vast repertoire of natural antibodies with a potent complement system provides the shark with a remarkable—and possibly unique—natural humoral immune system. Little is known about the evolution of natural immunity. It seems that phylogenetically younger species must rely increasingly on overt antigenic stimulation.

If the destruction of sensitized foreign structures may be considered the most basic and dramatic function of the complement system, 350 million years of phylogeny have left it nearly unaltered. The more recent development into a nine-component system could be viewed as a refinement, necessitated by a call for more sophisticated phlogistic activities and corresponding safety and control mechanisms. It appears that the classical pathway of complement activation indeed has a remarkable evolutionary survival value.

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References and Notes

1. Abbreviations and definitions: C, complement; C1, C2, . . . , C9, the various components of complement; E, sheep erythrocyte; EA, sensitized E; EAC1–7, EA reacted sequentially with the first seven mammalian C components; $DGVB^{2+}$, isotonic dextrose-gelatin-Veronal buffer containing divalent calcium (0.00015*M*) and magnesium (0.0005*M*); EDTA, ethylenediaminetetraacetate; functionally pure, not contaminated with another C component; sensitized, reacted with specific antibody; intermediate complexes, sensitized E reacted sequentially with various C components, each conferring reactivity for the next component.
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12. This work was in part reported at the First Congress of Developmental and Comparative Immunology, Aberdeen, Scotland, July 1980. We thank the Miami Seaquarium for providing and keeping the nurse sharks, and M. Dorsey for bleeding them.
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DNA Synthesis in Cultured Adult Cardiocytes

Abstract. *Trypsin-dissociated atrial cardiocytes from adult rats were exposed to [^3H]thymidine for sequential 24-hour periods from day 2 to day 12 of culture. On day 3 and each day thereafter, cells were prepared for ultrastructural radioautography and examined with an electron microscope. Maximal incorporation occurred on day 5, when 63 percent of the cardiocytes were labeled. Mitotic activity was never present in more than 0.5 percent of the cardiocytes examined. Incorporation of [^3H]thymidine and mitosis occurred only in immature cardiocytes characterized by subsarcolemmal primry filaments and Z bands with or without specific granules; more mature cardiocytes were never labeled.*

Long-term cultures of isolated myocardial cells from the ventricles of fetal and neonatal mammals have provided information on the contraction, metabolism, pharmacology, and electrical properties of heart muscle (*1, 2*). These cardiocytes are capable of DNA synthesis and mitosis both in vivo (*3, 4*) and in vitro (*5, 6*). The same is true for atrial cardiocytes from neonatal rats (*7*). Since DNA synthesis and mitosis are restricted in adult mammalian cardiocytes in vivo (*3, 4*), it is desirable to develop techniques for investigating these activities in adult mammalian cardiocytes in vitro. Ventricular cardiocytes isolated from adult mammals have survived only for short periods (*8, 9*) and have not shown mitotic activity (*10*). We now report that atrial cardiocytes from adult rats can be maintained in culture, where they exhibit intense DNA synthesis and moderate mitotic activity.

Atrial cardiocytes from 300- to 350-day-old female Sprague-Dawley rats (Biobreeding Laboratories) were cultured (*7*). The atria were excised from 40 to 50 hearts, placed in Eagle's minimal essential medium (Gibco), washed twice in Hanks solution (Gibco) without calcium and magnesium, and dissociated at 37°C in 0.1 percent trypsin (Difco). The cultures were enriched in cardiocytes by the selective plating technique of Kasten (*2*): the dissociated cells were preplated for three 1-hour periods, followed by one 24-hour period in gelatin-coated 25-cm culture flasks (Corning) to allow attachment of endothelial cells and fibroblasts.

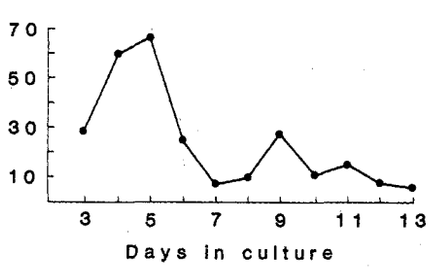

Fig. 1. Percentage of cardiocytes (N = 1000) showing labeled nuclei and subsarcolemmal filaments with forming Z bands or specific granules.

The final supernatant was plated at density of 2×10^6 to 3×10^6 cells in 6 ml of Eagle's minimal essential medium buffered with Hepes at *p*H 7.4 and supplemented with 10 percent fetal calf serum, 1 percent glutamine, and 1 percent penicillin and streptomycin (Gibco). The medium was renewed on days 4, 7, and 10. The cultures were incubated in air at 37°C in gelatin-coated 25-cm flasks.

Cardiocytes started to attach at the end of day 2, grew steadily, and reached confluence on day 8. At this stage they elongated and enlarged to form a criss-cross pattern of branching cells. For sequential 24-hour periods from day 2 to day 12, cardiocytes were exposed to [*methyl*-^{3}H]thymidine (1 μCi/ml; specific activity, 20.0 Ci/mmole; New England Nuclear) (Fig. 1). The exposed cells were prepared each day (starting on day 3) for ultrastructural radioautography by the flat substrate technique (*11*). At each time interval, 1000 cells through all layers of the plastic blocks (but not in adjacent sections) were scanned consecutively, first for the presence of silver grains over the nucleus and then for the presence in these labeled cells of filaments with forming Z bands or specific granules (secretory-like granules characteristic of atrial cardiocytes) (*12*). Only cells with these features (Fig. 2a) were considered to have synthesized DNA. The maximal incorporation of [^{3}H]thymidine occurred on day 5; at this time 63 percent of cardiocytes had labeled nuclei. This was followed by a sharp decline, with subsequent small peaks on days 9 and 11. Cells undergoing mitosis (Fig. 2b)—most of which were labeled—were always present but never exceeded 0.5 percent of the cardiocytes examined. A few labeled binucleate cells were encountered from day 5 on.

The entire spectrum of cell development, from very immature to nearly mature cardiocytes, was encountered in the cultures. Maturation of the cells was asynchronous; by day 7, cells with subsarcolemmal filaments accompanied by forming Z bands and an abundant rough endoplasmic reticulum were found side by side with more organized cells con-

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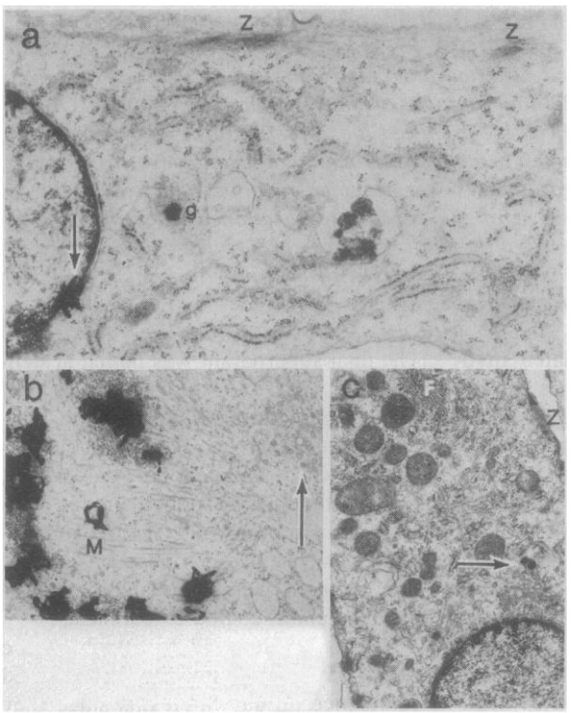

Fig. 2. Ultrastructural radioautographs. (a) Cell exposed to [^{3}H]thymidine from day 7 to day 8. The nucleus is labeled (arrow). Primary filaments with forming Z bands (*Z*) are parallel to the sarcolemma (*g*, specific granules) (×23,700). (b) Cell in metaphase, from a culture exposed to [^{3}H]thymidine from day 5 to day 6. Silver grains are visible over dividing DNA amidst spindle microtubules (*M*). The small Golgi vesicles contain progranules (arrow) (×9000). (c) Cell exposed from day 6 to day 7. The nucleus is unlabeled. Typical mature myofilaments (*F*), both thick and thin, are present in the cytosol far from the sarcolemma and are decorated with numerous ribosomes. Subsarcolemmal Z bands of a mature type and specific granules (arrow) are also present (×11,800).

taining adult-type myofilaments and Z bands, a much less conspicuous rough endoplasmic reticulum, a large Golgi complex, and specific granules (Fig. 2c). By day 13 most of the cardiocytes were of the latter type.

In these atrial cardiocytes sarcomerogenesis occurred as described by Legato (*13*) for cultured ventricular cardiocytes from neonatal rats. Specific granules were always present in small numbers and progranules were often visible in the Golgi area. Maturation seemed to impose stringent restrictions on DNA synthesis: no labeled cell contained myofilaments or mature Z bands away from the subsarcolemmal area. Specific granules, on the other hand, were observed in labeled and unlabeled cells. Most of the cells that did not meet the criteria for labeled cardiocytes were of the more mature type. Only a small proportion (5 percent) of the unlabeled cells examined on days 3 and 4 could have been fibroblasts. No other cell type found in the myocardium in vivo—such as endothelial cells, pericytes, smooth muscle cells, or macrophages (*14*)—could be identified. This may have been due, at least in part, to the long period (more than 24 hours) of preplating. Even at the most immature stage, the cells often contained specific granules that easily differentiated them from fibroblasts and smooth muscle cells (*15*); further evidence is the later maturation of most of these cells (more than 95 percent) into more typical adult-type cardiocytes. The discrepancy between the proportion of mitotic cells (0.5 percent) and the high degree of labeling observed on some days may be related to the long labeling periods and to differences in the length of the mitotic and synthetic phases of the cell cycle (*4*).

DNA synthesis and mitosis can be induced relatively easily in areas surrounding an injury in the adult ventricular myocardium of fish, amphibians, and reptiles (*4*). The cardiocytes immediately adjacent to a necrotic focus seem to partially dedifferentiate before DNA synthesis and mitosis, acquiring a striking resemblance to early embryonic cardiocytes. This is not the case, however, in mammalian ventricular myocardium (*4*). Despite some conflicting evidence, it is now generally agreed that there is only sluggish DNA synthesis in the nuclei of myofibers adjacent to an area of ventricular infarction in rats and mice, and very little mitotic activity (*4*). The picture is completely different in the atrial myocardium of adult mammals. Following infarction of the left ventricle in rats, intense DNA synthesis occurs in both atria and mitosis may be seen (*4*). Here again, preceding the reactive hyperplasia, most atrial cardiocytes undergo partial dedifferentiation, with morphologically evident protein synthesis. Breakdown of Z disks occurs during prometaphase, and released myofilament bundles are pushed toward the periphery (*4*). DNA synthesis can occur, however, in fully differentiated cardiocytes from neonatal rats (*16*). The presence of a small (2 to 3 percent) pool of proliferative atrial myocytes in normal adult rats is not sufficient to explain the intense DNA synthesis observed in the present study.

It is possible that the dissociation technique used was partly responsible for the initiation of DNA synthesis in the atrial cells. During exposure to trypsin some of the enzyme enters the cell (*17*) and damages myofilaments (*18*). In ventricular cardiocytes from neonatal rats this procedure is followed by an intense wave of protein synthesis and cell division (*1*). The same phenomenon is seen in cultured atrial cardiocytes from neonatal rats (*7*). Another factor that may trigger DNA synthesis is exposure to the growth-promoting effects of serum (*19*). Whether dissociation in trypsin and exposure to serum are triggering factors equivalent to injury in vivo remains to be determined. Apart from their intrinsic biological interest, the present results offer the opportunity to study in vitro the factors responsible for the initiation of DNA synthesis and mitotic activity in mature mammalian cardiocytes.

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