

Experimental Infection of Mice by Intrahepatic Inoculation of Oncospheres and Eggs of *Echinococcus multilocularis*

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Introduction

It is well known to produce primary cystic infection with *Echinococcus multilocularis* in laboratory animals by means of oral administration. It was reported by Yamashita et al.⁸⁾ that the primary cystic infection by oral administration with *E. granulosus* egg was produced at such a low infective ratio as 10% in laboratory animals. However, Heath¹⁾ and Williams and Colli⁶⁾ succeeded in an attempt establishing the primary cystic infection with *E. granulosus* in laboratory animals by intraperitoneal inoculation of egg or oncosphere.

For the electron microscopical observation on the initial histogenesis from oncosphere to initial echinococcal cyst, the authors determined to inoculate the *E. multilocularis* egg into the liver of laboratory mice as the means of getting many developing oncospheres in a small area injected. In this preliminary examination, the larval echinococcal tissue developing in the laboratory mice inoculated with artificially hatched oncospheres and the untreated intact eggs of *E. multilocularis* were observed light microscopically.

Materials and Methods

The adult tapeworms were collected from small intestine of a dog experimentally infected with *Echinococcus multilocularis*. Namely, the adult tapeworms were crushed in a mortar and then suspended in Hanks' solution. The suspension was filtered into a test tube through the sieves of 100 and 200 meshes per inch. The filtrate was allowed to stand for 15 minutes. The upper suspension containing tissue-debris was removed from the test tube. The sediment in the bottom of the test tube was dispersed in Hanks' solution. The suspension was removed into the centrifugal tube, was centrifuged for 5 minutes at 800 rpm, and was dispersed again in Hanks' solution. These procedures were repeated 3 times for the purpose of clearing the eggs. A part of the eggs was treated in saline solution containing 0.2% pepsin (1: 10,000 Difco) and 0.1% hydrochloric acid for 10 minutes at 37°C in 50 ml conical flask, agitated on a magnetic stirrer. The fluid was allowed to settle for 15 minutes. The sediment was digested again in 20 ml of Rinaldini solution containing 0.5% trypsin and 1% pancreatin for 15 minutes at 37°C on the magnetic stirrer. The hatched oncospheres were washed 3 times by the alternating repetition of centrifugation and dispersion in Hanks' solution containing 200 units penicillin G and 200 γ streptomycin sulfate per ml. Activity and viability of the hatched oncospheres were estimated by supravital staining with 0.2% neutral red and 0.2% Janus green. The inoculum was prepared in the concentration containing about 100 viable, hatched oncospheres or intact eggs per 0.05 ml. Prior to the intrahepatic injection, the mice were shaved

of their hair on hepatic region using a safety razor as a means of seeing the liver through the skin. The respective 60 mice of CF-1- and A/He-strain were divided three groups. The respective 30 mice of each strain were intrahepatically injected the hatched oncospheres or intact eggs. Of each strain, the respective 20 mice, which were orally administered with intact eggs and were intrahepatically injected with hatched oncospheres or intact eggs, were sacrificed at the desired intervals. All of the other mice remaining were dissected 200 days after inoculation. The echinococcal cysts in the liver and other organs were examined microscopically. The liver of each mouse used was weighed. The sections were made for histological investigation.

Results

1. The development of echinococcal tissue in CF-1- and A/He-mice orally inoculated with intact eggs

In the orally inoculated CF-1- and A/He-mice, small vesiculated oncospheres were found sporadically in their liver 3 days after inoculation. In the 5th day cases, unilocular cyst formed by syncytial monolayer cells was 50~70 μm in diameter. Multilocular vesiculation of the cysts without cuticular layer was recognized in the mice 10 and 15 days after inoculation. In the 20th and 30th day cases, a number of spongy multilocular cysts had the PAS-positive cuticular layer. The initial formation of brood capsule, which consists of an accumulation of proliferating undifferentiated cells forming a lumen in its center, was seen in the mice 50 and 100 days after infection. In the 120th day cases, initial formation of protoscoleces was recognized in the brood capsules. In the 160th day cases, a number of protoscoleces were found in the brood capsules. No remarkable difference in the development of echinococcal tissue was recognized between CF-1- and A/He-mice.

2. The development of echinococcal tissue in CF-1- and A/He-mice intrahepatically inoculated with hatched oncospheres and intact eggs

No essential difference in the development of echinococcal tissue was recognized between the two strains of mice and between hatched oncospheres and intact eggs. Accordingly, the findings of echinococcal tissue in those mice used will be described together in the following.

Considerable number of vesiculated oncospheres were found in the hemorrhagic legion of the liver of mice the next day after inoculation. In the 3rd day cases, the hemorrhage was nearly absorbed, and some dozen of small cysts, about 15~20 μm in diameter, distributed all over the liver. Active multilocular vesiculation of echinococcal cysts was manifested in the 10th and 20th day cases. The individual sizes of the multilocular cysts were about 200~300 μm in diameter. In the 30th day mice, the cysts were surrounded with proliferating connective tissue. About 1~22 μm thick, PAS-positive, cuticular layer was recognized outside the germinal layer of cysts. The initial formation of brood capsule was recognized in the mice 80 days after inoculation. Echinococcal cysts were found in mesentery in addition to liver in one of the 2 CF-1 mice and 2 of the 2 A/He mice. Some completely matured protoscoleces were recognized in the brood capsules of the cysts. In the 2 CF-1-mice and a A/He-mouse 160 days after inoculation with hatched oncospheres, echinococcal cysts were recognized in mesentery and spleen in addition to liver, respectively. In all of the respective mice of CF-1- and A/He-strain, formation of echinococcal lesions in mesentery was seen besides liver. Histologically, these echinococcal cysts in mesentery revealed the active multilocularization and the formation of protoscoleces. Especially, the cysts in spleen of the A/He-mouse showed very active multilocularization and the formation of abundant pro-

toscoleces. However, the echinococcal cysts in the liver of these cases revealed the poor multilocularization and initial formation of brood capsule. Most of the cysts were surrounded with thick cuticular layer and connective tissue. Some of the cysts surrounded with severe proliferated connective tissue showed the regressive change of echinococcal tissue.

3. Echinococcal tissue in CF-1- and A/He-mice infected by the oral or intrahepatic inoculation with hatched oncospheres or unhatched eggs

The infection of echinococcal cysts was recognized in the liver of all cases dissected 200 days after inoculation. In all of the mice orally inoculated with the intact eggs, the echinococcal cysts were found only in liver. On the contrary, considerable number of the mice intrahepatically inoculated with the hatched oncospheres and intact eggs revealed the development of echinococcal cysts in various visceral organs and on the surface of peritoneum. Namely, 3 CF-1-mice and 2 A/He-mice intrahepatically inoculated with hatched oncospheres showed the formation of echinococcal cysts in the mesentery and the cavity of pelvis. Each case of 5 CF-1-mice treated in the same manner as above revealed the development of echinococcal cysts in the mandibular gland, spleen, hypoderm, ovary and diaphragm, respectively. Each case of 3 A/He-mice infected by intrahepatic inoculation with the hatched oncospheres revealed the establishment of echinococcal cysts in the diaphragm and kidney and on the parietal peritoneum. Each of 4 CF-1-mice infected by intrahepatic inoculation with the intact eggs revealed the formation of echinococcal cysts in the spleen, mesentery, and kidney and on the serosal surface of intestine, respectively. Each of 5 A/He-mice infected by the same way with the intact eggs possessed the cysts in the diaphragm, mesentery, ovary and kidney and on the parietal peritoneum, respectively.

The echinococcal cysts in the mice orally given the intact eggs revealed to be obviously larger and well-developed in comparison with the cysts in the liver of the mice intrahepatically inoculated with oncospheres. In the intrahepatically inoculated mice, no difference in the size of echinococcal cysts and the degree of formation of protoscoleces was recognized between the hatched oncospheres and intact eggs administered.

In the histological observation, well-developed, multilocular cysts containing abundant brood capsules with protoscoleces were recognized in the liver of all of the CF-1- and A/He-mice orally inoculated with the intact eggs. In the intrahepatically inoculated cases, multilocular cysts were recognized in the liver of all of the cases. However, the formation of protoscoleces was seen in the liver of 3 of 10 CF-1-mice and in 2 of 10 A/He-mice intrahepatically inoculated with hatched oncospheres and in the respective 3 of 10 CF-1-mice and of 10 A/He-mice intrahepatically inoculated with intact eggs. In those cases, the degeneration, disruption and collapse of echinococcal tissue were seen in some of the cysts in the liver and on the peritoneum. The pieces of cuticular layer collapsed were recognized in some nodules of connective tissue in the liver and on the peritoneum. The hyperplasia and dilation of interlobular bile duct were seen in the liver with the nodules of connective tissue. Comparatively large, mild multiloculized cysts and small, highly multiloculate cysts were seen in the mesentery and the cavity of pelvis. The latter revealed the remarkable formation of protoscoleces. The echinococcal cysts in the ovary and spleen showed the high multilocularization and the remarkable formation of protoscoleces.

Discussion

Yamashita et al.⁷⁾ reported that the echinococcal cysts were classified into two types according

to morphological combination of the cyst and host tissue reaction. They stated that CF-1- and A-strain mice belonged Type 2 which showed the low development requiring more than 5 months for protoscolex formation, minute individual cysts and severe reaction of host tissue. In the present experiment, no essential difference in the development of echinococcal tissue was recognized between CF-1- and A/He-mice. Williams and Colli⁶⁾ succeeded in producing the primary cystic infection with *Echinococcus granulosus* in jirds, Swiss mice and golden hamsters inoculated with oncospheres and eggs. They considered that the peritoneal environment is by no means inimical to the development of embryos of *E. granulosus*. In the present experiment using the hatched oncospheres of *E. multilocularis*, however, the echinococcal cysts showed more active development in the mice orally inoculated than that in the mice intrahepatically inoculated. It is considered that this difference in the development of the echinococcal tissue is due to the difference of the species of the parasites used. It is a well-known⁷⁾ fact that in experimental animals, almost all echinococcal foci are established in the liver by oral inoculation of *E. multilocularis* eggs. It is considered that the extremely poor development of the hydatid tissue is due to the intense tissue-reaction such as proliferation of connective tissue against foreign substance induced with the hatched oncospheres or the intact eggs by intrahepatic injection. In the present experiment, the echinococcal cysts were frequently recognized to be established within other abdominal organs such as spleen, kidney, ovary, diaphragm and mesentery besides liver. The cysts revealed a more active growth in those organs than that of the cysts established in liver. It is known that metastatic multilocular echinococcosis occurs both in the human²⁾ and animals^{3,4,5)}. It is considered that the oncospheres invaded into the hematogenous and lymphatic route play a role in the production of metastatic foci, because the hemorrhage was recognized in the injected area of the liver.

Summary

The hatched oncospheres and unhatched eggs of *Echinococcus multilocularis* were inoculated into the liver of mice of CF-1- and A/He-strain. The development of echinococcal tissue in the mice intrahepatically inoculated was compared with that of the echinococcal tissue in the mice orally inoculated with the egg. The primary echinococcal infection was established in all of the mice. However, the development of the echinococcal tissue showed to be more active in the mice orally inoculated than in the mice intrahepatically inoculated. In the orally inoculated mice, the echinococcal tissue was established only in the liver. On the contrary, echinococcal cysts were recognized in the spleen, ovary, mesentery, diaphragm and the cavity of pelvis, besides liver. The development of the echinococcal tissue is more active in the other organ than in liver. No difference in the development of echinococcal tissue was recognized between the two strains of the mice and between the hatched and the unhatched oncospheres used.

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Explanation of plates

Figures 3~12 are photomicrographs of specimens stained with hematoxylin-eosin.

Plate I

- Fig. 1 Liver of CF-1 mouse 30 days after intrahepatic injection of eggs. Multilocular echinococcal cysts actively developing are recognized in an area injected.
- Fig. 2 Multilocular echinococcal cysts in spleen (left) and liver (right) of CF-1 mouse 200 days after intrahepatic injection of hatched oncospheres.
- Fig. 3 Echinococcal cysts in liver of CF-1 mouse 30 days after intrahepatic injection of eggs.
- Fig. 4 Echinococcal cysts in liver of CF-1 mouse 40 days after intrahepatic injection of eggs.
- Fig. 5 Echinococcal cyst in mesentery of CF-1 mouse 160 days after intrahepatic injection of hatched oncospheres.
- Fig. 6 Echinococcal cysts in liver of A/He mouse 100 days after intrahepatic injection of eggs.

Plate II

- Fig. 7 Echinococcal cyst in liver of CF-1 mouse 30 days after intrahepatic injection of eggs.
- Fig. 8 Regressive echinococcal cyst in liver of CF-1 mouse 100 days after intrahepatic injection of eggs. The cyst is surrounded with thickened connective tissue.
- Fig. 9 Degenerated echinococcal cyst in liver of CF-1 mouse 200 days after intrahepatic injection of eggs.
- Fig. 10 Proliferation of the bile ducts in liver of CF-1 mouse 200 days after intrahepatic injection of eggs.
- Fig. 11 Well-developed echinococcal cysts in liver of A/He mouse 200 days after intrahepatic injection of hatched oncospheres.
- Fig. 12 Well-developed echinococcal cysts in spleen of CF-1 mouse 200 days after intrahepatic injection of hatched oncospheres.



