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Freeze-fracture studies on the surface membranes of pleomorphic bloodstream and in vitro transformed procyclic *Trypanosoma brucei*

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Summary

The surface membranes of bloodstream long slender, short stumpy and culture procyclic stages of *Trypanosoma brucei brucei* were compared with respect to freeze-fracture electron microscopy, intramembrane particle (IMP) distribution and β-hydroxysterol content as shown by the characteristic intramembrane lesions induced by the polyene antibiotic, filipin. Little difference was observed between IMP density of long slender and short stumpy form body membranes: IMP's were more abundant on the protoplasmic face (PF) than on the exoplasmic face (EF). The procyclic culture form body membrane showed an increased density of PF IMPs and a decreased density of EF IMPs over their bloodstream short stumpy form predecessors. Flagellar membrane fracture faces displayed higher IMP densities than body membrane fracture faces of the same trypanosome. The numbers of filipin-induced lesions (FIL) indicated an increased level of β-hydroxysterols in the short stumpy forms relative to the level in the long slender bloodforms. FIL density was further increased in the body membrane of the procyclic culture form. FIL density was higher in the flagellar membrane than in the corresponding body membrane and FIL were excluded from flagellum to body attachment zones of the flagellar membrane of all stages. The polarity of the FIL in the surface membranes was reversed on transforming from bloodstream to culture procyclic stages. These observations indicate qualitative differences between the surface membranes of the three stages, independent of the presence or absence of the surface coat.

Key words: freeze-fracture electron microscopy; surface membranes; sterol cytochemistry; *Trypanosoma brucei.*

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Introduction

Changes in the character of the cell surface of the African (salivarian) trypanosomes during the life cycle have been revealed by lectin binding, complement activation and measurements of surface charge, as well as by conventional ultrastructural studies on sections of trypanosomes (Vickerman, 1985). The most striking changes accompany loss and reacquisition of the surface or variable antigen coat (Vickerman, 1969; Steiger, 1973). This coat consists of a monomolecular layer of glycoprotein; sequential replacement of one glycoprotein with others of different antigenic specificity is responsible for antigenic variation and thus evasion of the host's immune response by the parasite (reviewed Vickerman, 1978; Borst and Cross, 1982; Boothroyd, 1985). In *Trypanosoma brucei* the coat is present in all stages in the mammalian host, but is lost on entering the tsetse fly vector, and reacquired during differentiation to the metacyclic stage.

The focus of attention in recent years has been on molecular characterisation of the variable antigen coat (reviewed Cross, 1984). Less attention has been paid to the structure of the membrane itself; some information on its composition is available for the slender (monomorphic) bloodstream form only (Rovis and Baekkeskov, 1980; Voorheis et al., 1979). The freeze-fracture replica technique allows comparison of the internal structure of membranes. A detailed study of the membrane changes occurring during acquisition of the coat as seen in freeze-fracture replicas has already been published (Tetley and Vickerman, 1985). The present observations are of the intra-membrane differences between multiplicative long slender trypomastigotes and non-dividing short stumpy trypomastigotes from the mammalian host, and between these stages and the uncoated procyclic trypomastigotes which arise from them when they are cultivated in vitro at 26 °C.

Materials and Methods

Parasites. Slender and stumpy form bloodstream *Trypanosoma brucei brucei* of the AnTaR1 serodeme (AnTat 1.8 and 1.9 cloned stock EATRO 1125) were grown in female CFLP mice previously given 600 rads whole body irradiation to increase trypanosome yields (Luckins, 1972). All bloodstream forms were separated from blood by anion exchange chromatography on DEAE celluaose columns (Lanham, 1968) and harvested by centrifugation at 1000 *g* for 10 min in phosphate saline glucose.

Slender blood forms were obtained from ether-anaesthetised mice in blood withdrawn by cardiac puncture just prior to peak parasitaemia ($\sim 10^8$/ml). Thirty-six hours later when >90% of parasites in the remaining mice were of stumpy morphology, trypanosomes were recovered after similarly sacrificing the animals.

T. brucei procyclics, derived from AnTat 1.8 stumpy bloodforms, by transformation in vitro were maintained by serial culture in Cunningham's medium (Cunningham, 1977) at 26 °C and harvested in log phase by centrifugation for 10 min at 1000 *g*. Filipin treatments were performed on culture procyclics which had been twice-weekly sub-cultured in Cunningham's medium for at least one month after initial transformation.

Filipin treaments. Filipin solution in DMSO (dimethylsulphoxide) was added to 0.1 M cacodylate buffer, pH 7.4 to give a final filipin concentration of 50 μg/ml (final DMSO concentration 0.5% v/v). Fixed parasites were pelleted, then resuspended in filipin solution to a concentration of 10^6/ml and incubated in the dark at 20°C for 4–6 h. Parasites were then washed in 0.1 M cacodylate buffer, infiltrated overnight at 4°C with 25% glycerol in the same buffer and freeze-fractured.

Electron microscopy. Pelleted parasites were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer, pH 7.4 at 20°C. Half the pellet was taken for freeze-fracture electron microscopy and the remainder processed essentially as detailed previously (Brown et al., 1973) for embedding and sectioning.

Freeze-fracture electron microscopy. Fixed, centrifuged parasites in glycerol buffer were sandwiched between two Balzer's copper support plates and rapidly cooled to −180°C by plunging into liquified propane. The specimens were transferred to a complementary replica device under liquid nitrogen and subsequently fractured in a vacuum of 2×10^{-6} torr. at −100°C. Replicas were obtained of the exposed fracture faces immediately by unidirectional platinum-carbon shadowing at 45° followed by carbon evaporation at 90°. The replicas were then retrieved by thawing into glycerol buffer, washing in distilled water and cleaning with 40% chromic acid overnight at 20°C. Further repeated washings in distilled water preceded collection of replicas on 300 mesh grids and observation in an AEI 801 transmission electron microscope operating at 60 Kv.

Counting of intramembrane particles (IMPs) and filipin-induced lesions. IMPs and lesions were counted from prints at a final magnification of 100,000×. Counts made on micrographs were of regions evenly shadowed and a graphics digitising tablet was employed to obtain areas under irregular perimeter profiles. Filipin-induced lesions were identified and counted as 25–30 nm pits or protrusions.

Results

Intramembane particles. Examination of the inner, protoplasmic face (PF) and outer, exoplasmic face (EF) fractures of the surface membranes of freeze-fractured trypanosomes showed a greater density of IMPs on the PF compared with the EF in all forms studied. Figures for the IMP densities in the body membranes of the three forms studied are given in Table 1.

There was little difference between the IMP densities on corresponding halves of the plasma membrane of slender and stumpy parasites. However, in culture forms a marked change in IMP distribution between the two membrane

Table 1. Intramembrane particle (IMP) and filipin-induced lesion counts (FIL) on *T. brucei* fracture faces represented as the mean number per square micron±2 S.E., taken from the number of measurements given in parenthesis

T. brucei stage	IMP/μm^2		FIL/μm^2	
	Body membrane		Body membrane	Flagellar membrane
	Protoplasmic face	Exoplasmic face		
Bloodstream long slender	2353±150 (6)	600±57 (6)	5±2 (12)	36±13 (10)
Bloodstream short stumpy	2314±184 (6)	536±52 (5)	104±22 (17)	184±39 (13)
Culture procyclic	3332±213 (4)	241±12 (4)	162±52 (13)	258±46 (14)

halves became apparent; a decrease in EF IMP numbers was accompanied by an increase in PF IMP numbers (Figs. 1 and 2). This was paralleled by a loss of surface coat revealed by transmission electron microscopy of sectioned pellets (not illustrated).

A constant feature in all forms where a trypanosome's flagellum and body membrane fractured together was the higher IMP density on the body membrane than on the flagellar fracture faces (see Figs. 3–6). Clusters of IMPs corresponding to the macular attachment sites of the trypanosome's flagellum/body junction (Figs. 3 and 6) were seen on the PF of the flagellar membrane in all forms.

The number of filipin-induced lesions per square micron observed in the body and flagellar membranes of long slender blood forms was markedly fewer than in corresponding surface membrane regions of short stumpy forms (Figs. 3 and 4, and Table 1). An increase in lesion density was also seen after transformation of short stumpy forms into procyclic culture forms (Table 1).

Filipin-induced lesions appeared as pits on the PF and protuberances on the EF of both the body and flagellar surface membranes of short stumpy and long slender blood forms (Figs. 3 and 4). However, the reverse was seen for the corresponding procyclic surface membrane fracture faces: protuberances occurred on the PF and pits were found on the EF (Figs. 5 and 6).

Filipin-induced lesions were always at a higher density on the flagellar membrane fracture face compared with the adjacent body membrane fracture of the same trypanosome in all forms. The lesions were excluded from the IMP clusters of the flagellum to body macular attachment zones in the flagellar membrane PF (Figs. 3 and 6).

Discussion

In freeze-fracture replicas the short stumpy form body membrane appears little different from that of the long slender form with respect to IMP numbers. This could be explained on the basis that coated forms have similar surface membrane enzymes and transport sites necessary for physiological function in the blood environment and that alteration of the surface membrane protein composition may be unnecessary. In addition the architecture of the surface membrane may be constrained by the need to support the glycoprotein coat.

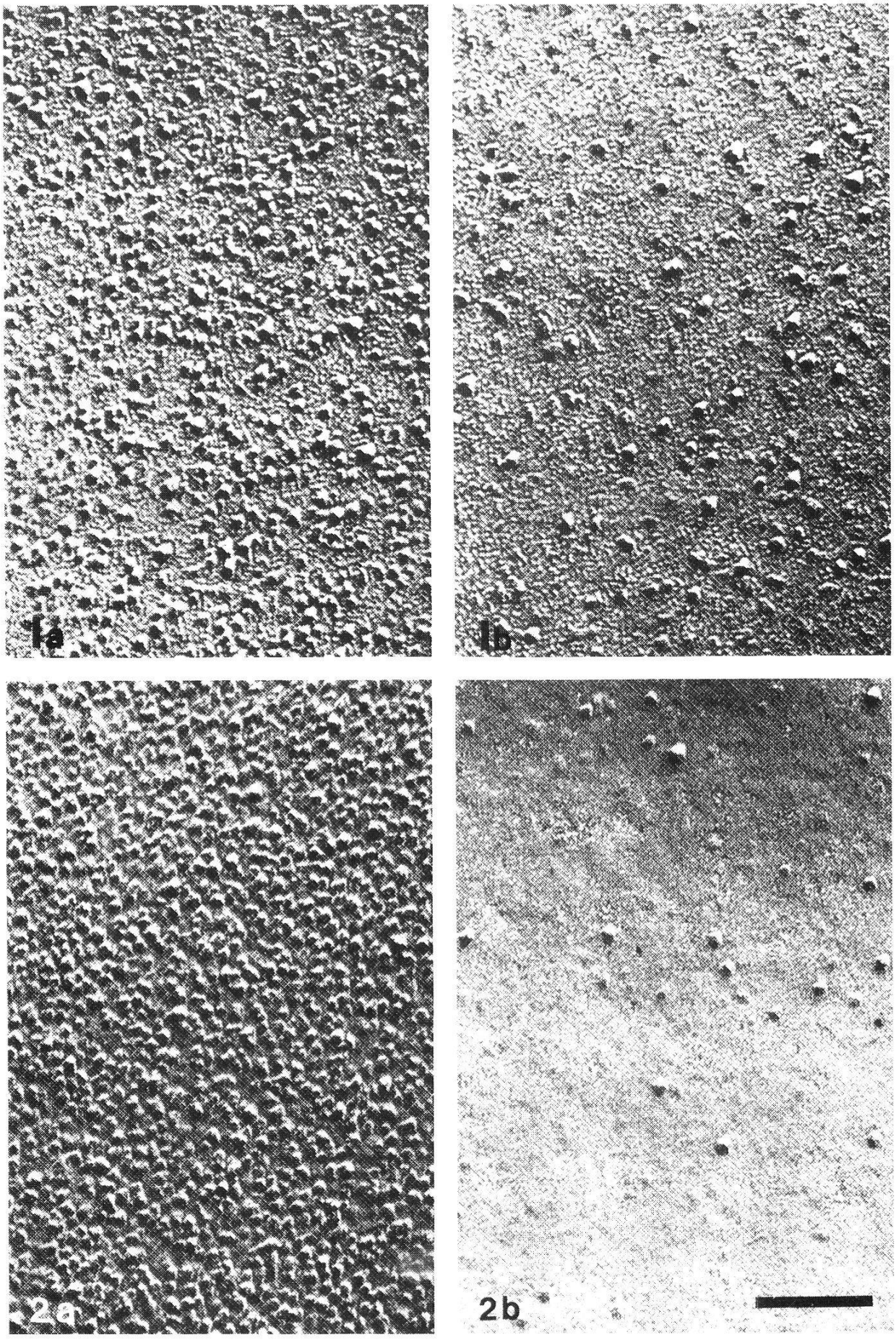
Figs. 1–6. Pt/C shadow direction is from the bottom to the top of all micrographs.

Abbreviations: PF_{fl} = flagellar membrane PF (protoplasmic face), EF_{fl} = flagellar membrane EF (exoplasmic face), PF_{bm} = body membrane PF, EF_{bm} = body membrane EF. Arrows = filipin-induced lesion (protuberance), Arrowheads = filipin-induced lesion (pit), IMP = intramembrane particle, fl = flagellum, fp = flagellar pocket, SC = surface coat.

Figs. 1 and 2. Fracture faces of the body membrane of *T. brucei* stages to show IMP distributions. Scale bar = 100 nm.

Fig. 1. Short stumpy blood form. a) PF, b) EF.

Fig. 2. Procyclic culture form. a) PF, b) EF.


1a
1b
2a
2b

Changes in body membrane ultrastructure accompanying transformation may reflect the differing requirements of respiratory substrate of bloodstream and procyclic forms. The necessity for replacement of transport sites and receptors during loss of the surface coat glycoprotein seems likely. Interestingly, as seen from Table 1, the PF IMP densities increase during progression from stumpy to procyclic form, whereas the EF IMP densities decrease.

There is, however, no evidence for EF IMPs being quantitatively related to the variant surface glycoprotein (VSG) molecules comprising the surface coat. The latter have a far higher packing density on the surface than is represented by particles in the membrane (600 IMPs/μm^2 compared with 60,750 VSG molecules/μm^2; Jackson et al., 1985). Only in the relatively simple systems such as the erythrocyte or bacterial membranes has analysis of specific IMP function been possible (Pinto da Silva and Nicolson, 1974; Verkleij and Vervegaert, 1978). It appears likely that IMPs are the morphological representations of intercalated globular proteins (possibly associated with lipids) within the membrane.

Freeze-fracture cytochemistry using the polyene antibiotic, filipin, shows the distribution of β-hydroxysterols through the morphologically identifiable 25 nm lesions produced where filipin and sterols interact within the membrane. The specificity, mode of action and use of filipin as a tool in determining membrane cholesterol have been evaluated (Norman et al., 1976; Robinson and Karnowsky, 1980; Friend and Bearer, 1981) and recently reviewed (Severs and Robenek, 1983; Miller, 1984). The marked increase in filipin-induced lesions during the slender-stumpy transformation contrasts with the lack of change in IMP density and indicates a marked increase in cholesterol content.

The general effect of increasing cholesterol in membranes is to decrease the fluidity and permeability thus resulting in a more rigid membrane structure. A cholesterol:phospholipid ratio of around 0.5 has been shown for monomorphic (long slender) *T. brucei* (Rovis and Baekkeskov, 1980). This ratio falls within the lowest end of the range observed for mammalian cells (0.49–1.04; Emmelot, 1977). Rovis and Baekkeskov (1980) predicted that stumpy forms might need a higher cholesterol:phospholipid ratio for entering the invertebrate host in order to prevent the phospholipid gel to liquid-crystal transition effect at the lower temperature encountered in the fly.

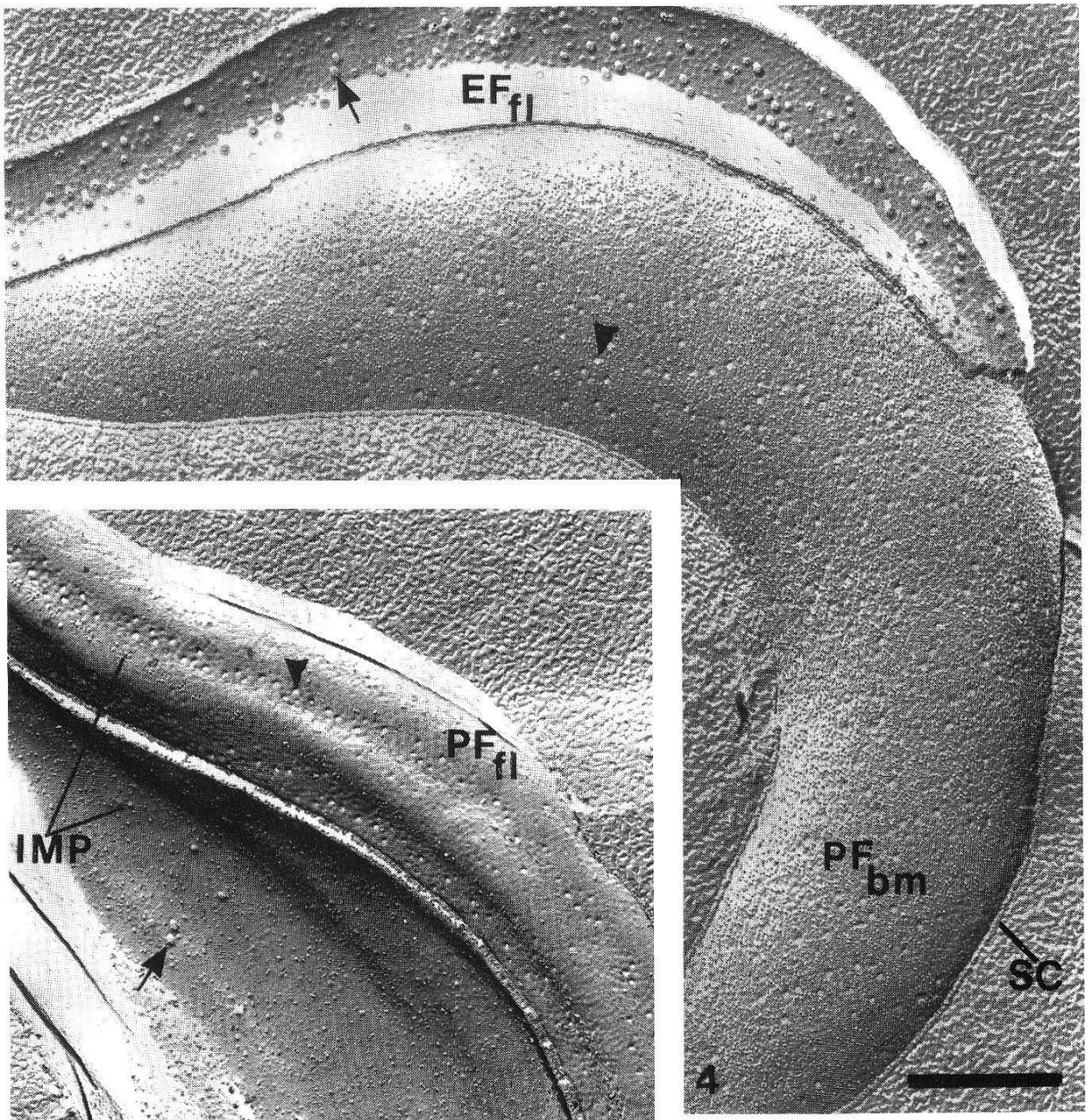
The increase in cholesterol content of stumpy *T. brucei* surface membranes may explain the ability of this stage to resist lysis for longer than long slender trypanosomes during the Blood Incubation Infectivity Test (BIIT) (Rifkin, 1978; Rickman and Robson, 1970).

A possible consequence of the higher surface membrane cholesterol levels in stumpy forms is reduced motility as this depends upon deformation of the body membrane by the beating flagellum to form the undulating membrane.

Figs. 3 and 4. Body and flagellar membrane fracture faces of blood forms of *T. brucei* showing filipin-induced lesions. Scale bar = 0.5 μm.

Fig. 3. Long slender form.

Fig. 4. Short stumpy form.


EF fl
PF fl
IMP
EF bm
3
PF bm
SC
4

Movement is more rapid in long slender forms perhaps because they have a less rigid body membrane due to decreased cholesterol content.

The increased density of filipin-induced lesions in the body membrane of culture procyclics over their stumpy progenitors may reflect a change in the sterol present or a change in permeability to filipin. Procyclic trypanosomes are likely to contain ergosterol rather than cholesterol in their membranes (Dixon et al., 1972), but since both are β-hydroxysterols, both react with filipin. Ergosterol is synthesized only by culture forms when cholesterol is limited and may be required for specialised functions for which cholesterol cannot substitute. Mammalian blood forms have no synthetic capacity for sterols, and rely on uptake of cholesterol from the abundant supply in the host circulation (Dixon et al., 1972; Venkatesan and Ormerod, 1976).

There is no potentially restrictive barrier to filipin penetration at the surface membranes of the procyclic as exists for the blood stages in the form of the 15 nm glycoprotein layer (Fig. 4) cross-linked by glutaraldehyde prior to incubation. This barrier might influence lesion density through steric hindrance of filipin-sterol interaction by limiting diffusion of the antibiotic across the fixed, coated surface of the blood forms. The low molecular weight of filipin (566 daltons), however, suggests that its penetration of the surface coat should not present a problem.

The increase in numbers of filipin-induced lesions in the flagellum over that in the body membrane of the trypanosome (Table 1) indicates a functionally less fluid membrane region which may be significant in the action of the beating flagellum. Flagellum to body attachments which appear as clusters of IMPs on the flagellar PF (Smith et al., 1974; Hogan and Patton, 1976; Vickerman and Tetley, 1979) (Fig. 6) are apparently insensitive to filipin in common with other intramembrane particle arrays studied so far (Severs and Robenek, 1983). During the attached stages of development of *T. brucei* in the salivary glands of the tsetse fly, flagellar attachment plaques to the host microvilli have also been shown to be devoid of filipin induced lesions (Tetley and Vickerman, 1985), so it appears that sterols are excluded from these regions also.

The tendency for lesions to occur mainly as pits and correspondingly as protuberances in the PF and EF respectively in blood forms (and conversely in procyclics) argues for an unequal partitioning of sterol according to current interpretations of the molecular interaction of filipin with membranes (Severs and Robenek, 1983; Miller, 1984). Sterol-filipin binding initiates deformation in one half of the bilayer due to re-orientation of sterol from a horizontal to a vertical position in the plane of the membrane (Severs et al., 1981). Consequent increase in surface pressure results in a bulge (lesion) towards the monolayer of

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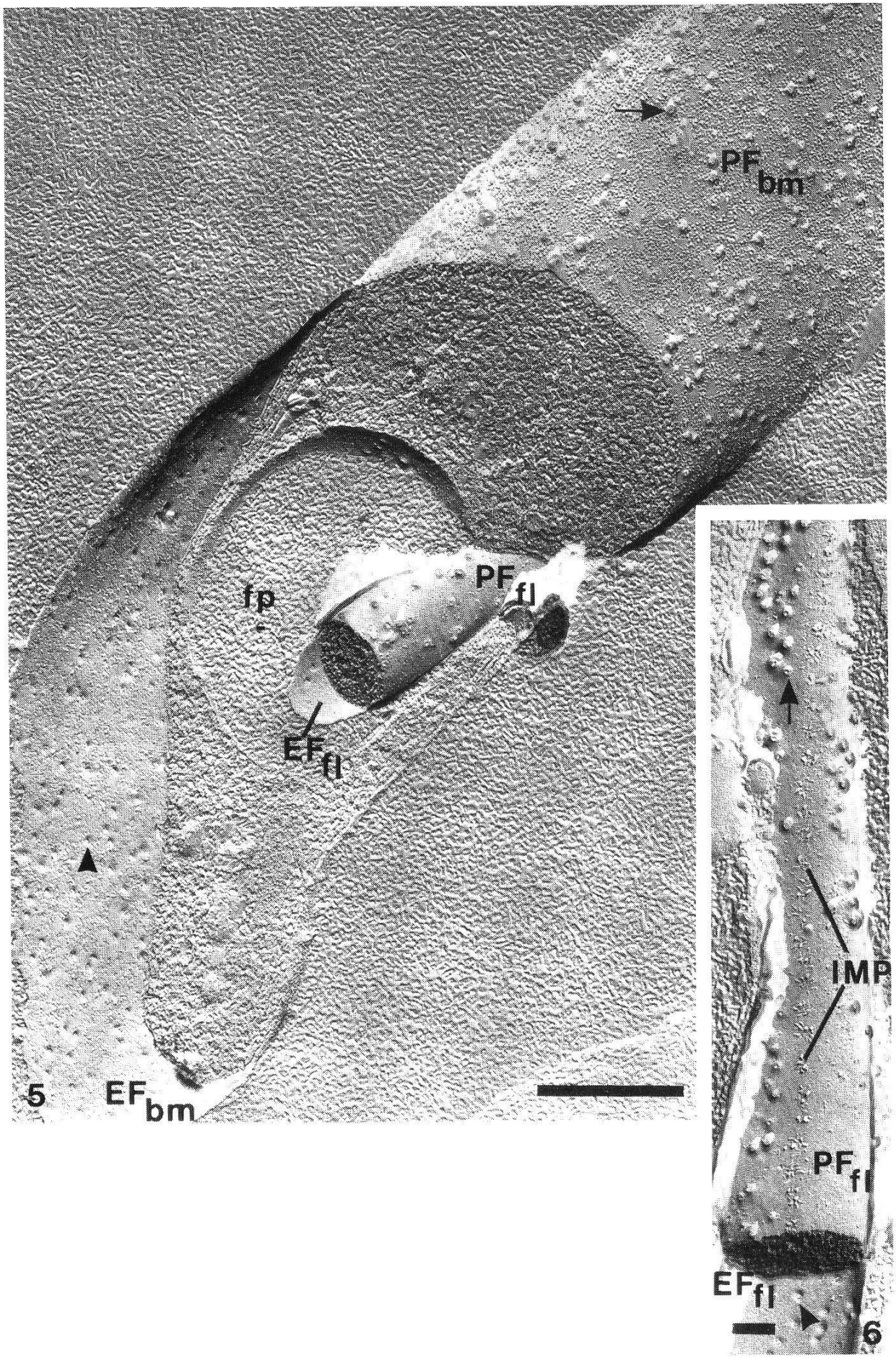
Figs. 5 and 6. Body and flagellar fracture faces of procyclic *T. brucei* showing filipin-induced lesions.

Fig. 5. PF and EF of surface membranes. Scale bar = 0.5 μm.

Fig. 6. Flagellar membrane showing absence of filipin-induced lesions from PF IMP clusters at flagellar attachment zone. Scale bar = 100 nm.


PF bm
fp
PF fl
EF fl
5
EF bm
IMP
PF fl
EF fl
6

origin of the filipin-sterol complex. The monolayer exhibiting most pits would thus seem to represent the membrane half with the highest sterol content. This model is complicated by the possibility of "flip-flop" of sterol from one membrane half to the other (Miller, 1984) and also by the effects of fixation on filipin-sterol complex symmetry: fixation can reverse the polarity seen in unfixed preparations (Robinson and Karnowsky, 1980).

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