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Effects of AK-form Inducing Substances on Mitomycin-C treated *Trypanosoma gambiense* and *Trypanosoma evansi* in Mice

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Introduction

Many investigators have demonstrated the presence of DNA in the kinetoplast of trypanosomes by Feulgen staining, radioautography, the formation of satellite band in CsCl density gradient centrifugation and the rupture of isolated kinetoplast by osmotic shock (Steinert and Steinert, 1962; Du Buy *et al.*, 1965; Inoki and Ono, 1969; Ozeki *et al.*, 1970; Ono *et al.*, 1971).

It is also known that the drugs inhibiting DNA synthesis or binding DNA, such as acriflavine (Neville and Davies, 1966), *p*-rosaniline (Cavalier and Angelos, 1950) and ethidium bromide (Le Pecq and Paoletti, 1967), show the trypanocidal action or the induction of the organisms devoid of kinetoplast (Akinetoplastic form or dyskinetoplastic form, hereafter referred to as AK form) by the inhibition of kinetoplast duplication (Werbitzki, 1910. Inoki, 1956. Sakamoto, 1963). Inoki and Matsushiro (1959) showed that the rate of appearance of AK form in mice after *p*-rosaniline treatment (AK induction test) can be employed to determine the inheritability and the degree of *p*-rosaniline resistance of the trypanosomes.

We are interested in testing the effects of compounds having the ability to interact with trypanosomal DNA. Previously, we examined the chemotherapeutic effect of Furazolidon in *T. gambiense* infected mice. Furazolidon was found to show therapeutic effect against both *p*-rosaniline sensitive and resistant clones, but apparently more effective against sensitive clone (Ono and Inoki, 1973). It was further found that in Furazolidon resistant clone obtained by treatment with Furazolidon, the percentage of AK forms induced by *p*-rosaniline was lower than that in the original clone (unpublished data).

Mitomycin-C which is an antitumor substance inhibits DNA synthesis in *Escherichia coli* just like a derivative of Furan (Shiba *et al.*, 1958; Endo *et al.*, 1963). Moreover, this antibiotic is able to bind DNA (Iyer and Szybalski, 1963) and to inhibit the genetic transformation of *p*-rosaniline resistance in *T. gambiense* (Inoki *et al.*, 1960).

In the present paper, the first experiment was done to observe if the survival days of infected mice could be prolonged by treatment with Mitomycin-C after the appearance of *T. gambiense* and *T. evansi* in the circulating blood.

In the second experiment, to examine the interaction between effects of the AK form inducing substances and Mitomycin-C on the kinetoplast of trypanosomes, the AK induction test in the clones of both species obtained by repeated treatments with Mitomycin-C was undertaken by the use of AK form inducing substances.

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Materials and Methods

The *Trypanosoma gambiense* (Wellcome

strain) and *Trypanosoma evansi* (Taiwan strain) employed in this study, were the same as those used in previous work (Inoki, *et al.*, 1961). Both species have been maintained in this laboratory by serial passages through ddo mice.

The counting of AK form was carried out according to the methods of Inoki (1956). The AK induction test by Inoki and Matsushiro (1959) was employed to determine degree of drug resistance of the parasites in mice.

The original clones of *T. gambiense* and *T. evansi* were sensitive to *p*-rosaniline, ethidium bromide and acriflavine, eliciting about 29 % of AK forms by the AK induction test (Inoki, 1956; Sakamoto, 1963).

The therapeutic effect of Mitomycin-C on mice was examined as follows: Mice inoculated with *T. gambiense* or *T. evansi* were used in groups of 8 to 12. Forty-eight hours after inoculation, the mice showing parasitemia were intraperitoneally given with 1.0–7.2 mg/kg Mitomycin-C and the number of survival days was recorded. The blood of the treated and untreated mice was examined microscopically for the presence of trypanosomes.

An attempt to obtain Mitomycin-C resistant clones of *T. gambiense* and *T. evansi* was done as follows: Mice inoculated subcutaneously with about 25,000 trypanosomes were immediately treated intraperitoneally with 1 mg/kg of Mitomycin-C. The parasites were transferred to new mice, when trypanosomes appeared in the peripheral blood of these mice three or four days after the infection. This procedure was repeated 10 times and then clones were isolated by the single cell isolation technique (Inoki, 1960).

The AK induction test in mice infected with Mitomycin-C treated clones was performed by intraperitoneal injection of *p*-rosaniline, ethidium bromide and acriflavine at a dose of 10 mg/kg in all cases except for 1 mg/kg of acriflavine in *T. gambiense*.

The AK induction test with the original and *p*-rosaniline resistant clones (Inoki and Matsushiro, 1959; Ono and Inoki, 1971) in infected mice was also performed as mentioned above to serve as control.

Mitomycin-C was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and acriflavine from Eli Lilly Research Laboratories (Indiana, U.S.A.). *P*-rosaniline was purchased from Chroma Geselschaft Schmid & Co. (Stuttgart-Unterturkheim) and ethidium bromide from Sigma Chemical Company (St. Louis, U.S.A.).

Results

Table 1 shows the effect of Mitomycin-C on mice infected with *T. gambiense*. All the untreated controls died of the infection on the fourth and fifth day. In Mitomycin-C treated mice, a slight delay in death was observed only at high doses more than 4.5 mg/kg. The cause of death in 3 of 13 mice treated with Mitomycin-C, three in dosage of 7.2 mg/kg, may be due to the toxicity of this drug.

Table 2 shows the effect of Mitomycin-C on mice infected with *T. evansi*. Doses of 1 to 4.5 mg/kg Mitomycin-C did not cause a delay in the death as compared with the control.

As mentioned above, Mitomycin-C does not show any therapeutic effect on mice infected with *T. gambiense* and *T. evansi*. The present experiment also shows that Mitomycin-C does not have the ability to induce the AK forms.

Table 3 shows the rates of appearance of AK forms in the original *p*-rosaniline sensitive clones, resistant clones and Mitomycin-C treated clones in *T. gambiense* and *T. evansi*. As shown in the table, the percentage of AK forms visible before dye injection in mice infected with each of all clones of *T. gambiense* did not exceed 1 % and the percentage with the all clones of *T. evansi* is about 5 %.

The parasite in all clones of both species appeared in the peripheral blood of mice at about the same time after inoculation. It is probable that the growth rate of these clones is almost the same. The rate of appearance

Table 1 The effects of Mitomycin-C administered after appearance of the parasite in the blood on survival of mice infected with *T. gambiense*.

Species	Doses /kg mice	Degree of infection	Day after the injection with Mitomycin-C										
			1	2	3	4	5	6	7	8	9	10	11
T. gambiense	7.2 mg	+	−	−	±	D							
	7.2 mg	+	−	−	−	−	−	+	D				
	7.2 mg	+	−	+	+	D							
	7.2 mg	+	−	−	+	##	D						
	7.2 mg	+	−	−	+	##	###	D					
	7.2 mg	+	+	##	D								
	4.5 mg	+	−	##	###	###	D						
	4.5 mg	+	−	+	##	##	###	D					
	4.5 mg	+	−	##	##	D							
	4.5 mg	+	+	##	###	D							
	1.0 mg	+	##	##	D								
	1.0 mg	+	+	+	##	D							
	1.0 mg	+	+	##	##	D							
	untreated	+	##	D									
		+	##	D									
		+	##	###	D								
		+	##	D									
		+	+	###	D								
		+	+	##	D								
		+	+	###	D								
		+	##	###	D								

The degree of infection was determined by the number of parasites which appeared in the blood before Mitomycin-C treatment.

−; trypanosomes are not found in 10 fields under the microscope (×400) +; less than one parasite per one field. ##; 2~10 parasites. ###; 11~50 parasites. ####; more than 51 parasites. D; Death

of AK forms after the AK induction with *p*-rosaniline, ethidium bromide and acriflavine was about 30 % in the original clones of the both species used as control. Moreover, the AK induction test with ethidium bromide and acriflavine in *p*-rosaniline resistant clones of *T. gambiense* and *T. evansi* bring about the same rate of appearance of AK forms as in control. However, in the clones of the both species treated with Mitomycin-C, the rate was low as being 16 to 21 % after the AK induction with *p*-rosaniline and ethidium bromide. On the other hand, the rate of appearance of AK forms induced with acriflavine was as high as 30 %, almost the same as control.

Discussion

Mitomycin-C is one of the most effective antitumor substances which exerts a strong inhibitory activity against DNA synthesis of microorganisms such as bacteria, virus and protozoa (Shiba *et al.*, 1958; Bent-Porat, 1961).

The drugs such as *p*-rosaniline, ethidium bromide and acriflavine cause the increase of AK forms and inhibit cell division in trypanosomes. They interact with DNA by way of binding with DNA and inhibit DNA

Table 2 The effects of Mitomycin-C administered after appearance of the parasite in the blood on survival of mice infected with *T. evansi*

Species	Doses /kg mice	Degree of infection	Day after the injection with Mitomycin-C										
			1	2	3	4	5	6	7	8	9	10	11
T. evansi	4.5 mg	+	+	##	###	D							
	4.5 mg	+	+	+	##	###	D						
	4.0 mg	+	+	+	+	##	###	D					
	4.0 mg	##	##	##	###	D							
	4.0 mg	+	+	+	+	##	###	D					
	2.0 mg	+	+	−	+	##	###	D					
	2.0 mg	+	+	+	−	##	D						
	2.0 mg	+	+	###	D								
	1.0 mg	+	+	##	D								
	1.0 mg	+	##	##	D								
	1.0 mg	+	##	###	D								
	1.0 mg	+	+	##	###	D							
	untreated	+	##	###	D								
		+	##	##	D								
		+	##	D									
		+	+	##	##	D							
		+	+	##	###	D							
		+	##	##	D								
		+	##	###	D								
		+	##	##	D								

The degree of infection was determined by the number of parasites which appeared in the blood before Mitomycin-C treatment.

− ; trypanosomes are not found in 10 fields under the microscope (×400) + ; less than one parasite per one field. ## ; 2～10 parasites. ### ; 11～50 parasites. ### ; more than 51 parasites. D ; Death

synthesis.

Sakamoto (1963) examined the effect of some antitumor substances on the induction of AK forms in *T. evansi*, but he could not find any effect with Mitomycin-C.

In the present experiment, also we found that Mitomycin-C has no ability to induce the AK form of *T. gambiense* and *T. evansi* in mice.

We also found that Mitomycin-C can not prolong the survival days even if such a high dosage as LD_{50} (Taguchi, 1967) of mice was employed. However, it was clearly demonstrated that the clones of both *T. gambiense* and *T. evansi* obtained by repeated treatment with Mitomycin-C were not highly affected by *p*-rosaniline and ethidium bromide. It is not clear that such clones have resistant to Mitomycin-C or not, because the degree of the resistance to Mitomycin-C in the clones can not be examined by the AK induction test and therapeutic experiment. However, the present experiment shows that the effect of Mitomycin-C on the trypanosomes can be detected by the use of AK form inducing substances and consequently Mitomycin-C was proved to possess some effects on the inhibition of kinetoplast duplication with *p*-rosaniline and etihidium bromide.

The *p*-rosaniline resistant clones of *T. gambiense* and *T. evansi* are easily isolated in mice (Inoki and Matsushiro, 1959; Ono

Table 3 The rate of appearance of AK forms in 3 different clones of *T. gambiense* and *T. evansi* in mice before and after treatment with AK form inducing substances.

Species	clone	Rate of appearance of AK forms (%)					
		before the AK induction test	after the AK induction test with AK-form inducing substances				
			p-rosaniline		ethidium bromide		acriflavine
T. gambinse	WS	0.5[a]	0.3≤m≤0.7[b]	29.4	28.3≤m≤30.5	30.3	29.5≤m≤31.1	30.7	29.5≤m≤31.9
	WR	0.6	0.3≤m≤0.9	15.9	14.6≤m≤17.2	30.3	29.0≤m≤31.6	29.7	28.9≤m≤30.5
	WM	0.4	0.1≤m≤0.7	17.6	15.7≤m≤19.5	21.1	20.1≤m≤22.1	29.6	28.9≤m≤30.3
T. evansi	TS	5.0	4.6≤m≤5.4	28.9	27.6≤m≤30.2	30.2	28.0≤m≤32.4	31.3	30.2≤m≤32.4
	TR	5.2	4.0≤m≤6.4	12.1	11.3≤m≤12.9	30.4	29.4≤m≤31.4	29.9	28.9≤m≤30.9
	TM	4.9	3.4≤m≤6.4	16.6	15.6≤m≤17.6	19.5	17.9≤m≤21.1	30.1	28.9≤m≤31.3

WS; *T. gambiense* *p*-rosaniline sensitive clone, WR; *T. gambiense* *p*-rosaniline resistant clone, WM; clone obtained from WS after treatment with Mitomycin-C, TS; *T. evansi* *p*-rosaniline sensitive clone, TR; *T. evansi* *p*-rosaniline resistant clone, TM; clone obtained from TS after treatment with Mitomycin-C. a; mean value, b; 99% reliability.

and Inoki, 1971). However, all attempts to make phenanthridinium resistant clone have been unsuccesful with one exception (Hawking, 1963) in which a clone of *T. congolense* resistant to prothidium was obtained. We also failed in getting ethidium bromide resistant *T. gambiense* (unpublished data). Nevertheless, it seems interestng that treatment with Mitomycin-C without exposure to *p*-rosaniline and ethidium bromide reduces the effectiveness of such dyes to induce AK form in *T. gambiense* and *T. evansi*. The same results were obtained with Furazolidon treatment of mice infected with *T. gambiense* (unpublished data). Therefore, the clones treated with the inhibitory compounds of DNA synthesis such as Nitrofurazone derivative and Mitomycin-C may have been changed to decrease the induction of AK forms by *p*-rosaniline and ethidium bromide. The result obtained from these experiments is, therefore, indicative of a close relationship between the effects of DNA synthesis inhibiting substances and AK form inducing substances on the kinetoplast duplication. The analysis of effects of the inhibitors of DNA synthesis on the AK form induction by AK form inducing substances may be useful to clarify the mechanism of DNA synthesis in the kinetoplast of trypanosomes.

In the AK induction with ethidium bromide, the rate of AK forms in *p*-rosaniline resistant clones of *T. gambiense* and *T. evansi* is the same as that in *p*-rosaniline sensitive clone. This result means that both clones are sensitive to ethidium bromide.

However, Inoki (1956) and Sakamoto (1963) found that *p*-rosaniline and ethidium bromide are capable of inducing AK form from kinetoplastic ones (K form) in equivant concentration with the same mode of action in both *T. gembiense* and *T. evansi*. Moreover, we found that the repeated treatment with ethidium bromide of *p*-rosaniline sensitive clone of *T. evansi* causes the 100 % change of K form to AK form. In contrast, ethidium bromide does not cause such a change in *p*-rosaniline resistant clone of *T. evansi* (unpublished data). Therfore, these data may show clearly the interaction between *p*-rosaniline and ethidium bromide on trypanosomes.

Inoki *et al.*, (1960) and Ono (1966, a) observed an inhibitory action of some antitumor substances including Mitomycin-C and cartinogenic substances upon the genetic transformation of *p*-rosaniline resistance in trypanosomes. Their experiments show the action of cartinogenic substances and an-

titumor substances on trypanosomal DNA. In the present experiment, the result of the AK induction test by *p*-rosaniline in the clones treated with Mitomycin-C may support the inhibitory effect of Mitomycin-C on the genetic transformation of *p*-rosaniline resistance in *T. gambiense* reported by Inoki *et al.* (1960).

But, Ono (1966, b) found that the clones of *T. gambiense* obtained by the repeated treatment with cartinogenic substances (3′-C1-DAB, 4′-OCH_3-MAB) are sensitive to *p*-rosaniline. Moreover, such clones are unable to acquire *p*-rosaniline resistance even by the repeated treatment with *p*-rosaniline. This results possibly indicate that cartinogenic substances do not react with trypanosomal DNA like as Mitomycin-C in the present experiment. Therefore, the further studies are necessary to clarify the relations between the actions of antitumor and cartinogenic substances on the kinetoplast of trypanosomes.

Summary

We examined the effect of Mitomycin-C on the prolongation of survival day of mice infected with *Trypanosoma gambiense* and *Trypanosoma evansi*. It was revealed that Mitomycin-C can not prolong the survival day even if a dosage as high as LD_{50} for mice was employed. We have demonstrated, however, that the induction of AK form in the clones of both *T. gambiense* and *T. evansi* obtained by repeated treatment with Mitomycin-C were not highly affected by *p*-rosaniline and ethidium bromide which have marked AK form inducing activity for trypansosmes.

References

1) Bent-Porat, T. (1961) : Effect of Mitomycin-C on the synthesis of infective virus and deoxyribonucleic acids in pseudorabis virus-infected rabbit kidney cells. Nature, 190, 33-34.
2) Cavalieri, L. F. and Angelos, A. (1950) : Studies on the structure of nucleic acids. I. Interaction of Rosaniline with deoxypentose nucleic acid. J. Am. Chem. Soc., 72, 4686-4690.
3) Du Buy, H., Mattern, C. F. T. and Riley, F. L. (1965) : Isolation and characterization of DNA from kinetoplasts of *Leishmania enrietti*. Science, 147, 754-756.
4) Endo, H., Ishizawa, M., Kamiya, T. and Kawano, M. (1963) : A nitrofuran derivative, a new inducing agents for the phage development in lysogenic *Escherichia coli*. B. B. A., 68. 502-505.
5) Hawking, F. (1963) : Chemotherapy of trypanosomiasis in "Experimental chemotherapy" (Schnitzer, R. J., and Hawking, F. ed.). I, 129-256. 1st ed., Academic Press, New York and London.
6) Inoki, S. (1956) : Origin of the akinetoplastic strain of *Trypanosoma gambiense*. Cytologia, Supplt. Vol. (Proceedings of international Gentics symposia, Tokyo, Japan), 550-554.
7) Inoki, S. and Matsushiro, A. (1959) : Relationship between kinetoplast elimination and pararosaniline resistance in *Trypanosoma gambiense*. Biken J., 2, 371-374.
8) Inoki, S., Ono, T. and Sakamoto, H. (1960) : Effect of anti-tumor substances on the drug-resistance transformation in *Trypanosoma gambiense*. Biken J., 3, 205-207.
9) Inoki, S. (1960) : Studies on antigenic variation in the welcome strain of *Trypanosoma gambiense*. I. Improvements in technique. Biken J., 3, 215-222.
10) Inoki, S., Taniuchi, Y., Sakamoto, H., Ono, T. and Kubo, R. (1961) : Interspecific transformation of drug-resistance between *Trypanosoma gambiense* and *Trypanosoma evansi*. Biken J., 4, 111-119.
11) Inoki, S. and Ono, T. (1969) : Studies on the DNA synthesis in *Trypanosoma gambiense* and *Trypanosoma evansi* by means of ^3H-thymidine radioautography. Jap. J. Parasitol., 18, 217-225. (in Japanese)
12) Iyer, V. N. and Szybalski, W. (1963) : A molecular mechanism of Mitomycin action : Linking of complementary DNA strands. Proc. Nat. Acad. Sci., 50, 355-362.
13) Le Pecq, J. B. and Paoletti, C. (1967) : A fluorescent complex between ethidium bromide and nucleic acids. J. Mol. Biol., 27, 87-106.
14) Neville, D. M. and Davies, D. R. (1966) : The interaction of acridine dyes with DNA : An X-ray diffraction and optical investigation. J. Mol. Biol., 17, 57-74.

15) Ono, T. (1966, a) : Effects of cartinogenic substances on the drug resistance transformation in *Trypanosoma gambiense*. (I) Jap. J. Parasitol., 15, 175-180. (in Japanese)
16) Ono, T. (1966, b) : Effects of cartiongenic substances on the drug resistance transformation in *Trypanosoma gambiense*. (II) Jap. J. Parasitol., 15, 181-187. (in Japanese)
17) Ono, T., Ozeki, Y., Okubo, S. and Inoki, S. (1971) : Characterization of nuclear and satellite DNA from trypansosmes. Biken. J., 14, 203-215.
18) Ono, T. and Inoki, S. (1971) : Studies on the effect of *p*-rosaniline resistant *Trypanosoma evansi*. Biken J., 14, 419-422.
19) Ono, T. and Inoki, S. (1973) : Chemotherapy and akinetoplastic form induction test with Furazolidon in *Trypanosoma gambiense* infected mice. Jap. J. Parasitol., 22, 320-330.
20) Ozeki, Y., Ono, T., Okubo, S. and Inoki, S. (1970) : Electron microscopy of DNA released from ruptured kinetoplasts of *Trypanosoma gambiense*. Biken J., 13, 387-393.
21) Sakamoto, H. (1963) : Genetic studies on the mode of akinetoplastic form induction in *Trypanosoma evansi* by means of injecting mouse with some chemical agents. Jap. J. Parasitol., 12, 1-12. (in Japanese)
22) Shiba, S., Terawaki, T., Taguchi, T. and Kawamata, J. (1958) : Studies on the effect of Mitomycin-C, on nucleic acid metabolism in *Escherichia coli* B. Biken J., 1, 179-193.
23) Steinert, M. and Steinert, G. (1962) : La synthése de l'acide désoxyribonucléique au cours du cycle de division de *Trypanosoma mega*. J. Protozool., 9, 203-211.
24) Taguchi, T. (1967) : The pharmacological action of Mitomycin in "Mitomycin-its experimental research and clinical application" (Shiba, S. and Taguchi, T. ed.) 51-79. 1st ed., Igaku Shoin Ltd, Tokyo. (in Japanese)
25) Werbitzki, F. W. (1910) : Über blepharoblastlose. Zentr. Bakteriol. I. Abt. Orig., 53, 303-315.

Mitomycin-C で処理して得た *Trypanosoma gambiense* および *Trypanosoma evansi* の Clone に対する AK 型原虫誘発物質の効果

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trypanosoma 原虫の kinetoplast は DNA をもつており，DNA と結合したり，DNA 合成を阻害したりする物質の中には kinetoplast に作用して，これを失つた原虫（AK 型原虫）を誘発するものがある．この実験で使用する *p*-rosaniline, ethidium bromide および acriflavine はいずれもこのような薬剤である．Mitomycin-C は DNA と結合する性質をもつた抗腫瘍性抗生物質であり，DNA 合成も阻害する．この実験ではまず，*T. gambiense* および *T. evansi* に感染したマウスについて Mitomycin-C の治療効果を調べたが，LD_{50} と同じ量を感染マウスに注射しても延命効果はあまりみられなかつた．また，AK 型原虫誘発効果もなかつた．しかし，Mitomycin-C は *T. gambiense* の *p*-rosaniline 耐性に関する形質転換を阻害する（猪木ら，1960）ことからも，この抗生物質が trypanosoma の kinetoplast に対して何等かの作用をもつことが予想される．そこで Mitomycin-C の trypanosoma に対する作用と AK 型原虫誘発物質による AK 型原虫誘発効果との関係を調べるため，今回の実験を行なつた．このため，in vivo で *T. gambiense* と *T. evansi* を 1 mg/kg の Mitomycin-C に接触させる操作を 10 回繰返して行い，それぞれの clone を得て，それらに感染したマウスについて，*p*-rosaniline, ethidium bromide および acriflavine による AK 誘発効果を調べた．その結果，acriflavine では control すなわち，*T. gambiense*, *T. evansi* の original clone および *p*-rosaniline 耐性 clone と同じ AK 型原虫出現率をみたが，*p*-rosaniline, ethidium bromide では AK 型原虫誘発効果が抑えられ，Mitomycin-C で処理しない original clone に比べて AK 型原虫の出現率が低かつた．この成績は Mitomycin-C がこれらの薬剤による AK 型原虫誘発効果に何等かの阻止作用を示したことを意味するとともに，Mitomycin-C は *T. gambiense* および *T. evansi* に対して顕著な抗 trypanosoma 作用をもたず，また AK 型原虫誘発効果もないが，*p*-rosaniline, ethidium bromide の存在下ではその効果が検出出来ることを示している．