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Pararosaniline-induced Akinetoplastic *Trypanosoma evansi*: Formation and Characterization

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ABSTRACT

A kinetoplastic strain of *Trypanosoma evansi* was induced to become akinetoplastic by pararosaniline. Loss of kDNA minicircles was analyzed by DAPI fluorescence and Southern blot analysis probed with cloned kDNA fragments. Qualitative changes in the kinetoplast prior to akinetoplastidy include various fragmented fluorescence patterns and formation of intermediate fluorescence intensity. Thus, kDNA loss induced by pararosaniline is preceded by a gradual decrease in its size. We show that trypanosomes with intermediate amount of kDNA are unstable tending to become akinetoplastic. We further demonstrate that the difference in population doubling time between kinetoplastic and akinetoplastic forms is 4.7 and 5.5 hrs, respectively. Our results indicate that although the mutants have maintained their ability to multiply, their slower doubling rate limits them to dominate in a given population. Moreover, this feature may be one of the possible factors responsible for their inferiority to those with kDNA and for their selective disadvantage in nature.

INTRODUCTION

The kinetoplast, which is a unique intramitochondrial structure situated near the base of the flagellum, characterizes the members of the Order Kinetoplastida. It is composed of DNA molecules collectively called the kinetoplast DNA (kDNA) which represents about 7-20% of the total cellular DNA (Stuart, 1983; Stuart and Feagin, 1992). There are two types of kDNA molecules, namely, the maxicircles (20-40 kb) numbering from 20 to 100, and minicircles (1-2.5 kb), from 5,000 to 10,000 (Englund et al., 1982; Stuart, 1983; Simpson 1986 1987; Stuart and Feagin 1992). Maxicircles are considered to be

analogous to eukaryotic mitochondrial DNA (Benne et al. 1983; Feagin and Stuart 1985; Benne 1990). Minicircles, on the other hand, have initially been assumed to play structural roles in providing a scaffolding for the maxicircles, particularly during replication, and in the ordered segregation of the unitary mitochondrion (Cosgrove 1973; Borst and Fairlamb 1976; Borst and Hoeijmakers 1979a, b). Recently, however, the minicircles have been shown to code for guide RNAs (gRNAs) involved in RNA editing of maxicircular transcripts (Blum et al. 1990; Sturm and Simpson 1990). Maxicircles and minicircles form independent catenanes which are extensively interlocked establishing an intricate and complex kDNA network (Shapiro 1993).

These unique features aside, the kDNA exhibits various phenomena which according to number and arrangement may either be polykinetoplastidy (several kDNA) or eukinetoplastidy (single kDNA), or pankinetoplastidy (dispersed kDNA) (Vickerman and Preston 1976; Kallinikova 1981). In addition, the presence of ancillary kDNA (aDNA) of unknown biological process has recently been demonstrated (Miyahara and Dvorak 1994). Furthermore, changes in kDNA pattern such as variation in the dominance or the copy number of different minicircles has been described as transkinetoplastidy (Lee et al. 1992). In extreme situations, however, the kDNA may apparently exhibit deletions in composition whether maxi- or minicircles as in dyskinetoplastidy, or deletions in both DNA as in akinetoplastidy. While mutants exhibiting deletions in kDNA have been referred to as I- or K- (Borst and Hoeijmakers 1979a, b), those lacking any kDNA at all have been called K° (Stuart and Gelvin 1980). Mutation may either be spontaneous or induced resulting to an impaired replication of the kDNA (Hajduk 1978, 1979; Kallinikova 1981).

Trypanosomes most susceptible to kDNA mutation are species of the Subgenus *Trypanozoon*. These kinetoplastids possess a repressed mitochondrial activity that may be a transient or permanent feature of their life cycle. Nevertheless, they are exceptional in that they may exist as viable dys- or akinetoplastids which are equally infective and prolific as their kinetoplastic counterparts (Borst and Hoeijmakers 1979b).

In the present paper, we induced the loss of the kinetoplast in *Trypanosoma evansi*, the causative agent of Surra disease in camels, by successive treatment of infected mice with a basic fuchsin dye, pararosaniline. We propose the name akinetoplastic to describe the mutants. Although we were not able to demonstrate the molecular events leading to mutation, we show the cellular changes and occurrence of intermediate stages leading to the loss of kDNA after treatment of the dye. Based on these qualitative changes in the kDNA, we describe the possible genesis of pararosaniline-induced akinetoplastic trypanosomes. We further demonstrate that athough the kDNA-deficient mutants have maintained the ability to multiply, they have a limited growth rate which may somehow significantly contribute to their selective disadvantage in nature.

MATERIALS AND METHODS

Parasites: The kinetoplastic *Trypanosoma evansi* used in the study is a Tansui strain isolated from a waterbuffalo in China. Trypanosomes were maintained by passage in 8-week-old ICR mice. For transmission electron microscopy and DNA analysis, infected blood was collected by cardiac puncture and parasites were isolated and purified by passage through DE52 anion exchange column (Lanham and Godfrey 1970).

Induction of akinetoplastidy: Akinetoplastidy was induced by successive injections of 10-20 μg/g of pararosaniline (Nacalai Tesque, Inc., Japan) following the method of Inoki et al. (1960). Loss of kinetoplast was assessed by fluorescence microscopic (Olympus Research Microscope Model AHBT VanoxT) examination of parasites labeled with 2,4-diamidino-6-phenylindole (DAPI) in smears of tail blood samples according to Hajduk (1976). To obtain purified akinetoplastic clones, parasites without kinetoplast were propagated by single cell inoculation into mice.

Electron microscopy: Purified kinetoplastic and akinetoplastic parasites were independently washed in phosphate-saline glucose buffer (PSG), pH 7.6, prior to fixation with 2.5% gluteraldehyde and post-fixation in 1% OsO_4 for 1 hr each at room temperature. Fixed samples were dehydrated in a graded series of ethanol solutions and finally embedded in Epon. Ultrathin sections mounted on copper grids were double stained with 2% lead citrate and 1% uranyl acetate. Examination of the sections was accomplished with a JEOL JEM-lOOCX Electron Microscope.

kDNA analysis: To confirm the lack of kDNA minicircles in cloned parasites, total DNA from normal and mutant forms was isolated, purified and analyzed by Southern blot using cloned fragments of kDNA from parental kinetoplastic strain as probes. Briefly, total DNA was extracted from parasites by the standard procedure (Sambrook et al. 1989) and whole kDNA from kinetoplastic parent was isolated by CsCl-Ethidium Bromide ultracentrifugation (TLN-100 vertical rotor, Beckman Optima Ultracentrifuge). Ethanol precipitated DNA samples were resusupended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at 4° C until use. Restriction enzyme digestion and Southern hybridization analysis likewise followed standard methods (Sambrook et al. 1989).

Examination of kDNA changes leading to akinetoplastidy: Tail blood sample from an infected mouse treated with 40 μg/g pararosaniline stained with DAPI. The differential count of cells according to various kinetoplast form and size per hundred parasites was recorded. Cells were grouped into 5, namely: (a) parasites with normal kDNA fluorescence intensity (Kn, Fig. lA); (b) trypanosomes with intermediate kDNA fluorescence intensity (Kintermed, Figs. 1B, C); (c) akinetoplastic forms (AK, Fig. lD); (d) kinetoplastic parasites (Kn) undergoing cell division (Kdiv, Figs. lI, J, K left); and (e) other trypanosomes with various kDNA fluorescent spots and those with intermediate kDNA fluorescence during cell division (Kothers, Figs. lE-H, K right). The difference in

the intensity of DAPI fluorescense among normal, medium and small kDNA was measured using an Olympus BH-2 microscope equipped with OSP-OPU/PMU/MBI accessory units for measuring fluorescense intensity. Likewise, fluorescence intensity of the nucleus, supposed site of kDNA in mutants, and background were noted for comparison.

To determine the stability and tendency of trypomastigotes with intermedate kDNA fluorescence intensity, parasites previously treated with 40 μg/g were inoculated into 2 groups of mice in triplicate: (I) without and (2) with daily pararosaniline (40 μg/g) injections at 24 hrs intervals for 2 days. The parasite population initially contained about 16% trypanosomes with intermediate kDNA and 32% akinetoplastic forms. Prior to drug treatment, subsequent differential cell counts were determined at 24 hrs intervals for 3 days.

Growth rate of kinetoplastic and akinetoplastic clones under normal conditions and pararosaniline supression: The growth behaviour of kinetoplastic and akinetoplastic clones in vivo was followed. Cell density of 10^6/ml was inoculated into triplicate samples of mice that (1) did not receive pararosaniline and (2) those that were subsequently injected with (a) 20 μg/g and (b) 40 μg/g pararosaniline daily for 3 days. The total number of parasites was counted for 3 days. Growth curves were established and the population doubling time was determined by interpolation of the graphs.

RESULTS

Pararosaniline was used to induce mutation in kDNA leading to its loss. DAPI facilitated the distinction between normal and mutant trypomastigotes. In Fig. 1, all fluorescent bodies represent DAPI-stained DNA. Kinetoplastids always have 2 fluorescent particles, a large nuclear DNA and a small kDNA (Fig. 1A). Akinetoplastic forms, on the other hand, have only the large nuclear DNA (Fig. 1D). Transmission electron micrographs showed the disk-shaped kDNA network typical of kinetoplastids (Fig. 2A). On the other hand, electron-opaque bodies were found within the mitochondrion of akinetoplastic clones (Fig. 2B). These electron-opaque bodies, however, were not seen in the kinetoplastic cells. The absence of DAPI fluorescence in the akinetoplastic parasites implies that these electron-opaque bodies do not necessarily contain DNA. To confirm the loss of the kDNA in the established purified akinetoplastic clones, total DNA was extracted and isolated from both kinetoplastic and akinetoplastic parasites. Total DNA was digested with enzymes (AluI, HinfI, MboI, TthHB8I) known to restrict *T. evansi* minicircles (Borst et al. 1987; Songa et al. 1990; Masiga and Gibson 1990; Ou et al. 1991; Lun et al. 1992). The parental kinetoplastic kDNA was linearized with TthHB8I (1-kb T fragment) or with MboI (I-kb M). The I-kb T fragment was further restricted with MboI yielding a 0.6-kb and a 0.4-kb MboI-TthHB8I fragments. All of these fragments were cloned in pBluescript SK+ and eventually labeled with digoxigenin to be used as probes for Southern hybridization analysis of inde-

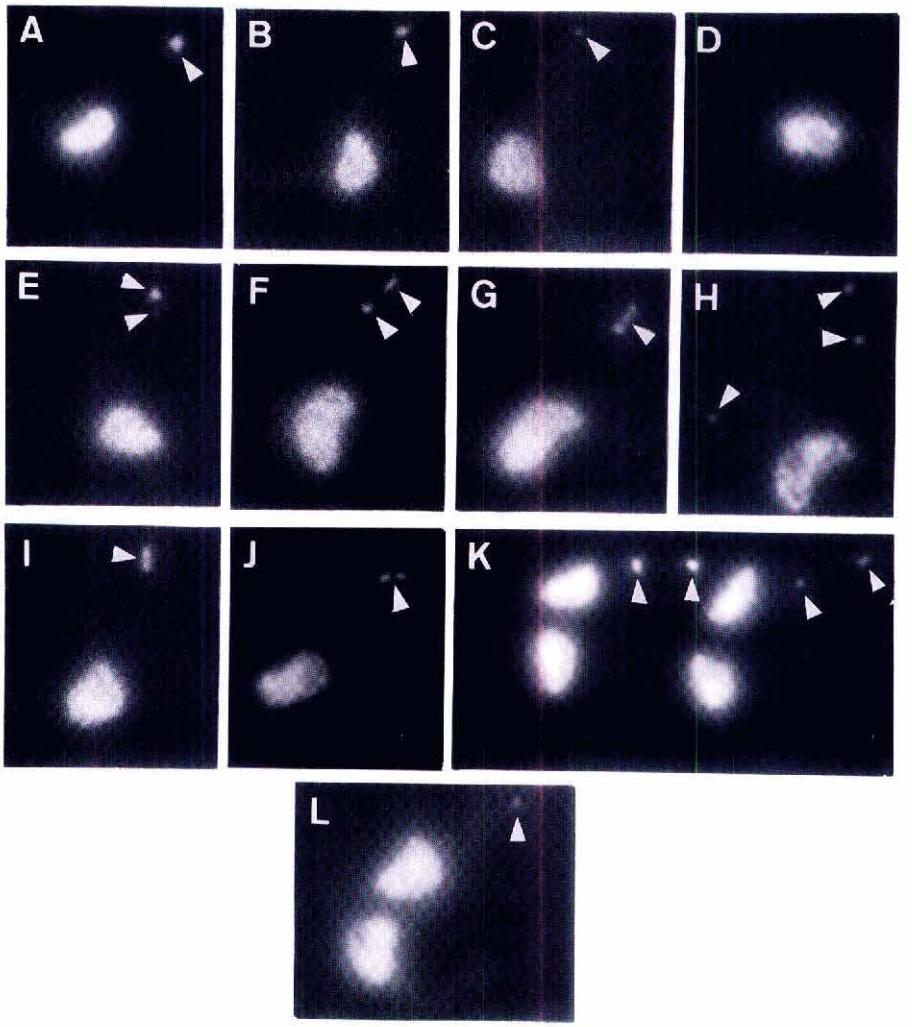

Figure 1. DAPI-stained *Trypanosoma evansi* showing large fluorescent nuclear DNA and small fluorescent kDNA (arrowhead). Shown are profiles of trypanosomes with (A) normal kDNA, (B) medium kDNA, (C) small kDNA, (D) without kDNA, (E-H) various patterns of kDNA fragmentation, (I, J) normal kDNA during initial stages of division, (K) segregated normal (left) and intermediate (right) kDNA prior to cytokinesis, and (L) small kDNA prior to "all or none" division. All forms were seen in a parasite population that was daily treated with 40 µg/g pararosaniline at 24 h intervals for 2 days. x12,000.

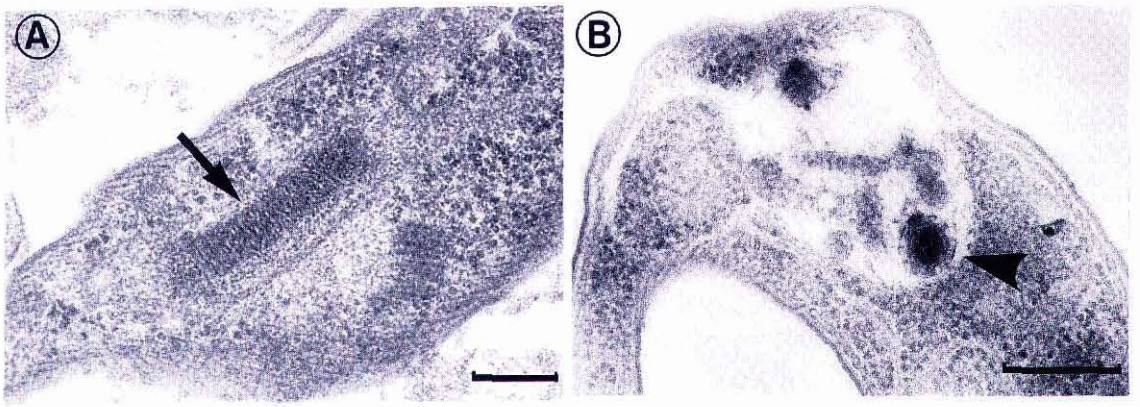

Figure 2. Electron micrographs of kinetoplastic (A) and akinetoplastic (B) parasites. Note the typical disk-shaped kDNA network (arrow) of the kinetoplastid in contrast to the electron-opaque bodies (arrowhead) within the mitochondrial vesicle of the akinetoplastic cell. Bars, 1 µm.

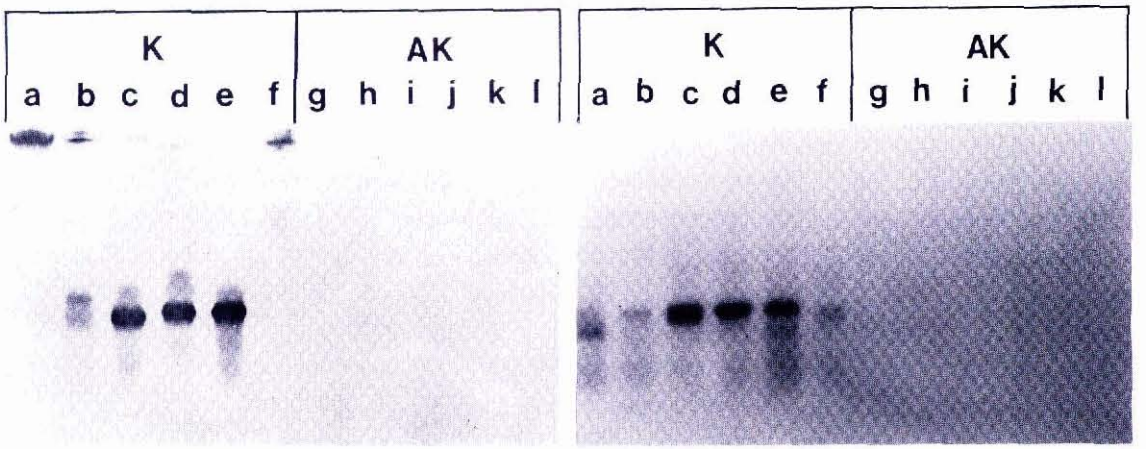

Figure 3. Southern blots of total DNA digests of kinetoplastic (K) and akinetoplastic parasites (AK). Each lane contains approximately 1 µg of enzyme restricted kinetoplastic total DNA and 4 µg of digested akinetoplastic total DNA probed with approximatley 50 ng of digoxigenin-labeled cloned kDNA minicircle fragments: (A) whole kDNA (1.0 kb) linearized with MboI and (B) 0.4 kb MboI-TthHB8I fragment. (a,g) undigested total DNA; (*b,h*) *AluI*; (*c,i*) HinfI; (d,j) MboI; (e,k) TthHB8I; (f,l) BamHI.

pendent AluI, HinfI, MboI and TthHB8I digests of total DNA. A BamHI digest of total DNA of both kinetoplastic and akinetoplastic parasites was included as a control. Representative blots probed with 1-kb total minicircle and 0.4-kb MboI-TthHB8I fragment, clones are shown in Fig. 3 wherein no apparent sequence homology was detected between akinetoplastic DNA with the kDNA probes. This result suggests that the mutant we induced does not have kDNA minicircles.

Various attempts to isolate and purify the residual kDNA of akinetoplastic parasites were not successful. Polymerase Chain Reaction (PCR) using primers based on published sequences of *T. evansi* kDNA minicircles (Songa et al. 1990; Ou et al. 1991; Artama et al. 1992) was negative for the akinetoplastic total DNA. Preliminary Southern analysis using CsCl-EB-ultracentrifugation-purified samples of whole kDNA from parental kineto- plastic forms demonstrated the presence of a small amount of homologous sequence in total DNA digests of akinetoplastic forms and was localized by electron microscopic in situ hybridization in the mitochondrion as well as in the nucleus (not shown). To rule out the possibility of nuclear contamination and assure purity of probes, kDNA cloning was accomplished as previously described. The use of these cloned minicircle probes eliminated cross-hybridization in subsequent Southern blots.

In the course of microscopic assessment of akinetoplastidy, various forms, shapes and sizes of the kDNA were observed in addition to different stages of cell division which may be due to the asynchronous nature of the initial population of bloodstream trypomastigotes. Fluorescence staining with DAPI facilitated the observation and distinction of the qualitative changes in the kDNA after pararosaniline treatment (Fig.1). A number of fragmented fluorescence patterns were observed (Figs. lE-H). Two to three equal or unequal fluorescent particles, attached or scattered, of kDNA were seen. These were smaller in size than the normal kDNA of kinetoplastids. Cells bearing smaller kDNA particles were considered to be intermediate between kinetoplastic and akinetoplastic forms. At least 2 intermediate kDNA sizes, medium (Fig. lB) and small (Fig. lC), were observed. The average intensity of DAPI fluorescence of normal, medium and small kDNA was measured to be 131, 67 and 36.8, respectively. In other words, the fluorescence intensity of normal kDNA was 1.95x more than the medium kDNA which was in turn 1.82x more intense than the small kDNA. In addition, these forms were observed to have been divided and segregated into 2 almost equal portions (Fig. 1K). Ocassionally, trypano- somes with a small kDNA and divided nuclear DNA were seen (Fig. 1L).

We attempted to select for trypanosomes with intermediate kDNA. However, differential cell count analysis of previously drug-treated (40 μg/g pararosaniline) parasites revealed that they constituted a minority (3-16%) in the entire population. In the absence of pararosaniline, trypanosomes with intermediate kDNA decreased in number (from 16% at 0 hr to 3% at 72 hrs)

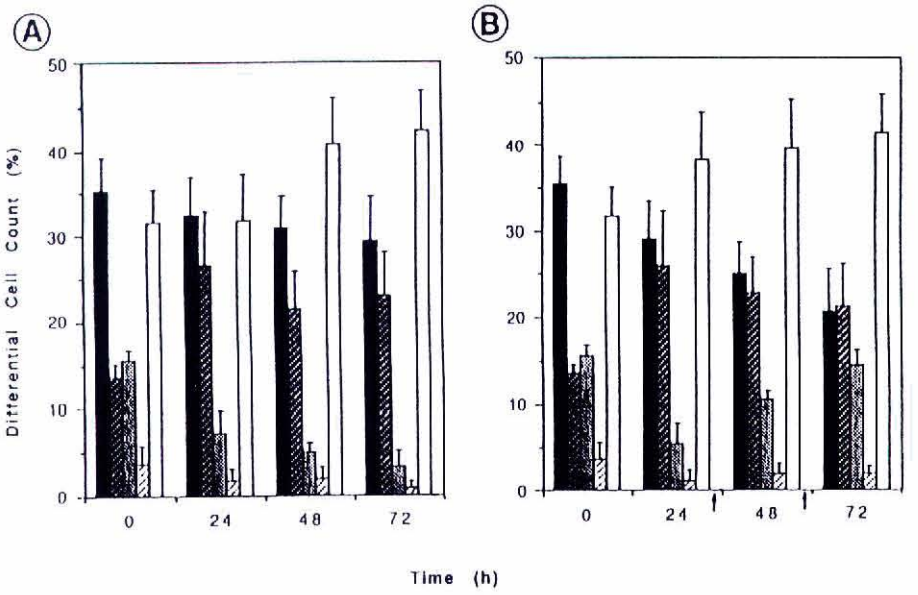

Figure 4. Graphical representation of the akinetoplastic tendency of trypanosomes with intermediate kDNA. Differential cell count of parasites without (A) and with (B) pararosaniline treatment. Drug-treated trypanosomes received 40 μg/g pararosaniline at 24 hrs intervals daily for 2 days. Arrows denote time at which drug was injected into mice. ■ normal kDNA; □ dividing normal kDNA; □ intermediate kDNA; □ dividing intermediate kDNA and various kDNA fragmentation patterns; □ akinetoplastic cells lacking kDNA. Values represent mean of triplicate samples. ± S.D. is designated by bars.

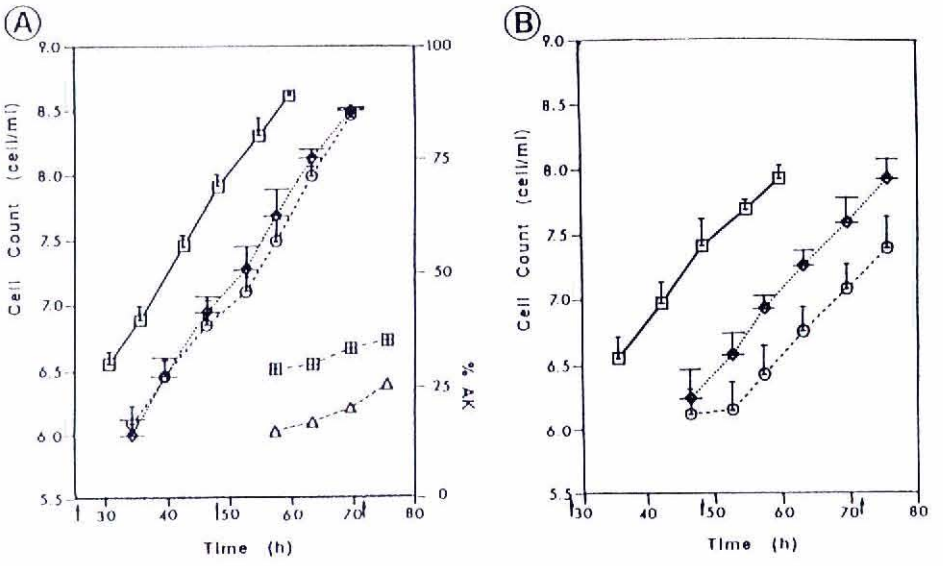

Figure 5. Logarithmic graphical representation of the growth rate of kinetoplastids (A) and cloned akinetoplastic forms (B) under normal conditions and with pararosaniline exposure. Arrows denote time at which the drug was injected into mice. —□— without pararosaniline; --- ◇--- 20 μg/g; --- ○--- 40 μg/g; --- △--- %AK at 20 μg/g; --- ⊞--- %AK at 40 μg/g. Values represent mean of triplicate samples. ± S.D. is designated by bars.

and gave rise to more akinetoplastic forms (from 32% at 0 hr to 42% at 72 hrs) (Fig. 4A). On the other hand, the presence of the drug appeared to maintain the number of trypomastigotes with intermediate kDNA in the population (from 16% at 0 hr to 15% at 72 hrs) (Fig. 4B). The presence of pararosaniline likewise enhanced the formation of more akinetoplastic forms (from 32% at 0 hr to 42% at 72 hrs). However, under both normal and drug conditions, it seemed that trypanosomes with intermediate kDNA were unstable and very transient. In lieu of their very low counts and instability, it was not possible to isolate and clone these trypanosome forms.

We finally focused our attention on the akinetoplastic clones to establish the biological significance of the absence of kDNA minicircles. We performed parallel experiments to determine the growth rate of kinetoplastic and akinetoplastic parasites. Kinetoplastids exhibited a shorter doubling rate (4.7 hrs) which was relatively constant even under pararosaniline supression (4.8 and 4.5 hrs at 20 and 40 μg/g respectively) despite a moderate increase in akinetoplastic population (Fig. 5A). In contrast, mutants reproduced at a slower rate (5.5 hrs) under conditions without pararosaniline treatment and the rate became more markedly retarded when they were subjected to increasing dosages of the dye (5.6 and 7.16 hrs at 20 and 40 μg/g, respectively) (Fig. 5B). Therefore, the growth of the akinetoplastic forms was slower by approximately 16-18% at normal and at 20 μg/g pararosaniline conditions, and even 60% at a drug condition of 40 μg/g.

DISCUSSION

In the present paper, loss of the kDNA was induced using pararosaniline and the term akinetoplastic is aptly proposed to describe these mutants. This is in contrast to previous studies in which pararosaniline-induced mutants of *T. evansi* still retained the DNA in their kinetoplast although not in a network conformation (Ono et al. 1971). Two lines of evidence show that our established purified clones of akinetoplastic parasites are devoid of kDNA: (a) the lack of a fluorescent kDNA in DAPI-stained parasites, and (b) the absence of sequence homology with cloned wildtype kDNA minicircle fragments in Southern blot analysis. *Trypanosoma evansi* is reported to have 5,000-10,000 homogeneous minicircles, the same number as *T. brucei* (Masiga and Gibson 1990). If 0.1% of the kDNA (approximately 5 minicircles) of a single parasite is the amount enough to be detectable (Degrave 1992), the presence of minicircles in the large excess of akinetoplastic total DNA in our Southern blots as well as PCR would have surely been detected. Likewise, a complete loss of kDNA sequences in *T. equiperdum* has been accomplished by Riou et al. (1980) using ethidium bromide and acriflavine while the absence of kDNA minicircles in other dyskinetoplastic trypanosomes has been shown by others as well (Stuart and Gelvin 1980; Ou et al. 1991; Lun et al. 1992). In addition, two strains of *T. evansi*, Zwart(-196) and AnTat-1, have been characterized as lacking in DAPI-stainable kDNA, organized network, and minicircles (Borst and

Hoeijmakers 1979a). Our akinetoplastic trypanosomes are similar with and may be related to these strains.

Pararosaniline is a basic fuchsin stain used in Feulgen reactions where it interacts with the aldehyde groups of DNA (Duijndam and Van Duijn 1975). However, its mode of action in vivo is as yet not understood. Nevertheless, like other trypanocidal drugs which may either be intercalating or nonintercalating, pararosaniline preferentially binds to and selectively represses the replication of kDNA as indicated by its ability to produce dyskinetoplastic forms (Inoki et al. 1962; Hajduk 1978). Qualitative changes such as fragmentation or disintegration occur in the kinetoplast resulting to its damage or loss (Delain et al. 1971; Kallinikova 1981). We have observed a number of fragmented fluorescence patterns (Figs. 1E-H) and intermediate kDNA fluorescence intensity (Figs. 1B, C) implying that the kDNA has several pararosanilinesensitive regions. These regions may be AT-rich as other drugs have been shown to have affinity to AT-rich segments of the kDNA (Hajduk 1978). The formation of medium and small kDNA in addition to their increase in number in the presence of pararosaniline (Fig. 3B) suggest that there may be several fragmentation cycles which may be enhanced by continuous drug exposure. The intermediate kDNA with decreasing fluorescence intensity indicates that the kDNA progressively diminishes in size prior to the production of an akinetoplastic parasite. The lower fluorescence intensity in addition to finding the segregated portions of the intermediate kDNA in a dividing cell (Fig. 1K) implies that it is still divisible despite its size. The small kDNA may not have a pararosaniline-sensitive site and may lack the ability to divide, therefore, an "all or none" mitotic process (Cosgrove 1966; Hajduk, 1978) ensues as suggested by Fig. 1L. In this "all or none" division of the parent cell, the small kDNA is presumably unequally distributed to daughter cells after longitudinal binary fission, thus, producing a kinetoplastic and a mutant akinetoplastic cell. However, although the intermediate kDNA may retain its ability to divide, the stability and viability of the trypomastigotes bearing this may be short lived as indicated by their low counts (Fig. 4). These forms soon become akinetoplastic.

The effect of pararosaniline on the kDNA of kinetoplastic *T. evansi* parallels that of acriflavine on the kDNA of *Leishmania tarentolae*, *T. cruzi*, *T. mega*, and *Crithidia lucilae* such that a gradual decrease in the size of the kinetoplast was observed to precede "all or none" division (Deane and Kloetzel 1969; Steinert and Van Assel 1967; Hajduk 1978). In addition, scanning electron microscopic study of pararosaniline-induced akinetoplastic parasites has revealed the presence of unique surface concavities which might be another possible effect of this dye (described elsewhere).

The kinetoplast network of *T. evansi* is distinct from the networks of other trypanosomes because of the absence of maxicircles and the presence of a homogeneous population of minicircles (Borst et al. 1987; Masiga and Gibson 1990; Songa et al. 1990; Ou et al. 1991; Lun et al. 1992). This is expected of

trypanosomes unable to develop cyclically in Tabanid flies, the insect vectors of *T. evansi*. Function-wise, respiratory processes of Krebs cycle and oxidative phosphorylation do not occur in the mitochondrion of these trypanosomes. Furthermore, although three putative guide RNA (gRNA) genes have been found in their minicircles (Gajendran et al. 1992), the lack of maxicircles sheds some doubt in the role of the minicircles in RNA editing. What is then the function of a minicircular kDNA network in *T. evansi*? The most plausible answer is a structural role in cell division (Cosgrove 1973; Borst and Fairlamb 1976; Steinert et al. 1976; Borst and Hoeijmakers 1979a, b).

The kDNA network is speculated to facilitate the division and segregation of the mitochondrion and its genome during cell division. We sought to establish any biological significance of the complete loss of the kDNA by determining the growth rate of both kinetoplastic and akinetoplastic clones under normal conditions as well as under pararosaniline supression. If the kDNA functions during cell division, then the rate of growth of kinetoplastids is expected to be faster under normal conditions and slower as pararosaniline, which selectively affects kDNA, increases in dosage; whereas the akinetoplastic forms should have a slower rate under normal conditions and a constant growth rate under pararosaniline treatment considering that they lack kDNA. We obtained the results expected under normal conditions and dissimilar results under drug supression such that the kinetoplastic population doubled at a relatively constant rate while marked retardation in the growth rate of akinetoplastic trypanosomes was observed (Fig. 5). Increasing pararosaniline dosage resulted to a moderate increase in the number of akinetoplastic forms in the kinetoplastic population. These data indicate that (1) the primary target of pararosaniline is indeed the kDNA resulting to production of akinetoplastic forms and (2) the kinetoplastid always overcomes the akinetoplastic parasite in a given population. These results agree with those of Inoki et al. (1962). On the other hand, in kDNA-deficient mutants, the dye exerts its effects upon other targets in the cell such as the nuclear DNA. As other drugs, there is a certain amount of pararosaniline required to affect the kDNA thereby increasing akinetoplastic population. In accordance with Inoki et al. (1960), the multiplication potential of akinetoplastic forms is retained despite the absence of kDNA implying that kDNA is not vital for the existence of these organisms. However, the rate at which they double is generally slower (by 16-18% in normal conditions or 18-60% under drug supression) than normal trypanosomes. This shows that although the fundamental function of multiplication is retained in akinetoplastic forms, they grow at a slower rate and are, therefore, dominated by their kinetoplastic counterparts in a given population. This may be one of the possible factors that significantly account for their inferiority to those with kDNA and for their selective disadvantage in nature. However, since kDNA mutation is irreversible and stable (Hajduk 1978; Kallinikova 1981), it is possible to select for the akinetoplastic parasites albeit their moderate number.

Light microscopy has demonstrated the proximity of the kDNA to the basal body of the flagellum (Vickerman and Preston 1976) whereas transmission electron microscopy has shown the presence of kinetoplast fibrils connecting to the inner mitochondrial membrane adjacent to the basal body (De Souza 1984). Weinman et al. (1984) and Takeo (1987) have also demonstrated by electron microscopy a microtubular connecting system between the flagellum and the mitochondrion of *T. lucknowi* and *T. evansi*, respectively. Furthermore, we have recently localized *T. cruzi* minicircles at the mitochondrial membrane juxtaposed to the basal body of the flagellum by in situ hybridization (described elsewhere). Notwithstanding, Robinson and Gull (1991), not only demonstrated in *T. brucei* the connection of the kDNA to the basal body but also provided evidence on the dependence of kDNA separation upon movement of basal bodies. Hence, based on these premises and if we assume that the basal body acts as a centriole and that a physical relationship exists between the flagellar apparatus and the mitochondrion, the kDNA network which is adjacent to the mitochondrial membrane facing the basal body may facilitate the proper division and segregation of the unitary mitochondrion of trypanosomes regardless of the presence or absence of maxicircles. We may, therefore, infer that the kDNA minicircles play a role in mitochondriogenesis and that its absence somehow impedes mitochondrial biogenesis thereby consequently delaying normal mitotic processes. It seems probable that in *T. evansi*, after pararosaniline has interacted with the kDNA followed by its loss, mitochondriogenesis in resultant akinetoplastic forms is not efficient enough for the parasites to multiply at the same rate as the kinetoplastids.

Based on published reports on related studies and our results put together, we, thus, propose the following: the kinetoplastic cell takes up pararosaniline at the flagellar pocket where endocytosis is documented to active (Vickerman and Preston 1976). Due to the strategic location of the kinetoplast, the drug is transported through the mitochondrial membrane followed by its interaction with pararosaniline-sensitive AT-rich regions in the kDNA. The effect of the drug on kDNA eventually results to fragmentation followed by a progressive decrease in its size. "All or none" division of the parent cell takes place when the smallest kDNA size, unable to divide, is achieved, thus, producing a normal and a mutant daughter cell. The effect of pararosaniline on the nuclear DNA is unknown. However, in lieu of the existence of a kinetoplast-flagellum complex, mutant cells without a kDNA network have slow division and segregation of their unitary mitochondrion (mitochondriogenesis), retarded cytokinesis, and, hence, delayed doubling time. It is perhaps because of this feature why the akinetoplastic trypanosomes cannot be selected in nature. Conversely, kinetoplastic *T. evansi* with intact kDNA minicircles is preadapted to survive.

We have demonstrated in the present paper the dynamics of the kDNA of *T. evansi* including its progressive reduction in size prior to loss after pararosaniline treatment. We have also shown the growth limitation of kDNA-deficient akinetoplastic parasites that may possibly account for their selective

disadvantage in nature. However, further studies on the mechanism of action of pararosaniline are necessary to render insights on its molecular effects on the kDNA as well as nuclear DNA of trypanosomes and the evolution and nature of transient intermediate kDNA forms.

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