[Jpn. J. Parasitol., Vol. 35, No. 5, 403–410, October, 1986]

Influence of X-ray Irradiation on the Proliferative Ability of the Germinal Layer Cells of *Echinococcus multilocularis*

KENJI OHNISHI

(Received for publication; March 17, 1986)

Key words: *Echinococcus multilocularis*, Germinal layer cell, X-ray irradiation, Chinese hamster

Introduction

Alveolar echinococcosis is caused by infection with the larval stage of *Echinococcus multilocularis*. The natural intermediate host of this parasite is small rodents and the human also plays the role of accidental intermediate host. In rodents, multilocular hydatids comprise non-cellular laminated layers, cellular germinal layers, brood capsules, protoscolices and calcareous corpuscles as parasitic components. In humans, brood capsules, protoscolices and calcareous corpuscles are virtually absent. Although alveolar echinococcosis is a uncommon disease, it is one of the most lethal human helminth infections and the natural course of this disease is progressively. Chemotherapeutics including mebendazole apparently does not kill the parasite (Schantz *et al.*, 1982) and only radical resection offers a hope for cure (Kasai *et al.*, 1980), however successful surgical treatment of alveolar hydatid disease is rare (Mosimann, 1980). Radiation therapy has not been attempted on the larval stage of *Echinococcus* spp., while the influence of X-ray irradiation on eggs or protoscolices of *Echinococcus* spp. has been observed by a few investigators. Williams and Coli (1972) studied the influence of X-ray irradiation on the infectivity of eggs of *Echinococcus granulosus* to jirds. Movsesijan *et al.* (1968) studied the immunogenic potential of irradiated protoscolices of *E. granulosus* to know the protective immunity to adult worm infection in dogs. Markell and Beal (1974) reported the influence of X-ray irradiation on eggs and protoscolices of *E. multilocularis* by using cotton rats as experimental intermediate host. In most cases, the only live component of the parasite in human multilocular hydatids is the germinal layer cells. The influence of X-ray irradiation on the echinococcal germinal layer cells has never been investigated. The present paper deals with the effect of different doses of X-ray irradiation on the proliferative ability of the germinal layer cells of larval *E. multilocularis*.

Department of Parasilogy, Asahikawa Medical College, Nishikagura 4-5, 078 Asahikawa, Japan.

Materials and Methods

Parasite

Larval *E. multilocularis* (isolate name: Asahikawa No. 1), used in the present investigation, was isolated from the liver of a female Japanese patient on January, 1984 (Ohnishi *et al.*, 1985) and has been maintained in our laboratory by intraperitoneal passage of larval suspension (homogenized echinococcal lesion) to Chinese hamsters.

Animals

Male 8 to 10-week-old Chinese hamsters (*Cricetulus griseus*), raised in the Asahikawa Colony (CHA), were provided by the Depart-

ment of Biological Science, Asahikawa Medical College and used in the experiments. All the animals were fed pelleted commercial food and water ad lib.

X-ray irradiation

A Toshiba therapy X-ray machine (KXC-19-2) was used operating at 220 kilovolts and 20 milliamperes, filtered with 0.7-Cu and 0.5 of Al. A dose rate of 117 R/min was employed.

Light microscopy

The hydatids were fixed in 10% formalin, dehydrated in graded series of ethanol and embedded in paraffin. Sections were cut on a Yamato Koki microtome and stained with periodic acid-Schiff solution (PAS).

Electron microscopy

The hydatids were fixed in 4% paraformaldehyde and 1% glutalaldehyde, buffered to pH 7.4 with 0.1 M sodium cacodylate for 30 to 60 min at room temperature and post-fixed in 1.33% osmium tetroxide in 0.067 M s-colidine buffer (pH 7.4) at 4°C for 60 min. The materials were then stained with 0.5% uranyl acetate in water at 4°C for 12 to 15 hr, dehydrated in graded series of ethanol, and embedded in Spurr's medium (Spurr, 1969). Sections were cut on a Reichert Om U3 ultramicrotome and stained with lead citrate for 5 min and examined under a electron microscope (Nihon Denshi JEM 100 S).

Experimental design

Experiment 1: Small sized and early stage sterile hydatids were removed aseptically from the peritoneal cavity of the Chinese hamsters inoculated with larval suspension intraperitoneally 20 days previously and were placed in sterilized 0.85% saline containing penicillin G 100 IU/ml and streptomycin sulfate 0.1 mg/ml. The vesicles of hydatids in the early stage consisted of a laminated and a germinal layer, containing no protoscolex (sterile) or brood capsule. The small hydatids were irradiated by X-ray at dose levels of 1,000 or 10,000 R. Two pieces of the irradiated or non-irradiated hydatids were implanted into each animal intraperitoneally (2 animals per each experimental and control group). The mean total fresh weight of the implanted hydatids was about 0.1 g per animal. All the animals were killed 150 days post implantation and the developed hydatids originating from the implanted hydatids were examined macroscopically and light microscopically.

Experiment 2: Small sized early stage hydatids were removed as same as experiment 1 and irradiated by X-ray at dose levels of 5,000, 15,000, 25,000, 35,000, 45,000 or 55,000 R. Non-irradiated small hydatids were kept under the same conditions as those of each irradiated groups. Subsequently the irradiated or non-irradiated hydatids were implanted into hamsters (2–6 animals per each experimental and control group), equally to the condition of experiment 1. All the hamsters were killed 113 days later and the hydatids in their peritoneal cavity were examined light and electron microscopically.

Results

Experiment 1.

Fully developed fertile hydatids were found in all the animals receiving the non-irradiated or irradiated early stage hydatids consisting of laminated and germinal layers. No significant difference was found between the weight of the hydatids derived from the implanted hydatids irradiated at 1,000, 10,000 R and non-irradiated control hydatids. All the recovered hydatids included vesicles comprising a laminated layer, a germinal layer, brood capsules, numerous mature protoscolices and calcareous corpuscles.

Experiment 2.

The influence of X-ray irradiation at dose levels between 5,000 and 55,000 R on the hydatids containing germinal layer cells but neither protoscolex nor brood capsule is given in Table 2. All the hamsters implanted with the

hydatids, non-irradiated or irradiated at 5,000, 15,000, 25,000 and 35,000 R, had fully developed hydatids which included a number of vesicles consisting of a non-cellular laminated layer, a cellular germinal layer, brood capsules, protoscolices and calcareous corpuscles (Figs. 2 and 3). The mean weight of the fully developed hydatids of each dose group irradiated at up to 35,000 R was not significantly different from that of non-irradiated control groups.

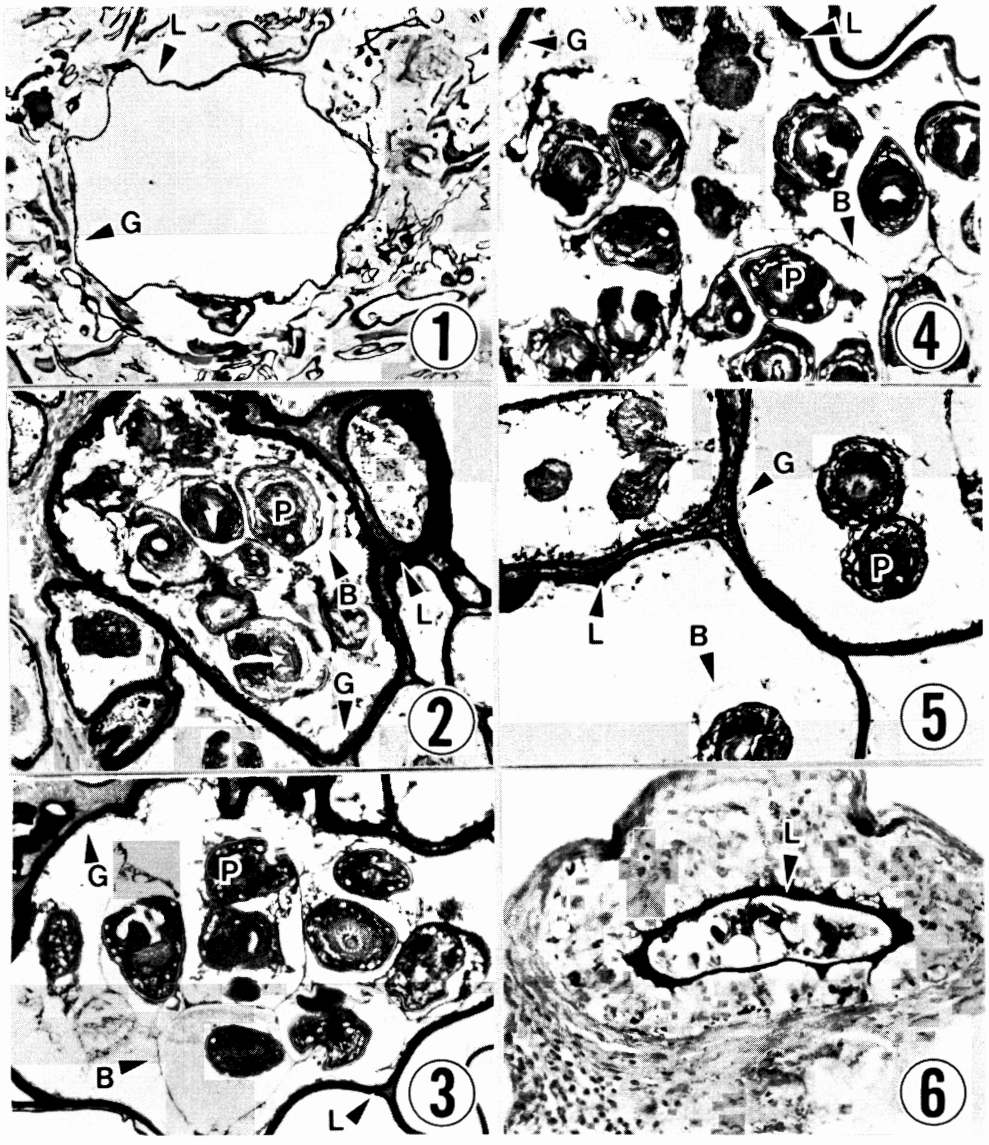
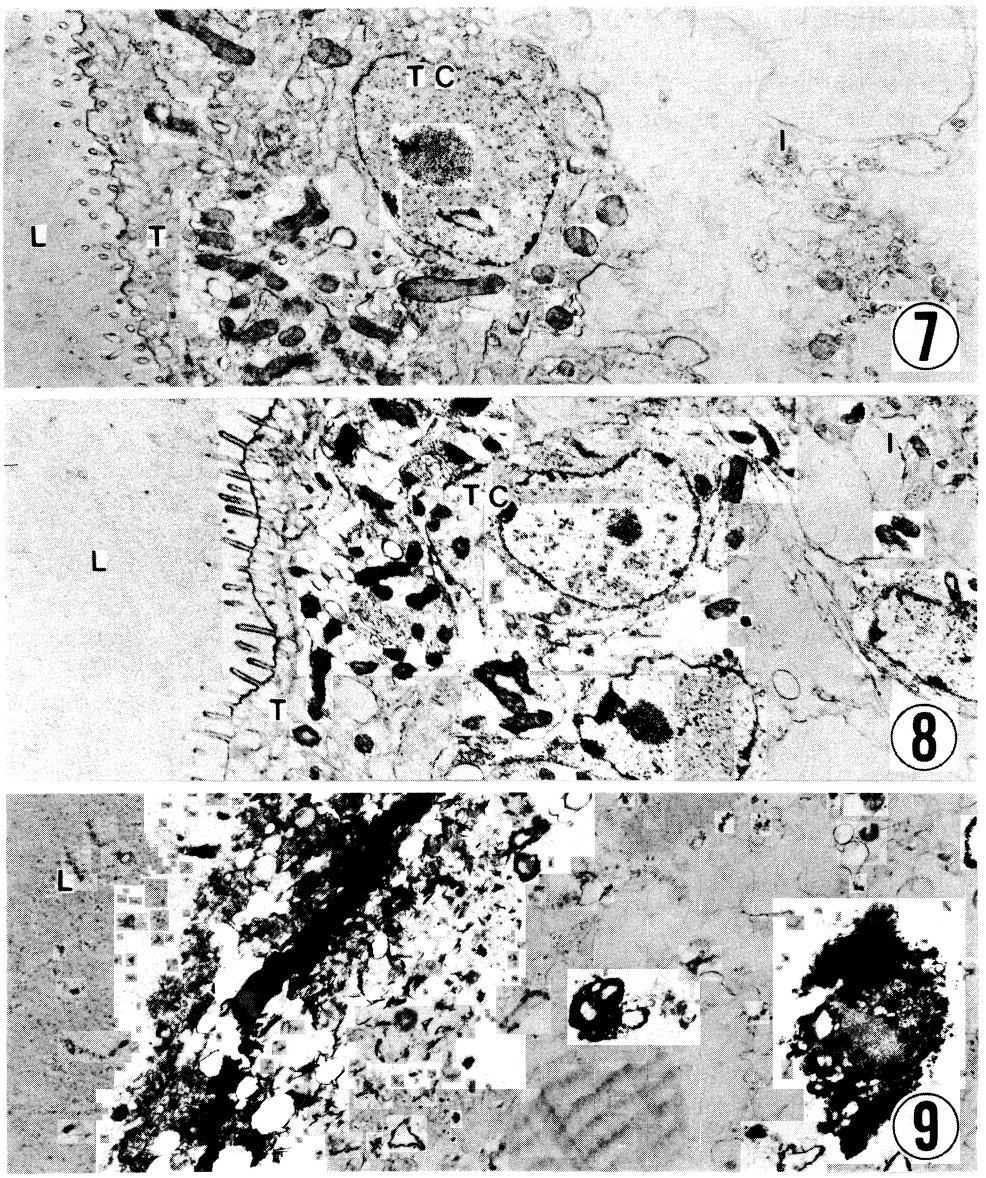


Fig. 1 Early stage sterile hydatid obtained from the animal 20 days after inoculation of larval suspension. Light micrograph. PAS, × 100.

Figs. 2–5 Fully developed hydatids originating from irradiated sterile hydatids at 5,000 R (Fig. 2) and non-irradiated ones (Fig. 3), from sterile hydatids irradiated at 45,000 R (Fig. 4) and non-irradiated ones (Fig. 5), 113 days after implantation. Light micrographs. PAS, × 100.

Fig. 6 Degenerated, implanted hydatid originating from the hydatid irradiated at 45,000 R, 113 days after implantation. There are no normal germinal layer, brood capsule and protoscolex. Light micrograph. PAS, × 100.



Figs. 7, 8 Germinal layer of vesicles in fully developed hydatids originating from the sterile hydatids irradiated at 45,000 R (Fig. 7) and non-irradiated ones (Fig. 8), 113 days after implantation. Electron micrographs. x 9,400.
Fig. 9 Germinal layer of the degenerated, implanted hydatid originating from the sterile hydatid irradiated at 45,000 R, 113 days after implantation. Note degenerated germinal layer and no microthrix. Electron micrograph. x 9,400.

Abbreviations:

L: laminated layer, G: germinal layer, B: brood capsule, P: protoscolex, T: tegument, TC; tegumental *cell region*, I: innermost area.

Two animals implanted with hydatids irradiated at 45,000 R possessed small but fertile hydatids. Out of 6 hamsters in this group of 45,000 R, 3 were found to have very small and degenerated hydatids and one was found to have no hydatids. All the animals receiving hydatids irradiated at 55,000 R had degenerated hydatids as seen in above described 3 animals irradiated at 45,000 R. These degenerated hydatids were shrunken and encapsulated by host cells. Only the non-cellular laminated layer could be identified (Fig. 6). Electron microscopically, the germinal layers of the vesicles in the developed hydatids originating from non-irradiated hydatids comprised the tegument with microtriches, the tegumental cell region and the innermost area (Fig. 8). Those structures were also seen in the developed hydatids derived from hydatids irradiated at up to 35,000 R and 45,000 R (Fig. 7), but they utterly collapsed in degenerated hydatids derived from irradiated hydatids at 45,000 R (Fig. 9) and 55,000 R. These results suggest that the irradiation at level of 45,000 R is the minimum dose level to inhibit the normal development of multilocular hydatids.

Discussion

The present investigation aimed to elucidate the influence of X-ray irradiation on the

Table 1 Effect of X-ray irradiation on early stage sterile hydatids (Experiment 1)

Hydatid used	Dose of X-ray (R)	No. of animals		Weight (g) of developed hydatid
		used	developed hydatid(+)	
Hydatids from	0	2	2	1.7, 1.0
hamsters 20 days	1,000	2	2	4.7, 1.5
post-inoculation	10,000	2	2	4.1, 1.5

Table 2 Effect of X-ray irradiation on the germinal layer cells in the early stage sterile hydatids (Experiment 2)

Dose of X-ray (R)	No. of animals		Weight (g) of developed hadatid	mean ± SD
	used	developed hydatid(+)		
5,000	4	4	13.2, 7.9, 5.4, 3.3	7.5 ± 4.3
0	4*	3	10.2, 3.0, 1.9	5.0 ± 4.5
15,000	4	4	3.1, 1.3, 1.2, 0.9	1.6 ± 1.0
0	4	4	4.8, 4.7, 2.5, 1.0	3.3 ± 1.8
25,000	4	4	1.8, 1.6, 1.1, 0.8	1.3 ± 0.5
0	4	4	5.1, 3.2, 2.1, 1.8	3.1 ± 1.5
35,000	4	4	2.8, 2.1, 1.4, 1.0	1.8 ± 0.8
0	4	4	2.9, 2.6, 2.0, 2.0	2.4 ± 0.5
45,000	6	2	0.5, 0.5	0.5 ± 0.0†
0	5	5	8.1, 5.0, 2.8, 2.1, 1.7	3.9 ± 2.7
55,000	2	0		–
0	2	2	2.1, 0.6	1.4 ± 1.1

*One animal died during the course of the experiment.
†Significant at $P < 0.05$.

germinal layer cells which may play the most important role in the proliferation or development of the larval *E. multilocularis*. Although the mechanism of proliferation or growth of larval *E. multilocularis* is only partially understood, several investigators have described the role of germinal layer cells in the proliferation of the hydatids. Rausch and Wilson (1973), Gamble *et al.* (1979) and Ohnishi *et al.* (1985) reported the development of multilocular hydatids in experimental animals receiving human pathological, sterile hydatids. Their reports strongly suggest the developmental potential of germinal layer cells for fertile hydatids because the materials used in their experimental infection included the germinal layer but not the brood capsule or protoscolex. It was confirmed that the vesicles in the small hydatids used in the present study (Experiments 1 and 2) were sterile. Hydatids implanted into hamsters constituted of cellular germinal layers and non-cellular laminated layers. Thus the fully developed hydatids in these hamsters must have originated from germinal layer cells. Mehlhorn *et al.* (1983) reported that the proliferation of the larval stage of this parasite occurred by the protrusion of solid cell columns filled with undifferentiated cells of the germinal layer and also that formation of the brood capsule might be initiated from massive accumulation of these cells. Ali-Khan *et al.* (1983) reported that the lysis of the laminated layer of alveolar echinococcal cysts occurred by the binding of macrophages, eosinophils and neutrophils to the layer and speculated that the released germinal layer cells might metastasize if they gain access to a blood stream. It is thus reasonable to suppose that the germinal layer cells develop into hydatids and may metastasize. Lascano *et al.* (1975) described the fine structure of the germinal layer of *E. granulosus* as having 3 components, the tegument, the tegumental cell region and the innermost area all of which were also recognized in developed hydatids originating from non-irradiated or irradiated hydatids in the present study. Generally Cyclophyllidea including *Echinococcus* spp. seem to be resistant to X-ray irradiation at various stage of their life cycle. Schiller (1959) reported that cysticercoids of *Hymenolepis nana* were found in beetles fed the eggs exposed to X-irradiation at 5 to 40 kr, and that the frequency of occurrence of abnormal cysticercoids was dependent on the X-ray dose. Beveridge and Rickard (1975) demonstrated that following the exposure of eggs of *Taenia pisiformis* to X-irradiation at 30,000 rads, hatching *in vitro* was unaffected. Relating to *Echinococcus* spp. a few investigators have studied the influence of X-ray irradiation on eggs or protoscolices, but the influence on germinal layer cells has not been investigated. Williams and Coli (1972) reported that the eggs of *E. granulosus* were markedly affected by X-ray irradiation at 20 kr or above and lost their infectivity at doses over 30 kr. Movsesijan *et al.* (1968) found that protoscolices of *E. granulosus* irradiated at 30 kr could infect the dogs. Markell and Beal (1974) demonstrated that the eggs of *E. multilocularis* lost their infectivity at 40 kr or above and that the protoscolices lost their developmental potential to hydatids by irradiation at 20 kr or above. The results obtained in the present studies show that X-ray irradiation at up to 35,000 R on the early stage hydatids containing germinal layer cells did not conspicuously affect their proliferative potential. In 4 out of 6 hamsters receiving hydatids containing germinal layer cells irradiated at 45,000 R and in all the animals implanted with those irradiated at 55,000 R, no development of hydatids was observed. From the results of the present studies, it would be appear that the tolerance limit to X-ray irradiation of the germinal layer cells was between 45,000 R and 55,000 R, and that exposure at 45,000 R or above is necessary to extinguish their proliferative potential or to kill them. The fact that multilocular hydatids developed from the irradiated early stage of hydatids implies the following possibilities: (a) the germinal layer cells were affected by irradiation but thus injury failed to disrupt the proliferative process,

(b) the cells were injured and then repaired or (c) the cells were not affected. Though the target region of ionizing irradiation on living cells is still obscure, Bernhard (1981) stated that the DNA base, especially thymine and cytosine, was damaged by ionizing irradiation and von Sonntag *et al.* (1981) explained the induction of strand breaks in DNA. In the present experiments, the nucleus especially the DNA of the germinal layer cells might have been injured by X-ray irradiation at dose levels of 45,000 R and 55,000 R. In conclusion, the present studies indicate that the cells of the germinal layer of larval *E. multilocularis* as well as eggs of this parasite are X-ray resistant, although the exact mechanism of loss of proliferative ability of germinal layer cells awaits further study.

Summary

Influence of X-ray irradiation on the proliferative ability of the germinal layer cells of larval *Echinococcus multilocularis* was studied by using small sterile hydatids containing vesicles composed of a non-cellular laminated layer and a cellular germinal layer. The small sterile hydatids were irradiated by X-ray at dose levels of 5,000, 15,000, 25,000, 35,000, 45,000 or 55,000 R and implanted into the peritoneal cavity of Chinese hamsters. Fully developed hydatids were recognized in all cases irradiated at up to 35,000 R, when assessed 113 days after implantation. At 45,000 R, 2 out of 6 animals showed small, fully developed hydatids. No such hydatid was found in the other 4 animals nor in any of the animals implanted with hydatids irradiated at 55,000 R. No structural differences were observed between fully developed hydatids originating from the irradiated and non-irradiated small hydatids. These results indicate that the tolerance limit of the germinal layer cells is between 45,000 R and 55,000 R.

Acknowledgments

I would like to thank Professor Kazuya Mikamo, Department of Biological Science, Asahikawa Medical College, for supplying Chinese hamsters. I am most grateful to Professor Haruhiko Kutsumi and Drs. Tohru Inaoka and Minoru Nakao, Department of Parasitology, Asahikawa Medical College, for reading this manuscript and Mr. John Sunley, Department of English, Asahikawa Medical College, for correcting English.

References

1) Ali-Khan, Z., Siboo, R., Gomersall, M. and Faucher, M. (1983): Cystolytic events and the possible role of germinal cells in metastasis in chronic alveolar hydatidosis. Ann. Trop. Med. Parasitol., 77, 497–512.
2) Bernhard, W. A. (1981): Solid-state radiation chemistry of DNA: The bases. Adv. Radiat. Biol., 9, 199–280.
3) Beveridge, I. and Rickard, M. D. (1975): The effects of X-radiation on *Taenia pisiformis*. Int. J. Parasitol., 5, 641–649.
4) Gamble, W. G., Segal, M., Schantz, P. M. and Rausch, R. L. (1979): Alveolar hydatid disease in Minnesota. First human case acquired in the contiguous United States. J. Am. Med. Ass., 241, 904–907.
5) Kasai, Y., Koshino, I., Kawanishi, N., Sakamoto, H., Sasaki, E. and Kumagai, M. (1980): Alveolar echinococcosis of the liver. Studies on 60 operated cases. Ann. Surg., 191, 145–152.
6) Lascano, E. F., Coltorti, E. A. and Varela-Diaz, V. M. (1975): Fine structure of the germinal membrane of *Echinococcus granulosus* cysts. J. Parasitol., 61, 853–860.
7) Markell, E. K. and Beal, C. B. (1974): Resistance of *Echinococcus multilocularis* to X-ray. J. Parasitol., 60, 729–730.
8) Mehlhorn, H., Eckert, J. and Thompson, R. C. A. (1983): Proliferation and metastases formation of larval *Echinococcus multilocularis*. II. Ultrastructural investigations. Z. Parasitenkd., 69, 749–763.
9) Mosimann, F. (1980): Is alveolar hydatid disease of the liver incurable? Ann. Surg., 192, 118–123.
10) Movsesijan, M., Sokoloć, A. and Mladenović, Z. (1968): Studies on the immunological potentiality of irradiated *Echinococcus granulosus* forms: Immunization experiments in dogs. Br. Vet. J., 124, 425–432.
11) Ohnishi, K., Nakao, M. and Kutsumi, H. (1985): Isolation of larval *Echinosoccus multilocularis* by injection of infected human hepatic tissue homogenate into the Chinese hamster. Z. Parasitenkd., 71, 693–695.
12) Rausch, R. L. and Wilson, J. F. (1973): Rearing

of the adult *Echinococcus multilocularis* Leuckart, 1863, from sterile larvae from man. Am. J. Trop. Med. Hyg., 22, 357–360.
13) Schantz, P. M., Van den Bossche, H. and Eckert, J. (1982): Chemotherapy for larval echinococcosis in animals and humans: Report of a workshop. Z. Parasitenkd., 67, 5–26.
14) Schiller, E. L. (1959): Experimental studies on morphological variation in the cestode genus *Hymenolepis* III. X-irradiation as a mechanism for facilitating analyses in *H. nana*. Exp. Parasitol., 8, 427–470.
15) Spurr, A. R. (1969): A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res., 26, 31–43.
16) von Sonntag, C., Hagen, U., Schön-Bopp, A. and Schulte-Frohlinde, D. (1981): Radiation-induced strand breaks in DNA: Chemical and enzymatic analysis of end groups and mechanistic aspects. Adv. Radiat. Biol., 9, 109–142.
17) Williams, J. F. and Colli, C. W. (1972): Influence of ionizing irradiation on infectivity of eggs of *Echinococcus granulosus* in laboratory rodents. J. Parasitol., 58, 427–430.

多包虫の胚層細胞の増殖能に対するX線照射の影響

大西健児
(旭川医科大学医学部寄生虫学教室)

多包虫の胚層細胞の増殖能に対するX線照射の影響を調べた．材料は胚層と角皮層だけから形成された感染後20日の無原頭節多包虫である．分離した多胞虫に5,000 R，15,000R，25,000R，35,000R，45,000Rおよび55,000RのX線照射を行い，直ちにチャイニーズハムスターの腹腔に移植して，その後の多包虫への発育をみた．移植113日後に剖検して多包虫を検索すると，35,000Rまでの照射量ではX線の影響は顕著でなく，対照群と同様に移植された包虫は内部に原頭節が形成されよく発育した多包虫となっていた．45,000Rの照射を受けた無原頭節多包虫を移植された6例の動物では，2例に原頭節を有する小型の多包虫が認められ，光顕的には無照射の材料から発育した多包虫と形態的には差がなく，その胚層の電顕像においても差はなかった．他の3例では，移植された多包虫は著しく萎縮，変性しており，残りの1例では多包虫を見い出せなかった．55,000R照射後に移植された多包虫は，全例が萎縮，変性していた．このように45,000Rまたは55,000Rの照射を受けたため，発育せずに変性した多包虫では，光顕的には繁殖胞，原頭節は存在せず胚層も崩壊していた．電顕的にも，胚層の tegument，tegumental cell region および innermost area の崩壊が認められた．以上のことから，多包虫の胚層細胞は45,000R以上のX線照射により増殖能力を失うことが示唆された．