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Effect of Berenil on the Kinetoplast of *Trypanosoma gambiense* Pararosaniline Sensitive and Resistant Clone in Mice

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Abstract

After the inoculation of Berenil into *Trypanosoma gambiense* infected mice, dyskinetoplastic forms appeared as a result of inhibition of kinetoplast duplication without any affect on nuclear and cytoplasmic duplication. The present study demonstrated that in some dividing forms with a nucleus appeared after the treatment with Berenil a flagellum in which the kinetoplast can not be seen near to it, was observed at the posterior end of the parasite and one kinetoplast with or without a flagellum migrated anteriorly. That is, a dividing form containing one kinetoplast, one nucleus and two flagella (1K1N2F), and a dividing form containing one kinetoplast, one nucleus and a flagellum (1K1N1F) were observed. In the latter, the kinetoplast without a flagellum migrated close to the nucleus or anteriorly far from it. Such trypanosomes appeared in the original clone after the treatment, but, not in the *p*-rosaniline resistant clone. We have never seen such migration of the kinetoplast without a flagellum in trypanosomes treated with various chemicals except for Berenil in the present study. The present study also indicated that the original clone was more sensitive than the *p*-rosaniline resistant clone to the effect of Berenil in inhibiting kinetoplast division.

Key words: Berenil, dyskinetoplastic form, kinetoplast migration, *Trypanosoma gambiense*

Introduction

Many chemicals interacting with DNA, such as acriflavine, pararosaniline, ethidium bromide, hydroxystilbamidine and furazolidon are known to be trypanocidal and to induce dyskinetoplastic trypanosomes and the deformation in the ultrastructure of the kinetoplast (Cavalieri and Angelos, 1950; Inoki, 1956; Neville and Davies, 1966; Le Pecq and Paoletti, 1967; Ono and Inoki, 1971, 1973 and 1975). These chemicals have no effect on nuclear and cell division at the concentrations that induce dyskinetoplastic forms.

Berenil (Diminazene aceturate, Hoechst, Germany) shows a preferential binding to kinetoplast DNA and inhibits DNA synthesis in *Trypanosoma mega* (Newton and Le Page, 1967). Therefore, when Berenil was inoculated into animals infected with various *Trypanosoma* species, *T. evansi, T. equiperdum, T. congolense* and *T. brucei*, dyskinetoplastic forms appeared in the peripheral blood (Killick-Kendrick, 1964: Riou and Benard, 1980: Chitambo et al., 1992). But, analysis of formation of dyskinetoplastic forms has not been attempted. Chemicals inducing dyskinetoplastic forms, such as *p*-rosaniline, hydroxystilbamidine and Berenil do not intercalate into DNA, but show a preferential binding to kinetoplast DNA. Therefore, there may be the interaction between effect of *p*-rosaniline and one of Berenil on trypanosomes. In the present studies, we examined morphologically the mode of formation of dyskinetoplastic forms and a difference of effect on these clones after injection of Berenil into mice infected with *T. gambiense* of *p*-rosaniline sensitive original and resistant clones.

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Materials and Methods

Trypanosoma sp.

The Wellcome strain of *Trypanosoma gambiense*

was used in the present study. The strain was derived from Dr. H. Takayanagi in 1989 (Department of Medical Zoology, School of Medicine, Nagoya City University). Since then it has been maintained in our laboratory by serial passages in mice and preserved by 10% DMSO in 0.01 M PBS (pH 7.2) in liquid nitrogen. Two clones of the Wellcome strain were used in the present study. One was the original clone (hereafter WS) and the other was the clone WR isolated from WS treated with 50 μg *p*-rosaniline per g mouse body weight. WS was sensitive to *p*-rosaniline, ethidium bromide and acriflavine, eliciting about 25% of dyskinetoplastic form (AK) 4 hr after intraperitoneal injection with these chemicals into infected mouse. WR which can still grow after injection of as much as 50 μg *p*-rosaniline/g mouse body weight, do not produce dyskinetoplastic trypanosomes even by a dose of 10 μg *p*-rosaniline/g body weight. WR was obtained from WS repeatedly treated with *p*-rosaniline (Inoki and Matsushiro, 1959). The WR line has been maintained by serial passages in ICR mice and preserved by 10% DMSO in 0.01 M PBS in liquid nitrogen for more than three years. Division of trypanosomes begins in the basal body. And then, it is followed by binary fission of the kinetoplast, and later by division of the nucleus and cytoplasm. Therefore, trypanosomes of WS and WR before the treatment with Berenil are undivided forms with one kinetoplast and one nucleus (1K1N), dividing forms with two kinetoplast and one nucleus (2K1N) and dividing forms with two kinetoplasts and two nuclei (2K2N). ICR mice weighing approximately 30 g were used in the present study. The mice were kept in the conventional condition.

Infection of mice, treatment and preparation of blood smears

When trypanosomes had reached a level of 5×10^8 trypanosomes/ml blood stream in ICR mice 3 days after intraperitoneal inoculation with approximately 1×10^5 trypanosomes, the mice were injected intraperitoneally with 10 μg/g of Berenil. Blood samples were taken at 1, 2, 3, 4 and 8 h after treatment. They were stained with Giemsa after hydrolysis with 1N HCl at 60°C for 2 min and examined under a light microscope. The numbers of trypanosomes of various forms were counted on 1,000 randomly selected parasites in each stained blood smear. All values were given as means± standard deviation of six replicate experiments.

Results

For analysis of effect of Berenil on WS and WR, the numbers of appearance of trypanosomes of various forms were examined at intervals after injection of 10 μg/g of Berenil into infected mice (Table 1). Figures 1 and 2 show dividing forms of untreated trypanosomes. When two kinetoplasts in 2K1N separated, a flagellum is always observed near to each kinetoplast (Fig. 1). Two kinetoplasts in 2K2N are seen posteriorly from two nuclei (Fig. 2). After the treatment with Berenil, however, trypanosomes of various forms appeared as shown in Figs. 3–6. In Table 1, the rate of appearance of trypanosomes of various forms is almost the same in both clones before the treatment with Berenil. The number of 2K1N in WS decreased greatly with the lapse of time after the treatment with Berenil, while that in WR till 4 hr did not decrease so much as in WS. The number of 2K2N also decreased in WS, but no decrease in number of this type are seen by 4 hr in WR. Dividing form with one kinetoplast and two nuclei (1K2N) are seldom produced in both clones before the treatment. But, the number of this type in WS increased 2 hr after the treatment. The increase in WR by 4 hr was not so much as in WS. Dyskinetoplastic form without the kinetoplast (Fig. 3) increased 1 hr in WS after the treatment and then increased remarkably with the lapse of time. While no increase was observed in WR by 4 hr after the treatment. The decrease in the numbers of 2K1N and 2K2N and the increase in those of 1K2N and of dyskinetoplastic forms which were demonstrated by 2 hr in WS, were observed 8 hr in WR after the treatment. The number of dyskinetoplastic forms in WS was greater than in WR. Anucleate forms with one kinetoplast (1KAN) were a few observed in both clones.

In some dividing forms with one nucleus appeared after the treatment with Berenil, one flagellum in which the kinetoplast can not be seen near to it, was observed at the posterior end of the parasite and one kinetoplast with or without a flagellum migrated anteriorly. That is, 1K1N with two flagella (1K1N2F, Fig. 4) and 1K1N with one flagellum (1K1N1F)

Table 1 Changes in the numbers of trypanosomes of various forms* appeared after injection of 10 μg/g Berenil into mice infected with *T. gambiense* of *p*-rosaniline sensitive and resistant clones

Forms	Hours after Berenil treatment					
	0	1	2	3	4	8
1K1N†	810.1 ± 38.2#	828.1 ± 33.5	789.8 ± 19.9	712.1 ± 42.6	658.6 ± 49.5	622.8 ± 59.1☆
	808.8 ± 18.4	839.5 ± 25.9	855.5 ± 42.8	837.2 ± 20.4	825.7 ± 23.1	739.8 ± 24.7★
2K1N‡	113.0 ± 21.9	74.1 ± 17.1	31.3 ± 10.5	25.8 ± 11.6	26.0 ± 13.1	28.0 ± 12.6
	116.0 ± 14.0	88.8 ± 18.4	83.6 ± 27.6	75.1 ± 10.6	54.1 ± 12.4	18.6 ± 10.1
2K2N§	64.5 ± 19.2	63.3 ± 16.6	36.0 ± 14.9	28.8 ± 10.4	30.3 ± 15.6	17.1 ± 6.5
	59.0 ± 6.4	59.3 ± 12.5	42.6 ± 16.3	49.1 ± 16.4	51.2 ± 9.3	20.6 ± 7.3
1K2N‖	0.8 ± 1.3	2.1 ± 1.9	16.0 ± 8.2	40.8 ± 16.2	43.1 ± 7.8	7.5 ± 5.2
	0.3 ± 0.5	0.6 ± 1.2	1.1 ± 1.1	4.5 ± 3.9	10.2 ± 5.7	46.8 ± 12.3
AK1N¶	8.8 ± 3.5	18.3 ± 4.2	93.0 ± 11.4	147.3 ± 18.6	179.5 ± 45.8	263.1 ± 54.8
	16.0 ± 4.3	10.0 ± 4.7	12.1 ± 4.7	16.8 ± 4.2	22.1 ± 7.3	162.8 ± 15.2
AK2N**	0.5 ± 0.8	0.5 ± 0.5	1.0 ± 0.6	1.3 ± 1.0	1.5 ± 1.8	9.1 ± 7.2
	0.3 ± 0.8	0.3 ± 0.5	0.3 ± 0.5	0	0.1 ± 0.3	2.5 ± 2.0
1K1N1F††	0.3 ± 0.8	4.1 ± 2.3	12.1 ± 6.1	10.6 ± 3.5	8.3 ± 3.0	48.6 ± 14.9
	0.2 ± 0.4	0	0	0.1 ± 0.3	0	1.5 ± 1.2
1K1N2F§§	0.3 ± 0.8	3.0 ± 2.3	19.0 ± 8.2	47.6 ± 25.9	53.3 ± 21.5	7.6 ± 3.0
	0	1.0 ± 2.0	2.5 ± 2.1	9.3 ± 7.8	33.4 ± 14.3	8.3 ± 4.1
1KAN◇	0.2 ± 0.4	0.2 ± 0.4	0	0	0	0.6 ± 1.0
	0	0	0	0.1 ± 0.3	0.2 ± 0.4	1.6 ± 1.9

*The numbers of trypanosomes of various forms are counted on 1,000 randomly selected parasites. †Undividing form with one kinetoplast and one nucleus. ‡Dividing form with two kinetoplasts and one nucleus. §Dividing form with two kinetoplasts and two nuclei. ‖Dividing form with one kinetoplast and two nuclei. ¶Dyskinetoplastic form with one nucleus. **Dyskinetoplastic form with two nuclei. ††Dividing form with one nucleus and one kinetoplast in which a flagellum is not observed near to it. §§Dividing form with one nucleus and one kinetoplast in which a flagellum is observed near to it. ◇Anucleate form with one kinetoplast.

#All values are given as means ± standard deviation of six replicate experiments.

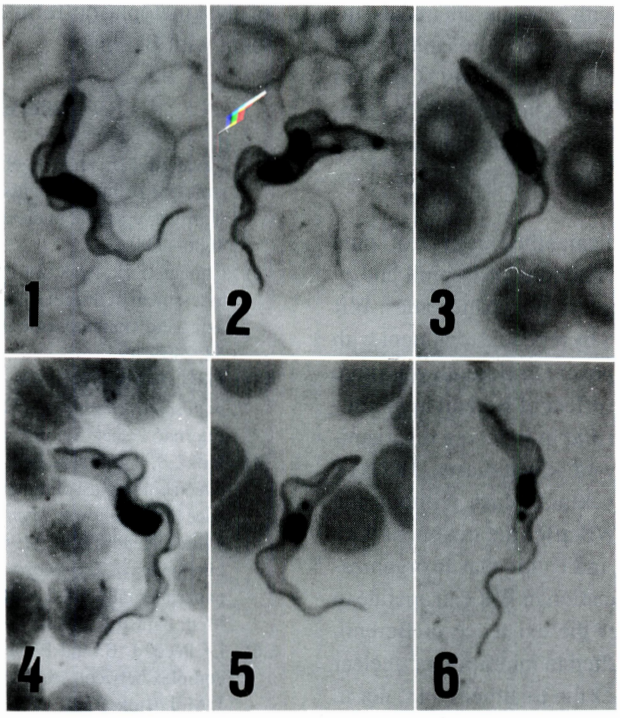
☆ The upper line in each form corresponds to WS. ★ The lower line corresponds to WR.

were observed. Such dividing forms increased with the lapse of the time. In 1K1N1F, the kinetoplast without a flagellum was seen close to the nucleus (Fig. 5) or anteriorly far from it (Fig. 6). These forms increased in WS after the treatment, but, not in WR.

Discussion

We have demonstrated that in *T. gambiense* treated with many kinds of dyskinetoplastic form inducing substances dyskinetoplastic forms were produced by the selective inhibition of kinetoplast duplication without any affect on nuclear and cytoplasmic duplication (Inoki 1956; Ono and Inoki 1971, 1973, 1975).

In the present study, the total number of trypanosomes having two nuclei, such as 1K2N, 2K2N and AK2N within 4 hr in WS and 8 hr in WR after treatment with Berenil was about the same as the number of trypanosomes having two nuclei before the treatment in these clones. This finding suggests that Berenil resulted in the production of



Figs. 1 and 2 Lightmicrographs showing a dividing form of WS untreated with Berenil. ×1,600

Figs. 3–6 Lightmicrographs showing trypanosomes obtained 4 hrs after injection of 10 μg/g of Berenil into mice infected with WS (all Figs. except for Fig. 4) or WR (Fig. 4). ×1,600

Figs. 1 and 2 When two kinetoplasts separated, a flagellum is already observed near to each kinetoplast. Two kinetoplasts are seen posteriorly from two nuclei.

Fig. 3 Dyskinetoplastic form. Figs. 4–6. Trypanosomes with one kinetoplast and one nucleus with two flagella (Fig. 4), and with one kinetoplast and one nucleus with a flagellum are observed. In the latter, the kinetoplast is seen close to the nucleus (Fig. 5) or anteriorly far from it (Fig. 6).

dyskinetoplastic forms without any affect on nuclear division. In 8 hr after the treatment, the total number of trypanosomes having two nuclei in WS was less than half of that in WR. It means that the nuclear division is inhibited in WS 8 hr after the treatment, but, not in WR.

The present study reported that in 1K1N1F appeared after the treatment with Berenil in WS, one kinetoplast without a flagellum was observed close to the nucleus or anteriorly far from it. In trypanosome replication, the daughter basal bodies separate before the kinetoplast, which has just divided, begins to separate, and then the nucleus and cytoplasm divide successively. Therefore, disorder in formation of basal body due to inhibition or delay of kinetoplast division might result in appearance of trypanosomes having the kinetoplast without a flagellum. *p*-rosaniline, hydroxystilbamidine and Berenil do not intercalate, but show a preferential binding to kinetoplast DNA and produce the dyskinetoplastic forms. Therefore, there may be a similarity in the effect of these chemicals on trypanosomes. However, we have never seen such migration of kinetoplast without a flagellum in

trypanosomes treated with various chemicals including hydroxystilbamidine and *p*-rosaniline and except for Berenil in the present study (Ono and Inoki, 1971, 1973, 1974, 1975; Ono and Nakabayashi, 1978, 1979, 1980, 1987), suggesting that the action mechanism of Berenil differs at least in a part from those of other chemicals.

The present study indicates the existence of some relationship between the actions of Berenil and *p*-rosaniline on the kinetoplast of trypanosomes. WR which obtained from WS by repeated treatment with *p*-rosaniline without exposure to Berenil, was more resistant than WS to the effect of Berenil in inhibiting kinetoplast division and in inducing disorganization and abnormality of kinetoplasts. 1K1N1F showing migration of kinetoplast without the flagellum appeared in WS after the treatment with Berenil, but, not in WR, indicating that an organization of kinetoplast duplication in WR can operate to some extent even under the existence of Berenil. The chemicals also resulted in inhibition of nuclear division in WS 8 hr after the treatment, but, not in WR, suggesting the existence of a relationship between the actions of Berenil and *p*-rosaniline on the nucleus as well as the kinetoplast of trypanosomes. Studies on the effect of DNA synthesis inhibitor and DNA interacting chemicals on trypanosome is useful to clarify differences in the mechanism of DNA synthesis in the nucleus and the kinetoplast of trypanosomes.

It is interesting to examine whether dyskinetoplastic forms appeared after the treatment with berenil are able to multiply or not. Treatment of infected mice with 10 μg Berenil/g body weight induced the high percentage of the dyskinetoplastic forms and caused a temporary clearance of parasite followed by relapsing parasitemia. Although not shown in the data, the percentage of dyskinetoplastic forms in relapsing mice always returned to the initial level prior to the treatment with Berenil. After the treatment of infected mice with Berenil, the selection of kinetoplastic forms as a result of the extinction of dyskinetoplastic forms and the conversion from dyskinetoplastic form to kinetoplastic form due to restoration of the kinetoplast DNA might occur in the latent period in which trypanosomes can not be seen in the peripheral blood. Therefore, the further studies are necessary to clarify multiplicability of dyskinetoplastic form appeared after the treatment with Berenil.

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