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Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*

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A semi-defined medium for the cultivation of bloodstream forms of the African trypanosome brucei subgroup was developed. Out of 14 different strains tested, 10 could be cultured including *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*. The presence of a reducing agent (2-mercaptoethanol or thioglycerol) was found to be essential for growth. The standard medium consisted of Hepes buffered minimum essential medium with Earle's salts supplemented with 0.2 mM 2-mercaptoethanol, 2 mM pyruvate and 10% inactivated serum either from rabbit (*T. brucei, T. equiperdum, T. evansi* and *T. rhodesiense*) or human (*T. gambiense*). Although a general medium could be defined for the long-term maintenance of trypanosome cultures, the initiation to culture nevertheless required particular conditions for the different strains. The cultured trypanosomes had all the characteristics of the *in vivo* bloodstream forms including: morphology, infectivity, antigenic variation and glucose metabolism.

Key words: infective African trypanosomes/*in vitro* culture

Introduction

The *Trypanosoma brucei* subgroup of African trypanosomes includes important pathogens of humans (*T. rhodesiense, T. gambiense*) and domestic animals (*T. brucei, T. evansi, T. equiperdum*) (Hoare, 1972). The human pathogens and *T. brucei* are cyclically transmitted from mammal to mammal by an insect vector the tse-tse fly. In the mammalian host, the trypanosomes are covered by a dense coat which consists of a single glycoprotein responsible for the antigenic identity of an individual trypanosome clone. Each clone can give rise to variants expressing structurally and immunologically distinct cell surface glycoproteins. Upon ingestion by the vector, trypanosomes transform into the insect midgut form which lacks the glycoprotein coat and is not infective for mammals. They regain their coat and infectivity when trypanosomes reach the metacyclic stage in the fly saliva (Vickerman, 1978). No cyclical development occurs with *T. evansi* which is transmitted mechanically through a vector, and *T. equiperdum* which is transmitted venereally.

Although insect forms of the trypanosomes can be cultured in a variety of axenic media (Taylor and Baker, 1978), mammalian forms have been culturable only in the presence of feeder cells (Hirumi *et al.*, 1977; Hill *et al.*, 1978; Brun *et al.*, 1981; Balber, 1983). It has thus been difficult to perform *in vitro* metabolic studies on these forms. Here we present an *in vitro* culture system which supports the rapid growth of infective forms of *T. brucei, T. evansi, T. rhodesiense* and *T. gambiense* without using a feeder layer. The parasites cultured in this medium exhibit the major properties of bloodstream trypomastigotes.

Results

Trypanosome culture conditions were first optimized with *T. equiperdum* and then extended to other trypanosome strains. The axenic culture of most strains usually required three stages: an initiation stage and an adaptation stage followed by the maintenance stage where the trypanosomes could be maintained for very long periods of time.

Culture conditions supporting the growth of T. equiperdum bloodstream forms

Numerous media and sera conditions were tested for the initiation and maintenance of *T. equiperdum* BoTat 1 cultures. Cultures could be initiated by inoculating 1 ml of medium A based on Hepes buffered minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal calf serum (FCS) and 0.1 mM 2-mercaptoethanol with 5×10^3 trypanosomes isolated from a mouse. After 3 days of culture at 37°C in a 4% CO_2 atmosphere, cell densities of $1-3 \times 10^5$ trypanosomes were obtained. Thereafter, the culture medium was changed every day for 1 week using medium A supplemented with either FCS or rabbit serum and always containing 2 mM pyruvate and 2-mercaptoethanol. The presence of a reducing agent was essential for the initiation and maintenance of trypanosome growth with both sera. Trypanosome growth was optimal in the range of 0.125 – 0.25 mM mercaptoethanol (Figure 1); thioglycerol gave similar results while dithiotreitol did not support trypanosome growth and mercaptoethaloamine was toxic at all concentrations tested. Therefore, a concentration of 0.2 mM mercaptoethanol was used in all subsequent experiments. To optimize further the culture conditions, the effect of rabbit and fetal calf serum concentration was tested and both were found to give optimal growth yields at 10 and 15% (data not shown). Two other variants of the BoTat serodeme, BoTat 4 and BoTat 28 which lack kinetoplast DNA, were also adapted to culture, using these conditions.

Conditions for culturing other trypanosome subspecies

Initiation stage. Having established culture conditions adequate for the growth of *T. equiperdum*, we tested their ability to support the growth of other trypanosome subspecies. The conditions for *T. equiperdum* did not allow the initiation and long-term growth of most of the other strains tested. Therefore, we tested a variety of other parameters including the influence of pyruvate, nucleotide precursors (hypoxanthine and thymidine), sera origin and macrophage feeder layers on the initiation and growth of a variety of different trypanosome subspecies.

Three different basic media were tested. These included (i) medium B (see Materials and methods), (ii) medium B supplemented with 2 mM pyruvate and (iii) medium B supplemented with 2 mM pyruvate plus 0.1 mM hypoxanthine and 0.016 mM thymidine. Each of these different media was tested with four different sera added to a final concentration of 10% and in addition the effect of macrophage feeder layers was investigated in each medium.

Of the 14 strains tested, 10 could be established in culture. Eight of the strains could be adapted to culture very rapidly: *T.*

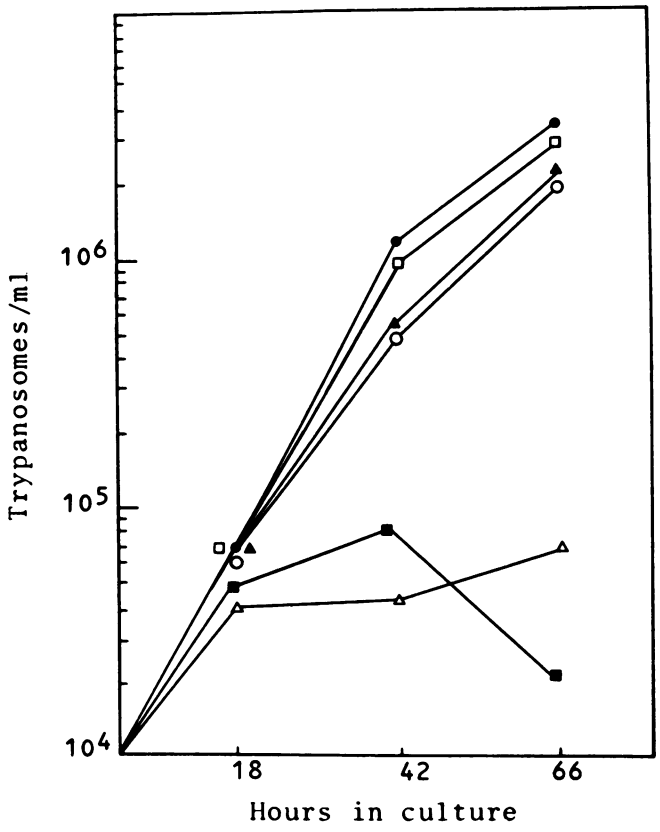

Fig. 1. Effect of 2-mercaptoethanol concentration on the growth of *T. equiperdum* bloodstream forms BoTat 1 at 37°C. 10^4 trypanosomes maintained in culture for 2 weeks were inoculated in 1 ml of medium A (Materials and methods) supplemented with 2 mM pyruvate, 10% rabbit serum and different concentrations of 2-mercaptoethanol: 1 mM (■–■), 0.5 mM (▲–▲), 0.25 mM (●–●), 0.125 mM (□–□), 0.062 mM (○–○), 0.031 mM (△–△). The number of trypanosomes was determined 18, 42 and 66 h after inoculation.

equiperdum (BoTat 1), *T. evansi* (Antat 3), *T. brucei* (Antat 2, 17, 1), *T. rhodesiense* (Etat 2, Utat 1) and *T. gambiense* (Litat 1). A cell density of 1–6 x 10^5 trypanosomes/ml was reached after 3 days of culture with all of the strains tested, except the *T. rhodesiense* strain Utat 1, which required 6 days to reach this level. The two other rhodesiense strains, Antat 25 and Antat 12, first showed a dramatic decrease of the population, falling to < 100 trypanosomes/well within 5 days. After 2–3 weeks, the population began to increase and reached a density of 0.5–1 x 10^5 parasites/ml in the wells supplemented with horse serum and containing a feeder layer. The population was probably the result of a selection of variants that could adapt to the new *in vitro* culture conditions and may not be representative of the trypanosomes in the inoculum. Using these conditions, we were unable to initiate growth of three of the *T. gambiense* strains (Antat 13, 8, 22) and one *T. evansi* strain (stock ketri). In these cases, no trypanosome multiplication was observed and the parasites died within 5 days.

The results in Table I show that different strains required different conditions to initiate their growth in culture. These differences included the origin of the sera necessary to supplement the basic medium and the presence of other factors such as pyruvate, nucleotide precursors or a macrophage feeder layer.

Though there is not a universal medium, these data make it possible to suggest generalized conditions for optimal initiation of trypanosome growth. (i) The use of a feeder layer is preferred although not always absolutely necessary, showing that bloodstream trypanosomes can multiply in a semi-defined medium. Therefore macrophages should always be used for initiation of the growth of trypanosomes isolated from animals. (ii) Several sera should be tested, in particular fetal calf, rabbit and horse sera for the trypanosome strains pathogenic to domestic animals and *T. rhodesiense*. Rat and human sera should be used for the initiation phase of *T. gambiense* strains. (iii) The use of a particular serum determines the necessity for the presence of pyruvate and nucleotide precursors. Human serum must always be supple-

Table I. Determination of optimal initiation conditions for the growth of African trypanosome bloodstream forms in the presence and absence of a macrophage feeder layer

Serum 10%	Medium	*T. equiperdum*		*T. evansi*		*T. brucei*						*T. rhodesiense*								*T. gambiense*	
		BoTat-1[a]		Antat-3[a]		Antat-17[a]		Antat-2[a]		Antat-1[a]		Etat-2[a]		Utat-1[b]		Antat-25[d]		Antat-12[c]		Litat-1[a]	
		Feeder	None	Feeder	None	Feeder	None	Feeder	None	Feeder	None	Feeder	None	Feeder	None	Feeder	None	Feeder	None	Feeder	None
Fetal calf	B	170	180	90	68	50	9	100	10	450	230	530	0	120	85	0	0	0	0	–	–
	B,pyr.	31	36	27	11	25	<1	–	–	70	44	490	350	140	220	0	0	0	0	–	–
	B,pyr.,H.T.	20	27	24	4	34	<1	18	30	28	44	660	500	110	135	0	0	0	0	0	0
Rabbit	B	5	8	120	66	430	240	360	100	500	480	400	390	<1	<1	0	0	0	0	0	0
	B,pyr.	5	24	110	140	350	370	–	–	180	350	410	330	<1	<1	0	0	0	0	0	0
	B,pyr.,H.T.	13	56	88	70	420	290	230	74	200	800	480	450	<1	<1	0	0	0	0	0	0
Horse	B	150	20	90	26	0	0	18	20	70	68	0	0	60	<1	0	0	40	0	0	0
	B,pyr.	126	15	70	40	70	90	–	–	24	42	120	40	26	11	0	0	35	0	7	<1
	B,pyr.,H.T.	6	8	90	60	390	250	75	27	470	360	260	240	32	12	50	0	95	0	90	20
Rat	B	14	<1	42	<1	20	–	8	3	60	<1	–	–	–	–	–	–	–	–	<1	<1
	B,pyr.	24	20	33	<1	5	5	–	–	54	<1	–	–	–	–	–	–	–	–	<1	<1
	B,pyr.,H.T.	12	7	90	<1	580	210	110	18	250	270	–	–	–	–	–	–	–	–	220	280
Human	B	–	–	–	–	–	–	–	–	–	–	0	0	0	0	0	0	0	0	0	0
	B,pyr.	–	–	–	–	–	–	–	–	–	–	0	0	0	0	0	0	0	0	180	220
	B,pyr.,H.T.	–	–	–	–	–	–	–	–	–	–	0	0	0	0	0	0	0	0	200	190

Number of trypanosomes (x10^{-3}/ml) after 3,[a] 6,[b] 18[c] and 24[d] days of culture. Cultures were initiated in 24-well tissue culture clusters filled 24 h before trypanosome inoculation with 1 ml/well of the different media: medium B (Materials and methods); medium B, 2 mM pyruvate (B,pyr.); medium B, 2 mM pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine (B,pyr.,H.T.); supplemented with 10% of one of several different sera: fetal calf, horse (Boehringer); rabbit, rat and human (prepared in the laboratory). Half of the wells received a mouse peritoneal macrophage feeder layer as described in Materials and methods. 5 x 10^3 bloodstream form trypanosomes isolated from a mouse were inoculated per well. The clusters were returned to the CO_2 incubator at 37°C and the cultures examined and counted daily.

mented with pyruvate while horse and rat sera need the presence of pyruvate and nucleotide precursors.

Adaptation stage. The conditions described above were developed for the initiation of trypanosome growth in culture. However, it became clear that these conditions were not always sufficient for the subsequent maintenance of cultures. Most of the strains required an additional adaptation phase before they could be efficiently maintained in culture for long periods.

Cultures were considered to have completed the initiation stage when the trypanosomes reached $1-5 \times 10^5$ parasites/ml. At this point, optimal adaptation conditions were tested by replacing 3/4 of the trypanosome suspension with new medium. Several general observations were made. (i) The adaptation phase for most strains was more reproducible when the initiation stage was carried out in the presence of a macrophage feeder layer which was thus still present in the cultures during the adaptation phase. Nevertheless, the presence of a feeder layer was unnecessary for BoTat, Etat 2 and Litat 1. (ii) All trypanosome strains with the exception of *T. gambiense* could be optimally adapted by using basic medium B (see Materials and methods) supplemented with 2 mM pyruvate and 10% inactivated rabbit serum. For *T. gambiense*, rat and human sera were equally good for the adaptation stage of culture. (iii) Pyruvate was absolutely required for continuous growth of all strains tested. The nucleotide precursors were still required when rat serum was used. (iv) 4 – 8 days growth in adaptation medium was optimal for allowing long-term growth in medium lacking feeder cells.

Maintenance in the absence of a feeder layer. After adaptation, the various strains were subcultured by transferring ~0.05 – 0.1 ml of the trypanosome suspension into 1 ml of fresh medium in the absence of a feeder layer.

Basic medium B supplemented with rabbit serum and 2 mM pyruvate was optimal for the subculture of all strains except *T. gambiense*, the growth of which was best supported by human and rat sera. During the first week of growth in the absence of a feeder layer, the cells were maintained at a density of $0.5-10 \times 10^5$ cells/ml by diluting the culture in fresh medium. After the first week, the trypanosomes could be diluted as desired for subculturing the strains. It should be noted that after this first week of culture, pyruvate could be omitted from the medium supplemented with rabbit serum without affecting growth. However, subcultures, particularly of *T. brucei* strains, could occasionally suddenly stop growing in medium lacking pyruvate. Pyruvate could not be omitted from medium supplemented with rat or human serum. By subculturing the trypanosomes, every 1 – 3 days in the appropriate medium, all strains have been continuously cultivated *in vitro* for >4 months.

Effect of serum origin on growth of trypanosome in the maintenance phase. To optimize the maintenance culture conditions for each strain, trypanosome growth was tested in basic medium B supplemented with pyruvate, hypoxanthine, thymidine and 10% serum of different origin (listed in Materials and methods), either prepared in the laboratory or obtained from commercial sources. Table II summarizes the growth characteristics, growth yield and generation time, obtained with commercial fetal calf serum and with the sera prepared in the laboratory. These fresh sera always supported parasite growth better than the commercial ones (data not shown). As observed above, rabbit serum proved to be the best for all strains except *T. gambiense* where development is good with horse, rat or human serum. Horse and pig sera prepared in the laboratory supported the growth of most strains but always less efficiently than rabbit serum. Human serum was cytotoxic not only for all non-human infective strains, but also for the *T. rhodesiense* strain Utat 1. In contrast, human serum supported the growth of the other *T. rhodesiense* strains Etat 2, Antat 12 and 25 even though their growth could never be initiated in its presence. Although Antat 12 and 25 would grow in the maintenance stage in medium supplemented with human serum, the inoculum first underwent a precipitous drop in viable cells, much as observed in the initiation phase, and then started growing.

Table II. Effect of serum origin on bloodstream form trypanosome growth characteristics

Trypanosomes	Origin of sera tested					
	Fetal calf	Rabbit	Horse	Pig	Rat	Human
BoTat 1	2.8(7.8)	3.6(6.7)	1.4(8.2)	1(10)	0.8(8.7)	0
Antat 3	0.01	0.2(11.2)	0.12(17)	0.1	0.02	0
Antat 17	0.01	1.2(11.3)	0.7(16.5)	1.1(12.3)	0.8(14)	0
Antat 2	0.5(16)	2.5(8.4)	0.7(14)	0.4	0.7(13)	0
Antat 1	0.02	1.2(10.5)	1.1(13.3)	0.7(12.6)	0.2	0
Etat 2	0.01	4(6.1)	2(6.3)	1.7(6.7)	3.2(6.5)	2.6(7.3)
Utat 1	1.2(8.8)	3.5(6.7)	1.5(8.2)	1.1(7.8)	2.2(7.5)	0
Antat 12	0	0.4(21)	0.5(19)	0.03	<0.02	+
Antat 25	0	0.1(32)	0.1(22)	0.14(20)	<0.02	+
Litat 1	0	0.4(16)	1.5(11)	0.2	1.8(10.5)	2.1(10.4)

10^4 trypanosomes maintained in culture for 8 – 10 weeks in medium B, 2 mM pyruvate supplemented with 10% human serum (*T. gambiense*) or rabbit serum (all other strains) were inoculated in 1 ml medium (B, 2 mM pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine) supplemented with 10% of one of several different sera: commercial fetal calf serum, or laboratory prepared sera (rabbit, horse, pig, rat, human). The 24-well tissue clusters were returned to the CO_2 incubator at 37°C and the trypanosomes counted daily. The generation time was determined between 24 and 48 h of culture. (+) Trypanosomes developed 2 – 3 weeks after inoculation. Maximum growth is expressed as 10^{-6} trypanosomes/ml followed by the generation time (hours) in parentheses.

Using the optimal serum, the generation time ranged from 6.1 to 11.3 h for the trypanosomes which develop an acute infection in mice and 19 – 32 h for strains that induce a chronic infection (Antat 12, 25). The maximum growth yield differs from one strain to another ranging from 10^5 to 4×10^6 trypanosomes/ml. The growth yield is not simply dependent on the availability of a limiting nutrient since it can increase as the cells are maintained in culture. For instance, with *T. evansi* after 2 months, the maximum yield was 2×10^5 cells/ml and after 4 months of subculture, the yield increased to 9.6×10^5 cells/ml. In some cases, Antat 3 and 17, the growth yield could also be increased by increasing the rabbit serum concentration to 15 or 20% (data not shown).

The cultured trypanosomes could be cloned with a high efficiency by growing the diluted cells in a medium with a macrophage feeder layer (see Materials and methods). Six to eight positive wells out of 10 inoculated with a single variant of the BoTat serodeme were routinely obtained. The cultured trypanosomes gave >90% viable cells after 1 month at −80°C when frozen as described in Materials and methods.

Properties of trypanosomes cultured in a semi-defined media

After >3 months of culture, the trypanosomes retained all the characteristics of bloodstream forms. (i) The morphology was the long slender form. (ii) A variant specific antigen was demonstrated at the surface of several strains by indirect immunofluorescence using either monoclonal antibodies (BoTat 1, 4, 28), or polyclonal variant specific antisera (Litat 1, Etat 2, N.Van Meirvenne, personal communication). (iii) Antigenic variation could

Table III. Changes in glucose metabolites during growth of *T. equiperdum* BoTat 1

	Concentration in mM	
	Fresh medium	Medium after 48 h culture
Glucose	10.8	4.2
Pyruvate	0.12	6.1
Glycerol	0.35	5.8
Lactate	1.8	1.9
Succinate	0.02	0.03
Cell number	–	3.5×10^{-6}/ml

5×10^5 trypanosomes maintained in culture for 2 months in medium B 2 mM pyruvate 10% rabbit serum, were centrifuged, resuspended in 10 ml of the same medium without pyruvate and cultured at 37°C in a T-25 tissue culture flask. 48 h after culture, trypanosomes were counted and centrifuged at 1000 *g* for 10 min. The supernatant was then assayed for the presence of glucose and some of its metabolites.

be observed *in vitro* in cloned populations of *T. equiperdum* variants. We were able to detect the presence of new variants as determined by indirect immunofluorescence with variant specific monoclonal antibodies, 14 days after cloning. (iv) All the cultured trypanosome strains were infective in cyclophosphamide-treated mice. The inoculation of 10^4 parasites/mouse killed the mice within 1 week. Furthermore, when mice were each injected with a single cultured organism of BoTat 1 or Antat 2, 5/10 and 6/10, respectively, developed a lethal infection. This efficiency of infectivity is comparable with that obtained with *in vivo* grown trypanosomes. (v) The cultured trypanosomes had the metabolic characteristics of bloodstream (as opposed to insect) forms. As shown in Table III, pyruvate and glycerol were the end-products of glycolysis, no increase in tricarboxylic acid cycle metabolites (lactate, succinate) was detected. It should be noted that the presence of glycerol as an end-product of glycolysis was unexpected since it is normally observed only when bloodstream trypanosomes are maintained in anaerobic conditions (Bowman and Flynn, 1976).

Discussion

We have studied the culture conditions necessary for the growth of African trypanosomes in axenic culture and shown that under the appropriate conditions the cultured trypanosomes maintain their bloodstream characteristics. Several culture conditions were tested and among these three components proved to be of major importance. These included the presence of a reducing agent, of pyruvate and the type of serum used.

The presence in the culture medium of a reducing agent such as mercaptoethanol or thioglycerol was crucial. The optimal concentration for growth of all strains tested was found to be 0.02 mM which represents four times the concentration normally used for mammalian cells such as lymphoid tumor lines or hybridoma (Fazekas de St Groth and Scheidegger, 1980). The probable role of the reducing agents is to stabilize different essential components of the medium such as vitamins and glutathione. It is known that trypanosomes are very susceptible to damage by activated oxygen species because they possess only a very low concentration or none of the enzyme such as glutathione peroxidase and catalase which destroy hydrogen peroxide in mammalian cells (Meshnick *et al.*, 1978). Thus, reducing glutathione may function as a scavenger of free radicals. The important role of glutathione is supported by the observation that inhibition of glutathione synthesis was found to be curative in mice infected with *T. brucei* (Arrick *et al.*, 1981). The need to eliminate the activated oxygen species could explain why feeder layer cells had previously been essential for the *in vitro* culture of trypanosomes.

The addition of pyruvate is important for long-term trypanosome culture despite the fact that parasites themselves are able to synthesize large amounts of this component. Although pyruvate is sometimes inhibitory in the initiation phase, a minimum concentration seems to be required for proper growth once the parasites are adapated to culture. The reasons for this pyruvate dependence are not clear.

Optimal growth of all strains, except *T. gambiense*, was obtained with rabbit serum which also gave the best results with feeder layers (Brun *et al.*, 1981). These observations are surprising since most of the trypanosome strains develop a very low parasitemia in rabbits. Furthermore, cultures with rabbit as well as with human serum did not require nucleotide precursor supplement, in particular purines, upon which trypanosomes depend absolutely, since they are unable to synthesize them *de novo* (Fish *et al.*, 1982). On the other hand, horse and rat sera required the addition of nucleotide precursors. These observations may indicate important variations in the concentration of these components among the different sera. It therefore seems probable that the serum preferences observed, particularly in the initiation phase, reflects similar variation regarding other important, though undefined, factors. It is worth emphasizing that regardless of what factor in specific sera is essential for adaptation, most strains are able to grow with similar requirements. Another possible factor, affecting the ability of some trypanosome strains to use certain sera, is the presence of host specific trypanocidal factors, since it has been shown that human high-density lipoprotein lyses all trypanosome strains non-infective to man (Rifkin, 1978).

The semi-defined media described in this article allows culture of infective trypanosomes which retain *in vitro* their specific surface antigens and their morphological and biochemical characteristics. This culture system will help the elucidation of the physiology, biochemistry and antigenic variation mechanisms of the parasites and will facilitate the development and testing of new trypanocidal drugs.

Materials and methods

Mice

Six- to ten-week-old female Swiss mice (purchased from Evic Ceba, Zone Industrielle, Blanquefort, France) were used in all experiments. Mice were infected by i.p. injection of trypanosomes from a freshly thawed capillary stabilate.

Trypanosomes

All strains, except *T. equiperdum*, were kindly sent to us by N.Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium).

T. equiperdum. Three variants were used: BoTat 1 (Bordeaux-Trypanozoon-antigenic-type 1), BoTat 4 (Capbern *et al.*, 1977) and BoTat 28 a variant lacking kinetoplast DNA (Riou *et al.*, 1980).

T. brucei variants: Antat 1/1 (Antwerp-Trypanozoon-antigenic-type), Antat 2/1 (Van Meirvenne *et al.*, 1977) and Antat 17/1 are descendants of strains isolated respectively in Uganda from a gazelle, in Nigeria from a tse-tse, and in Zaïre from a sheep.

T. rhodesiense variants: Etat 1/2 (Edinburgh-Trypanozoon-antigenic-type) (Lumsden and Herbert, 1975), Utat 1/8 (Uganda-Trypanozoon-antigenic-type), Antat 12/1 (Magnus *et al.*, 1982) and Antat 25/1 originated from stocks isolated respectively from *Glossina pallidipes* in Uganda and from patients in Uganda and Rwanda. The potential infectivity to man of the Etat strain has been demonstrated by an accidental laboratory infection (Van Meirvenne *et al.*, 1976).

T. gambiense variants: Litat 1/1 (Lille-Trypanozoon-antigenic-type) (Afchain *et al.*, 1975), Antat 13/1, Antat 22/1 and Antat 8/1 are descendants of strains isolated from patients in the Ivory Coast, Zaïre, the Congo and Cameroun, respectively.

T. evansi. Antat 3/1 is a variant which derives from a South American capybara isolate (Van Meirvenne *et al.*, 1977). The stock Ketri 2480 was isolated from

a camel in Kenya.

All strains and variants induce an acute infection in mice, except three of the *T. gambiense* variants (Antat 13, 22 and 8) and two *T. rhodesiense* clones (Antat 12 and 25) which produce chronic infections. Therefore with the latter strains mice were injected with 300 mg/kg body weight of cyclophosphamide 24 h before trypanosome inoculation.

Culture media, sera and feeder layer

The standard medium consisted of: MEM powder medium (for 1 l) with Earle's salts, without sodium bicarbonate with L-glutamine (Serva No 47360), 10 ml MEM non-essential amino acid concentrate (100x), 5.95 g Hepes, 1 g glucose, 2.2 g sodium bicarbonate, 1100 ml double-distilled water.

After adjustment of the pH to 7.3 with 5 N NaOH, the medium was filter sterilized (Millipore 0.22 μm), stored at 4°C and used within 10 days. Before use, the medium was supplemented with 30 μg/ml kanamycin (medium A) and 0.2 mM 2-mercaptoethanol (medium B). 1 M Na pyruvate (pyr), 10 mM hypoxanthine (H) and 1.6 mM thymidine (T) were made up in double-distilled water, filter sterilized and stored in 1 ml aliquots at −20°C. Saline contained 10 mM phosphate buffer, pH 7.4, 8.5% w/v NaCl, 0.1% w/v glucose.

Rabbit and rat sera were obtained from blood taken respectively from the ear vein and by heart puncture of adult animals. Fresh horse and pig blood was obtained from a slaughterhouse. Human blood came from AB^+ volunteers. After coagulation at room temperature for 2 − 4 h, the blood was stored overnight at 4°C. The sera were centrifuged for 15 min at 4000 *g*, inactivated by incubation at 56°C for 30 min, filter sterilized (Millipore 0.22 μm) and stored in 10 ml portions at −20°C. Commercial sera included fetal calf, horse, newborn calf (Boehringer), pig and chicken (Gibco), were inactivated and stored in the same manner as for the fresh sera.

The feeder layer consisted of peritoneal macrophages. They were obtained from normal Swiss mice by flushing the peritoneal cavities with ~4 ml of 0.34 M sucrose. After centrifugation (10 min at 200 *g*), they were resuspended in culture medium B at a cell density of 5 x 10^5 macrophages/ml. The cells (0.05 ml) were distributed into 24-well tissue culture clusters (Nunc) containing 1 ml of the appropriate media per well and incubated at 37°C in a CO_2 incubator (4% CO_2 − 96% air). A cell density of 5 x 10^4 macrophages/ml was used for cloning trypanosomes in flat-bottomed microtrays (Falcon Microtest III) containing 0.2 ml medium per cup.

Culture system, maintenance of cultures, cloning and cryoconservation

All cultures were grown at 37°C in a CO_2 incubation (4% CO_2 − 96% air).

Initiation of trypanosome cultures was carried out in 24-well tissue culture clusters. On the day before trypanosome inoculation, the wells were filled with 1 ml of the appropriate media with or without a feeder layer and returned to the CO_2 incubator.

Bloodstream trypanosomes were obtained from a mouse with a rising parasitemia (1 − 5 x 10^8 parasites/ml) by heart puncture in the absence of anti-coagulant. Depending on the parasitemia, 1 − 10 needle drops of heart blood were rapidly mixed with 4 ml of saline and centrifuged for 5 min at 300 *g* to sediment most of the red blood cells. The supernatant fluid was removed to another tube, the trypanosomes were counted, adjusted to 10^5/ml with saline and aliquots of 0.05 ml were placed in the pre-filled tissue culture wells and incubated in the CO_2 incubator. The cultures were regularly examined with an inverted microscope and when the trypanosome density reached 1 − 5 x 10^5 trypanosomes/ml, the medium was gently mixed, 3/4 of the trypanosome suspension was removed and replaced by new medium. Serial subcultures were made by transferring 0.05 ml of the log phase culture into 1 ml of fresh medium.

For cloning, trypanosomes were diluted in saline and distributed in Microtest III tissue culture plate wells (Falcon) to get one parasite in one out of three wells. To prevent evaporation, the external wells were filled with distilled water. If two observers could each detect only one trypanosome, 0.1 ml of medium was added to the well and the contents were transferred into a well containing a macrophage feeder layer in 0.1 ml of medium. Macrophage feeder layers, cultured in medium B supplemented with 10% fetal calf serum, could be used for 3 weeks if the medium was replaced 24 h before cloning by trypanosome culture medium. Subcultures were made into the larger 2 cm^2 wells without feeder layers as soon as the supernatant fluid contained trypanosomes.

For cryopreservation, one volume of the cultured trypanosome suspension (0.1 − 1 x 10^6 parasites/ml) was mixed with one volume of freezing medium composed of 70% serum (the same type used for culture) and 30% glycerol. The tubes are placed in a closed polystyrene container at −80°C overnight before being transferred to liquid nitrogen. For culture initiation, tubes were thawed and two drops of the trypanosome suspension are inoculated into a cup of 24-well tissue culture cluster containing a macrophage feeder layer previously cultured in medium B supplemented with 10% serum. After 3 − 4 days culture, subcultures were made.

Cell counting and immunofluorescence

Trypanosomes were counted in a Malassez haemocytometer. Variant surface antigens were detected at the surface of formaldehyde fixed trypanosomes by indirect immunofluorescence (Nantulya and Doyle, 1977).

Glucose, pyruvate, glycerol, lactate and succinate determination

All assays were done by enzymatic methods using test kits or reagents from Boehringer: glucose (kit cat. No 263826), pyruvate (kit cat. No 124982), lactate (kit cat. No 149993), glycerol (kit cat. No 148270) and succinate (Beutler *et al.*, 1976).

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