

Survival of *Miamiensis avidus* (Ciliophora: Scuticociliatia) from antibody-dependent complement killing

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Previously, we had reported that some *Miamiensis avidus*, a major pathogen of scuticociliatosis in cultured olive flounder, strongly agglutinated by flounder immune sera could escape from the agglutinated mass within a few hours. In the present study, we observed that *M. avidus* not only escaped from the agglutinated mass but also conducted division(s) before shedding its old covering. Furthermore, ciliates that survived the antibody-dependent complement killing (ADCK) assay were not killed even when re-exposed to a freshly prepared ADCK assay. This result suggests that the liberated ciliates from the ADCK assay might change not only their i-antigen types but also the epitopes of major surface antigens, which debilitate antibody-mediated complement killing ability.

Key words: *Miamiensis avidus*, Agglutination, Antibody-dependent complement killing, Division, Survival

A protein called immobilization antigen (i-antigen) is known as the major protein covering ciliates surface including cilia. Although free-living ciliates such as species in the genera of *Tetrahymena* and *Paramecium* have a repertoire of genes encoding several i-antigens, the expression of the genes in protein level is limited to only one type in a time (Leeck and Forney, 1996; Simon and Schmidt, 2007), and the i-antigen type can be switched into other i-antigen type by environmental changes, such as temperature (Sommerville, 1970). In facultative parasitic ciliates, changes in i-antigen type attendant on infection may be crucial for survival against host's immune attacks. *Miamiensis avidus* (Syn. *Philasterides dicentrarchi*)

is a facultative parasitic ciliate and has been a culprit of mass mortalities in cultured marine fish, such as turbot (Iglesias *et al.*, 2001), sea bass (Dragesco *et al.*, 1995), and olive flounder *Paralichthys olivaceus* (Kim *et al.*, 2004).

Iglesias *et al.* (2003) reported that turbot immunized with formalin-fixed or lysated *P. dicentrarchi* showed serum agglutinating activity and partially protected against *P. dicentrarchi* infection, suggesting the usefulness of i-antigen as a target for vaccine against scuticociliatosis. They also observed that some agglutinated ciliates escaped from the agglutinated mass by sloughing off their old outer covering. Similarly, we had previously demonstrated that some *M. avidus* strongly agglutinated by flounder immune sera could escape from the agglutinated mass within a few hours (Lee and Kim, 2008).

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In the present study, we have observed that *M. avidus* not only escaped from agglutinated mass but also conducted division(s) before shedding off old covering. Furthermore, ciliates survived and liberated from the agglutinated mass during antibody-dependent complement killing (ADCK) assay were not killed even when re-exposed to the freshly prepared ADCK assay.

Materials and Methods

Culture of ciliates and production of rabbit antiserum

Culture of *M. avidus*, antigen preparation, and olive flounder immunization were performed as described in Lee and Kim (2008). To obtain rabbit immune serum against *M. avidus*, specific pathogen free New Zealand white rabbits weighing 2 kg were used in the experiment. Two rabbits were intramuscularly immunized with either phosphate buffered saline (PBS) alone (Control) or 1 mg of cultured *M. avidus* lysate emulsified with an equal volume of Freund's complete adjuvant (FCA, Sigma). Two booster injections of either PBS alone or the antigen with identical dose of ciliate lysate emulsified with Freund's incomplete adjuvant (FIA, Sigma) were done at two weeks interval. Two weeks after the second booster injection, the rabbits were sacrificed and bled. The collected sera were stored at -80°C .

Agglutination and Antibody-dependent complement mediated killing (ADCK) assays

Ciliates cultured from Chinook salmon embryo (CHSE)-214 cells were washed three times in Hank's balanced salt solution (HBSS, Sigma), before being incubated with olive flounder sera or rabbit sera in 96-well flat-bottom micro titration plates. For agglutination assay, the sera were previously heat inactivated at 50°C (olive flounder) or 56°C (rabbits) for 30 min to inactivate complement. All the sera were diluted serially ranging from 1/4 to 1/4096 in HBSS. After

addition of ciliates to the wells (1×10^3 ciliates/well), the plates were incubated at 20°C and were observed every 10 min for 2 hrs to analysis agglutination. In all assays, control wells containing HBSS and ciliates were included. After 24 hrs incubation in agglutination assay, the ciliates were separated from the media by centrifugation, washed with HBSS, and re-exposed to newly prepared heat-inactivated immune sera for agglutination or exposed to heat-inactivated immune sera plus freshly prepared control sera for ADCK activity. To confirm whether agglutination activity was still remained in the used media of agglutination assay, freshly cultured naïve ciliates were exposed to the used media.

For ADCK assay, 500 ciliates were added to each well containing 50 μl of serially diluted (1/4-1/4096) heat-inactivated immune or control sera. The plates were incubated at 20°C for 10 min, and then added 50 μl of naïve olive flounder or rabbit sera as complement source. The concentrations of added naïve sera were one half of the last dilution showing scuticocidal activity. The plates were observed every 1 hr for over 16 hrs. After 24 hrs incubation ADCK assay, the ciliates were separated from the media by centrifugation, washed with HBSS, and re-exposed to newly prepared media for ADCK assay.

Results and Discussion

Both flounder and rabbit antisera produced by immunization of CHSE-cultured ciliates showed strong agglutination activity against cultured ciliates. Interestingly, we firstly observed that *M. avidus* strongly agglutinated by immune sera conducted division(s) before sloughing off the old outer covering (Fig. 1). In the rabbit sera (Fig. 1. A,B), the cyst-like formation and escaping were lasted over 48 hrs, whereas in the flounder sera (Fig. 1. C,D), this phenomenon was lasted only 24 hrs. This phenomenon was not occurred in ciliates weakly agglutinated by immune sera, but occurred only in strongly agglutinated ciliates, sug-

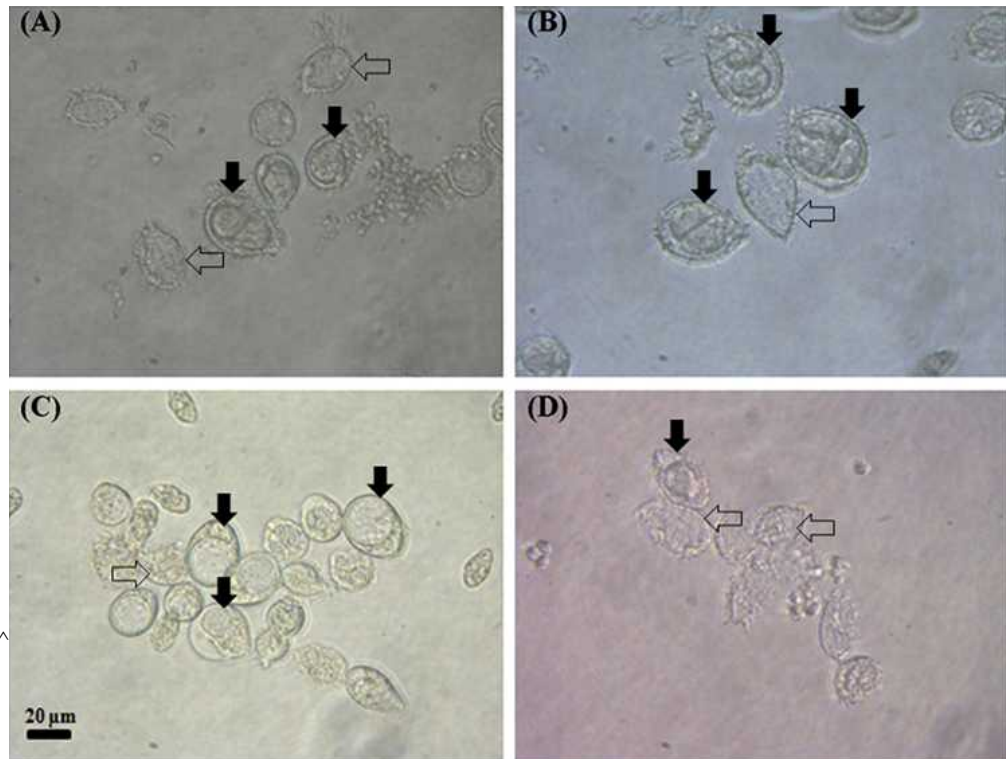


Fig. 1. Escaping *Miamiensis avidus* from agglutinated mass by sloughing off old outer covering. Some ciliates conducted division before escaping from the old outer membrane when incubated in the rabbit (A, B) and flounder (C, D) immune sera at higher concentrations in which strong agglutination activities were shown. Arrows indicate cyst-like containing single or dividing ciliates (black arrows) or empty cyst-like after escaping of ciliates (empty arrows).

gesting that heavy agglutination might be a drive force to form cyst-like and to initiate division of *M. avidus*.

In agglutination assay, liberated ciliates from the aggregated mass freely moved in the freshly prepared heat-inactivated antiserum, but most of all were killed by addition of complement source. This result suggests that the liberated ciliates might change i-antigen type, which conferred the ciliates to move freely in the heat-inactivated antiserum. However, as other major surface antigens' epitopes, which provide binding sites to antibodies and activate classical complement pathway, might not be changed, most of the liberated ciliates were killed by exposure to complement.

In ADCK assay, ciliates liberated from the aggregated mass and survived from the ADCK media

showed free movement in spite of the presence of immune sera in the assay media which still had activity to kill the freshly cultured ciliates by ADCK. Furthermore, the survived ciliates were not killed even by re-exposure to the freshly prepared ADCK assay media. This result suggests that the survived ciliates from ADCK assay might change not only i-antigen type but also epitopes of major surface antigens, which debilitate antibody-mediated complement killing ability. Further studies are needed to determine whether *M. avidus* can evade previously established adaptive humoral immune responses.

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