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Studies on the mechanisms supporting the continuous growth of *Trypanosoma (Trypanozoon) brucei* as bloodstream-like form in vitro[1]

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Summary

The conditions which enable growth of *Trypanosoma (T.) brucei* stock S 427 as bloodstream-like form over bovine fibroblast-like cells (BHF) were analyzed. The experiments with different BHF conditioned media indicated that there is no stable factor produced by the BHF which can trigger growth as bloodstream-like form. Only living, actively metabolizing BHF could support continuous growth. The intercellular localization of trypanosomes in the BHF feeder-layer was a main feature of an intact culture. The number of trypanosomes per mm^2 BHF-layer was fairly constant. Light- and scanning electron microscopy revealed that there is no regular arrangement of the intercellular trypanosomes. No contact structures such as desmosomes or hemidesmosomes could be observed between trypanosomes and the BHF by transmission electron microscopy. Whenever trypanosomes were separated from the BHF by micropore membranes in different chamber systems the continuous growth as bloodstream-like form was no longer possible. An obligatory short-term trypanosome/BHF association is therefore assumed as growth trigger. Whether this inductive short-range signal is transmitted by an exchange of unstable, low concentrated factors or by cell contact interactions is not yet clear. The fibroblast surface antigen, fibronectin, did not seem to be involved in mediating the trypanosome/BHF association.

Key words: Trypanosoma (T.) brucei; bloodstream form; in vitro conditions; bovine fibroblast-like cells; growth stimulation.

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Introduction

The development of in vitro conditions which enable the continuous growth of vertebrate-infective, bloodstream-like *T. brucei* has been attempted for many years without success. Hirumi et al. (1977a, b) were the first to achieve continuous cultivation of bloodstream-like forms of *T. brucei* stock S 427 in a system consisting of bovine fibroblast-like cells in RPMI 1640 with 20% inactivated foetal bovine serum. These culture conditions also enabled cloning (Hirumi et al., 1978), and the trypanosomes could undergo antigenic variation in vitro (Doyle et al., 1979). The same monomorphic derivative of stock S 427 which has lost the ability for cyclical transmission, could also be cultivated over various other mammalian cells (Hill et al., 1978a; Hirumi et al., 1980; Brun et al., 1979). In addition an analogous in vitro system for the long-term cultivation of vertebrate-infective, bloodstream-like forms of pleomorphic, tsetse fly-transmissible stocks of *T. brucei* and *T. rhodesiense* has been described recently (Brun et al., 1979). It clearly emerges from these studies that bloodstream-like forms might need living feeder-layer cells for in vitro growth. Hirumi et al. (1977a, b), Hill et al. (1978a, b) and Brun et al. (1979) have pointed out the obvious preference of the trypanosomes for localization and growth in the spaces between the tissue culture cells. Whether diffusable growth factors or inductive cell to cell contacts are initiating and supporting growth is not known. In the present study we have attempted to analyze the conditions which enable growth of *T. brucei* stock S 427 as bloodstream-like forms over bovine fibroblast-like cells.

Material and methods

Trypanosomes

Trypanosoma (T.) brucei stock S 427 was received in 1977 from Dr. H. Hirumi (ILRAD, Nairobi) as cultured bloodstream-like forms in the bovine fibroblast culture system, as described by Hirumi et al. (1977a, b).

Culture medium

The medium used for this study was Rosewell Park Memorial Institute (RPMI) 1640 medium with 300 mg/l L-glutamine (Gibco Bio-Cult, Irvine, Scotland) supplemented with 2 g/l Na-bicarbonate, and 25 mM HEPES (Calbiochem, San Diego, CA, USA). The pH was adjusted to 7.3. Before use 20% inactivated foetal bovine serum (iFBS, Gibco Bio-Cult) and 150 units/ml/μg/ml of a penicillin/streptomycin mixture (Difco Lab., Detroit, Mi., USA) were added. This medium is subsequently described as "complete medium".

Bovine fibroblast-like cells

The bovine fibroblast-like cells ILR-BHF 476 (Hirumi et al., 1977b; subsequently described as BHF) were obtained from ILRAD, Nairobi. They were maintained with complete medium in T-25 tissue culture flasks (Falcon Inc., Oxnard, CA, USA) or in 24-well tissue culture plates ($\varnothing$ 16 mm, Costar Inc., Cambridge, Mass., USA) at 37° C in a 5% CO_2/air-atmosphere. Subcultures were made every 6–12 days when the cells became confluent.

Maintenance of cultures

Cultures were initiated in T-25 flasks (5 ml medium) or in wells of tissue culture plates (1 ml per well) containing almost confluent BHF-layers. 10^6 (T-25 flask) or 10^5 (per well) cultured bloodstream-like forms were used as inoculum. After 24 and 48 h half of the medium was replaced. Later on, when an intercellular trypanosome population had been fully established, the medium was exchanged completely every day. The cultures were kept at 37° C in a 5% CO_2/air-atmosphere.

Trypanosomes were recovered from the culture supernatant by centrifugation (10 min, 350 *g*). Where nothing else is stated, such supernatant trypanosomes were used as inoculum for all the experiments described below. Intercellularly localized trypanosomes were obtained after the BHF had been detached by a "rubber policeman". The trypanosomes were separated from the BHF by centrifugation (20 min, 50 *g*).

All counts were made in a Neubauer haemocytometer. To check the infectivity of trypanosomes approx. 5×10^4 motile forms were injected (i.p.) into female Swiss-ICR mice (25 *g*). The mice were examined every second day by the haematocrit technique.

BHF conditioned media (BCM)

Complete medium (4 ml) was incubated in T-25 flasks with a confluent BHF-layer for 3, 6, 12, 24, 48 and 72 h. BCM were subsequently filter sterilized (0.45 μm, Millipore) and the pH was adjusted to 7.3 with 1 N NaOH. Concentrated BCM were prepared by incubating only 3.75 ml (instead of the normally used 15 ml) complete medium over a confluent BHF-layer in a T-75 tissue culture flask for 12 or 24 h. The BHF-layer was covered in this case with a medium layer which was only 500 μm thick.

Media conditioned by trypanosomes and BHF (TBCM) were obtained by the incubation of 10^6 trypanosomes in 4 ml complete medium over a BHF-layer for 12 or 24 h.

Chamber experiments

The following chamber systems were designed to separate trypanosomes from the BHF-layer by micropore membranes:

- 10^6 trypanosomes in 2 ml complete medium were brought into a dialysis bag (20 DM, pore size 15–20 Å, Union Carbide), and the bag was subsequently placed directly over the BHF-layer in a T-25 flask containing 4 ml medium.
- The plexiglass chamber as shown in Fig. 1 was developed. A 0.45 μm Millipore filter (type SSWP, ∅ 25 mm) separated the upper compartment (1500 mm^3, holding medium + trypanosomes) from the lower compartment (630 mm^3, holding medium + BHF). The distance from the BHF-layer to the filter was 1.5 mm. The chamber was clamped to make it water-tight and then sterilized by dry heat (75° C for 2 days).
- Millipore chambers (Millipore Corp., Bedford, Mass., USA) with 0.45 μm pore size (type THWP) were assembled as described by Weiss and Tanner (1979) and loaded with 10^5 trypanosomes or 5×10^4 BHF. The chambers were inserted into the wells of a tissue culture plate containing 1 ml medium and BHF or trypanosomes (Fig. 2 B).
- A comparable system was developed using acrylglass tubes (16 mm length, ∅ 15/11 mm). A 0.4 μm Nucleopore membrane was cemented (MF-cement, Millipore Corp.) onto one end (Fig. 2 A). These inserts could hold 750 μl medium and were inoculated with 10^5 trypanosomes. They were placed into the wells of the tissue culture plate, directly on top of the BHF-layer (Fig. 2 A).

BHF in agar

BHF-layers were trypsinized (1× trypsin-EDTA-solution, Gibco Bio-Cult). The cell suspension was transferred into 2 ml of 2× concentrated complete medium, warmed up to 38° C and subsequently mixed with 2 ml 1.5% Bacto-Noble-agar (Difco Lab.). This mixture was plated onto the bottom of a T-25 culture flask, overlayed with 4 ml complete medium and inoculated with 10^6 trypanosomes.

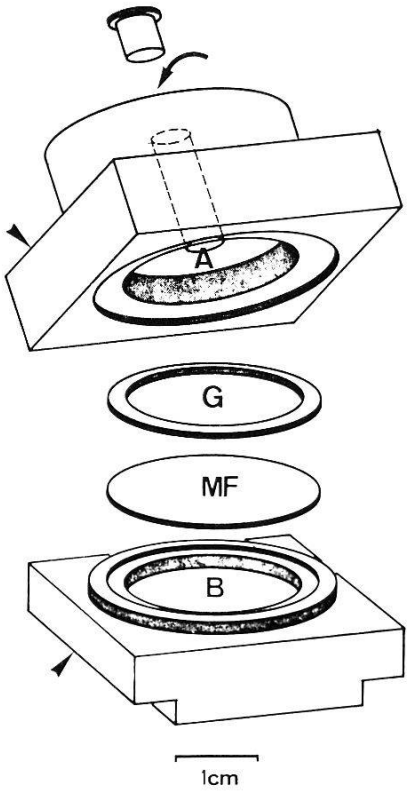


Fig. 1

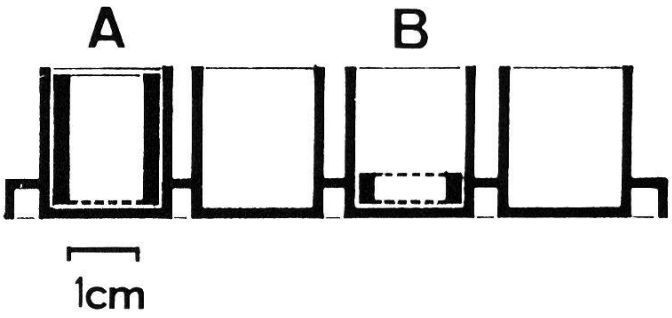


Fig. 2

Fig. 1. Plexiglass chamber separating the BHF-layer in the lower compartment (B) from the trypanosomes in the upper compartment (A) by a 0.45 μm Millipore filter (MF), gasket (G), filling-hole ($\lceil$), attachment sites of clamps ($\blacktriangleright$).

Fig. 2. Sectional view of the acrylglass tube (A) and the Millipore chamber (B) inserted into the wells of a tissue culture plate, ------ indicates 0.4 μm micropore membranes.

BHF extracts

BHF were removed from the culture flask with a "rubber policeman", washed extensively with serum-free RPMI 1640 and subsequently lyophilized. In another experiment washed BHF were forced open in 5 ml medium by freeze-thawing (3 times, $-70°$ C). The suspension was ultracentrifuged (2 h, 120 000 *g*) and the supernatant was tested for trypanosomal growth promoting activities. The pellet was washed once (2 h, 120 000 *g*) and also tested.

Light microscopy

Cultures were routinely examined using an inverted phase contrast microscope. Morphological observations were made on methanol-fixed and Giemsa-stained smears.

Electron microscopy (EM)

BHF-layers containing intercellular trypanosomes were fixed with 2½% glutaraldehyde for 90 min at 4° C and further processed for EM following standardized methods (Hecker et al., 1972). However, as the bottom of the tissue culture flask had to be included in the preparation, it was necessary to replace the acetone-propylene oxide dehydration by dehydration with ethanol.

For scanning electron microscopy (SEM), BHF were cultured on coverslips (∅ 15 mm). After fixation with 2½% glutaraldehyde (30 min, 4° C), the coverslips were washed overnight in 0.2 M Na-cacodylate buffer containing 2% sucrose (pH 7.3) and postfixed in 2% OsO_4 (2 h, 4° C). The specimens were dehydrated in graded acetone (50–70–90–3 × 100%, 20 min each) and critical point

dried using CO_2 as transition fluid (Cohen, 1974). After mounting, the dried specimens were sputtered with gold (20 nm) and subsequently examined in a Stereoscan Mark IIA (accelerating voltage 10 kV, specimen tilt 20–30°).

Fibronectin

The BHF were examined for the presence of fibronectin using the indirect immunofluorescence antibody test (IFAT). Coverslip cultures were fixed with 4% formalin (in phosphate-buffered saline, PBS, pH 7.2) for 1 h, washed with PBS and then incubated in a 1 : 50 dilution of a rabbit anti-fibronectin antiserum[2] for 40 min at 37° C. After washing with PBS, the cells were incubated with a 1 : 10 dilution of a fluorescein isothiocyanate coupled Ig-fraction of a goat anti-rabbit IgG (Miles-Yeda Ltd., Israel) for 40 min at 37° C, washed with PBS and mounted in buffered glycerol.

Statistics

As a normal distribution was not always evident between the results of different experiments, the median ($\tilde{x}$) ±standard error (S.E.) had to be calculated for the cell counts. Consequently, the results were compared using the U-(rank)test.

Results

Table 1 summarizes the growth characteristics of S 427 bloodstream-like trypanosomes in the bovine fibroblast culture system maintained in T-25 flasks and in tissue culture plates. These data are consistent with the results of Hirumi et al. (1977a, b). The same mean number of intercellular trypanosomes (approx. 1000 per mm^2 BHF-layer) could be found in both culture systems at day 11 and 12, respectively.

A cell-free system had to be designed in order to test possible effects of conditioned media or BHF components on the growth of bloodstream-like forms (Fig. 3). When 10^6 trypanosomes were inoculated into 4 ml of complete medium in a T-25 flask, they died within 3 to 6 h. Interestingly, a slightly higher number of inoculated trypanosomes survived as infectious bloodstream-like forms up to 48–72 h. The higher amount of pyruvate produced by the higher number of trypanosomes seems to account for this inoculum-dependent survival effect (Tanner et al., 1980a). Thus, in all the following experiments where the growth mechanisms of the bloodstream-like forms were studied an inoculum of 2.5×10^5/ml was used in T-25 flasks and 10^5/ml in wells (⌀ 16 mm) of tissue culture plates to avoid the pyruvate-dependent survival effect.

Conditioned media

Fig. 4 shows the survival of bloodstream-like trypanosomes in BCM. The trypanosomes could not survive in 3-h BCM and died within 3 to 6 h as did the controls. The 6-h BCM had only a slight effect on the survival of the trypanosomes, while ≥12-h BCM supported survival as infectious bloodstream-like form for up to 48 h. An initial doubling of the trypanosome population was sometimes observed in 24-h and 48-h BCM. However, these divisions cannot be compared

[2] Kindly provided by Dr. K. Ballmer, Biocenter, University of Basel

Table 1. Growth of S 427 bloodstream-like trypanosomes in the bovine fibroblast (BHF) culture system

System	Growth area[a] mm^2	ml medium	N[b]	Trypanosomes $\times 10^5$ (median $\pm$ S.E.)		
				inoculum	intercellular of BHF	supernatant number/24 h
T-25 flask	2500	5	8 (4)	10	25.5 ± 1.5 (CD 11)	93.0 ± 3.0 (CD 4–11)
Costar tissue culture plate	200	1	10 (5)	1	2.0 ± 0.3 (CD 12)	10.9 ± 0.5 (CD 4–12)

[a] Area covered by a confluent BHF-layer
[b] Number of cultures examined, number of experiments in brackets
CD = Day of culture

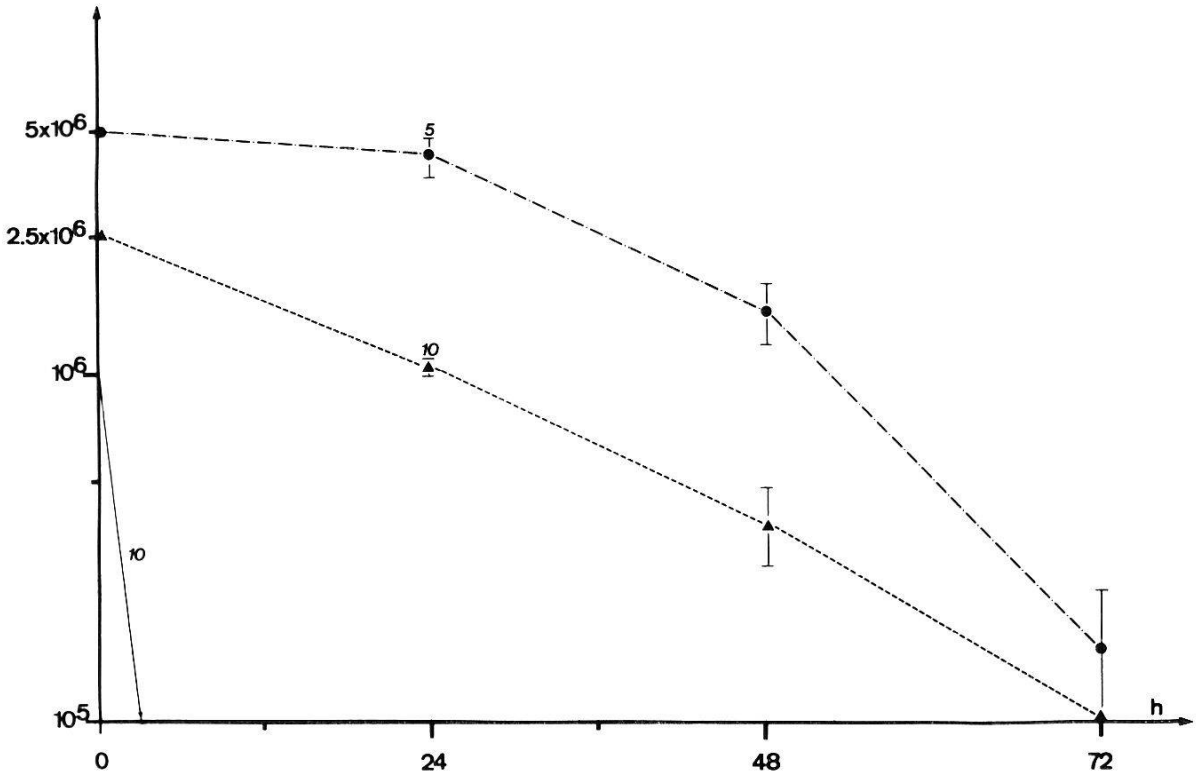


Fig. 3. Survival of bloodstream-like trypanosomes in 4 ml RPMI 1640 + 20% inactivated foetal bovine serum without feeder layer cells. Median ± S. E., number of cultures examined in italics, T/C = trypanosomes per culture.

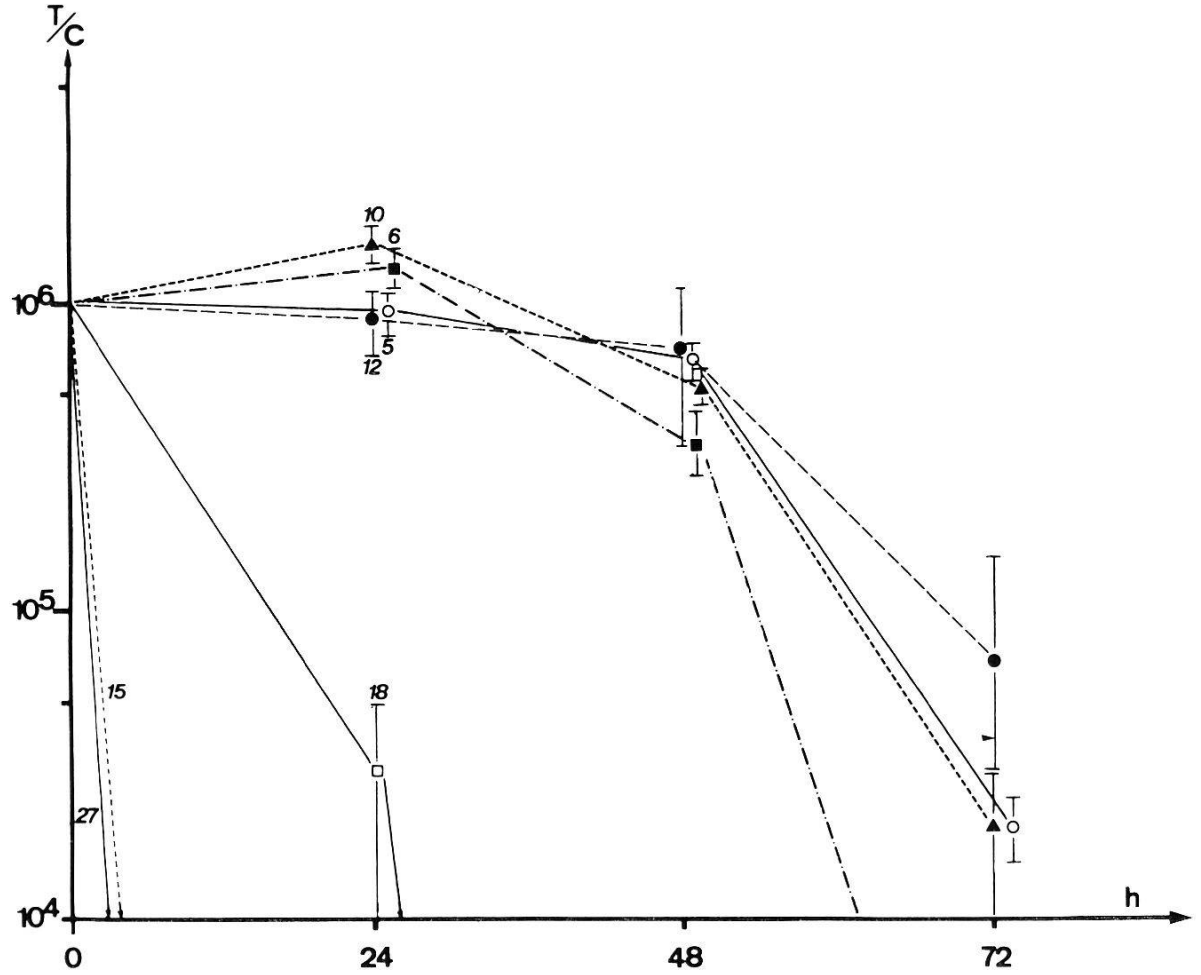


Fig. 4. Survival of bloodstream-like trypanosomes in BHF-conditioned media (BCM). Median ± S.E., number of cultures examined in italics, T/C = trypanosomes per culture. ——— = Controls; ---------- = 3-h BCM; ——□—— 6-h BCM; ---●--- 12-h BCM; ------▲------ = 24-h BCM; -·-·-■-·-·- = 48-h BCM; ——○—— = 72-h BCM.

with the growth characteristics of trypanosomes over a BHF feeder-layer (cf. Table 1). The inoculated trypanosome population displayed the well known (Hirumi et al., 1977a) monomorphism of the slender-like cultured bloodstream forms. During the first 24 h in BCM, the trypanosome population was still monomorphic and the infectivity was retained (Table 2). The monomorphism was gradually lost after 24 h parallel to the decreased cell number. Granulated thick dying forms became predominant after 48 h. This situation was also reflected in the infectivity tests (Table 2). The reversibility experiments were consistent with this interpretation, i.e. trypanosomes were reinoculated onto a BHF-layer after 24, 48 and 72 h in BCM. After 24 h in 24-h BCM, a trypanosome population could easily be reestablished in the BHF-system, whereas after 48 and 72 h in 24-h BCM, only some or even single trypanosomes started to grow giving rise to a new bloodstream-like trypanosome population.

Concentrated BCM also had no growth supporting effect for the trypanosomes. Again, only prolonged survival could be observed, comparable to that observed with BCM (Fig. 4). The question now arose as to whether the trypanosomes first had to stimulate the BHF to produce and release trypanosomal growth supporting factors. In several experiments with 24-h TBCM (also in combination with fresh medium) evidence for such a mechanism could not be found. Again only survival up to 48 h was observed.

Lyophilized and freeze-thawed BHF

Lyophilized BHF were added to 10^6 trypanosomes in concentrations equivalent to one or two confluent BHF-layers (3 experiments each). No prolonged survival or induction of cell divisions were observed in these assays. Freeze-thawed BHF were further tested for trypanosomal growth stimulating effects. Neither the BHF-lysate nor the lysed cell bodies containing sediment induced divisions in trypanosome populations. Trypanosomes incubated with the lysate died within 15 h. The sediment slightly supported survival ($\bar{x}$ after 24 h: 3.05×10^5, after 48 h: 2.0×10^4, from two experiments with an inoculum of 10^6).

As BCM, concentrated BCM, TBCM, lyophilized BHF and BHF extracts did not support any growth of the trypanosomes as bloodstream-like forms, the question arises as to whether a direct trypanosome/BHF association is a prerequisite for growth.

The intercellular trypanosome population

The observations of the intact BHF-system revealed that 15 min after the inoculation the trypanosomes localized intercellularly (single or in clusters with >50 trypanosomes). They seemed to have a predilection for the caverns formed by BHF. Intercellularly localized trypanosomes were motile and hardly stuck to one place. Therefore, the contact of the trypanosomes with one particular site of the BHF surface was extremely short, but could take place countless times. Scanning electron microscopy demonstrates the topography in a confluent

Table 2. Infectivity of S 427 bloodstream-like trypanosomes after different times in BHF-conditioned media (BCM), 10^4 motile forms were injected i.p. per mouse

ID:	2	3	4	5	6	7	8	9	10	11	12	13	21[a]
Inoculum[b]	14/15[c]	15/15											15/15
24-h BCM 24 h[d]	0/8	1/8	5/8	6/8		7/8					8/8		8/8
48 h	0/7							1/7		2/7		3/7	3/7
72 h	0/8												0/8
48-h BCM 24 h	0/3				1/3	3/3							3/3
48 h	0/3											1/3	1/3
72 h	0/3												0/3
72-h BCM 24 h	1/5	5/5											5/5
48 h	0/5		1/5			3/5	4/5						4/5

[a] Mice where no trypanosomes could be found 21 days after infection (ID) were considered as negative
[b] Cultured bloodstream-like trypanosomes used as inoculum
[c] Number of positive / total number of mice
[d] Time in BHF-conditioned media (BCM)

BHF-layer (Figs. 6, 7). It clearly revealed that there was no distinct arrangement of the trypanosomes within the intercellular spaces. The flagella were not found in a regular relationship with the BHF. Nevertheless, the number of trypanosomes per mm^2 BHF-layer was found to be very constant (cf. Table 1). As a single trypanosome could hardly be followed for a long period, we do not know how long a trypanosome remains intercellularly. Transmission electron microscopy did not reveal desmosomes or hemidesmosomes between the trypanosomes and the BHF or the plastic bottom of the tissue culture flask (Fig. 5).

The proportion of dividing forms among the intercellular trypanosome population was not significantly (χ^2-test) higher than that among trypanosomes of the supernatant (Table 3). However, the absolute number of trypanosomes in the supernatant was 12 times higher. T-25 flasks with a confluent BHF-layer were brought into the upright position, i.e. the BHF-layer was then vertical. Trypanosomes inoculated in such a system did not localize intercellularly. Only single forms were found in the layer and the majority of the forms was found on the BHF-free bottom of the flask. There was no continuous growth of the inoculated trypanosome population observed in such cultures where there was no established, intercellular trypanosome population. When these cultures were brought back into the horizontal position, an intercellular population was established and continuous growth was again possible.

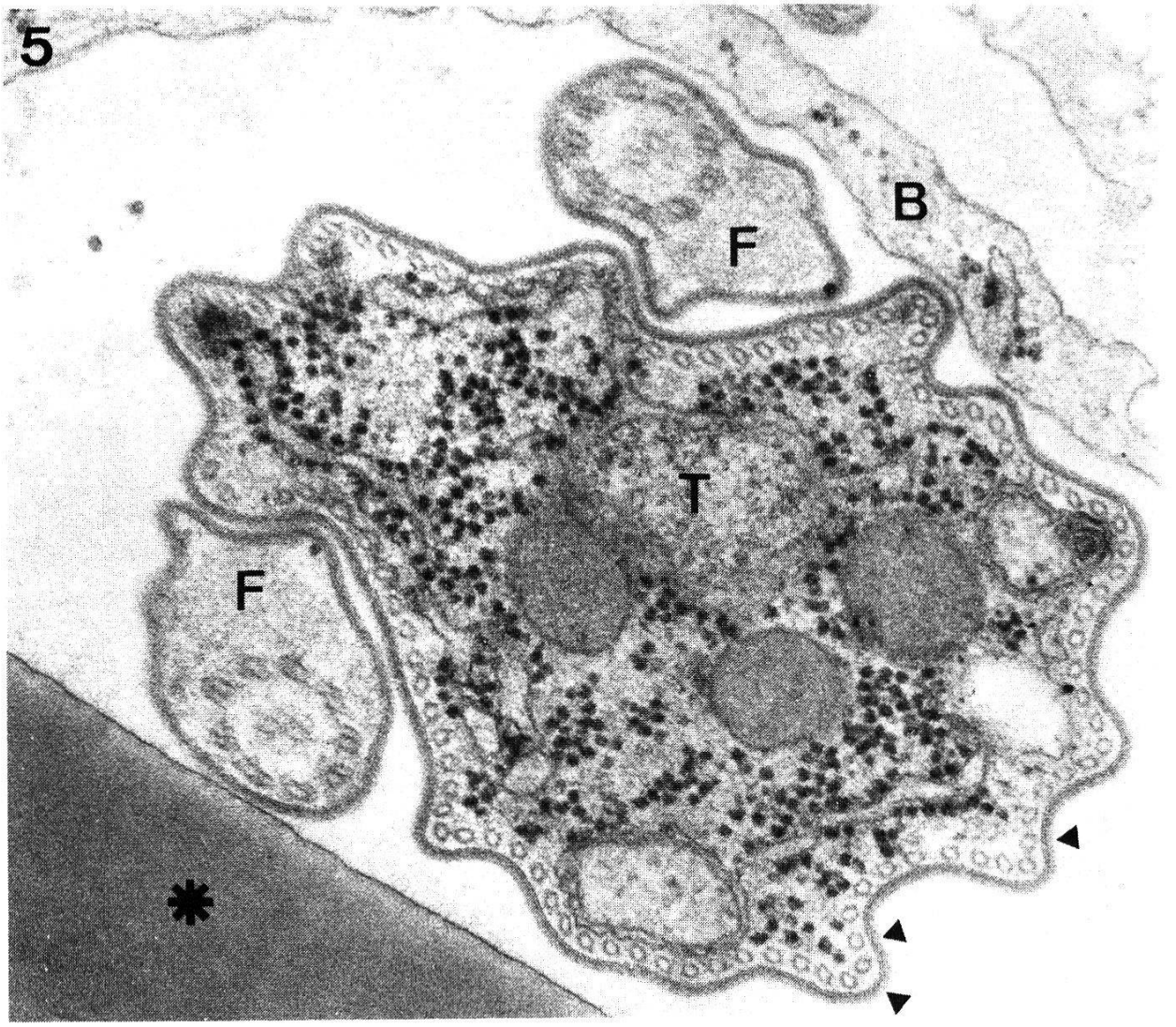
Separation of the trypanosomes from the BHF by micropore membranes

Trypanosomes (10^6) inoculated into a dialysis bag and placed directly onto a confluent BHF-layer survived as infectious forms without growth for 48 h ($\tilde{x} \pm$ S.E. 48 h: $0.9 \pm 0.55 \times 10^6$, n = 11). Control cultures (10^6 trypanosomes on a BHF-layer) continuously grew during 48 h ($\tilde{x} \pm$ S.E. 48 h: $15.9 \pm 1.9 \times 10^6$, n = 12).

When trypanosomes (2.5×10^5) were separated from the BHF-layer by an 0.45 μm Millipore filter in a specially designed plexiglass chamber (Fig. 1), growth of the bloodstream-like trypanosomes was no longer observed. No live trypanosomes were found 24 h later. There was continuous growth within control chambers lacking the filter. This clearly shows that growth inhibition and death of the trypanosomes was not caused by the nature of the plexiglass. Using 0.45 μm Millipore chambers loaded with either trypanosomes or BHF and inserted into the wells of a tissue culture plate (cf. Fig. 2 B), no growth of the trypanosomes (inoculum 10^5) was observed inside the chambers or around the BHF-containing chambers. After 24 h no live trypanosomes were found.

Fig. 5. Bloodstream-like trypanosome (T) in the spaces between the bovine fibroblast-like cells (BHF), surface coat (▶), flagellum (F). There are no contact structures between the trypanosome and the BHF (B) or the bottom of the tissue culture flask (★). 54 200×.

Figs. 6/7. Scanning micrographs of a BHF-layer with intercellular trypanosomes. There is no regular arrangement of the trypanosomes within the intercellular spaces. d = dividing form. 2400×.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B
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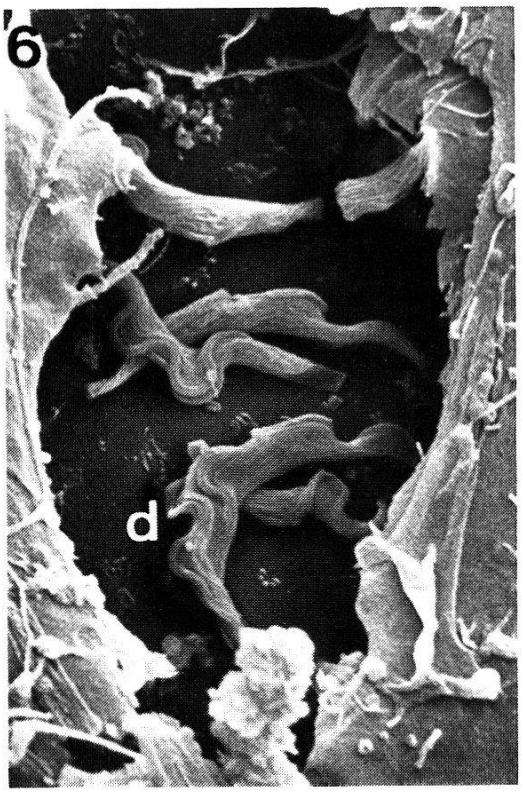
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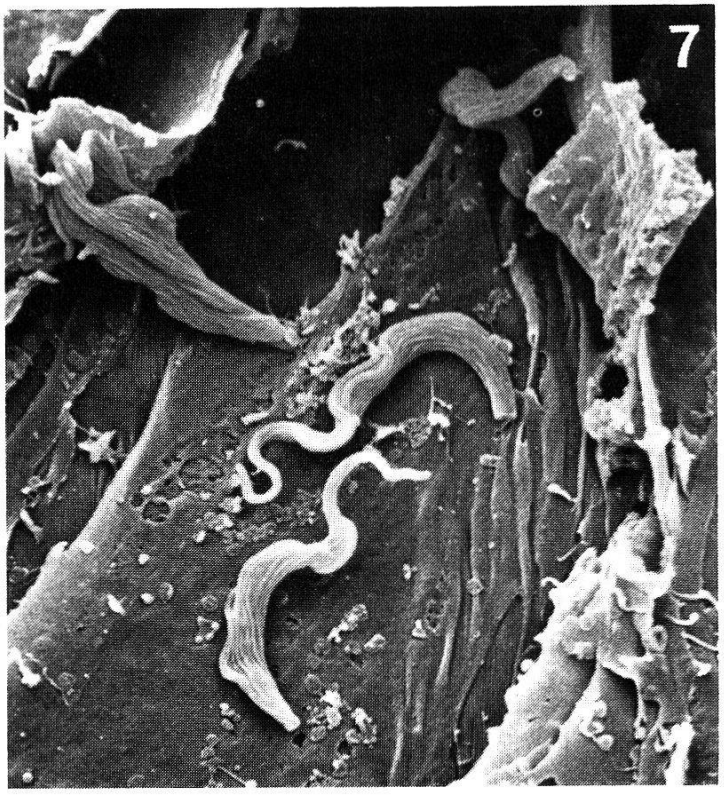
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Table 3. Proportion of dividing forms among trypanosomes found in the supernatant and in the BHF-layer

Trypanosome population	N[a]	Trypanosomes $\times 10^6$ ($\bar{x} \pm$ S.E.) after		% Dividing forms[b] $\bar{x} \pm$ S.D.
		inoculum	48 h	
Supernatant	12 (4)	1.0	14.5 ± 1.50	18 ± 5 (200)
BHF-layer[c]	12 (4)	–	1.2 ± 0.05	25 ± 6 (200)

[a] Number of cultures examined, number of independent experiments in brackets
[b] Number of trypanosomes counted per culture in brackets
[c] Intercellularly localized trypanosomes

As in all these chamber systems the path for diffusion between the trypanosomes and the BHF might have been too long for an adequate exchange of growth supporting factors, acryltube inserts (Fig. 2 A) were directly placed over BHF-layers in wells of tissue culture plates. Table 4 summarizes the results with this chamber system. For an appropriate evaluation of these results the following points have to be considered. First, the counts in the control cultures (control, control + filter, control + tube) represent the number of trypanosomes in the supernatant, while in the other systems the counts represent the total trypanosome number. Secondly, the daily medium exchanges were difficult to standardize due to the characteristics of the system (+ or − intercellular forms). In addition, the inserts reduced the growth area for the BHF. Nevertheless, the results are comparable during the first three days. It became evident that the Nucleopore membrane alone exerts no growth inhibition compared to the control. The acryltube was only inhibitory from 72 h onwards, but the cultures did not die out. Whenever a membrane separated the trypanosomes from the BHF-layer, growth was only minimal and significantly reduced to all the controls (Table 4). In addition, the trypanosomes died after 5 days. No continuous maintenance was possible within the inserts. Another mode of medium exchange (10% daily) also did not result in continuous maintenance or even growth. Trypanosomes could keep their infectivity within the inserts for 72 h (5×10^4 i.p. → prepatent period 4 days). The inserts did not damage BHF-layers, as it was possible to inoculate wells after the removal of the inserts (i.e. after 5 days) and a continuous growth comparable to the controls could be observed.

BHF which were enclosed into a 1.6 mm thick 0.75% agar-layer remained rounded and did not support growth of bloodstream like trypanosomes.

Fixed BHF-layers

BHF-layers were fixed with 1% formalin or with 0.25% glutaraldehyde in order to obtain indications as to the nature of the apparent trypanosome/BHF

Table 4. Growth of bloodstream-like trypanosomes within inserts (0.4 μm pore size) placed over BHF-layers in wells of tissue culture plates

System[a]		N[b]	Trypanosomes $\times 10^5$ ($\bar{x} \pm$ S.E.) after					Culture[c]	% medium exchanged			
			inoculum	24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h
Control	(200)[d]	11 (4)	1.0	2.0 ± 0.4 A	5.1 ± 0.8 E	10.9 ± 1.9 I	6.8 ± 1.9	+(>20 CD)	50	50	100	100▶
Control + filter	(200)	6 (2)	1.0	2.8 ± 0.4 B	8.0 ± 0.5 F	14.2 ± 1.0 K	5.5 ± 1.6	+(>20 CD)	50	50	100	100▶
Control + acryltube	(105)	6 (2)	1.0	2.9 ± 0.2 C	3.3 ± 1.0 G	2.4 ± 2.1 L	0.6 ± 0.5	+(>20 CD)	50	50	100	50▶
Acryltube inserts	(105)	6 (2)	1.0	1.7 ± 0.3 D	1.6 ± 0.6 H	1.4 ± 1.2 M	0.8 ± 0.8	−(5 CD)	50	50	50	50
Acryltube inserts	(105)	7 (3)	1.0	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	<0.01	−(5 CD)	10	10	10	10

[a] Culture system as described in Fig. 2 A

[b] Number of cultures examined, number of experiments in brackets

[c] Indicates if the cultures could be kept, number of days in culture (CD), respectively days of death in brackets

[d] mm^2 BHF-layer in the system

▶ = exchange of medium in this way until the cultures were terminated

U-(rank) test: 2 $P > 0.1$: A–B, A–C, E–F, E–G, I–K, L–M
2 $P < 0.05$: G–H
2 $P < 0.01$: C–D, I–L

association as trypanosomal growth trigger. These fixations are reported optimally to preserve antigenic determinants on cell surfaces (Nantulya and Doyle, 1977; Stocker and Heusser, 1979). However, the trypanosomes did not grow over these previously fixed BHF-layers and no intercellular populations were established (4 experiments for each fixation).

Fibronectin

The fibroblast surface antigen, fibronectin (reviewed by Yamada and Olden, 1978), could be detected on and within BHF using a specific antiserum in the IFAT. The well known fibrillar arrangement of fibronectin was observed. The examination of BHF-layers containing intercellular trypanosomes did not reveal any correlation between the occurrence of fibronectin and the localization of intercellular trypanosomes. The anti-fibronectin serum did not react with the trypanosomes.

Discussion

Since its isolation in 1960 (Cunningham and Vickerman, 1962) stock S 427 trypanosomes have been subpassaged, cloned and stabilized numerous times (cf. Cross and Manning, 1973). This has resulted in monomorphic trypanosome populations which no longer display pleomorphism and are no longer cyclically transmitted by the tsetse fly (Dr. L. Jenni, personal communication). It was such a trypanosome population which was propagated in vitro by Hirumi et al. (1977a, b). This raises the question as to what extent loss and/or blocking of certain features might have enabled continuous in vitro growth as bloodstream-like forms.

When trypanosomes were removed from the feeder-layer and transferred into complete or serum-free medium they died within 3 to 6 h (Fig. 3). This shows that the iFBS exerts little direct trypanosomal growth stimulating effect. It further indicates that growth-stimulating serum factors known from the mammalian tissue culture (reviewed by Rudland and Jimenez de Asua, 1979) or from the cultivation of *Leishmania* species and *T. cruzi* (O'Daly, 1980) do not play an important role in the BHF-system. The results from the cell-free system allow us further to assume that the RPMI 1640 + 20% iFBS is quite unfavourable for the trypanosomes and that it rather stimulates the BHF feeder-layer optimally.

The experiments with conditioned media (Fig. 4) suggest that there is no stable factor produced by the BHF which can trigger growth as bloodstream-like form. The initial doubling of the trypanosome population which was sometimes observed in 24-h and 48-h BCM, can only be considered as the completion of previously initiated divisions. If there are existing triggers in BCM, far more than a twofold increase of the trypanosome number would have to occur on the basis of the observed in vitro growth characteristics of bloodstream-like

forms (cf. Table 1). Nutrients are not limited in BCM at an initial dose of 2.5×10^5/ml, as many experiments with the intact BHF-system (e.g. Table 3) clearly showed that continuous growth can occur within 48 h without any medium exchange.

Conditioning of the medium did not seem to deplete the medium of essential nutrients or to accumulate inhibitory factors. It was still possible successfully to inoculate BHF overlayed with 48-h or even 72-h BCM.

Prolonged survival of the trypanosome population was the only effect observed in $\geq$12-h BCM (Fig. 4). A prolonged survival was also found when $>2.5 \times 10^5$/ml trypanosomes were inoculated into complete medium (Fig. 3.). This short-term survival appears to be supported by pyruvate, either metabolized by the BHF or the trypanosomes themselves (Tanner et al., 1980a). It was found that $\geq$0.1 mM was the critical initial pyruvate concentration in RPMI 1640 + 20% iFBS.

The present results show that only living, actively metabolizing BHF can support continuous growth of bloodstream-like forms in vitro. Earlier attempts to cultivate *T. brucei* as bloodstream forms have already indicated the need for metabolic active feeder-layer cells (Le Page, 1967; Hawking, 1971). Analogous requirements have been reported for *T. lewisi* (Dougherty et al., 1972; El On and Greenblatt, 1977), for *T. acomys* (El On and Greenblatt, 1977) and for *T. musculi* (Viens et al., 1977). In addition, the co-cultivation of host tissue and trypanosomes was found promising in attempts to cultivate *T. congolense* as bloodstream form (Gray et al., 1979).

As confluent BHF-layers as well as 2000 rad irradiated BHF (results not presented) supported growth of the bloodstream-like forms, multiplying BHF do not seem to be essential. A short-range inductive signal must be considered as the prerequisite for any growth of bloodstream-like forms in the BHF-system. The presence of an intercellular trypanosome population was one of the main growth characteristics in the intact BHF-system. Hirumi et al. (1977a, b) and Hill et al. 1978a) have already pointed out this phenomenon. In addition, intercellular trypanosomes are also an obligatory feature of culture systems developed for pleomorphic *T. brucei* stocks at 37° C (Hill et al., 1978b; Brun et al., 1979).

Although light- and electronmicroscopy did not reveal any regular arrangement of intercellular trypanosomes (Figs. 6, 7), the number of trypanosomes per mm² BHF-layer was surprisingly constant. As it can be assumed that all confluent BHF-layers offer the same possibilities for an intercellular localization, the constant number of intercellular trypanosomes per mm² might indicate an optimal exploitation of the available space. This supports the idea of an obligatory BHF/trypanosome association.

There was no relative and no absolute higher number of dividing trypanosomes among the intercellular trypanosome population (Table 3). Therefore, the dynamics between supernatant and intercellular trypanosomes appears as a

crucial point, i.e. how long trypanosomes remain close to the BHF and if there is any correlation with the cell cycle of the trypanosomes and/or of the BHF.

Besides these growth characteristics, the chamber experiments strongly support a growth inductive short-range signal. Whether this is transmitted by an exchange of unstable, low concentrated factors or by cell contact interactions is not yet clear. The experiments where BHF were separated from the trypanosomes by micropore membranes favour the latter possibility. Nevertheless, slight growth of trypanosomes within the acrylglass inserts could be observed for 3 days before the cultures died out (Table 4). The short-range exchange of inductive molecules might have been partially prevented in our system by the physico-chemical phenomenon of the diffusion boundary layer (Levich, 1962). The structure of the BHF-layer (Fig. 6) facilitates this process. Distinct microclimates would therefore be created within the countless caverns which could harbour growth factors. According to this, the negative results with conditioned media as well as with the chamber experiments would no longer be surprising. In this connection, Stoker (1973) has pointed out the significance of the diffusion boundary layer as possible cause of the density-dependent growth inhibition of mammalian cells in vitro.

The postulated BHF/trypanosome association does not seem to have any morphological expression. No contact structures such as desmosomes or hemidesmosomes could be observed (Fig. 5). This is in contrast to the division-associated, long-term contact interactions of different trypanosomatid species in their vector host (reviewed by Molyneux, 1976). However, adhesive interactions are not always reflected morphologically (Curtis, 1972). The motility and the arrangement of intercellular trypanosomes favour the idea of cell/cell contacts through non-average, low charge density, surface regions (Weiss, 1977). Our experiments with fixed BHF could support this view, as the fixatives probably altered the surface charge situation. Growth triggers mediated through specific cell surface constituents which function as intercellular ligands can presumably be excluded. Such interactions can still take place with fixed cells (Burger, 1977). It is also unlikely that this is due to the fact that bloodstream-like forms undergo in vitro antigenic variation (Doyle et al., 1979), which includes fundamental surface conversion. Furthermore, S 427 trypanosomes grow as bloodstream-like forms over different feeder-layer cell types (Hill et al., 1978a; Hirumi et al., 1980; Brun et al., 1979). It is hard to understand how the same receptor structures could have convergently evolved to this extent.

Although fibronectin is known as an adhesive and organizing protein (Yamada and Olden, 1978), it does not seem to play a role in mediating BHF/trypanosome interactions. There was no correlation between the occurrence of fibronectin and the localization of intercellular trypanosomes.

T. brucei sub-group trypanosomes multiply not only in the bloodstream but also extravascularly in various tissues (Losos and Ikede, 1972). Interestingly, intercellular trypanosomes from the lymphoid tissue were found to be mono-

morphic with a high proportion of dividing forms compared to the simultaneously isolated pleomorphic bloodstream trypanosomes (Tanner et al., 1980b) Analogies to the intercellular trypanosomes in the BHF-system may be assumed. It remains to be established with the in vitro system for pleomorphic stocks (Brun et al., 1979), whether feeder-layer forms can be compared with the intercellular trypanosome populations in vivo and if so, whether only trypanosomes from the supernatant display pleomorphism.

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