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Isolation of *Theileria parva* schizonts from infected lymphoblastoid cells

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Summary

This study set out to develop a rapid method for the isolation of schizonts from *Theileria parva*-infected bovine and buffalo lymphoblastoid cells. Parasitized lymphoblastoid cells were lysed by treatment with the cytolytic toxins, aerolysin and Ah-1 hemolysin, produced by *Aeromonas hydrophila,* and the schizonts were separated by Percoll density-gradient centrifugation. Light and electron microscopic examination showed that the isolated schizonts from lymphoblastoid cells infected with *T. parva* (Muguga) retained their normal morphology and were essentially free from host cell components. The schizonts also retained antigens as recognized by a series of anti-schizont monoclonal antibodies. The concentrations of toxin and Ficoll 400 in the lysis buffer which gave optimal cell lysis varied for 10 different infected cell lines tested.

Key words: *Theileria parva;* schizonts; aerolysin; hemolysin; *Aeromonas hydrophila.*

Introduction

Theileria parva is the etiological agent of East Coast fever (ECF), a tick-borne, lymphoproliferative disease of cattle in East and Central Africa. Theilerial sporozoites invade bovine lymphocytes and develop into multinucleated

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schizonts. The presence of the schizont induces uncontrolled proliferation of host cells. Lymphoid cells taken either from *Theileria*-infected cattle or infected in vitro with sporozoites can be cultured continuously in vitro (Brown, 1983).

Although *Theileria*-infected cell lines have been used for immunological and limited biochemical studies (Creemers, 1983; Minami et al., 1983; Allsopp et al., 1985), the intracellular location of the schizonts has hindered molecular biological studies on these parasites. A method described previously for isolating and extracting *T. parva* schizonts (Nyormoi et al., 1981) is unsuitable for these studies due to the complexity of the procedure. Recently, Frevert et al. (1986) reported a method for the isolation of *T. annulata* schizonts from bovine lymphoblastoid cells, but examination of the isolated schizonts revealed ultrastructural damage.

Aeromonas hydrophila, a Gram-negative bacterium is known to produce potent cytolytic toxins and both aerolysin and hemolysin have been purified and well characterized (Bernheimer and Avigad, 1974; Buckley et al., 1981; Asao et al., 1984). Pearson et al. (1982) showed that erythrocytes and leucocytes in blood from rodents infected with African trypanosomes could be lysed by aerolysin without any obvious deleterious effects on the viability or motility of the parasites. Recent studies by Grab et al. (1986) indicated that this toxin could also be used to isolate schizonts from *Theileria*-infected bovine lymphoblastoid cells.

This report describes in more detail a simple and rapid procedure for the purification of schizonts from *T. parva*-infected lymphoblastoid cells using toxin-mediated cell lysis and Percoll gradient centrifugation.

Materials and Methods

Theileria cultures

Theileria-infected lymphoblastoid cell lines were cultured essentially as described by Brown (1983) in RPMI 1640 medium (Gibco Ltd., Paisley, UK) supplemented with 10–20% (v/v) heat-inactivated foetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine and 50 μg/ml gentamycin sulfate. The cell lines used for purification of schizonts are listed in Table 1. A cloned bovine T lymphoblastoid cell line (G6) infected with the Muguga stock of *T. parva parva* (G6 TpM) (Brown and Logan, 1986) was used throughout these experiments unless otherwise stated.

Bacterial toxins

Aerolysin was purified according to the method of Buckley et al. (1981) from *A. hydrophila* strain Ah-65. Hemolysin produced by the Ah-1 strain of *A. hydrophila* was purified according to the method of Asao et al. (1984) except that the culture medium was supplemented with 10 mg/ml of yeast ribonucleic acid (R.6625, Sigma Chemical Co., St. Louis, MO, USA). The purified hemolysin was then dialysed against 0.03 M borate buffer (pH 8.2) to remove urea from the solution.

Cell lysis

The cells were centrifuged at 200 g for 10 min and resuspended to a concentration of 4×10^7 cells/ml in HEPES buffer [10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), 150 mM NaCl, 20 mM KCl, pH 7.4] containing 1 mM $CaCl_2$, protease inhibitors [20 μg/ml each of

Table 1. Optimal concentrations of toxins and Ficoll 400 used for schizont isolation from different *Theileria*-infected cell lines

Cell line	Cell line reference	Host cell	Aerolysin[1]		Ah-1 hemolysin[2]	
			Toxin (μg/ml)	Ficoll 400 (% w/v)	Toxin (μg/ml)	Ficoll 400 (% w/v)
G6 *T. parva* (Muguga)	Brown and Logan (1986)	Bovine	10	2	23	2
G6 *T. parva* (Marikebuni)	Conrad et al. (1987a)	Bovine	5	5	23	2
G6 *T. parva* (Mariakani)	Conrad et al. (1987a)	Bovine	2.5	5	NT[3]	
T.19.4 *T. parva* (Muguga)		Bovine	10	2	11.5	2
T.19.4 *T. parva* (Marikebuni) clone 1		Bovine	10	5	23	2
D.120.4 *T. parva* (Marikebuni)		Bovine	10	5	NT	
T. parva (Kilifi)	Minami et al. (1983)	Bovine	2.5	2	NT	
T. parva (Kiambu 5)	Minami et al. (1983)	Bovine	5	5	NT	
T. parva (Mavueni)	Minami et al. (1983)	Bovine	5	2	NT	
T. parva (Muguga) clone H 12	Kurtti et al. (1981)	Bovine	5	2	69	2
6834II subclone 1C11 1E8[4]	Conrad et al. (1987b)	Buffalo	10	2	46	2

[1] Cells were treated with the toxin at room temperature for 15 min.
[2] Cells were treated with the toxin at room temperature for 25 min.
[3] NT = not tested.
[4] Subclone of 6834II clone 5.

leupeptin, antipain, chymostatin and L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64)], and varying concentrations of Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden). To optimize conditions for toxin-mediated cell lysis, we tested a range of toxin concentrations (0.5 to 100 μg/ml), Ficoll concentrations [0 to 10% (w/v)], reaction temperatures (0 to 37°C) and reaction times (5 to 30 min). After treatment with the toxin, ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 5 mM to minimize aggregation of the schizonts.

Schizont separation

A stock solution of Percoll (Pharmacia Fine Chemicals) was prepared by mixing 8.5 parts of Percoll with 0.5 part of 20× HEPES buffer (pH 7.2) and 1 part of 50 mM EDTA (pH 7.4). The cell lysate was added to 3.8 ml of this stock solution and the volume was adjusted to 5 ml with HEPES buffer containing 5 mM EDTA (HEPES-EDTA buffer) to give a final Percoll concentration of 64.6% (v/v). The mixture was gently overlayed with a 45% Percoll solution in HEPES-EDTA buffer and centrifuged at 26,000 rpm for 30 min at 4°C in a SW 41 Ti rotor (Beckman, Palo Alto, CA, USA). After centrifugation, visible bands were collected, diluted with HEPES-EDTA buffer and centrifuged for 5 min at 13,000 g in a microcentrifuge (Eppendorf Gerätebau, Hamburg, FRG).

Light and electron microscopy

For light microscopic examinations, smears were prepared on glass slides using a cytocentrifuge (Cytospin, Shandon Southern Instruments, Cheshire, UK). The smears were air dried, fixed with methanol and stained with 10% Giemsa solution (Merck, Darmstadt, FRG) in phosphate buffered saline (pH 7.2).

For electron microscopy, infected lymphoblasts, lysed cells or purified schizonts were fixed with an equal volume of a fixative containing 5% glutaraldehyde, 4% formaldehyde and 0.5% picric acid in 200 mM phosphate buffer, pH 7.2 (Ito and Karnovsky, 1968). After 1 h at room temperature, the samples were centrifuged for 1 min in a microcentrifuge, washed in 100 mM cacodylate buffer (pH 7.2) and post-fixed with 1% OsO_4 in cacodylate buffer. The pellets were treated with 1% uranyl acetate in maleate buffer (pH 5.0), dehydrated in cold ethanol and embedded in Epon-araldite. Thin sections were stained with uranyl acetate and lead citrate prior to examination with a transmission electron microscope (EM-10A, Carl Zeiss, Oberkochen, FRG).

Indirect fluorescent antibody test (IFAT)

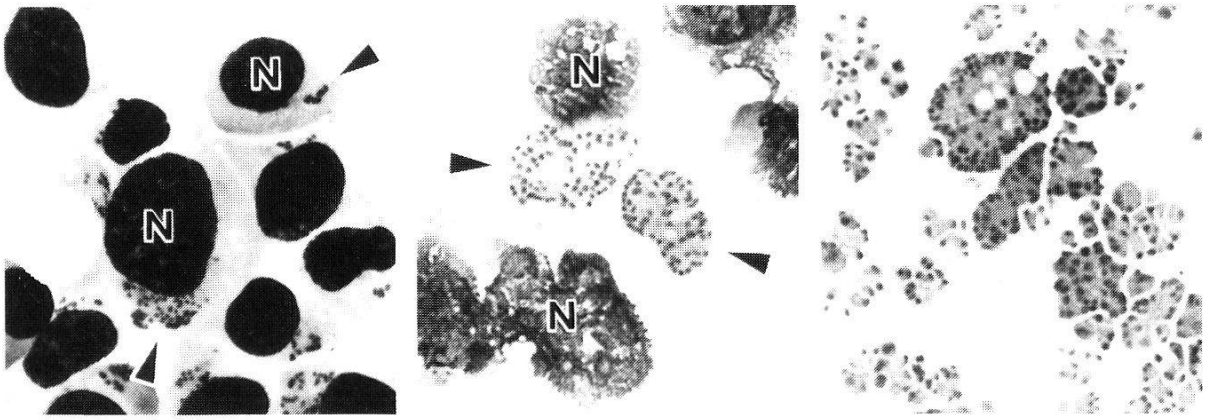
Theileria-infected lymphoblastoid cells and isolated schizonts from these cells were tested for reactivity with ILRAD's anti-schizont monoclonal antibody numbers 1 to 6 and 12 to 16 (Minami et al., 1983).

Metabolic labelling of schizont proteins

After centrifugation at 200 g, for 10 min, *T. parva*-infected lymphoblastoid cells were resuspended to a concentration of 10^6/ml in 10 ml of RPMI-1640 medium containing 0.75 μg/ml methionine, 10% (v/v) dialysed heat-inactivated foetal bovine serum and 200 μCi of ^{35}S-methionine (SJ 204, Amersham International, Buckinghamshire, UK). The cells were incubated in a tissue culture flask for 18 h at 37°C with 5% CO_2. Cells were then lysed under the conditions given in Table 1 and schizonts were purified as described above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified schizonts and infected lymphoblastoid cells were solubilized in sample buffer [62 mM Tris-HCl, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 7.5% (v/v) glycerol] and then incubated for 5 min at 100°C. SDS-PAGE was performed on slab gels containing a 5–15% (w/v) polyacrylamide gradient (Laemmli, 1970). To detect ^{35}S-labelled proteins, the gel was processed for fluorography (using Amplify; Amersham International), dried and then exposed to Fuji RX100 film (Fuji Photo Film Co., Tokyo, Japan) at −80°C for 5 days. A mixture of ^{14}C-methylated proteins (Amersham International) was used as molecular weight markers.



1 2 3

Fig. 1. Light micrograph of a Giemsa-stained cytocentrifuge smear of G6 lymphoblastoid cells infected with *T. parva* (Muguga). Host cell nuclei (N) and intracellular schizonts (arrowhead) are shown. ×650.

Fig. 2. Light micrograph of a Giemsa-stained cytocentrifuge smear of G6 lymphoblastoid cells infected with *T. parva* (Muguga) after treatment with aerolysin. Host cells are lysed while schizonts (arrowhead) remain morphologically intact. Host cell nuclei (N) are shown. ×760.

Fig. 3. Light micrograph of a Giemsa-stained cytocentrifuge smear of schizonts isolated from G6 lymphoblastoid cells infected with *T. parva* (Muguga) by aerolysin treatment and Percoll density-gradient centrifugation. Schizonts retain their normal morphology and are essentially free from host cell material. ×760.

Immunoblotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane as described by Dunn (1986), and detected immunologically using anti-schizont monoclonal antibody No. 4 and ^{125}I-labelled anti-mouse Ig (Amersham International) as described by Shapiro et al. (1987) except that the renaturing treatment with urea was omitted. For quantitation of bound ^{125}I-labelled antibody, the protein bands detected by autoradiography after immunoblotting, were cut from the nitrocellulose membrane and counted in a gamma scintillation spectrometer (Batteiger et al., 1982).

Protein measurement

Lymphoblastoid cells and purified schizonts were washed twice with saline and protein concentrations determined by using the BCA protein assay reagent (Pierce Chemical Co., IL, USA). Bovine serum albumin was used as a standard.

Results

Cell lysis by the bacterial toxins

The optimal concentrations of toxins and Ficoll 400 for schizont isolation varied for different cell lines as shown in Table 1. In general higher toxin concentrations and longer incubation times were required to lyse cells with the Ah-1 hemolysin, as compared with aerolysin.

Under the conditions described in Table 1, about 80% of cells were lysed without altering the normal morphology of the schizonts as determined by light microscopic examination (Figs. 1 and 2). The ultrastructural features of

T. parva schizonts before and after cell lysis with aerolysin, are shown in Figs. 4 and 5. Schizonts in lysed lymphoblastoid cells retained their normal ultrastructural appearance while the host cell membrane was disrupted and the cytoplasmic matrix lost. The nuclear envelope of the host cell generally lost its normal appearance of double membrane, and the nuclear material was dispersed.

Schizont separation

After discontinuous density gradient centrifugation of the aerolysin lysate, bands formed on top of the 45% Percoll and at the interface of 45% and 64.6% Percoll solutions. The upper band consisted of host cell nuclei, unlysed cells, cell debris and some trapped schizonts. Intact schizonts, free from host cell fragments and unlysed cells, were found in the lower Percoll interface band (Fig. 3). Electron microscopic examination indicated that this fraction contained virtually pure schizonts which retained their intralymphocytic morphology, and were essentially free from host cell membranes, nuclei or mitochondria (Figs. 6 and 7). Using the same conditions for separation after treatment with Ah-1 hemolysin, pure and intact schizonts were also obtained, as determined by light microscopy (data not shown).

Indirect immunofluorescent test

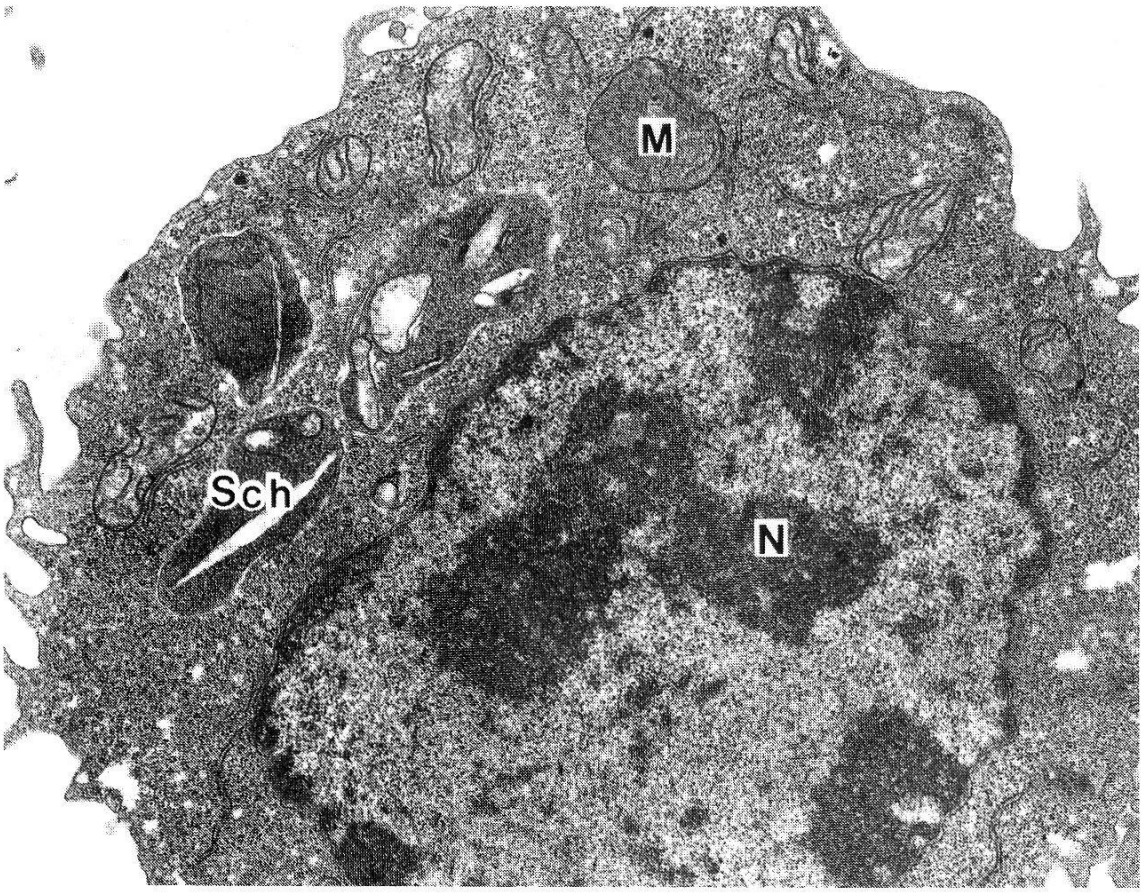
The isolated schizonts from G6 TpM reacted with monoclonal antibodies 1 to 5, and 12 to 14 but not with 15 and 16. This profile was identical to the profile seen with other *T. parva* (Mugua)-infected cell lines (Minami et al., 1983). The monoclonal antibody reactions and the intensity of fluorescence seen with the isolated schizonts was similar to that of the schizonts in intact lymphoblastoid cells (data not shown).

Schizont protein analysis by SDS-PAGE and immunoblotting

The major ^{35}S-labelled proteins of schizonts purified from metabolically labelled infected cells have molecular masses of 93, 79, 65, 59, 56, 51, 48, 47, 35 and 31 kDa (Fig. 8, lane A). By immunoblotting, an 84 kDa schizont protein was recognized by anti-schizont monoclonal antibody No. 4, and there appeared to be no difference between intracellular and purified schizonts in the molecular mass of this molecule (Fig. 8 lanes B and C). The 84 kDa protein was detected as a faint band in ^{35}S-labelled schizont proteins (Fig. 8, lane A, arrow).

Fig. 4. Electron micrograph of a G6 lymphoblastoid cell infected with *T. parva* (Muguga). Host cell nucleus (N), mitochondria (M) and schizont (Sch) are shown. ×11,800.

Fig. 5. Electron micrograph of a G6 lymphoblastoid cell infected with *T. parva* (Muguga) treated with aerolysin. The schizont (Sch) retains its morphology while damage to host cell membrane, mitochondria (M) and nucleus (N) are apparent. ×9,850.



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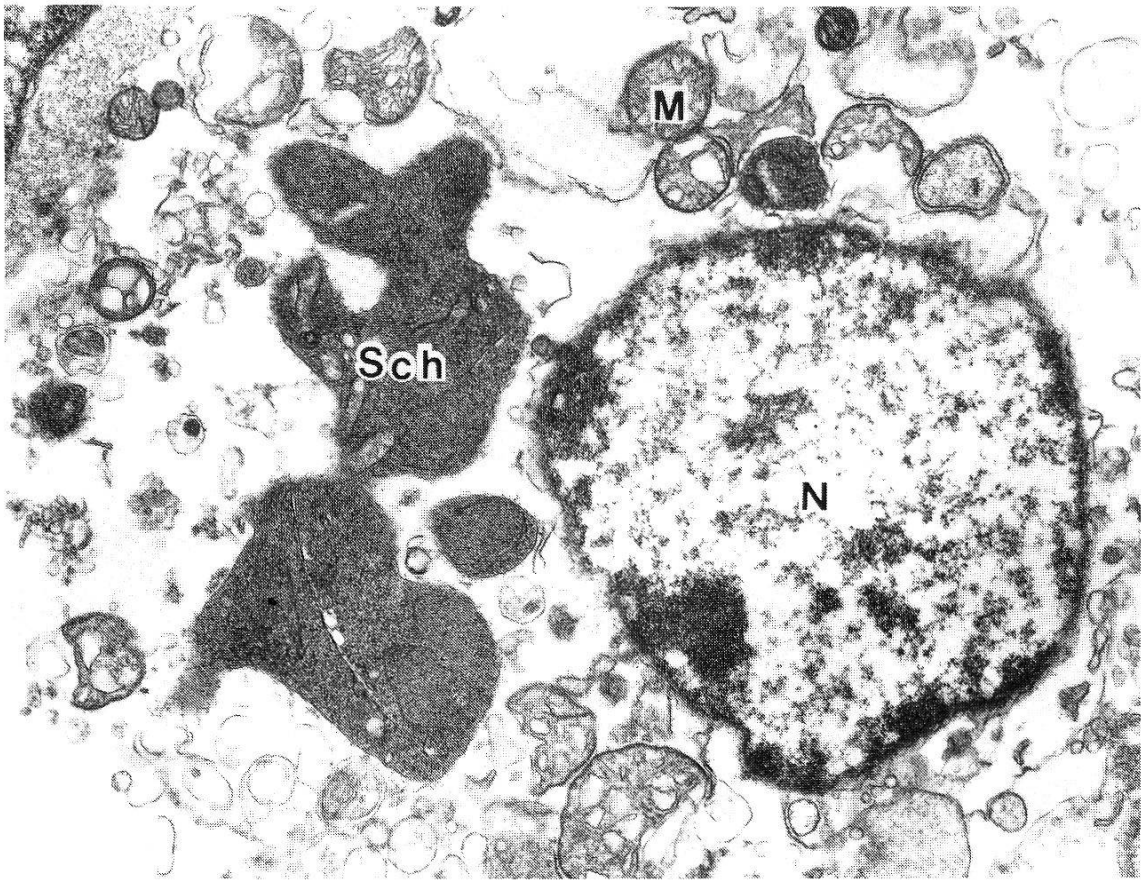

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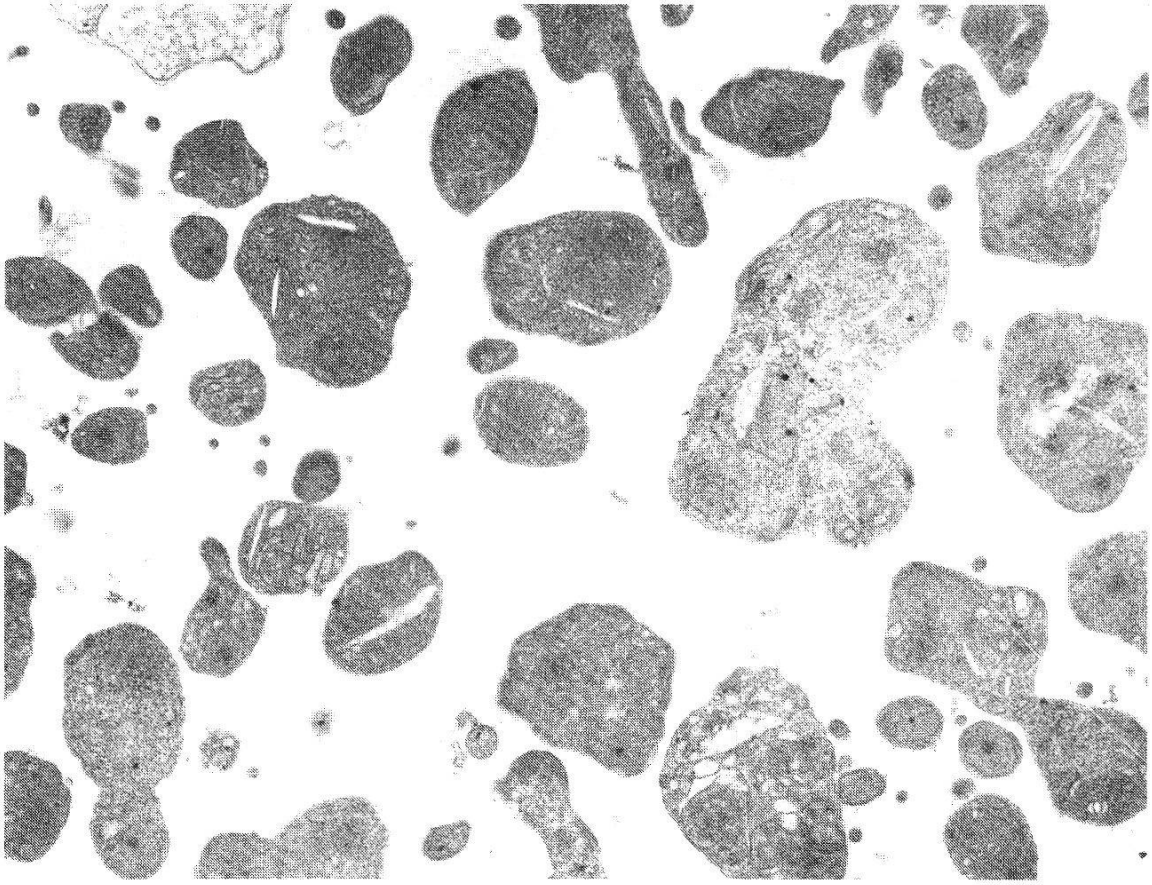

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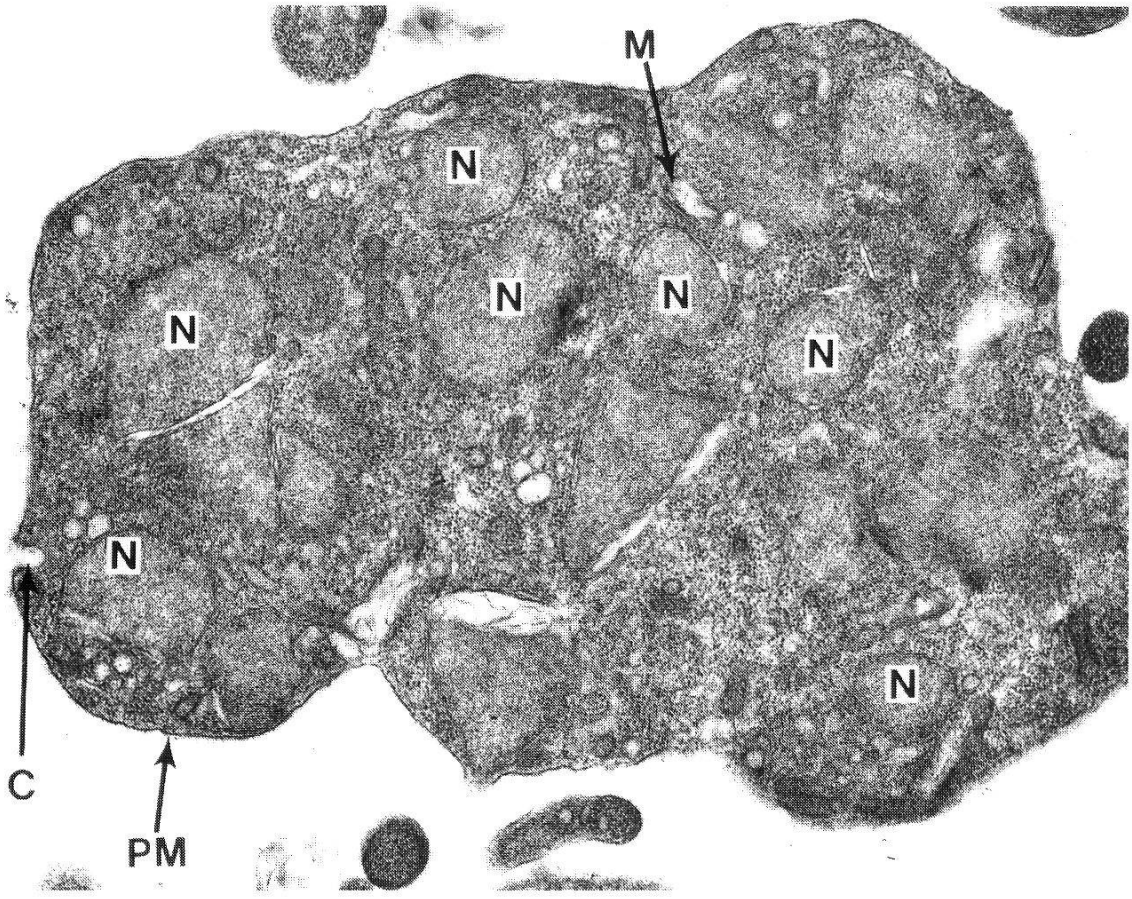

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To evaluate the recovery and purity of schizonts, the 84 kDa antigen band of starting material (cell homogenate) and various schizont preparations were excised from nitrocellulose membranes which had been treated with anti-schizont monoclonal antibody No. 4 followed by ^{125}I-labelled anti-mouse Ig. Radioactivity of secondary antibody bound to the 84 kDa antigen was quantified. As shown in Table 2, better recovery of purer schizonts was obtained from the G6 TpM cell line when infected cells were lysed in buffer containing 2% Ficoll 400 as compared with those lysed in buffer containing 0 or 5% Ficoll 400. The yield of schizonts under these conditions was estimated to be about 18%.

Discussion

In order to isolate and purify schizonts from *Theileria*-infected cells for molecular biological studies, it is essential that the cell disruption method used causes minimal damage to the parasite. We found that toxins produced by *A. hydrophila* were suitable for this purpose. Howard and Buckley (1982) reported that the binding of aerolysin to erythrocyte membranes was facilitated by a specific glycoprotein receptor, and caused the formation of discrete holes of defined size, which resulted in cytolysis by a colloid-osmotic process. A similar process is probably involved in lymphoblastoid cell disruption and release of intact schizonts. It is likely that aerolysin does not affect the intracellular and released schizonts because schizonts do not have the glycoprotein receptor for the toxin. Hemolysins of *A. hydrophila* may also bind to membrane phospholipid (Kozaki et al., 1987) but their capacity to bind to phospholipid is less than that to the receptor glycoprotein (Howard and Buckley, 1982). Another possible explanation for the selective lysis of host plasma membrane is that irreversible binding of the aerolysin to membrane receptors on the cell surface (Howard and Buckley, 1982) reduces the concentration of free toxin to a level insufficient to lyse schizonts released from the cells. The damage to host cell nuclei appeared to be due to the removal of Ca^{2+} by EDTA, rather than due to the binding of aerolysin, since smears prepared before the addition of EDTA revealed intact host cell nuclei.

Aerolysin and Ah-1 hemolysin have similar molecular masses of approximately 50 kDa, but they differ in other biochemical characteristics such as their isoelectric points and sensitivity to dithiothreitol (Buckley et al., 1981; Asao et al., 1984 and 1986). Nonetheless, under optimal conditions the two toxins

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Fig. 6. Electron micrograph of schizonts isolated from aerolysin treated G6 lymphoblastoid cells infected with *T. parva* (Muguga). The schizonts are free from host cell membranes, mitochondria and nuclei. ×5,680.

Fig. 7. Electron micrograph of a schizont isolated from aerolysin treated G6 lymphoblastoid cells infected with *T. parva* (Muguga). The ultrastructural features of the schizont, including the plasmalemmal membrane (Pm), cytostome (C), acristate mitochondria (M) and nuclei (N) appear intact. ×17,600.

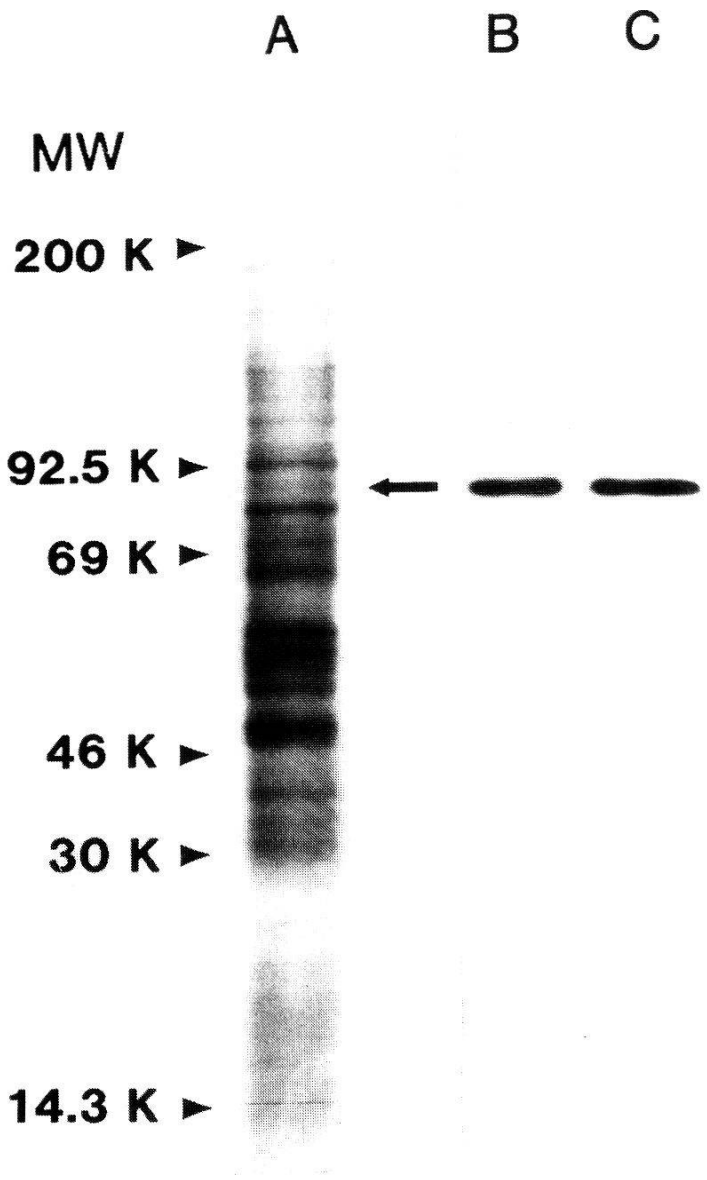

Fig. 8. SDS-PAGE and immunoblotting of schizont proteins. ^{35}S-labelled schizont proteins (lane A, 5×10^5 cpm) were detected by fluorography. Immunoblotting of purified schizonts (lane B, 0.7 μg protein) and schizont-infected lymphoblastoid cells (lane C, 21.5 μg protein) was performed using anti-schizont monoclonal antibody No. 4 and ^{125}I-labelled anti-mouse Ig. The protein band recognized by the monoclonal antibody is indicated by arrow in lane A. Molecular weight (MW) markers on the left.

worked equally well for obtaining pure schizonts from *T. parva*-infected lymphoblasts.

The lysis method described appears to be better than the method of Nyormoi et al. (1981) which requires mechanical disruption of cells resulting in a high incidence of schizont-ghost formation. Attempts to apply the glycerol-lysis method, which was developed to lyse platelets (Barber and Jamieson, 1970) and cultured lymphoid cells (Jett et al., 1977), gave unsatisfactory results because

Table 2. Quantitation of 84 kDa schizont antigen by immunoblotting using anti-schizont monoclonal antibody

Sample[1]	Total protein (μg)	Specific activity ±S.E.[2] (cpm/μg)	Degree of purification[3]	% Recovery of 84 kDa protein ±S.E.
Schizont-infected cell homogenate	6900	83±12.8 (5)	1.0	100
Schizonts (0% Ficoll)[4]	60.8	1195±150 (3)	14.4	12.7±1.6
Schizonts (2% Ficoll)[4]	26.2	3912±190 (6)	47.1	17.8±0.9
Schizonts (5% Ficoll)[4]	19.7	1800±160 (3)	21.7	6.2±0.6

[1] Each sample was prepared from 4×10^7 G6 TpM cells.

[2] Schizonts protein separated by SDS-PAGE were immunoblotted with anti-schizont monoclonal antibody No. 4 and ^{125}I-labelled anti-mouse Ig. The band of 84 kDa protein detected by autoradiography was cut from the nitrocellulose membrane and its radioactivity was quantified. S.E. = Standard error. Numbers in parenthesis represent numbers of determinations.

[3] Degree of purification = specific activity of schizont preparation/specific activity of cell homogenate.

[4] Purified schizonts obtained by Percoll centrifugation of G6 TpM cells lysed at room temperature for 25 min in buffer containing 23 μg/ml of Ah-1 hemolysin and 0%, 2% or 5% (w/v) Ficoll 400.

schizont fractions obtained from the lysate after Percoll density-gradient centrifugation were contaminated with unlysed cells and nuclear debris. Other methods of cell lysis, including the use of anti-lymphocyte serum plus complement, detergents, or osmotic shock, were also unsatisfactory because schizonts were either destroyed or damaged (unpublished data).

The addition of up to 5% (w/v) Ficoll 400 in the lysis solution increased the yield of schizonts and reduced the contamination of host cell nuclear debris. The optimal concentration of Ficoll 400 varied for different cell lines (Table 1). Why Ficoll 400 has a favourable effect for schizont purification is not clear, but its protective effect on subcellular organelles (Rickwood, 1984) may also apply to schizonts.

The discontinuous Percoll density-gradient centrifugation was found to be the most suitable method for obtaining pure and intact schizonts. Nyormoi et al. (1981) purified schizonts from mechanically disrupted cells by DEAE cellulose ion exchange chromatography after lysing host cell nuclei with a high salt concentration buffer. This method was modified by Frevert et al. (1986) to isolate *T. annulata* schizonts. Although schizonts were obtained by employing these methods, the techniques are not ideal for biochemical or molecular biological studies as they cause release of large quantities of host cell DNA which is difficult to remove, and the long and complex procedure involved may result in the loss of biological activities of the parasites.

Other gradient media tested in our studies included sucrose, metrizamide, Ficoll-Paque and Percoll solutions containing 0.25 M sucrose instead of NaCl. All of these caused some damage to the free schizonts (data not shown). We also found that the addition of divalent cations, Ca^{2+} or Mg^{2+}, to the Percoll solutions in place of EDTA also resulted in poor separation of schizonts.

Quantitation of schizonts based on optical counting was difficult. Unstained schizonts were too small to be distinguished from other cellular particles and to be counted accurately. Counting schizonts in Giemsa-stained smears is not reliable because schizonts might be lost during processing and because they tended to clump. For these reasons, we quantified schizont antigen by immunoblotting. Although this method was complicated and time consuming, the results gave satisfactory information on recovery of schizonts as well as degree of purification. The antigen detected by the monoclonal antibody No. 4 was identified on the surface of the intracellular schizonts, but not in host cell cytoplasm (Shapiro et al., 1987). If this antigen is not released from the schizont surface, its recovery may correlate with recovery of schizonts. The best recovery of schizonts was approximately 17% of total intracellular schizonts. The remaining schizonts were probably trapped within the upper band in the Percoll gradient with the cell nuclei.

Optimal concentrations of toxins and Ficoll 400 varied between *Theileria*-infected cell lines as shown in Table 1. This is probably due to differences in toxin binding to the cells or to differences in resistance of the cells to osmotic

shock. Thus, the optimal concentrations of toxins and Ficoll 400 must be determined experimentally for each cell line.

Since the schizonts purified by the method described in this paper retained their characteristic antigens as well as normal morphology, these parasites can be used for biochemical and immunological studies. Schizonts isolated by this method are currently being used for two-dimensional gel electrophoresis to characterize parasite proteins and to obtain parasite DNA and RNA for molecular biological studies. The use of *A. hydrophila* toxins may prove to be an important aid in the isolation of other intracellular microorganisms.

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