[Jpn. J. Parasitol., Vol. 36, No. 3, 179–182, June, 1987]

Research Note

Virulence of *Toxoplasma gondii* (RH-cyst III strain) Cultured in Mouse Embryo Cells

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(Received for publication; November 14, 1986)

Key words: *Toxoplasma gondii*, Cell culture, Cyst-like body

Toxoplasma gondii (Tg), an obligate intracellular protozoa, grows in mammalian and avian cells by endodyogeny. The virulence of the parasite has been evaluated on the basis of different criteria such as invasiveness, dividing time, cyst formation and the mortality and survival time of animals given graded inocula of parasites. Virulent strains cause rupture of host cells due to unrestricted multiplication within short periods. On the other hand, avirulent strains grow relatively slowly in the host cells and the infected hosts can survive for a long period, harboring cysts in organs.

The RH strain is a virulent strain which can kill a mouse within a few days post infection. It has been passaged for over 30 years in mice and other hosts and is commonly used for sero-diagnosis and research works. Recently, Yano (1986) isolated an avirulent strain of the parasite from immune mice infected with the RH strain. He reported that mice infected with the isolate could survive asymptomatically for more than 30 days post inoculation and cysts were found in their brains and other organs. To investigate the characteristics of this newly isolated strain (designated as RH-cyst III strain), the parasites were cultured in mouse embryo cells (MEC). MEC were obtained from embryos of BALB/c mice at 12–14 days of pregnancy.

Cysts of the RH-cyst III strain were obtained from chronically infected mice and inoculated to mice intraperitoneally (i.p.). On the 7th day post inoculation the peritoneal exudate was harvested and centrifuged at 3,000 rpm for 10 min. The sediments with trophozoites were suspended in Eagle's minimum essential medium containing 10% fetal calf serum, 2 mM glutamine, penicillin G-potassium and streptomycin sulfate (MEM10FCS) and added into MEC monolayered tissue culture flasks (Corning Ltd., 75 cm^2 cat. 25110). They were incubated overnight at 37°C after which the supernatant was agitated and discarded together with nonadherent cells and cell debris. Fresh MEM10FCS was added and further incubated for several days under daily observation using an inverted microscope. When parasite multiplication was observed, the culture medium was harvested and filtered with a filter paper (S&S Ltd., FR030/1). After centrifugation at 3,000 rpm for 10 minutes, the sediment was resuspended in MEM10FCS and the number of parasites was adjsuted to 5×10^5/ml. Each 0.2 ml of the parasite suspension was mounted on 15 mm × 15 mm cover glasses monolayered with MEC and incubated at 37°C for 11 hours. Then, the cover glasses were washed and transferred into petri dishes containing MEM10 FCS. After incubation at 37°C for 6, 12, 24, 36, 48 hours and 7 days, cover glasses were fixed with meth-

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anol and stained with Giemsa. Cyst-like structures observed were classified according to the number of parasites enclosed within the membrane.

To examine the virulence of RH-cyst III parasites cultured in MEC for at least 4 months, ICR adult mice were i.p. inoculated with 0.5 ml of 5×10^6 parasites. On the 7th and 30th day post inoculation, the peritoneal exudates and brains were examined for the presence of parasites.

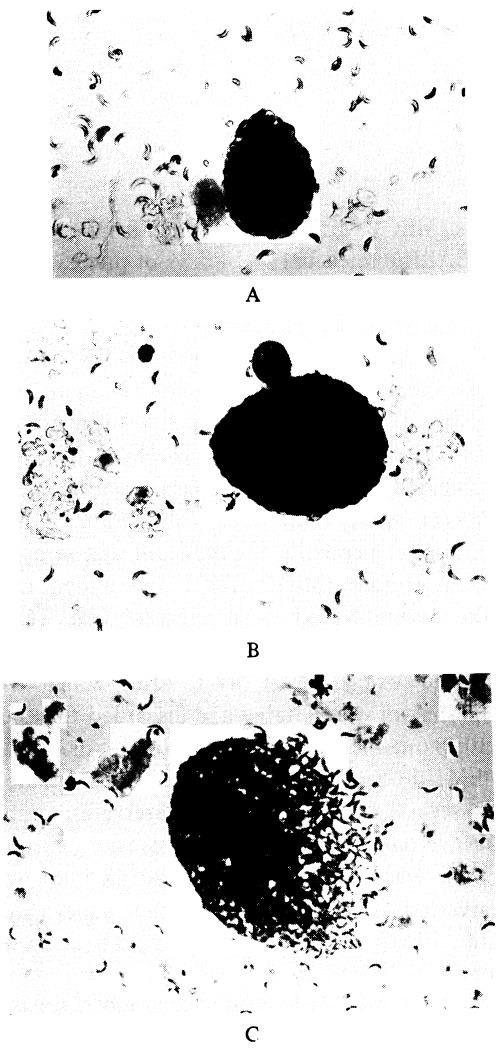

Fig. 1 Cyst-like body from MEC culture medium (Giemsa stain).
A: The nucleus of host cell adheres to the cyst-like body.
B: Note different sizes of cyst-like bodies.
C: Note burst membrane of cyst-like body and subsequent release of the parasites into the medium.

Results show that on the 7th day post inoculation with RH-cyst III strain obtained from chronically infected mice, many mononuclear cells and only a few (less than 1×10^4/ml) parasites were observed in the peritoneal exudates of mice. When a suspension of such mononuclear cells and parasites was added in the MEC monolayer culture, the colonies consisting of burst MEC and clumps of parasites appeared on the monolayer about 5–7 days later. Moreover, it was interesting to note that within the MEC cytoplasm were membrane-bound structures full of tightly packed parasites. Some of these structures remained intact even when the host cells were denatured or broken (Fig. 1A). Such structures, tentatively designated as "cyst-like body", have been maintained in culture for more than 4 months. The extracellular parasites were separated by filtration from the cyst-like bodies and showed a viability of more than 95% by trypan blue staining test. Table 1 shows the progressive development of the RH-cyst III parasites in culture. Parasites penetrated MEC from 0 to 11 hours post challenge. Subsequently, they began to divide in the parasitophorous vacuoles from 12 hours onward at approximately 12 hour intervals. Ninety-six hours later some of the infected cells ruptured. In some cells the limiting membrane of the cyst-like bodies were observed to have become thicker.

Table 1 Growth of *Toxoplasma* RH-cyst III strain in MEC culture

No. Parasites in a parasitophorous vacuole	No. parasitophorous vacuole			
	11	23	35	47 hr. p. i.*
1	200	243	62	32
2	0	69	178	84
4	0	0	4	106
8	0	0	0	0

p.i.: post inoculation
*On the 7th day p.i., cyst-like bodies were detected in culture media.

Such bodies were frequently observed not only in cultured cells but also in culture media in repeated experiments. The membrane of the cyst-like bodies could be digested with 0.02% trypsin solution at 37°C for 15 minutes. The parasites in the cyst-like bodies were stained partly with periodic acid-schiff's reagent.

Mice which were inoculated with RH-cyst III parasites cultured in MEC for at least 4 months could survive without any clinical sign for more than 30 days post inoculation. However, cysts were detected in their brains (Fig. 2, Table 2). On the other hand, mice inoculated with 5×10^4 trophozoites of RH strain succumbed and died within 6 days post inoculation (data not shown).

The observation in this report indicate that the RH-cyst III strain isolated from RH strain, maintains a low virulence and forms cyst-like bodies continuously for a long time under cell culture conditions without the presence of specific antibodies against Tg parasites. In our laboratory, we have never found cyst-like body in cell culture inoculated with RH strain parasites. The doubt of the contamination of cyst-forming protozoa in RH-cyst III parasites would be negligible, because the mice inoculated with RH-cyst III parasites showed the increase of the titer of anti Tg antibodies (Yano *et al.*, 1986).

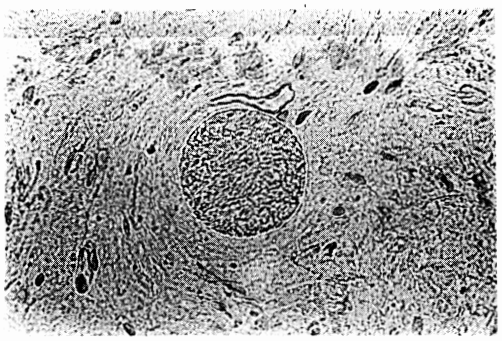

Fig. 2 Squash preparation of an infected mouse brain (unstained).
Note the tissue cyst is round and the cyst wall in composed thin layer membrane.

Table 2 *Toxoplasma* infection with RH-cyst III strain in mice

No. mice	days p. i.	Detection of parasites	
		peritoneal cavity	brain
3	7	less than 5×10^4 (T)	ND
6	30	ND	Detected (C)

ND: Not detected.
T: trophozoite
C: cyst

There have been reported the observation of cyst-like bodies found in cell cultures inoculated with RH strain and that the presence of anti Tg antibodies in the culture medium enhanced the production of cysts (Hogan *et al.*, 1960; Shimada *et al.*, 1974). Dubey reviewed that even RH strain, which has been passaged as trophozoites for long periods can form cysts under suitable conditions without immune factors (Dubey, 1977). On the other hand, Matsubayashi and Akao observed cyst like clusters in HeLa cells and L cells infected with Beverley strain but they did not find cyst-like body in the case of cell culture of RH strain (Matsubayashi and Akao, 1963).

It is presumed that RH-cyst III strain would be selected by the protective immune responses against Tg infection from the population of RH strain parasites or would be transformed from the vilurent parasite to the slow-dividing and cyst forming parasite during the period of passages in mice immunized with Tg antigen.

In the present study, it was observed that both the free trophozoites and the cyst-like bodies developed concomitantly for some duration in the same cell-culture flasks. However, although the free trophozoites could be separated from the cyst-like bodies by means of filtration technic, the possibility of coexistence by cyst-forming parasites freed from cyst-like bodies cannot be completely ruled out (see Fig. 1C). Trophozoites with cyst-forming ability released from broken cyst-like bodies might have been floating in the culture medium. This experiment, however, was not done with use of a cloned parasite line. Accordingly, further minute studies would be necessary to obtain

a possible explanation of the cyst-like body formation by RH-cyst III strain.

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