into the cornea should act differently from other materials rich in this protein. It is likely, however, that the explanation lies in some alteration of the collagen used in the membrane. Collagen is composed of a repeating "monomer," tropocollagen, which is itself composed of three chains of amino acids arranged in a coiled helix (*2*). Peptides of low molecular weight, termed telopeptides, extend from the body of tropocollagen (*4*). There is increasing evidence that these telopeptides determine species difference of collagen and play a determinative role in its molecular biology (*1, 3, 4*). The feature distinguishing the collagen membrane from other collagen-rich materials used in the cornea is that most of the telopeptides were removed during preparation of the membrane by enzymatic proteolysis (*10*). A further important point is the extensive introduction of intra- and intermolecular cross-links into the collagen membrane by heat or ultraviolet irradiation (*1*). It is known that cross-links stabilize the tropocollagen molecule and play an important role in determining the resistance to reabsorption (*11*).

MICHAEL W. DUNN, TOMIO NISHIHARA
KURT H. STENZEL
A. WHITLEY BRANWOOD
ALBERT L. RUBIN

Departments of Surgery (Ophthalmology) and Pathology and *Rogosin Laboratories, Department of Medicine, New York Hospital-Cornell Medical Center, New York*

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Vertebrate Regeneration System: Culture in vitro

Abstract. *With standard tissue-culture techniques and media, various components of the lizard tail regenerate have been maintained in culture for 8 months. Differentiation of two cell types, melanophores and striated muscle, has been obtained. Myoblast proliferation and fusion can be selectively controlled by altering the culture medium.*

The details of tail regeneration in a number of lizard species have been described (*1, 2*). As in other regeneration systems, the developmental capacities of these regeneration cells are in dispute. In addition, the various interactions involved in the initiation of the regenerate, as well as those occurring during its differentiation, have not been adequately resolved. These basic aspects of vertebrate regeneration have proven difficult to clarify with available techniques in vivo. Studies of regeneration in vitro have been hampered by the general paucity of techniques for the culture of cells from lower vertebrates. Although considerable progress along these lines has been made in recent years (*3*), all reported attempts to culture the components of a regenerate have achieved, at best, maintenance and limited outgrowth (*4*). We describe techniques for the culture in vitro of various components of the tail regenerate in the lizard *Anolis carolinensis*.

The portion of a tail bearing a regenerate of known age was first swabbed with ether to remove fungal spores. The regenerate was then removed and placed in Hanks's salt solution containing antibiotics (*5*). The regenerate was cut into circular segments approximately 1 to 2 mm thick and placed in dissociation medium, consisting of growth medium (GM-I) containing 2.0 mg of collagenase per milliliter (*5*). The *p*H was maintained at 7.2 with a bicarbonate buffer and gassing with a mixture of 95 percent air and 5 percent CO_2. Dissociation was carried out at 26°C for various lengths of time (2 to 8 hours) depending on the component to be isolated for culture. Following this preliminary loosening of the components, the tissue slices were carefully dissected with the aid of tungsten needles to yield the material for culture (that is, wound epithelium, promuscle aggregates, procartilage aggregate, or general mesenchymal cells of the regenerate). The isolated components were then placed in fresh dissociation medium, gassed, and dissociated with occasional pipetting for an additional 2 to 4 hours until suspensions of single cells were obtained. The fully dissociated cells were filtered through several layers of sterile lens paper to remove cell clumps, spun down in a clinical centrifuge, and then resuspended in a known volume of growth medium. The cells were counted in a hemocytometer, diluted to appropriate concentrations, and plated in either 60-mm Falcon plastic petri dishes or in Falcon 30-ml flasks.

During the initial phases of this study, pooled cultures of dissociated cells obtained from early regenerates were used to screen various media. Although several commercially available media and various combinations of added biologicals were tested for their ability to support growth of the cells, two formulations were found to support growth for long periods of time and in some cases additionally support differentiation of the cultured cells. Growth Medium I (GM-I) consisted of Coon's (*6*) modification of Ham's F-10 medium containing 5 percent chick embryo extract, 10 percent fetal calf or horse serum, 1 percent GIBCO antibiotic mixture (*5*), and 0.07 percent bicarbonate. Growth Medium II (GM-II) contained Eagle's basal medium with glutamine and the same concentrations of embryo extract, fetal calf or horse serum, antibiotic, and bicarbonate. Although both media supported long-term proliferation of the lizard cells, a higher plating efficiency and more rapid growth was consistently obtained with GM-I. However, GM-I failed to support differentiation of striated muscle, whereas GM-II did support muscle formation. All cultures were incubated at 26°C in sealed chambers gassed with 95 percent air and 5 percent CO_2 and maintained at 100 percent humidity. Every 4th day half of the medium in each dish was exchanged for fresh medium.

Presumptive muscle cells, obtained from 3- to 6-mm regenerates, were plated at a concentration of 2×10^3 to 3×10^3 cells per dish. In all cases the

culture dishes had been previously coated with a thin film of rat tail collagen. A plating efficiency of 3 to 5 percent was obtained. The cells began attaching to the culture surface within an hour, and attachment was complete by 24 hours. The cells underwent a lag period of about 2 weeks before rapid proliferation commenced. Near the beginning of the 3rd week large numbers of mitotic figures were present, and by 21 days large colonies of several thousand cells were present. This rapid growth continued, and by day 28 the first indications of fusion into myotubes was evident in some of the colonies. By day 31 of culture, these colonies exhibited large numbers of multinucleate myotubes (Fig. 1A) having the characteristic morphology of developing skeletal muscle. Although longitudinal myofibrils were well formed by day 31, formation of typical cross-striations usually did not appear until day 50 (Fig. 1B). The fusion of the promuscle cells to form myotubes is dependent on the culture medium. Promuscle cells grown continuously in GM-I, or grown in GM-II and switched to GM-I before fusion, never formed myotubes. Instead, after an extended period of proliferation, the cells took on a long, attenuated morphology, and no fusion could be detected (Fig. 1C). Duplicate cultures that were grown in GM-I well past the time when companion cultures in GM-II had myotubes, were switched to GM-II. In these cultures, myotubes formed within 3 to 5 days. This differential response to the two media might well offer a convenient system both for analyzing the relationships between cell division and differentiation and for studying the fusion process. The time required for muscle differentiation in this system is longer than that reported for embryonic chick systems (*7*); however, it follows rather closely the time sequence within the developing lizard-tail regenerate in vivo (*2*). Recent results indicate that this time period in culture can be substantially reduced if GM-II in which horse serum is substituted for fetal calf serum is used and the incubation temperature is elevated to 31°C.

At the time of original screening of media, long-term cultures were initiated. One such culture, AB-2, has now been cultured in GM-I for a period of 8 months. During this time the cells have been subcultured three times. The primary culture was plated at high density (6×10^4 cells per flask), and the medium was changed at weekly intervals. The cells underwent a tremendous amount of multilayering within the first 3 weeks of culture. At this time there was no morphological evidence of differentiation.

The first subculture was initiated at 74 days, and the harvested cells were plated at a density of 4×10^3 cells per flask in GM-I. These subcultures formed monolayers within 30 days, and three distinct colony types were evident within the monolayer: (i) colonies of plump, spindle-shaped cells; (ii) colonies of differentiated melanocytes (Fig. 1D); and (iii) colonies of fibroblast-like cells. No other phenotypes were recognized even though some of the cultures were maintained for an additional 2 months. When cultures were fed with GM-II, however,

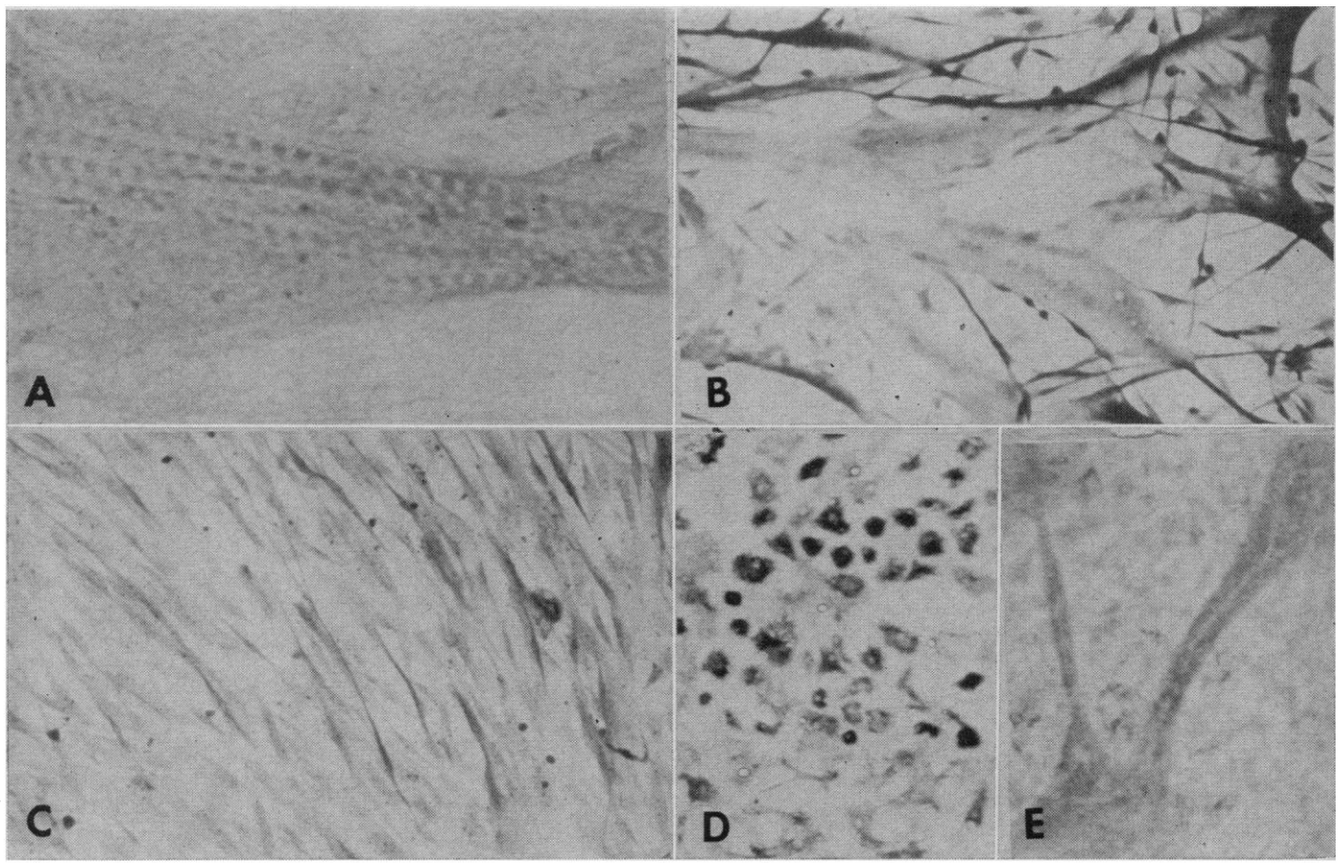

Fig. 1. (A) Muscle fiber from a colony growing in GM-II, showing cross-striations (× 900); (B) developing myotubes from a colony growing in GM-II (× 100); (C) companion culture to (B) growing in GM-I, showing lack of fusion (× 200); (D) portion of a melanocyte colony (× 200); (E) explant of ependymal tube growing upon a substrate of its connective tissue (× 35).

those colonies composed of the plump, spindle-shaped cells exhibited fusion and myotube formation. The melanocyte and fibroblastic colonies remained unchanged following the change of medium.

A second subculture was initiated 30 days later. In addition to passage cultures, 20 dishes were plated in GM-I at a density of 4×10^3 cells per petri dish. By 21 days these subcultures had macroscopic colonies (30 to 40 per plate) which were either fibroblastic or "myoblastic" in morphology. At this point ten of the cultures were switched to GM-II. Within 7 days the "myoblastic" colonies exhibited myotube formation, while all cultures remaining in GM-I failed to form myotubes. When the latter cultures were terminated (58 days after subculture), those colonies, originally designated as being "myoblastic," were composed of large, elongate mononucleate cells. Thus these cells which had been carried in culture for a period of 104 days without any overt expression of muscle, could form muscle when plated in a permissive medium.

Other components of the regenerate and mature tail have also been cultured. The ependymal tube, for example, has been cultured in both media as: (i) dissociated cells; (ii) partially dissociated explants of the tube and its associated connective tissue; and (iii) organ cultures on Millipore filters. When grown as dissociated cells, the ependyma forms large plaques of monolayered cells. Intact ependymal tube growing in association with its connective tissues retained its tubular morphology and underwent branching (Fig. 1E).

In a number of the pooled cultures of early regenerates, some wound epithelial cells were inadvertently included. These gave rise to colonies which expanded over the underlying multilayer of mesenchymal cells and produced sheets of keratinized cells for a period of 60 days. Cultures of dissociated mature cartilage tube have been carried in GM-I for a total of 4 months, during which they have been subcultured four times. During this period they have shown no decline in growth. Attempts to obtain overt differentiation of these cells has so far been unsuccessful as judged by studies of S^{35} incorporation and metachromasia after Azure-A staining.

These results, although preliminary, demonstrate the feasibility of conducting both short- and long-term studies on the components of the lizard-tail regenerate in vitro. With these techniques one may be able to approach many of the problems of regeneration that have thus far eluded analysis.

S. B. Simpson, Jr.
Prentiss G. Cox
Departments of Anatomy and *Biology, Western Reserve University, Cleveland, Ohio 44106*

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Reserpine: Effect on Structure of Heart Muscle

Abstract. *We examined the ultrastructure of hearts from dogs given reserpine intramuscularly for 4 days, and from untreated dogs. Sections of the myocardium from treated dogs invariably revealed mitochondrial abnormalities at the 5th and 14th days. These included fragmentation and loss of structure of the cristae, and cyst formation. The appearance at 25 days in the treated as well as in all the untreated dogs was normal. We concluded that reserpine in the dose used produces marked structural changes in the mitochondria of heart muscle, and that these changes are reversible. These changes may account for the myocardial depression sometimes seen after administration of reserpine.*

We have found evidence of impaired myocardial function in dogs given a total of 100 μg of reserpine per kilogram of body weight in divided doses over 4 days (*1*). Uncoupling of oxidative phosphorylation in heart mitochondria has been reported to occur after administration of large doses of reserpine (5 mg/kg) (*2*). In this study we examined the ultrastructure of the heart to see if there were changes in the mitochondria which might explain our own findings.

Twelve dogs weighing 7 to 29 kg were given intramuscular injections of 25 μg of reserpine (Serpasil, Ciba) per kilogram of body weight per day for 4 days (days 1 to 4). Specimens of myocardium were obtained on either the 5th day (six dogs), 14th day (three dogs), or 25th day (three dogs). There were six untreated dogs.

The dogs were anesthetized with intravenous injections of sodium thiopentone and a mixture of nitrous oxide and oxygen (2 : 1). A midline thoracotomy was performed. In nine dogs a fine nylon catheter was introduced into the anterior descending branch of the left coronary artery near the apex, pushed in a retrograde direction for about 1.5 cm, and tied in place. Of these, four were untreated and five had been given reserpine (three at 5 days, one at 14 days, and one at 25 days after treatment). Perfusion was begun immediately with a freshly prepared solution of 0.1 percent osmium tetroxide in 0.1*M* phosphate buffer (*p*H 7.45) and 0.02 percent calcium chloride, at a pressure of 150 to 180 mm-Hg. As the perfusion was begun the artery was clamped just above a bifurcation about 2.5 cm proximal to the end of the catheter, and in this way only a small area of the myocardium was perfused; this area received a normal blood supply until the instant the perfusion was begun.

Perfusion was maintained for 3 minutes; while the heart was beating strongly an area of muscle changed color as blood was replaced by the fixative. A small strip of muscle was excised from this area and immediately placed in 1 percent osmium tetroxide in Palade's buffer (*3*), cut into cubes of approximately 1 mm^3, and rapidly transferred to fresh osmium tetroxide (1 percent) in Palade's buffer. After 2 hours at 5°C we removed the fixative by washing the muscle in tap water.

In the remaining nine dogs (two untreated and seven treated) fixation by