some animals while remaining free of pathology in others. Animals receiving parathyroid extract displayed none of the gross skeletal abnormalities described by Umanskii and Kudokotsev (16).

JUVENILES: Young (postweanling) animals adjusted readily to life with a single forelimb, modifying gait and feeding behavior in the process. Often these animals were observed to employ the limb stump, much as they would an intact limb, to secure the food pellet while it was gnawed upon. We have related this use of the stump while feeding to the appearance of prominent cushions of dense, regular fibrous connective tissue on the posteromedial surfaces of the amputated limbs of some rats (12). All rats undergoing forelimb amputation displayed hypercellular humeral bone marrows for the first three weeks of the postsurgical interval, reflecting blood loss at the time of surgery. Thereafter the appearance of the marrow was essentially normal.

Gross Appearance of Postsurgical Limbs

At autopsy postoperative limbs were examined *in situ* and subsequently during blunt dissection after their removal. Limb specimens removed from animals operated upon as weaned juveniles displayed variable degrees of scarring and adhesion, accounting in some for moderate adduction and immobilization of the limb.

Among neonates and juveniles those limbs displaying distinct evidence of regeneration terminated in an expanded or bulbous segment brought about by the presence of augmented quantities of bone and/or cartilage. Those limbs displaying no regenerative responses tapered to a point and their humeri were often flattened mediolaterally so as to form a bladelike terminus extending from the prominent deltoid tuberosity to the end of the humeral remainder.

Microscopic Appearance of Postsurgical Limbs

Control (Simple Amputation) Animals

Four days after amputation, microscopic examination of sections through the long axis of the limb revealed the presence of new subperiosteal bone along the periphery of the distal humerus and the presence of blood clot in the distal position of the medullary cavity (Fig. 23-1). Muscle

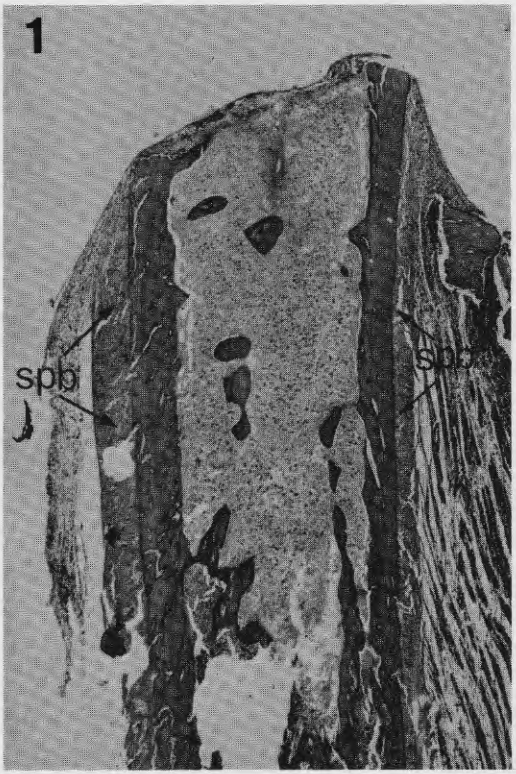

Figure 23-1. Control (simple closure) limb, four days postamputation. New subperiosteal bone (spb) surrounds the humeral shaft. The medullary cavity is filled with clot substance in its distal portion. More proximally it is occupied by hypercellular marrow (not illustrated). H&E, ×18. (From P. Pearson et al., *J. Morph.*, *159*:427-452, 1979. Courtesy of The Wistar Institute, Philadelphia.)

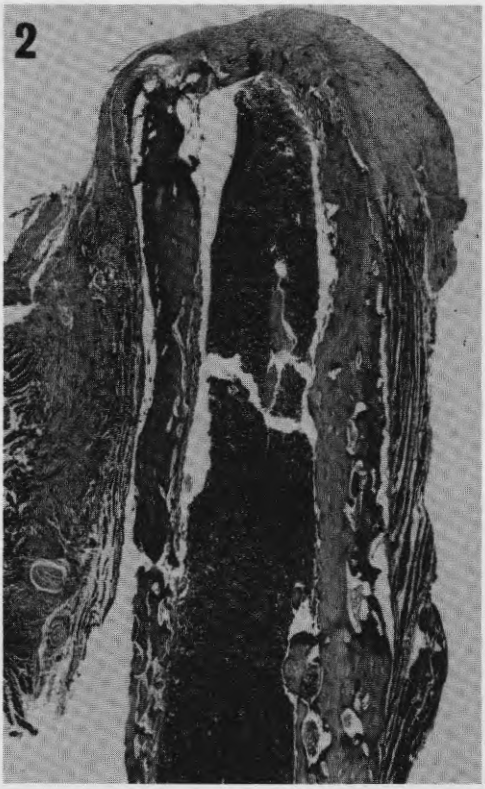

Figure 23-2. Control (simple closure) limb, seven days postamputation. Note that the humeral defect has been closed by an interrupted lamina of compact bone. Clot substance occupying the distal portion of the medullary cavity has been replaced by hypercellular marrow. H&E, ×18.

tissue displayed neither myogenesis nor disuse atrophy. The specimens were unexceptional in all other respects.

Fourteen days after amputation new subperiosteal bone growth resulted in closure of the terminal humeral defect (Fig. 23-2). The new bone thus formed was often fenestrated by blood vessels entering and leaving the medullary cavity. Distal to the bridge of bone sealing the medullary cavity were observed several laminae or tunics of mixed connective tissue separated in most instances from the humerus by a bursa, and transmitting blood vessels of small diameter (not illustrated). Among this group several terminal humeri were provided with moderately large cushions of dense fibrous connective tissue, which, on the basis of placement within the limb as well as general appearance, suggest a mode of formation related to mechanical stresses to which the limb termini may have been exposed during feeding and locomotor activities.

Neither extended growth of the humerus nor myogenesis were

observed, and only a single specimen among sixteen animals comprising the control (simple amputation) group displayed significant osteochondrogenesis at the humeral terminus beyond that required for closure of the medullary cavity.

Twenty-eight days after amputation, specimens displayed only closure of the humeral defect and nonregenerative healing. Regenerative responses were not observed nor was extended growth of the operated humeri.

Experimental Responses: Muscle Covering

Four days postamputation, limb termini displayed closure of the humeral medullary cavity by formation of a bridge or plug of bone that sealed the terminal defect (Fig. 23-3). Thus, when compared with similar healing processes in the control group, it is apparent that the presence of the

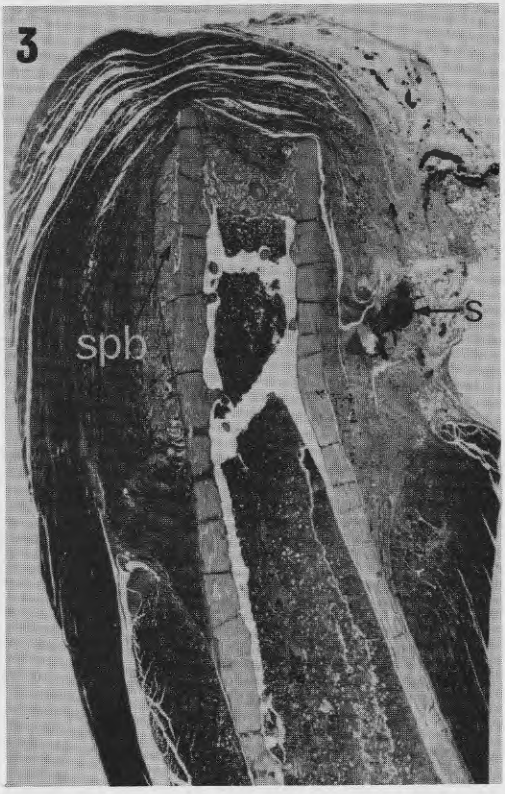

Figure 23-3. Experimental (muscle covering) limb, four days postamputation. The humeral defect has been sealed by a plug a new bone and osteogenic tissue. New subperiosteal bone (spb) is present distally. Note that the divisions of the triceps brachii forming the muscle covering pass from the left, over the transected humerus, to the right where the muscle is united with the biceps brachii by a suture (s). H&E, × 13.5. (From P. Pearson et al., *J. Morph, 159*:427-452, 1979. Courtesy of The Wistar Institute, Philadelphia.)

Figure 23-4. Experimental (muscle covering) limb, fourteen days postamputation. A large cartilage mass (ch) has arisen between a newly formed column of subperiosteal bone (spb) beneath, and the muscle covering (mc) above. Note that the substance of the muscle covering undergoes fibrous replacement as it passes over the cartilage mass. The humeral defect is sealed by a plug of cancellous bone. H&E, × 14. (From P. Pearson et al., *J. Morph.*, *159*:427-452, 1979. Courtesy of The Wistar Institute, Philadelphia.)

muscle covering accelerated the repair of the defect. At this interval, new subperiosteal bone formation was also observed. The muscle covering was retained in place, surrounding the humeral terminus on three sides.

Seven days following amputation, islets and foci of hyaline cartilage were observed in close apposition to the humeral terminus and within the connective tissue immediately distal to it. The plug of bone sealing the medullary cavity displayed increased thickness and architectural complexity as compared with four days of postsurgical repair.

Fourteen days after amputation, a prominent component of the limb terminus was a large cap of hyaline cartilage distal to the transected humerus, with a spongiosium often intervening between the humerus and the cartilage (Fig. 23-4), and resting upon a column of new subperiosteal bone. The presence of this mass, together with new subperiosteal bone

growth, extends the length of the humerus significantly beyond the plane of original transection. The appearance of this cartilage mass at a later interval suggests that at least its basal (proximal) components may participate in subsequent growth plate formation.

Twenty-eight days after amputation the regeneration process had continued and in several specimens the cartilage mass observed at the fourteen day study interval had been reorganized into a structure possessing a growth plate (Fig. 23-5). It is evident from examination of Figure 23-5 that the reshaping of the cartilage mass, together with the deposition of new subperiosteal bone, extends the length of the humerus significantly, but it has been observed that this elongation process may sometimes occur on a single aspect of tbe humeral transection plane rather than from the entire humeral surface, suggesting that an as yet unidentified growth stimuli may be unevenly supplied to all regions of the wound surface.

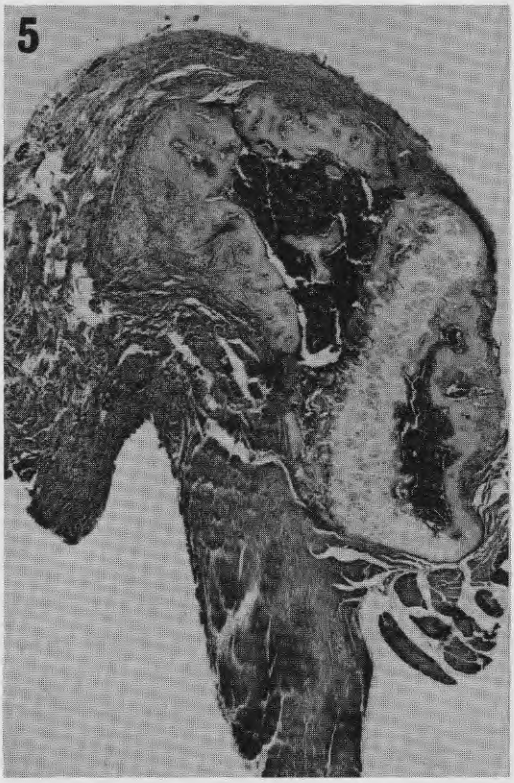

Figure 23-5. Experimental (muscle covering) limb, twenty-eight days post amputation. The specimen has been rotated 90° clockwise. The marrow-filled bony cavity to the left is the tangentially sectioned humeral shaft. The regenerated distal growth plate lies in contact with a regenerated, marrow-filled epiphysis to the right. Note that the growth plate is formed of columns of chondrocytes. Remnants of the muscle covering may be observed arching over the skeletal tissues and passing along the right margin of the illustration. H&E, ×29.

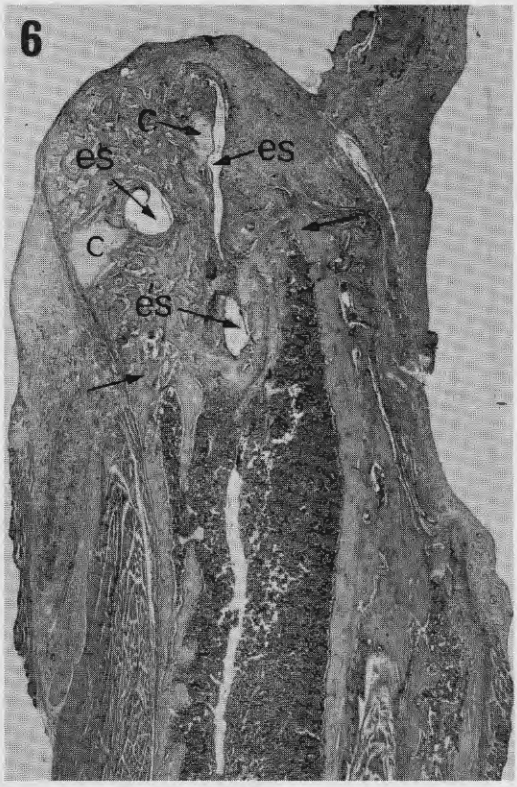

Figure 23-6. Experimental (ineffective device) limb, fourteen days after enplacement of an electrical device delivering no current to amputation plane tissues. The spaces occupied by the ineffective electrode tip (es) are bordered in their transit through the limb stump by several small foci of chondroblastic cartilage (c). Note that while the foci make contact with the electrode space, they fail to extensively line it as they do in limbs into which active devices were implanted (Figs. 23-7, 23-8, and 23-10). While some new bone is observed in the vicinity of the electrode spaces, thereby accounting for the lengthwise extension of the limb beyond the level of amputation (unlettered arrows), osteogenesis in the area of the limb adjaceut to the electrode tip is significantly diminished when compared with that observed to form in response to effective device stimulation (Figs. 23-7, 23-8, 23-10). H&E, ×21. (From P. Pearson et al., *J. Morph., 159*:427-452, 1979. Courtesy of The Wistar Institute, Philadelphia.)

Among specimens bearing muscle coverings, myogenesis was commonly observed in contrast to limb specimens of the control (simple amputation) group where the process was rarely noted.

Experimental Responses: Electrical Devices

Seven days after amputation followed by immediate implantation of effective electrical devices, limb specimens sectioned through their long axes displayed elongated spaces that had been occupied by the platinum wire of the bimetallic couple. Areas in closest contact with the electrodes

were lined almost throughout their lengths with immature, chondroblastic cartilage (Figs. 23-7 and 23-8). At points where the apices of the platinum wires extended furthest into the humeral medullary cavities, new bone was actively formed, both about the electrode tip and dissecting proximally for a variable distance into the medullary cavity (Fig. 23-8). New subperiosteal bone growth was quite vigorous in some specimens, but failed to constitute a prominent feature of others. Of particular interest was the heightened myogenesis frequently observed in specimens of the seven day group (Fig. 23-9), which was distinctly more active and developmentally complete than that noted in the preceding groups, both experimental and control. Moreover, among seven day specimens, newly formed muscle fasciculi displaying continuing myogenesis were frequently observed to lie adjacent to other fasciculi, demonstrating degenerative changes indicative of disuse atrophy.

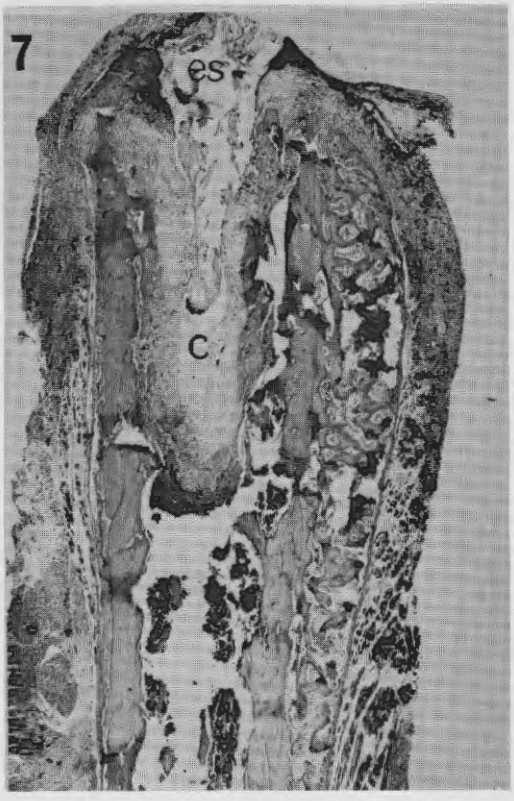

Figure 23-7. Experimental (effective device) specimen after seven days of electrode stimulation followed by seven days with device removed. Note that the electrode space (es) is lined on all sides by cartilage (c) and that at the base of this cartilage (proximally) there is a plug of bone that makes incomplete contact with the cut surfaces of the humerus at the level of transection. New subperiosteal bone is closely applied to the humeral shaft along the right side of the illustration. H&E, ×23.

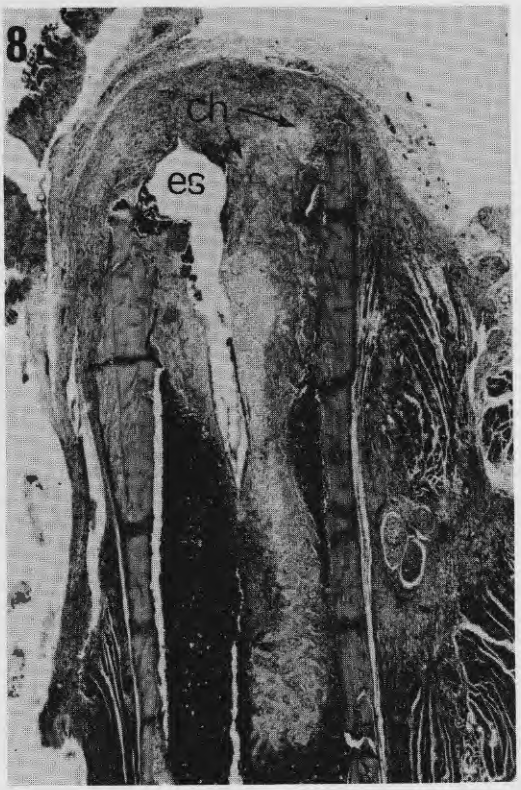

Figure 23-8. Experimental (effective device) specimen after seven days of electrode stimulation. Foci of chondrogenesis (ch) are indicated by arrows. The electrode space (es) is surrounded by chondroblastic cartilage on both sides. The lightly staining intramedullary mass beneath it is formed of new osteogenic tissue (ot). H&E, ×21. (From P. Pearson et al., *J. Morph., 159*:427-452, 1979. Courtesy of The Wistar Institute, Philadelphia.)

Another seven day specimen is illustrated in Figure 23-10. In this instance the platinum electrode tip was inadvertently inserted into the tissues of the limb at a right angle to its long axis and then bent proximally to assume its intramedullary location. It is noted that the appearance of the distal half of the humerus is much modified by the presence of the device, suggesting that skeletal tissue responses to bimetallic couple implantation are not restricted to the area surrounding the electrode tip but may extend proximally for a considerable distance, thus involving at least the distal half of the portion of the humerus remaining after amputation.

Fourteen days after amputation, limb specimens continued to elaborate new bone principally via the mechanism of subperiosteal growth. At this time, the space occupied by the platinum wire that had been removed either seven or eleven days earlier was obliterated and the humeral terminus was extensively modified, assuming the general shape of the ampu-

Figure 23-9. Experimental (effective device) specimen after seven days of electrode stimulation and illustrating myogenesis in muscle fascicule lying adjacent to the humeral shaft and several mm proximal to the amputation plane. H&E, ×432.

tated distal extremity. This recovery of terminal contour and shape was accompanied by the appearance of cartilage or cartilages, generally resting upon spongiosa, and leading through their proliferation to the extension of bone length. Articular cartilage formation was not observed in this series.

Regenerative Responses Obtained with Rats of Varying Age

NEWBORN RATS: Rats exposed to forelimb amputation during the first day of life displayed the most extensive regenerative responses we have obtained to date including, in several instances, the formation of entire new bones, the appearance of new growth plates and the emergence of articular cartilage at bone termini. Forty-five one-day-old specimens, killed between five and 271 days after amputation, have been examined to date. In all instances the surgical procedure, as noted above, consisted of simple amputation with no attempt to effect closure through suturing of the wound. Operated pups generally displayed retarded statural and pelt

growth, probably as a result of initial blood loss combined with reduced locomotor efficiency. Of forty-five pups operated upon, twelve developed multifocal abscesses in the operated limb that persisted throughout the postsurgical study interval. Only one of these, however, has been included in this report because of the novel response it displayed (Fig. 23-14A).

Figure 23-11 illustrates the appearance of a specimen obtained from a rat thirty days after amputation, which had been carried out on the first day of life. Soft tissues were removed by mealworms over two days. Following photography, the specimen was fixed and prepared for serial sectioning. The figure illustrates the presence of two new long bones adjacent to the transected region of the humerus. Figure 23-12 illustrating a serial section through a region of this specimen presents the appearance of

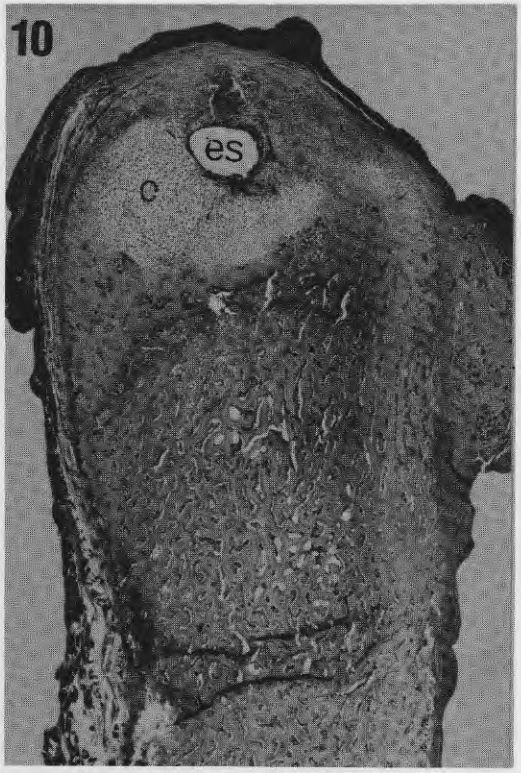

Figure 23-10. Experimental (effective device) specimen after seven days of electrode stimulation. In this limb the electrode penetrated soft tissues perpendicularly to the humeral long axis and did not enter the medullary cavity. Thus the electrode space (es) is seen in cross section. Hyaline cartilage (c) forms an incomplete boundary partially delineating the electrode space. Much of the skeletal tissue substance of the humeral shaft is composed of new, actively forming bone displaying an intensely spicular architecture and lacking a well-formed cortical component. H&E, ×23. (From P. Pearson, et al., *J. Morph.*, *159*:427-452, 1979. Courtesy of The Wistar Institute, Philadelphia.)

portions of three growth plates associated with these bones as well as of a single, anatomically correct epiphysis. A region of putative articular cartilage is indicated, hut it is to be noted that in this specimen fixation was delayed for forty-eight hours, thus rendering this identification somewhat precarious. The polarization of the cellular components of the marrow to the periphery of the medullary cavity is also considered to be an artifact of delayed fixation.

Two similar specimens obtained from the newborn surgery group were recovered approximately nine months after amputation and each exhibited a newly formed long bone lying adjacent to the transected humerus. Figure 23-13 illustrates the x-ray appearance of one of these specimens and reveals the presence of an epiphysis, and also a degree of growth in the transected humerus that compared favorably with that observed in the contralateral (control) humerus (27.5 mm versus 31.0 mm). The appearance of a representative serial section prepared from this specimen is

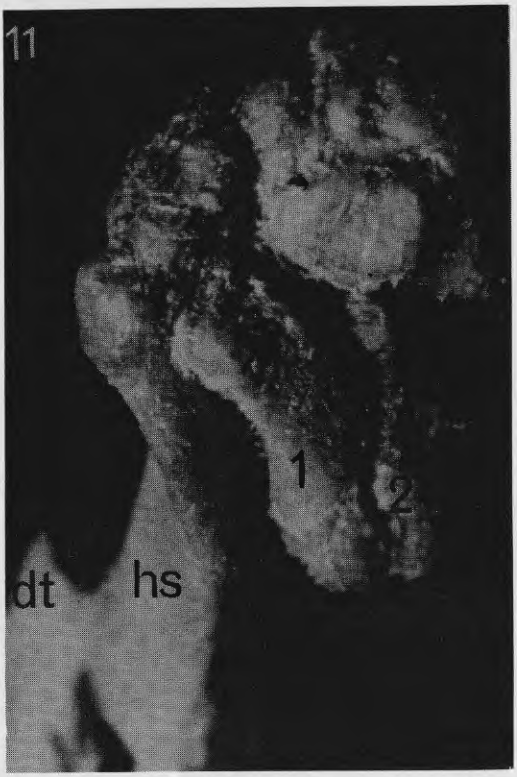

Figure 23-11. Specimen from a rat undergoing amputation on day 1 of life and killed twenty-nine days later. Soft tissues were removed by mealworms over several days. The deltoid tuberosity (dt) lies to the left. Immediately to its right is the humeral shaft (hs). Two accessory bones (1, 2) are indicated. Approximately ×8.

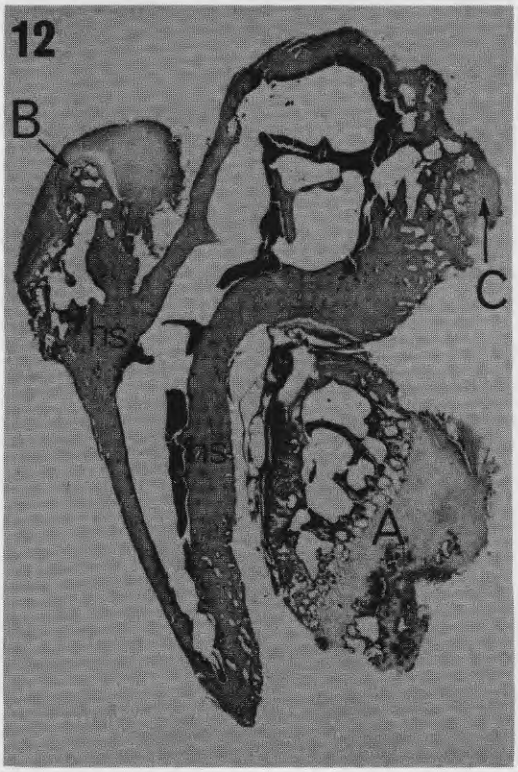

Figure 23-12. Microscopic appearance of a serial section through the gross specimen illustrated in Figure 23-11. This specimen possesses three growth plates indicated by the letters A, B, and C. Growth plate C, while only fragmentary in this serial section, is more extensively represented in adjacent regions of the specimen. Growth plate A, adjacent to a regenerated epiphyseal structure, is a portion of bone 1 illustrated in Figure 23-11. The humeral shaft (hs) lies next to it. Delayed fixation accounts for the polarizatiou of the marrow to the periphery of the medullary cavity. H&E, ×15.

illustrated in Figures 23-14A and B. It should be noted that the accessory long bone terminates in an unspecialized manner at approximately the level of transection of the humerus and does not articulate with it.

The appearance of a second specimen possessing a newly formed long bone is illustrated in Figure 23-15. As in the previous specimen, the newly formed bone possesses a proximal epiphysis and growth plate. Here, however, the distal terminus of the bone is partially overlain with articular cartilage and is closely applied to the transected portion of the definitive humerus, the distal, transected surface of which is also provided with articular cartilage. Moreover, connective tissue suggestive of ligamentous attachments unites the two bones although no joint capsule structures are readily identified.

Juvenile Rats of Increasing Age: As the age of the rat at the time of

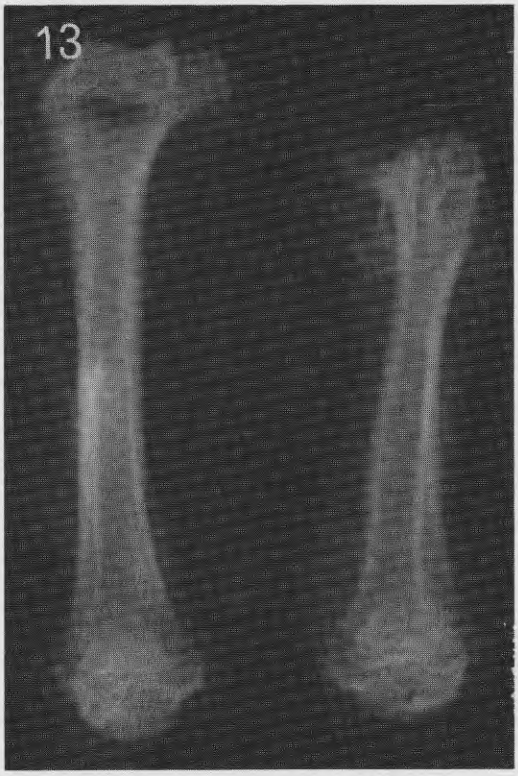

Figure 23-13. X-ray of control (left) and operated (right) humeri obtained from a rat undergoing amputation on day 1 of life and killed nine months later. Note the outline of an accessory bone lying to the left of distal portion of the operated humerus and in close apposition to it. Approximately ×3.

amputation was increased to two weeks, then to three to four weeks, and finally to four weeks, regenerative responses were observed with decreasing frequency. Preliminary observations made to date and continuing at this time reveal that juvenile animals in the two to four week category at amputation displayed more vigorous longitudinal growth and more active periosteal osteogenesis than did older animals. Additionally, the new bone thus formed at the limb terminus appeared more fenestrated. Of interest is the observation that in these young animals, as distinct from rats undergoing amputation at four to five weeks (100 gm), muscle covering does not apparently evoke regenerative responses of magnitude. Although myogenesis was not actively stimulated in these groups and distally placed, newly formed growth plates were not observed, it is essential to view this segment of the study with certain reservations, for observations upon all tissue components have not been completed.

Effects of Parathyroid Extract Administration

Animals receiving PTE followed by forelimb amputation at either fifteen or twenty-five days after birth developed none of the gross skeletal abnormalities described by Umanskii and Kudokotsev (16). Moreover amputated humeri continued to grow in length at a rate that closely kept pace with the rate of growth of contralateral (unoperated) humeri.

Limb specimens examined while relatively transparent following *in situ* methyl green staining of cartilage followed by clearing in methyl salicylate were divided into two groups. Those specimens that displayed no terminal cartilaginous structures and whose humeral extremities tapered into a point were studied no further. Those remaining specimens in which cartilage could be visualized at the limb terminus (nine of thirty-six specimens) were sectioned serially in the longitudinal plane and examined microscopically. It is of interest to note that those nine specimens were obtained from only two litters and, moreover, that four of the nine pups were from a single litter of nine young. Of eight males born to this litter and receiving forelimb amputations on the twenty-fourth day of life, four pups failed to respond to amputation in a manner indicative of heightened regenerative activity.

Microscopically, the pattern of response most frequently observed was one which we have noted upon occasion in the past but which, here, constituted the commonly observed response of the group. It was characterized by a slightly expanded humeral terminus formed of cartilage cells and highly metachromatic fibrous connective tissue. Between the two a spongiosum generally intervened (see Fig. 23-16). The cartilage component of the limb terminus was notable in some instances for its diffuse appearance, cellular heterogeneity, and the intense metachromasia imparted to the connective tissue surrounding it (Fig. 23-16). In several specimens more compact terminal cartilages bore some of the characteristics of poorly organized growth plates, such as palisading of chondrocytes at oblique angles to the long axis of the bone, the arrangement of collagen bundles between these columns of cells, and the invasion of the bone-cartilage interface by blood vessels. However, proliferating and calcifying chondrocytes and the forms intermediate to them were not present, and the architecture of the cartilages was only suggestive of that of growth plates.

Among several of these specimens, enhanced myogenesis was observed. By this it is meant that the extent of myogenesis noted was greater than that which might have been anticipated in animals of the same age not receiving PTE. In this experiment those animals displaying heightened myogenesis had received both muscle coverings and PTE and had undergone forelimb

Figure 23-14A. Section through the distal portion of the operated humerus illustrated in Figure 23-13. Intramedullary inflammatory foci are indicated by arrows. Observe that the accessory bone is provided with a medullary cavity, a growth plate, and an epiphysis. Moreover, tendon and muscle insert into its epiphysis and metaphysis. H&E, ×10.

amputation at twenty-five days of life. Moreover, they had all been obtained from the same litter.

Of thirty-six rats comprising this study, twenty-seven animals derived from all four experimental and control groups (see Table 23-I) displayed no responses indicative of regenerative activity. Of the remaining nine animals, five received PTE and the muscle covering, one received the muscle covering and administration of vehicle, one received vehicle in the absence of muscle covering (simple surgical procedure) and two rats (one from each of the two latter groups) displayed borderline responses by microscopic criteria, although identified as of interest when examined in the cleared state. It is thus apparent that PTE administration to animals undergoing forelimb amputation at twenty-five days of life followed by muscle covering had significantly contributed to the formation of the tissue responses described above. The observation that a single rat receiving vehicle plus the simple surgical procedure displayed a qualitatively as

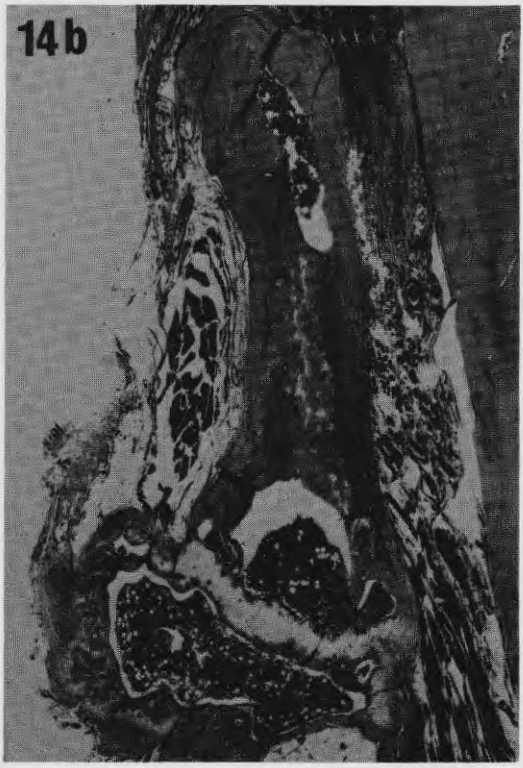

Figure 23-14B. Higher magnification of the specimen illustrated in A. Note that the humeral shaft of this accessory bone is formed of compact and cancellous bone and that portions of the epiphysis are covered with articular cartilage (lightly staining areas). The growth plate contains columns of cells displaying zones of division, maturation, and calcification and surrounded by abundant matrix. H&E, ×21.

well as a quantitatively similar response to those rats receiving PTE prior to formation of muscle coverings corresponds with our previously reported observations (12) in which a single 100 gm male rat from among sixteen displayed tissue regeneration responses similar to those observed in rats in which muscle coverings had been formed following a forelimb amputation and in which regenerative responses were observed.

The results of this study make possible a preliminary approach to the question of the significance of extensions of cartilage from the distal humeral growth plate proximally into the amputation plane and the potential of these cartilaginous extensions for augmentation of significant limb regeneration as noted by Nunnemacher (11). In this context it is of some interest to note that, of the three amputations, the planes of which transected such cartilage extensions, only one resulted in a significant response of the type described. This response was obtained in an animal

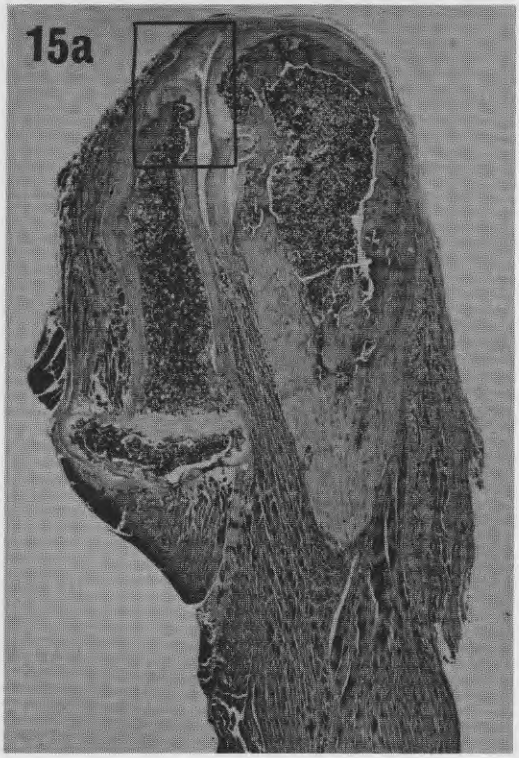

Figure 23-15A. Section through the distal portion of the humerus of a rat undergoing amputation on day 1 of life and killed three months later. As in the previous specimen, the accessory bone is closely applied to the surface of the humerus, which was sectioned tangentially. Note that the accessory bone is capped distally with a circular knob of cartilage. H&E, ×10.

that had undergone forelimb amputation fifteen days after birth and was characterized by the formation of two or three bodies of cartilage underlain by two or three discrete spongiosa.

The purpose of PTE administration, as set forth by Umanskii and Kudokotsev (16) who reported dramatically enhanced limb regeneration in young rats in response to it, was to evoke skeletal tissue dedifferentiation prior to amputation and thus to establish an environment favorable for heightened regenerative activity. On the basis of this anticipated result, we have begun an examination of serially sectioned histological preparations seeking evidence of dedifferentiation in the skeletal and connective tissues of those limb termini selected for detailed examination. While this assessment continues, it may be stated that no patterns of tissue reorganization suggestive of dedifferentiation and blastema formation were noted. In

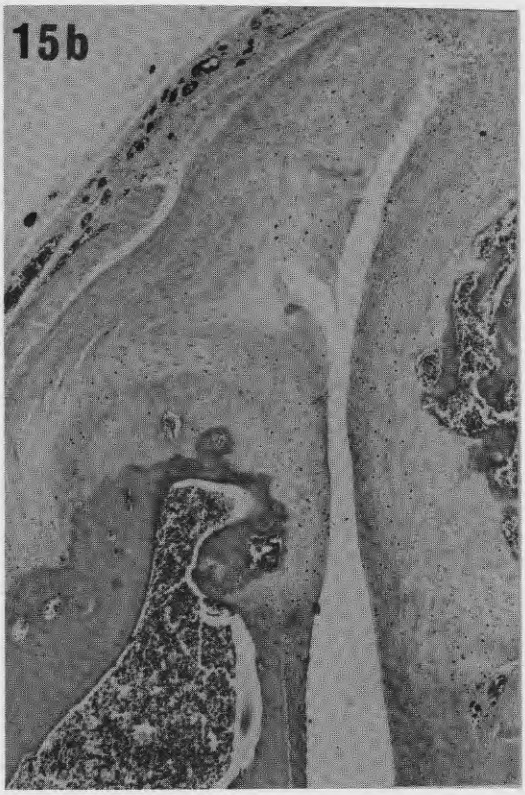

Figure 23-15B. Detail of the area enclosed within the rectangle in A and illustrating the distal extremities of the accessory bone and the humerus. Observe that the surfaces of both bones are covered with articular cartilage at their points of closest contact. Congested venules are seen in the upper left. H&E, ×62.

some specimens, however, diminished numbers of chondrocytes were embedded in a highly fibrous matrix, which, in some areas, was caseous and appeared to be disintegrating. Often such areas of chrondroid, as it might be termed, were arrayed as a series of bodies lying between the transected humerus and more distal and intensely metachromatic fibrous tissue, which represented the remnant of the deeper layers of the muscle covering and the superficial fascia of the limb (Fig. 23-16). In these limbs, as well as in others displaying few skeletal tissue responses to amputation and/or hormone administration, there was considerable muscle degeneration with fatty infiltration and replacement by fibrous connective tissue. At present we are unable to assign to any of these observations a place in an integrated dedifferentiation response, but we continue to review the histological preparations with this possibility in mind.

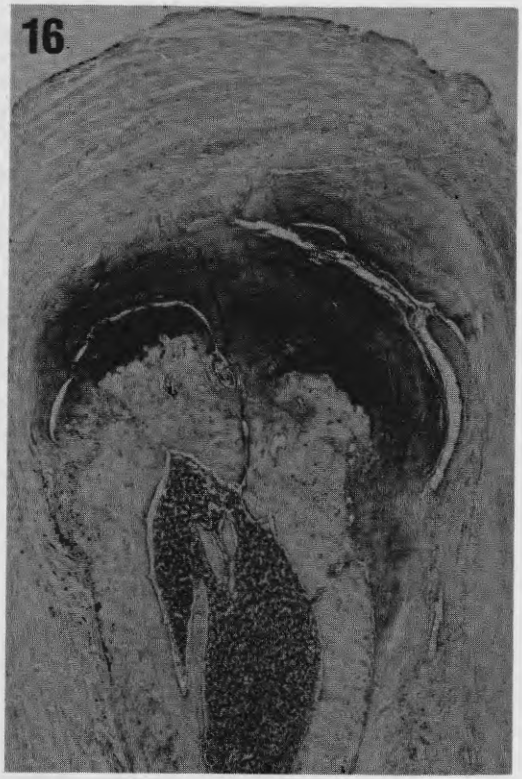

Figure 23-16. Experimental (muscle covering) limb obtained from a rat receiving PTE for ten days prior to amputation performed on the twenty-fifth day of life. Observe that the humeral terminus is bifid and that each ramus is capped by an intensely metachromatic cartilage body. Note also that the connective tissue, which has replaced the muscle covering, is also strongly metachromatic. Toludine blue O, ×19.

DISCUSSION

Effects of Muscle Covering and Relation to PTE Administration

We have previously described the use of the muscle covering procedure for the augmentation of inherent regenerative capacity in the 100 gm male albino rat (12). This response is characterized by heightened subperiosteal bone growth at the amputation plane, and by the extension of the length of the limb via the formation of a cartilage body, sometimes of considerable size, composed of evenly dispersed hyaline cartilage cells applied to tbe humeral surface by an intervening spongiosum, and restricted on all other sides by the muscle covering itself. Although we are uncertain as to the nature of the muscle covering stimulus, we presently incline to the view that both mechanical and chemical factors may exert interrelated actions, so that a cooperative effect is observed. With regard to mechanical effects, we have noted that chondrogenesis occurring beneath the muscle cover-

ing, and even growth plate formation, may take place at a time when many of the skeletal muscle fasciculi forming the muscle covering are degenerating or have already undergone substantial replacement by fibrous connective tissue. Thus, it may be suspected that the specific muscle-bone-periosteum relationship that is established by formation of the muscle covering may he required only early in the incomplete regeneration process we have observed and is not essential for sustained growth of the regenerate. However, cartilage cells organized in columns and thus resembling a growth plate may arise wholly from muscle and the connective tissue components normally associated with it. In this context Carlson (2) has described the formation of a growth platelike cartilage body arising within a field of minced muscle relocated *in situ*. Moreover, amputations performed upon young rats often give rise to regenerated distal growth plates (15). That these regenerations occur in the absence of muscle coverings indicates that while muscle may evoke formation of well-organized cartilage bodies in the vicinity of the amputation plane, these bodies may arise from other pools of progenitor cells as well. Nunnemacher (11) has asserted that these cartilage bodies and, particularly, the regenerated growth plates he observed were derived directly from the substance of the distal growth plate, portions of which project proximally in the form of tails or pegs of cartilage ascending into the amputation plane. We have observed several of these cartilaginous growth plate prolongations (three among thirty-six rats in a single study). Heightened regeneration was not observed in animals bearing them.

In further assessing the role of the muscle covering in the elicitation of augmented regenerative responses, it is of some interest to note that while only a single animal from among nine bearing a muscle covering and receiving not PTE but vehicle displayed significant elaboration of cartilage and bone after amputation, five of eleven animals receiving both PTE and muscle covering displayed equivalent responses. Thus, it appears that daily PTE administration prior to forelimb amputation may stimulate cartilage and bone precursors to heightened proliferative activity, resulting in "sensitization" of the amputation field to mechanical or other effects of the muscle covering. The terminal skeletal tissue structures formed in these forelimbs through the interaction of PTE and muscle covering appear somewhat aberrant when compared with counterparts evoked by muscle covering alone. While the PTE-induced terminal cartilages are also formed of chondrocytes together with abundant matrix and fibrous components, the distribution and general arrangement of these constituents results in cartilage bodies irregular in outline and often applied directly to the subperiosteal bone sealing the humeral defect and surrounded by laminae of fibrous connective tissue, the more proximal layers of which are intensely metachromatic. Some areas of the cartilage bodies may be com-

posed principally of matrix with but few chondrocytes, while adjacent regions of the cartilage may be hypercellular. In yet other regions fibrous components may predominate with the exclusion of most matrix. On the basis of preliminary examination of this material, it is not possible to state whether these alterations reflect processes of tissue dedifferentiation, of aberrant skeletal and connective tissue regeneration, or of the intermixture of wound healing and tissue regeneration in an uncommonly observed pattern.

Age at Amputation as a Determinant of Regenerative Capacity

The experiments performed to date attempt to relate regenerative capacity to the age of the animal at the time of amputation. We have not yet examined the regenerative capacities of the late term fetuses and of presenile adults. On the basis of results obtained with one-day-old rats, we speculate that forelimb-amputated fetuses retained in utero until birth might possess greatly heightened regeneration potential.

The study of Mizell of limb regeneration in opossum fetuses (10) is of interest here, not only because it constitutes an example of essentially complete mammalian limb regeneration induced via a neurotrophic mechanism but also because it enabled him to make fundamental observations upon cellular phenomena occurring within the regenerate and led to the description of a series of cell and tissue responses that he has considered to be indicative of cellular dedifferentiation. In this context, we are currently examining the possible operation of epimorphic phenomena in our most advanced regenerates, especially those where skeletal reduplication has occurred, giving rise to entire new long bones. Such studies may also be relevant to possible mechanisms underlying digital regeneration in children, now widely reported (4, 5, 6, 14) and of great interest.

The importance of regeneration capacity of late term rat fetuses would relate to the nature of the animal model itself. One may speculate on the insights that might be obtained from a system in which regeneration phenomena may occur readily and completely in fetal life, but with diminished vigor and possibly somewhat different results during early life, and not at all during juvenile life and into adulthood.

Studies upon one-day-old rats disclose a generally heightened capacity for regeneration and, in those animals in which transections were made through the distal growth plate, a tendency for formation or duplication of long bones. These newly formed long bones generally lie parallel to the transected humoral shaft but, in a single instance where several bones were formed, were observed to assume other orientations. We refer to these structures as accessory bones and are uncertain whether they represent duplicated humeri, bones of the distal forelimb osseous chain (radius,

ulna, etc.), or simply newly formed bones arising in response to injury and lacking assignment to the skeleton of the limb. Becker (1) has described similar accessory bone formation in rats twenty-one days of age at amputation.

It is noted that the several accessory bones we have identified were somewhat unusual in appearance, displaying in one instance an expanded proximal extremity, and in all specimens lacking articulation with the definitive humerus and distal growth plates and epiphyses. As a result of these anatomical deficiencies, comparisons of the specimens with rat radii and ulnae of varying developmental ages have not led to their identification. Supporting the proposition that these bones may represent definitive distal skeletal elements is the observation that in a single instance the distal end of the amputated humerus and the distal end of the accessory bone were faced with articular cartilage at their point of contact and were united by a continuous field of connective tissue (Fig. 23-15b). While it is apparent that a synovial joint had not formed, we are unable to exclude the possibility that the distal portion of the accessory bone was not in actuality the proximal portion of a regenerated radius or ulna.

As already noted, Nunnemacher (11) and Selye (15) have described the regeneration of the distal growth plates following amputation of limbs of neonatal rats. Nunnemacher has observed that its reformation occurred most frequently among younger age groups (younger than sixteen days) with decline in regenerative capacity between fifteen and twenty-five days of life and complete loss of this capacity if amputations were performed after twenty-five days. Significantly when forelimb amputations were carried out between fourteen and sixteen days of life, regeneration of the distal humeral growth plate was not observed even though its regeneration could be evoked by amputations performed earlier in life. Our experience with forelimb-amputated rats of the same age (approximately two weeks old) fully supports this observation and may be interpreted to indicate that innate regenerative capacity diminishes sharply after birth. Nunnemacher attributes this negative response to forelimb amputation to the early union of the distal humeral growth plate apparatus which, in the rat, closes at thirty-one to forty-two days (3) whereas the inactivation of the distal femoral growth plate occurs considerably later. Such an analysis emphasizes the importance of the developmental history of the bone in the design of experiments that examine its regenerative capacity.

Level of Amputation as a Determinant of Regenerative Potential

Nunnemacher (11) has also directed attention to the level of amputation as a critical factor for prediction of regenerative response. He believed that even small proximally directed prolongations of growth plate cartilage

present in the amputation plane could give rise to the regenerated growth plates he observed. As indicated above, we have noted that in several instances such cartilaginous prolongations did not confer upon the limb remainders of young animals the capacity for either regeneration of the distal growth plate or of the distal humeral extremity that had been extirpated. However, when the level of transection passed through the full thickness of the distal growth plate (as determined by examination of the portion of the limb that had been amputated after its cartilage had been stained *en bloc* and rendered transparent), accessory bones were sometimes formed. Transections performed in the adjacent metaphysis rarely evoked significant regenerative responses, and in eighteen specimens never led to the formation of accessory skeletal elements. It appears, therefore, that a substantial and perhaps critical volume of growth plate cartilage must be retained in the amputation stump for the organization of these accessory bones, but it should be emphasized that no evidence currently available convinces one that these accessory bones do indeed arise from cartilage components of the growth plate divided in this matter.

Dissociation of Electrical and Mechanical Components of Electrical Stimulation

In evaluating responses to electrical stimulation, it is necessary to distinguish between those tissue responses that may be attributed to the delivery of current and those effects that occur in response to the mechanical effects of device implantation. This problem has been addressed through the use of uncoupled bimetallic couples that produce no electrical stimulation of amputation plane tissues but, nevertheless, evoke bone and cartilage formation, albeit with greatly reduced effectiveness. This control device approach indicates that some of the effects of electrode implantation may be directly attributed to mechanical as opposed to electrical responses.

Although uncoupled himetallic couples evoked minimal enhancement of bone and cartilage formation in the tissues surrounding the platinum electrode tip, no stimulation of myogenesis was observed. This suggests that significantly enhanced myogenesis observed in response to implantation of effective devices was evoked by the electrical component, whereas osteochondrogenic responses were of mixed origin.

The question of electrical versus mechanical responses to device implantation has thus been substantially answered. A more complete proof of this answer may be sought through application of wholly noninvasive electrical stimulation procedures now in use in the clinical setting, i.e. through use of external coils operating by inductive coupling. The efficacy of such a regimen of stimulation indicates, as do our comparisons between effective and ineffective bimetallic couples, that the prepotent effects of

device implantation upon the osteogenic tissues are primarily electrical in nature, and that the mechanical stimulus component is only a minor one. To test this position further it will be essential to examine the effects of external coil stimulation in a system which duplicates that employed by Becker (1) and by ourselves (8).

The Target of the Electrical Stimulus

At this stage of our work little of definitive nature can be said concerning the cells of origin of the regenerative responses reported. However, some discussion is warranted to focus upon significant possibilities. As already noted, one of the targets of the electrical stimulus must be skeletal muscle progenitor cell(s). A second target resides within the pool of cells giving rise to cartilage and bone and presumed to include cell types of the periosteum and perhaps endosteum. This latter conclusion is arrived at because part of the observed osteogenesis in response to bimetallic couple stimulation appears to arise from proliferative activity of periosteum, which also appears to give origin to cartilage tissue identified at the transection surface shortly after initiation of electrical stimulation. In this connection, the osteogenic layer of the periosteum, while principally composed of osteoblasts, may possibly harbor a small population of embryonic cells of great potentiality, presumably established during fetal life.

In our use, platinum electrode tips were directed into the humeral medullary cavity for a distance of 1-2 mm. In virtually all instances, the marrow in contact with the electrode tip was replaced with osteogenic tissue, poorly organized bone, and chondroblastic and mature cartilage. Since direct marrow cell responses to the presence of the electrode tip were not observed, possible origins of the above tissues might involve endosteum or other as yet unidentified precursor cells.

Identification of Specimens Exhibiting High Regenerative Activity

Formerly we have found it necessary to prepare thin serial paraffin sections of all limb specimens, a time-consuming process that has severely limited the number of experiments which could be carried to completion and which has greatly increased the cost of such research. Recently, this situation has been ameliorated by application of preliminary screening methods for the visualization of internal structural detail so that only those limbs bearing distinct regenerative responses might be selected in advance of histological preparation. These methods involve standard radiological examination of all limb specimens followed by fixation and exposure of the specimens to dyes and solvents, which first stain cartilage tissue *en bloc* and then render the entire limb transparent. We have observed that overnight exposure of limb specimens to methyl salicylate, the clearing agent used, renders the removal of folds from paraffin sections floating on water more

difficult and often impossible. However vexing this artifact may be, it rarely interferes with the interpretation of tissue structure. By these means it has been possible to exclude most specimens in which regenerative responses were not present with a very considerable saving in sectioning and staining time as well as in materials.

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