THE POLAR RING OF COCCIDIAN SPOROZOITES: A UNIQUE MICROTUBULE-ORGANIZING CENTRE

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SUMMARY

The infective stages, or 'zoites, of coccidian parasites possess an organized network of spirally arranged microtubules that closely follow the helical body shape of these vermiform cells. These subpellicular microtubules are anchored anteriorly by insertion into a highly structured circular microtubule-organizing centre (MTOC) known as the polar ring.

This MTOC has been examined both in situ and in isolated, critical-point-dried whole cytoskeletons. The 24 microtubules attach laterally to the MTOC through shallow depressions on the inner face of the ring; the ends do not appear to be physically capped. The polar ring has no obvious or regular substructure, although it has a faintly fibrous appearance. The polarity of the microtubules, determined by 'hook decoration', is such that the plus or fast-growing end is distal to the MTOC.

The coccidian 'zoite MTOC is unique both in its highly defined structure and in the degree of organization it confers upon the developing cell in terms of the number, spacing, orientation and polarity of the subpellicular microtubules.

INTRODUCTION

The shape and polarity of eukaryotic cells are controlled by the cytoskeleton, which in many protozoa, such as flagellates, ciliates and sporozoans, is stable and changes only during cell division or differentiation. The accurate formation and organization of this cytoskeleton is obviously paramount to the success of the cell. Microtubules represent a key element of such cytoskeletons, and their organization is controlled, at least at the time of assembly, by microtubule-organizing centres (MTOCs).

MTOCs exhibit a high degree of structural diversity. In Physarum, the nucleating area is a single structure (Roobol, Havercroft & Gull, 1982), whereas in Polytomella the cytoskeletal microtubules originate from a complex or battery of nucleation sites on the flagellar rootlet fibres (Brown, Massalski & Patenaude, 1976; Stearns & Brown, 1981). The majority of MTOCs in protozoan and animal cells that have been described are associated with centrioles or basal bodies, although the axopodia of heliozoans (Cachon & Cachon, 1982; Euteneur & McIntosh, 1981a,b; Jones & Tucker, 1981) are a notable exception to this general rule.

MTOCs also exhibit functional differences, the most obvious being the degree of influence on the resulting microtubule array. The diffuse MTOCs of Echinosporeaum appear merely to seed microtubule assembly, as the number of microtubules is variable, and their direction, spacing and packing appear to be governed by

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a sequestering mechanism that 'zips' the microtubules into their characteristic spiral pattern (Jones & Tucker, 1981). By contrast, the cytopharyngeal baskets of Nassula (Pearson & Tucker, 1977) have template-organizing centres that dictate the direction, polarity, spatial organization and, to some extent, the number of microtubules in the resulting organelle.

A common feature of many MTOCs is that they can nucleate the assembly of microtubules in vitro and this property has been exploited to identify presumptive MTOCs. Kinetic analysis of such assembly studies indicate that the fast-growing end of the microtubule (the plus end) is invariably distal to the MTOC (Bergen & Borisy, 1980; Bergen, Kuriyama & Borisy, 1980). Kirschner (1980) has argued the importance of this polarity by pointing out that cytoplasmic microtubules would be inherently unstable at tubulin concentrations close to the critical concentration, unless the microtubules were capped at their minus end by an anchorage site such as a MTOC. The assembly studies of microtubule polarity have been augmented by the elegant hook-decoration technique of Heidemann & McIntosh (1980) and by in vitro decoration with dynein (Haimo, Teltzer & Rosenbaum, 1979). These studies have shown, with only one major exception, that the plus end is distal to the MTOC in a variety of systems (discussed by Euteneur & McIntosh (1981a) and Heidemann (1980)). The exception concerned the polarity of the microtubules associated with kinetochores, where hook-decoration and in vitro assembly experiments yielded opposite results (Euteneur & McIntosh, 1980a; Bergen et al. 1980; Summers & Kirschner, 1979).

We present here the first description of a novel MTOC that associates with the lateral surface of the microtubule. The MTOC we describe is that of the coccidian Eimeria, which differs from that described in other organisms both in terms of the clarity of the structure and in the specific geometry of the attachment between the MTOC and the microtubules.

As the ultrastructure of Eimeria is not commonly known, we shall first briefly describe the cell and the organization of its cytoskeleton. The life cycle of these parasites involves several metamorphic stages, including the production of motile invasive stages, instrumental in the spread of infection within the host. Ultrastructural examination of these invasive stages, known as sporozoites or merozoites depending upon their position in the life cycle, has revealed a highly conserved structural organization (shown diagrammatically in Fig. 1) common to all members of the Phylum Apicomplexa (Jensen & Edgar, 1976; Porchet-Hennere & Vivier, 1971; Roberts & Hammond, 1970; Sinden, 1978). The motile stages of coccidian parasites, referred to collectively as 'zoites, are small (7–15 μm long), vermiform cells with an extensive subpellicular microtubule cytoskeleton that terminates at the anterior in a polar ring (D’Haese, Mehlhorn & Peters, 1977; Russell & Sinden, 1982). This microtubular cytoskeleton lies immediately beneath a trimembranous pellicle, the innermost layer of which bears linear arrays of intramembranous particles (Dubremetz & Torpier, 1978; Porchet & Torpier, 1977) that follow the paths of the subpellicular microtubules. Within the polar ring lies another microtubular structure known as the conoid. This is a basket-like meshwork of spirally woven microtubules
Fig. 1. A diagrammatic representation of a typical coccidian 'zoite. This cell has a complex pellicle (p) consisting of a plasmalemma (pl) and a double membrane layer with subtending microtubules (mt). The double membrane layer terminates near the anterior at the polar ring (pr) and near the posterior polar ring (pp). These parasites contain secretory bodies called micronemes (m) and rhoptries (r), which emerge anteriorly through the conoid (c) and the preconoidal ring (pc). Also present are refractile bodies (rb), the nucleus (n), mitochondria (mo) and rough endoplasmic reticulum (er).

(D'Haese et al. 1977; Roberts & Hammond, 1970). There is no apparent connection between the conoid and the rest of the subpellicular microtubule cytoskeleton.

The structural conformity of these 'zoites' is reflected in the manner in which they are formed from their stem cells during merogony and sporogony. The 'zoites grow as a bud from the mother stem cell, and Heller & Scholtyseck (1971) demonstrated that budding is preceded by the formation of the conoid and the polar ring. Desser and co-workers (Desser, 1980; Desser & Allison, 1979; Paterson & Desser, 1981; Wong & Desser, 1976) have observed that during 'zoite formation the subpellicular microtubules develop from the polar ring and extend back to an amorphous band that also marks the posterior limit of the double inner membrane layer. Several workers (Heller & Scholtyseck, 1971; Sinden, 1978; Wong & Desser, 1976) report 'arms' or
'bridges' between the subpellicular microtubules and the innermost membrane of immature zoites, although none is visible in fully formed cells.

In this study, the structure of the polar ring and its associated microtubular cytoskeleton were extensively examined in situ and in isolated specimens, and the polarity of the attached microtubules was determined. The structure of the polar ring exhibits features that have not been described in other MTOCs. Our findings support Kirschner's (1980) proposal that one of the functions of the MTOC is to prevent the adventitious assembly of non-nucleated microtubules.

MATERIALS AND METHODS

Cell isolation

Sporozoites of *Eimeria acervulina* and *E. tenella* were kindly supplied as sporulated oocysts, isolated from chicken intestine and stored in 2% potassium dichromate, by Dr Ray Williams (Burroughs-Wellcome, Berkhamsted, Berks.) and Mr John Spelman (May & Baker, Ongar, Essex). The parasites were excysted as required by treatment with 14% (w/v) sodium hypochlorite, agitation with 500 μm glass beads, and incubation at 41 °C with 2-5 mg ml⁻¹ trypsin (Difco) and 5 mg ml⁻¹ bile salts (Difco) in Hanks' buffered saline solution (Davis, 1973). The sporozoites were washed with Hanks' solution, and the cyst debris was removed by sieving the suspension through a 10 μm pore bolt cloth filter.

Ultrastructural methods

Cells were fixed for 45 min in 2.5% glutaraldehyde containing 2.5% tannic acid and buffered with 0.1 M-cacodylate (pH 7.2), post-fixed for 45 min with 1% osmium tetroxide in 0.1 M-cacodylate buffer (pH 7.4), and dehydrated through an acetone series. Samples were embedded in Spurr's resin, sectioned, sequentially stained with uranyl acetate and lead citrate, and examined using a Philips 300 transmission electron microscope at 60–100 kV.

Glycerol extraction

Isolated sporozoites were extracted for 12 h at 4°C with a microtubule/microfilament stabilizing buffer consisting of 50% glycerol, 5 mM-Pipes, 5 mM-EGTA, 2 mM-MgCl₂, 30 mM-KCl (pH 6.9). Before fixation, the glycerol was removed by sequential washing for 15 min in 25% glycerol (3 times), 10% glycerol and 5% glycerol prepared in the stabilization buffer.

Critical-point-dried, detergent-extracted cells

The procedure for preparing whole mount preparations of *Eimeria* sporozoites has been described previously (Russell & Sinden, 1982). Briefly, the sporozoites, in Hanks' medium were allowed to settle on 'holey' Formvar-coated grids, and then treated for 10–20 s at 21°C with 0.1–1.0% Triton X-100 in an extraction buffer containing 50 mM-Pipes, 5 mM-EGTA, 3 mM-MgCl₂, 30 mM-KCl, 2% polyethylene glycol 6000 (pH 6.9). The cytoskeletons were then fixed with 2.5% glutaraldehyde prepared in the extraction buffer, and stained with 1% uranyl acetate in 30 mM-KCl, 2 mM-MgCl₂ (pH 6.9). Finally, the samples were dehydrated through an acetone series, critical-point-dried, and examined by transmission electron microscopy. The presence of the membranous pellicle interferes with the clear visualization of the polar ring and associated microtubules. Clearer images can be obtained by separating the microtubule cytoskeleton from the pellicles by 2–5 passages through a drawn-out Pasteur pipette while the cells are in the detergent extraction buffer and prior to settling on the Formvar-coated grids.

Hook decoration

We have used the procedure described by McIntosh, Euteneur & Neighbours (1980) and Heidemann (1980). Pig brain microtubules were purified through two cycles of assembly–disassembly.
Following a third cycle of assembly, the microtubules were dissociated by cold in a buffer containing
0·5 M-Pipes, 1 mM-EDTA, 1 mM-MgCl₂, 1 mM-GTP (pH 6·9), and were centrifuged for 3 h at
250 000 g at 4 °C to minimize any self-nucleation. The sporozoites were incubated for 20 min at 37 °C
in a solution containing 0·5 M-Pipes, 1 mM-EDTA, 1 mM-MgCl₂, 1 mM-GTP, 5% dimethylsul-
phoxide, 1% Triton X-100, 0·1% deoxycholate, 0·02 sodium dodecyl sulphate and 1–3 mg ml⁻¹
tubulin (pH 6·9). We found that hook decoration did not occur with buffer prepared at pH 6·4, and
that it was important to use pig and not chick brain microtubule protein.

RESULTS

Ultrastructure of the microtubular cytoskeleton

Eimeriine sporozoites have a complex pellicle consisting of a plasmalemma and an
underlying layer of two unit membranes (Fig. 2). The spacing between the plas-
malemma and this double membrane layer is constant (20 ± 2 nm). While the plasma-
lemmma is continuous, the inner membranes are formed of leaflets or ‘plaques’
(Dubremetz & Torpier, 1978; Porchet & Torpier, 1977) and terminate anteriorly at
the polar ring, and posteriorly short of the polar body (see Fig. 1).

The complex pellicle is subtended by the subpellicular microtubules (Fig. 2) that
lie close to the innermost membrane. There are no observable crossbridges linking the
microtubules and the pellicle in these mature cells. Glycerol-extracted sporozoites
stained with tannic acid (Fig. 3) demonstrate that the microtubules have the 'normal'
13 protofilaments, and earlier negative-stain studies have shown the characteristic
8 nm periodicity (Roberts & Hammond, 1970; Russell & Sinden, 1981). The number
of subpellicular microtubules is constant within a species: both E. acervulina and E.
tenella possess 24, but the number varies greatly from genus to genus (see

The three-dimensional organization of the subpellicular microtubules was deter-
mined using detergent-extracted, critical-point-dried sporozoites of E. acervulina
(Fig. 4). The microtubules are firmly anchored at the anterior end to the polar ring,
and then pass through the collar region to extend approximately half the length of the
parasite. Thin-section (Figs 2, 3) and negative-stain studies (Roberts & Hammond,
1970; Russell & Sinden, 1981) show no detectable side projections, and no apparent
terminal caps of anchoring structures at the posterior ends of the microtubules
(Russell & Sinden, 1982). The helical arrangement of the microtubules parallels the
'serpentine' body shape typical of these cells (Fig. 4).

Ultrastructure of nucleation rings, in situ and after isolation

Thin sections through the nucleation ring of glycerol-extracted E. acervulina
sporozoites (Fig. 5) show the 24 subpellicular microtubules of the cytoskeletal basket
are associated with the polar ring. They appear to be held in position by the ring, and
in particular by its inner surface.

The polar ring and its associated microtubules can be isolated by gentle lysis (Fig.
6), although the conoid is invariably lost. Critical-point drying and positive staining
reveals that all the microtubules terminate at the ring, and that the ring has a rigid and
highly defined structure. It has a diameter of 350–400 nm, varies in thickness between
Figs 2–4
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40 and 55 nm, and the spacing between inserted microtubules is 17–25 nm, dimensions that have been observed for both eimeriine species. No regular substructure has been observed, but the polar ring has a fibrous appearance (Figs 5, 6), as though there are many filaments oriented circumferentially.

Although the microtubules clearly terminate at the ring, it is difficult to resolve the precise geometry of their attachment from critical-point-dried specimens. Consequently, both glancing sections through intact cells and negative stained preparations of isolated polar rings have been examined. The glancing section (Fig. 7) shows that the microtubules lie within the inner surface of the ring (open arrow), and as the microtubules terminate at the ring (Fig. 5) the ends of the microtubules must be associated with the ring's inner surface.

However, this does not distinguish between an insertion of the end of the microtubule into the ring, as is commonly found for other MTOCs, and a lateral interaction between the ring and a region close to the microtubule end. The ends of the microtubules are clearly outlined in high-resolution negatively stained isolated cytoskeletons (Fig. 8), indicating a lateral association with the ring rather than an end-on insertion into the MTOC matrix. Furthermore, the microtubules bear no obvious or developed capping structure at their ends (solid arrow). Critical-point-dried isolated MTOCs (Fig. 9) indicate that the points of attachment of the microtubules appear to be small deformations, or hoops, in the wall of the ring into which the microtubules are slotted.

A second, more diffuse ring situated within the polar ring is observed in some preparations (Fig. 9). Similar structures have been found in negative-stained preparations (D'Haese et al. 1977) and have been suggested as representing the remains of the anterior limit of the inner membrane complex.

Hook decoration of microtubule cytoskeletons in situ

The hook-decoration technique of Heidemann & McIntosh (1980) for determining

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Fig. 2. A thin section from a sporozoite of *E. tenella*. The microtubules (arrows) and the complex multilamellar pellicle are clearly visible. The dense spherical bodies lying in the cytoplasm are micronemes. ×164 000.

Fig. 3. A high-resolution electron micrograph of a sub-pellicular microtubule in a glycerol-extracted, tannic acid-stained sporozoite of *E. tenella*. The 13 protofilaments of the microtubule are clearly revealed. ×383 000.

Fig. 4. A sporozoite of *E. tenella* treated in 0.5 % Triton X-100 in extraction buffer. The cytoplasmic contents have been 'drained' from this cell. The procedure exposes the conoid (c), the collar region (co) and the micropores. The subpellicular microtubules are closely associated with the pellicle, describe a helical path, and extend approximately half the length of the sporozoite. ×19 000.

Fig. 5. A thin section from the anterior of the sporozoite of *E. acervulina* extracted in glycerol and fixed in glutaraldehyde/tannic acid. The 24 subpellicular microtubules insert into the inner face of the polar ring, which has a vaguely fibrous appearance. ×215 000.

Fig. 6. An isolated, critical-point dried polar ring and associated microtubular cytoskeleton from a sporozoite of *E. acervulina*. Most of the microtubules remain attached to the polar ring. ×119 000.
Figs 5 and 6. For legend see p. 199.
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Fig. 7. A glancing section through the anterior region of an intact sporozoite of *E. tenella*. The sub-pellicular microtubules (solid arrow) lie in contact with the inner surface of the polar ring (open arrow). ×129 000.

Fig. 8. A negative stained isolated cytoskeleton of *E. acervulina* showing the association of the microtubules with the inner surface of the polar ring. The ends of the microtubules are clearly outlined (arrows) against the background of the polar ring, suggesting a lateral interaction between the microtubules and the polar ring. No structure is evident that caps the ends of the microtubules. ×206 000.
the microtubule polarity was employed on the cytoskeleton of eimerine sporozoites. The electron micrographs illustrated (Fig. 10) were taken of sections from the same organism. Great care was taken to ensure that the correct orientation of these sections was maintained throughout processing and examination. In the anterior, or collar, region of the sporozoite (Fig. 10a) much of the internal structure has been removed by the detergent treatment, though some amylopectin granules persist. The microtubules still show a close association with the pellicle. In the nuclear region of the cell (Fig. 10b) this association has apparently broken down. The microtubules in both sections show hook-like addition of brain tubulin. These hooks have an anticlockwise rotation when viewed from the anterior of the organism. According to the rules of Heidemann & McIntosh this demonstrates that the plus or fast-growing ends of the subpellicular microtubules are situated posteriorly, distal to the polar ring.

**DISCUSSION**

The polar ring of eimerine sporozoites is a clearly defined, highly organized structure (Figs 5–9), which lacks the amorphous granularity commonly associated with the MTOCs of other cells. It is the most highly organized MTOC yet described, with the notable exception of the basal body, even though its detailed substructure is ill-defined (Figs 5, 9). The capacity of this polar ring to organize the sub-pellicular microtubules is highly developed, as it appears to control the number, spacing, directionality and orientation of the assembled microtubules. Furthermore, unlike the MTOCs of many cytoplasmic microtubule systems, such as *Physarum* (Roobol et al. 1982) and *Polytomella* (Brown et al. 1976), it lacks, at least in the mature sporozoite, any connection to basal bodies or centrioles.

The linkage between the microtubules and the polar ring is also unusual. Here, the ends of the sub-pellicular microtubules lie inside the polar ring and are laterally attached to the MTOC through shallow depressions (Figs 8, 9). Thin-sectioned and negatively stained material indicates that the ends of the microtubules lack any apparent terminal cap (Figs 7, 8). This is in striking contrast to previously described MTOCs in which the microtubules insert into the MTOC in such a way that the end of the microtubule appears physically capped. This peculiar lateral association of the microtubules with the polar ring therefore presents a unique test for Kirschner's (1980) model of the relationship between microtubule polarity and stability.

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**Fig. 9.** A high-resolution electron micrograph of an isolated polar ring of *E. acervulina*. The microtubule attachment sites appear as slight depressions in the matrix of the ring. The remains of another, less-structured ring (arrow) lie within the polar ring. ×183 000.

**Fig. 10.** a. A thin section through the collar region of a sporozoite of *E. acervulina*, fixed following incubation in hook-decoration buffer. Microtubules and the amylopectin granules (a) have survived the treatment. ×59 500. b. A section taken posterior to a from the same cell. ×53 000. Both micrographs reveal subpellicular microtubules, many of which show 'hook-like' addition of brain tubulin. All hooks observed had an anticlockwise rotation when viewed from the anterior end of the cell, suggesting that the plus end of these microtubules is at the posterior end of the sporozoite.
The sub-pellicular microtubules are stable, yet we find that the microtubules share a common polarity with the plus end, as predicted by Kirschner's model, distal to the MTOC (Fig. 10). As the microtubules do not extend beyond the polar ring (Figs 6, 9), this suggests that the minus end may be capped by a mechanism that either is undetectable at the ultrastructural level or has been selectively lost during glycerol extraction. Neither thin sectioning of intact cells nor negative staining of isolated cytoskeletons reveals any developed capping structure. This does not, however, eliminate the possible presence of, for instance, a specific capping protein such as the 64 000 Mr protein proposed as the cause of the cold-stability of some brain microtubules (Job, Rauch, Fischer & Margolis, 1982).

Of course, from the determination of microtubule polarity by hook decoration in vitro we cannot ascertain with ultimate certainty from which end(s) microtubule assembly occurs in vivo. Unfortunately, the development of the sporozoite occurs within the host tissue and subsequently within a protective cyst, so preventing the use of labelled precursors to determine directly the site of assembly in vivo. However, the ultrastructural organization during development imposes considerable restraints on the two possible models; namely, assembly of the sub-pellicular microtubules at the minus (MTOC) end or from the free plus end.

The development of the sporozoite from the sporoblast is initiated by mitosis and the migration of a daughter nucleus to the plasmalemma. The polar ring forms at this site, and the sporozoite develops as an outgrowth of the sporoblast (Dubremetz, 1973; Paterson & Desser, 1981; Wong & Desser, 1976). Consequently, as the sporozoite is extruded, the polar ring (and minus end of the sub-pellicular microtubules) is distal to the sporoblast and at the anterior end of the sporozoite, while the boundary between the sporozoite and sporoblast is proximal and contains the plus ends of the microtubules.

This extrusion is accompanied by the assembly of the trimembranous pellicle and underlying microtubule cytoskeleton. However, throughout the formation of the sporozoite, the sub-pellicular microtubules and the double inner membrane complex always terminate opposite the fold in the sporoblast membrane (Paterson & Desser, 1981; Sinden, 1978; Wong & Desser, 1976). The crosslinks that have been observed between the innermost membrane and the subtending microtubules of developing zoites (Heller & Scholtyseck, 1971; Sinden, 1978; Wong & Desser, 1976), together with the linear arrays of intramembraneous particles that run parallel to the microtubules, strongly suggest that the two structures are connected, and that their assembly is coordinated. Furthermore, a band of amorphous osmiophilic material (Sinden, 1978; Wong & Desser, 1976) is present throughout sporozoite development and is located in close proximity to the plus ends of the sub-pellicular microtubules and the growing inner membrane complex. Consequently, the boundary of the 'extruding' sporozoite and the sporoblast is marked during development by the proximal end of the inner membrane complex and by this amorphous band. Significantly, in the mature sporozoite the inner membrane complex, which lies beneath the plasmalemma (Fig. 1), extends beyond the ends of the sub-pellicular microtubules and the amorphous osmiophilic band is absent, implying that it is utilized only during development.
This developmental sequence suggests that both the microtubules and the membrane complex, assuming they are not constantly sliding past each other, are both assembled at either the anterior (− end) or posterior (+ end) of the sporozoite. However, the continuation of membrane growth after the cessation of microtubule assembly, indicates that growth must be occurring at the posterior (free +) end rather than in the region of the polar ring.

If, however, assembly were occurring at the polar ring (− end) then the amorphous osmiophilic band, which is coincident with the free plus end, would have to migrate or be pushed along the sporozoite at a rate equal to that of the assembly of both the membrane and the microtubules, only to be lost on maturation. In contrast, if assembly is at the plus end this band may represent microtubule/membrane precursors. Assembly at the kinetically unfavourable minus end would, furthermore, necessitate that the plus end was capped. No such capping structure is evident apart from the amorphous band, yet such a capping structure would need to be retained even after maturation of the sporozoite, while the amorphous band is lost on differentiation.

A final objection to the assembly of microtubules in vitro at the minus end is that this would require the growing microtubules to slide past the polar ring.

We therefore conclude, in the absence of direct kinetic observations, that the subpellicular microtubules are assembled at their free plus ends, and that the polar ring effectively caps the microtubules. The in vitro determined polarity, and probably also the in vivo assembly, is therefore consistent with Kirschner’s (1980) model, despite the unorthodox structure of the MTOC.

The eimeriine polar ring may provide a unique system for determining the structural and biochemical organization of a MTOC, since it can be viewed at high resolution (Figs 8, 9) and the cytoskeleton complete with the polar ring can be readily isolated (Fig. 6). Furthermore, as different coccidian species contain various, but constant, numbers of sub-pellicular microtubules, the polar ring may prove to be invaluable in determining the molecular control of microtubule number and their spacing.

Dr Russell is grateful to the Wellcome Trust who funded much of this research, and for the support and encouragement of Dr R. E. Sinden. The authors also acknowledge useful discussions with Drs S. S. Desser and J.-F. Dubremet.

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(Received 13 May 1983–Accepted 1 August 1983)