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Simple Techniques for Preparation of Small Vesicles from *Echinococcus multilocularis* Metacestodes and Colorimetric Quantitation of the Viability of Germinal Cells

TAMOTSU KANAZAWA[1)], HIROKO ASAHI[1)] AND KEIZI MOCHIDA[2)]

[1)]Department of Parasitology and [2)]Department of Veterinary Science, National Institute of Health, Toyama 1-23-1, Shinjuku-ku, Tokyo 162, Japan.

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Abstract

A method of preparation for the small vesicles from *Echinococcus multilocularis* metacestodes and a method of colorimetric quantitation of the viability of germinal cells in the vesicles were reported. Percoll step gradient was used to prepare the vesicles. They could be found at 16% and 28% of the Percoll interfaces. The vesicles freshly recovered from metacestodes took up MTT and were stained blue. On the contrary, heat-killed or formalin-fixed vesicles did not absorb MTT. Albendazole sulfoxide suppressed the absorption in a dose-dependent manner. Furthermore, the vesicles which absorbed MTT could infect gerbils with great efficiency, but those not taking up MTT had poor infectivity. It is concluded that these techniques are of practical value for the preparation of vesicles and the assessment of the viability of germinal cells.

Key words: *Echinococcus multilocularis*; vesicle; germinal cell; Percoll; MTT.

Introduction

Alveolar and cystic hydatid diseases are serious parasitic zoonoses, undoubtedly becoming increasingly important in the world. After peroral infection with eggs of *Echinococcus multilocularis* or *E. granulosus*, metacestodes develop in organs of intermediate hosts including humans, especially in their liver. Metacestodes of *E. multilocularis* consist of numerous small vesicles and a network of filamentous solid cellular protrusions of germinal cells which are responsible for infiltrating growth and transformation into tube-like and cystic structures. Furthermore, the detachment of germinal cells from infiltrating cellular protrusions and their subsequent distribution via lymph or blood can give rise to distant metastatic foci (Ali-Khan *et al.*, 1983; Eckert *et al.*, 1983; Mehlhorn *et al.*, 1983). Therefore, more intensive studies focusing on vesicles and germinal cells should be carried out. In this paper, we report simple techniques for the preparation of small vesicles from metacestodes of *E. multilocularis* and for the assessment of the viability of germinal cells.

Correspondence: Tamotsu Kanazawa
金澤　保[1]，朝日博子[1]，持田慶司[2]（[1]国立予防衛生研究所寄生動物部，[2]国立予防衛生研究所獣医科学部）

Materials and Methods

Parasite

E. multilocularis, Alaskan strain, kindly supplied from Hokkaido Institute of Public Health, Japan, has been maintained in our laboratory using Mongolian gerbils, *Meriones unguiculatus* as secondary alveolar hydatid cysts. The animals were supplied by the Department of Veterinary Science, National Institute of Health, Japan. Hydatid cysts were recovered aseptically from the peritoneal cavity of the animals three months after infection. Mixed suspensions of small vesicles and protoscoleces of the parasite were prepared by the methods described previously (Kanazawa *et al.*, 1993, Kanazawa *et al.*, 1994). Briefly, hydatid cysts were minced, digested with dispase (Godo Shusei Co. Ltd., Japan) for one hour, and passed through 16-, 35- and 60-mesh wire screens. The suspensions

were washed several times in RPMI 1640 with glutamine (Gibco BRL, Life Technologies, Inc., USA).

Purification of protoscoleces and small vesicles

Isotonic solutions of Percoll (Pharmacia Biotech, Sweden) of different densities (16%, 28% and 38%) were prepared by diluting the stock suspension with the appropriate volumes of 10× concentrated minimum essential medium (Nissui Pharmaceutical Co. Ltd., Japan) and distilled water. These solutions were stored at 4°C before use. To achieve sharp interfaces, 10 ml of each Percoll solution was layered from the bottom of 50-ml plastic-conical centrifuge tubes. The parasite samples suspended in a volume of 3 ml of medium were layered at the top of the gradient. The tubes centrifuged at 100×*g* for 10 min at 5°C. The collected fractions were diluted in RPMI 1640 containing 200 U/ml of penicillin G and 200 μg/ml of streptomycin and sedimented several times before further use. The numbers of organisms in each fraction were counted microscopically.

Viability Assay

The vesicle preparations were suspended in RPMI 1640 with glutamine containing 10% FCS and the antibiotics. One ml of the parasite suspensions each containing about two hundred vesicles was placed individually into wells (24-well plates). A simple colorimetric quantitation assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was used to assess the parasite viability. MTT was purchased from Sigma Chemical Co., (USA). A stock solution of MTT was prepared beforehand by dissolving 5 mg of MTT in one ml of distilled water. The filtered stock solution was stored at 4°C before use. The MTT solution was added to each well. The final concentration of MTT was 100 μg/ml. The suspensions were incubated in a 5% CO_2 incubator. The supernatant incubated for 16 hours were carefully removed and replaced with 500 μl of dimethyl sulfoxide (DMSO) and allowed to stand at room temperature for one hour. Following gentle agitation to disperse the color, the absorbance of the DMSO supernatant was read at 546 nm (ELISA analyzer ETY-96, TOYO Sokki, Japan) relative to DMSO blank. Heat-killed and formalin-fixed vesicles were used as controls.

Evaluation of drug effects

Albendazole sulfoxide and praziquantel were kindly supplied by Smith Kline & Fujisawa (Japan) and Bayer Yakuhin, Ltd. (Japan), respectively. Albendazole sulfoxide was investigated at concentrations of 50 μg/ml, 100 μg/ml and 150 μg/ml. Praziquantel was investigated at concentrations of 50 ng/ml, 100 ng/ml and 150 ng/ml. These concentrations were reported to reveal enough protoscolicidal activity (Taylor and Morris, 1988). These drugs were dissolved in DMSO and added to each well to the final concentrations. The concentration of DMSO was 0.75% in the experiments using albendazole sulfoxide and negligible (0.75×10^{-3}%) in the experiments using praziquantel. The parasites were treated in the medium containing each concentration of the drugs for four days and then their viabilities were assessed by the method described above. Furthermore, twenty vesicles stained in blue or not, respectively, were injected intraperitoneally to gerbils for examining their infectivity. Two or three months after the injections, they were sacrificed and examined to determine whether they were infected with the parasite.

Results

Separation of protoscoleces and small vesicles

The protoscoleces and small vesicles were separated using a Percoll gradient, as shown in Fig. 1. Most of the mature protoscoleces were found at the bottom of the tube. In contrast, the small vesicles were observed at the interface between 16% and 28% Percoll. Immature protoscoleces were recovered mainly at the interface between 28% and 38%. However, destroyed vesicles, necrotic tissue and others were found at each interface. A similar pattern of separation was achieved when non-digested minced metacestodes were applied to the gradient, but the amounts recovered were small.

Colorimetric assessment of the viability of germinal cells

These experiments were based on the observations that freshly recovered small vesicles took up MTT and rapidly reduced it to formazan, thereby staining themselves blue. In contrast, heat-killed vesicles were observed not to take up MTT and were

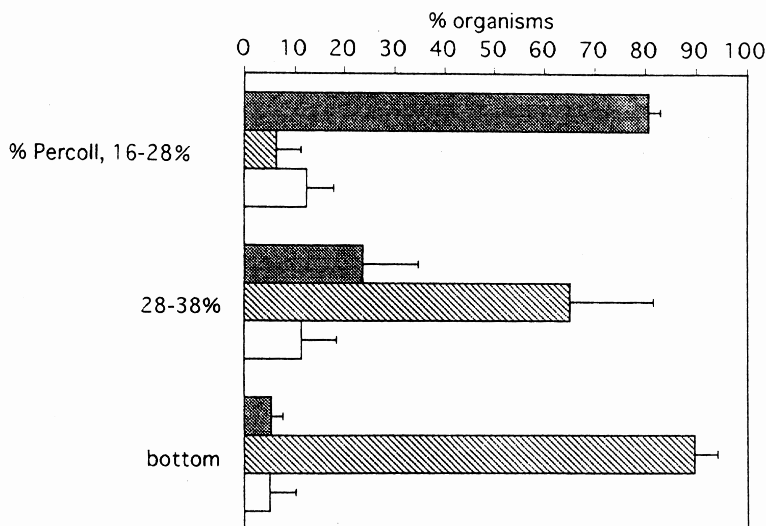

Fig. 1 Separation of *Echinococcus multilocularis* metacestodes by centrifugation on Percoll.
The metacestodes were minced and digested and then passed through a wire mesh. The preparation separated on Percoll.
▩ : vesicles ▨ : protoscoleces □ : destroyed vesicles, necrotic tissue and others

not stained. By microscopic examinations, a color difference could already be observed between freshly recovered and heat-killed vesicles two hours after incubation with MTT. A marked color difference was then observed 16 hours after the incubation (Fig. 2). Quantitative viability assessment was achieved by solubilizing formazan with 100% DMSO 16 hours after incubation. Albendazole sulfoxide is an effective chemical against alveolar hydatid disease. As shown in Fig. 3, 100 μg/ml or more of the drug concentration suppressed the MTT absorptions. In contrast, praziquantel which is uneffective against alveolar hydatid disease did not suppress them. The small vesicles stained in blue two hours after incubation with MTT and those not stained 16 hours after incubation were separately injected into the peritoneal cavity of gerbils. Two or three months after infection, the gerbils were sacrificed and examined for infection (Table 1). The small vesicles which took up MTT were able to infect all of the gerbils. On the contrary, the vesicles showing no staining had a much lower infectivity rate.

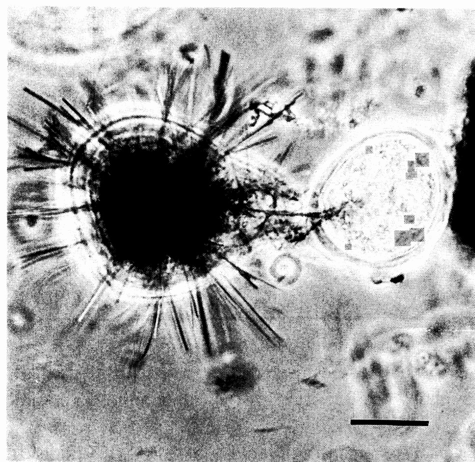

Fig. 2 Vesicles which absorbed MTT (left) and did not (right) 16 hours after incubation in the medium containing MTT.
Bar = 100 μm

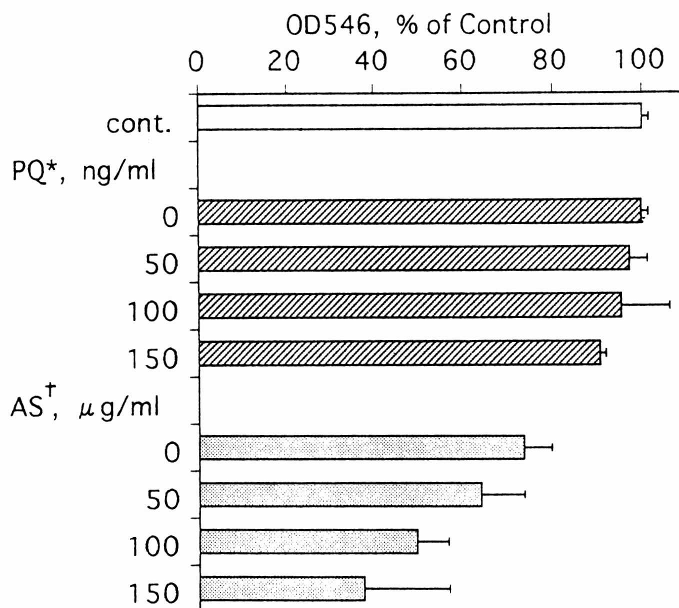

Fig. 3 Viability of germinal cells of *Echinococcus multilocularis* located in the vesicles, measured using the colorimetric MTT assay. Albendazole sulfoxide (AS) and praziquantel (PQ) were dissolved in DMSO.
Control medium contains no DMSO.
*DMSO concentration in the medium was negligible (0.00075%).
†DMSO concentration in the medium was 0.75%.
Heat-killed vesicles showed 25% of control OD.

Table 1 The results of experimental infection by either stained or unstained vesicles to Mongolian gerbils

	Vesicles stained in blue	Vesicles not stained
Exp. 1	4/4*	1/4
Exp. 2	4/4	2/6

*No. of infected animals/No. of used animals.
Twenty vesicles were injected intraperitoneally to gerbils and the animals were sacrificed two or three months after injection.
The vesicles stained in blue were assessed two hours after adding MTT to the medium. The vesicles not stained were assessed 16 hours after adding MTT to the medium.

Discussion

Germinal cells of *E. multilocularis* are drawing considerable attention because they play an important role in alveolar hydatid disease in humans. Production of new brood capsules and protoscoleces is initiated by germinal cells inside the vesicles. Preparation of protoscoleces and assays for their viability are not difficult (Smyth and Barrett, 1980). Studies on *in vitro* drug efficacy against protoscoleces have been reported (Sakamoto, 1973; Duriez *et al.*, 1992), and research on host defence mechanisms using protoscoleces has been carried out (Kassis and Tanner, 1977; Kamiya *et al.*, 1980; Kanazawa *et al.*, 1993). Protoscoleces develop into adult worms in the small intestine of the final hosts, and they could not directly induce alveolar hydatid disease in humans. In order to clarify drug efficacy or host defence mechanisms in humans against the parasite, investigations of germinal cells are essential. However, *in vitro* culture techniques of germinal cells have not been established.

A method of preparing vesicles including germinal cells from metacestodes has been reported

(Hemphill and Gottstein, 1995). Vesicle suspensions were made by pressing metacestode material through a sterile metal sieve. Although good materials are obtained by this technique, our method has certain characteristics. We could collect many vesicles and prepare both vesicles and protoscoleces at the same time. Furthermore, because the metacestode of *E. multilocularis* is a multivesicular, infiltrating structure with no limiting host-tissue barrier, it is contaminated with a lot of host cells. We used a protease to disperse them. Therefore, the vesicles prepared by our technique were contaminated with only a small amount of host cells. We also confirmed that the protease did not affect the viability of the germinal cells (data not shown).

The metabolism of MTT to formazan has been widely applied as a quantitative measure of cell viability (Mosmann, 1983). The application of MTT assay to helminths has been reported in investigations concerning *Dipetaronema viteae* and *Brugia pahangi* (Comley *et al.*, 1989), *Onchocerca gutturosa* and *O. volvulus* (Towson *et al.*, 1990), *Schistosoma mansoni* (Nare *et al.*, 1991) and *Toxocara canis* (Akao *et al.*, 1995). Other parameters for parasite viability, especially microscopically evaluating motility, morphological integrity and dye exclusion of the parasite, have also been used. These microscopical assessments are sometimes sophisticated and time-consuming. On the other hand, the MTT assay was simple and could be performed rapidly.

In the present study, this assay was applied to the assessment of the viability of germinal cells located in the vesicles of *E. multilocularis*. We observed that freshly prepared small vesicles took up MTT at 37°C and that either heat-killed or formalin-fixed small vesicles did not take up the chemical. Albendazole sulfoxide, which is considered to be an effective chemical against alveolar hydatid disease, suppressed the absorption of MTT in a dose-dependent manner. DMSO itself was also thought to be harmful to germinal cells because the MTT absorption by the vesicles cultured in the medium containing 0.75% DMSO was suppressed in comparison with the vesicles cultured in the medium containing no DMSO. The vesicles stained in blue could infect gerbils without fail, but the ones not stained could hardly infect the animals. Therefore, we concluded that this assay was an appropriate tool for the assessment of the viability of germinal cells. Nevertheless, because a few animals were infected with alveolar hydatid disease by the injection of unstained vesicles, we must not forget that germinal cells in parts of unstained vesicles are also alive and still retain their infectivity.

The culture conditions used in these experiments were not favorable for germinal cells because their absorption of MTT decreased day by day (data not shown). To investigate germinal cells inside vesicles *in vitro*, we have to carry out experiments under the best culture conditions. Hemphill and Gottstein (1995) recently reported about the maintenance and proliferation of *E. multilocularis* metacestodes as well as the formation of protoscoleces in a chemically defined medium devoid of host influence. This culture conditions and the methods reported here will be expected to help toward carrying out studies on metacestodes.

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