are involved (*14*). Similarly the site of action of hydrocortisone in the induction of tyrosine transaminase is far from clear, although it seems possible from our results that the hormone affects the nucleocytoplasmic barrier, allowing the transport to the cytoplasm of certain RNA species which are normally restricted to the nucleus. Hybridization studies do suggest that certain nuclear RNA species are not normally present in liver cytoplasm (*15*), and that the nucleocytoplasmic barrier may be defective in several hepatomas (*15*).

Whether the use of such analogs as 8-azaguanine will provide a general functional test for the presence of inactive messenger RNA must await further study. However, our results and those of others (*16*) suggest that the investigation of cellular controls (other than the half-life of cytoplasmic RNA) by means of actinomycin D alone may be insufficient; a comparison of the effects of the latter drug with those of 8-azaguanine and 5-azacytidine might be more meaningful.

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Growth in vitro of Cells from Hyperplastic Nodules of Liver Induced by 2-Fluorenylacetamide or Aflatoxin B_1

Abstract. *Cell suspensions obtained from hyperplastic nodules induced in rat liver by either of the two hepatic carcinogens, 2-fluorenylacetamide or aflatoxin B_1, show growth when cultured in vitro. No growth of cells from liver adjacent to the hyperplastic nodules or from liver of control rats has been obtained so far under comparable conditions. Hepatocarcinoma cells induced by 2-fluorenylacetamide grow readily in vitro but behave differently. These findings suggest that some nonmalignant cells capable of growth in vitro arise during liver carcinogenesis prior to the appearance of unequivocal cancer. Cultures of such cells may offer new avenues for the study of liver carcinogenesis.*

The appearance of overt experimental liver malignancy is preceded by the occurrence of a new population of hepatocytes organized into discrete hyperplastic liver nodules (HLN) (*1, 2*) and showing reproducible biochemical and morphologic characteristics (*2*). Such nodules appear to be one site of origin of liver cancer (*1, 2*), since (i) they are seen regularly during carcinogenesis with 2-fluorenylacetamide (2-FAA), aflatoxin B_1, and virtually every hepatic carcinogen, (ii) unequivocal cancer can be observed within nodules in livers without identifiable cancer elsewhere, and (iii) glycogen containing a bound metabolite of 2-FAA is present in nodules and in cancers but not in liver surrounding these lesions. The latter finding is consistent with the hypothesis that the nodules are part of a linear sequence between original liver and hepatic cancer cells. Since these nodules grow and become labeled with radioactive thymidine to a degree far in excess of the surrounding liver (*3*), it became important to study the growth potential of such cells under better controlled conditions in vitro in the hope of not only defining some of the parameters of their growth characteristics but also developing an in vitro system that can be exploited for the biochemical analysis of carcinogenesis.

Male white Wistar rats (Carworth Farms) weighing 150 to 200 grams were used. We induced HLN by feeding the rats a 2-FAA dietary regimen for 15 weeks (*2*) or aflatoxin B_1 for 15 weeks followed by the basal diet. Control animals were given only the basal diet (*2*). Hepatocellular carcinomas were obtained from animals maintained on the 2-FAA regimen (*2*) for 9 months.

In 14 separate experiments conducted over a 1½-year period, rats (16 on 2-FAA regimen and 3 control for each experiment) were killed by ether anesthesia or decapitation. Specimens of readily identifiable HLN, hepatocellular carcinomas, liver adjacent to both these lesions, and control rat liver were aseptically collected. We also obtained HLN and adjacent liver from two rats ingesting 3 parts per million of aflatoxin B_1 and from six fed aflatoxin B_1 at 2 parts per million. Multiple aliquots of tissues used for culture were taken for histologic study (*2*) with no evidence of malignancy observed in nodules by light microscopy. Suitably prepared tissue aliquots were repeatedly trypsinized and suspended in Roswell Park Memorial Institute medium 1629 with added 20 percent fetal calf serum, 1 percent glutamine, penicillin (100 unit/ml), and streptomycin (100 mg/ml) and were inoculated into 4-oz (118-ml) sterile plastic flasks (Falcon) or glass prescription bottles. Collagen-coated cellulose sponges were prepared and used as described by Leighton *et al.* (*4*). The antibiotic tylosin tartrate (*5*) was added to prevent mycoplasma contamination. The procedures of Stulberg *et al.* (*6*) and of Lovelock *et al.* (*6*) were used to preserve the HLN cells at low temperatures.

All cell cultures were incubated at 37°C. Cultures were examined twice weekly when the mediums were changed. The multiplication rate of the cells

grown on plastic flasks was determined by counting triplicate trypsinized cell cultures at appropriate time periods (*7*).

In all experiments, both control rat liver (basal diet only) and liver obtained from nonnodular hepatic loci of rats fed the 2-FAA or aflatoxin B_1 regimens degenerated after 48 hours of culture, irrespective of whether or not the growth medium was supplemented with 10 or 20 percent fetal calf serum.

The HLN cells harvested by trypsinization at each passage grew slowly on plastic surfaces, forming a typical monolayer in 14 days. The rapidity of monolayer formation was directly proportional to the number of cells inoculated. After 14 days of growth with four changes of medium, the cultures were split 1 to 2 at 2-week intervals. Starting with a population of 10^5 cells, a maximum population of 3.0×10^6 cells was reached after 16 days of growth. This represented a 30-fold increase with a generation time of 25.7 hours. The log phase of growth was reached on the 9th day. The addition of 15 to 20 percent fetal calf serum did not influence the generation time. Plating efficiency in plastic bottles inoculated with 100 cells was 70 to 80 percent.

Cytopathogenic effects were not observed in any passages of the 14 experiments. The cultures of HLN survived for 4 months with a maximum of six passages. These cells could be preserved with glycerol or dimethylsulfoxide (*6*). Cells could be regenerated after storage at –70°C for 2 months. However, HLN cells could not be regenerated beyond 1 week when stored at 4° to 6°C in growth medium containing 20 percent calf serum. Frequent examinations for contamination by mycoplasma, bacteria, and fungi were negative.

Cells from liver cancer induced by 2-FAA grew rapidly when initially cultured in glass prescription bottles. A maximum population of these cancer cells was reached after 11 days of growth, whereas the HLN failed to replicate when placed on glass in the primary passage. The maximum population of nodular hepatocytes cultivated on plastic was not attained until 16 days of growth.

The cultures of the HLN cells were composed predominantly of polygonal cells showing frequent cytoplasmic bridging. Occasional perinuclear aggregates of granules, multinucleated cells, and frequent cytoplasmic vacuoles were observed. When cultured on collagen-coated sponges, the HLN cells from monolayer culture assumed a much more organoid arrangement with the reproducible formation of ductlike structures (Fig. 1).

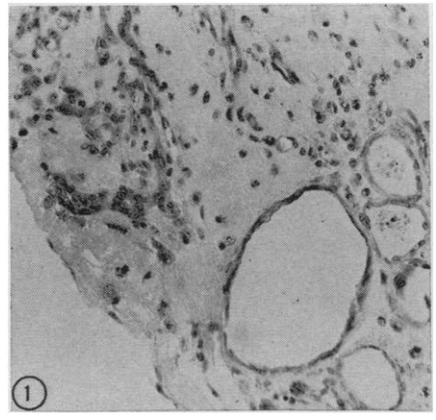

Fig. 1. Collagen-coated sponge culture of HLN cells, 8 days. Epithelial cells forming variable-sized ducts are evident. Hematoxylin and eosin (× 150).

Cells from HLN were prepared for electron microscopy (*8*). Survey electron micrographs (Fig. 2) of HLN cells following 11 days of growth in vitro showed that the organoid pattern observed in Epon-embedded 1-μ-thick sections was due to interdigitation of microvilli between adjacent cells, and to cellular attachment sites morphologically similar to "tight junctions" (*9*) (Fig. 2, inset). Frequently, where three HLN cells came into close apposition, a region simulating a bile canaliculus was observed (Fig. 2). Membranous arrays of occasionally dilated rough endoplasmic reticulum, vesicles of smooth endoplasmic reticulum, mitochondria, lysosomes, centrioles, perinuclear Golgi apparatus, and occasional delicate filaments were noted in the cytoplasm.

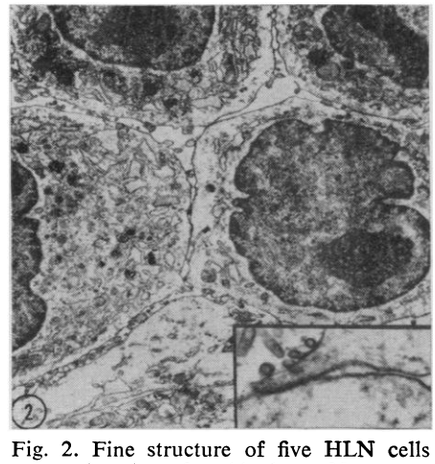

Fig. 2. Fine structure of five HLN cells grown in vitro for 11 days. Numerous microvilli of adjacent cells interdigitate and form canaliculus-like structures. An organoid pattern is evident. Uranyl acetate and lead citrate (× 4140). Inset shows a tight junction between adjacent cells (× 14,670).

These observations indicate that, as a tissue, the HLN has a growth potential in vitro different from both the nonnodular and nonmalignant liver and the 2-FAA-induced hepatocellular carcinoma. Histologically, the HLN is composed of hepatocytes, a few ductular components, and supporting tissue in proportions roughly comparable to normal liver with no obvious new type of cell (*2*). Thus, the cells which show the in vitro growth are probably one or more of these types which have acquired new biological properties. The most critical question relates to whether or not they are hepatocytes. The formation of ductlike structures and the epithelial-like growth including the presence of tight junctions (*9*) are suggestive of an origin from liver parenchymal or ductular cells. However, it must be emphasized that morphologic criteria for cell identification, especially in vitro, are not conclusive. More definitive identification must rest upon the analysis of the cultures for the presence or production of biochemical components considered to be typical of hepatocytes or other types of cells in liver.

The potential availability of an in vitro population of HLN cells offers new possibilities for exploring several facets of carcinogenesis. The mechanism of the successful replication of HLN cells in the presence of grossly distorted DNA (*2*), the comparison of the metabolism of continuously dividing nonmalignant HLN cells with that of a self-limiting replicating population (as in regenerating liver) and that of neoplastic population, and the development of a much more rapid screening procedure for potential hepatocarcinogens are but three such possibilities.

Finally, if the further study supports the identification of the HLN cells growing in vitro as hepatocytes, then the earliest cell showing obvious growth in vivo and in vitro during the carcinogenic regimen is not an overt malignant cell but a cell which has yet to acquire the acceptable earmarks of a cancer cell. Consistent with this formulation are the findings that the HLN have failed so far to transplant to liver or other sites under conditions in which hepatocellular carcinomas regularly do (*3*). This system might well enable the

separation of those biochemical properties of a malignant cell associated with growth from those related to invasion and metastasis.

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Prevention of Metamorphosis by Exposure of Insect Eggs to Juvenile Hormone Analogs

Abstract. *Metamorphosis of the bugs* Pyrrhocoris apterus *and* Oncopeltus fasciatus *is blocked by the application of juvenile hormone analogs to the eggs 4 weeks earlier. One or more supernumerary larval molts occur to form "giant" larvae which routinely die without undergoing metamorphosis. When the corpora allata were excised at the outset of the fifth larval stage, the entire phenomenon vanished and all individuals underwent normal metamorphosis. The inhibition of metamorphosis can therefore be attributed to a continuation of the secretion of endogenous juvenile hormone by the corpora allata of mature larvae derived from eggs treated with juvenile hormone.*

The capacity of juvenile hormone to block the embryonic development of insects was discovered by Sláma and Williams (*1*) in their study of the sensitivity of the eggs of the bug *Pyrrhocoris apterus* to the juvenile hormone analog "juvabione." Topical application of juvabione to mated females or to the freshly oviposited eggs prevented the eggs from hatching. In both cases embryonic development proceeded to a certain stage and then stopped. Similar effects have been observed after application of other juvenile hormone analogs to the eggs of silkworms (*2*) and locusts (*3*).

Studies of the effects of juvenile hormone (JH) analogs on silkworm eggs (*2, 4*) provided the first indication that JH can also have latent effects which are realized days or weeks later during postembryonic development. In *Pyrrhocoris* and *Oncopeltus*, latent effects of JH are also seen. Deaths during larval life, especially at the time of molting, are prevalent among larvae hatching from eggs treated during the first 4 days after oviposition or from the eggs of females treated with JH (*2, 5, 6*). I report here that metamorphosis can be completely blocked by exposure of the late embryo to JH.

I used a mixture of JH analogs that was synthesized as described (*7*). The JH was dissolved in acetone (Mallinckrodt "Nanograde") and dispensed from a 30-gauge needle fitted with a polyethylene tip and sealed onto a 50-μl microsyringe (Hamilton Co.); the latter was operated by an Agla micrometer (Burroughs Wellcome & Co.).

Eggs were collected from mated female *Pyrrhocoris apterus* and *Oncopeltus fasciatus* and incubated at 27°C and 60 percent relative humidity under 17 hours of daily illumination. Specific amounts of JH were topically applied in 0.05 μl of acetone to eggs anchored on Scotch tape. Control eggs received either pure acetone or no treatment. The eggs were carefully removed from the tape and placed in petri dishes at 27°C. Unhatched eggs and egg shells were removed. A maximum of 20 hatched larvae were reared in each dish to which a cotton-plugged vial of water was added along with linden seed for *Pyrrhocoris* and milkweed seed for *Oncopeltus* (*8*). About a month later, adultoids and sixth instar larvae were separated according to type and reared until death.

Application of 2.5 μg of JH to *Pyrrhocoris* eggs 121 to 168 hours after oviposition (about 6 to 30 hours before hatching) was fully effective in preventing the metamorphosis of fifth instar larvae (Table 1). Nearly all molted into perfect sixth stage larvae, and, of these, 16 percent molted to perfect seventh stage larvae. All ultimately died without initiating metamorphosis. This dose is about three times the amount (0.8 μg) which, when applied to a freshly molted fifth instar, produced a supernumerary sixth instar larva (*7, 9*). Treatment of the eggs with a dose of 0.25 μg usually produced adultoids of types I through III (*10, 11*).

The latent effects of JH were studied by applying 1 μg of H^3-juvabione (*12*) to *Pyrrhocoris* eggs at hour 144 of incubation. After the material was homogenized in 15 ml of toluene scintillation fluid containing 10 percent Biosolve (Beckman Instruments, Inc.), the total radioactivity were measured in triplicate samples of eggs and of individual larvae after hatching and after each larval molt. Radioactivity showed a rapid decline and none was detectable after the molt to the third instar.

A conspicuous feature of embryonic development is the onset of eye pigmentation which occurs immediately after blastokinesis. In *Oncopeltus* pigmentation begins after 105 hours of incubation at 27°C, and hatching occurs after 156 hours; the corresponding values for *Pyrrhocoris* are 115 and 174 hours. Prior to blastokinesis the effects of JH were mainly on embryogenesis and larval development (*1, 2, 5*). Thus, when 0.25 μg of JH was applied to 20 *Oncopeltus* eggs at hour 84, four failed to hatch and two failed to complete the molt which occurs immediately after hatching. Five of the remaining larvae subsequenlty died during one of the larval molts.

When the exposure to JH was post-