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Structural and Molecular Differences in the Host Parasite Relationship between the Enteric (Coccidian) and Exoenteric Forms of *Toxoplasma gondii*

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ABSTRACT

The host parasite relationship of the enteric (coccidian) and exoenteric (tachyzoite, bradyzoite) forms of *Toxoplasma gondii* was examined in vivo by electron microscopy and immunocytochemistry. Significant structural and molecular differences were observed between the enteric and exoenteric forms. The expression and distribution of certain rhoptry and all the dense granule proteins was examined by immunocytochemistry. It is was observed that, while the antibody recognising ROP2,3,4 positively stain the anterior of the infective stages (merozoite, tachyzoite and bradyzoite) of both the enteric and exoenteric stages, ROP1 appeared to be absent from the merozoite. It was found that GRA8, like GRAs1-6, was present in both the tachyzoite and bradyzoite stages but did not appear to be expressed by the enteric stages. The only dense granule proteins expressed by the enteric (coccidian) stages were GRA7 and NTPase. By electron microscopy, the parasitophorous vacuole (PV) containing the enteric forms in the enterocytes of the cat was limited by a laminated wall consisting of three fused unit membranes. This is in contrast to the exoenteric stages where the PVs are limited by a single unit membrane. These differences point to a unique host/parasite relationship for the enteric stages of *T. gondii*.

INTRODUCTION

Toxoplasma gondii is an unusual coccidian parasite with the cat as its

definitive host but any warm-blooded animal, including humans, acting as intermediate hosts. In the intermediate host, infection results in an acute phase where the tachyzoites reproduces rapidly followed by a chronic phase when the bradyzoites reproduces slowly giving rise to tissue cysts which are found predominantly in muscle and brain. These tissues cysts are believed to remain viable for long periods and can transmit the infection if eaten by other intermediate hosts or the cat (Tadros and Laarman 1982). In the case of the cat, infection results in a unique form of asexual (endopolygony) and sexual development (Sheffield 1970; Ferguson et al. 1974; 1975) in the small intestine with the production of oocysts that are passed in the faeces.

In all phases of the life cycle, *T. gondii* is an obligate intracellular parasite and therefore the inter-relationship with the host cell is vital for parasite survival. The host/parasite relationship of the tachyzoite has been extensively studied and it is proposed that the PV is modified by various parasite proteins secreted initially by the rhoptries and subsequently by the dense granules (Cesbron-Delauw 1994; Joiner and Dubremetz 1993). The vacuoles are also characterised by being limited by a unit membrane and the collecting of host cell mitochondria and rough endoplasmic reticulum (rER) adjacent to the membrane (Sheffield and Melton 1968; Jones and Hirch 1972; Sinai et al. 1997). In contrast, little is known about the host/parasite relationship of the enteric forms in the small intestine of the cat although it had been reported that a thickened, laminated membrane limited the PV (Pelster and Piekarski 1972; Ferguson 1975; Ferguson and Wright 1998). In addition, it has recently been reported that there are major differences in the expression of various dense granule proteins between the enteric and exoenteric forms (Ferguson et al. 1999a; 1999b). In the present study we compare the ultrastructural features of the PVs and have extended the immunocytochemistry to examine the expression and distribution of the molecules recognised by antibodies to ROP1 (Leriche and Dubremetz 1991) and ROP2,3,4 (Sadak et al. 1988) plus the recently described GRA8 (Carey et al. 1999) in the enteric and exoenteric forms of *T. gondii*.

MATERIAL AND METHODS

Toxoplasma

The M3 (sheep isolate) and RRA strains of *Toxoplasma gondii*, maintained in chronically infected mice, were used in the experiments.

Animals

Cats, barrier maintained and guaranteed free for *T. gondii*, were obtained from the Animal Unit, Newcastle University, Newcastle, UK. The mice used were

BALB/c strain.

Antibodies

GRA proteins: the antibodies to GRA1-7 and NTPases were as described previously (Ferguson et al. 1999a; 1999b). In addition, a monoclonal antibody which recognises GRA8 (Carey et al. 1999) was examined. Rhoptry proteins: a monoclonal antibody to ROP1 (T5 2A3, Leriche and Dubremetz 1991) and a monoclonal antibody which recognises ROP2,3,4, (T3 4A7, Sadak et al. 1988) were examined.

Infection and Autopsy

Cats and mice were infected periorally with tissue cysts of *T. gondii*. Cats at 7 days post-infection (PI), and mice at 10, 15, 21, 28 and 56 days PI, were autopsied and various tissues processed for immuno-light and electron microscopy as described previously (Ferguson et al. 1999a).

Immunocytochemistry

Sections of cat small intestine and of mouse lung and brain were stained with various concentrations of anti-GRA8, anti-Rop1 and anti-Rop2,3,4 by a standard two stage protocol using peroxidase as chromagen for light microscopy and colloidal gold particles as the label for electron microscopy (Ferguson et al. 1999a).

Electron microscopy

For routine electron microscopy samples of infected cat small intestine were processed as described previously (Ferguson at al. 1999a). Tachyzoites in the peritoneal exudate of acutely infected mice and tissue cysts in the brains of chronically infected mice were processed as described previously (Ferguson and Hutchison 1981; Ferguson and Hutchison 1987).

RESULTS

Immunocytochemistry

When sections of the mouse lungs and brain were stained with anti-ROP1, positive staining was observed within the tachyzoites (Fig. 1a) and the bradyzoites (Fig. 1b). The location of the of the staining within the rhoptry was confirmed by immuno-electron microscopy (not shown). When similarly strained sections of the cat intestine were examined, no staining was observed within the enteric forms with the apical cytoplasm of the merozoites of mature schizonts being unstained (Fig. 1c). In sections stained with anti-ROP2,3,4, there was positive staining of the

tachyzoites (Fig. 1d) and bradyzoites (Fig. 1e). In addition, within the enteric forms, there was granular staining of the apical cytoplasm of the merozoites within mature schizonts (Fig. 1f). By immuno electron microscopy, it could be shown that the antibody was staining the rhoptries of the bradyzoite (Fig. 2a) and merozoite (Fig. 2b).

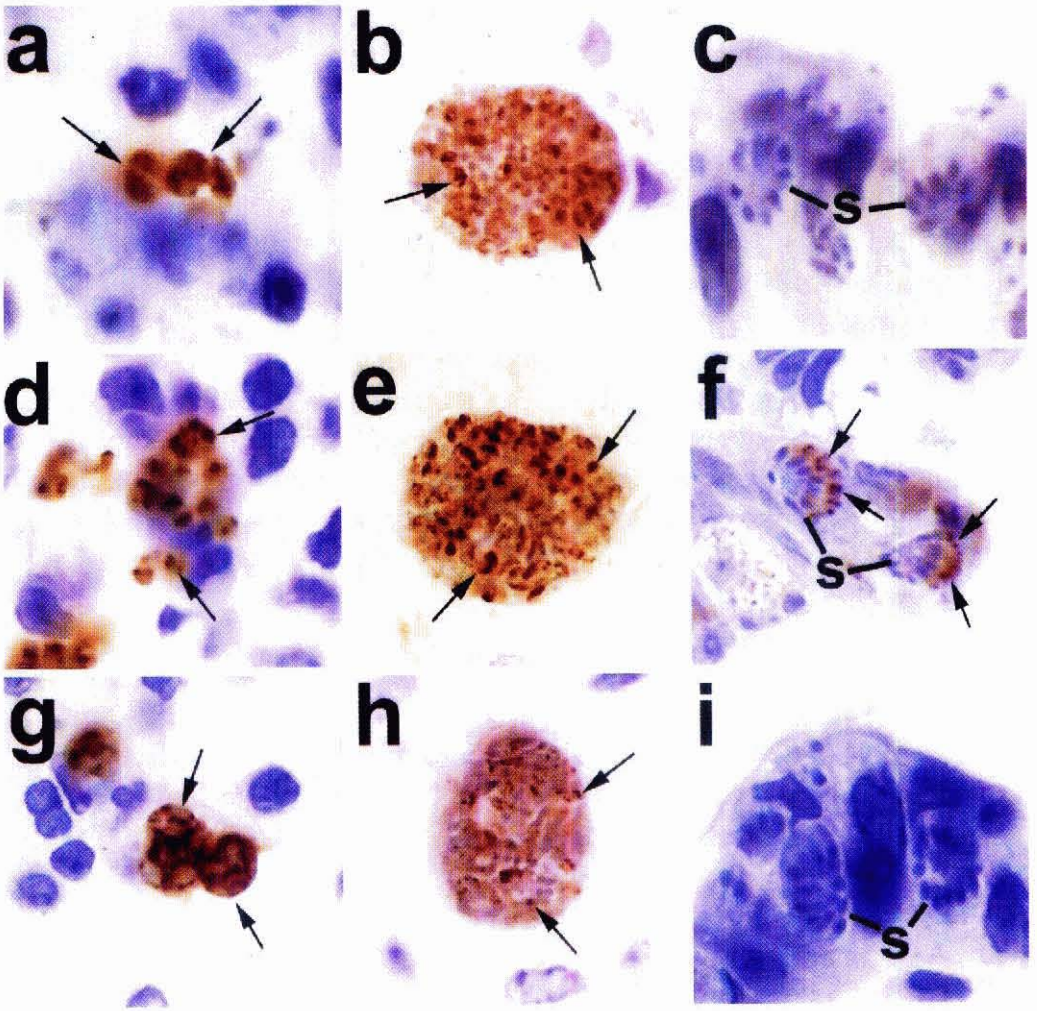

Figure 1. Sections of the lung of an acutely infected mouse (a, d, g), the brain of a chronically infected mouse (b, e, h) and the small intestine of an infected cat (c, f, i) immunostained with anti-ROP1 (a, b, c), anti-ROP2,3,4 (d, e, f) and anti-GRA8 (g, h, i). Note the positively stained tachyzoites (a, d, g - arrows) and bradyzoites (b, e, h - arrows) but that only the merozoites stained with anti-ROP2,3,4 show positive apical staining (f - arrows). S - schizont. ×1,500.

As described previously, while the tachyzoites and bradyzoites stained positively for all the dense granule proteins (GRA1-7 and NTPases), only GRA7 and NTPases were observed in the enteric forms (Ferguson et al. 1999a; 1999b). When stained with anti-GRA8, it was observed that the tachyzoites (Fig. 1g) in the mouse lung and the bradyzoites (Fig. 1h) within tissue cyst in mouse brain stained positively for GRA8. By immuno electron microscopy it could be confirmed that the GRA8 stained was located in the dense granules (Fig. 2c). The vacuole containing the tachyzoites was also strongly stained (Fig. 1g) but the wall of the tissue cyst appeared to be unstained (Fig. 1h). In contrast, the PVs containing the enteric forms were unstained. In addition there was no staining of the merozoites of mature schizonts by immuno-light (Fig. 1i) or electron microscopy (Fig. 2d). The staining patterns for the dense granule proteins in the various infectious stages are shown in Table 1.

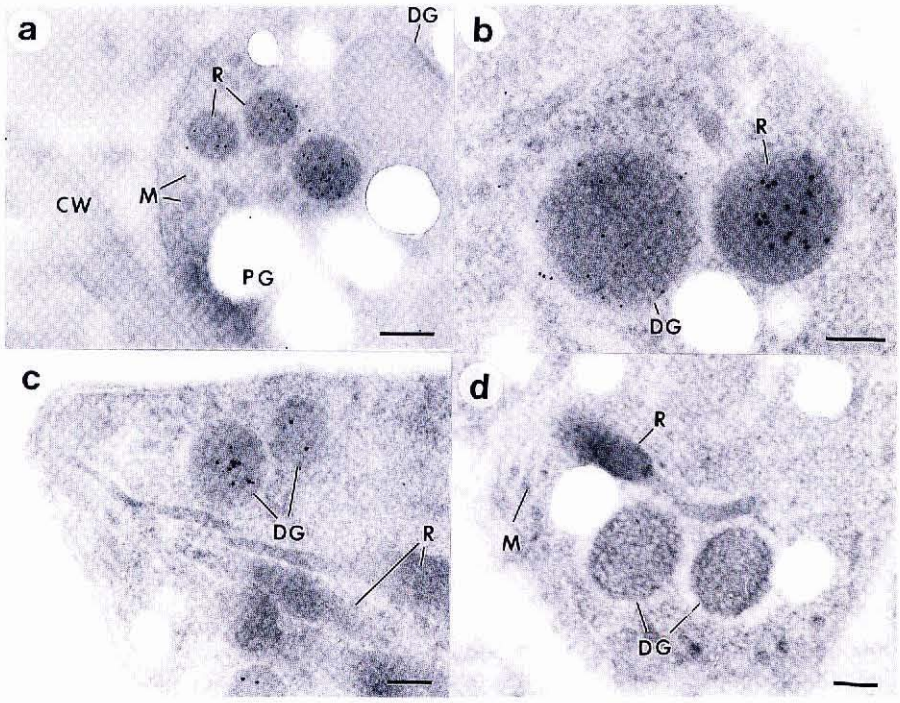

Figure 2a. Immuno-electron micrograph of a bradyzoite labelled with anti-ROP2,3,4 showing staining of the rhoptries (R) while the dense granule (DG) is unstained. CW - cyst wall.; M - microneme. b A merozoite within a cat enterocyte double labelled with anti-NTPases/anti-ROP2,3,4 and visualised using 5- and 10-nm gold particles, respectively. Note the separate labelling of the rhoptry (R) and the dense granule (DG). c, d Apical cytoplasm of a tachyzoite (c) and a merozoite (d) stained with identical concentrations of anti-GRA8. Note the positive staining of the dense granules (DG) in the tachyzoite (c) while those of the merozoite (d) are unlabelled. R - rhoptry; M - microneme. Bars are 100nm.

Electron microscopy

Parasites at various stages of asexual and sexual development were observed within the epithelial cells of the villi of the small intestine of the cat (Fig. 3a). The parasites were present within a tight fitting parasitophorous vacuole (PV) predominantly located in the apical cytoplasm of the enterocytes (Fig. 3a). The PV was limited by a thickened wall, which on detailed examination appeared to have a laminated substructure (Fig. 3b). In certain areas, the wall could be resolved into three unit membranes (Fig. 3c). The myelin-like substructure appeared to result from the fusion of these membranes. In tangential sections through the wall there appeared to be circular structures associated with the membranes. In cross-section these appeared as conical structures which were closely applied to both the PV membrane and the plasma membrane of the parasite (not shown).

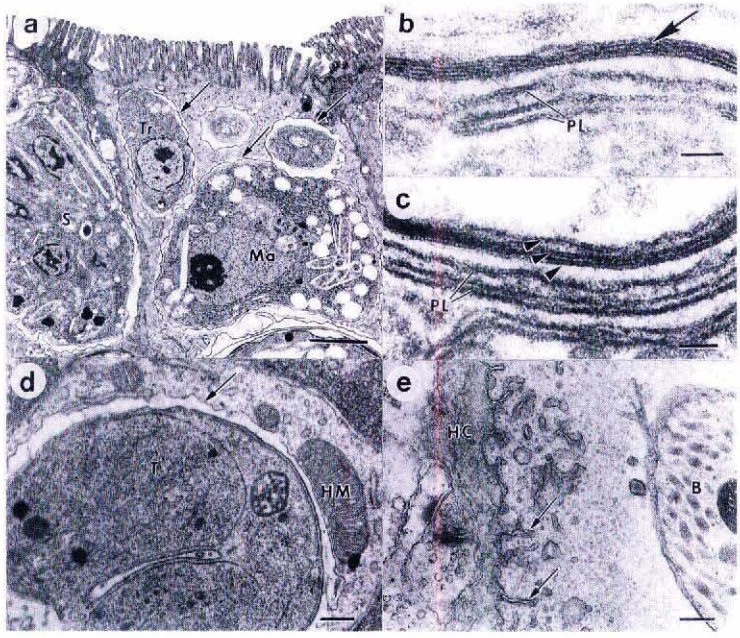

Figure 3. **a** Electron micrograph through the small intestine of a cat showing various coccidian stages within the apical cytoplasm of the enterocytes. Note the thickened membrane (arrows) limiting the PV. Ma - macrogamete; S - schizont; Tr - trophozoite. Bar is 2μm. **b** Enlargement of the periphery of the PV showing the laminated appearance (arrow) of the limiting membrane. PL - pellicle enclosing the parasite. Bar is 50nm. **c** Part of the PV membrane where separation of the laminated structure shows it to consist of three unit membranes (arrowheads). PL - parasite pellicle. Bar is 50nm. **d** Section through a marcophage from the peritoneum of an acutely infected mouse showing a developing tachyzoite (T) within a PV limited by a thin membrane (arrow). Note the host cell mitochondrion (HM) closely apposed to the membrane of the PV. Bar is 200nm. **e** Section through the periphery of a tissue cyst in the brain of a chronically infected mouse. Note that the cyst wall is limited by a thin membrane with numerous invaginations (arrows). HC - host cell; B - bradyzoite. Bar is 200nm.

In contrast, the tachyzoites were located in a PV limited by a single unit membrane (Fig. 3d). The PV also contains a tubular network and the host cell mitochondria and rough endoplasmic reticulum are closely associated with the limiting membrane (Fig. 3d). A unit membrane also limited the wall of the tissue cysts in mouse brain. There were numerous invaginations of the membrane into an underlying homogeneous matrix forming the cyst wall (Fig. 3e). However, there was no evidence of host cell organelle rearrangement.

Table 1. The expression of dense granule proteins in the various infectious forms of *T. gondii*

Infectious stage	RA1	GRA2	GRA3	GRA4	GRA5	GRA6	GRA7	GRA8	NTPase
Tachyzoite	+	+	+	+	+	+	+	+	+
Bradyzoite	+	+	+	+	+	+	+	+	+/- [a]
Merozoite	-	-	-	-	-	-	+	-	+
Sporozoite [b]	+	+ [a]	-	+	+	+	+	ND	-

[a] Reduced expression
[b] From Tilley et al. (1997)

DISCUSSION

In previous studies of the tachyzoite it has been shown that rhoptry and dense granule proteins are released into the developing parasitophorous vacuole during or shortly after invasion (Saffer et al. 1992; Cesbron-Delauw 1994). It is believed that these proteins have an important role to play in adapting the PV for parasite development although the exact function of the various ROP and GRA proteins is still unknown. Interestingly it has been shown using parasites in which the ROP 1 or GRA2 genes have been knocked out that the parasite is still capable normal development in vitro although there may be some in vivo effects (Soldati et al. 1995; Mercier et al. 1998). It is possible that this reflects some redundancy among the rhoptries and dense granule proteins associated with tachyzoite development but this will require further investigation. The recently described GRA8 is a proline rich molecule with a trans-membrane domain (Carey et al. 1999). The present study shows that it has a similar distribution to the majority of other GRA proteins (GRAs1-6) in that it is associated with the PV of the tachyzoites but is absence from the coccidian stages of the parasite (Table 1). To date, the only dense granule proteins expressed in the enteric stages are GRA7 and NTPases (Ferguson et al. 1999a; 1999b). This is the first study to examine the expression of certain of the rhoptry proteins in the both the enteric and exoentric

forms. It was observed that the antibody which recognises the family of related proteins (ROPs 2, 3 and 4) positively stained the infectious stages of both the enteric and exoenteric forms (tachyzoite, bradyzoite and merozoite). However, because the antibody cannot differentiate between the three proteins, it is not possible to say whether ROPs2, 3 and 4 are all expressed in the enteric forms. This work is at a preliminary stage and will require further investigation with a more extensive range of anti-rhoptry antibodies. In contrast, it was also observed that while ROP1 was expressed in the exoentric stages (tachyzoite and bradyzoite), the merozoites of the enteric stages appeared to be negative. Therefore the present study provides evidence that not only are there differences in the expression of the dense granule proteins but also in the rhoptry proteins between the exoenteric and enteric forms of *T. gondii.*

It was proposed that since many of the GRA proteins and now ROP1 are limited to exoenteric forms, which are ubiquitous in the hosts and cells type which they can infect, that this may reflect specific adaptations to exoenteric development (Ferguson et al. 1999a). The absence of these molecules in the enteric forms may limit their development to the enterocytes of the cat which would be advantageous to the coccidian stages. There is recent evidence in support of this hypothesis in that it was shown that isolated gut stages could not infect cultured fibroblasts but could infect enterocytes of the small intestine of the cat (Omata et al. 1999).

The molecular differences in rhoptry and dense granule protein expression appeared to be reflected in significant structural differences in the parasitophorous vacuole. The coccidian stages within the enterocytes of the cat gut are located in a PV limited by a laminated membrane while in all other situations, both in vivo and in vitro, the parasite is found within a vacuole limited by a single unit membrane. This includes mouse enterocytes where the parasite undergoes tachyzoite-like development (Speer and Dubey 1997). In addition, the lack of rearrangement of the host cell organelles is markedly different from the collecting of strands of rER and mitochondria around the PV during tachyzoite development (Sheffield and Melton 1968; Jones and Hirch 1972; Sinai et al. 1997). In this situation, although the PV membrane and the rER and mitochondrial membranes are closely appossed, and appear to be physically attached (Sinai et al. 1997), there is a distinct zone (12-20 nm wide) of separation.

The structure of the PV also distinguishes the coccidian stages of *T. gondii* from other closely related members of the Apicomplexa such as *Eimeria* spp. (Pittilo and Ball 1979; Chobotar et al. 1975; Ferguson et al. 1976) in which develop occurs within enterocytes in PVs limited by a unit membrane. However it is similar to the structure of the PV described for *Isospora felis* and *Isospora*

rivolta which also infect the cat intestine (Pelster 1973; Ferguson et al. 1980). In the descriptions of the PV for *Isospora* sp, spherical structures were associated with indentations of the PV membrane (Pelster 1973; Ferguson et al. 1980) which are different from the conical structures observed for *T. gondii* (Ferguson and Wright 1998). The possible function of these structures is at present unclear. *Isospora* spp and *T. gondii* produce similar oocyst (two sporocysts each with four sporozoites) and the similarity of their PVs would be consistent with their close phylogenic relationship.

Therefore, while the tachyzoites represent an excellent model for the study of intracellular parasites (Joiner and Dubremetz 1993) it must be remembered that this stage represents only one aspect of the complex life cycle of *T. gondii*. From the results of the present study it would appear that care should be taken in extrapolating observations on the host parasite relationship of the tachyzoite to other stages in the complex life cycle, in particular the coccidian stages.

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