Giardia: highly evolved parasite or early branching eukaryote?

David Lloyd and Janine C. Harris

The phylogeny of the commonest protozoal agent of intestinal disease, *Giardia*, is unclear. Although recent intensive research suggests this important human parasite is an early branching eukaryote that evolved before the endosymbiotic origin of mitochondria, there is also evidence to suggest that, as a highly evolved parasite, it has lost many of its ancestral characteristics. In this case, these organisms might have arisen much more recently from aerobic free-living flagellates.

Giardia intestinalis (syn. *lamblia*, *duodenalis*) is the most common of the water-borne protozoal parasites infecting humans and causes an estimated ~10^8 cases per annum. Almost all the current literature [1–3] and even the most up-to-date textbooks [4] place *G. intestinalis* at the base of the eukaryotic tree, as its lowest branch. However, although this microaerophilic organism [5] (Fig. 1), which lacks both mitochondria and hydrogenosomes, shows many metabolic features that make it similar to bacteria [6], a growing body of evidence suggests that its organelles and other subcellular structures are highly reduced [7]. Several genes encoding proteins typically localized in mitochondria in other organisms have been cloned from *Giardia*, indicating that evolution from a mitochondria-containing ancestor might have occurred [7]. Thus, *Giardia* might have evolved as a parasite comparatively recently from a typical aerobic free-living flagellate [8].

This article debates these two contrasting and highly controversial viewpoints and focuses on those specific areas of recent research that indicate there are uncertainties regarding the phylogenetic origins of this important parasite.

Subcellular organization

The pervasive philosophy that the diplomonads are 'early branching' protists became an established idea, and several protein sequences, including those of elongation factors 1 and 2, seemed to confirm the view that *G. intestinalis* should be placed on the deepest branch of the eukaryotic phylogenetic tree. The size and sequence of both the large and small subunit rRNAs suggest a basal eukaryotic status [9,10]. These proteins also show numerous bacterial-like metabolic similarities [6] and appear to be ultrastructurally undifferentiated. No structures that can be identified as mitochondria, hydrogenosomes or peroxisomes are present in *Giardia*, and their amitochondriate nature has been regarded as a strong indication of their 'lowly' evolved status. However, the more closely they are examined, the more complex their intracellular organization appears to be, with an impressively specialized constellation of cytoskeletal elements dedicated to effective interaction with the host jejunal mucosa (Fig. 1).

It is now clear that the earliest subcellular-fractionation studies, which apparently confirmed their structural simplicity in terms of membrane organization and organelle content [11], were carried out using traditional methods. Thus, harsh disruptive forces (mechanical breakage of cells using a motor-driven Potter homogenizer, which generates enormous liquid shear forces) were used, followed by a standard centrifugation protocol to fractionate the homogenate into the size ranges (sedimentation coefficients) expected for particles sedimenting into nuclear, mitochondrial and 'microsomal' fractions. When more gentle breakage procedures were used (20 seconds shaking with glass beads at 4 kHz), quite different results were obtained [12]. Thus, whereas several enzyme markers characteristic of eukaryotic organelles (e.g. nicotinamide nucleotide oxidoreductase, pyruvate-ferredoxin oxidoreductase and malate dehydrogenase) had previously been designated as being 'non-sedimentable' in extracts of *G. intestinalis*, in the newer procedure considerable proportions of the total recovered enzyme units were sedimentable after centrifugation at 10 000 g for 60 min.

Mitochondria

Mitochondria can have highly variable morphologies [13]; in lower eukaryotes they are rarely the regular sausage-shaped organelles displayed in textbooks. Thus, they can be highly reticulate or 'giant' multilobate structures (the megamitochondrion) as in *Saccharomyces cerevisiae* or *Chlamydomonas reinhardtii*, where serial sectioning of an entire organism is necessary to elucidate their complicated structures. Confirmation of this complexity has occasionally been achieved by using high-voltage electron microscopy, which produces an electron beam of energy sufficient to penetrate a whole cell and, after specimen tilting, gives a 3-D-reconstructed image directly. Moreover, the form of this 'mitochondriome' often varies during the cell division cycle; fragmentation to multiple organelles could occur at a specific stage during cellular growth and division. Recent elaboration of tomographic-imaging methods also highlighted the physical continuity of the mitochondrial outer membrane with the endoplasmic reticulum (ER) [14].

Anaerobic growth conditions impose limits on mitochondrial biogenesis. In the best-studied system, *S. cerevisiae*, anoxia-induced mitochondrial regression yields organelles with very poorly organized inner membranes, changes that are reversible within tens of minutes during respiratory adaptation. Under anaerobic conditions, the mitochondria lose most of their cytochrome components and unsaturated fatty acids; only cytochrome *b* remains, together with drastically lowered levels of ATP synthase. The capacity of the resulting 'promitochondria' to

David Lloyd*
Microbiology (BIOSI), Cardiff University, PO Box 915, Cardiff, Wales, UK CF10 3TL.

Janine C. Harris
Cultech Biospeciality Products, York Chambers, York Street, Swansea, Wales, UK SA1 4NA.
*e-mail: LloydD@Cardiff.ac.uk

 0966-842X/02/$ – see front matter PII: S0966-842X(02)02306-5

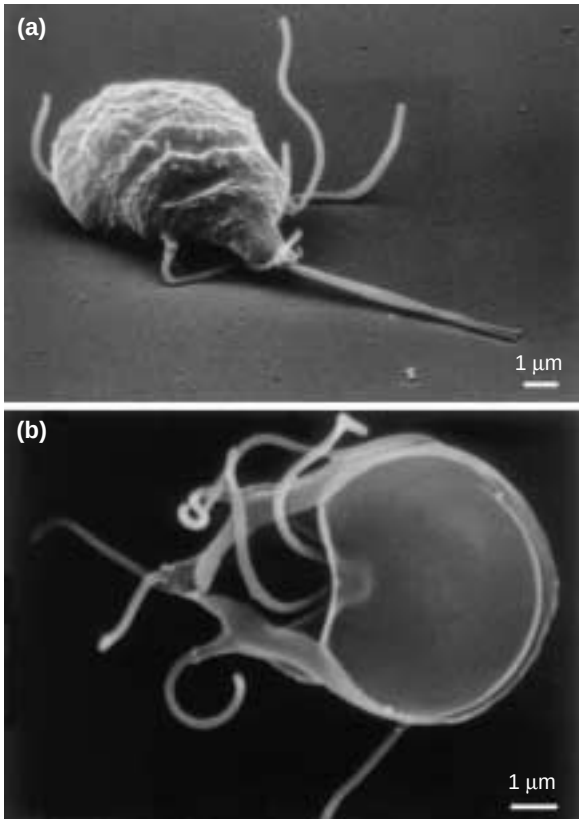

Fig. 1. Scanning electron micrographs of *Giardia intestinalis*: (a) dorsal surface; (b) ventral surface.

accumulate cationic dyes (e.g. rhodamine 123 or cyanine dyes) is almost completely lost, suggesting that the transmembrane electrochemical potential of the inner membrane is greatly attenuated. Ion transport via active processes (e.g. through the action of K^+ and Ca^{2+} pumps) is still carried out by the promitochondrion, as is proton translocation.

In an early, and largely ignored, cytological study of *Giardia* [15], Cheissin noted the presence of subpellicular bodies, which he thought might be mitochondria. He suggested that 'the ultrastructure of mitochondria may be related with the oxygen deficiency in the *Lamblia* environment', and that they might represent 'nothing but changed mitochondria with a few cysts or tubules'; he also refers to Nach and Dutta's even earlier observations [16] of filament-like mitochondria in cysts. Cheissin noted fuchsin-stained granules under the body surface, which also gave positive nitroblue tetrazolium (NBT) and neotetrazolium reduction. These optically observed granules appeared to be identical in location to the subpellicular granules observed in electron micrographs of thin sections.

The absence of conventional mitochondria in *G. intestinalis* is clearly evident from the many recent ultrastructural studies that have yielded high-resolution electron micrographs. Scrutiny of these produces no convincing evidence for characteristic mitochondrial profiles with outer and cristate inner membranes. However, this does not preclude the presence of a membrane system adapted to carry out some mitochondria-like functions. We have tested two of these: electron transport and the generation of membrane potential (D. Lloyd *et al.* unpublished). For the former, reduction of a tetrazolium salt of suitable redox potential has long been an excellent method for cytological location of membrane-associated electron transport chains, provided that the salt is reducible and that the reduced formazan product is insoluble and hence deposited at the site of the electron donor. The fluorogenic tetrazolium CTC (5-cyano-2,3-ditolyl tetrazolium chloride) provides an excellent artificial electron acceptor for studies using confocal scanning laser microscopy. In *G. intestinalis*, this dye is reduced by endogenous sources of reducing power (e.g. metabolic intermediates ultimately provided by the breakdown of stored glycogen), and gives a picture of multiple specialized reduction areas associated with areas of membrane, just within the plasma membrane of the organism. Gently disrupted organisms (three cycles of freezing and thawing) give a similar picture of membrane-bound reduction sites. In this case, either pyruvate or NADPH can be used as an electron donor once the permeability barriers have been removed (Fig. 2a). Other tetrazolium salts can also be reduced by the metabolic pools of endogenous electron donors in live *Giardia* (Fig. 2b,c) and also in cysts [17].

Use of the cationic dye rhodamine 123 for specific fluorescent staining of mitochondria has been widespread for animal cells [18], and this fluorophore is accumulated by *Giardia* (Fig. 2d). With yeast, the intensity of uptake reflects the state of development of mitochondria. This can be assessed in 3-D reconstructions of organisms after confocal laser scanning microscopy. The cationic voltage-sensitive dye responds to the membrane potentials of the membranes it encounters and crosses; it is accumulated 100-fold in the cytosol, and hence through the inner mitochondrial membrane, to reach a final concentration 10^4 times that in the extracellular fluid. Anaerobic conditions for growth produce yeast that take up rhodamine 123 only weakly into the cytosol and promitochondria (Fig. 2e). In *S. cerevisiae* grown aerobically, the megamitochondrion and smaller ovoid organelles are heavily stained and brightly fluorescent (Fig. 2f). In *G. intestinalis* after anaerobic growth in stoppered tubes in the traditional way, rhodamine 123 is weakly accumulated into membrane-bound compartments seen adjacent to the inner-face of the plasma membrane in serially sectioned organisms (as revealed by confocal laser scanning optical microscopy). In most cells there are four or five of these locations (Fig. 2d). These sites do not appear to be the same as those where formazan deposition occurs, and the relationship between the generation of membrane potential and electron transport in this organism requires further elucidation.

Hydrogenosomes

We have recently attempted to locate hydrogenosomes, now generally regarded as being evolved from mitochondria [19], in *Giardia* using immunofluorescent labelling [20]. Brugerolle [21] has

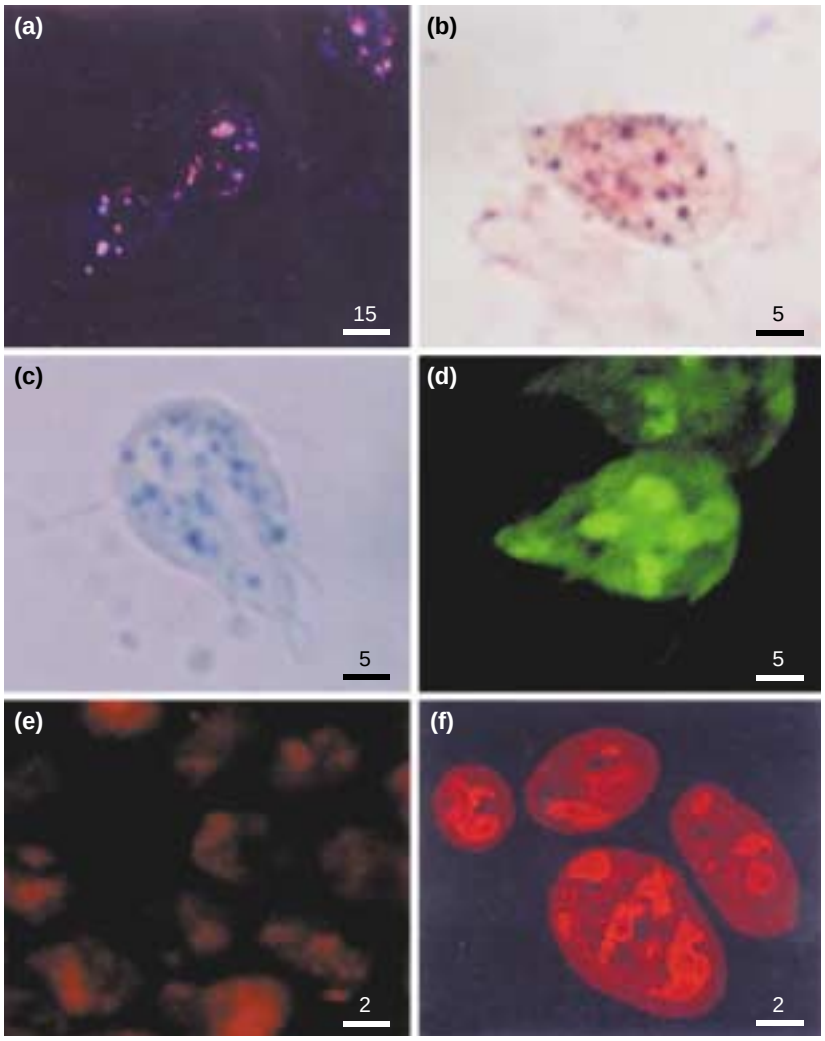

Fig. 2. *Giardia intestinalis* has membrane systems specialized for electron transport and transmembrane potential generation. (a) The fluorogenic tetrazolium salt CTC (5-cyano-2,3-ditolyl tetrazolium chloride) in the presence of NADPH is reduced inside freeze-thawed *Giardia* at discrete locations. Reduction of this dye has also recently been demonstrated in cysts [17]. (b) Triphenyltetrazolium chloride reduction in intact *Giardia*; the insoluble pink formazan is deposited at sites where the redox reaction occurs. (c) 3(4,5-dimethylthiazolyl-1-2)2,5 diphenyltetrazolium bromide reduction also occurs at particular locations in *Giardia*. (d) *Giardia* after exposure to rhodamine 123. (e,f) *Saccharomyces cerevisiae* after anaerobic and aerobic growth, respectively, treated with the cationic fluorophore rhodamine 123. The growth conditions employed for *Giardia* cultures are similar to those for anaerobic yeast cultures; the unstructured mitochondria of anerobically grown yeast are not easily identifiable. Scale bars indicate μm.

prepared antibodies to hydrogenosomal malic enzyme, the α- and β-subunits of succinyl synthetase, and also to the hydrogenosomal-enriched fraction from *Trichomonas vaginalis*. Application of these antibodies to *G. intestinalis* did not give any discrete localization within the organisms. The motivation for this research arose from the discovery that an early report [11] asserting that *G. intestinalis* possesses no detectable hydrogenase is incorrect. Although many studies of the metabolism of this organism have used membrane-inlet mass spectrometry [22], the use of a silicone rubber membrane did not reveal H_2 accumulation, as the organism produces ethanol, which is fragmented to give a signal at 2, the same mass number as H_2. The use of a Mylar membrane (permeable only to H_2 and CO_2, but not to ethanol), has clearly revealed that H_2 is a product of *G. intestinalis* fermentation under strictly anaerobic conditions [19]. Careful assay of extracts prepared and tested under strict anaerobiosis confirms the presence of low levels of hydrogenase activity. The presence of a gene for an iron-hydrogenase has recently been confirmed (Genbank Acc. no. AF242293); as this organism has no hydrogenosomes, this class of enzyme is expected to be membrane-associated and it now remains to be seen where in the organism it is located.

Golgi apparatus, ER and lysosomes

Although the Golgi complex is not so well elaborated in the trophozoites of *G. intestinalis* as in many other eukaryotes, developmental expression of the enzyme activities associated with this organelle occurs in parallel with the appearance of a morphologically identifiable Golgi apparatus during the process of encystation [23]. Protein secretion, in both trophozoites and in encysting organisms, is sensitive to brefeldin A, and the membrane association of ADP-ribosylation factor and β-COP (a subunit of coatomer) was observed [24]. Further evidence for the presence of a Golgi body came from work on Bip, a heat shock protein 70 (Hsp70) analogue that resides in the lumen of the ER in higher eukaryotes, where it functions as a molecular chaperone in protein folding and translocation of proteins across the membrane. Giardial Bip was found to contain a carboxy-terminal KDEL ER-retention signal, indicating the presence of an ER in this organism [25]. As the KDEL signal is employed in the retrieval of ER proteins that have entered the Golgi, a functional Golgi probably exists. An antibody specific for giardial Bip made fluorescence detection of ER possible by optical microscopy and, in conjunction with gold-labelled cryosectioning for electron microscopy, it became possible to identify ER cisternae with tubules, stacked perinuclear membranes, and the presence of Bip in the nuclear membrane [26]. Specialized sub-regions suggested the presence of a differentiated endomembrane system associated with microtubule structures (axonemes and the adhesive disc). The use of a C_6-NBD ceramide probe, which is widely used to identify the Golgi in mammalian cells, has recently given positive results with trophozoites and indicates the presence of Golgi membranes in the perinuclear region, often as a crescent pattern of staining in confocal microscopy images [27]. Post-translational glycosylation of variant specific surface proteins, a Golgi-specific type of protein modification of plasma membrane proteins, although not always detected, further suggests a Golgi function in trophozoites of *G. intestinalis* [28].

Typical lysosomal organization is not evident in *G. intestinalis*; however, peripheral vesicles located just below the plasma membrane show acid phosphatase activity and are isolated intact in homogenates [29]. These organelles also accumulate macromolecules ingested by the protozoon, thus they can accumulate horseradish peroxidase or lucifer yellow. Connections between the peripheral vesicles and ER (marker enzyme, glucose-6-phosphatase) suggest that *G. intestinalis* trophozoites have an endosomal–lysosomal

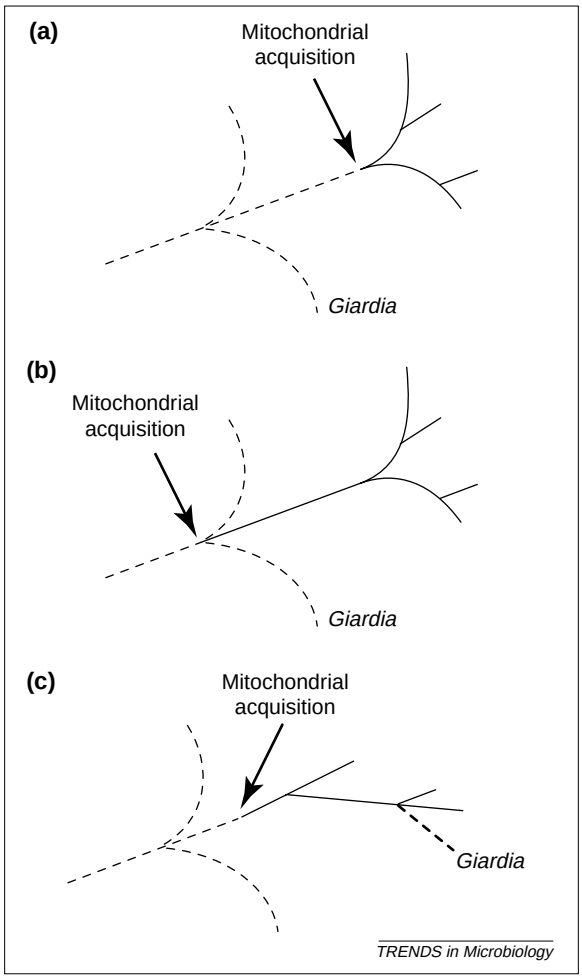

Fig. 3. Major hypotheses for *Giardia* phylogenetic positions: (a) early branching and ancestrally amitochondriate; (b) early branching with lost (or modified) mitochondria (secondarily amitochondriate); (c) as a sister taxon to mitochondriate eukaryotes that lost (or have modified) mitochondria; secondarily amitochondriate.

system represented by these peripheral vesicles. It is suggested that this is 'an ancient and primitive antecedent' of the more highly organized compartmentation (early and late endosomes, and lysosomes) typical of higher eukaryotes. Elegant subcellular-fractionation studies [30] have successfully separated and characterized the membrane-associated enzymes of the Golgi–ER–lysosome system.

Unusual biochemistry: bacterial and archaeal enzymes

G. intestinalis exhibits an entirely fermentative metabolism [31] and therefore lacks cytochrome-mediated electron transport and chemiosmotically coupled proton-translocation-driven ATP synthesis. It also lacks enzymes of the tricarboxylic acid cycle [11] and thus ADP phosphorylation is mediated entirely by substrate-level processes. However, *G. intestinalis* does have genes coding for mitochondrial proteins: the presence of genes encoding both mitochondrial Cpn60 and valyl-tRNA synthetase and, very recently, a mitochondrial-like Hsp70 [32] has been confirmed. Glucose is converted to phosphoenolpyruvate by the glycolytic pathway. Gene sequences of several of the enzymes involved in glycolysis have been examined. Eukaryotic enolase sequences contain several insertions and deletions when compared with one another and with Bacteria and Archaea. The *Giardia* enolase gene is not exceptional in this respect [33]. The triose phosphate isomerase gene is believed to be of α-proteobacterial origin [34]. A key regulatory step of this sequence in most organisms, the ATP-dependent phosphofructokinase, is replaced in *G. intestinalis* by a pyrophosphate-dependent enzyme. Pyruvate phosphate dikinase also replaces pyruvate kinase, and ^{31}P nuclear magnetic resonance studies indicate a high intracellular concentration of pyrophosphate.

The use of pyrophosphate instead of ATP doubles the energy efficiency of glucose fermentation [35]. The major products are alanine (anaerobically), ethanol and CO_2 (at O_2 concentrations between 0.1 and 1.0 μM), or acetate and CO_2 (above 1.0 μM O_2) [22]. The production of acetate occurs via pyruvate:ferredoxin oxidoreductase (PFOR) [6]. This enzyme is a homodimer of 135 kDa subunits which preferentially uses pyruvate, oxaloacetate and α-ketobutyrate. It is inhibited by SH-reacting inhibitors, and resembles the 2-ketoacid oxidoreductases in anaerobic mesophilic bacteria. It is completely different from the pyruvate dehydrogenase multienzyme complex (containing thiamine pyrophosphate and lipoic acid) responsible for oxidative decarboxylation in mitochondria and in aerobic and facultatively anaerobic bacteria and yeast, all of which also lack PFOR. A second enzyme, α-ketobutyrate oxidoreductase, which metabolizes this substrate more rapidly than pyruvate, makes *G. intestinalis* resemble *Pyrococcus furiosus* and *Halobacterium halobium* and provides an alternative energy-generating pathway.

Studies on the iron-sulfur proteins, PFOR [36] and hydrogenases [37] of *Spironucleus barkhanus* and the obligately anaerobic *Clostridium pasteurianum* in comparison with that of *G. intestinalis* indicated a complex evolution, including possible gene duplications and horizontal gene transfers among bacteria. A common origin for eukaryotic cytosolic and hydrogenosomal PFORs from a single bacterial source seems likely, rather than many separate horizontal gene transfers. Several fungi and protists encode proteins with domains typical of PFOR likely to be monophyletic with those from anaerobic protists. These can be fused with other redox proteins (e.g. that responsible for methionine synthesis in *S. cerevisiae*). The overall picture from this study is of a PFOR as a common ancestor of the PFOR of contemporary eukaryotes that was retained during the divergence of amitochondriate and mitochondria-bearing lineages.

Acetate production in *G. intestinalis* is accompanied by ATP generation. The enzyme catalysing this reaction is an unusual nucleoside diphosphate-dependent acetyl CoA synthetase [38]. It is known only in one other eukaryote, *Entamoeba histolytica*, and in a few anaerobic prokaryotes and it is more similar to archaeal rather than bacterial homologues. Alternative metabolism of acetyl CoA to ethanol in *G. intestinalis* occurs via an alcohol dehydrogenase; the gene encoding this enzyme shows extensive homology to those in *Escherichia coli*, *Clostridium acetobutylicum* and *E. histolytica* [39].

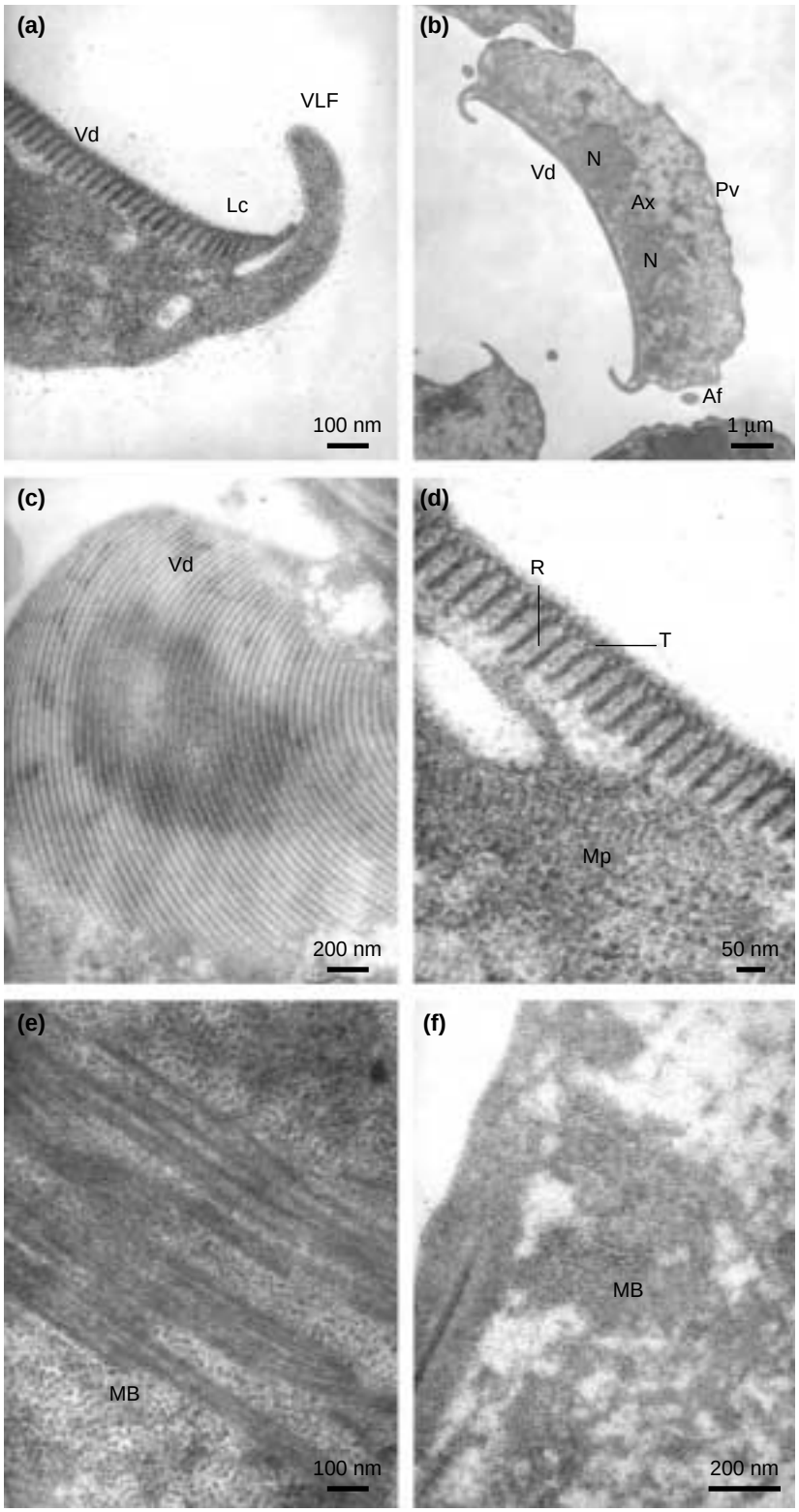

Fig. 4. Transmission electron microscopy of ultra-thin sections of *Giardia intestinalis*. (a) Ventral disc and associated structures. (b) Horizontal cross-section through the trophozoite at the level of the nuclei and ventral disc. (c) Section through the ventral disc in the ventral plane showing the coiling of the microtubules. (d) Section through the ventral disc at high magnification. (e,f) Median bodies. Abbreviations: Af, anterior flagella; Ax, axonemes; Lc, lateral crest; MB, median bodies; Mp, margin plates; N, nucleus; Pv, peripheral vesicles; R, microribbons; T, microtubules; Vd, ventral disc; VLF, ventrolateral flange.

In *G. intestinalis*, arginine is an important energy source. The arginine dihydrolase pathway yields 1 mol of ornithine and 2 mol of ammonia, and the last enzyme of the pathway, carbamate kinase, generates 1 mol of ATP by substrate-level phosphorylation [6,40]. *G. intestinalis* can produce energy more rapidly by this route than by glycolysis, and its redox-neutral character could provide advantages in that, unlike glycolysis, no regeneration of oxidized nicotinamide nucleotides is necessary. Phylogenetic comparisons of the gene sequences of the *Giardia* carbamate kinase with those of the carbamoyl-P-synthetases of the urea cycle in a variety of higher organisms suggest that the carbamate kinase gene, predominately present in prokaryotes (and also in trichomonads and the diplomonad *Hexamita inflata*), is its progenitor [41].

An antiporter similar to that which transports arginine in exchange for ornithine in *Giardia* has also been characterized in some anaerobic bacteria that contain the arginine dihydrolase pathway (e.g. *Lactococcus lactis*) as well as in the aerobic *Pseudomonas aeruginosa*. Arginine uptake in eukaryotes generally proceeds by a completely different mechanism. Polyamine synthesis from ornithine provides another function for this pathway.

Alanine, which is present at high concentrations (~50 mM) in *G. intestinalis* serves as an osmoticant; efflux via a volume-activated alanine transporter enables a fast recovery (measured in minutes) following hypotonic swelling [42].

Oxygen consumption by *G. intestinalis* in the absence of a respiratory chain terminated by cytochrome oxidase is predominantly mediated by a monomeric, 46 kDa flavoprotein NADH oxidase. Whereas most flavoprotein oxidases produce H_2O_2, this enzyme is rather unusual in that it produces H_2O, making it like those of anaerobic intestinal bacteria [43]. Thus, the amino-terminal sequence of the *Giardia* enzyme has high similarity with those from *Enterococcus faecalis* and *Serpulina hyodysenteriae*.

The usual enzymes for the detoxification of partially reduced O_2 products ('reactive oxygen species'), superoxide dismutase, catalase and peroxidase are not present in *G. intestinalis* although the activity of a NADH peroxidase has been reported [6]. The intracellular thiol pool, responsible for redox buffering of the cytosolic environment and protection against oxidative stress, is highly atypical [44]. Whereas glutathione is the major player in this role in most organisms, this thiol is absent from *G. intestinalis*, and its function is assumed by cysteine. The thiol reductase in *G. intestinalis* is a soluble dimeric (2×35 kDa) NADPH-dependent thioredoxin reductase-like disulfide reductase that is a flavoprotein with a non-flavin thiol component at its active centre. This enzyme has 75% similarity with those from *E. coli* and *S. cerevisiae*; it seems likely that thiol-cycling and regulation of ribonucleotide-reductase activity as well as maintenance of the redox state are the key functions necessary in the microaerophilic homeodynamics of the organism.

Conclusions

Many unusual features of *Giardia* ultrastructure, metabolism and gene sequences have led to its

description as an 'enigmatic, interesting, simple, anaerobic, primitive, ancient, early diverging and deep-branching eukaryote', and to a phylogenetic location at the base of the eukaryotic tree (Fig. 3). The hypothesis that amitochondriate eukaryotes should be regarded as the earliest eukaryotes that once inhabited the anaerobic earth and constitute a distinct 'kingdom', the archeozoa [45], is not sustained by current discoveries [46–48]. Thus, it has been realized that many other organisms of this 'kingdom', [Microsporidia and probably the Parabasalia (i.e. the Trichomonads)] are recently derived from highly aerobic, mitochondria-containing protists. The chimeric acquisition of genes by lateral gene transfer processes and the artefacts inherent in fast-evolving lineages [49] make the construction of phylogenies complex [50]. Depending on which macromolecule is used as a marker, fast-evolving groups are not always the same, and artefacts result in misplacing taxa at the base of the evolutionary tree. Incongruent phylogenies can often be explained in this way and the current paradigms must be re-examined.

Giardia is yet another example of a highly reduced parasite, fantastically well-attuned to its host's characteristics and the changing challenges it encounters on its journey from the intestine to the natural environment and back [30]. Its peculiarities are uniquely suited to its ecology, and its ubiquity and pathogenic prevalence attest to its success and highly evolved status. These organisms are certainly not simple [51] nor primitive (Fig. 4) and perhaps they are not so early diverging either! The rapidly advancing *Giardia* genome project [52] might help further assessment.

Acknowledgements
We thank A. Hayes and M. O'Reilly of Cardiff University and R. Wadley of the University of New South Wales for their expert help with microscopy.

References

1 Adam, R.D. (2001) Biology of *Giardia lamblia*. *Clin. Microbiol. Rev.* 14, 447–475
2 Lloyd, D. and Wallis, P. (2001) A *Giardia* feast. *Trends Parasitol*. 17, 115–117
3 Lane, S. and Lloyd, D. Current trends into the waterborne parasite *Giardia*. *Crit. Rev. Microbiol.* (in press)
4 Madigan, M-T. *et al*., eds (2000) *Biology of Microorganisms* (9th edn), Prentice Hall International Inc
5 Lloyd, D. *et al.* (2000) The microaerophilic flagellate *Giardia intestinalis*: oxygen and its reaction products collapse membrane potential and cause cytotoxicity. *Microbiology* 146, 3109–3118
6 Brown, D.M. *et al*. (1998) Anaerobic bacterial metabolism in the ancient eukaryote *Giardia duodenalis*. *Int. J. Parasitol*. 28, 149–164
7 Embley, T.M. and Hirt, R.P. (1998) Early-branching eukaryotes? *Curr. Opin. Genet. Dev.* 8, 624–629
8 Fenchel, T. and Finlay, B.J. (1995) *Ecology and Evolution in Anoxic Worlds*, pp. 108–121, Oxford University Press
9 Sogin, M.L. *et al*. (1989) Phylogenetic meaning of a kingdom concept: an unusual ribosomal RNA from *Giardia lamblia*. *Science* 243, 75–77
10 Sogin, M.L. (1997) History assignment: when was the mitochondrion founded? *Curr. Opin. Genet. Dev*. 7, 792–799
11 Lindmark, D. (1980) Energy metabolism of the anaerobic protozoon *Giardia lamblia*. *Mol. Biochem. Parasitol*. 1, 1–12
12 Ellis, J.E. *et al*. (1993) Electron transport components of the parasitic protozoon *Giardia lamblia*. *FEBS Lett*. 325, 196–200
13 Lloyd, D., ed. (1974) *The Mitochondria of Microorganisms*, Academic Press
14 Mannella, C.A. (2000) Introduction: our changing views of mitochondria. *J. Bioenerg. Biomembr.* 32, 1–4
15 Cheissin, E.M. (1965) Ultrastructure of *Lamblia duodenalis* 2. The locomotory apparatus, axial rod and other organelles. *Arch. Protistenkd.* 108, 8–18
16 Nach, V. and Dutta, G.P. (1962). Cytochemistry of protozoa, with particular reference to the Golgi apparatus and the mitochondria. *Int. Rev. Cytol.* 13, 323–355
17 Iturriaga, R. *et al*. (2001) Detection of respiratory enzyme activity in *Giardia* cysts and *Cryptosporidium* oocysts using redox dyes and immunofluorescence techniques. *J. Microbiol. Methods* 46, 19–28
18 Chen, L.B. (1988) Mitchondrial membrane potential in living cells. *Annu. Rev. Cell Biol.* 4, 155–181
19 Biagini, G.A. *et al*. (1997) Evolution of the hydrogenosome. *FEMS Microbiol. Lett.* 153, 133–140
20 Lloyd, D. *et al*. *Giardia intestinalis*, a eukaryote without hydrogenosomes produces hydrogen. *Microbiology* (in press)
21 Brugerolle, G. *et al*. (2000) Immunolocalization of two hydrogenosomal enzymes of *Trichomonas vaginalis*. *Parasitol. Res*. 86, 30–35
22 Paget, T.A. *et al*. (1993) The effects of oxygen on fermentation in *Giardia lamblia*. *Mol. Biochem. Parasitol*. 57, 65–72
23 Reiner, D.S. *et al*. (1990) Sorting of cyst wall proteins to a regulated secretory pathway during differentiation of the primitive eukaryote *Giardia lamblia*. *Eur. J. Cell Biol*. 53, 142–153
24 Lujàn, H.D. *et al*. (1995) Developmental induction of Golgi structure and function in the primitive eukaryote *Giardia lamblia*. *J. Biol. Chem*. 270, 4612–4618
25 Gupta, R.S. *et al*. (1994) Cloning of *Giardia lamblia* HSP70 homologs: implications regarding origin of eukaryotic cells and of endoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A.* 91, 2895–2899
26 Soltys, B.J. *et al*. (1996) Identification of endoplasmic reticulum in the primitive eukaryote *Giardia lamblia* using cryoelectron microscopy and antibody to BiP. *J. Cell Sci.* 109, 1909–1917
27 Lanfredi-Rangel, A. *et al*. (1999) Trophozoites of *Giardia lamblia* may have a Golgi-like structure. *FEMS Microbiol. Lett*. 181, 245–251
28 Hitpold, A. *et al*. (2000) Glycosylation and palmitoylation are common modifications of *Giardia* variant surface proteins. *Mol. Biochem. Parasitol*. 109, 61–65
29 Lanfredi-Rangel, A. *et al*. (1998) The peripheral vesicles of trophozoites of the primitive protozoan *Giardia lamblia* may correspond to early and late endosomes and lysosomes. *J. Struct. Biol.* 123, 225–235
30 Jarroll, E.L. *et al*. (2001) Regulation of carbohydrate metabolism during *Giardia* encystment. *J. Eukaryot. Microbiol*. 48, 22–26
31 Lloyd, D. and Williams, A.G. (1993) Biological activities of symbiotic and parasitic protists in low oxygen environments. *Adv. Microb. Ecol*. 13, 211–262
32 Morrison, H.G. (2001) *Giardia lamblia* expresses a proteobacterial-like DnaK homolog. *Mol. Biol. Evol*. 18, 530–541
33 Keeling, P.J. and Palmer, J.D. (2000) Phylogeny: parabasalian flagellates are ancient eukaryotes. *Nature* 405, 635–637
34 Keeling, P.J. and Doolittle, W.F. (1997) Evidence that eukaryotic triose phosphate isomerase is of α-proteobacterial origin. *Proc. Natl. Acad. Sci. U. S. A.* 94, 1270–1275
35 Mertens, E. (1993) ATP vs. pyrophosphate: glycolysis revisited in parasitic protists. *Parasitol. Today* 9, 122–126
36 Horner, D.S. *et al*. (1999) A single eubacterial origin of eukaryotic pyruvate:ferredoxin oxidoreductase genes: implications for the evolution of anaerobic eukaryotes. *Mol. Biol. Evol.* 16, 1280–1291
37 Horner, D.S. *et al*. (2000) Iron hydrogenase and the evolution of anaerobic eukaryotes. *Mol. Biol. Evol*. 17, 1695–1709
38 Sanchez, B. *et al*. (2000) Acetyl-CoA synthetase from the amitochondriate eukaryote, *Giardia lamblia* belongs to the newly-recognized superfamily of Acyl CoA synthetases (nucleoside diphosphate-forming). *J. Biol. Chem*. 275, 5974–5803
39 Rosenthal, B. *et al*. (1997) Evidence for the bacterial origin of genes encoding fermentation enzymes of the amitochondriate protozoan parasite *Entamoeba histolytica*. *J. Bacteriol*. 179, 3736–3745
40 Edwards, M.R. *et al*. (1992) Arginine metabolism during culture of *Giardia intestinalis*. *Mol. Biochem. Parasitol*. 53, 97–103
41 Minotto, L. *et al*. (1999) Characterisation and expression of the carbamate kinase gene for *Giardia intestinalis*. *Mol. Biochem. Parasitol*. 98, 43–51
42 Maroulis, S.L. *et al*. (2000) Role of K^+ in the response of *Giardia intestinalis* to hypoosmotic stress. *Mol. Biochem. Parasitol*. 108, 141–145
43 Brown, D.M. *et al*. (1996) A H_2O-producing NADH oxidase from the protozoan parasite *Giardia duodenalis*. *Eur. J. Biochem*. 241, 155–161
44 Brown, D.M. *et al*. (1996) Thiol redox management by disulphide reductase in the protozoan parasite *Giardia duodenalis*. *Mol. Biochem. Parasitol*. 83, 211–220
45 Cavalier-Smith, T. (1993) Kingdom protozoa and its 18 phyla. *Microbiol. Rev.* 57, 953–994
46 Hashimoto, T. *et al*. (1995) Phylogenetic place of mitochondria-lacking protozoan *Giardia lamblia* inferred from amino acid sequence of elongation factor 2. *Mol. Biol. Evol*. 12, 782–793
47 Keeling, P.J. (1998) A kingdom's progress: archezoa and the origin of eukaryotes. *Bioessays* 20, 87–95
48 Roger, A.J. (1999) Reconstructing early events in eukaryotic evolution. *Am. Nat*. 154, 5146–5163
49 Phillipe, H. *et al*. (2000) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. *Proc. R. Soc. London Biol. Sci*. 267, 1213–1221
50 Harvey, P.H. *et al*., eds (1996) *New Uses for New Phylogenies*, Oxford University Press
51 Biagini, C.A. and Bernard, C. (2000) Primitive anaerobic protozoa: a false concept? *Microbiology* 146, 1019–1020
52 Adam, R.D. (2000). The *Giardia lamblia* genome. *Int. J. Parasitol*. 30, 475–484