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Characterization of cyclically transmitted *Trypanosoma (T.) brucei* isolates from man

D. RICHNER, L. JENNI

Summary

Four different trypanosome isolates from human patients isolated in 1979 during the epidemic of sleeping sickness in Busoga, south-east Uganda, were characterized by the following methods: isoenzyme analyses of bloodstream forms by isoelectric focusing; in vitro tests of human serum resistance of bloodstream as well as metacyclic forms; tsetse fly transmission through *Glossina morsitans centralis* and *Glossina morsitans morsitans* to compare the above characteristics of parasite populations before and after the cyclical passage; the indirect immunofluorescent antibody test (IFAT) to analyse the antigenic relationship between metacyclic populations of the four different stocks. The results of the isoenzyme analyses and the in vitro tests of human serum resistance separated one stock (TH K 86) from the 3 other isolates which behaved similarly. The tsetse fly transmission did not influence the characteristics except that a temporary switch to human serum sensitivity occurred in the metacyclic population of isolate TH K 86. The indirect immunofluorescent antibody test (IFAT) revealed a close antigen relationship between the four metacyclic populations. Mice immunized against metacyclic forms of two isolates exhibited solid cross-protection against the infection with one different isolate.

Key words: *Trypanosoma (T.) brucei*; field isolates; isoenzymes; human serum resistance.

Introduction

The changing characteristic of East-African *Trypanosoma (T.) brucei* isolates with respect to man-infectivity is of fundamental importance in sleeping

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sickness epidemiology. Changing patterns of sensitivity to normal human serum of trypanosome populations have been observed by different authors (Van Meirvenne et al., 1975, 1976; Rickman and Kolala, 1979). The induction of human serum resistance among trypanosome populations initially exhibiting serum sensitivity has been achieved in syringe passaged bloodstream forms (Rickman and Kolala, 1979).

For epidemiological studies of East-African isolates, one further parameter, the isoenzyme patterns after starch gel electrophoresis of various enzymes, has been used to discriminate different trypanosome isolates from man and animals (Gibson et al., 1980; Gibson and Gashumba, 1983; Tait, 1980; Tait et al., 1985). Ebert (1982) was the first who applied isoelectric focusing as a new qualitative and quantitative method to determine the isoenzyme patterns of different *T. cruzi* isolates. The application of this technique for the characterization of *Trypanozoon* stocks has been reported by Betschart et al. (1983).

The blood incubation infectivity test, BIIT (Rickman and Robson, 1970) distinguishes potentially man-infective trypanosome populations from non-infective populations. A new in vitro test for human serum resistance has been introduced recently (Jenni and Brun, 1982).

Tsetse fly transmission studies give further information regarding the degree of adaptation of one *Trypanozoon* stock to a certain vector species and subspecies expressed by successful transmission and concomitant salivary gland infection rates. Moreover the metacyclic forms can thus be tested for human serum resistance.

The major aims of this study were to investigate the influence of cyclical transmission on different characteristics (isoenzyme pattern and human serum resistance) and the search for trypanosome clones which could be used for mixed-transmission studies and the analysis of genetic recombination.

Materials and Methods

Trypanosome stocks and vertebrate host

The origin and history of the stocks TH K 14/79, TH K 28/79, TH K 64/79, and TH K 86/79 are presented in Table 1.

The cryopreserved bloodstream form stabilates were rapidly thawed and diluted with PSG 6:4, pH 8.0 (Lanham and Godfrey, 1970). Female ICR mice (25 g) were infected intraperitoneally. Rapid blood passages in mice were carried out every 3 to 4 days in order to obtain sufficient numbers of parasites for isoenzyme analysis, in vitro cultivation, cyclical transmission and antigenic analysis.

Isoenzyme analysis of bloodstream form parasites

Bloodstream forms of each stock were harvested from mice by cardiac puncture. The parasites were separated from the blood cells according to Lanham and Godfrey (1970). Lysis of the trypanosomes was carried out as described by Betschart et al. (1983). The lysates were immediately frozen as beads of 5 µl in liquid nitrogen and the protein concentrations were estimated with the modified Lowry method of Peterson (1977).

In contrast to the isoelectric focusing method of Betschart et al. (1983), the Servalyt Precotes, pH 3-10 (Serva, Heidelberg) were prefocused for 2 h before applicator slits were loaded with the

Table 1. Origin of trypanosome stocks

Host	Location in Uganda	Year of isolation	Stabilate designation at		No. of passages in rodents since isolation
			UTRO Uganda*	Trop. Inst. Hamburg	
Man	Bukanga	1979	UTRO 2530	TH K 14/79	4
Man	Nambale	1979	UTRO 2525	TH K 28/79	7
Man	Nakalama	1979	UTRO 2528	TH K 64/79	4
Man	Bulongo	1979	UTRO 2526	TH K 86/79	4

* Gibson and Gashumba (1983)

lysates (Fig. 1). In all further experiments a Pharmacia power supply (ECPS 3000/150) with a Pharmacia Volthour integrator (VH-1) was used. The precotes were pre-focused for about $\frac{1}{2}$ h (150–200 Vh) and focusing was stopped after reaching 2000 Vh (Figs. 2–4).

For both enzymes analysed, isocitrate dehydrogenase (ICD; E.C. 1.1.1.42) and phosphoglucomutase (PGM; E.C. 2.7.5.1), 5 μ l of each lysate, diluted to a final protein concentration of 10 μ g/ μ l were loaded.

In vitro test for human serum resistance

Bloodstream forms were harvested by taking a heparinized haemocrit capillary of tail blood from an infected mouse. Subsequent separation of parasites from the blood cells was carried out by centrifugation in a haemocrit centrifuge. The capillary was cut between the pelleted blood cells and the plasma. The trypanosomes on top of the pellet were rinsed into 200 μ l of MEM (supplemented with Earle's salts, 6 g HEPES, 1 g Glucose, 10 ml MEM Non-essential amino acids (100 \times) and 10 mg Gentamycin per litre). This medium is designated as MEM 109–4. The number of parasites per volume was determined, using an improved Neubauer hemocytometer.

Extravascular forms from lymph nodes were isolated according to Tanner et al. (1980).

Metacyclic forms were harvested from infected flies by encouraging them to salivate into a drop of warmed medium (MEM 109–4 containing 20% of heat-inactivated horse serum).

The *in vitro* test system for human serum resistance used, was as described by Jenni and Brun (1982) but the medium was supplemented with 20% instead of 15% of the corresponding sera. For all controls, heat-inactivated horse serum was used. Besides feederlayers of *Microtus montanus* fibroblast-like cells (MEF), monolayers of human embryonic skin and lung cells were used as well. The initiation and maintenance of the cultures was carried out according to Brun et al. (1981). Normally 2×10^5 trypanosomes were inoculated per well. The duration of the individual tests was limited to about 10 days and the experiments were repeated at least three times for each stock.

Cyclical transmission

Puparia of *G. m. morsitans* were obtained from the Tsetse Research Laboratory, Department of Veterinary Medicine, Langford House, Langford, Bristol; those of *G. m. centralis* were received from ILRAD, Nairobi, Kenya.

Teneral flies were either infected immediately after hatching or after being cooled for up to 96 h in a humid chamber at 7°C. Twenty to 30 infected flies were kept in Geigy-cages at 26°C and 80–90% relative humidity. They were maintained by membrane feeding three times a week on rehydrated pig blood (Bauer and Wetzel, 1976). After two weeks, infected tsetse flies were transferred into single tubes and subsequently probed on a warmed slide. Saliva samples with metacyclic forms were fixed with acetone and served as antigen for subsequent indirect fluorescent antibody test (IFAT). Antigens were stored at -70°C in desiccated plastic bags until use.

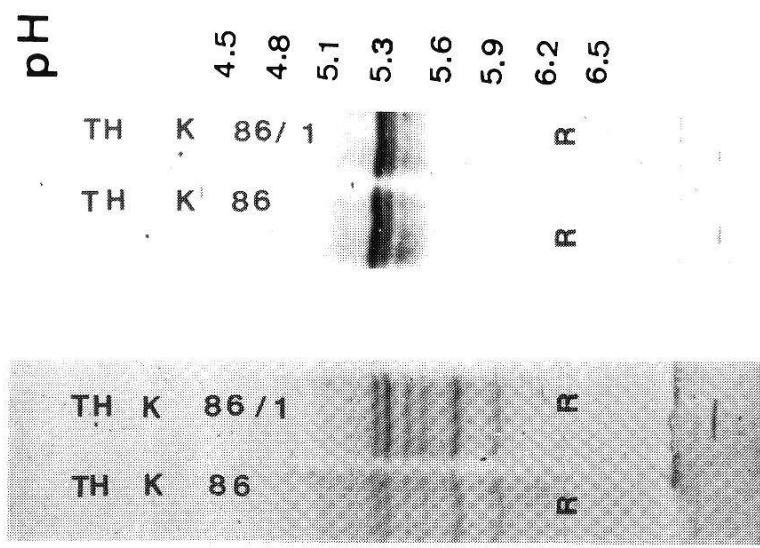
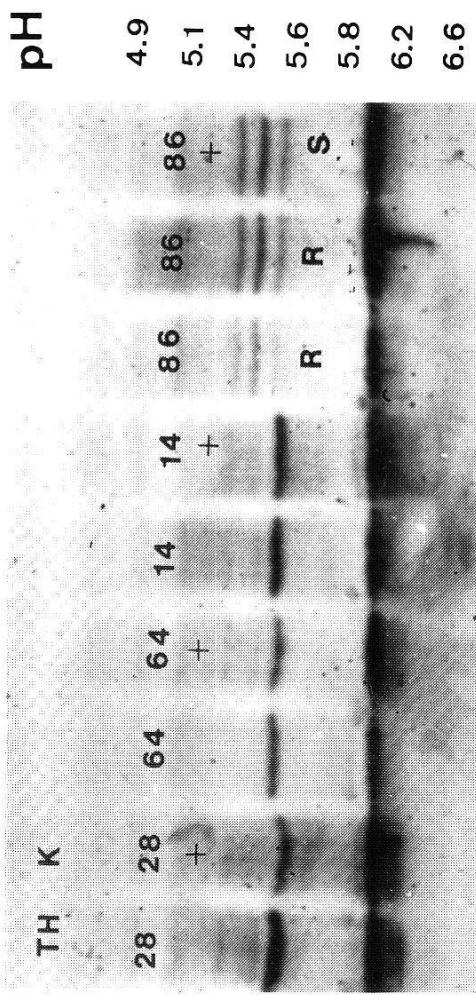
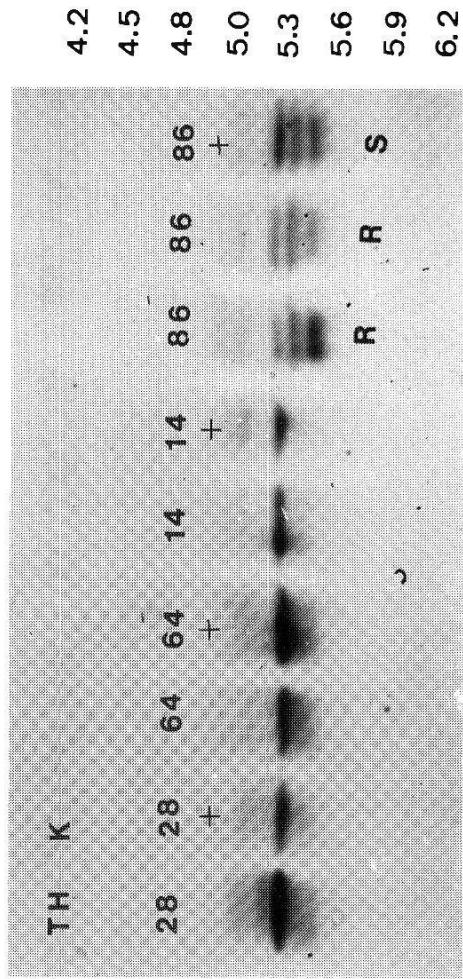


Fig. 1. Isocitrate dehydrogenase (ICD; E.C. 1.1.1.42), Lanes left to right: (1) TH K 28, (2) TH K 28+, (3) TH K 64, (4) TH K 64+, (5) TH K 14, (6) TH K 14+, (7/8) TH K 86 R, (9) TH K 86+ S. + = bloodstream form population after tsetse fly transmission; R = human serum resistant; S = human serum sensitive.

Fig. 2. Phosphoglucomutase (PGM; E.C. 2.7.5.1). Left to right: same stocks, same order as in Fig. 1.

Fig. 3. Isocitrate dehydrogenase (ICD; E.C. 1.1.1.42). Comparison of TH K 86 clone 1 with the parent population TH K 86.

Fig. 4. Phosphoglucomutase (PGM; E.C. 2.7.5.1). Comparison of TH K 86 clone 1 with the parent population TH K 86.



4
3
2

Indirect fluorescent antibody test (IFAT)

Antisera against metacyclic forms of each stock were raised in female C 57 BL mice by cyclical infection followed by a subsequent treatment with Berenil (Hoechst; 25 mg/kg body weight) 48 h after infection. The treated mice were challenged with infective tsetse fly bites at intervals of five days. After four infective fly bites the immunized mice were bled and the antisera were harvested and heat-inactivated.

The indirect fluorescent antibody tests were carried out according to Van Meirvenne et al. (1975). Fluorescein isothiocyanate (FITC) conjugated rabbit anti mouse IgG (Miles-Yeda Ltd., Israel) diluted 1:40 with Evan's Blue/PBS (1/10⁴) was used.

Immunization tests

Mice were immunized against TH K 14 or TH K 64 by cyclical infection and treatment with Berenil 48 h after the infectious fly bite. These mice were challenged with metacyclic forms 3–6 times on different days postinfectious bite. Crossprotection-tests *in vivo* were then carried out by feeding positive flies infected with TH K 86 on these mice at various intervals.

Results

Isoenzyme patterns

a) *Isocitrate dehydrogenase* (ICD; E.C. 1.1.1.42). The stocks TH K 14, TH K 28 and TH K 64 showed identical isoenzyme patterns, characterized by one strong band at a pI of 5.6. Stock TH K 86 differed completely, presenting three clearly distinguishable bands at pIs of 5.4, 5.5 and 5.6. The samples were applied at the position pH 6.0 (Fig. 1).

b) *Phosphoglucomutase* (PGM; E.C. 2.7.5.1). Stock TH K 86 showed again three different major bands at pIs of 5.3, 5.4 and 5.5. The 3 other stocks exhibited a single dominant band at a pI of 5.3 (Fig. 2). One or three bands of PGM activity were thus detected by isoelectric focusing, this is in contrast to the PGM isoenzyme pattern exhibiting 1 or 2 bands of activity after starch gel electrophoresis (Gibson et al., 1980; Tait, 1980; Gibson and Gashumba, 1983).

One clone of TH K 86 showed exactly the same ICD- and PGM-isoenzyme patterns as the population it was derived from (Figs. 3 and 4). In Figs. 2–4 the samples were applied at the position pH 7.0. For both enzymes, no qualitative difference occurred between the isoenzyme patterns within each of the four stocks before and after tsetse fly transmission.

Human serum resistance

Results obtained from the *in vitro* tests for human serum resistance are summarized in Table 2. Only a very small proportion (<1%) of TH K 86 metacyclic forms showed resistance to human serum. After an adaptation period of up to 5 days in the presence of human serum, these resistant trypanosomes began to multiply rapidly.

The human cell line used as a feederlayer showed an increased attractivity to all the characterized trypanosome stocks. The invasion of vertebrate forms into the intercellular spaces of the fibroblastoid monolayer could be accelerated if lymph node forms were used as inoculum instead of bloodstream forms.

Table 2. In vitro test for human serum resistance

Stock	Bloodstream forms	Lymphnode forms	Metacyclic forms
TH K 14	S	nd	
TH K 14+	S	S	
TH K 28	S	nd	
TH K 28+	S	nd	
TH K 64	S	nd	
TH K 64+	S	nd	
TH K 86	R	R	partly R
TH K 86+	R/S	nd	<1%

R = hs-resistant; S = hs-sensitive; + = after cyclical transmission; nd = not done

Table 3. IFAT with metacyclic forms

Antisera	Metacyclic antigens			
	TH K 14	TH K 28	TH K 64	TH K 86
TH K 14				
reciprocal end-titer: 40	(+)	(+)	(+)	+
TH K 28				
reciprocal end-titer: 80	(+)	(+)	(+)	(+)
TH K 64				
reciprocal end-titer: 80	(+)	((+))	(+)	(+)
TH K 86				
reciprocal end-titer: 160	(+)	(+)	(+)	+

+= More than 50% of the trypanosomes reacted positively.

(+)= Half of the trypanosomes reacted positively.

((+))= Less than 50% of the trypanosomes reacted positively.

Antigenic relationship

All antisera raised against metacyclic antigens of one isolate crossreacted with a certain percentage of metacyclic forms of the three other isolates. All metacyclic populations revealed heterogenous reactions with regard to the expressed variable surface antigens even when homologous antisera and antigens were tested. The number of metacyclic forms in the saliva samples of infective flies used as antigens for the IFAT was very small (20–50 only). Therefore the percentage of positively reacting trypanosomes could only be estimated (Table 3).

Discussion

In this study, isoelectric focusing (IEF) has successfully been extended to the characterization of further East-African isolates from man. The method was improved by introducing a prefocusing period, which resulted in a higher resolution of the different bands.

For the two enzymes isocitrate dehydrogenase (ICD; E.C. 1.1.1.42) and phosphoglucomutase (PGM; E.C. 2.7.5.1) the system has been established with high reproducibility on bloodstream form parasites and thus may become an important tool for the characterization of unknown *Trypanozoon* isolates in epidemiological research. It was possible to differentiate between TH K 86 and the other three isolates according to their PGM- and ICD-isoenzyme patterns. No alteration of these patterns occurred in bloodstream forms after tsetse fly transmission. Whereas uncloned and cloned TH K 86 showed heterozygous patterns for both enzymes, the three other isolates exhibited a homozygous single banded pattern. Trypanosome isolates from the Lake Victoria area have been analysed previously (Gibson et al., 1980; Tait, 1980; Gibson and Gashumba, 1983). These authors found that many of the isolates from this region showed multibanded PGM- and/or ICD-patterns. They argued that heterozygous patterns may occur due to hybridization of originally distinct parasites during their life-cycle (Gibson et al., 1980; Tait, 1980). Recently, this phenomenon has also been observed by a group at the Swiss Tropical Institute in *T. (T.) brucei* metacyclines obtained from 1:1 mixed infection experiments with two isoenzymatically distinct parental clones (Jenni et al., unpublished results). It is interesting to note that TH K 86 clone 1 shows the same pattern for ICD with three major isoenzyme bands focused at exactly the same pH as the hybrids produced in the laboratory.

The in vitro test for human serum resistance introduced by Jenni and Brun (1982) proved to be very reliable and easy to perform as the parasites' resistance or sensitivity against normal human serum can be observed directly using an inverted microscope. Using this method it has been found that East-African human isolates, in contrast to West-African isolates from man, may lose their resistance to human serum when they are syringe-passaged from one vertebrate host to the other, or during cyclical transmission (Jenni and Brun, 1984). In the case of TH K 86, less than 1% of metacyclic forms were human serum resistant. If no resistant metacyclic forms were present, human serum resistance always developed in the presence of horse serum among bloodstream forms derived from metacyclic forms in vitro after 72 h. On the other hand, it was possible to obtain a hs-sensitive bloodstream form population of TH K 86 when the supernatant of a culture containing hs-resistant trypanosomes was injected into mice followed by several syringe passages. No change in ICD- and PGM-isoenzyme pattern occurred in this hs-sensitive population, when compared with the pattern of the originally hs-resistant isolate.

The results obtained with the IFAT and cross-challenges in vivo indicate a high degree of similarity among the four different isolates concerning the expressed m-VATs. This similarity may be considered as a momentary snapshot in the dynamics of rapid evolution of the *T. b. rhodesiense* metacyclic variable antigen repertoire as described by Barry et al. (1983). Although the cross-reactions (IFAT) were positive in each case they were of heterogenous character with only a certain percentage of metacyclics showing fluorescence. As procyclic forms did not react with the antisera raised against metacyclic forms, the VSG-specificity of the antibodies was confirmed. The restricted extent of mVSG-diversity within these 4 isolates is supported by the results of the in vivo cross-protection tests: Mice immunized against metacyclic VAT's of TH K 14 and TH K 64 were completely protected against cross-challenge with metacyclics of TH K 86.

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