J. Protozool. Res., *8*, 227-232(1998)
Copyright©1998, The Research Center for Protozoan Molecular Immunology

Chemically Induced Akinetoplastic *Trypanosoma evansi*

H. UEMURA, M.R.A. SILVA-TAHAT, T. YANAGI and H. KANBARA

Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan.

Received 24 Feburary, 1999

Key words: *Trypanosoma evansi*, minicircle DNA, pararosaniline, akinetoplastidy.

ABSTRACT

We have induced the loss of kinetoplast DNA in *Trypanosoma evansi* by successive treatment of infected mice with a basic fuchsin dye, pararosaniline.

The "akinetoplastic" clones induced from a "kinetoplastic" strain were propagated by single cell inoculation into mice. In the course of assessing akinetoplastidy by DAPI staining after pararosaniline treatment, various fragmentation patterns and smaller-sized kDNA were detected.Semi-quantitative measurement of kDNA fluorescence indicated that pararosaniline causes a gradual decrease of the minicircle DNA. We attempted to obtain the parasites with intermediate kDNA, however these were unstable and tending to become akinetoplastic.

We then compared the growth rate of the akinetoplastic parasites with the original kinetoplastic strain. The growth of the kinetoplastics was relatively constant in the presence or absence of pararosaniline. On the other hand, the kDNA-deficient mutants showed a delayed growth rate and it was retarded when they were subjected to increasing dosage of the dye. According to these results we speculate that minicircle DNA of *Trypanosoma evansi* is not essential for the parasite growth, however it may facilitate the division and segregation of the mitochondrion and the genome during cell division.

INTRODUCTION

The kinetoplast, an unusual mitochondrial feature of the Order Kinetoplastida, is the organized network of DNA molecules (kinetoplast DNA). It is composed of approximately 50 copies of the maxicircles (20-40 kb) and 5,000 to 10,000 of minicircles (1-2.5 kb). The maxicircles are the homologues of other mitochondrial genomes (Stuart 1983; Simpson 1986), and minicircles have been shown to encode guide RNA messages involved in RNA editing of maxicircle DNA transcripts (Sturm and Simpson 1990). Sequence heterogeneity of minicircle DNA in several species of Trypanosomes is explained by RNA editing; genetic information of different minicircle sequences is requires to provide sufficient genetic information for extensive editing (Stuart and Feagin 1992). The kinetoplast of *T. evansi* is unique among those of parasitic protozoa. It lacks maxicircles and absence of minicircle sequence heterogeneity (Borst et al. 1987). *Trypanosoma evansi*, the causative agent of the disease known as surra and under various local names, is transmitted only mechanically by biting flies, and can not complete cyclical development in the insect vectors. According to the hypothesis that molecular heterogeneity in minicircle is for extensive editing of guide RNA to produce mature mRNA of some mitochondrial proteins, loss of mitochondrial function by missing the maxicircle DNA would result in loss of necessity of heterogeneity in minicircles and hence to minicircle homogeneity.

We are interested in the role of minicircle DNA in this parasite, which may not need functional mitochondria and does not have maxicircle DNA. Is there any advantage to the parasites in keeping homogeneous minicircle DNA? We have treated infected mice using

pararosaniline to induce akinetoplastic parasites by following the method of Inoki et al. (1960), and compared the growth rates of kinetoplastic and akinetoplastic parasites.

Induction of akinetoplastic *T. evansi*:

Trypanosoma evansi (Tansui strain isolated from a water buffalo in Taiwan) was induced to akinetoplastic by successive treatments of infected mice with 10-20 μ/g of pararosaniline. Loss of kinetoplast DNA was assessed by fluorescence microscopy after staining the parasite smear with 2,4-diamidino-6-phenylindole (DAPI). The mutant akinetoplastic clones, which are the parasites without kinetoplast, were purified and established by single cell inoculation into mice. The complete absence of kinetoplast DNA was confirmed by DAPI staining (Fig.1) and Southern hybridization using probes of cloned kDNA fragments (Fig.2). The minicircle DNA fragment could be detectable in the kinetoplastic samples less than 25 ng loading of whole parasite DNA. No corresponding band could be seen in akinetoplastic parasite DNA even in the lane of 4μg loading.

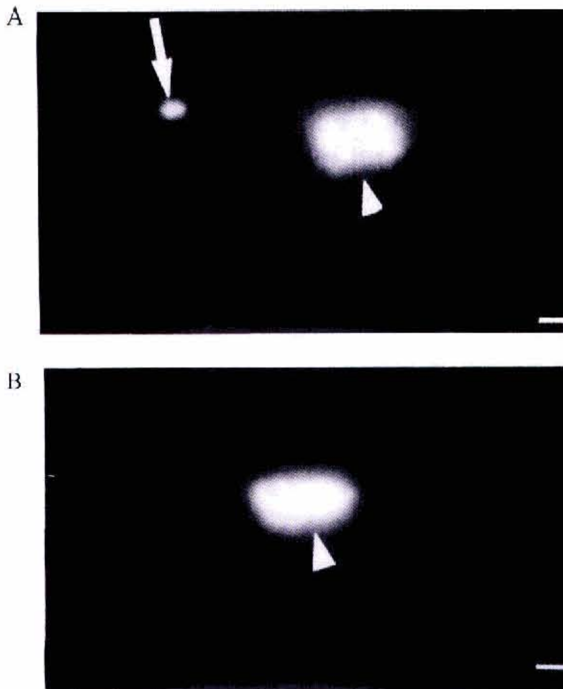

Figure 1. DAPI-staining fluorescence micrographs. Bars, 1 μm.

(A) *T. evansi* kinetoplast clones with a large nuclear DNA (arrowhead) and a small kDNA (arrow).

(B) An akinetoplastic mutant with a nuclear DNA (arrow) only.

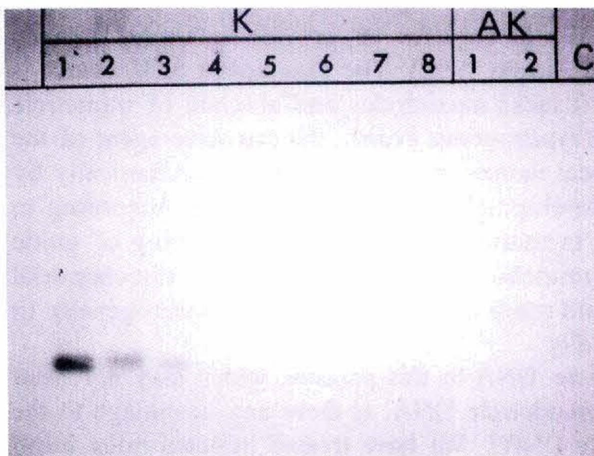

Figure 2. Southern blot hybridization of kinetoplastid and akinetoplastid using total kDNA clone as a probe. Each lane was loaded 0.4 μg, 0.1 μg, 0.025 μg, 0.006 μg, ..., 4 times series of dilutions of Hinf I linearlized whole DNA from kinetoplastic parasites (K-1, 2, 3, 4, ..., 8). Total DNA of akinetoplastics were loaded, 4 μg and 1 μg (AK-1, 2).

The kinetoplastic and akinetoplastic parasites were applied for electron microscopical observations. The typical disk-shaped kDNA network of kinetoplastids (Fig. 3A) was not present in akinetoplastic cells. On the other hands, electron opaque bodies were observed within the mitochondrion of the mutant parasites (Fig. 3B). This difference in mitochondrial structure may reflect the total loss of minicircle DNA in the akinetoplastic mutant, because both of the parasites examined were grown and obtained in the absence of the drug. However, further studies on electron microscopy of spontaneous akinetoplastic cells may help to rule out the possibility of unexpected actions of pararosaniline.

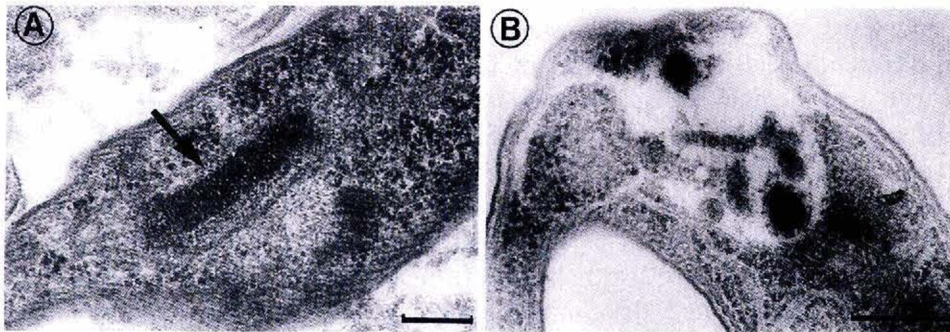

Figure 3. Transmission electron micrographs of kinetoplastic (A) and akinetoplastic (B) *T. evansi*. The disk-shaped kDNA network (arrow) of kinetoplastid and the electron-opaque bodies (arrowhead) within the mitochondrion of the akinetoplastic parasite.

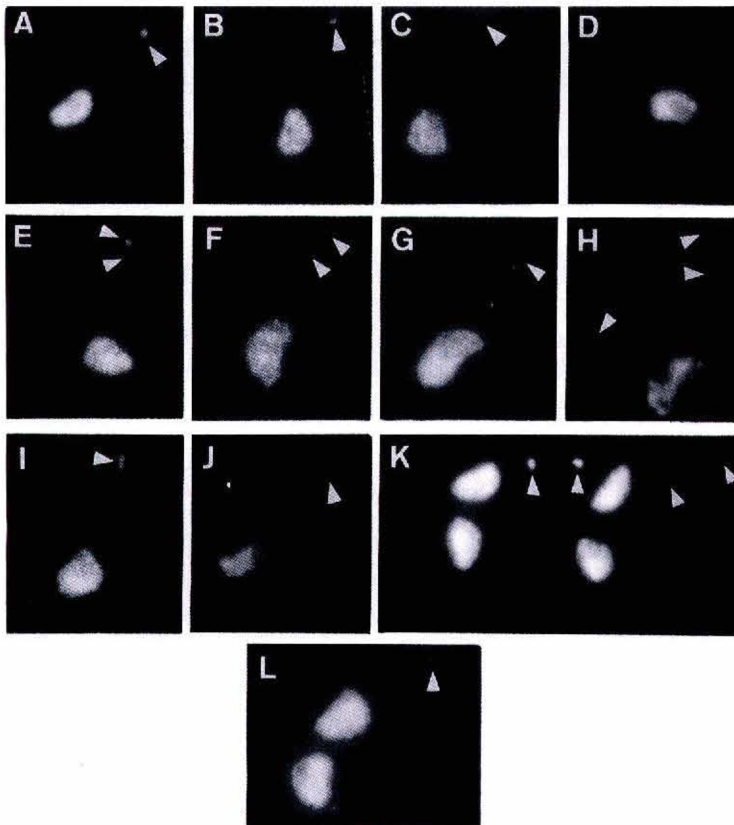

Figure 4. Qualitative changes of kinetoplast DNA. DAPI-stained *T. evansi* showing the large fluorescent nuclear DNA and small fluorescent kDNA (arrowhead). Trypanosomes with normal kDNA (A), medium kDNA (B), small kDNA (C), without kDNA (D), various patterns of kDNA fragmentation (E-H), normal and intermediate kDNA of initial stage of cell division (I and J), normal and intermediate segregated kDNA (left and right in K).

Instability of trypanosomes with intermediate kDNA:

In the course of microscopic assessment of akinetoplastidy, various forms, shapes, sizes of kDNA were observed. Typical fluorescence microscopical pictures with different intensity of kDNA are shown in Figure 4. DAPI fluorescence intensity was measured using an Olympus BH-2 microscope equipped OSP-OPU/PMU/BMI accessory units. The average intensity of

normal (Fig. 4A), medium (Fig. 4B) and small (Fig. 4C) kDNA were 131, 67 and 36.5 units respectively. In other words, relative intensities of these intermediate kDNA were half and quarter of the normal one. We also observed patterns of kDNA fragmentations as shown in Fig. 4E-J; various weaker kDNA fluorescent spots were seen in some of the parasites during initial stage of cell division. In addition, parasites with two nuclear and two equal intensities of kDNA (Fig. 4K), small intensity of one kinetoplast with two nuclear (Fig. 4L) could be detectable. From these observations, we understand that parasites were gradually decreasing kDNA in contents during the process of cell division, and then finally lost kDNA completely.

We attempted to select trypanosomes containing intermediate contents of kDNA. However, differential cell count analysis of previously drug-treated parasites revealed that parasites with intermediate kDNA were always minority in the entire population. In the absence of pararosaniline (Fig. 5A), trypanosomes with intermediate kDNA decreased in number from 16% at time 0 to 3% at 72 hrs. later, even akinetoplastic forms were increased from 32% to 42%. On the other hand, trypanosomes kept in the drug pressure (Fig. 5B) maintained the population of parasites with intermediate kDNA (16% at 0 hr. and 15% at 72 hrs.). Akinetoplastic forms were also increased from 32% to 42% in this condition. In either case, under both normal and drug conditions, trypanosomes with intermediate kDNA seemed to be unstable and tend to lose minicircle DNA completely.

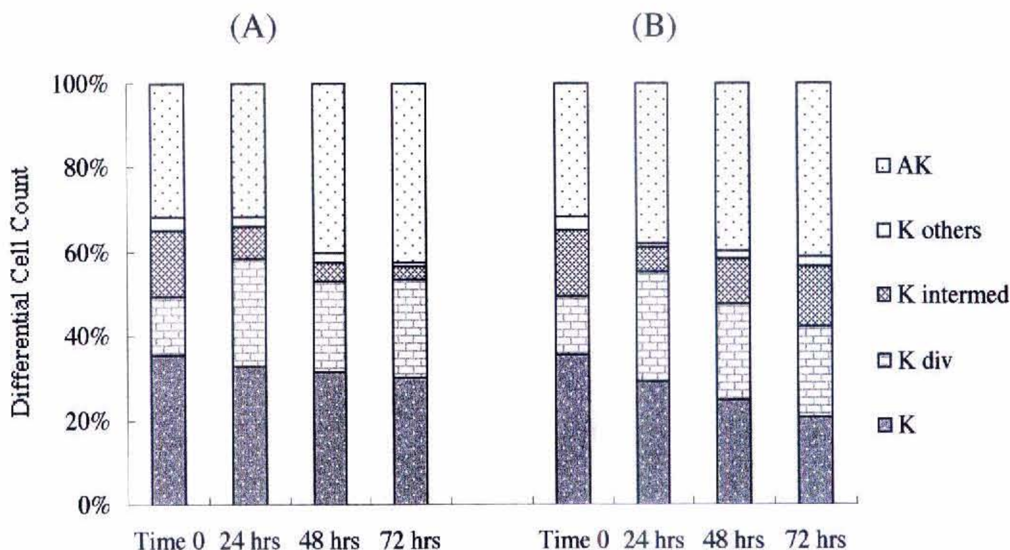

Figure 5. Akinetoplastic tendency of trypanosomes with intermediat kDNA. Differential cell counts of previously drug-treated parasites, kept in the absence (A) or presence (B) of pararosaniline. AK, akinetoplastic parasite lacking kDNA; K others, dividing intermediate and variously fragmented kDNA; K intermed, intermediate kDNA; Kdiv, dividing normal kDNA; K, normal kDNA.

Growth rate of akinetoplastic clone:

We then considered the biological significance of kDNA minicircles by comparing growth behavior of kinetoplastic and akinetoplastic parasites. Normal kinetoplastic parasites exhibited a shorter doubling rate (about 4.7 hrs.), compared to akinetoplastic ones. This was relatively constant even in the presence of pararosaniline (Fig. 6A). In contrast, mutant parasites duplicated at a little slower rate (5.5 hrs.) under normal condition, and the rate became more delayed when they were exposed to increasing dosage of the drug (Fig. 6B). This indicated that the parasites containing minicircle DNA might have some advantage in

nature.

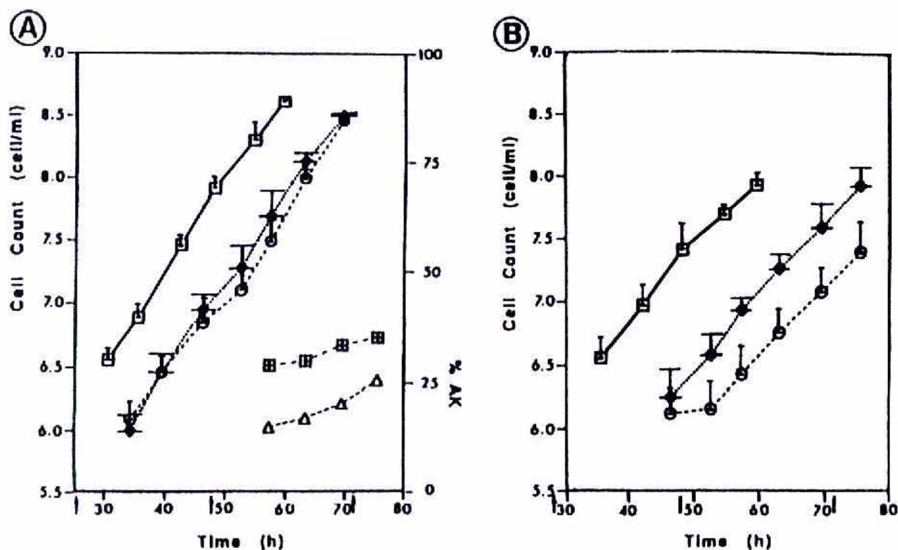

Figure 6. Growth rate of kinetoplastic (A) and akinetoplastic (B) clones under normal conditions and with pararosaniline exposure. At the time of 0 and 48 hrs.(arrows), the drug was injected into mice. —□— without pararosaniline; —◇— 20 μ g/g and —○— 40 μg/g of pararosaniline. The percentage of newly appeared akinetoplastic parasites was also shown, at the condition of —△— 20 μg/g and —⊞— 40 μg/g.

DISCUSSION

We have presented induction and characterization of akinetoplastic form of *T. evansi* using pararosaniline (Silva-Tahat et al. 1995b). Pararosaniline is a basic fuchsin stain dye used in Feulgen reactions and it is known to interact with aldehyde groups of DNA (Duijndam and van Duijn 1975). It had been also shown that some of trypanocidal drugs, pentamidine, diminazene aceturate, isometamidium chloride, and ethidium bromide could produce cleavage in minicircle DNA of *Trypanosoma equiperdum*, and selective inhibition of topoisomerase type II is involved in this breakage (Shapiro and Englund 1990). Our observation of some weaker fragmentation patterns of minicircle DNA suggested similar mechanism of pararosaniline. We then anticipated that akinetoplastic parasites would be less sensitive to the drug and grow faster than the original kinetoplastic ones. However, the parasites missing kinetoplast minicircle DNA showed slower growth behavior in the presence and absence of pararosaniline. Similar effect was reported using isometamidium chloride, an effective trypanocidal drug, that dyskinetoplastic *T. evansi* and *T. equiperdum* were more susceptible to the drug than their parental kinetoplastic strains (Kaminsky et al. 1997). The primary target of these drugs is thought to be kDNA resulting to production of akinetoplastic forms. In the produced akinetoplastic mutants, the dye exerts its effect on the other targets such as nuclear DNA.

We now understand our observations as on a following. Pararosaniline is incorporated into parasites and interfere in the process of kinetoplast replication. It produces smaller contents of minicircle DNA. Once such a parasite of decreased numbers of kDNA minicircle is appeared, it is unstable and tend to loose minicircle DNA and finally complete loss will produce the akinetoplastic trypanosome. Homogeneous population of kinetoplast minicircle DNA is not essential for *T. evansi* growth, however the parasites containing this organelle have some advantages in the division and segregation of mitochondrial and cytosolic

proteins and genome structures.

Further characterization of akinetoplastic clones using scanning electron microscopy revealed the marked morphological differences in surface topography (Silva-Tahat et al. 1995a). Unique concavities or pockets could be seen on the surface of more than 90 % of the mutant parasites. The cavities vary in size, depth, number and location on the surface membrane. Most were found at the posterior potion of the trypomastigote. We could not explain the biological significance of these cavities, however it may be produced as the absence of the content minicircle DNA in the kinetoplast position.

ACKNOWLEDGEMENTS

This is based on the presentation at the International Symposium on Strategies for Research and Control for Surra: *Trypanosoma evansi* Infection, in Obihiro, Japan, 19-22 August 1998. We are grateful to Professor H. Hirumi and the organizing committee for giving us the opportunity to present our work. Our research on trypanosomes is supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Borst, P., Fase-Fowler, F. & Gibson, W.C. 1987. Kinetoplast DNA of *Trypanosoma evansi*. *Mol. Biochemical. Parasitol. 23: 31-38.*

Duijndam, W.A.L. & van Duijn, P. 1975. The interaction of purinic acid aldehyde groups with pararosaniline in the Feulgen-Schiff and related staining procedures. *Histochemistry 44: 67-95.*

Inoki, S., Taniuchi, Y., Matushiro, A. & Sakamoto, H. 1960. Multiplication ability of akinetoplastic form of *Trypanosoma evansi*. *Biken J. 3: 123-127.*

Kaminsky, R., Schmid, C. & Lun, Z. R. 1997. Susceptibility of dyskinetoplastic *Trypanosoma evansi* and *T. equiperdum* to isometamidium chloride. *Parasitol. Res. 83: 816-818.*

Silva-Tahat, M.R.A. Ichinose, A., Uemura, H. & Kanbara, H. 1995a. *Trypanosoma evansi*: Unique concavities on the surface membrane of pararosaniline-induced akinetoplastic clones as revealed by scanning electron microscopy. *Jpn. J. Trop. Med. Hyg. 23: 9-13.*

Silva-Tahat, M.R.A. Uemura, H. & Yanagi, T. 1995b. Pararosaniline-induced akinetoplastic *Trypanosoma evansi*: formation and characterization. *J. Protozool. Res. 5: 10-22.*

Shapiro, T.A. & Englund, P.T. 1990. Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. *Proc. Natl. Acad. Sci. USA 87:950-954.*

Simpson, L. 1986. Kinetoplast DNA in trypanosomatid flagellates. *Int. Rev. Cytol. 99: 119-179.*

Stuart, K. 1983. Minireview: kinetoplast DNA, mitochondrial DNA with a difference. *Mol. Biochem. Parasitol. 9: 93-104.*

Stuart, K. & Feagin, J.E. 1992. Mitochondrial DNA of kinetoplastids. *Int. Rev. Cytol. 141: 65-88.*

Sturm, N.R. & Simpson, L. 1990. Kinetoplast DNA minicircles encode guide RNAs for editing of cytochrome oxidase 3 subunit III mRNA. *Cell 61: 879-884.*