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Experimental Study of the Effects of *Clonorchis sinensis* Infection on Induction of Cholangiocarcinoma in Syrian Golden Hamsters Administered 0.03% N-2-fluorenylacetamide (FAA)

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Key words: liver fluke, *Clonorchis sinensis*, liver tumor, cholangiocarinoma, carcinogenicity, N-2-fluorenylacetamide (FAA)

Introduction

Association of liver tumors, especially cholangiocarcinoma, with *Clonorchis sinensis* infection in human cases has been reported by Katsurada (1897), Ch'in (1955), Hou (1956), Chou *et al.* (1976), Purtilo (1976) and many other investigators, but the factors governing the association remain uncertain. In Hong Kong, an endemic area of *C. sinensis* (Gibson and Sun, 1971), cholangiocarcinoma is often found in association with *C. sinensis*, which is thought to be an important etiological factor in the induction of the cholangiocarcinoma (Hou, 1956; Purtilo, 1976). Hou (1956) has observed the direct transformantion of hyperplastic bile-ducts to cholangiocarcinoma in human clonorchiasis and reported that all transitional stages in the development of carcinoma are clearly demonstrable. Experimental studies by Thamavit *et al.* (1978) have shown that *Opisthorchis viverrini*-infected hamsters fed the hepatocarcinogen, dimethylnitrosamine invariably develop cholangiocarcinoma, whereas non-infected hamsters fed the same carcinogen did not. This study suggests that there is a synergism between the liver fluke and carcinogen in the induction of the bile duct carcinoma; the presence of the parasite in the bile ducts possibly promotes a carcinogenicity of the biliary epithelial cells.

The present study deals with the effects of *C. sinensis* infection on the induction of cholangiocarcinoama with N-2-fluorenylacetamide (FAA), which is highly carcinogenic (Porta *et al.*, 1959; Miller *et al.*, 1964; Guthman *et al.*, 1972).

Materials and Methods

Preparation of metacercariae: Fresh-water fishes, *Pseudorasbora parva*, which haboured various kinds of metacercarial cysts, were taken from the basin of Nagdong River, an endemic area of *C. sinensis* in Korea (Dong, 1974). The fishes were cut into small pieces, which were digested in artificial digestive fluid for two hours at 37°C (Okabe *et al.*, 1970). This was then filtered and the sediment was washed, and resedimented, repeatedly. Metacercarial cysts of *C. sinensis* in the final sediment were identified under a stereoscopic microscope, and collected.

Animals and experimental procedure: A total of 110 female syrian golden hamsters (Kyudo Inc., Kumamoto, Japan) were used, at 8 to 10 weeks old at the beginning of the experiment. The animals were housed in plastic cages, at four per cage. They were divided into two groups; 60 *C. sinensis* infected-ani-

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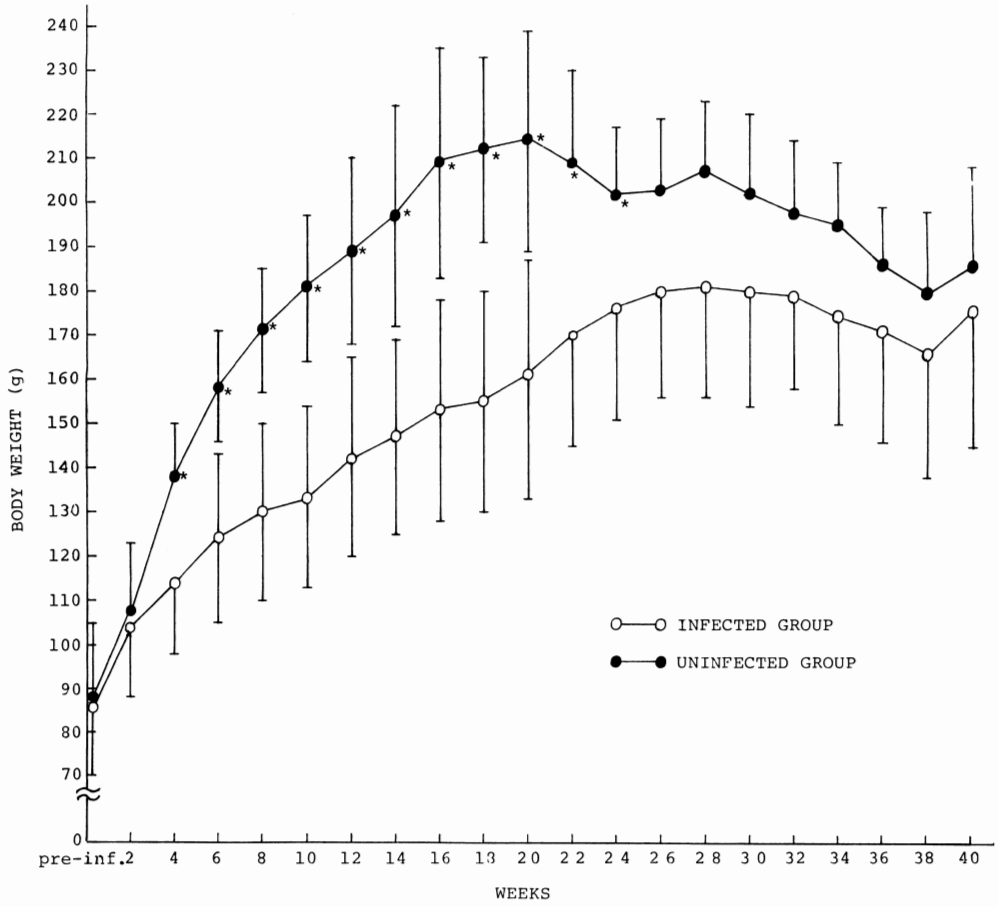


Fig. 1 Growth curves for infected and uninfected animals administered 0.03% FAA.
*Significantly different from the infected group ($p<0.05$)

mals, and 50 uninfected. The infected group was orally given with 40 metacercariae per hamster. Both groups were given similar experimental diet (CE-2, Clear Japan Inc., Tokyo, Japan) containing 0.03 % FAA (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) at the time of the infecting and this diet was continued for 40 weeks thereafter. Then, after 40 weeks, the surviving animals were fed the normal diet without FAA. All animals were weighed at two-week interval for 40 weeks after the administration of FAA.

All animals were autopsied or necropsied at certain intervals (see Table 1) throughout the experimental period. Nine animals lost due to cannibalism were excepted. As a result of the macroscopic examination of internal organs, especially the liver, then worms were recovered from the extra- and intra-hepatic bile ducts and the gall bladder of infected animals, and were counted. The internal organs of all animals were fixed in 10 % buffered formalin, and representative sections were embedded in parafin for the histological study. Tissue sections were stained with hematoxylin and eosin, and periodic acid Shiff (PAS).

Results

Animal body weight : Body weight changes of the infected, and uninfected, hamsters (mean±S. E.) for 40 weeks after the administration of FAA are shown graphically in Figure 1. The animals in the infected group showed a lighter body weight than those in

Table 1 Incidence and appearance time of liver tumor and recovery worm number at various periods to autopsy

Period to autopsy (weeks)	Infected group			Uninfected group	
	No. of hamsters examined	Mean No. of worms recovered	No. of hamsters with liver tumor	No. of hamsters examined	No. of hamsters with liver tumor
2- 4	4	26(19-29)*	0		
5- 8	4	25(18-32)	0		
9-12	8	27(14-31)	0	3	0
13-16	9	23(11-30)	0	3	0
17-20	4	24(16-28)	0	5	0
21-24	10	22(12-31)	0	20	0
25-28	2	21(16-25)	1	4	0
29-32	1	16	1	1	0
33-36	2	13(9-16)	2(1)†	2	1
37-40	2	13(10-15)	1(1)	1	1
41-44	2	10(7-12)	2	4	1
45-48	2	14(13-14)	2(1)	1	1
49-54	3	13(11-16)	2(2)	4	2
Total	53		11(5)	48	6

* Range of worms recoverd.
† No. of hamsters with metastasis.

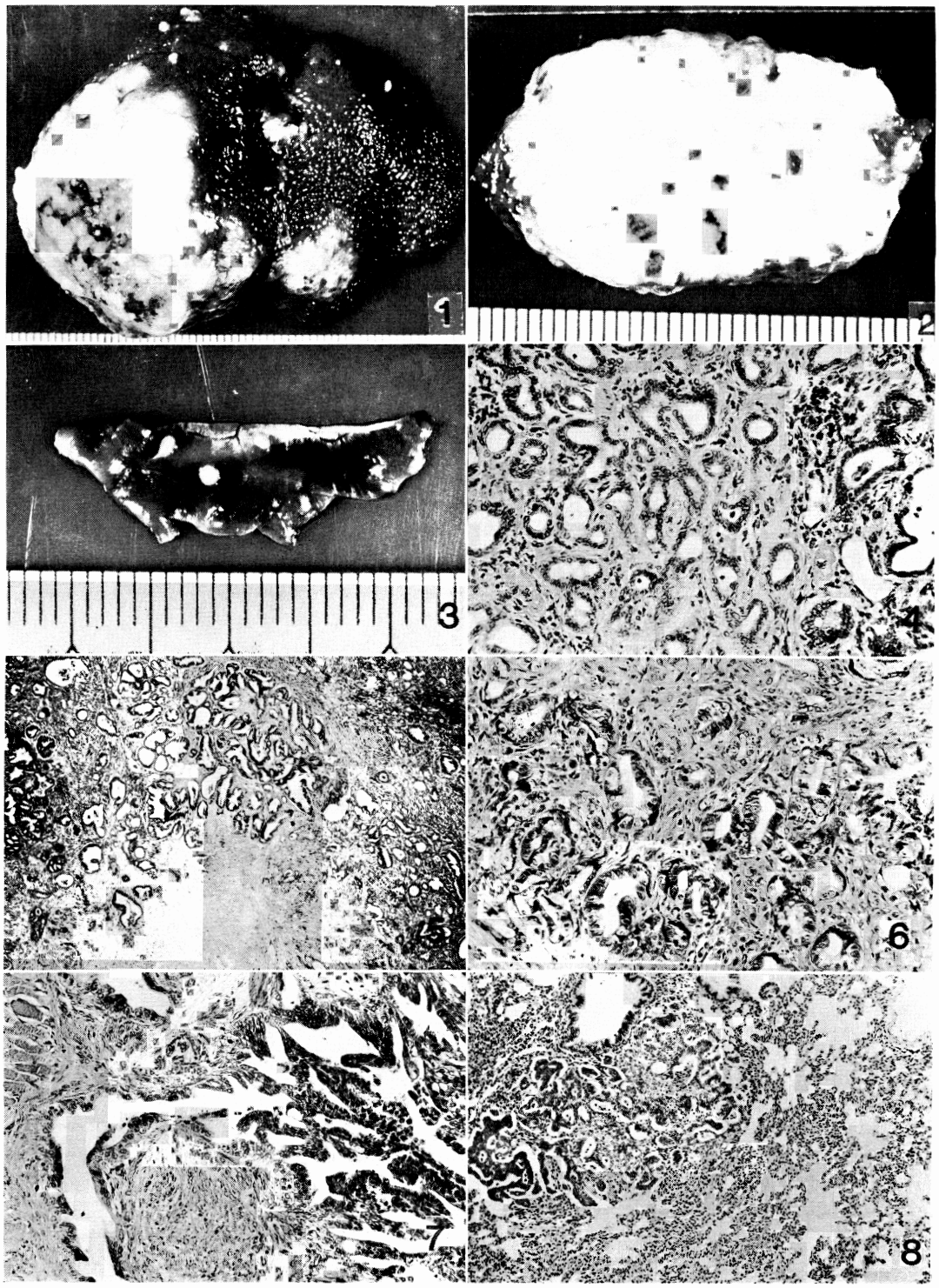
Table 2 Incidence of liver tumor in the period from 25 to 54 weeks during which the liver tumors were observed

Period to autopsy (weeks)	Infected group		Uninfected group	
	No. of hamsters examined	No. of hamsters with liver tumor	No. of hamsters examined	No. of hamsters with liver tumor
2-24	39	0	31	0
25-54	14	11(78.6%)*	17	6(35.3%)
Total	53	11(20.6%)	48	6(12.5%)

* Significantly different from uninfected group ($p<0.05$).

the uninfected group during the 40 weeks, and the growth rate was significantly lower in the infected group than in the uninfected group, during 4 to 24 weeks.

Incidence and appearance time of liver tumor: The incidence and appearance time of liver tumors in infected, and uninfected animals are summarised in Tables 1 and 2, together with the presence, or not, of metastasis and the recovery worm number. In both the infected and the uninfected groups, liver tumors were noted not until 24 weeks. One animal autopsied at 25 weeks in the infected group was first to have developed any liver tumor. Overall then liver tumors were observed in 11 (20.6 %) of 53 animals by the end of the experimental period (54 weeks). In the uninfected group, the first liver tumor was observed in one animal autopsied at 35 weeks, and totally 6 (12.5 %) of 48 animals had developed a tumor by 54 weeks. The incidence rate of liver tumors in the total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period of 54 weeks was not significantly different between the infected and the uninfected group. However, the incidence rate between 25 to 54 weeks, when the liver tumors were mainly noted, was significantly higher in the infected group (78.6%) than compared with in the uninfected group (35.3 %).

Metastses of tumor were present in the infected group, but not in the uninfected group. Of 11 animals with liver tumors, then 5 (45.6 %) had some metastases to diaphragm, retroperitonium, and to lungs.

There was no significant correlation between the worm number and the incidence of liver tumors.

Macroscopic findings: In the infected group, animals showed characteristic gross changes other than liver tumors in the bile ducts and livers caused by the infection with *C. sinensis* after 5 weeks. The extra- and intra-hepatic bile ducts were markedly dilatated, and the surface of the livers with jaundice was irregular and nodular with small white firm areas. On the contrary, cysts with mucus measuring 0.5 to 1.0 cm were seen on the surface of the livers with or without liver tumors after 20 weeks in both groups. However, the cystic lesions in the infected group were smaller in size and less in number in comparison with those of the unifected group.

The liver tumors noted in the two groups showed closely similar gross features. The tumors were greyish-white and firm or cirrhotic with mucus on the surface. Most of the tumors showed a massive growth and occupied one lobe of the liver small satellite nodules on other lobes (Photo. 1). On a cut surface, the tumors revealed abundant mucus and included areas of necrosis, sometimes with hemorrhage (Photo. 2).

In the infected group, disseminated metastases of the tumor to the diaphragm and retroperitoneum, identified by microscopic examination, were noted. Greyish-white and firm nodules measuring about 0.2 cm similar to the appearnce of primary tumors, were scattered on the tissues (Photo. 3). The metastasis to lungs was not macroscopically detected.

Ascites were seen in 23 (43.4 %) of 53 animals in the infected group and in 3 (6.3 %) of 48 animals in the uninfected group. The animals with tumors generally showed a tendency to bear large amounts of ascites.

Microscopic findings: As shown in Table 4, the histological changes in the livers induced with FAA were classified into four grades by degree of mainly biliary changes according to the criteria (Table 3), which were based on the scheme of Edwards and White (1941), Stewart and Snell (1954), and Reddy *et al.* (1977). Grade 1 generally showed a slight degree of bile ductle proliferation in the infected group, whereas in the uninfect-

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Photos. 1-8 are of infected animals

Photo. 1 A greyish-white and firm cholangiocarcinoma showing a massive growth, occupying one lobe of the liver with small satellite nodules in all lobes, and hemorrhage on the surface.

Photo. 2 Cut surface of the liver of Photo. 1, showing abundant mucus, and areas of necrosis and hemorrhage.

Photo. 3 Metastasis to diaphragm showing greyish-white and firm nodules similar to an appearance of the same primary tumor.

Photo. 4 Microscopic appearance of cholangiofibrosis consisting of regular ducts or glands surrounded with low dense fibrous stroma. H. and E. ×100.

Photo. 5 Microscopic appearance of cholangiocarcinoma with necrotic areas. The tumor consists of various-sized irregular ducts and glands surrounded with uniformly dense fibrous stroma, and shows an invasive growth pattern. H. and E. ×40.

Photo. 6 Higher magnification of a portion of Photo. 5. The neoplastic cells show stratification, high nuclear cytoplasmic ratio, nuclear hyperchromatism, prominent nucleoli and abundant mitotic figures. H. and E. ×200.

Photo. 7 Microscopic appearance of metastasis on the diaphragm shown in Photo. 3. H. and E. ×100.

Photo. 8 Microscopic appearance of metastasis in lungs. H. and E. ×50.

Table 3 Criteria for grade of histological changes in the livers of experimental animals

Feature	Grade-1	Grade-2	Grade-3	Grade-4*
Bile ductle proliferation	− or +	+ or ++	++	+++
Cystic and glandular lesion	− or +	+	++	+++
Glandular pattern	Regular	Regular	Irregular	Irregular
Invasion	−	−	−	+++
Stratification	−	−	+	+++
N/C ratio	−	−	+	+++
Mitosis	−	−	+	+++

* Grade-4 is cholangiocarcinoma.
− : negative ; + : slight ; ++ : moderate ; +++ : severe

Table 4 Grade of histological changes in the livers of experimental animals at various periods to autopsy

Period to autopsy (weeks)	Infected group					Uninfected group				
	No. of hamsters examined	No. of hamsters with Grade No.				No. of hamsters examined	No. of hamsters with Grade No.			
		G-1	G-2	G-3	G-4*		G-1	G-2	G-3	G-4*
2- 4	4	4								
5- 8	4	4								
9-12	8	4	2	2		3	3			
13-16	9	1	3	5		3	3			
17-20	4		1	3		5	5			
21-24	10		2	8		20	2	18		
25-28	2			1	1	4	1	3		
29-32	1				1	1		1		
33-36	2				2	2			1	1
37-40	2			1	1	1				1
44-44	2				2	4			3	1
45-48	2				2	1				1
49-54	3			1	2	4			2	2
Total	53	13	8	21	11	48	14	22	6	6

* Grade 4 is cholangiocarcinoma.

ed group, there was usually a slight degree of cystic lesions lined by flattened cells. The cysts were mostly empty, and occasionally contained a fine granular material. In addition, proliferation of oval cells was seen in the portal areas in both groups (Emmanuel, 1956 ; Minase *et al.*, 1975). In grade 2, the cystic lesions and bile ductle proliferation were more evident, and glandular lesions were also observed. The glands, which formed pseudotubules with histological characteristics of the bile duct, were lined by irregularly arranged epithelial cells with respectively large nuclei. The bile ductle proliferation and glandular lesions were prominent in the infected group, while the cystic lesions were prominent in the uninfected group. In grade 3, similar lesions to those observed in grade 2

were seen in more extensive areas than in grade 2 and furthermore, cholangiofibrosis was noted, consisting of regular ducts or glands surrounded with low dense fibrous stroma. The cells lining the ducts or glands showed large nuclei resembling the nuclei of oval cells, and also a higher nuclear cytoplasmic ratio than those of normal bile ducts (Photo. 4). The cholangiofibrosis was prominent in the infected group. Grade 4 corresponded with cholangiocarcinoma.

All liver tumors noted in both groups were mucus-producing cholangiocarcinoma which fulfilled propounded histological criteria (Reddy *et al.*, 1977). The liver tumors were composed of various-sized irregular ducts and glands surrounded with uniformly dense fibrous stroma, and showed an invasive growth pattern (Photo. 5). Most of the tumors contained necrotic areas, sometimes with hemorrhage (Photo. 5). The neoplastic cells lining the ducts and glands were generally cuboidal and columnar in form, and showed stratification, a high nuclear cytoplasmic ratio, nuclear hyperchromatism, prominent nucleoli and abundant mitotic figures (Photo. 6). Sometimes, goblet cell metaplasia was present in the glandular epithelium. In the glandular lumens, amorphous and eosinophilic material, with or without fragmented neutrophils and mononuclear cells, was observed. Histochemically, it was a periodic acid Schiff-positive material and was seen especially in those glands with goblet cell metaplasia. Small nodules macroscopically found on the diaphragm and retroperitoneum, in the infected group, showed the same histological features as the primary liver tumor, and also metastasis to the lungs was detected in two of the infected animals (Photo. 7 and 8).

Liver parenchyma was progressively degenerated by the development of biliary lesions subsequently after the experimental period, so that it was greatly obliterated in some lobes. This was prominent in the infected group. Liver cells of the intact parencymal tissues showed megalocytosis and eosinophilic intranuclear bodies of various sizes and occasionally large enough to occupy the nucleus almost completely. Although hyperplastic nodules of liver cells, types 1 and 2 (Tsuda *et al.*, 1979), were noted in some animals in both of the groups, hepatocellular carcinoma was not found.

In the infected group, besides the lesions described above, the histological changes of the bile ducts which are characteristic of clonorchiasis were observed. Epithelial hyperplasia, marked dilatation, and periductal fibrosis, were noted in the large bile ducts associated with either pericholangitis or cholangitis. Bile ductle proliferation associated with periportal fibrosis, together with the connection between portal areas by fibrous bands, gave the appearance of multi-lobular cirrhosis in most hepatic lobes, from 9 weeks after the infection.

Discussion

Cholangiocarcinoma associated with clonorchiasis has been reported by many investigators, and the interrelationship has been suggested but not satisfactorily proved. Cholangiocarcinoma has been observed (Hou, 1964 and 1965) in cats and dogs which have been experimentally or spontaneously infected with *C. sinensis*, and it has been concluded that *C. sinensis* had acted as a causative agent in inducing the liver carcinoma. However, these results do not elucidate the relationship between *C. sinensis* and cholangiocarcinoma. The interaction between a liver fluke, *O. viverrini*, and carcinogen in hamsters, has been studied and evidence put forward that the liver-fluke infection promotes the carcinogenesis of the intra-hepatic bile duct neoplasm induced with the carcinogen (Thamavit *et al.*, 1978). The evidence suggests that a close relationship exists between liver fluke and cholangiocarcinoma, though indirectly. There are few known investigations with regard to *C. sinensis*. The effects of *C. sinensis* infection on the induction of cholangiocarcinoma with FAA were accordingly investigated in the present study.

The results of the present study show that *C. sinensis* infection has two important effects on the induction of cholangiocarcinoma with

FAA ; (1) it increases the incidence of liver tumor, and ; (2) reduces the latent period for neoplastic formation.

The animals infected with *C. sinensis* and administered FAA (the infected group) showed a significantly higher incidence rate of tumors (78.6%) than the animals administered FAA only (the uninfected group) (35.3%) in the period from 25 to 54 weeks during which the tumors were observed (Table 2). As has been suggested (Thamavit *et al.*, 1978), this increase in the incidence rate of liver tumors in the infected group is considered to show that the combination of *C. sinensis* with FAA has a synergistic effect on the induction of cholangiocarcinoma. Moreover, it is notable that the metastases of the primary tumor to the other organs were detected in 5 of the 11 animals with the tumor in the infected group, though it is not established that the evidence is closely related to the synergism of *C. sinensis* with FAA. With reference to liver-fluke infection and bile-duct carcinoma, it may be that *C. sinensis*, and *O. viverrini*, act as a promoter in a carcinogenic process requiring an initiator (Flavell, 1981). However, it is not clear whether *C. sinensis* acted as promoter or initiator in the induction of cholangiocarcinoma.

The animals in both groups developed cholangiocarcinoma. There was a significant difference in the latent period for neoplastic formation. The tumor appeared 10 weeks earlier in the infected group than in the uninfected group ; in the infected group the first tumor was noted at 25 weeks and in the uninfected group at 35 weeks (Table 1). The difference in appearance time of the tumor suggests that the *C. sinensis* infection has an effect of reducing the latent period for neoplastic formation. Such a difference between the two groups was also noted in the real-time histological changes in the bile ducts. The animals in both groups revealed the same series of biliary lesions as observed (Porta *et al.*, 1959) in hamsters administered FAA ; epithelial hyperplasia, cystic and glandular lesions, and cholangiofibrosis. In the infected group, the high-grade biliary lesions were observed at an earlier stage compared with the uninfected group (Table 4). This evidence is presumed to indicate that a high turnover of hyperplastic biliary epithelial cells due to persistent stimulus of *C. sinensis* may increase their suscebility to action by FAA, and result in increasing the number of carcinogen-susceptible epithelial cells. This also gives an explanation for the reduction in the period for the neoplastic formation.

Although a histogenesis of cholangiocarcinoma associated with *C. sinensis* infection has not yet been clearly established, there are some reports suggesting possible precursors of cholangiocarcinoma : the hyperplasia of biliary epithelial cells (Hou, 1956), mucus cell hyperplasia and goblet cell metaplasia (Ch'in, 1955 ; Chou and Gibson, 1970 ; Chou *et al.*, 1977), and cholangiofibrosis (Reddy *et al.*, 1977). These precursors were observed in this study, but direct transformation of them to cholangiocarcinoma was not detectable. Both direct, and indirect transformation via intermediates, are being researched.

On the other hand, etiological factors such as nutritional, genetic, environmental and immunological as possibly (Flavell, 1981) are also involved in induction of the cholangiocarcinoma associated with *C. sinensis*. In regard to the nutritional factor, the infected animals showed significantly lower growth rate than uninfected ones (Fig. 1), and furthermore, 23(43.4 %) of the 53 animals beared ascites. These results are indicative of malnutrition in the infected animals. Such malnutrition due to the *C. sinensis* infection may develop the biliary lesions (Flavell *et al.*, 1980) and provide a favorable condition for tumorigenesis.

Summary

The effects of *Clonorchis sinensis* infection on induction of cholangiocarcinoma with N-2-fluorenylacetamide (FAA) were investigated using female syrian golden hamsters. The hamsters were divided into two groups ; one was infected with 40 metacercariae per hamster together with administration of 0.03 % FAA (infected group), and the other group

was administered 0.03 % FAA only (uninfected group). In both groups, cholangiocarcinoma was observed after 25 weeks of the administration. The incidence rate of the liver tumors was significantly higher in the infected group (78.6 %) compared with the uninfected group (35.3 %) in the period from 25 to 54 weeks during which the tumors were observed. Metastases to other organs were observed in 5 (45.6 %) of the 11 animals with liver tumors in the infected group, but not in the uninfected group. The first liuer tumor was noted at 25 weeks in the infected group and at 35 weeks in the uninfected group. Histologically, in the infected group, the high-grade biliary lesions due to FAA were observed at an earlier stage compared with the uninfected group. These results suggest that *C. sinensis* infection has two important effects on the induction of cholangiocarcinoma with FAA ; (1) it increases the incidence of the tumor, and ; (2) it reduces the latent period for neoplastic formation. It is also suggested that *C. sinensis* infection may provide a favorable condition of tumorigenesis in human clonorchiasis.

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N-2-fluorenylacetamide (FAA) 投与ハムスターにおける胆管癌発生に及ぼす肝吸虫感染の効果に関する実験的研究

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発癌剤を用いた胆管癌発生に及ぼす肝吸虫感染の効果を検索する目的で，肝吸虫感染動物と非感染動物に発癌剤を投与し，両者における胆管癌発生の頻度とその発生時期について比較検討した．

実験動物は8～10週令 Syrian golden hamster の雌を用い，これを肝吸虫メタセルカリア40隻を経口感染させた感染群 (53匹) と非感染群 (48匹) の2群に分けた．肝吸虫感染と同時に両群に0.03% N-2-fluorenylacetamide (FAA) の経口投与を開始し，40週間継続投与した．その後は，FAA を含まぬ飼斜を与え，54週まで観察した．

両群において，25週以後肝腫瘍の発生がみられた．肝腫瘍発生頻度は，肝腫瘍発生後の25週から54週の間において，感染群が78.6%，非感染群が35.3%で，両群間に有意の差を認めた．また，感染群においては肝腫瘍の発生した11例中5例 (45.6%) に他臓器への転移を認めたが，非感染群においては認めなかつた．肝腫瘍の発生時期は，感染群 (25週) が非感染群 (35週) に比較して10週早かつた．両群に認められた肝腫瘍はすべて組織学的に胆管癌であった．発癌前における FAA の影響による肝内胆管の変化は，感染群において，非感染群に比較して早期に悪性度の高い病変が認みられた．

これらの結果から，肝吸虫感染が FAA を用いた胆管癌発生に及ぼす効果として，発癌頻度を高め，発癌までの期間を短縮することが考えられる．