

## Lipofuscin pigment in adult *Clonorchis sinensis*

Seung-Yull Cho, Kye Yong Song\* and Bong-Jin Rah\*\*

*Departments of Parasitology, Pathology\* and Histology\*\*, College of Medicine,  
Chung-Ang University, Seoul 151, Korea*

### INTRODUCTION

When alive, adult *Clonorchis sinensis* is normally pinkish and transparent. The intestine, uterus, vitellaria and sex glands are plainly visible. However, some of adults may contain black pigment in their parenchyme, when collected from infected human cases. Adults with the black pigment are usually larger, and may have discontinuous vitellarian follicles. Such worms with black pigment could hardly be found in experimental infection especially when the infection period was relatively short.

In the past, the worms in the genus *Clonorchis* were once separated into two species; *C. sinensis* and *C. endemicus* (Looss, 1907). One of the differential characters of two species was whether the black pigments were present or not. However, such speciations were later denied by Kobayashi(1917); and larger adults with black pigment and with discontinuous vitellarian follicles were considered as either those of intra-specific variation or aged worms of *Clonorchis sinensis* (Kobayashi, 1917).

Though the earlier workers recognized the presence of the pigment, its nature and/or origin has never been studied in detail. Kobayashi (1917) mentioned that the pigment might have originated from yolk cells because the shell material in vitellaria closely resembled the black pigment in its morphology. Later, Faust and Khaw(1927) described the colour of adult *Clonorchis sinensis* as follows: "The flukes, when alive, are opalescent gray in color, but in bile fluid, usually golden brown. If they are allowed

to die *in situ* preceding autopsy, they absorb the bile pigment, which discolors them permanently and can not be washed out---" By these descriptions, it appeared likely that Faust and Khaw thought the black pigment was bile, and stained them just before death. Komiya(1966) mentioned that the minute black pigment granules in *Clonorchis* might be derived from host blood. In this connection, Chu *et al.*(1982) demonstrated that host blood is included in the diet of *C. sinensis*.

We paid attention to the nature of the black pigment of adult *Clonorchis sinensis* collected from infected human cases, and made some histochemical and electron microscopic observations to find its nature.

### MATERIALS AND METHODS

#### 1. Adult worm of *Clonorchis sinensis*:

Adult *Clonorchis* were collected from human cases either at autopsy or at surgical operations. The infected cases were all Koreans who had lived in different endemic localities. At the time of collection, adult worms moved actively in warm saline or in bile juice. The source of examined adults were shown in Table 1.

The collected adult worms were fixed either by cover glass pressure or at natural posture in 10% formalin.

#### 2. Histochemical stainings:

Parts of formalin-fixed *Clonorchis* which have black pigments, were embedded in paraffin, cut at 6 $\mu$ m, and stained by the following techniques. All of the stainings were followed by the methods described in Manual of Histologic

**Table 1.** The origin of adult *Clonorchis sinensis* from human cases

Case No.	Date of collection	Age (year) and sex	Method of recovery	No. of worms collected	No. of worms with pigment
1	October, 1968	34, male	Autopsy	4	4
2	July, 1971	40, male	Surgery	1,441*	16
3	November, 1973	22, male	Autopsy	462	0
4	February, 1977	39, male	Surgery	307	307**
5	June, 1981	26, male	Surgery	57	0
6	July, 1981	60, female	Surgery	2,954	Most of them

\* Out of 4,569 worms collected, 1,441 were examined in detail.

\*\* All of the worms were less pigmented; pigments were found at microscopic sections.

Methods of the Armed Forces Institute of Pathology (1960).

- 1) Haematoxylin-eosin staining
- 2) Gomori method for iron
- 3) Fontana Masson silver staining for melanin
- 4) Hall's method for bilirubin
- 5) Periodic acid-Schiff (PAS) staining
- 6) Amylase-PAS staining
- 7) Ziehl-Neelsen method for acid-fast bacteria
- 8) AFIP method for lipofuscin

If necessary, control tissues for relevant methods were stained concomitantly along with the *Clonorchis* section.

### 3. Electron microscopy:

A part of formalin-fixed *Clonorchis* which had conspicuous black pigment, was refixed with 2.5% glutaraldehyde in PBS (pH 7.4). The specimens were postfixed by 1% OsO<sub>4</sub> solution. The ultrathin sections were made, and stained by uranyl acetate and lead citrate. The sections were observed using JEOL-200CX transmission electron microscope.

## RESULTS

### 1. Distribution of black pigment in adult *Clonorchis*:

As shown in Fig. 1 in the Plate, the black pigment may distribute all over the body, however, the degree of pigmentation varied worm by worm. In less pigmented worms, the anterior and posterior ends were more pigmented than the central parts of the body.

By microscopic examination, the pigments

situated at parenchymal cells of loose connective tissue (Fig. 2) and of suckers outside the muscle cells. No pigment particles were observed at peripheral syncytial tegument, subtegumental cells, muscle cells, wall and lumen of uterus, vas deferens, intestine and excretory canal. The pigments were also not found at testes, ovary, vitellaria and seminal receptacle.

The black pigment particles were similar with granules in cytoplasm of vitelline cells, by their colour and shape, when observed by H & E staining. The particle size, colour and shape of black pigment varied in certain degree. The larger particles, in 4-9 $\mu$ m, were round, oval or elliptical and usually blackish; and they appeared to exhibit ill-defined lamination. The smaller particles, 1-2 $\mu$ m in radius, were yellowish and aggregated in cluster.

### 2. Histochemical stainings:

The stainability of the black pigment was shown in Table 2. The pigments were positive in lipofuscin (Fig. 3) and Ziehl-Neelsen AFB stainings (Fig. 4); and was weakly positive in PAS and amylase-PAS stainings. But, iron, bilirubin and Fontana Masson stainings were negative. The staining characteristics strongly indicated that the black pigments were clumps of lipofuscin.

The tissues of *Clonorchis*, other than the pigment, did not show positive reaction by prussian blue (Gomori method) and bilirubin (Hall's method). By Fontana Masson silver staining, however, vitelline cells and shell of eggs in uterus stained in black. By PAS staining,

**Table 2.** Histochemistry of the black pigment from adult *Clonorchis sinensis*

Staining techniques	Stainability in				Black pigment in <i>Clonorchis</i>
	Hemosiderin	Bilirubin	Melanin	Lipofuscin	
Haematoxylin & eosin	Golden brown	Yellow	Dark brown	Yellow	Yellowish brown
Prussian blue	+	—	—	—	—
Fontana Masson silver	—	—	+	—	—
Bilirubin	—	+	—	—	—
P A S	—	—	—	+	+
Amylase-PAS	—	—	—	+	+
Ziehl-Neelsen	—	—	—	+	+
AFIP for lipofuscin	—	—	—	+	+

parenchymal cells and parts of sex gland cells stained red in varying degree.

### 3. Electron microscopy:

As shown in Fig. 5, the pigment particles were scattered throughout the cytoplasm of a parenchymal cell. The numbers of particles varied cell by cell. Their sizes were in range of 1.2-3.3  $\mu\text{m}$  in cross, and their shapes were round to elliptical. The pigments have limiting membrane.

The ultrastructure of a pigment was shown in Figs. 6 and 7. Various sized electron dense granules aggregate at the centre of a particle (Fig. 6) or irregularly distributed throughout a particle (Fig. 7). Round, elliptical or polygonal shaped membrane structure (Fig. 8) varied in their size from 0.05-0.24  $\mu\text{m}$ .

## DISCUSSION

The findings from the histochemistry and from the electron microscopy clearly indicated that the black pigment in adult *Clonorchis* from human cases was lipofuscin (Porta and Hartroft, 1969; Trump and Jones, 1979). Unlike previously speculated, the pigments were not derived from host blood. The pigment was not melanin as well, but originated from lysosome of the worm.

Kobayashi(1917) was correct in considering that the black pigment was an aging product of long-lived adult *Clonorchis*. The lipofuscin pigment is believed to represent the indigestible residues of autophagic vacuoles of lysosomes, formed during aging and atrophy. It is derived

through lipid peroxidation of polyunsaturated lipids of subcellular membranes. Lipofuscin production is presumed from the lack of dietary anti-oxidants that help prevent auto-oxidation (Robbins and Cotran, 1979).

The histologic localization of lipofuscin pigment in *Clonorchis* is very difficult to explain. So far, the pigments did not exist in the vital tissues such as tegument and intestine, and in sex organs. Such preferential localization might be due to the fact that our specimens were all living worms moving actively in saline. There might be present dying worms which were heavily loaded with lipofuscin even at the vital tissues and sex organs. Though lipofuscin is believed to be innocuous material, it is also evident that lipofuscin is associated with dying process.

One of epidemiologic characters of clonorchiasis in Koreans is that most of cases began to be infected since 20 years old. Our specimens with lipofuscin were collected from aged people older than 34 years (Table 1). Therefore it is cautiously speculated that the pigment might begin to appear in at least 15 years or older worms. Lipofuscin might be a useful indicator in determining the relative age of *C. sinensis* collected from infected human cases.

## SUMMARY

Adult *Clonorchis sinensis* from infected human cases may have black pigment in their parenchyme. In present paper, the nature of the

pigment was investigated.

The pigment distributed in cytoplasm of parenchymal cells of the worms. Histochemically the pigment was positive by periodic acid-Schiff, Ziehl-Neelsen, and lipofuscin stainings. The pigment particle contained many, various sized membrane structure as well as electron dense granules when observed by electron microscopy.

Staining characteristics of the black pigment, together with electron microscopic findings, strongly indicated that the pigment was lipofuscin.

### ACKNOWLEDGEMENT

We thank to Associate Professor Je G. Chi of Seoul National University for his encouragement at the initial stage of this study; to Professor Y.T. Yang of Chung-Ang University for his review of manuscript. Dr. S.T. Hong provided us the worms from case No. 6.

### REFERENCES

- Chu, D.S., Jeong, K.H. and Rim, H.J. (1982) Studies on the blood intake activity by *Clonorchis sinensis* in experimental animals. *Korea Univ. Med. J.*, 19(1):71-80(in Korean).
- Faust, E.C. and Khaw, O.K. (1927) Studies on *Clonorchis sinensis* (Cobbold). *Am. J. Hyg.* (Monograph), 8:90.
- Kobayashi, H. (1917) On the life-history and morphology of the liver distome (*Clonorchis sinensis*). *Mitteilungen d. Med. Hochschule zu Keijo*, 1:251-284.
- Komiya, Y. (1966) *Clonorchis* and clonorchiasis. In: *Advances in Parasitology*, (Dawes, B. ed.), 4:53-106, Academic Press, London.
- Looss, A. (1907) Some parasites in the Museum of School of Tropical Medicine, Liverpool. *Ann. Trop. Med. & Parasit.*, 1:121-154. (cited from Kobayashi, 1917)
- Luna, L.G. (1960) Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd ed., McGraw Hill Book Co., New York.
- Porta, E.A. and Hartroft, W.S. (1969) Lipid pigments in relation to aging and dietary factors (Lipofuscin). In: *Pigments in Pathology* (Wolman, M. ed.), 191-235, Academic Press, New York.
- Robbins, S.L. and Cotran, R.S. (1979) Pathologic basis of diseases, 2nd ed., p. 46, W.B. Saunders Co., Philadelphia.
- Trump, B.F. and Jones, R.T. (1979) Diagnostic electron microscopy, Vol. 2, John Wiley & Sons, New York.

==우리말 요약==

### 간흡충에 나타나는 리포푸신 색소

중앙대학교 의과대학 기생충학교실, 병리학교실\* 및 조직학교실\*\*

조 승 열·송 계 용\*·라 봉 진\*\*

실험동물에서 짧은 기간 감염시킨 후 얻은 간흡충(*Clonorchis sinensis*)에서는 거의 볼 수 없지만, 간, 담도 수술 또는 부검을 실시할 때 사람에서 얻은 간흡충충에는 검은 색소를 갖는 것을 관찰할 때가 있다. 이 색소는 살아 있는 간흡충에서도 명백하며, 색소가 없는 간흡충은 색이 분홍빛이고 투명한데 비하여 색소가 있는 것은 검은색을 띠고 불투명하다.

사람에서 얻은 간흡충 모두가 검은 색소를 갖고 있는 것은 아니며, 이제까지의 경험으로는 30세 이상 어른에서 얻은 간흡충중 그 길이가 약 1cm 이상인 것에서 나타난다. 이 색소의 성분에 대하여 과거에는 빌리루빈, 또는 숙주 혈구세포에서 유래한 철을 포함하는 색소일 것이라고 생각하고 있었다. 저자들은 이 색소가 간흡충의 노쇠현상과 관련된 것으로 생각하고, 그렇다면 lipofuscin일 것이라고 생각하였다.

위의 가정을 확인하기 위하여 육안적으로 분명히 검은 색소가 있는 충체의 조직절편을 만들고, H & E, prussian blue, bilirubin, Fontana-Masson, PAS, amylase-PAS, Ziehl-Neelsen 및 AFIP method for lipofuscin 등 8가지 조직화학적 염색을 실시하였다. 색소는 H & E, prussian blue, bilirubin, Fontana-Masson 염색으로 착색되지 않았으며, PAS, amylase-PAS, Ziehl-Neelsen, 및 AFIP method for lipofuscin에 염색되어 이것이 조직화학적으로 리포푸신 색소임을 확인하였다.

색소를 다시 전자현미경으로 관찰한 바 색소과립안에는 모양이 둥글거나 타원형이고 크기가 0.05~0.24 $\mu$ m인 세포막구조가 산재하였음을 발견할 수 있어 간흡충의 검은 색소는 리포푸신 색소임을 다시 확인할 수 있었다.

### EXPLANATION FOR PLATES

- Fig. 1.** Formalin fixed, unstained adult worms of *Clonorchis sinensis*. On the left, is an adult without black pigment, which was collected from case No. 5 in Table 1. The worm on the right is the adult laden with black pigment, which was from case No. 6.
- Fig. 2.** Lipofuscin pigment at the parenchymal cells of *Clonorchis sinensis*. H & E staining,  $\times 430$ .
- Fig. 3.** Staining with AFIP method for lipofuscin. The pigment particles were stained red by carbol fuchsin, whereas the background was stained yellow by picric acid.  $\times 430$ .
- Fig. 4.** Staining with Ziehl-Neelsen method. The cytoplasm of vitelline cells were stained blue by methylene blue. But lipofuscin pigments were stained purple by carbol fuchsin and by methylene blue.  $\times 430$ .
- Fig. 5.** Membrane bound lipofuscin particles in cytoplasm of a parenchymal cell of *C. sinensis*. Lead citrate and uranyl acetate (=L & U) staining.  $\times 5,400$ .
- Fig. 6.** High magnification of a lipofuscin pigment from *Clonorchis*. Various sized electron dense granules and a few of vacuoles are aggregated in the centre, and somewhat homogeneous lysosomal matrix situated in periphery. Due to poor fixation of the sample, limiting membrane is mostly denuded. L & U staining.  $\times 28,000$ .
- Fig. 7.** *Ibid.* Many laminated granules have electron dense core of varying density. L & U staining.  $\times 28,000$ .
- Fig. 8.** In this specific site of a particle, a lot of membrane bound granules are found. L & U staining.  $\times 54,000$ .





