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Fine structural localisation of lipid material in *Theileria parva* (Muguga) schizonts

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ABSTRACT: The presence and distribution of lipidic materials within Theileria parva-infected lymphoblasts has been studied using a number of modified fixation procedures. The addition of either malachite green or tannic acid to glutaraldehyde resulted in the retention of substantial amounts of lipidic materials both within the Theileria schizont and throughout the host cell cytoplasm. In contrast, post fixation with imidazole-buffered osmium only produced an increase in the density of various parasite and host cell membranes.

1. INTRODUCTION

The tick-borne, protozoan parasite Theileria parva causes an acute and often fatal disease in cattle known as East Coast Fever. The infectious stage of the parasite, the sporozoites, enter bovine lymphoid cells where they differentiate into the schizont stage. Infected cells undergo cell transformation with the schizont and host cell dividing synchronously leading to a rapid clonal expansion of parasitized cells. During a study of the development of the intracellular schizont stage, loose, lipid-like membranous material was consistently observed within the schizont, associated with the schizont plasma membrane and, localized throughout the host cell cytoplasm particularly in association with the host cell mitochondria.

It is now well established that the conventional fixation, dehydration and embedding procedures used for the preparation of biological specimens for electron microscopy result in the substantial extraction of lipidic materials from the specimen. In recent years, however, a number of modified fixation methods and procedures have been devised in an attempt to both reduce the extraction of lipidic materials during specimen preparation, and to increase the electron density of such materials in thin sections.

In the present study a number of modified fixation protocols have been used to investigate the nature of these membranous structures and to study the distribution of lipidic materials in general within both the schizont and transformed host cell.

2. MATERIALS AND METHODS

Lymphoblastoid cell lines infected with Theileria parva (Muguga) were established by mixing peripheral blood mononuclear cells (PBMs) with sporozoite suspensions in vitro. The cell cultures were grown in RPMI 1640 medium with 10 mM HEPES buffer, supplemented with 10% heat-inactivated foetal bovine serum (Gibco Ltd., Paisley, Scotland), 5 x 10^{-5}M 2-mercaptoethanol, 2 mM L-glutamine and 50 ug/ml gentamycin sulphate.

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For routine electron microscopy, cells were fixed from D10 post-infection onwards in one of the following primary fixatives:
a) 2.5% glutaraldehyde in either 0.1M phosphate, cacodylate or PIPES buffer (pH 7.2),
b) in a mixture of 2.5% glutaraldehyde, 2% formaldehyde and 0.02% picric acid in either 0.1M phosphate or cacodylate buffer (pH 7.2).

After a brief wash in 0.2M cacodylate buffer, the specimens were post-fixed in cacodylate-buffered, 1% osmium tetroxide and "en bloc" stained with 1% uranyl acetate in 50 mM maleate buffer (pH 5.2).

To localize lipidic materials, infected cells were fixed by one of the following modified fixation protocols:
a) 2.5% glutaraldehyde in 0.1M phosphate, cacodylate or PIPES buffer (pH 7.2) containing 0.1 - 1.0% malachite green (MG) (Sigma Chemical Co. Ltd.). The fixative was freshly made up immediately before use. The specimens were post-fixed in osmium tetroxide and "en bloc" stained with uranyl acetate.
b) 2.5% glutaraldehyde in 0.1M cacodylate buffer containing 1-2% tannic acid (TA) (Mallinckrodt Inc., USA) and 0.5 mg/ml saponin (Sigma Chemical Co. Ltd.). The samples were washed in several changes of 0.2M cacodylate buffer, post-fixed in osmium tetroxide and 'en bloc" stained with uranyl acetate.
c) Infected cells were fixed in 2.5% glutaraldehyde in 0.1M phosphate or cacodylate buffer, post-fixed for 30 - 120 min in imidazole-buffered, 1% osmium tetroxide (Angermuller and Fahimi 1982), and subsequently "en bloc" stained with uranyl acetate.
d) Cells were fixed in one of the routine aldehyde fixatives, post-fixed in osmium tetroxide and "en bloc" stained with uranyl acetate. The samples were then stained with 1% p-phenylenediamine (Sigma Chemical Co. Ltd.) in 70% ethanol for 1 h during dehydration (Ledingham and Simpson 1972).
Samples were also fixed using the tannic acid - p-phenylenediamine technique of Guyton and Klemp (1988).

All samples were dehydrated and embedded in an Epon-Araldite mixture. Ultrathin sections (average thickness 70 nm) were either examined unstained or double stained with uranyl acetate and lead citrate and examined on a Zeiss EM10A electron microscope.

3. RESULTS

In Theileria-infected cells fixed using standard procedures, profiles of loose membranous material were observed within the schizont associated with the schizont mitochondria and plasma membrane, and localized throughout the host cell particularly in association with the host cell mitochondria (Figure 1).

The addition of malachite green (MG) to glutaraldehyde in any of the buffer systems used, invariably resulted in the appearance of numerous, osmiophilic droplets within both the schizont and host cell cytoplasm (Figure 2). In general, these MG-stainable droplets were composed of a moderate to extremely dense, finely granular material. The density of the droplets varied between the different samples and was dependent upon both the amount of MG present in the fixative and with the duration of primary fixation (1-18 h). The higher amounts of MG and/or longer fixation times generally resulted in an increase in the density of the droplets. However, some extraction of MG from the samples during both prolonged dehydration and embedding was observed (see also Lawton 1989).

Within the schizont, MG-stainable droplets were observed associated with the nuclear envelopes and within or closely associated with the mitochondria. Droplets

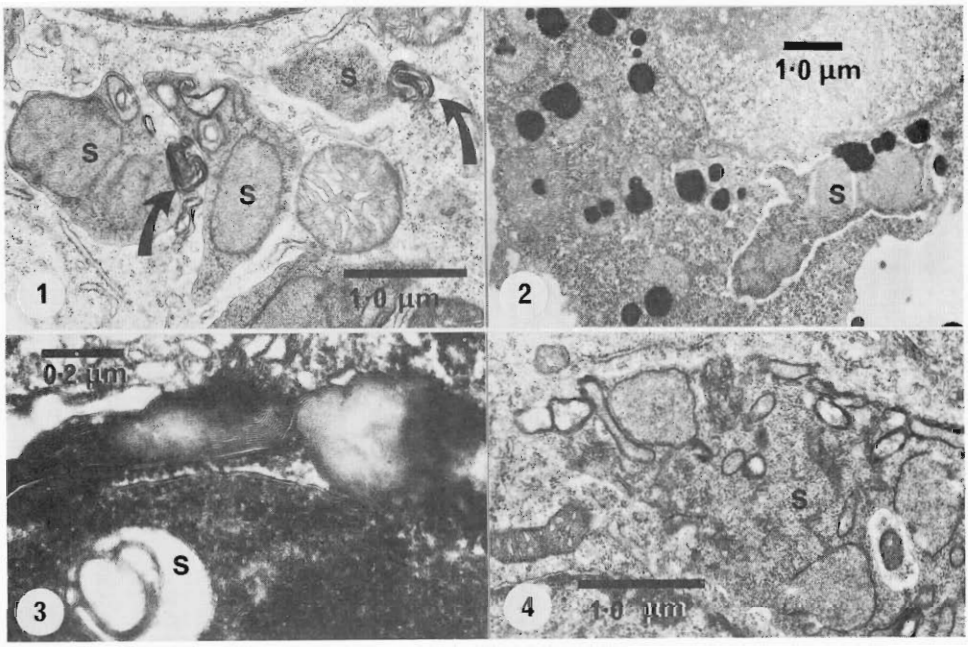

Fig. 1. Conventionally fixed Theileria parva-infected cell showing presence of loose membrane-like structures (arrows) associated with the schizont (S).
Fig. 2. Unstained section of MG-glutaraldehyde fixed cell showing numerous osmiophilic droplets associated with the schizont (S) and throughout the host cell.
Fig. 3. Part of a Theileria schizont fixed in TA-glutaraldehyde showing the presence of multilamellar structures associated with the schizont (S).
Fig. 4. Part of a Theileria schizont (S) post-fixed with imidazole-osmium showing an increase in the density of certain parasite membrane systems.

were also found in the apparent process of being extruded from the schizont and/or lying outside on the surface of the schizont in direct contact with the schizont plasma membrane. In the host cell, MG-stainable droplets were observed within the nucleus and in parts of the nuclear envelope, scattered throughout the cytoplasm and in association with various organelles, in particular, the mitochondria.

In cells fixed in TA-glutaraldehyde, multilamellar membranous structures were observed both inside the schizont, in the process of being extruded from the schizont and also closely associated with the outer surface of the schizont (Figure 3). The periodicity of these multilamellar structures ranged from 5 to 8 nm, although extruded material having no substructure was occasionally observed. In the host cell, multilamellar membraneous structures were found scattered throughout the cytoplasm, particularly associated with the mitochondria.

In cells post-fixed with imidazole-buffered osmium tetroxide, the membranes of the host cell mitochondria and the nuclear and mitochondrial membranes of the schizonts showed a marked increase in electron density (Figure 4). Dense inclusion bodies, some of which had a distinct lamellar substructure were seen within and in the process of being extruded from the schizont, although, in general, the schizont plasma membrane did not show any marked increase in electron density.

The use of either ethanolic p-phenylenediamine after normal osmication (Ledingham and Simpson 1972) or the sequential TA - p-phenylenediamine method of Guyton and Klemp (1988) did not produce any marked increase in the retention and visualization of lipidic materials as compared with conventionally processed material.

4. DISCUSSION

The main result of the present study has been the demonstration of substantial amounts of MG- and TA-stainable lipidic materials both within the Theileria schizont and throughout the host cell cytoplasm. The addition of either MG or TA to glutaraldehyde during primary fixation has been previously shown to preserve certain classes of phospholipids in mammalian tissues (Teichman et al. 1974; Pourcho et al. 1978; Kalina and Pease 1977), whilst post-fixation with imidazole-buffered osmium produces prominent electron opaque staining of lipids containing unsaturated fatty acids (Angermuller and Fahimi 1982). Thus, based on these observations, the present results indicate that the Theileria schizont contains appreciable amounts of phospholipids and that there is also an accompanying substantial increase in the phospholipid contents of the infected host cell. Other studies using the MG-glutaraldehyde fixation protocol on uninfected lymphocytes and on Con A-stimulated lymphoblasts have shown that whilst some increase in cellular phospholipids occurs as a result of cell transformation (data not shown), the substantial amounts of phospholipid present in Theileria-infected cells appear to be directly related to the presence of the parasite.

The differentiating Theileria schizont induces and maintains host cell transformation leading to a rapid clonal expansion of parasitized cells. At present, however, little is known about the mechanism(s) whereby the parasite induces and maintains the continuous proliferation of infected lymphoid cells (see Ole MoiYoi 1989, for a brief review of the possible mechanisms). The presence of significant amounts of phospholipid both within the Theileria schizont and throughout the host cell may, therefore, be of potential interest to our understanding of Theileria-induced host cell transformation and blastogenesis, given the increasingly important role(s) of phospholipids in cell biology and, in particular, their role as modulators in many biological processes (Pagano and Sleight 1988). Also the presence of substantial amounts of lipid within both the parasite and host cell may indicate that lipid metabolism plays a crucial role in the growth and development of the Theileria schizont and could, therefore, be a potential area for chemotherapeutic intervention.

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