RESEARCH ARTICLE

Isolation and Purification of the Schizont Stage of *Theileria annulata* from Host Leukocytes through Novel Biochemical Techniques

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Abstract

Background: The protozoan intracellular parasite, *Theileria annulata*, induces uncontrolled proliferation and transformation in bovine B lymphocytes and monocytes in the blood circulation and lymph nodes of host cells in macroschizont stage of life cycle. The development of a rapid and efficient technique is likely to necessitate for isolation purified schizonts from host cells for incorporation in isolation highly purified RNA, protein, or glycoproteins of schizonts from host cells. **Materials and Methods:** This study approved based on the aerolysin - nocodazole. Aerolysin that purified from gram-negative pathogen *aeromonas hydrophila* has an ability to form discrete channels and unstable a eukaryotic cell membrane in 0°C and low concentration. Nocodazole used for parasite separation from Microtubule network of infected lymphocytes and monocytes. **Results:** In purified schizonts, no large nucleus of host cell visualize in Giemsa and 4',6-diamidino-2-phenylindole staining. Schizonts are free and intact from host cells. **Conclusions:** intact *T. annulata* schizonts suitable for RNAs, proteins, glycolipids, and glycoproteins of schizonts free of host debris.

Key words: 4',6-Diamidino-2-phenylindole, aerolysin, nocodazole, percoll, *Theileria annulata*

INTRODUCTION

Theileria annulata is an obligate intracellular, protozoan parasite that causes tropical theileriosis, a devastating lymph proliferative disease of cattle in developing countries.[1] Schizont stage of *T. annulata* lives in B lymphocytes and monocytes of cattle blood circulation and lymph nodes. Devastating changes in gene expression and protein production constrain by macroschizonts. These changes induced lymph proliferation and immortality in host cells. These alterations in infected cells facilitated increasing in number and finding of new host cells without egressing from previous host cells. The capacity of *T. annulata* to induce leukocyte transformation implies within the infected leukocyte express "oncogenic" substances, and as a step toward their identification, we must develop a procedure lead to the isolation of the parasite gene(s) responsible.[2-5] Although parasite DNA can be readily obtained from infected erythrocytes due to the absence of host cell nuclei, identification of putative oncogenes requires purification of parasite-derived peptides, proteins, and/or the construction of parasite-specific cDNA libraries made from the transforming stage of the parasite's life cycle.

For many researches and experiments needfulness to have purified protein, RNA or DNA of intracellular parasite is obviously required.

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Observation multinucleated macroschizonts are common a few hours later subsequently sporozoites entered leukocytes of cattle blood circulation especially B lymphocytes and monocytes. The most critical aspect of invasiveness of *T. annulata* correlated with altered expression of downstream target genes in cell life cycle arrangement in this stage.[6,7] A strong interaction between some parasite surface proteins and microtubules of host cells obviously important.[3,7,8] Microtubules are the primary components of mitotic spindles and are, therefore, essential for mitotic cell division. Moreover, in concert with actin and intermediate filaments, microtubules organize the cytoplasm and control trafficking, and their disruption leads to cell cycle arrest and loss of cellular architecture.[9,10]

The strong parasite-microtubule interaction prevents efficient separation of the parasite cells from their host cells, so if we would like separate schizonts from host cells efficiently, we must isolate the association between microtubules and parasites in leukocytes.[6,11] There are various microtubule inhibitors in pharmacology, *nocodazole* is one of them that is in the family of colchicine-site binders that interact with tubulin dimers, inhibit their assembly into microtubules, and enhance GTPase activity in the absence of polymerization, and addition of nocodazole to mammalian cells cultured *in vitro* results in the loss of most of the cytoplasmic microtubules including spindles.[12,13] At sufficient and tested concentrations to disrupt host cell microtubules by nocodazole, there is no effect on the schizont of *T. annulata*.[14-16] After schizont isolation, if we did not use nocodazole in schizont purification, we will have incomplete synchrony in established cell lines, and there will no clear results to obtain studies on how the parasite induces host cell proliferation to involve cell cycle analysis.[16]

Aerolysin is a channel-forming cytolysis toxin that is secreted as a proaerolysin form by a gram-negative rod shape microorganism, *Aeromonas hydrophila*.[17-19] The ability of destruction membrane and permeability barrier of aerolysin caused with 3 nm holes of proaerolysin polymerization.[20,21]

Lower concentrations of a lethal concentration of aerolysin on eukaryotic cells cause sensitive cells to osmotic changes without entire destruction[16,20,21]

In this study, we used aerolysin that we had isolated from *A. hydrophila*. The concentration used in this study was lower concentrations than can lysis RBC of cattle in hemolysis test.

MATERIALS AND METHODS

Parasite strain and culture conditions

The vaccine cell line, strain S15 Iran, of *T. annulata* used in this study was obtained from the *Razi Vaccine and Serum Research Institute*. The parasites were cultured in RPMI-1640 medium (Sigma, Chemical Co., St. Louis, U.S.A.) supplemented with 10% FBS (Sigma, Australia), 292 µg/ml L-glutamine (Sigma, WKG, Germany), 4.5 mg/ml glucose, 100 µg/ml penicillin (Sigma, WKG, Germany), and 100 µg/ml streptomycin (Sigma, WKG, Germany).

Microtubule destruction

2×10^8 S15 cells were grown to a density of approximately 8×10^5 cells/ml. About 3 µM nocodazole (Sigma. WKG, Germany) used to destruct microtubules of the host cell for 2 h at 37°C. Cells were harvested by centrifuge 200 g for 5 min at 37°C. After washing one time by ice-cold PBS, cells resuspended 5×10^7 cells/0.9 ml ice-cold 1X HEPES buffer (10 Mm HEPES, 150 Mm NaCl, and 20 Mm KCl, pH 7.4) containing 1 Mm CaCl2. Resuspended cells in HEPES buffer and transferred into 1.5 ml microtubes (Eppendorf).

Aerolysin

Activated aerolysin destructs infected cells and schizonts completely by direct treatment.

However, at 0C•, aerolysin only bind to receptors on the plasma membrane of target cells and is not active; therefore, incubation on icy aerolysin causes enzyme binds to cell membrane but prevents rapid cell lysis of host cell and subsequently destruction of parasite cells.

After isolation, aerolysin of *A. hydrophila*, with hemolysis test, calculated 1 mg isolated enzyme must be used for 2×10^8 cells. About 1 mg activated enzyme is soluble in 1.5 ml ice-cold PBS. Cells in HEPES buffer added to the activated enzyme in ice-cold PBS and incubated the tubes under rotation on ice for 30 min. After this time, the enzyme binds so tightly that washing the cells is not able to remove enzyme from receptors. By two gentle washing (1200 rpm, 5 min at 4°C) in ice-cold PBS, unbound enzyme removed. Cells resuspended in ×1 HEPES containing 1 Mm CaCl2 and incubated at 37C• for 30 min (Per 10 min tested trypan blue exclusion when seen trypan blue is penetrated to cells, stopped incubation). Finally, by centrifuge at 200 g, pelleted cells (by sensitive membrane) and 5×10^7 cells/mL were resuspended in 1X HEPES containing 5 Mm EDTA.

Centrifugation for schizont separation from host cells

Host cell debris and nuclei were separated from schizont of *T. annulata* by percoll centrifugation essentially as described by Baumgartneret *et al.*, with several changes. In brief, a stock solution of percoll was prepared by mixing 8.5 parts of percoll with 0.5 part of 20 x HEPES (200 Mm HEPES,

3 M NaCl, 400 Mm KCl, pH 7.4) and one part of 50 Mm EDTA (pH 7.4). We in this study didn't use ultracentrifuge, so we made solution in 2 ml microtube (Eppendorf). The cell lysate (138 µL) was added to 520 µl of this percoll stock solution, and the volume was adjusted to 680 µL by the addition of ×1 HEPES containing 5 Mm EDTA, giving rise to 64.6% (vol/vol) final percoll concentration. The percoll-cell lysate mixture was transferred into 2 mL microtubes and carefully overlaid with a 45% percoll solution in 1X HEPES and 5 Mm EDTA.

The mixture was thereafter centrifuged at 12,000 rpm for 1 h at 10C˚. During centrifugation, a gradient is established that separates parasites by density from cellular debris and nuclei, after centrifugation, we have two bands, and parasite cells are between two layers. It can be collected with a Pasteur Pipette. Next, the parasites from the individual samples were collected in a 2 ml microtube. In this stage, in Giemsa staining, there are a lot of percoll crystals, so it is necessary to wash once in a large volume of PBS and pelleted by centrifugation at 5000 rpm, for 10 min at 4°C.

Giemsa staining

Giemsa staining is a simple method to evaluate Purified schizonts. In Giemsa staining, nuclei of the host cell and schizonts visible in infected leukocytes. After purification, in Giemsa staining, there are no visible nuclei of host cells [Figure 1].

4',6-Diamidino-2-phenylindole staining

A simple analysis to check the quality of purified parasites is staining the parasite fraction with 4',6-diamidino-2-phenylindole (DAPI), a DNA intercalating agent. In DAPI staining, nuclei stained and under a mixture of UV and standard illumination, seen nuclei of purified free schizont without nuclei of the host cell [Figure 2].

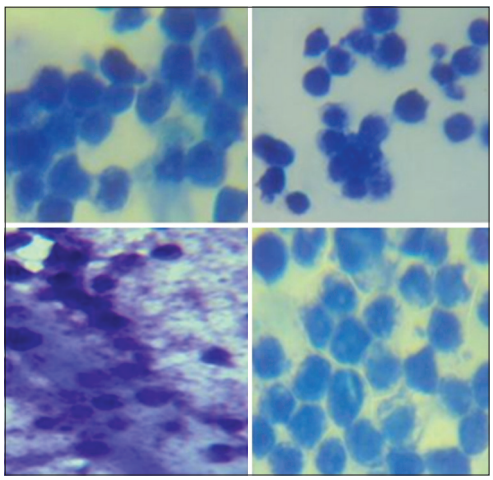

Figure 1: Light microscopic images of Giemsa staining of intact schizont stage of *Theileria annulata* free of host nuclei (×1000)

RESULTS AND DISCUSSION

The most important point that draws attention in the image of purified schizonts in Giemsa and DAPI staining is lack of large nuclei of host cells.

The macroschizonts are very clear in DAPI and Giemsa staining within miniature nuclei of microschizonts.

These purified schizonts are ready for any experiment that aims only RNA, cDNA, protein… of schizont without impurity and debris of host cells.

In this study, nocodazole elevated the quality and quantity of purification. In the early phase of study, nocodazole did not use in purification process and in comparison to sample that used nocodazole, acceptable results not appeared.

There is a close association between *T. annulata* and the host cell microtubule network. In mitotic cell division a task of microtubules is the separation of chromosome pairs, in infected host cells with *T. annulata*, schizonts bind to microtubules and divide between two daughter cells, soused the colchicine site-binder, nocodazole, improve schizonts separation and purification. In addition, this is confirmed that

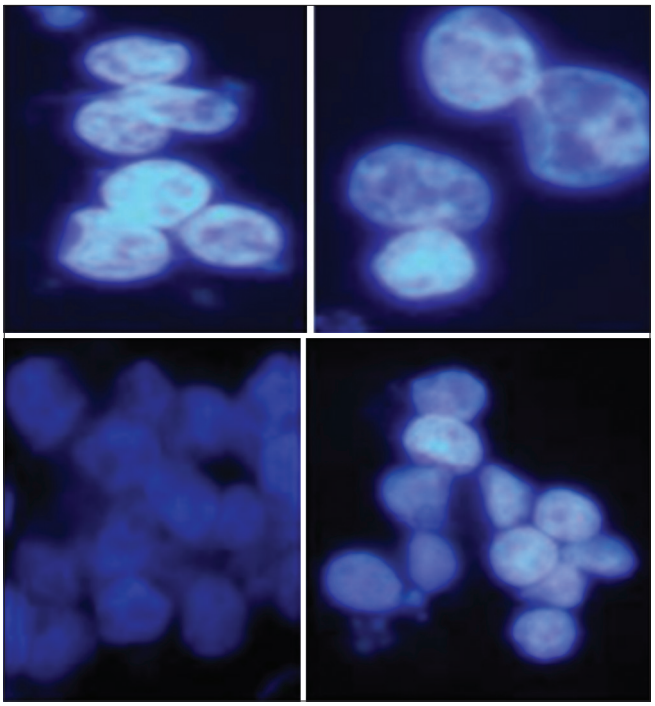

Figure 2: 4',6-Diamidino-2-phenylindole staining of purified schizont of *Theileria annulata* free of host nuclei under ultraviolet light microscopy (×1000)

nocodazole provokes reversible growth arrest in mammalian cells so in this study we had cell cycle synchronization of asynchronously growing *T. annulata* infected host cells.

Intact schizonts without any damage in cell structures was a principal purpose in this study thus some materials were not suitable for host cell membrane permeability, detergents and some enzymes enable to permeable cell membrane; however, controllability is very significant.

Aerolysin in lethal dose can damage to the plasma membrane of the host cell and kills host and schizont, but in lower concentration of lethal dose, and in low temperature, this enzyme enables to the permeable cell membrane of host cells without killing schizonts.

If we used aerolysin directly at 37°C not only host cells but also parasites lysed, but if infected cells along with icy aerolysin pre-incubated on ice, aerolysin bound to glycoproteins of host cells, and in the following, washing (remove) unbound enzyme, there will not be free enzyme to lysis schizont cells.

In this study, isolation and purification schizont stage of *T. annulata* was performed with nocodazole and aerolysin. Ultracentrifugation is a common step in published papers about this project but in this study did not use ultracentrifuge but schizonts isolated from host nuclei and debris completely with a centrifuge in longer time and lesser volume in 2 ml microtube.

Purified schizonts of *T. annulata* applicated for glycosylphosphatidylinositol (GPI) anchors and free GPIs isolation from schizont cell membrane for the first time.

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