Post Amputation Partial Blastema Formation In Adult Mice.

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Francis X. McCormack

This thesis for honors recognition has been approved for the Department of Biology

Dr. James Manion Advisor

Mr. Guido Bugni

Dr. Joseph Ward

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Abstract

The formation of a partial blastema in adult mice was achieved through treatment of the amputation surface of toes with saturated solution of sodium chloride and a one mM equimolar solution of db-cAMP and theophylline. This study represents an improvement on the study first successful in stimulating partial blastema formation in mice (Neufeld, 1980). The innovation employed in this study involved the use of db-cAMP and theophylline as an inhibitor of epidermal cell migration (epidermal migration onto the amputation plane inhibits regeneration). The advantages of chemical inhibition over surgical removal of epidermal tissue is discussed.

List Of Illustrations

Introduction

Although ingenious devices have been constructed in an effort to replace lost body parts, the ideal prosthesis would no doubt be the regenerated body part itself. Unfortunately for man, his ability to regenerate is severely limited as is the case with all mammals. Members of the class Mammalia regenerate severed foretoes fully when the amputation plane is no more distal than the base of the most distal phalange (Borgens, 1982). Developmental biologists hope that an understanding of biochemical signals and controls associated with limb development will lead to full regeneration of mammalian limbs. Recent studies indicate that the aforementioned signals and controls are manifested in cell surface reactions (Bryant et al., 1980). Research has only recently progressed to this stage, primarily through experimentation upon organisms with great regenerative capability. The most studied vertebrate taxa being the order Urodela.

This thesis was undertaken in an attempt to contribute to a current trend in regeneration research. This trend applies the findings gathered from studies on urodeles to the class Mammalia through experimentation. The scope of this paper encompasses the study of how the blastema is formed and how it functions as precursor to the regenerate.

Literature Review

The blastema is difined as the mass of cells occupying the region distal to the amputation plane.

The forelimb blastema -- forthwith to be referred to as "blastema", consists of labile, pluripotential tissue occupying the space previously at which dissociated soft tissues of the stump were located (Chalkley, 1956). The blastema persists for approximately 25 days in amphibians. After 25 days the blastema begins to redifferentiate, subsequently the number of blastema cells decrease.

The histological origins of the blastema has been the center of much controversy. The debate can be thus delineated: 1) Is the blastema formed from cells from all parts of the body of local tissue? 2) Is the blastema transformed into the regenerate through de novo differentiation of reserve cells or dedifferentiation and redifferentiation of functional tissue cells? 3) Is the blastema a mix of precommitted cells differentiating to particular cell types or a mass of pluripotent cells capable of all cell types? (Slack 1980). These questions have been treated through various theories.

One such theory defines the process of blastema formation as the release of living cells from the confines of their previous organization. This release is accompanied by an increase in the mitotic activity of these cells (Fischman and Hay, 1961). Developmental biologists long regarded "confines of previous organization" to mean apical limb epidermis. This concept arose from the observation that epidermis rapidly migrated to close the amputation wound. It is now generally agreed upon that the blastema results from the transformation of inner tissues such as muscle and cartilage into mesenchyma-like cells (Fischman and Hay, 1961). This transformation is termed metaplasia -- the dedifferentiation and subsequent redifferentiation of functional cells into different histological cell types. Inner stump tissue derived from embryonic mesoderm exhibits extensive metaplasia. Epidermis never contributes to inner stump tissues. It therefore appears that lineage restrictions exist for tissues of different embryonic origin (Slack, 1980).

The Hematological Origin Theory purported the idea that the blastema arose from blood cells. This theory has since been refuted (Fischman and Hay, 1960). This was done by labeling blood cells with a radioactive isotope while in the liver and spleen. Although labeled blood did appear in wound tissue of the limb, it had all disappeared by the twelfth or fifteenth day. Blastema cells from such wound tissue did not exhibit the label (Fischman and Hay, 1961). Had the label remained then it could have been deduced that blood cells contributed structurally to the formation of the blastema.

Confirmation of the theory that the blastema arises from inner stump tissues was achieved by again employing the use of the radioactive labeling technique. The experiment indicated the high level of mitotic activity of muscle, epimysium, endomysium, periosteum, nerve sheaths, and other connective tissues of the limb stump. This mitotic activity is evidence that these tissues are engaged in the generation of blastema tissue. The mitotic activity of these tissues easily accounts for the increased cell mass and thus is most probably the sole source of cells for the blastema (Fischman and Hay, 1961).

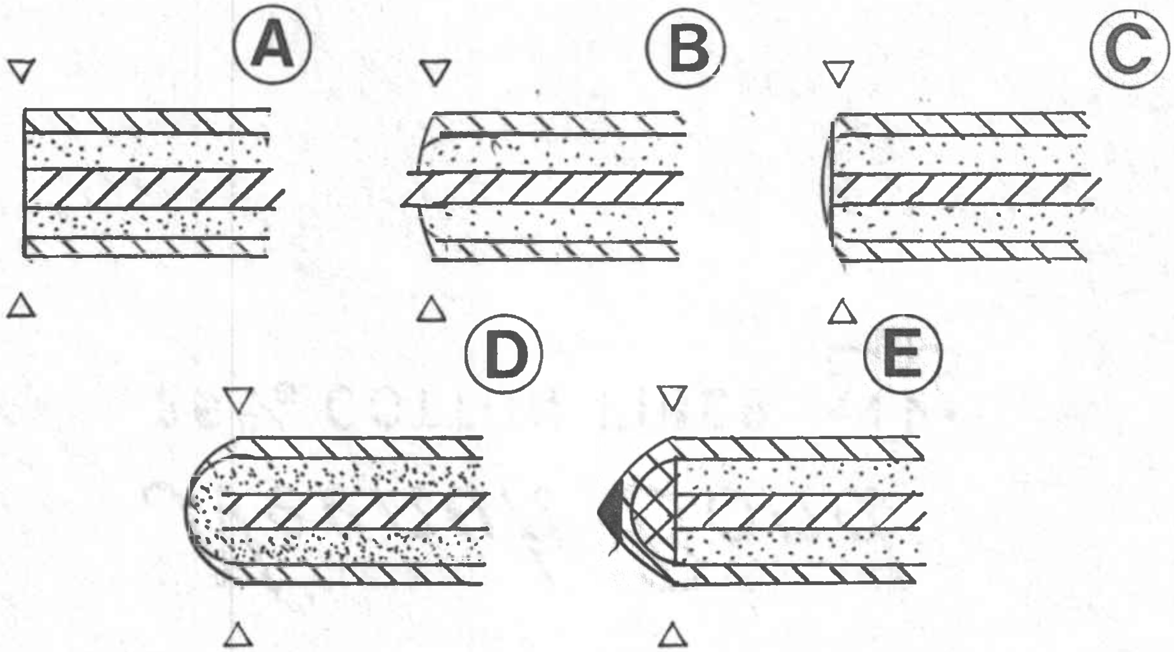
Correlation studies produced results that support the above study of mitotic activity. Proliferation rates of stump tissues and appearance rates of new cells in the regenerate were compared. These rates were found to correspond closely (Chalkley, 1956).

The Reserve Cell Theory challenged the concept that the blastema arose from tissues of the inner stump. This theory was based on the inference that there were present in all tissue, reserve cells capable of differentiating into muscle, cartilage, and possibly other connective tissue. This theory lost support when it was discovered that cells cultured and in vitro displayed physiological conservatism (Chalkley, 1956). Further evidence against the theory resulted from experiments in which urodele limbs were severed through a previously x-irradiated cross section of the limb. As a result the amputation surface contained no cells capable of division, and subsequently the stump was incapable of contributing to the formation of the blastema. If the Reserve Cell Theory were correct, a blastema would form as a result of the migration of reserve cells to the wound surface from non-x-irradiated parts of limb and body. No blastema was formed, however. This showed that local tissue is necessary for blastema formation (Slack, 1980).

Current theory holds, therefore, that the blastema originates as a result of transformation of inner limb tissues, into mesenchyma-like cells.

> Morphological and cytological development of the blastema is another area of active research.

There exists a specific sequence of morphological events following the amputation of the amphibian limb (fig. 1). The first of these events is the contraction of the soft tissues of the stump. This is followed by the formation of the blood clot. Fifteen to forty-five



Postamputation Morphological Events

Figure 1. Severing the limb (A) is followed by contraction of soft tissues (B). A blood clot is then formed (C). Epithelial and mesenchymal migration then commences (D). Migration is completed with the formation of the apical cap (black) and subjacent blastema (cross hatched) in (E). Epidermis is indicated by hatch marks (descending from left to right), bone by hatch marks (ascending from left to right) and mesodermal stump tissues by stippling. The thickness of the epidermal region is greatly exaggerated. The amputation plane is indicated by arrows.

minutes later the adjacent epithelium begins to migrate over the wound. This migration is completed in 24 hours. It is important to note that this migration is achieved without mitotic proliferation of the migrating cells. If such mitotic activity were to take place this would indicate that the epidermis is a source of originating tissue, but current theory contradicts this.

The migration of the first 24 hours is culminated in the formation of a thickened tissue layer termed the apical cap. The cap is observed to increase in size from the 10th to the 15th day postamputation. After 20 days the blastema elongates and begins to thin out. It is also at this point that the blastema begins to redifferentiate (Fischman and Hay, 1960). Resumption of normal cell division rate in the apical cap also accompanies blastema elongation. The phenomena of suspended mitotic activity is unique to limb regeneration (Fischman and Hay, 1960).

While epidermal tissue is migrating over the wound, mesenchymal tissue starts to migrate beneath the wound surface. This migration commences with the emergence of dissociated cells from the stump tissues. These mesenchymal cells accumulate between the wound epithelium and the subjacent tissues of the inner stump. This migration spans a time interval of 72 hours. (Chalkley, 1956).

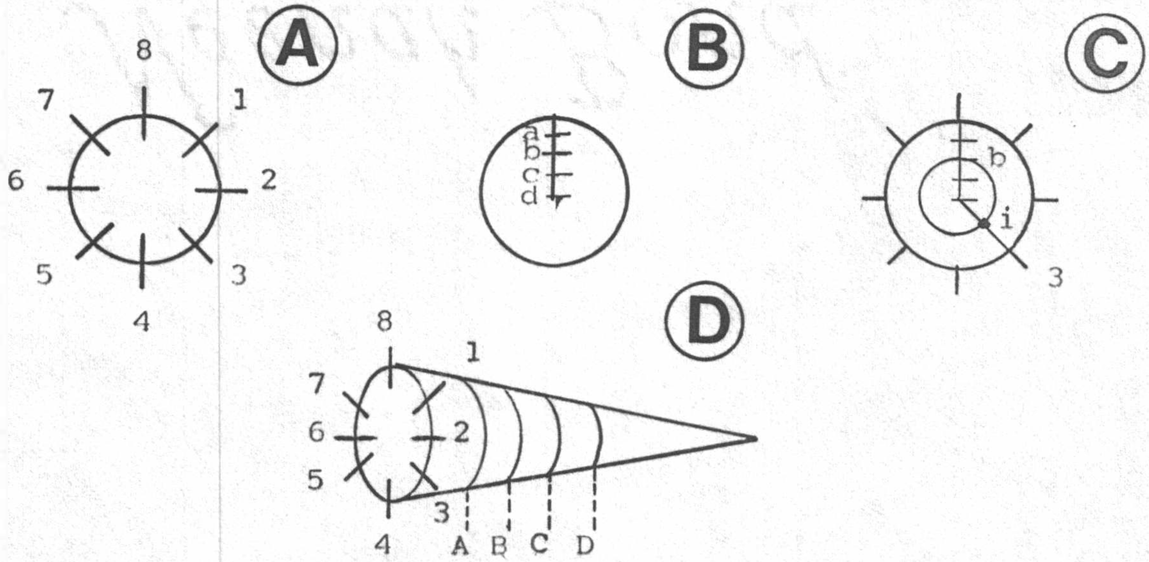
Also to be considered are the cytological events that accompany the morphological phenomena discussed above. One such event is the transformation of formed cell types into blastema cells. This occurs in three stages. The first stage is the loss of specialized intracellular and extracellular products formed during cellular differentiation. The second stage consists of the enlargement of cell nuclei and nucleoli indicative of increased nucleic acid synthesis (Fischman and Hay, 1960).

Accompanying the cytological and morphological changes are variations in DNA synthesis. These variations follow a specific timetable. In tissue proximal to the amputation plane DNA synthesis ceases two days after commencement of migration in the wound epidermis. DNA synthesis is initiated at a point 1 mm proximal to the amputation surface four to five days postamputation in the following tissues: muscle, endomysium, epimysium, periosteum nerve sheaths, and loose connective tissue. On the eighth day the amount of DNA being formed by migrating tissue and the proximal epitheleum peaks. Ten to twenty days postamputation there is increased DNA synthesis in the inner tissues of the stump (Faber, 1965). Synthesis of DNA in the blastema itself ceases on the 15th day to be resumed on day 20. This resumption coincides with elongation of the blastema (fischman and Hay, 1960).

Induction and differentiation are the mechanisms by which the blastema is transformed into the fully regernerated limb.

The events discussed in the previous paragraphs bring the blastema to the point at which differentiation occurs. Biologists have attempted to discern the patterning mechanisms which coordinate induction and differentiation. The stump acts as an inductive template for the differentiating blastema. Evidence for this concept stems from experiments in which labeled tissue was grafted onto x-irradiated stumps incapable, therefore, of regenerating independently. Blastemas formed as a result of this procedure consisted only of labeled tissue from the graft. The stump made no cellular contributions. The stump thus exerted an inductive influence on the grafted tissue without cellular proliferation (Slack, 1980).

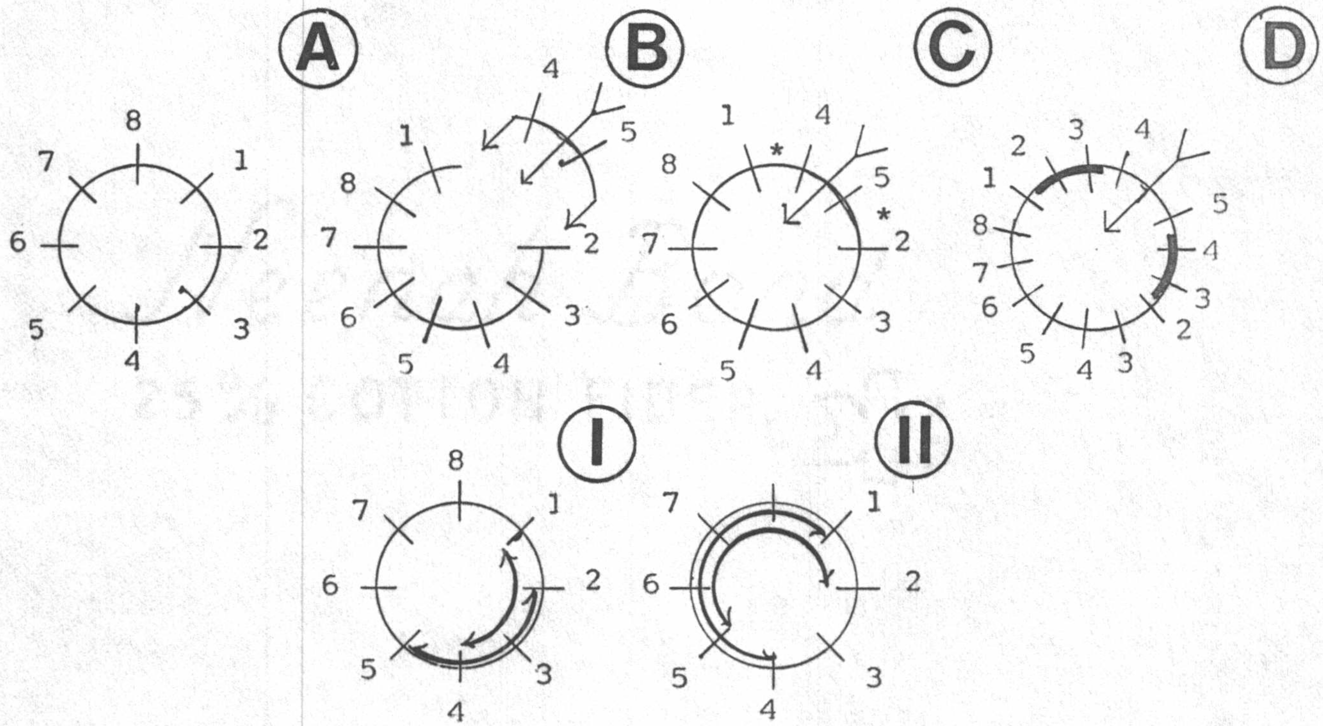
The Polar Coordinant Model offers a theoretical patterning mechanism believed to be at work in the stump. This model is based on the assumption that spatial patterns result from cells gaining information about their positions within the limb. These positions lie along polar coordinants in a two dimensional cell layer forming a cross sectional plane. One of these coordinants is termed circumferential and denotes the position of a cell around the perimeter of a cross section (fig. 2). The circumferential coordinants can be likened to the relative positions indicated by the numbers signifying the hours on the face of a clock. The other coordinant is termed radial and denotes the position of the cell relative to the center of the cross section. The radial coordinants are a measure of distance much like the annual rings are a measure of age on a tree stump.



The Polar Coordinant Model

Figure 2. Circumferential coordinants (1-8) designate relative positions about the perimeter of a cross sectional surface (A). Radial coordinants (a-e) denote distances relative to the central axis of the appendage (B). The polar coordinant model is employed in indicating the positional value of the point i which is 3 circumferentially and b radially (C). A three dimensional map of a tapering appendage with polar coordinants indicated is shown in (D).

The first rule of the Polar Coordinant Model is the Property of Intercalation. This rule states that when normally nonadjacent positions come in contact as a result of wound healing or grafting, cell division is stimulated which results in the generation of tissue normally separating the nonadjacent positions. The property further states that if the nonadjacent positions are circumferentially juxtaposed, then positions representing the shortest circumferential route between



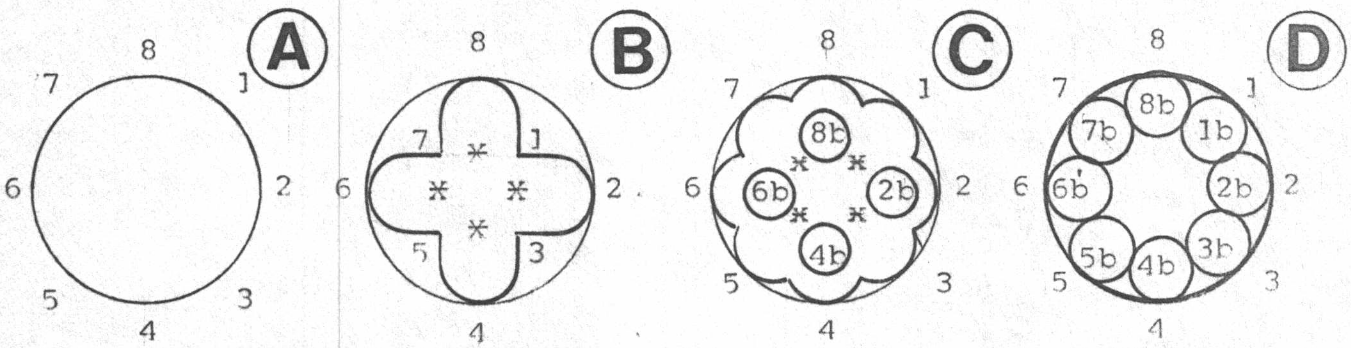
Properties of Intercalation and the Shortest Circumferential Rule

Figure 3. A normal cross section (A) of circumferential values 1-8 receives a graft in (B). The graft (arrow) results in confrontations (*) of nonadjacent circumferential positions (C). Thickened perimeter indicates intercalations generated (D). Diagram I indicates the shortest circumferential routes generated as intercalations. Longest circumferential routes (II) are offered for comparison.

A graft produces supernumerary growth when this shortest circumferential route represents positional values which later differentiate into the hand, foot, or digits. In this way additional extremities can be added to the limb.

The second rule of the Polar Coordinant Model is the Complete Circle Rule which states that whenever a complete circumference of positional values is exposed, as is the case with amputation, growth occurs in which all presumptive parts are generated, ie. distally directed metaplasia takes place.

The Distalization Rule is the third rule of the Polar Coordinant Model. This rule states that as circumferential tissues converge toward the wound center confrontations occur. These confrontations produce intercalations in accordance with the Shortest Intercalation Rule. These intercalations produce positional values that are repeats of values in the cell layer immediately proximal to the converging layer. The repeated value cannot occupy the same physical space as the original value and is thus forced distally (Bryant et al., 1980). This last rule then, offers a mechanism which explains the proximo-distal orientation of limb growth (fig. 4).



The Polar Coordinant Model Distilization Rule

Figure 4. Healing of the cross sectional amputation plane (A) brings nonadjacent circumferential positions into contact (*) as the epidermis invades the wound surface (B). These confrontations generate intercalations denoted 2b, 4b, 6b, and 8b; which in turn generate more confrontations (*) as shown in (C). These confrontations result in additional intercalations (D) denoted 1b, 3b, 5b, and 7b which complete a new monolayer of positional values (1b-8b) distal to the original amputation plane (1-8).

Although the Polar Coordinant Model was formulated to explain embryonic limb growth, experiments have demonstrated that the model can be applied to regenerating limbs as well (Maneoka and Bryant, 1982). Supernumerary growths have been induced in both embryonic limb buds and developing regenerates. Grafts of regenerate tissues on embryonic buds and vice versa produce identical responses. Therefore, the same patterning mechanism is at work. Further experiments have shown that this patterning mechanism may be common to all vertebrates. When tissues of evolutionarily diverse species are grafted onto chick wing buds, normal host limbs will develope unless the grafts are misaligned. In the latter case supernumeraries are observed to form (Maneoka and Bryant, 1982).

Thus the patterning effect of the stump may be understood in light of the Polar Coordinant Model. Not always, however, is the stump exclusively determinitive nor the blastema totally passive in regard to induction. This concept was demonstrated in experiments in which blastemas were transplanted to the head or back of amphibians. It was found that even the youngest of the blastemas were capable of self-differentiation (Faber, 1965). It is now accepted that upon separation from the stump, directive reorganization occurs autonomously, thus the blastema shows no inductive dependency on the stump (Faber, 1965). This is not to deny, however, that when present, the stump fails to exert an inductive effect.

Other experiments which isolated blastemas demonstrated that younger blastemas differentiated into proximal components of the limb. The older the blastema was upon separation from the stump, the more distal components were differentiated (Faber, 1965). These findings have since been expanded. It is now held that transplants of blastemas are able at any stage, of independently organizing into all the skeletal and muscular elements that would normally regenerate distal to the amputation plane in situ (Stocum, 1968).

Specific parts of the wholly differentiated regenerate were then traced to regions of origin in the undifferentiated blastema. Using carbon marking techniques it was discovered that the early blastema will form the upper arm and part of the lower arm close to the elbow in urodeles. In these studies a new structure was noted: the Apical Proliferation Center was attributed the ability of laying down the more distal limb parts after the proximal had appeared (Faber, 1965). Further experiments attribute the ability of the distal half of the blastema with the formation of the hand structure, the proximal half with proximal structure formation. When the blastema is separated from the stump and then sectioned longitudinally, a proximal-distal sequence of skeletal elements differentiates (Stocum, 1968).

The proximal-distal organization observed in the experiments above is induced by the Apical Organization Center. This organizing center is independent of the stump's morphogenetic influences and remains functional and intact when the blastema is remobed from the stump (Faber, 1965).

Innervation of the stump is the primary vehicle by which the blastema is initiated following amputation.

Aside from the morphogenetic fields discussed above, the primary inductive element is the nervous tissue in the stump. Indeed the blastema will fail to even form immediately post-amputation if the innervation is not proper. It appears that all somatic sensory nerve fibers have the necessary quality to induce limb regeneration. This inducive quality is seemingly unrelated to the conduction of nerve impulses in light of the fact that conduction of these impulses occurs in a centripetal direction -- for sensory neurons, while induction "moves" in a centrifugal direction (Singer, 1981).

In order for this inducive quality to be manifested, however, the innervation must be adequate. The amount of nervous tissue present at the amputation plane is a function of the total tissue surface area at the amputation surface. This can be seen by serially sectioning limbs and noting that despite the fact that amounts of tissue differ depending upon the level of the section, the nerve requirements are the same per unit amount of tissue (Singer, 1981).

The individual nerves exert their inductive effect through their axonal end source. A single neuron is incapable of inducing regeneration of itself. Combined actions of many neurons are required to induce regeneration. The number of axons required to achieve this constitute a threshold (Singer, 1981).

Methods And Materials

Fifteen adult mixed breed mice, and fifteen adult white mice, all female and with an average weight of 20 grams, were separated into three groups of ten and maintained on Purina lab chow. Following anaesthesia with ether, the toe immediately lateral to the middle toe was wiped with alcohol and severed with a scalpel. The amputation plane was through the diaphysis of the proximal phalanx on the right hind foot of each mouse. The wound was then dressed to prevent infection as the blood clot formed. The mice were placed in clean cages following surgery and allowed to recover. Oxytetracycline HCl (trade name: Terramycin), a water soluble antibiotic, was administered through automatic waterers. Treatment was not commenced until 32 days postamputation to allow for osteoclastis (Neufeld, 1980). Animals in group I were untreated, their wounds allowed to heal without interference. Animals in group II were treated solely with NaCl following surgical removal of the scar pad on day 32 and again on day 38 postamputation. This was achieved using a scalpel; asepsis was maintained. Group III also had scar pads removed on day 32 postamputation. These mice were then treated with a saturated solution of NaCl and an equimolar solution of one mM db-cAMP and theophylline.

Saturated salt solution was prepared and maintained at room temperature (25°C). Fresh one mM db-cAMP/theophylline solution (forthwith referred to as db-cAMP solution) was prepared daily by mixing equimolar amounts of solute into distilled water. The db-cAMP solution was maintained at approximately five degrees Celsius.

NaCl solution was administered by filling a container (dimensions: 12" high, base: 5"x8") to a depth of one cm.. Animals were then placed into the container for five minutes. Dibutyryl-cAMP solution was administered by filling ten 120 ml. to a depth of one cm.. Mice were placed individually into the beakers; escape being prevented by perforated lids. Treatment duration was five minutes.

Treatment of group II consisted of five minutes NaCl exposure, while group III consisted of five minutes NaCl exposure followed by five minutes db-cAMP exposure. Total treatment time for group III was therefore ten minutes. Treatments were administerd five times a day for a total of 25 minutes daily for group II and 50 minutes daily for group III.

At 47 days postamputation the animals were sacrificed in a CO_2 chamber. The severed toe was then excised from the rest of the foot using a scalpel.

Tissues were fixed in Bouin's fixative for ten days and decalcified using commercial decalcifier (trade name: RDO, produced by Dupage Kinetic Lab Inc.). Serial sections were cut at five micrometers, orientated sagittaly. Slides were then prepared for light microscopy (fig. 5).

1. Melt paraffin off slides
2. Deparaffinize
   a) xylene...3 min.
   b) xylene...3 min.
3. Clearing in isopropyl alcohol
   4 separate baths...10 dips each bath
4. Wash slides in running water till clear
5. Hematoxylin...10 min.
6. Wash in running water till clear
7. Acid Alcohol
   watch for pink coloration, avoid overbleaching to colorlessness
8. Was in tap water till clear
9. Lithium Carbonate till dark blue
10. Tap water...5 min.
11. Eosin...1 min.
12. Clearing
    a) 95% isopropyl alcohol
    b) 100% isopropyl alcohol
    4 separate baths...10 dips each
13. Xylene...10 dips
14. Xylene...10 dips
15. Mount with permount

Staining Protocol For Light Microscopy

Figure 5. Unless the numbered step calls for dipping, it is understood that the tissue specimen remain totally immersed for the time period indicated.

Results

The untreated stump of group I displays features denoting differentiated tissue. Hair follicles and sebaceous glands indicate a differentiated epidermis and endodermis. Subepithelial tissue appears layered and sparse. A definite bursa or cavity separates the bone from the skin. Loss of wound epithelium is demonstrated by the dense layered strata of subepithelium immediately subjacent to the darkly staining epidermis.

The stump of NaCl treated animals of group II demonstrate some properties of a blastema. Tissue is seen closely associated with the bone indicating that remodeling is in progress. The bursa has narrowed. Subepithelium is in greater quantity and demonstrates greater irregularity in comparison to group I. Persitance of the bursa and the presence of hair follicles and sebaceous glands, however, indicate differentiated tissue imcompatible with structural characteristics associated with blastemas.

The group III animals treated with db-cAMP solution have traits typical of blastema formation. Subepithelial tissue is great in quantity and irregularly orientated, moreso than in group II. The bursa has been replaced by a sparse concentration of cells. A large, dense concentration of randomly oriented cells is associated with the bone. Persistance of wound epithelium

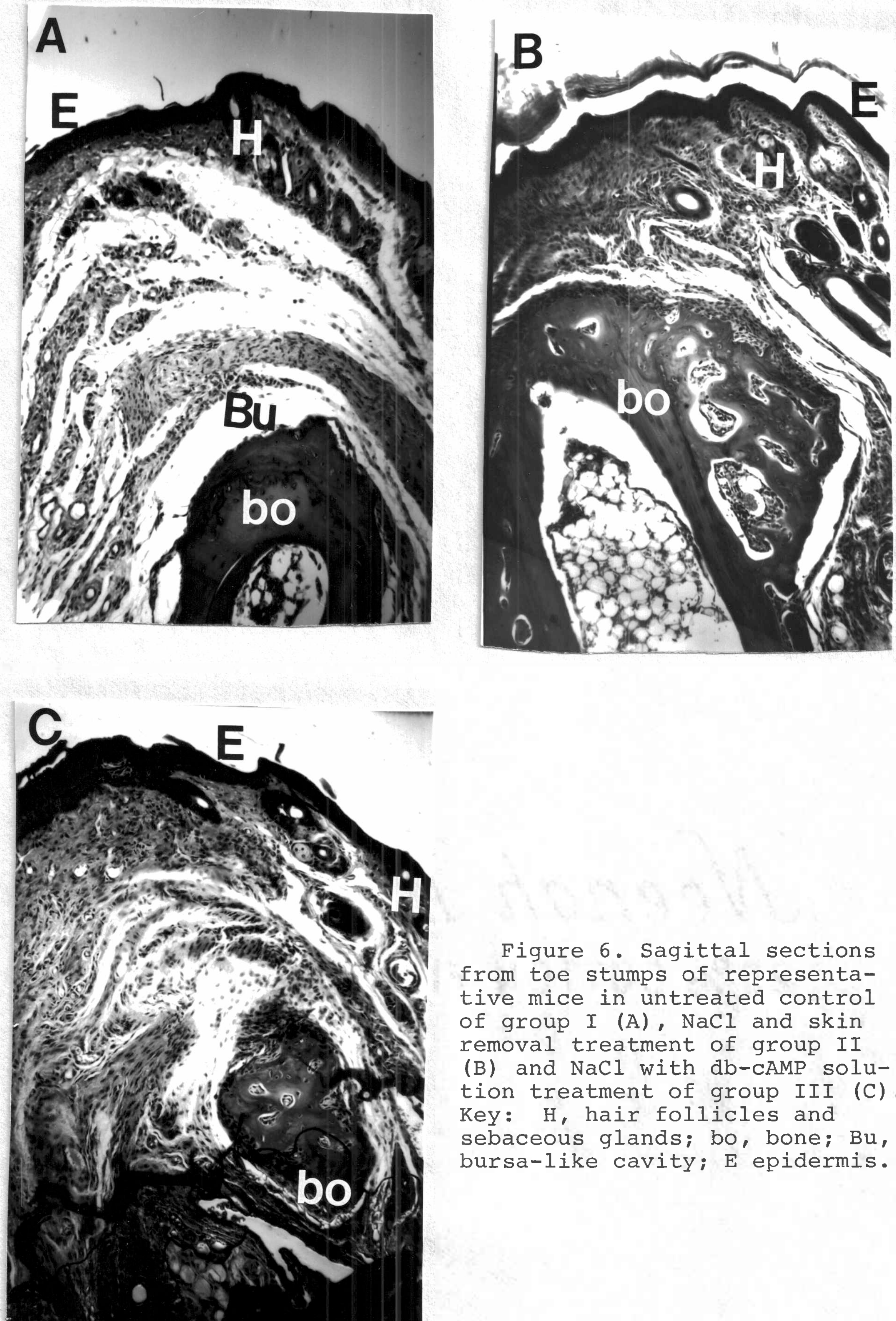


Figure 6. Sagittal sections from toe stumps of representative mice in untreated control of group I (A), NaCl and skin removal treatment of group II (B) and NaCl with db-cAMP solution treatment of group III (C). Key: H, hair follicles and sebaceous glands; bo, bone; Bu, bursa-like cavity; E epidermis.

is indicated by the presence of loosely associated, nonstratified cells immediately subjacent to the darkly staining epidermis. Presence of hair follicles and sebaceous glands once more indicate differentiated tissue.

Therefore, no partial blastema was formed in group I, yet, a partial blastema was generated in group II and III.

Discussion

The new method tested in this study involved the exposure of the amputation site of mouse appendages to a saturated solution of sodium chloride and an equimolar solution of one mM db-cAMP and theophylline. This technique was a modification of that used by Neufeld (1980) in which sodium chloride application along with repeated skin removal resulted in the formation of a partial blastema in adult mice. Repeated skin removal was employed by Neufeld to counteract the contraction of skin over the amputation site. The presence of skin over the wound surface, a common healing response in mammals, probably represses regeneration (Schotte and smith, 1959). The method used in this study attempted to counteract the contraction of skin over the amputation site through the use of a chemical inhibitor of epidermal migration. The use of N^6, $O^{2'}$-dibutyryl cyclic adenosine monophosphate (db-cAMP) was successful in inhibiting epidermal cell migration both in vivo and in vitro when used with equimolar concentrations of theophylline during wound closure in adult newts (Donaldson and Dunlap, 1980). Theophylline, an inhibitor of 3', 5'-cyclic nucleotide phosphodiesterase acted as a potentiator for the db-cAMP. The dibutyryl form of the nucleotide is believed to better penetrate the cell and be less susceptible to enzymatic breakdown.

The procedure used in this study used the same concentrations as Donaldson and Dunlap did It was hoped that a chemical inhibitor would provide a more consistent absence of encroaching skin and would traumatize underlying tissue less than repeated surgical removal of skin. Ultimately this would lead to a better partial blastema than that achieved by Neufeld.

The results indicate that treatment with db-cAMP and theophylline can significantly alter the morphology of mammalian stump tissue. Results also indicate that this treatment is an effective substitute for treatment by repeated skin removal as similar morphologies were attained from both methods. Procedural limitations precluded the determination of whether or not the db-cAMP treatment was more effective than the soduim chloride treatment. Overall results indicate that db-cAMP was more effective, yet these observations are qualitative and therefore prone to bias. Results are encouraging enough to warrant further research along these lines.

A partial blastema was achieved through application of db-cAMP. A complete blastema has the following characteristics: 1) mitotically active subepithelial tissue; 2) persistance of wound epithelium accompanied by absence of a basement membrane; 3) numerous subepithelial cells -- morphologically undifferentiated, irregularly orientated and capable of DNA synthesis; 4) a base of viable remodelled bone and 5) lack of collagen accumalation in subepithelia (Neufeld, 1980). The partial blastemas formed as the result of db-cAMP treatment demonstrated all these characteristics except persistance of wound epithelium as indicated in the presence of hair follicles and sebaceous glands. Such parameters as mitotic activity, presence of basement membrane, and DNA synthesis capability could only be fully characterized through the use of radioactive labels. This procedural adjunct proved financially prohibitive to the present study.

The biochemical basis for the regenerative effect of NaCl, db-cAMP, and theophylline on postamputation wound surfaces is largely unknown. NaCl may stimulate regenertion indirectly by its effect on nerve endings present in the amputation plane. The simplest explanation attributes the compound's regenerative effect to the fact that NaCl is a tissue irritant. This irritation engenders increased nervous stimulation which brings about augmented innervation and subsequent regeneration. Another explanation as to why NaCl is capable of altering wound surfaces identifies electrolytic character as the property that induces blastema formation. Impulses may travel from nerve endings through the wound tissue into the conducting solution. Nerves are better able, therefore, to exert their inductive effect in the presence of an electrolyte. Experiments using CaCl -- also an electrolyte, have not yielded blastema-like tissues (Kudokotsev and Kuntsevich, 1965; Scharf, 1961 and 1963). These findings shed doubt on this theory. Finally NaCl in such high concentrations may induce partial blastema formation due to its mildly toxic effect on migrating epidermis. Differentiated skin thus denied contact with wound epithelium would not be able to induce differentiation in the amputation plane.

Cyclic adenosine monophosphate may exert its effect as a mediator of cellular responses brought about by the presence of Nerve Growth Factor (Schubert et al. 1978). The presence of Nerve Growth Factor (NGF) may cause increases in intracellular levels of cAMP which in turn cause an increase in movement of calcium ions. Mobilization of these ions is necessary for the release of neurotransmitters at nerve terminals. Evidence for this lies in observed increases in specific activity of choline acetyl transferase and an increase of intracellular acetyl choline. This biochemical activity has been correlated with structural events in the plasma membrane. Cell-cell and cell-substratum adhesion is enhanced and neurite growth is observed (Schubert et al. 1978).

A more simple approach may be to attribue cAMP's salutary effect to its mitogenic activity with regard to Schwann cells. It is possible that substantial increases in levels of cAMP may act as an intracellular signal that causes Schwann cells to divide during regeneration (Brockes et al., 1978). If Schwann cells proliferate readily, then, the axon is quickly surrounded by myelin sheath and nerve conduction is greatly enhanced.

Finally, cAMP may induce formation of the partial blastema by inhibiting epidermal migration over the wound surface, (Donaldson and Dunlap, 1980). In doing this, the wound epithelium is not induced to differentiate and the blastema remains. This theory formed the basis of this study.

The presence of theophylline is necessary for cAMP to have its inhibitory influence on migrating epidermal tissue (Donaldson and Dunlap, 1980). The biochemical basis for this phenomenon is theoretically due to theophylline's activity as a phosphodiesterase inhibitor. In this capacity theophylline prevents metabolism of cAMP, thus safeguarding the nucleotide's chemical activity.

Theophylline may simply induce partial blastema formation through its activity as a vasodilator. In allowing more blood to reach the wound, a pluripotent mass of cells is better maintained.

In summary, the biochemical behavior of NaCl, cAMP, and theophylline is not fully understood. Current theory attempts to tie biochemical mechanisms with enhanced innervation. If these phenomena are related, then the deeper mystery is how neurogenesis induces blastema formation.

This study was carried out on a morphological rather than a biochemical level. A definitively better blastema-like structure than that achieved by Neufeld was not realized. In successfully producing a partial blastema, however, the methodology employed in this study becomes one of but three that can claim such results. Neufeld's work, and a study performed on nerve transplants in opossum hindlimbs (Mizzel and Isaacs, 1970) are the only other experiments involving the class Mammalia that achieved formation of blastema-like structures. Twenty such experiments had been performed by April 1980. Neufeld questions the validity of the results attained by Mizzel and Isaacs on the grounds that their experiments utilized immature specimens (Neufeld, 1980). If Neufeld's criticisms are justified, then it can be concluded that NaCl treatment accompanied by epidermal cell migration inhibition is the only means of producing partial blastema formation in mammals to date.

The present study provides a means of inhibiting the encroachment of epidermis over the wound surface that has advantages over the technique that involves repeated skin removal. The application of db-cAMP solution is less traumatic for the animal as there is no need for anaesthization or wound reopening. Treatment is more easily applied. The chance of infection is reduced through maintenance of the wound epithelium formed after osteoclastis. Surgical removal of invading epidermis allows this migrating tissue to exert its differentiating influence. This is done in the time interval between invasion of the wound surface to surgical removal thereof. The wound surface is thus exposed to successive waves of epidermal inductive influence. Treatment with db-cAMP provides continuous rather than periodic absence of encroaching skin. Subsequently the wound surface is exposed but gradually to epidermal inductive influence allowing for a more stable environment conducive to blastema formation.

Several improvements on the present study could enhance the salutary effect of db-cAMP on limb stump tissues in mammals. The suspension of db-cAMP, theophylline and NaCl in a gel that could be applied along with protective dressings would allow continuous medication. The present study utilized concentrations of db-cAMP found effective in inhibiting newt epidermal migration. Experiments could be carried out to ascertain the optimal concentration for epidermal migration inhibition in mice. Once the optimal concentration were discovered it could be incorporated into the procedure of this study. The effect of db-cAMP on mesenchymal cell migration -- a morphological event conducive to blastema formation, is unknown. Studies performed on this phenomena could produce data that would alter the value for the aforementioned optimal db-cAMP concentration.

Another area of related study might involve the search for alternatives to saturated NaCl solution. Potassium and sodium are ions of similar charge and permeability. If KCl were substituted for NaCl, more light might be shed on how NaCl is able to stimulate partial blastema formation.

The data obtained in this study could be augmented through use of radioactive labeling. Tritiated thymidine, for example, could be used to make autoradiographs. These would provide quantitative data with regard to the amount to mitotic activity within the partial blastema. Studied statistically this data would form the basis for an objective comparison of both procedures. The degree to which the basement membrane is formed could also be quantified through use of autoradiographs. These two additional parameters -- mitotic activity and presence of basement membrane, would allow more complete evaluation of the degree of blastema formation.

Most research on mammalian regeneration has occurred within the last 15 years. The field is young. This study represents a "brute force" approach to the study of this phenomenon. Basically the experiment consisted of mixing one potion with another to see whether or not they go "poof." Despite the rude design, this experiment details additional phenomena that may produce clues as to the biochemical basis for regeneration. The action of db-cAMP has been suspected of playing a role in blastema formation in urodeles. This study is the first to link this nucleotide with regeneration phenomenon in mammals.

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