ANTI-TUBULIN ANTIBODIES LOCATE THE BLEPHAROPLAST DURING SPERMATOGENESIS IN THE FERN PLATYZOMA MICROPHYLLUM R.BR.: A CORRELATED IMMUNOFLUORESCENCE AND ELECTRON-MICROSCOPIC STUDY

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SUMMARY

The discovery that the monoclonal anti-tubulin antibody YOL 1/34 recognizes a microtubule organizing centre, the blepharoplast (which arises de novo during the latter stages of spermatogenesis in the fern, Platyzoma microphyllum), has enabled us to follow it and associated microtubules throughout most of its ontogeny. By correlating electron-microscopic and immunofluorescence observations, YOL 1/34 is seen to stain the blepharoplast uniformly at a time when no microtubules are present within the organelle. Later, staining becomes intense at the surface, concomitant with the re-location of cylindrical channels to the periphery of the blepharoplast. During anaphase of the ultimate division of the spermatid mother cell the blepharoplast moves to the spindle poles and sharpens the otherwise barrel-shaped mitotic apparatus. Prior to this stage the blepharoplast is, however, off-centre and at variable positions around the poles.

Later still, in the differentiating spermatids, the blepharoplast is the focus for radiating cytoplasmic microtubules that abut directly onto the electron-dense organelle, penetrating the ribosome-free halo.

The three main conclusions are: (1) that tubulin in a pre-microtubular form is associated with the cylindrical channels that arise de novo within the previously amorphous blepharoplast and act as a template in basal body formation; (2) that the late appearance of the blepharoplast as a focus for the spindle poles during the final mitosis provides strong argument against its functioning during spindle pole initiation (despite its ability to sharpen the poles at anaphase); (3) that the blepharoplast does seem to act as a microtubule organizing centre in the mitotically quiescent spermatid.

INTRODUCTION

In higher plant cells traversing the cell cycle, microtubules sequentially form four different microtubule arrays. By allowing many cells to be screened, and with the three-dimensional view it affords, the technique of indirect immunofluorescence (Lloyd et al. 1979; Wick et al. 1981) has greatly helped the analysis of the microtubule cycle. It has, in particular, provided information on the way in which each phase of the cycle emerges from its predecessor (Wick & Duniec, 1984; Clayton & Lloyd, 1984; Doonan, Cove & Lloyd, 1985), but its use has so far been largely

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confined to cells containing amorphous microtubule nucleation sites. These sites are probably involved in the temporal and spatial regeneration of microtubule assembly and have only recently been identified in a peri-nuclear location, using a human scleroderma serum, in onion meristematic cells (Clayton, Black & Lloyd, 1985). However, not all microtubule arrays in plant cells are associated with amorphous nucleation sites. For instance, structured MTOCs such as centrioles, which are absent during most of the life of embryophytic plants, can be found during one particular stage of the life cycle of bryophytes, pteridophytes, cycads and Ginkgo (see reviews by Duckett & Carothers, 1982; Duckett, Carothers & Miller, 1983). During spermatogenesis in ferns and Equisetum the blepharoplast (a highly structured spherical organelle) appears de novo and upon fragmenting gives rise to basal bodies that are found only at this stage (Hepler, 1976). In addition to the flagellar microtubules, the motile male gametes of archeogoniate plants possess a cytoskeletal microtubule system, the multilayered structure (MLS), which also originates in close proximity to the fragmenting blepharoplast in young spermatids. It comprises a single band of regularly spaced microtubules, the spline, which circumscribes the characteristic spiral shape of the cells. Underlying the anterior rim of the spline is a lamellar strip, putatively identified on the basis of inhibitor studies (Miller, Duckett, Sheterline & Carothers, 1983) and ultrastructural analyses of mutant spermatozoids (Duckett, Klekowski & Hickok, 1979), as a highly structured MTOC. In the mature spermatozoids of ferns a second or accessory band of microtubules overlies the anterior rim of the spline (Duckett, 1975).

We have used anti-tubulin antibodies on the polypodiaceous fern Platyzoma microphyllum to follow the distribution of tubulin antigens from isodiametric thin-walled spermatogenous cells through the development of the naked, motile, spiral gamete. By correlating immunofluorescence images with those obtained from transmission electron microscopy we have found that tubulin antigens are present in the blepharoplast even though no microtubules are visible therein ultrastructurally. The discovery that anti-tubulin antibody stains the blepharoplast before procentriole assembly has enabled us to trace the precise relationship between the blepharoplast and mitotic cycle, and division of the spermatogenous cells.

MATERIALS AND METHODS

Plant material

Antheridial gametophytes of Platyzoma were produced in agar cultures as described previously (Duckett & Pang, 1984). To facilitate the recognition of the stages in spermatogenesis from electron micrographs and in cells separated for antibody treatment, individual antheridia were squashed under a coverslip and monitored on a Leitz Dialux 20 microscope using interference-contrast optics.

Transmission electron microscopy

Gametophytes were fixed in glutaraldehyde and osmium tetroxide as described previously (Miller et al. 1983). Sections, post-stained with lead citrate and uranyl acetate were observed in a JEOL 100S electron microscope operating at 80 kV.
Fixation and extraction of gametophytes for immunofluorescence

Antheridial gametophytes were fixed as described by Doonan et al. (1985). The staining of later stages of antheridial development was improved by the addition of 0.1% (v/v) Nonidet NP40 to the fixative. Cell walls were softened by incubating in 5 mM-EGTA containing 5% (w/v) Driselase (Sigma) and 50 μg ml⁻¹ leupeptin for 3–4 h. After washing in microtubule stabilizing buffer (MTSB) (Doonan et al. 1985) cells were released by gentle squashing and air dried to polylysine coverslips (Wick et al. 1981). To aid penetration of antibodies and to reduce autofluorescence cells were extracted for 2–3 h in MTSB containing 5% (v/v) dimethylsulphoxide (DMSO) and 3% (v/v) Nonidet NP40. Coverslips were washed free of detergent then stained by indirect immunofluorescence (IIF) using monoclonal antibodies against yeast tubulin (Kilmartin, Wright & Milstein, 1981) as described (Doonan et al. 1985). The coverslips were mounted in Citifluor Antifade Mountant (City University, London) containing 0.5 μg ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI) and viewed using a Zeiss epifluorescence microscope.

RESULTS

In order to interpret blepharoplast ontogeny and spermatid metamorphosis from anti-tubulin immunofluorescence images, it is crucial to be able to recognize the various developmental stages of cells after antheridia have been squashed for immunocytochemistry. We have circumvented this problem by using the fluorescent DNA stain, (DAPI) together with measurements of nuclear diameters. DAPI enables the recognition of stages in mitosis and nuclear metamorphosis for immunofluorescence, whilst nuclear size provides the means for discriminating between spermatogenous cells and young spermatids in both immunofluorescence and electron-microscopic observations.

Cytological observations on antheridium ontogeny and the origin of the blepharoplast

As in Marsilea (Hepler, 1976) antheridium development in polypodiaceous ferns involves a fixed number of cell divisions, which ultimately produce 32 spermatids and three sterile jacket cells. The first division delimits a unicellular antheridial initial from the subjacent vegetative cell. Divisions 2–4 yield the three jacket cells and, internally, a primary spermatogenous cell. Divisions 5–9 comprise synchronous mitoses of the spermatogenous cells ultimately producing 32 spermatids. In sections and squashes of excised antheridia a particular spermatogenous cell generation can be identified merely by counting the number of cells (e.g. 2, 4, 8, 16 or 32).

From sections and squashes of individual excised antheridia it is apparent that nuclear diameter remains constant through all the generations of spermatogenous cells (15–18 μm at interphase, up to 20 μm at prophase) but is significantly reduced (10–12 μm) in young spermatids where the nucleoli are also far less prominent.

Using a combination of sections and squashes we have attempted to establish the time of blepharoplast formation. In squashes the first structure that we identify as a blepharoplast, by extrapolating from Hepler's (1976) incisive observations on Marsilea, is a small phase-bright dot in antheridia containing 16 spermatogenous cells (i.e. the spermatid mother cells). At the electron-microscopic (EM) level this dot (Fig. 24) corresponds to a densely stained spherical or slightly oval body approximately 0.2 μm in diameter, lying in the cortical cytoplasm. The body has a
granular matrix with no obvious substructure. We have never observed similar structures in the cortical cytoplasm of earlier generations of spermatogenous cells.

Interphase spermatid mother cells from other antheridia (for IIF micrographs, see Figs 6–10) contain two phase-bright dots, lying either side-by-side or at varying distances from each other around the nucleus and ultimately at opposite poles.

These observations are in accord with cytological studies reported by Sharp (1926) for closely related ferns.

**Correlated immunofluorescence and electron-microscopic observations of spermatogenous cells**

*Prothallus cells of Platyzoma.* During interphase, the vegetative cells of *Platyzoma* gametophytes contain cortical arrays of microtubules that are orientated approximately transversely to the long axis of the cells (Fig. 1). These cells are large (70 μm long, 20 μm wide), their chloroplasts and cytoplasm being mostly peripheral, with a large central vacuole.

*Spermatogenous cells.* The early generations of spermatogenous cells contain both cortical and endoplasmic microtubule bundles that form a complex cagework around the nucleus (Fig. 2). Electron micrographs (Figs 25, 26) demonstrate that these bundles of microtubules contain up to eight microtubules. Stained with DAPI, the nucleus has a rather granular appearance and a prominent nucleolus. Unlike vegetative cells, the chloroplasts are no longer located by DAPI staining and cannot be located cytologically. Therefore the plastids are either lost or begin to degenerate at an early stage.

We have found no evidence for the existence of pre-prophase bands in the spermatogenous cells, but otherwise mitosis proceeds very much as in flowering plants (Wick et al. 1981; Wick & Duniec, 1984; Clayton & Lloyd, 1984). All cytoplasmic microtubules disassemble, and spindles develop, initially with narrow poles (data not shown) that widen to produce typical barrel-shaped spindles with broad poles. The spindle fibres associate laterally into sub-groups as shown in the

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**Abbreviations used in Figs.**

- ac, anterior complex in mature spermatozoids;
- bb, basal body;
- c, centriole;
- cc, cell wall;
- f, flagella;
- l, lamellar strip;
- m, mitochondrion;
- ma, anterior mitochondrion;
- mt, microtubules; mta, accessory band of microtubules in mature spermatozoids;
- n, nucleus;
- oc, osmiophilic crest;
- or, osmiophilic ridge;
- pl, plaque associated with blepharoplast;
- s, spline.

**Figs 1–4.** Immunofluorescence micrographs of *Platyzoma* stained with anti-tubulin and DAPI.

**Fig. 1.** Vegetative gametophyte cells showing transversely arranged cortical hoops of microtubules. ×700.

**Fig. 2.** A. Endoplasmic arrays of microtubules in interphase spermatogenous cells. These cells are highly autofluorescent. B. The same cells stained with DAPI. Note the prominent nucleoli (arrows). ×700.

**Fig. 3.** A. Barrel-shaped spindles characterize metaphase in early generations of spermatogenous cells. B. DAPI image of the same cells, the chromosomes have yet to congress at the metaphase plate. ×1500.

**Fig. 4.** A. Phragmoplast between the sister nuclei of early generation spermatogenous cells. The nuclei are slightly autofluorescent. B. DAPI image of the same cell showing a typical telophase configuration of chromosomes. ×1500.
pro-metaphase spindles (Fig. 3). The phragmoplast also develops as in flowering plant cells, appearing first during telophase in the mid-zone between the sister nuclei (Fig. 4), growing centrifugally to divide the cell.

Microtubules often radiate from foci near the nucleus of some spermatogenous cells towards the exterior of the cell (Fig. 5). Through-focussing usually reveals only one focus per cell, but occasional cells may contain two foci that occupy positions near the nucleus and are various distances apart (data not shown). Often the foci occupy polar positions, and may represent stages just prior to mitosis.

**Blepharoplast development**

Blepharoplasts are first visualized by anti-tubulin antibodies in spermatid mother cells (SMCs) as a brightly stained pair of dots (Figs 6, 7) approximately 0.2 μm in diameter. At this stage they do not appear to act as foci for the cytoplasmic microtubules. The microtubule foci described above for earlier stages are no longer found.

Electron micrographs of this stage show the presence of a densely stained granular body lying in the peripheral cytoplasm (Fig. 25). Other cells, at this stage, contain two ovoid blepharoplasts associated with a multilayered plaque-like structure.

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Figs 5–14. Immunofluorescence micrographs of *Platyzoma* stained with anti-tubulin and DAPI.

Fig. 5. A complex network of microtubules surrounds the nucleus and form a focus near the nuclear surface. ×800.

Fig. 6. Interphase spermatid mother cells showing random arrays of microtubules and a pair of nascent blepharoplasts (arrows) (cf. Fig. 30). ×850.

Fig. 7. Paired blepharoplasts (arrows) in a spermatid mother cell displaying increased staining intensity with respect to Fig. 6.

Figs 8–14. Blepharoplast (arrows) separation and positioning at opposite sides of the nucleus.

Fig. 8. Blepharoplasts (arrows) moving towards polar positions, microtubule arrays radiating from each. ×800.

Fig. 9. The blepharoplasts (arrows) have reached opposing sides of the nucleus. Through-focussing allows microtubule bundles to be traced from one blepharoplast to the other. In this cell and that shown in Fig. 8 the centre of blepharoplast stains only dimly relative to the outside. ×800.

Figs 10–14. The final antheridial mitosis (blepharoplasts marked by arrows).

Fig. 10. The blepharoplasts at opposite ends of the nucleus, probably pre-prophase. Most cytoplasmic microtubules have disassembled and the blepharoplasts stain more densely, although the upper blepharoplast here demonstrates a slightly paler centre. ×1400.

Fig. 11. Metaphase showing blepharoplasts near the spindle poles. ×800. The lower blepharoplast (lower arrow) lies to the left of the lower spindle pole.

Fig. 12. A. Early anaphase showing blepharoplasts at the now sharply focussed pole. B. DAPI image of the same cell. The chromosomes are just beginning to leave the metaphase plate. ×1300.

Fig. 13. Early telophase showing blepharoplasts (arrows) at the foci of the spindle microtubules and also associated with MTs (arrowhead), which run out into the cytoplasm. MTs partly obscure the blepharoplasts. ×1200.

Fig. 14. A. Late telophase showing the phragmoplast and blepharoplasts, the latter with identical staining properties to those seen at prophase (Fig. 10). B. DAPI image of the same cell showing telophase configuration of chromosomes. ×1300.
Microtubules during fern spermatogenesis
The granular matrix is similar in both cases and it is possible that the blepharoplast may be derived from these granular bodies (Fig. 25). Each blepharoplast measures 0.5 μm × 0.7 μm and the densely stained matrix is penetrated by cylindrical channels ('pro-centriolar cores'; Duckett & Carothers, 1982) about 70 nm in diameter. The channels are composed of a darkly stained central core (similar in texture to the granular matrix), about 20 nm diameter, surrounded by a lightly stained region. Between or close to the two blepharoplasts lies a multi-layered plaque-like structure about 0.1 μm wide (Fig. 30). On each side of the plaque's mid-line, marked by a layer of densely stained material 10 nm thick, is a layer of lightly stained material 25 nm thick, which in turn gives way to another densely stained layer. Outside this layer, there are further layers, alternately lightly and densely stained, but less clearly defined. The earliest stage at which the blepharoplast can be stained in the SCM with anti-tubulin antibodies is when it exists as a doublet - the single granular body (its presumptive precursor) has never been observed to stain. The commencement of staining appears to correlate with the appearance of the cylindrical channels within the granular matrix of the paired blepharoplasts.

The paired blepharoplasts increase in both size (to 0.7 μm) and staining intensity (Figs 6, 7), before migrating to the polar positions on the nuclear envelope (Figs 8–10). The blepharoplasts become the centre of radiating microtubules as they move apart (Figs 8, 9). The centre of these blepharoplasts does not appear to stain as brightly as stages before (Figs 6, 7) and after (Fig. 10) migration. Unfortunately, this stage occurs rapidly and we have been unable to find migrating blepharoplasts by

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Figs 15–21. Immunofluorescence micrographs of young spermatids of *Platyzoma*. (all ×1300).

Fig. 15. Through-focus series showing microtubule arrays extending over the nuclear surface and lining a deep indentation in the nucleus. The microtubules come together forming a focus near the mouth of the indentation (A) and run across the nuclear surface into the indentation (B). Lobes of the nucleus surround the indentation (C, D), while at a deeper focal plane the nuclear outline becomes approximately spherical again. The flattened side of the nucleus is on the same side as the nuclear furrow (cf. Figs 27, 28).

Fig. 16. Microtubule arrays radiating from the vicinity of the blepharoplast. The microtubules, having decreased in numbers, appear to form bundles.

Fig. 17. Slightly older spermatid than that in Fig. 16 showing far fewer microtubule arrays radiating from the blepharoplast and these remaining microtubules are stained in a dotted fashion.

Fig. 18. A. Spherical blepharoplasts lying adjacent to the nuclear surface. The DAPI-stained nuclei (A) display a flattened face, but the blepharoplast does not always associate with this face. The nuclei lack nucleoli and the chromatin has begun to condense, giving a mottled appearance.

Fig. 19. First stage in the break up of the blepharoplast, which now contains dots slightly brighter than the surrounding material. Through-focusing reveals that now only the surface of this ovoid blepharoplast cross-reacts with the antibody (cf. Fig. 31).

Fig. 20. Staining at a stage corresponding to the differentiation of centrioles over the blepharoplast. The centrioles give the structure a slightly knobbley appearance (cf. Figs 31, 33).

Fig. 21. A. Individual centrioles become visible as brightly staining dots in an approximately triangular area corresponding to that of the MLS overlying the nucleus (Figs 32, 33). A similar group of centrioles occupies the area outlined in a second spermatid but at a different focal plane, as shown by the hatched area.
Microtubules during fern spermatogenesis
Figs 22–24. Immunofluorescence micrographs of spermatid metamorphosis.

Fig. 22. Through-focus micrographs of a spermatid at the onset of flagellogenesis. The growing axonemes appear as bristle-like stubs (A) sticking out from the cell surface above the anterior region of the MLS (arrow). Careful focussing reveals other more closely packed axonemes within an approximately triangular area of intense staining (B) corresponding to the area of the spline overlying a notch in the DAPI-stained nucleus (C) (cf. Figs 37, 38). ×1500.

Fig. 23. Through-focus micrographs of late spermatid showing (A) backwardly directed axonemes apressed to the body of the cell; a, and p, approximate positions of the anterior and posterior tips of the lamellar strip. B. Anterior view showing the flagella obliquely inserted adjacent to an arc of staining that extends round three quarters of the circumference of the cell corresponding to the surface of the crescent-shaped nucleus stained with DAPI (C). ×1500.

Fig. 24. A. Anterior view of mature spermatozoïd fixed in the presence of 1% (v/v) Triton X-100. The flagella are splayed from an arc of staining corresponding to the anterior complex but the spline overlying the nucleus is unstained. a, p as in Fig. 39.
B. DAPI image of the same cell demonstrates the coiled nature of the nucleus; a, anterior tip of the nucleus. ×1600.
Fig. 25. Two spermatid mother cells containing spherical granular bodies interpreted as nascent blepharoplasts. ×.

Fig. 26. Cortical microtubules in spermatogenous cells (cf. Fig. 2). ×.

Fig. 27. Early spermatid. The blepharoplast lies above the deep channel in the nucleus with the intervening cytoplasm containing abundant microtubules (cf. Fig. 15). ×.
EM, and therefore cannot say if their substructure changes during migration. The nucleus of cells containing polar blepharoplasts generally show signs of DNA condensation (data not shown) and are therefore presumed to be approaching mitosis. None possesses a pre-prophase band. Some, with more intensely stained blepharoplasts, lack all cytoplasmic microtubules as far as can be detected by IIF (Fig. 10). In these cells the blepharoplasts are intensely stained by anti-tubulin antibody, but in some the centre appears slightly less stained.

The spindle assembles without precise reference to the blepharoplasts, although the blepharoplasts occupy approximately polar positions from pre-prophase until metaphase (Figs 10, 11). The metaphase spindle is barrel-shaped, with broad poles, and the blepharoplast can be some distance off-centre from the poles (Fig. 11). However, in early anaphase (Fig. 12), each blepharoplast becomes positioned precisely at the spindle poles, which are now compact rather than broad. The blepharoplasts remain in that position throughout mitosis and, although they may be partially obscured by the brightly stained spindle fibres, they remain stained by anti-tubulin antibody as discretely spherical objects. In telophase (Fig. 13), microtubules run from the blepharoplast into the cytoplasm. The phragmoplast (Figs 13, 14) is similar in appearance to that found in other cell types. At this stage the blepharoplast occupies a position near the nuclear surface distal to the cell plate. DAPI staining of the daughter nuclei reveals a decreased nuclear diameter (about 10 \( \mu m \)) compared to SMC nuclei (up to 18 \( \mu m \)).

**Spermatid cell development**

Abundant cytoplasmic microtubules appear after the final mitosis. They radiate from the vicinity of the blepharoplast, which lies at the mouth of a deep microtubule-lined indentation in the nuclear surface that can be seen by through-focussing (Fig. 15). Interpretation of the IIF images is difficult due to the density of microtubules associated with the nuclear indentation, but electron micrographs (Figs 27, 28) clearly demonstrate the close association between microtubules extending from the blepharoplast surface, and the nuclear envelope within the indentation.

The indentation in the nucleus disappears in subsequent stages, although DAPI staining reveals that the nucleus is no longer quite spherical, one hemisphere having a slightly flattened appearance (Figs 18B, 21B). The significance of the indentation and its relevance to eventual nuclear shape change is unclear from ultrastructural studies. From IIF micrographs, the blepharoplast does not subsequently show any constant association with the flattened side of the nucleus (Fig. 18).

Following the disappearance of the nuclear indentation, cytoplasmic microtubules begin to decrease in numbers (Figs 15–18). The size and staining intensity of the blepharoplast remain relatively constant during this period (Figs 16–18) and in the later stages the remaining microtubules can be clearly seen to radiate from the blepharoplast. In *Marsilea* (Hepler, 1976) microtubules end upon a layer of amorphous material, immediately around the blepharoplast, within a ribosome-free zone. In *Platyzoma* the peri-blepharoplast zone is also ribosome-free but no amorphous layer can be resolved. Microtubules penetrate the ribosome-free zone to
Fig. 28. Blepharoplast in young spermatid lying above the microtubule-lined channel in the nucleus (cf. Fig. 15). ×32000.

Fig. 29. Blepharoplast in young spermatid, showing abundant radiating microtubules. Within the lightly staining channels the central hubs of the procentriolar cores are clearly visible (cf. Fig. 16). ×58000.

Fig. 30. Spermatid mother cell showing the plaque-like structure associated with the blepharoplast. Serial sectioning revealed a second blepharoplast associated with this plaque (cf. Figs 6, 7). ×72000.

Fig. 31. The break up of the blepharoplast, showing a cluster of procentrioles and the nascent multilayered structure (cf. Figs 19, 20). ×45000.
end in close association with the structured portion of the blepharoplast (Figs 27–30, see especially Fig. 29). Another difference at the ultrastructural level is that the central cartwheels within the channels of the blepharoplast of *Platyzoma* are much more clearly defined than those in *Marsilea*.

The cytoplasm of mid-spermatid to mature spermatozoid stages displays high levels of autofluorescence, and to achieve better quality staining with antibodies of these later stages, we have found it necessary to include Nonidet NP40 in the fixative. We have used well-characterized onion root cells as controls and found no significant differences between microtubule arrays fixed in the presence or absence of detergent. The detergent, does not seem, therefore, to perturb microtubules, but enables us to remove substances that contribute to the autofluorescence.

**Blepharoplast fragmentation**

In previous stages the blepharoplast stains homogeneously, but upon the disappearance of cytoplasmic microtubules it appears by through-focussing to be hollow: only the surface stains (Fig. 18). Perfectly spherical blepharoplasts in *Platyzoma* spermatids are rarely observed to be hollow, this characteristic being much more obvious when the blepharoplast becomes oval-shaped (Fig. 19). This is unlike *Pteridium* (unpublished observations), in which surface-stained spherical blepharoplasts are frequent.

The oval-shaped structures increase in length, lying tangential to the nuclear surface (Fig. 19). They correspond to the differentiation of pro-centrioles at the surface of the blepharoplast. The interior of the blepharoplast is devoid of cylindrical channels and is uniformly granular (Duckett, 1975; Bell, 1979, for further details). The staining of the blepharoplast surface with anti-tubulin antibodies coincides with the position of these cylindrical channels.

The relationship between the timing of blepharoplast fragmentation and the disappearance of cytoplasmic microtubules shows some variation from one antheridium to another. Some cells may contain fragmenting blepharoplasts (at a similar or slightly later stage than that shown in Fig. 19) as well as some cytoplasmic microtubules (data not shown). In these cases the microtubules are always poorly preserved (as shown in Fig. 17) and may be in the process of disassembly. Electron microscopy demonstrates the presence of a few microtubules near the cluster of pro-centrioles (Fig. 30).

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Figs 32, 33. The initial association between the nucleus and multilayered structure.

Fig. 32. Transverse section through the spline overlain by granular material in which the centrioles are embedded. ×47 000.

Fig. 33. Tangential section through the nucleus showing the centrioles scattered over the surface of the spline (cf. Fig. 21). ×28 000.

Figs 34–36. Details of the multilayered structure.

Fig. 34. Longitudinal section through the anterior complex showing almost complete occlusion of the lamellar plates. ×45 000.

Fig. 35. Transverse section through the anterior complex. ×55 000.

Fig. 36. Details of the lamellar plates in a young spermatid. ×103 000.
Microtubules during fern spermatogenesis
Figs 37, 38. Flagellogenesis in mid-spermatids (cf. Figs 21, 22).

Fig. 37. The spline encircles about three quarters of the circumference of the nucleus, and the basal bodies are scattered over its surface. ×12,600.

Fig. 38. Section through the lamellar strip. The appearance of stellate transition profiles signals the transformation of centrioles into basal bodies and initiation of axoneme formation. ×45,000.
Fig. 39. Median longitudinal section of a mature spermatozoid. The nucleus, coiled through just over two gyres, is sectioned five times (labelled n1–n5, anterior to posterior), and the anterior complex, sectioned twice, (ac1 and ac2), is connected to the nucleus by the broad ensheathing spline (s1–s5) outside which lie the flagella. The central cytoplasm contains several amyloplasts. X15 000.

Various intermediate stages between the oval blepharoplast (Fig. 19) and the mature spermatozoid are shown in Figs 20–24. The blepharoplast appears to break up into a cluster of brightly staining dots, corresponding in electron micrographs to the appearance of short centrioles lying in an irregular aggregation adjacent to the nascent multilayered structure (MLS) (Fig. 31).

As this deltoid configuration of dots increases in size, the centrioles become individually discernible (Fig. 21). In cells fixed in the presence of detergent, the deltoid configuration (which corresponds to the MLS) is recognizable only by the presence of stained centrioles, but in cells fixed without detergent a more solidly stained strip overlying the nucleus is observed (data not shown).
The centrioles spread out uniformly over the MLS, which lies along the nucleus, the latter begins to change shape with the formation of an anterior beak behind the mitochondrion (Fig. 22). In thin sections the appearance of stellate profiles signals the transformation into basal bodies and the initiation of axoneme formation (Fig. 33). In immunofluorescence images the growing axonemes appear as bristle-like stubs radiating from the cell surface (Fig. 22). It should be noted that the mitochondria never appear to stain with DAPI in any of the later generations of antheridial cells. In earlier generations, lightly stained, poorly defined bodies are formed near the nucleus.

It is evident from Figs 37 and 38 that the spline (comprising 150–160 microtubules in Platyzoma) at this stage encircles about three quarters of the nuclear circumference as a broad band over 3 $\mu$m wide. The lamellar strip, about 10 $\mu$m in length, runs along its anterior edge ahead of the anterior ridge on the nucleus. Neither the spline nor the lamellar strip can be discerned during this stage in the immunofluorescence images of cells fixed in the presence of detergent.

In mid-spermatids, with comma-shaped nuclei, fully formed axonemes about 8 $\mu$m in length can be seen adpressed to the body of the cell in Fig. 23. Directed backwards, the axonemes lie approximately parallel to the axis of the spline tubules and thus at 45° to the long axis of the lamellar strip. This orientation is maintained throughout the later stages in nuclear metamorphosis. In mid-spermatids and mature spermatozoïds, individual cells that are fortuitously orientated with their longitudinal axes perpendicular to the direction of illumination present the best staining patterns. The crescent shape of the DAPI-stained nucleus of the mid-spermatid in such a disposition is shown in Fig. 23. A circumferential line that stains with DAPI separately from the central bulk of the nucleus can also be seen in this micrograph. Anti-tubulin staining of this cell reveals the flagella inserted obliquely around the circumference of the cell (Fig. 23). Through-focussing indicates that these are regularly inserted at the anterior edge of the nucleus. In addition, around the rim of the cell is a hoop of staining almost exactly in register with the circumferential line around the nucleus seen in the DAPI stage.

In mature Platyzoma spermatozoïds, the nucleus is coiled through about two gyres (Fig. 24) with the 50–60 flagella inserted over the spline lying anterior to the nucleus. Since their overall morphology corresponds closely to that already described in detail in Pteridium and Ceratopteris (Duckett, 1975; Duckett & Bell, 1976; Duckett et al. 1979), only those features pertinent to the anti-tubulin staining patterns are discussed here. The forward edge of the spline, circumscribing just under one gyre, is overtopped by a ridge of osmiophilic material, which joins up with the lamellar strip internally and an osmiophilic crest externally (Figs 34, 35). Between the latter and the cell membrane an accessory band of microtubules, about 10 $\mu$m in length, runs parallel to the axis of the lamellar strip (Fig. 34). From a maximum of 24 at the anterior end the number of tubules in this band declines to about 14 or 15 opposite the extreme posterior tip of the lamellar strip. Whereas in young spermatids the plates of the lamellar strip are clearly visible (Fig. 36), in the mature gametes (Fig. 39) these are almost completely occluded.
In contrast to the enhanced staining with anti-tubulin of moss spermatozoids fixed in the presence of Triton X-100 (Miller et al. 1983) similar treatment had no effect on the staining properties of Platyzoma spermatozoids. However, the discovery that this detergent significantly increases the splaying of the axonemes greatly facilitates investigation of possible staining of components within the body of the gametes (Fig. 24). The only non-flagellar staining is a hoop corresponding in position and dimensions to the anterior complex of accessory microtubules, osmiophilic crest and lamellar strip (Figs 34, 35).

**DISCUSSION**

**Staining of the blepharoplast with anti-tubulin antibodies**

The most significant finding to emerge from this investigation is that the blepharoplast - a structure within which microtubules cannot be discerned in electron micrographs - is stained by a monoclonal anti-tubulin antibody. Blepharoplasts of Pteridium (Doonan, unpublished) and Equisetum (Duckett, unpublished) that, ultrastructurally, are virtually identical to those of Platyzoma, show similar cross-reactivity with anti-tubulin. It could well be that this is a property of all archegoniate blepharoplasts.

The present study considerably strengthens the notion that the blepharoplast arises *de novo* during spermatogenesis in archegoniate plants, since neither ultrastructure nor immunocytochemistry reveals any obvious precursor structures, except for a granular body in SMCs that lack a normal blepharoplast (see below).

Developmental ultrastructural studies on the origin of centrioles and their subsequent transformation into basal bodies during flagella formation in land plant spermatogenesis point to a prominent role for the central cartwheel as a template that orders the regular spacing and assembly of the peripheral doublet and triplet microtubules (Duckett & Carothers, 1982). The recent demonstration of the self-assembly of cartwheel complexes in extracts from Tetrahymena basal bodies (Gavin, 1984) supports the notion that these set the basic architecture for the subsequent assembly of microtubules. The cylinders permeating the granular matrix of the blepharoplast appear to be the progenitors of the central cartwheels that later elaborate the peripheral microtubules as the blepharoplasts fragment in the early spermatids. The variations in staining properties of the blepharoplasts observed here suggest that the anti-tubulin is more probably binding to the cartwheels than to the granular matrix. In the spermatid mother cells and during the last antheridial mitosis the blepharoplast is uniformly fluorescent, but subsequent to the disappearance of cytoplasmic microtubules in young spermatids the structure appears hollow, staining brightly at the surface. This change in staining corresponds with ultrastructural data that confirm the re-distribution of cartwheels to a peripheral location. That these changes during late blepharoplast development coincide with re-location of tubulin is further reinforced by the changes occurring within early stages of blepharoplast formation. Initially, the SMCs contain single granular bodies. These 'pre-blepharoplasts' are formed of a granular component but the cylinders have not yet formed. As
single structures they have not been stained with anti-tubulin antibodies. However, in the same cell generation the pre-blepharoplast replicates; the twin body now contains cylinders and at this stage does stain with antibodies. It is therefore highly likely that tubulin, in a non-tubular form, is built into the construction of the cylindrical channels and these act as initiating sites for the precise assembly of the triplets during blepharoplast fragmentation.

The relationship between the blepharoplast and spindle formation

The timing of the appearance of the blepharoplast during spermatogenesis in Marsilea (Hepler, 1976) differs from that of Platyzoma and the comparison provides information on the role of this organelle.

In Marsilea, the blepharoplast arises on two separate occasions: in telophase of the second to last division of the spermatogenous cells and again during telophase of the penultimate division. In Platyzoma, however, it appears only once: just prior to the final division, when it divides and moves to the poles so that each is segregated to a daughter spermatid. Hepler (1976) concluded that the blepharoplast in Marsilea appeared to act as a microtubule organizing centre only during spindle formation in prophase of the final division. He therefore cast doubt upon its role as a MTOC. In Platyzoma the organelle becomes a focus for spindle microtubules only during early anaphase, even though it is present from prophase to metaphase, thereby allowing any possible function in spindle formation to be much more strongly negated. The anaphase spindle has very narrow, focussed poles that are quite unlike the barrel-shaped spindles with broad poles seen in earlier generations or in plant cells, which do not possess centroiles or blepharoplasts (Wick & Duniec, 1984; Clayton & Lloyd, 1984; Doonan et al. 1985). This underlines the point that the blepharoplast does not initiate spindle assembly and does not fulfil an indispensable role in the mitosis, except that when present it is capable of drawing the otherwise diffuse spindle pole organizing material into focus (but see below), much in the way that centroiles behave in other cells. There, it is the amorphous peri-centroileal material that nucleates microtubules (Telzer & Rosenbaum, 1979), which is in agreement with Pickett-Heaps' (1969) view that the amorphous component is the MTOC, whilst the centroile (and here, the blepharoplast) is an independent template for flagella formation during interphase. Separation of chromatin is as successful in barrel-shaped stages (without blepharoplasts) as in spindle-shaped mitoses (with blepharoplasts), from which it seems unlikely that the blepharoplast helps in the proper distribution of the amorphous component or in any other subsidiary function in mitosis. However, the blepharoplast also acts as a focus for microtubules (MTs) in the non-dividing spermatids but in this case the MTs appear to penetrate the ribosome-free halo and to end upon the blepharoplast. The growth habit of Platyzoma is unlike that of Marsilea (Hepler, 1976) and we have been unable to synchronize spermatogenesis in Platyzoma; therefore, we are unable to say from ultrastructural studies whether such a picture also holds for the blepharoplasts when present at the spindle poles. Nevertheless, as we have argued, the position of this
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organelle during early mitosis makes it unlikely that it acts as a MTOC per se at that stage but our ultrastructural evidence (see Fig. 29) still allows the possibility that it may act as a MTOC later, in the spermatid. The cytoplasmic MTs originating from the spermatid's blepharoplast are — in their stellar distribution — similar to the array seen by Brown & Lemmon (1983) in spores of the moss *Tetraphis pellucida* and, like these authors, we suggest that the MTs at this stage are involved in establishing the polarity of a terminally differentiated cell.

We conclude that while the positioning of the blepharoplast relative to the spindle poles rules out its role as a spindle pole organizer (even though it becomes associated with sharpened poles from anaphase onwards), its tight association with interphase MTs in the spermatid and the spermatid mother cell allows for MTOC function during interphase of those stages.

The different staining patterns of the multilayered structure obtained in the presence or absence of detergent deserve comment. In the absence of detergent, the spermatid is highly autofluorescent but a strip lying along the nucleus, corresponding in position to the spline plus overlying centrioles, stains intensely with anti-tubulin. Detergent reduces autofluorescence; the centrioles can now be discerned clearly but the spline stains only faintly. It is possible that the bright strip seen in the absence of detergent represents the 'joined-up' overlying centrioles, not the underlying spline, for this is the staining pattern when detergent is added. However, it seems unlikely that the spline is not composed of tubulin since the tubular appearance of its components and sensitivity to drugs known to depolymerize MTs (Cave & Bell, 1979; Myles & Hepler, 1982) argue against this. It is possible that detergent affects the presentation of antigens to the anti-tubulin antibodies. Such a conclusion is supported by the findings of Cherry & Hsu (1984) on animal spermatids: they showed that optimum staining of the manchette (a layer of aligned MTs running along the nucleus) was incompatible with optimum staining of other tubulin-containing components of the spermatid.

Alternatively, antigenic sites could be blocked by accessory structures. The spline tubules are associated with a layer of dense osmiophiolic material on one side and the nuclear envelope on the other. Mammalian and certain invertebrate sperm axonemes are associated with similar material and, as we find for the spline of fern spermatozoa, show poor cross-reaction to anti-tubulin antibodies (Fawcett & Bedford, 1979; Baccetti et al. 1984). Detergent treatment of the spermatozoid may cause this material to collapse around the spline tubules and physically exclude the antibody.

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