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# Generation of High Yields of Syrian Hamster Cholangiocellular Carcinomas and Hepatocellular Nodules by Combined Nitrite and Aminopyrine Administration and *Opisthorchis viverrini* Infection

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**Combined administration of 0.1% nitrite and 0.1% aminopyrine in the drinking water for eight to ten weeks resulted in subsequent development of both hepatocellular nodules and cholangiofibrotic lesions/cholangiocellular carcinomas in Syrian golden hamsters. Additional prior dosing with *Opisthorchis viverrini* metacercariae (100/animal) induced inflammatory and proliferative changes in the livers of infected hamsters and was associated with a significant increase in yields of hepatocellular and cholangiocellular preneoplastic and neoplastic lesions. Thus, environmental factors thought to be casually related to the high levels of human liver cancer observed in the Northeastern provinces of Thailand were sufficient to bring about development of equivalent tumors in experimental animals. The results indicate that parasite associated liver injury and non-specific compensatory regeneration may play an important role in generation of both hepatocellular and cholangiocellular carcinomas in man.**

Key words: Liver flukes — Liver cancer — Nitrite and aminopyrine

The wide variation in levels of liver cancer incidence in different parts of the world[1] is considered to be largely due to the presence or absence of environmental factors responsible for the development of the disease. Thus, attention has been concentrated on the influence exerted by cirrhotic proliferative lesions caused by viral hepatitis, chronic alcohol abuse and mycotoxin or other carcinogen contamination.[1-3] Although possible integration of viral DNA and exposure to aflatoxins or nitrosamines may have direct 'initiating' potential, non-specific toxic and regenerative lesions have been assumed to play an additional role in determining populations at risk of developing liver cancer. For example, in Thailand where both hepatocellular and cholangiocellular neoplasms are very common[4, 5] an association between liver fluke infestation and the latter type of tumor has long been established for man[6-9] and also experimentally in the Syrian golden hamster.[10-13]

Furthermore, earlier pointers to a link between opisthorchiasis and hepatocellular lesions[5, 14] have been supported by more recent experimental and epidemiological data.[15, 16] Infestation with the parasite *Opisthorchis viverrini* is responsible for similar histopathological changes in both Syrian hamsters and man[17, 18] and the susceptibility of this animal species to various hepatocarcinogenic nitrosamines[19-21] presents advantages for its use in experimental models of liver neoplasia. In Thailand exogenous or endogenous nitrosamines have been demonstrated to be formed from nitrates and nitrites in the local food[22, 23] and it is well known that secondary amino compounds can react with nitrites to give rise to potent carcinogens, including dimethylnitrosamine (DMN), in the stomach.[24, 25] In particular, aminopyrine and sodium nitrite administration has been demonstrated to cause cholangiocellular tumor development in hamsters.[26] The present investigation was performed with the aim of assessing any possible potentiation of aminopyrine/nitrite carcinogenesis by infection with *Opisthorchis* liver flukes.

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## Materials and Methods

Cyprinoid fish harboring metacercarial cysts were purchased from market places and towns of Northeastern Thailand. The muscles from the base of the pectoral fins and tail, as well as the fins and tails themselves, were removed and digested in pepsin solution overnight at 37°. The whole digested content was then filtered, washed and resedimented several times. Cysts of *Opisthorchis viverrini* were identified under a dissecting microscope and collected.

A total of 150 male Syrian hamsters (Armed Forces Research Institute of Medical Science, Bangkok, Thailand) aged 3 to 4 weeks at the commencement were maintained 5 to a cage in a temperature controlled room at 27°. They were fed a stock diet (Zuelling, Gold Coin Mills PTE, Ltd., Singapore) and tap water *ad libitum* throughout the experimental period. The animals were divided into eight groups: group 1, untreated; group 2, 0.1% sodium nitrite (N) in the drinking water; group 3, 0.1% aminopyrine (4-dimethylaminoantipyrine) (A) in the drinking water; group 4, N+A; group 5, 100 *Opisthorchis viverrini* metacercariae (P) by single intragastric intubation of a suspension in saline; group 6, P+N; group 7, P+A; group 8, P+N+A. In the relevant groups, administration of nitrite and/or aminopyrine (both chemicals from Sigma Chemical Company, St. Louis, MO) freshly prepared daily, was commenced four weeks subsequent to dosing with parasites and was continued for 8 or 10 weeks, the animals then being returned to the basal diet. Hamsters receiving the 8-week treatment were sacrificed 12 weeks later and animals receiving the chemicals for 10 weeks, 20 weeks later. Hamsters which became moribund and were sacrificed before this final time point were also included in the effective numbers of animals investigated histopathologically. After complete autopsy, the livers and other organs were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 4 $\mu$m and stained with hematoxylin and eosin (H-E) as well as Alcian blue/PAS for mucins.[27] Incidence data for preneoplastic and neoplastic lesions in the livers from the two experiments (8 or 10 weeks of chemical administration) were combined and statistically compared using $\chi^2$. No tumor development was observed in other organs.

## Results

Experimental data including animal grouping, body and liver weights (the latter expressed as percentage of body weight) and incidences of hepatocellular nodules, cholangiofibrosis and cholangiocellular carcinomas are given in the table. Significant increases for lesions of both cell types (hepatocyte and ductal/ductular) were evident in the P+N+A groups as compared to the N+A alone group. Hepatocellular lesions were limited to hamsters receiving both nitrite and aminopyrine and included clear (glycogen-storing) and basophilic (Figs. 1 and 2) foci and mixed cell or basophilic nodules (Fig. 3) demonstrating compression of the surrounding parenchyma. PAS staining revealed the clear and mixed cell lesions to be rich in glycogen. Non-specific depletion of glycogen and variation in the extent of intrahepatic bile duct changes, however, precluded accurate assessment of focus development and therefore attention was concentrated on the well-defined nodules.

With the exception of small cysts composed of flattened bile duct cells evident in all

Table I. Quantitative Data for Animal and Liver Weights and Neoplastic/Preneoplastic Lesions

| | Effective No. animals | Body weight (g) | Liver % body weight | Incidence (%) data | | |
|---|---|---|---|---|---|---|
| | | | | Cholangiocellular Carcinoma | Cholangiofibrosis | Hepatocellular nodules |
| Control | 15 | 171±15 | 4.6±0.2 | 0 (0) | 0 (0) | 0 (0) |
| N | 15 | 135±25 | 4.5±0.7 | 0 (0) | 0 (0) | 0 (0) |
| A | 15 | 167±24 | 4.7±0.9 | 0 (0) | 0 (0) | 0 (0) |
| N+A | 17 | 118±21 | 7.4±2.1 | 3 (18) | 7 (41) | 2 (12) |
| P | 14 | 126±15 | 4.6±1.0 | 0 (0) | 1 (7) | 0 (0) |
| P+N | 13 | 150±19 | 5.3±1.1 | 0 (0) | 2 (15) | 0 (0) |
| P+A | 14 | 139±18 | 4.5±0.5 | 0 (0) | 1 (7) | 0 (0) |
| P+N+A | 18 | 115±11 | 8.3±1.7 | 14 (78)** | 18 (100)* | 8 (44)* |

Significantly different from N+A values: * $P<0.05$, ** $P<0.01$.

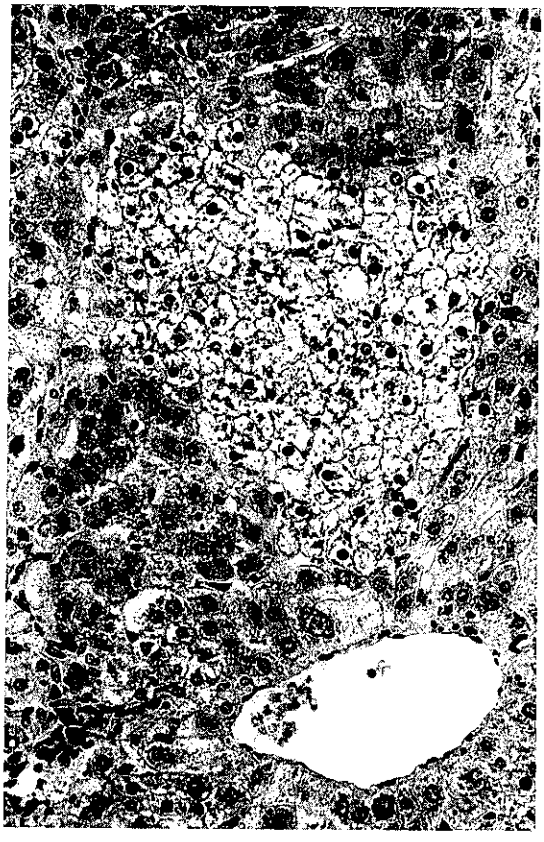

Fig. 1. Clear cell (glycogen storing) focus induced by aminopyrine and nitrite administration. H-E ×250.

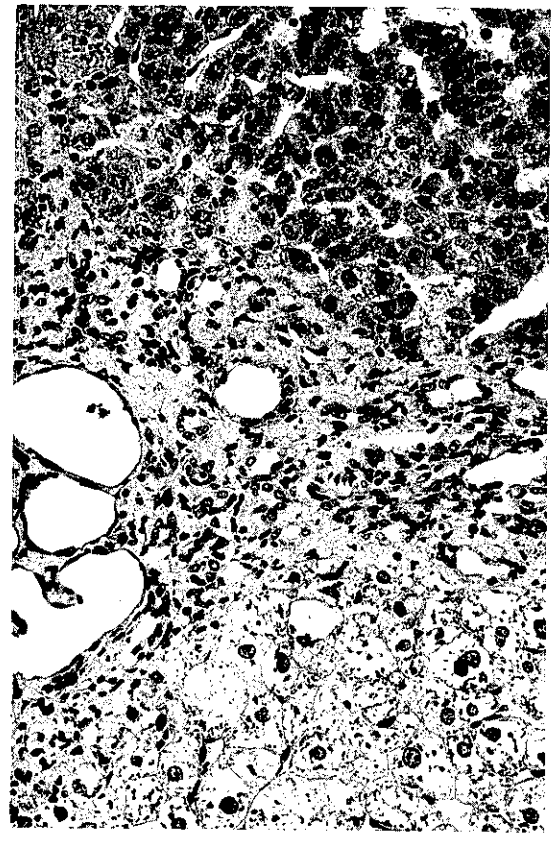

Fig. 2. Basophilic (upper right) and clear cell (bottom) foci observed adjacent to a flattened cyst. H-E ×250.

groups, including untreated controls, proliferative cholangiocellular lesions were limited to hamsters receiving parasites and/or both nitrite and aminopyrine. These included extensive areas of cholangiofibrosis (see Fig. 4), especially in the parasite-treated animals where duct atypia and first-order ductular proliferation (Fig. 3) were also pronounced. Essential similarities were observed between the cuboidal or columnar epithelium exhibiting goblet cell metaplasia in cholangiofibrotic lesions and the larger, locally invasive cholangiocellular carcinomas (Fig. 5). In the latter, however, the collagenous connective tissue elements were better developed, cellular and nuclear atypia were common and areas of epithelium lacking mucous production were also observed. Liver cell nodules could be clearly distinguished from background parenchyma (Fig. 6). Examination of lung and other organ tissue did not reveal the presence of metastases in any case.

## Discussion

The results of the present investigation confirm and extend the earlier report of cholangiocellular lesion development to include hepatocellular nodules after administration of combined aminopyrine and nitrite[26] to Syrian hamsters, while clearly demonstrating the potential of infestation with fluke parasites for promotion of liver tumor induction in both hepatocellular and cholangiocellular compartments. The fact that lesions of both types were induced suggests that a mixture of

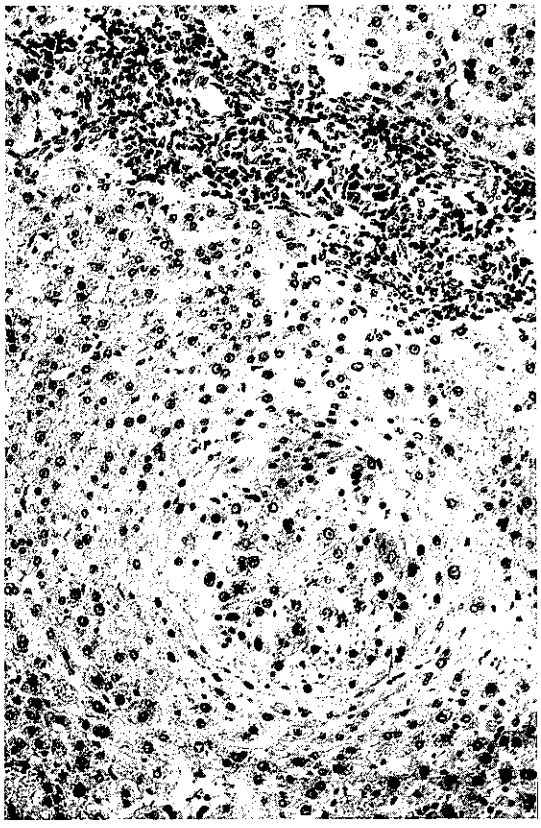

Fig. 3. Edge of a mixed cell nodule demonstrating compression of the surrounding parenchyma. Note parasite-induced first-order ductular proliferation (above). H-E ×150.

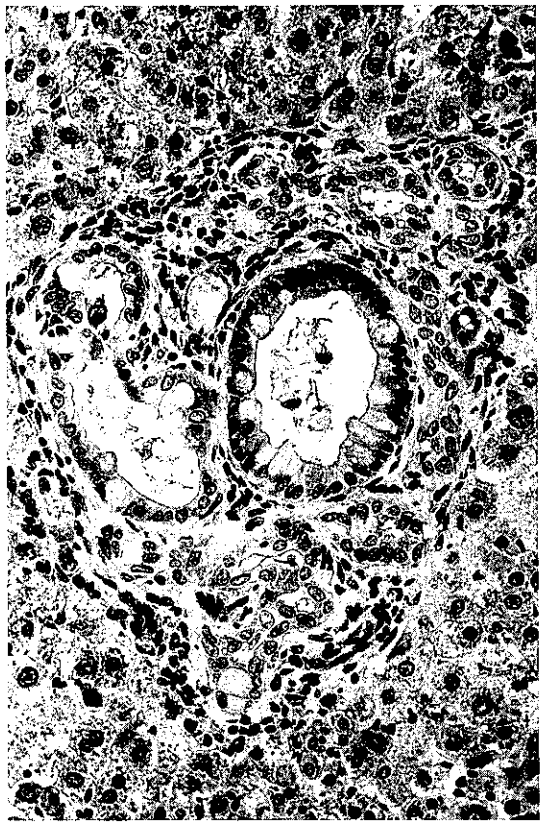

Fig. 4. Small area of cholangiofibrosis in a hamster given parasites and combined aminopyrine and nitrite. Note goblet cells within the atypical epithelium. H-E ×250.

carcinogens may be formed from nitrite and aminopyrine in the gut since the DMN which is reported to be produced *in vivo*[25] has usually only been associated with cholangiocellular carcinoma development in the Syrian hamster.[11, 13] However, low yields of hepatocellular nodules have been reported by one group after DMN administration[28] and thus this may only be a question of dose. While target cell specificity might differ between species as indicated by the preferential induction of hemangiosarcomas in rats administered aminopyrine and nitrite orally[29] the hamster hepatocyte does appear to be susceptible to a wide range of carcinogens.[20, 21, 30]

The finding of a direct association in the present experiments between cholangiocellular carcinoma and cholangiofibrosis development in the hamsters administered carcinogen precursors and parasites suggests an essentially similar histogenesis of intrahepatic bile duct neoplasms to that reported earlier for the rat.[31–33] Thus, Bannasch and coworkers[31, 32] revealed a sequence leading from early ductular proliferations through mucous cholangiofibrosis and benign cholangiomas to malignant carcinomas. This is in agreement with the conclusion drawn from results of an earlier investigation of carcinogen and parasite dose-dependency in the Syrian hamster model.[13] The abundant PAS and Alcian/blue-positive goblet and other mucin-secreting cells evident within the presently described cholangiofibrotic areas are presumably equivalent to the cholangiolar mucopolysaccharidosis described previously after carcino-

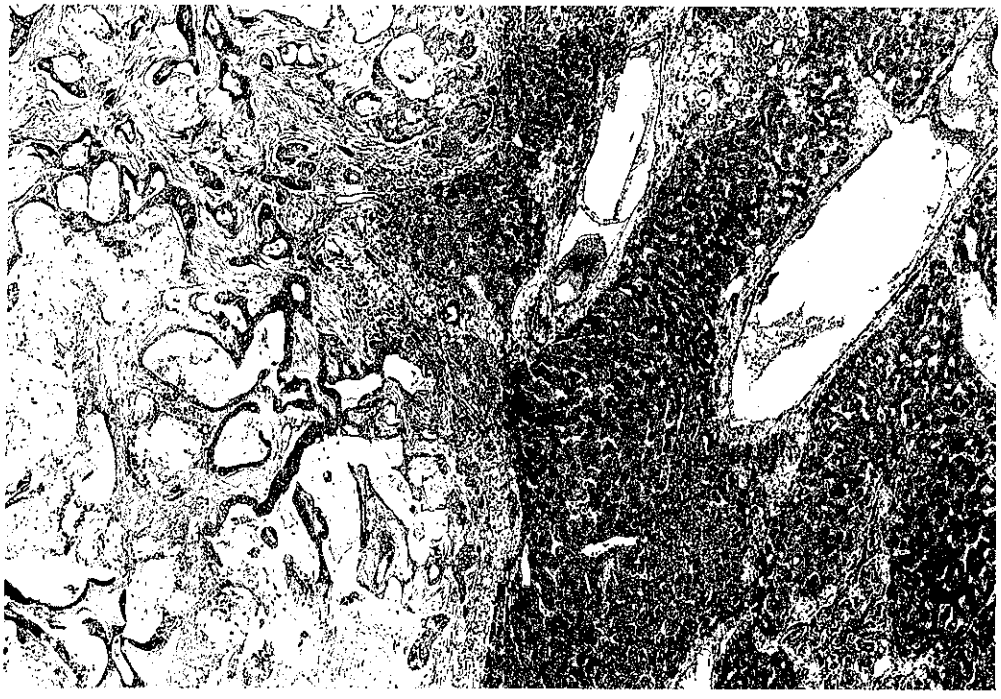

Fig. 5. Interface between a cholangiocellular carcinoma and background parenchyma demonstrating areas of ductular proliferation. H-E ×40.

genic[31–33] but not non-carcinogenic insult[34] to bile duct/ductular cells. While the essential significance of transient intestinal metaplasia for gastrointestinal neoplasia remains unclear (see reference 35 for discussion) the fact that a similar alteration of mucopolysaccharide metabolism is evident in human cholangiocellular lesions[36] suggests its general importance. A transient increase in glycogen has also been well documented in preneoplastic foci and nodules during rat hepatocarcinogenesis[31, 37] and the clear, mixed and basophilic cell lesions described here and in earlier publications dealing with hamster liver[16, 28] indicate essential similarities between the process of liver tumor induction in the hamster and rat.

Although it remains unclear whether direct physical trauma or chemical mediation is responsible for the damage and regenerative lesions observed in the duct system after dosing with *Opisthorchis viverrini*, a role for proliferation in the documented promotion of neoplasia appears unequivocal. Since the hamsters were infected prior to carcinogen application, effects at both 'initiation' and secondary 'modulation' levels[38, 39] might be expected. Replicative synthesis before chemically-induced macromolecular lesions can be repaired has been considered to play an important if not essential role in initiation of tumor development[40] and since parasite infestation in the hamster brings about early proliferation of both intrahepatic bile duct cells and hepatocytes[17] this could result in an enhancement of carcinogen effectiveness at the initiation level, during the period of carcinogen formation from nitrite and aminopyrine. On the other hand, the continued presence of the parasite might act as a secondary promoting stimulus through chronic increase in cell turnover in the liver. By analogy with reported hepatocellular lesion promotion by repeated partial hepatectomy or chemically-induced necrosis,[41–43] continued proliferation within the ductular/ductal tree might exert a non-specific enhancing stimulus for cholangiocellular lesions, whereas necrosis and re-

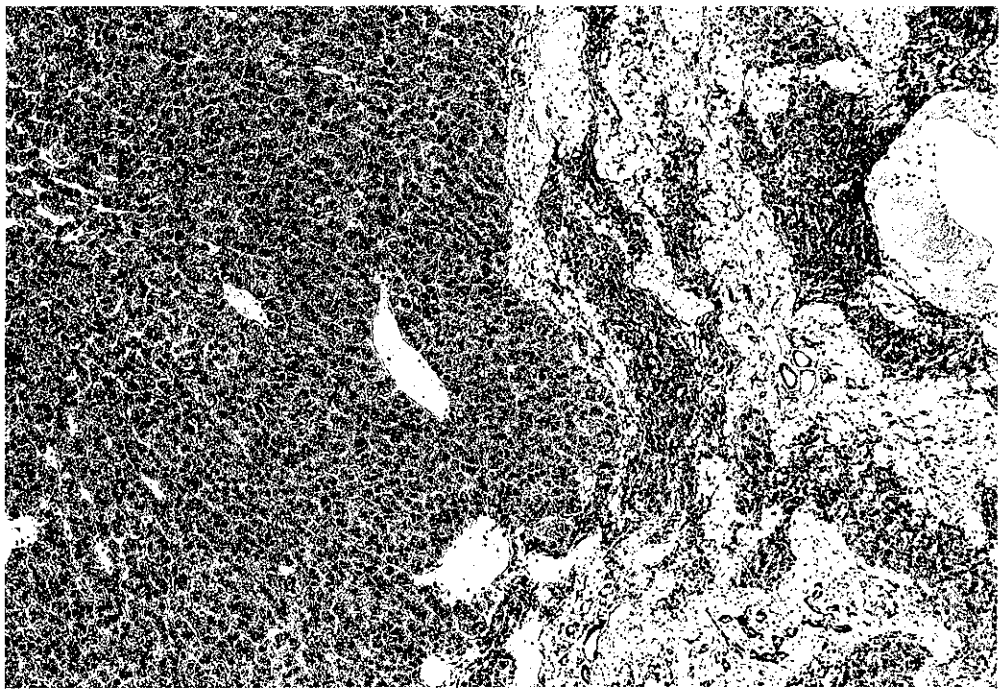

Fig. 6. Overview of liver tissue from an *Opisthorchis viverrini*-infected hamster treated with aminopyrine and nitrite. Note the distinction between expansively growing nodule (left) and liver parenchyma broken by fibrotic ductular proliferations. H-E ×40.

generation of parenchymal cells brought about by expanding cholangiofibrosis would have the same effect on hepatocellular foci and nodules. The finding of a strong positive association between chronic intrahepatic cholangitis and bile duct cancer in man[44, 45] and the evidence of hepatocellular carcinoma in conjunction with primary biliary cirrhosis[46] viewed in the light of the accepted links between cirrhosis and liver cancer[2, 3] might imply a general significance for chronic proliferation in tumor development in this organ for both man and experimental animals.

In conclusion, the present data strongly suggest that the removal or reduction of carcinogen precursors and *Opisthorchis* parasite contamination from the environment in Thailand might result in a parallel reduction of both hepatocellular and cholangiocellular neoplasms in man. The Syrian hamster model appears an appropriate vehicle for future research into the interdependency of contributory factors and the efficacy of potential control measures.

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