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TRYPANOSOMA EVANSI: UNIQUE CONCAVITIES ON THE SURFACE MEMBRANE OF PARAROSANILINE-INDUCED AKINETOPLASTIC CLONES AS REVEALED BY SCANNING ELECTRON MICROSCOPY

MARY ROSE AGNES SILVA-TAHAT*, AKITOYO ICHINOSE, HARUKI UEMURA AND HIROJI KANBARA

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Abstract: Pararosaniline-induced akinetoplastic clones of *Trypanosoma evansi* which lack DAPI-stainable kDNA network were characterized by scanning electron microscopy. Independent batches of akinetoplastic parasites from two passages in mice were observed to have similarities with parental kinetoplastic strain with respect to shape, form, and pleomorphism. The marked difference in surface topography was noted between the wildtype and the mutant as unique concavities on the cell surface of the latter. These concavities are variable in size, number and extent and may be inheritable. In addition, akinetoplastic cells were found to undergo active longitudinal binary fission and filopodia formation as reported by others. These observations suggest that the kDNA-deficient mutants of *T. evansi* have maintained their basic functions of cell division and infectivity and, therefore, the concavities on their surface are not detrimental to their existence.

Keywords: Scanning electron microscopy, *Trypanosoma evansi,* akinetoplastic form, surface concavity

INTRODUCTION

Trypanosoma evansi is a haemoflagellate which causes a wasting disease called surra (also as desrengadera or murrina in local language) in domestic animals particularly camels, horses and cattle. It has a wide geographical distribution ranging from North Africa, Asia and South America. Although it is most closely related to *Trypanosoma brucei,* it has deviated to a very simplified life cycle without invertebrate-related stages and, hence, morphology, as an adaptation to a mechanical mode of transmission by horseflies and other biting insects (Hoare, 1967, 1972).

However, like all other members of the order Kinetoplastida, *T. evansi* possesses a kinetoplast DNA (kDNA) albeit its reduced size which reflects the lack of respiratory processes in the mitochondrion (Borst and Hoeijmakers, 1979). In lieu of this feature, this trypanosome is susceptible to mutate spontaneously into forms with altered kDNA, the so-called dyskinetoplastic and akinetoplastic forms (Hoare, 1954). Spontaneous mutants account for about 3-6% of a given population (Inoki *et al.*, 1962). These mutants are morphologically similar to the wildtype except for the marked difference revealed by transmission electron microscopy as the replacement of the kDNA network by an electron-dense body referred to as the kinetoplast remnant (Vickerman and Preston, 1976). In addition, although the fundamental functions of the akinetoplastic cells are not much different from the kinetoplastids with respect to viability, infectivity, and ability to proliferate, we have previously observed that the akinetoplastic parasites have a delayed rate of cell division and, therefore, a slower growth (silva-Tahat et al., Res., in press).

In the present paper, we further characterize pararosaniline-induced akinetoplastic clones of *T. evansi* by scanning electron microscopy and describe their morphological features. We show that only the mutants have unique surface concavities which may be inheritable and further demonstrate that they undergo active longitudinal binary fission and filopodia formation indicating that the concavities are not harmful for the parasites.

Department of Protozoology, Institute of Tropical Medicine, Nagasaki University, Japan 852
*To whom all correspondence should be addressed.

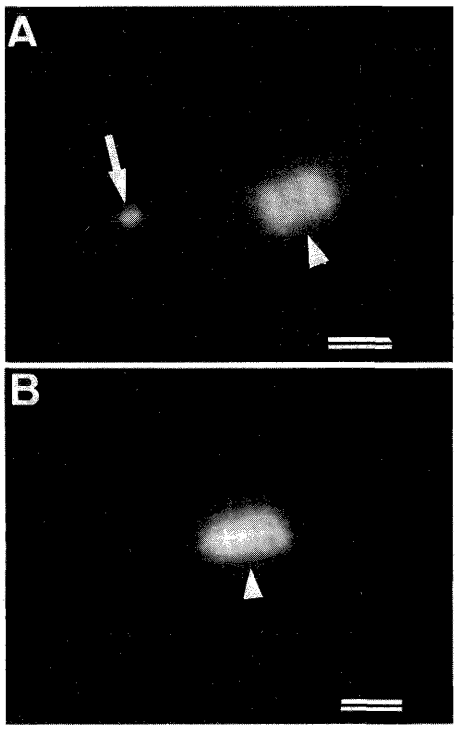

Figure 1 DAPI fluorescence micrographs. (A) Kinetoplastid with 2 fluorescent particles, a large nuclear DNA (arrowhead) and a small kDNA (arrow). (B) An akinetoplastic form with a single large fluorescent body, i.e., the nuclear DNA. Bars, 1 μm.

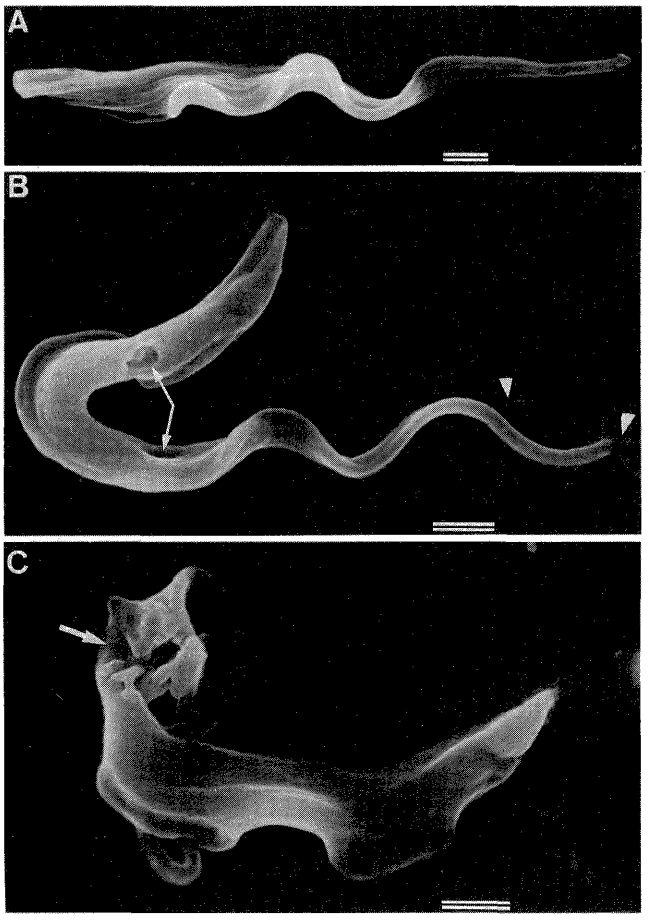

Figure 2 Scanning electron micrographs. (A) A representative photomicrograph of the kinetoplastid. Note typical trypanosome surface topography. (B) Akinetoplastic clone bearing concavity at the lower third of the cell body and a more shallow one at the anterior region (conjoined arrows). Short processes of segmented filopodia (arrowhead) arise from the surface of the free flagellum. (C) A number of deep potential concavities (arrow) gathered together at the anterior portion of the akinetoplastic body. Bars, 1 μm.

MATERIALS AND METHODS

Parasites

A kinetoplastic Tansui strain of *Trypanosoma evansi* isolated from a waterbuffalo was kindly provided by Dr. K. Fujisaki (National Institute of Animal Health, Tsukuba) and was used in the study. Parasites were cultured and maintained by passage in 8-week-old ICR mice.

Induction and cloning of akinetoplastic parasites was accomplished according to Inoki (1960). Briefly, the kinetoplastic strain was induced to become akineto plastic by successive injections of infected mice with 10-20 μg/g of pararosaniline. Akinetoplastidy was assessed by 2,4-diamidino-6-phenylindole (DAPI) staining of tail blood samples following the method of Hajduk (1976). Mutants were eventually cloned by single-cell isolation and propagated in mice. The akinetoplastic nature of the mutants was further confirmed by South ern hybridization analysis using kDNA probes (silva-Tahat et al., in press).

Electron Microscopy

Infected blood sample was collected by cardiac puncture. Parasites were isolated and purified by passage through DE52 anion exchange column. Both kinetoplastic (passage 5, K5) and established akinetoplastic clones (passages 3 and 4, AK3 and AK4, respectively), which have been maintained in the absence of the dye, were suspended and washed in 0.16M phosphate-saline-glucose (PSG) buffer (pH 7.4) consisting of 0.0113M $NaH_2PO4.2H_2O$, 0.0486M Na_2HPO_4, 0.0436M NaCl, and 0.0555M dextrose prior to fixation in 2% gluteraldehyde. Bloodstream trypomastigotes were

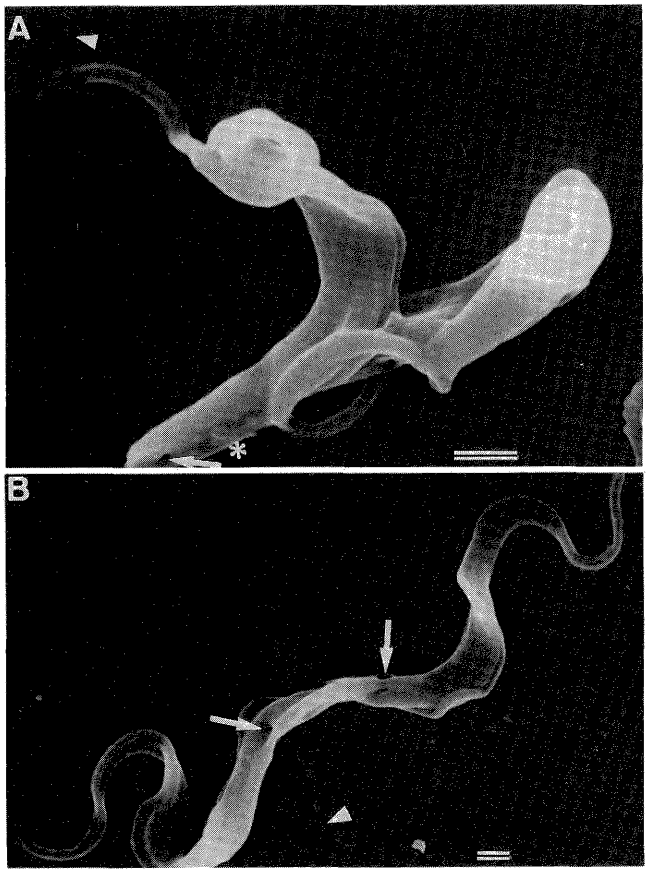

Figure 3 Scanning electron micrographs of akinetoplastic forms during cell division. (A) A trypanosome which bears a small concavity (arrow) at the posterior end is in the process of longitudinal binary fission. One of the daughter cells extends a filopodium (arrowhead) from the flagellum. Vesicular structures (asterisk) on the surface of the posterior cell body may denote primordial filopodia. Both daughter cells exhibit a slight degree of torsion at the anterior regions. (B) Presumably the final stage of cell division whereby daughter trypanosomes are still attached at their posterior broad terminal prior to complete division and separation. Note the presence of concavities (arrow) at the fused broad ends of both trypomastigotes and filopodium (arrowhead) in the same region of the lower trypomastigote. The upper trypanosome is very slightly twisted. Bars, 1 μm.

subsequently prepared for scanning and transmission electron microscopy following standard procedures.

Results and Discussion

Pararosaniline caused the deletion of a DAPI-fluorescent kDNA in *Trypanosoma evansi* (Figure 1). The absence of the kDNA in the akinetoplastic parasites was

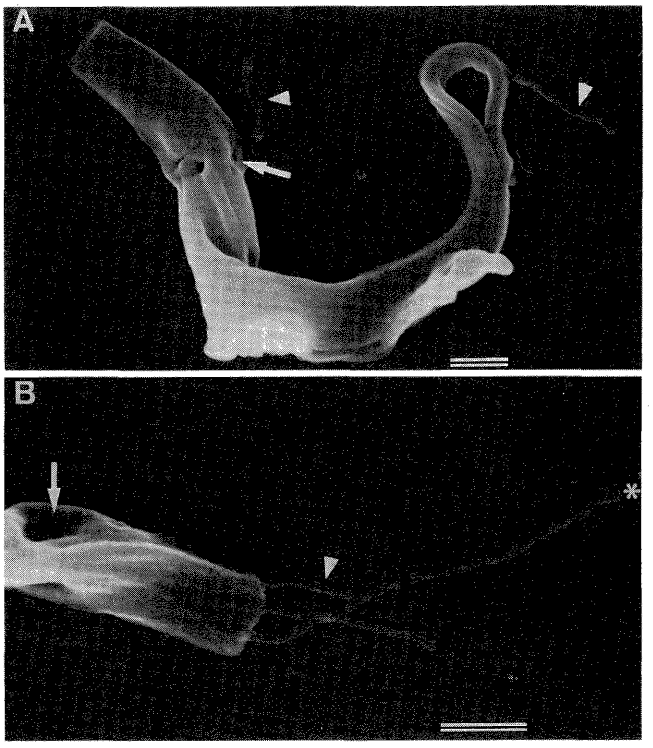

Figure 4 Scanning electron micrographs of akinetoplastic trypanosomes showing filopodia (arrowhead). (A) Filopodia arise from the flagellum at the anterior region and from the posterior portion where it is in close proximity with one of the concavities (arrow) present. The former seems relatively smooth while the latter appears to be segmented. (B) Several filopodia of various length extending from the posterior end of the parasite. The longest appendage appears to be segmented and terminates with a vesicular structure (asterisk). Continuations of the filopodia over the cell surface are shown adjacent to the concavities. Bars, 1 μm.

further confirmed by Southern Blot analysis (Silva-Tahat et al., in press). The mutants were established and cloned in mice, and processed for scanning as well as transmission electron microscopy.

In comparison with *T. brucei,* bloodstream trypomastigotes of *T. evansi* exhibited similar shape, form and pleomorphism as documented by Hoare (1972). While some of them may be long and slender having elongated flagella, some of them are short and stumpy possessing short flagella. These forms have been observed in both kinetoplastic and akinetoplastic clones of *T. evansi.* According to Hoare (1972), the mean measurements of the stumpy, intermediate, and slender forms are 16.8-19.6 μm, 19.5-20.7 μm, and 23.0-24.9 μm, respectively. In the kinetoplastic population,

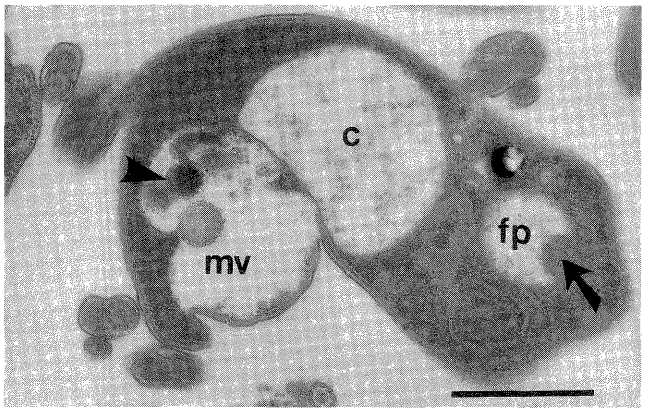

Figure 5 A transmission electron micrograph of an akinetoplastic parasite showing electron-opaque bodies (arrowhead) within the mitochondrial vesicle (mv), the hollow space of the concavity (c), and the flagellar pocket (fp). Note that although the concavity and flagellar pocket appear to be filled with the same amorphous material, the presence of the flagellum (arrow) distinguishes the latter from the former. Bar, 1 μm.

the stumpy form measured 16.8-19.3 μm in length with a mean of 18.3±0.69; the intermediate form was 19.7-21.6 μm with a mean of 20.8±0.65; and the slender form was 22.9-26.1 μm with a mean of 24.1±2.01. On the other hand, within the akinetoplastic population, the stumpy form measured 13.8-19.5 μm with a mean of 17.6±2.94; the intermediate form was 19.5-21.8 μm with a mean of 20.8±0.63; and the slender form was 22.5-24.7 μm with a mean of 23.2±0.99 (representative trypomastigotes are shown in Figure 2). Although there is some degree of variation, these measurements fall within the range of those recorded by Hoare (1972).

The surface of the streamlined akinetoplastic parasite body was essentially smooth and relaxed although a very slight degree of torsion was observed in dividing forms (Figure 3). Of prime interest, however, scanning electron microscopy has illustrated for the first time the presence of concavities or pockets on the surface of more than 90% of akinetoplastic cells in the sample. The orientation and extent of twisting of the remaining akinetoplastic forms did not allow observation of the presence of concavities. The concavities varied in size, depth, number and location on the surface membrane (Figures 2-4). Most were found at the posterior portion of the trypomastigote. An extreme case with respect to size, depth and number of concavity formation was observed in some akinetoplastic cells (AK4). Under the transmission electron microscope, however, they are seen as hollow spaces filled by an amorphous material and lined by the cell membrane (Figure 5). These concavities were apparently absent on the kinetoplastic body surface (Figure 2).

The functional significance of the concavities found on the surface of the akinetoplastic cells is not understood. Nevertheless, pararosaniline was able to induce mutation in the kDNA of the kinetoplastid thereby producing akinetoplastic forms in agreement with the results of Inoki et al. (1962). Other dyes (e.g., acriflavine, ethidium bromide) and antitrypanosomal drugs (e.g., berenil, samorin) have likewise been shown to exert the same effect on other trypanosomes (Hajduk, 1978; Shapiro and Englund, 1990). Notwithstanding, it is also possible that pararosaniline could have produced the concavities. We cannot rule out the possible membrane destabilizing effects of this dye. Drugs such as adenine nucleoside trypanocides (e.g., Puromycin, Cordycepin) and diamidines have been shown to interact with membrane biosynthesis in *T. rhodesiense* (Macadam and Williamson, 1969, 1972). Further studies on the mechanism of action of pararosaniline and scanning electron microscopy of spontaneous mutants may help clarify this observation.

However, despite the presence of concavities, a significant number of akinetoplastic trypanosomes were observed to be in the process of cell division. Fission appears to be initiated at the anterior portion bearing the flagellum and culminated at the posterior broad terminal of the parasite (Figure 3). Longitudinal binary fission is evident. In addition, attached to the posterior (proximal to the concavities) and anterior (usually from the free flagellum) regions of the akinetoplastic bloodstream trypomastigotes are thread-like structures known as filopodia (Figure 4). Filopodia were likewise seen in kinetoplastic parasites (not shown). The filopodia are known to possess the variant surface glycoproteins, or VSGs, (Vickerman and Luckins, 1969) and their formation has been implicated as the shedding of surface antigens (Cherian and Dusanic, 1977). The terminal vesicular structure at the tip of the filopodium (Figure 4B) appears to indicate that it is in the process of rounding off to be subsequently detached from the appendage. Similar vesicles were found in transmission electron micrographs of akinetoplastic forms (not shown).

The concavities were found in (1) established mutants collected from two passages in mice, AK3 and AK4, which have been grown and maintained in the absence of the dye, and (2) both daughter trypomastigotes (Fig. 3B) indicating that they are more likely to be inheritable features of the akinetoplastic clones. Our

observations of akinetoplastic trypomastigotes undergoing cell division and filopodia formation clearly imply that they still perform the fundamental activities of multiplication and infection comparable to the normal parasites. In conclusion, therefore, pararosaniline-induced akinetoplastic clones of *T. evansi* possess harmless unique cell membrane concavities.

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REFERENCES

1) Borst, P. and Hoeijmakers, J.H.J. (1979): Extrachromosomal DNA, In: ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. 15, Cummings, D.J., Borst, P., David, I.B., Weissman, S.M., and Fox, C.F. (Eds.), Academic Press, Inc., New York, pp. 515-531.
2) Cherian, P.V. and Dusanic, D.G. (1977): *Trypanosoma lewisi*: immunoelectron microscopic studies on the surface antigens of bloodstream forms, Exp. Parasitol., 43: 128-142.
3) Hajduk, S.L. (1976): Demonstration of kinetoplast DNA in dyskinetoplastic strains of *Trypanosoma equiperdum,* Science, 191:858-859.
4) Hajduk, S.L. (1978): Influence of DNA complexing compounds on the kinetoplast of trypanosomatids, In: Progress in Molecular and Subcellular Biology, Vol. 6, Hahn, F.E. (Ed.), Springer-Verlag, Germany, pp. 159-200.
5) Hoare, C.A. (1954): The loss of the kinetoplast in trypanosomes with special reference to *Trypanosoma evansi,* J. Protozool., 1:28-33.
6) Hoare, C.A. (1967): Evolutionary trends in mammalian trypanosomes, Adv. Parasitol., 5:47-91.
7) Hoare, C.A. (1972): The trypanosomes of mammals: a zoological monograph. Oxford and Edinburgh: Blackwell Scientific Publications. 749 pp.
8) Inoki, S. (1960): Studies on antigenic variation in the Wellcome strain of *Trypanosoma gambiense,* Biken J., 3: 215-222.
9) Inoki, S., Sakamoto, H., Ono, T. and Kubo, R. (1962): Studies on the Ak forms of *Trypanosoma evansi*: II. Effect of p-rosaniline on the appearance of Ak forms, Biken J., 5:127-131.
10) Macadam, R.F. and Williamson, J. (1969): Lesions in the fine structure of *Trypanosoma rhodesiense* specifically associated with drug treatment, Trans. Roy. Soc. Trop. Med. Hyg., 63:421-422.
11) Macadam, R.F. and Williamson, J. (1972): Drug effects on the fine structure of *Trypanosoma rhodesiense*: diamidines, Trans. Roy. Soc. Trop. Med. Hyg., 66(6):897-904.
12) Shapiro, T.A. and Englund, P.T. (1990): Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs, Proc. Natl. Acad. Sci. U.S.A., 87:950-954.
13) Silva-Tahat, M.R.A., uemura, H. and yanagi, T. 1995. Pararosaniline-indeed akinetoplastic *Trypanosoma evansi* : formation and characterization. J. Protozool. Res. (In press).
14) Vickerman, K. and Luckins, A.G. (1969): Localization of variable antigens in the surface coat of *Trypanosoma brucei* using ferritin-conjugated antibody, Nature, 224: 1135-1136.
15) Vickerman, K. and Preston, T.M. (1976): Comparative cell biology of the kinetoplastid flagellates. In: Biology of the Kinetoplastida, Vol. 1, Lumsden, W.H.R. and Evans, D.A. (Eds.). London: Academic Press Inc., Ltd. pp. 35-130.