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In vitro culture and synchronous release of *Sarcocystis neurona* merozoites from host cells

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Abstract

The growth of *Sarcocystis neurona*, isolate UCD1, in continuous culture was examined in 10 cell lines to identify growth conditions and methods for the preparation of parasites free of gross host cell contamination for molecular studies. The unpredictable, slow release of merozoites in most cell lines prompted development of a method to synchronously release the parasites from infected host cells. The calcium ionophore A23187 at a concentration of 1 μM was found to release intracellular merozoites with a 40 min treatment at 37°C. The release of merozoites en masse from attached host cells allowed for the rapid collection of relatively pure parasites from the culture supernatant. This release of merozoites occurred in five different host cell lines. The ionophore-released parasites were highly infectious for host cells and appeared to be morphologically identical to naturally released merozoites, except that the treated merozoites had an increased number of micronemes when examined by electron microscopy. The ionophore did not enhance the release of sporozoites from sporocysts, but freezing in the presence of 5% DMSO released sporozoites that were infectious to bovine monocytes in in vitro culture.

Keywords: *Sarcocystis neurona*; In vitro culture; Method; Ionophore; Equine protozoal myeloencephalitis

1. Introduction

Cyst-forming coccidia of the genus *Sarcocystis* are among the most prevalent parasites of livestock and are responsible for considerable economic losses (Jakel et al., 1999). Equine protozoal myeloencephalitis (EPM) is a debilitating central nervous system disease of the horse resulting from infection at this site with merozoites of *Sarcocystis neurona*. Merozoites of *S. neurona* are obligate intracellular parasites that can be cultured in several host cell lines (Davis et al., 1977; Lindsay et al., 1999; Speer et al., 2000). It is difficult, however, to isolate merozoites in suitable quantity and purity from host cells for molecular studies

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because *S. neurona* is a comparatively slow-growing, and an obligate intracellular parasite where the separation of this parasite from host cell debris is problematic. Further, there are multiple developmental forms of the parasite seen in cell culture (Speer et al., 2000). Preparing immunoreagents and nucleic acids from this organism requires the most pure samples obtainable.

The calcium ionophore A23187 mediates the exit of mature *Toxoplasma gondii* tachyzoites from infected host cells by increasing the Ca^{2+} in the parasitophorous vacuole (Endo et al., 1982; Stommel et al., 1997). It is likely that the spike of Ca^{2+} transduces the signal that ultimately stimulates exit of the parasite; however this final signal is still unknown (Pingret et al., 1996). The synchronous egress of *T. gondii* tachyzoites from the host cell under this stimulus appears to require complete maturation of the parasite, but parasite release from the vacuole occurs within minutes after addition of the ionophore. Although it has not been observed that *S. neurona* forms a parasitophorous vacuole (Dubey et al., 1991), the use of this technique for synchronous release of *S. neurona* merozoites and sporozoites was examined.

In this report, we have described culture conditions and host cell effects on the growth of *S. neurona*, and the use of the calcium ionophore, A23187, to release parasites from host cells. Effects of the ionophore treatment on sporocysts and sporocyst induced primary cultures were also examined.

2. Materials and methods

2.1. In vitro culture of S. neurona merozoites and host cell lines

The UCD1 isolate from the spinal cord of a horse diagnosed with EPM was the gift of Dr. Antoinette Marsh. This isolate has been maintained in bovine monocyte cells (BM) cultured in RPMI media supplemented with 10% bovine serum at 37°C in a 5% CO_2/air atmosphere. Established cell lines and primary cultures were maintained in plastic culture flasks incubated under the same conditions containing media plus serum supplements as described in Table 1. Host cell lines tested for supporting the growth of *S. neurona* were: BM [laboratory stock culture and BM 0617 (American Type Culture Collection, Rockville, MD) CRL 0617], bovine turbinate cells (BT cells, ATTC CRL 1390), human lung cells (HL cells, ATTC CCL 201-8Lu), human foreskin fibroblasts (HFF, ATTC CRL 2450), Chinese hamster ovary cells (CHO cells, ATTC CCL 61), bovine kidney (MDBK cells, ATTC CCL 22), goat tumor cells (GT cells, a gift from Dr. Jack Gaskin), equine dermal cells (ED cells, ATTC CRL 6288), and equine monocytes (EM, primary culture from peripheral blood). Host cells were released from the culture flask surface by trypsin treatment and transferred to fresh culture flasks at a density of 2×10^4 cm^{-2} resulting in a monolayer that was ~60% confluent. Parasites were immediately added at a density of 2×10^3 cm^{-2}. After 3 days of incubation, growth was monitored by counting infective foci under the microscope. Parasites were routinely harvested using methods described below when their density approached an average of 5–10 parasites developing within cells visible in a single microscopic field (400×). Free parasites in the culture medium were monitored by preparing a slide by adding 150 μl culture medium to a cytospin chamber, centrifuged for 6 min at 800 rpm followed by Geimsa staining.

Table 1
In vitro cultivation of *S. neurona*

Days to confluency[a]	Cell type	Media	Additives	#IF/HPF[b] 800×	Release by A23187
2	BM 0617	RPMI	10% bovine serum	5–10	Positive[c]
2	CHO	F12	10% fetal calf serum	3–5	Negative
2	MDBK	RPMI	10% bovine serum	3–5	Negative
2	Baby Hamster Kidney (BHK)	RPMI	10% bovine serum	3–5	Negative
2	GT cells	DMEM	10% bovine serum	1–2	Negative
4	HFF	DMEM	10% horse serum	1–2	Negative
4	ED cells	RPMI	10% bovine serum	3–5	Positive
5	HL cells	DMEM	10% bovine serum[d]	5–10	Positive
5	BT	DMEM	10% horse serum[d]	3–5	Positive
7	BM	RPMI	10% bovine serum	5–10	Positive
ND	EM	DMEM	10% horse serum	3–5	Negative

[a] Cells were plated at a density of 2×10^4 cm^{-2}.

[b] This is the average number of developing parasite forms (intracellular merozoites, schizonts or rosettes) per microscopic field where 10 randomly selected fields were counted on day 3 after initiating the culture of *S. neurona*, UCD-1 in the cell line indicated.

[c] A positive response to the calcium ionophore A23187 is defined by the release of free merozoites into the medium.

[d] Parasite growth in these cell lines was enhanced by the addition of 1 mM pyruvate and 1 mM glutamate to the growth medium.

Studies of the replication of the parasite were performed as follows. Approximately 220 *S. neurona* merozoites recovered from a culture supernatant were added to 5000 HL cells seeded and growing on Thermonax coverslips in 24 well plates to evaluate the growth and natural release of parasites. Every 3 days, the 2 ml supernate was removed and evaluated by cytospin and the number of merozoites present counted. The corresponding coverslip was fixed in methanol and stained with Geimsa and the number of intracellular parasites and those extracellular, but associated with the cover slip, were counted.

2.2. *Preparation of sporocysts for in vitro culture*

Sporocysts of *S. neurona* were obtained by scraping the mucosa of feral opossums that had been killed on the roadways in Florida. Mucosal scrapings were stored in antibiotic media at 4°C until used (Murphy and Mansfield, 1999). *S. neurona* sporocyst isolates were selected from among those identified using DNA marker analysis (Tanhauser et al., 1999). Prior to being placed in culture, sporocysts were treated in 5% sodium hypochlorite (bleach) for 5 min and washed in tap water by repeated cycles of centrifugation (300*g*, 10 min) and resuspension until the smell of bleach was gone. Sporocysts were floated on a 20/30/60% isosmotic colloidal silica step gradient (Percoll®), washed twice in PBS, as described above. Sporozoites were excysted by either of two methods: (1) ~100 sporocysts (quantified by counting sporocysts present in 25 μl hanging drop observed at 400×) were resuspended in 100 μl horse bile containing 2 μl trypsin (5 units/ml) and incubated for 4–6 h at 37°C under a 5% CO_2 95% air atmosphere. Sporozoites and unexcysted sporocysts were collected

by centrifugation as above, washed once in phosphate buffered saline, pH 7.2 (PBS) and resuspended in a final volume of 100 μl PBS. (2) ~100 sporocysts were resuspended in PBS containing proteinase K (1 mg/ml) and 1% SDS and incubated for 10 min at 37°C. Sporocysts were pelleted by centrifugation at 300*g* for 10 min and washed by resuspension in PBS without additives three times. Finally, the pellet was resuspended in 200 μl dimethyl sulfoxide (DMSO) freeze media (Fisher) and incubated for 2 h to overnight at −20°C.

One-half of the excysted sporocyst preparation was added to a 25 cm^2 flask with a freshly trypsinized, 60% confluent monolayer of BM cells in Dulbecco's medium containing 10% horse serum, 100 units/ml penicillin, 100 units/ml streptomycin, 1 mM pyruvate and 1 mM glutamate. The culture was maintained at 38°C in an atmosphere of 5% CO_2, 95% air. The culture medium was changed at 24 h and then at 2–4 day intervals. Parasite development was monitored by direct microscopic observation with a Nikon inverted microscope at 1–3 day intervals.

2.3. *Ionophore A23187-stimulated release and parasite purification*

A stock solution of calcium ionophore A23187 (Sigma) was prepared in DMSO to a final concentration of 1 mg/ml and stored at −20°C. Infected cell monolayers at 12 days post infection were washed three times with PBS or Hanks buffered salts solution (HBSS), and 10 ml of A23187 (1 μM in HBSS) was added to the washed monolayer and incubated at 37°C for 40 min in 5% CO_2, 95% air. Free merozoites were collected by centrifugation and washed in PBS as above. Parasites released by this method were examined by density gradient centrifugation, but further separation from host cell debris by density gradient centrifugation was not routinely necessary. For examination by electron microscopy, parasites were washed in PBS and suspended in 2% glutaraldehyde, dehydrated through alcohol, and embedded in epoxy using standard procedures in transmission electron microscopy by the ICBR Electron Microscopy Core at the University of Florida.

For further purification, parasites recovered from the supernatant were isolated from host cell debris on a discontinuous buoyant density gradient using Iodixanol (Optiprep) in PBS or HBSS. Merozoites resuspended gently in 1.0 ml PBS were layered onto a preformed, three step discontinuous gradient with layers of 1.03, 1.04 and 1.06 g/ml in a 15 ml round bottom centrifuge tube. The gradient was centrifuged at 1000*g* for 25 min at 20°C. Fractions containing particulate material at each interface were collected and examined microscopically.

3. Results

S. neurona merozoites replicated in two different BM lines, BT cells, HL cells, HFF, CHO cells, MDBK cells, GT cells, ED cells, and EM. Parasite growth in each of these host cell lines was observed over a 30 day period starting from an inoculum of 2000 parasites collected from the culture supernatant of BM cells. Formation of rosettes was first observed 3 days post infection with release and reinvasion of new cells occurring at 5 days post infection. The relative rate of growth of the UCD1 isolate in each of these host cell lines was estimated by determining the average number of developing intracellular parasites

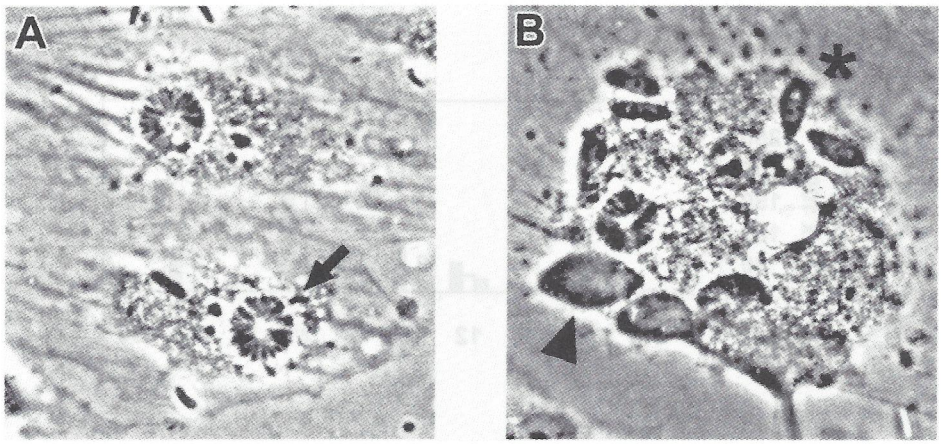

Fig. 1. *S. neurona* growing in BM cells. These parasites show typical prolific growth with rosette formation at 5 days of post infection when sub-cultured from BM 0617 cells. Three sizes are illustrated which correlate with nominal densities determined from gradient fractions. (A) Two intracellular rosettes and one extracellular parasite are shown at the arrow. This form appears to have a density of ~1.06 g/ml as determined from our gradient density studies. With ionophore treatment the individual merozoites from this mature rosette would be available for harvest. (B) The larger, less dense forms are unresponsive to ionophore release. These forms have nominal densities of ~1.03 g/ml, developing schizont (arrowhead) and 1.04 g/ml, M3 transforming into a schizont (asterisk) as determined in our density gradient studies.

visible per microscopic field (averaged over 10 randomly selected fields) on day 3 after initiating the culture. The results are summarized in Table 1. Of some interest was the observation that considerably more parasites developed in HL and BT cells than in the line of BM cells from which the inoculum was derived. However, the BM 0617 line was the host cell line best suited for the rapid proliferation of the parasite (See Table 1 and Fig. 1). When merozoites were introduced into a new host cell type that sustained normal growth of the parasite, the replicating forms of the parasite were noticeably larger.

The parasites grew unusually slowly in GT cells and HFF cells producing a morphologically distinct form. Electron micrographs revealed that the merozoites did not have prominent micronemes, but have many electron dense membrane bound structures (data not shown). When propagated in HFF cells, no merozoites were seen outside the host cells until 120 days of incubation, however, detached host cells filled with parasites were seen free in the media. Despite their poor growth in HFF cells, the parasite population remained infectious for BT cells. Parasites transferred from this culture reverted to a normal growth rate and morphology once transferred to a BT cell monolayer.

The efficiency of infection was increased when freshly trypsinized host cells were placed in sufficient numbers in the culture flask to establish a 60% confluent monolayer immediately before merozoites were added. This increase in numbers of merozoites entering host cells, improved the yield of parasites, and shortened by 2 weeks the length of time required for culture prior to harvesting the merozoites. Infection of BT cells by merozoites was increased by 50% using scraped cells from 30 day post infection as inoculum rather than

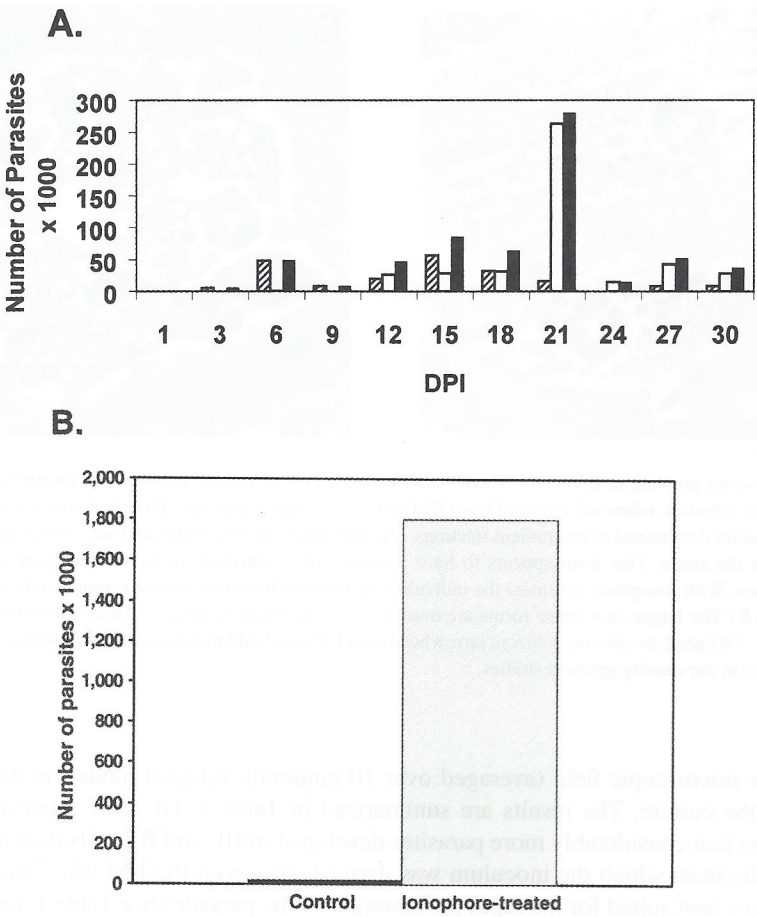

Fig. 2. (A) Parasites cultured in HL cells were counted every 3 days (black bar) and categorized as either extracellular (white bar) or intracellular (striped bar). The majority of the parasites were released at 21 days in this experiment. (B) Extracellular parasites in infected monolayers 12 days post infection. Duplicate 75 cm^2 flasks were grown for 12 days in HL cells and treated with HBSS (black bar) or HBSS with 1 μM A23187 (stippled bar). Free parasites were collected by centrifugation and counted with a hemocytometer.

the parasites in the supernatant from the same cultures. This result may be understood since most infectious merozoites are intracellular 28–30 days post infection and the release of the parasites from host cells at this point in time is asynchronous. By scraping the monolayer, a proportion of the host cells bearing mature rosettes were disrupted releasing additional merozoites that were able to enter other host cells.

Cultures of parasites replicating in HL cells were examined to follow the number of parasites free in the culture supernatant as compared with those found inside host cells during normal parasite growth as shown in Fig. 2A. Lung cells at 60% confluence were seeded at a density of 0.1 merozoites per host cell and parasite growth was monitored

microscopically for 30 days. Free parasites were not observed until 10 days post infection. During most of the period of observation, the percentage of extracellular parasites was less than 10%. On day 28, the largest number of free parasites was counted; but, the next day the numbers of extracellular parasites was back to less than 10%.

Parasite forms were distinguished by three different buoyant densities 1.03, 1.04, and 1.07 (g/ml) when Iodixanol (Optiprep) was used. The morphology of these forms are represented in Fig. 1. The form that separated with a buoyant density of 1.03 was tear shaped to oblong, had a large round central nucleus with an apparent nucleus:cytoplasm ratio of ~2. The 1.04 form was large, rounder, and had a nucleus:cytoplasm ratio of ~1. Early merozoites had a buoyant density greater than 1.06 and usually separated with host cells. This form was long and thin with a small central nucleus and an apparent nuclear:cytoplasmic ratio of ~0.5. This was also the form seen leaving the rosettes. Isolation of a pure population of merozoites free of host cell debris was possible using a buoyant density gradient, but this was difficult when host cells detached from the culture flask. Further, there was a significant reduction in parasite recovery.

Collection of parasites free in the culture medium was enhanced 90-fold by incubation of infected host cells for 40 min in 1 μM A23187 prior to collecting the culture supernatant. Parasitized host cells that released merozoites in response to ionophore treatment were BM, BM 0617, HL, BT, and ED cells. Infected GT, HFF, CHO, BHK, and primary EM cells were refractory for the release of parasites under the same conditions. The release of parasites in response to ionophore treatment was optimal at 10–12 days post infection, just after a few parasites were first observed free in the culture supernatant (Fig. 2B). Although difficult to accurately determine the percentage of individual merozoites released by this treatment, it was a large proportion. No mature rosettes were visible in a microscopic examination of treated cultures. The mechanism of release was not clear, but the selective disruption of the parasitized-host-cell membrane was seen in electron micrographs (data not shown). Initially, the host cell increased in size, became vacuolated, and had small breaks in the membrane. As the plasma membrane became more permeable, empty membrane bound vacuoles or vacuoles with peripheral ribosomes were released into the media. The host cell became long and cytoplasmic volume decreased. Parasites were observed to move beginning at 10 min and continuing until their release at 40 min. The merozoites undulated hyperactively in this media, but with the removal of the ionophore by addition of culture media, released parasites and the host cells recovered a normal appearance and activity. Ionophore-treated parasites remained animated and readily infected new cells when incubated with a fresh monolayer. The only difference noted in electron micrographs of ionophore-treated parasites was that they appeared to have more prominent micronemes than untreated parasites (Fig. 3).

Using parasites collected following release by A23187 provided improvement over both of the methods described above for inoculating cultures. Parasites released from the host cell monolayer by A23187 were single, hyperactive, and entered cells readily. When A23187-treated parasites were used as the inoculum, the infected host cells harbored an abundance of mature rosettes in 3–5 days. The extracellular parasites remaining after washing off the ionophore did not re-invade ionophore-treated host cells but increased in size while the few merozoites that remained in host cells formed rosettes in 5 days continuing the infection. Ten days after ionophore treatment, the parasites in the monolayer were

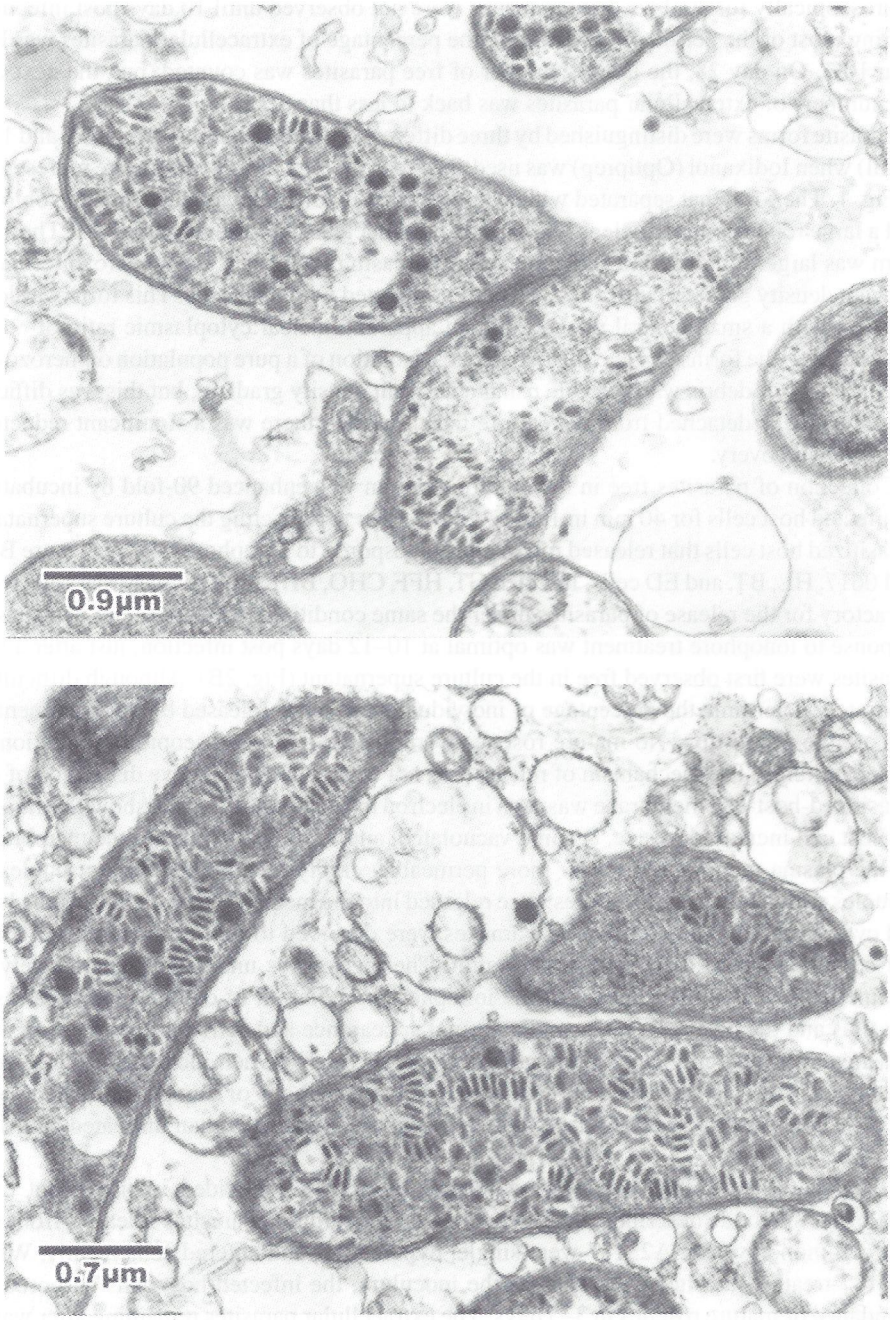

Fig. 3. Transmission electron micrographs comparing spontaneously released merozoites (Panel A) with those collected following treatment with A23187 (Panel B). An increase in micronemes is apparent following a 40 min treatment with 1 µM A23187.

unresponsive to calcium ionophore, whereas at 30 days, ionophore treatment again elicited parasite release. During this second ionophore treatment, many host cells were released into the supernatant.

A23187 did not elicit excystation of sporocysts, however the addition of 5% DMSO followed by one freeze thaw cycle did release sporozoites for culture. Sporocyst isolates of *S. neurona* have been successfully placed in in vitro culture in BM cells and propagated for more than 120 days by both methods described (either enzymatic excystation or by freeze thaw in DMSO).

4. Discussion

Cultivation of *S. neurona* in the laboratory requires the infection of host cell monolayers (Dubey et al., 1991; Dubey et al., 1998; Murphy and Mansfield, 1999; Lindsay et al., 1999). Multiplication rate differences exist which depend on the parasite isolate and the host cell (Speer et al., 2000). *S. neurona*, strain UCD1, was able to invade 11 different host cell lines but was found to thrive in BM 0617, BM or HL cells. Whereas, after invasion infection was non-progressive in HFF or GT cells. Moderate progressive growth was observed in ED, EM, MDBK, CHO, and BT cells.

S. neurona seemed better able to invade cells that were not confluent when placed on a monolayer or preferably seeded onto freshly trypsinized cells. Hermentin and Aspock (1987) reported an improved method for the cultivation of *T. gondii* when host cells were infected immediately after sub-culture observing 2–3-fold increase in multiplication rates. They suggested that there was a facilitated invasion of host cells by *Toxoplasma* when the cell's glycocalyx was irritated by trypsinization. Also, there was a more far-reaching exploitation of young separated host cells in contrast to cells of monolayers. This appeared to be similar with *S. neurona*, UCD-1. Clearly host membrane components important in invasion by *S. neurona* were not destroyed by trypsin treatment. All of the changes that take place in the host cell metabolism once the monolayer is confluent are not known, but growth rate ceases and cells eventually grow old and die. *S. neurona*, UCD-1 thrived in young cells that were actively multiplying and became more static in quiescent cells. It will be important to examine the growth characteristics of other isolates of *S. neurona* comparing these parameters between strains that have been isolated from diseased horses and those derived from opossum feces.

We observed the earliest release of merozoites from rosettes at day 5 followed by their entry into adjacent host cells and subsequent rosette formation. With rapidly growing host cells, the monolayer became confluent on day four, a day before maturation of the rossettes, whereas the slower growing host cells were not confluent at the first release of merozoites. In the slower host cell lines, BM and HL cells, parasites were seen free in the media sporadically starting at day 10–12, indicating that some rosettes had matured. Whereas in the rapidly growing cell lines, MDBK and CHO cells, free parasites were not observed until day 30 or greater. Thus, the rate of maturity of the *S. neurona* parasite appeared to coincide with the metabolism of the host cell. Had they continued to develop and invade new host cells every 5 days, there would have been an exponential increase in parasite numbers. That was not seen.

The use of calcium ionophore on monolayers that harbored mature merozoites allowed the synchronous release of parasites with little host cell contamination. The optimum time for parasite release was when a merozoite was observed sporadically in a confluent monolayer from day 10 to 12 post infection. It is possible that use of this method may also further enhance mass production of parasites especially if it works with the most prolific isolates such as SN6 (Speer et al., 2000).

Perhaps the most promising use of this phenomenon may be to synchronize development of the parasite. We found that a homogenous population of highly infective parasites could be released from the host cell monolayer. Little is known about how different developmental forms of *S. neurona* vary antigenically. Dubey et al. (1999) found the development of SN6 asynchronous with merozoite-shaped schizonts, immature schizonts, free-floating merozoites, and schizonts present in the culture and suggested the need for standardization of culture conditions to control antigenic variation of cultured *S. neurona*.

The mechanism by which the calcium ionophore stimulates this release is worthy of further study. Apicomplexan parasites possess a calcium storage compartment known as the acidocalcisome (Moreno and Zhong, 1996). When the calcium concentration rises in the parasitophorous vacuole of *T. gondii* this stimulates microneme discharge and parasite release (Carruthers and Silbey, 1999). It is unknown if the release of parasites from the host cell by A23187 is dependent on the presence of a parasitophorous vacuole. *T. gondii* is able to invade a wide range of cells in many different hosts by active penetration by the invasive form resulting in the formation of a parasitophorous vacuole. Only the asexual stages of *S. neurona* are known and are described as residing free in the cytoplasm of tissues from horses and cell culture without a parasitophorous vacuole (Dubey et al., 1991).

Calcium ionophore did not elicit excystation of sporocysts; however, the addition of DMSO and one freeze thaw cycle did release sporozoites for culture. BM were well suited for initiating cultures from sporozoites. The ability to cultivate *S. neurona* from the sporocyst stage will allow us the opportunity to compare parasites at this stage in development with those recovered from equine CNS tissues. Further, it will also allow us to compare many more isolates.

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