Effect of Monosaccharides on the Growth of *Trypanosoma brucei gambiense in vitro*[1)]

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Abstract: In a well-established culture of bloodstream forms of *Trypanosoma brucei gambiense*, trypanosomes usually adhere on the surface of feeder layer cells (FLC) and form clusters between these cells. The failure of trypanosome adherence on the surface of FLC will always lead to elimination of the parasites. To investigate this phenomenon, the effects of monosaccharides and anti-mouse brain cell rabbit serum (AMBRS) on the growth of bloodstream and procyclic forms of trypanosomes over new born mouse brain cells (NMBC) were assayed. Different concentrations of each of nine monosaccharides (α-D−(+)-glucose, D-(+)-galactose, D-(+)-mannose, α-D-(+)-fucose, D-(−)-ribose, D-(+)-xylose, D-(−)-arabinose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine) and various dilutions of antiserum were added to culture medium and used for cultivation of trypanosomes. At a concentration of 1.25 mM and above, mannose inhibited the growth of bloodstream forms on FLC but ineffective on procyclic forms. No inhibitory effect was observed on the remaining monosaccharides and antiserum tested. However, at 100 mM and above most of the monosaccharides had inhibitory effect. The present experiment showed that mannose inhibited specifically the growth of bloodstream forms of *T. b. gambiense* on FLC although its actual mechanism was not clarified.

Key words: *Trypanosoma brucei gambiense* (W) bloodstream forms, Feeder layer, *In vitro cultivation*, Monosaccharides, Cell-parasite adherence

INTRODUCTION

Bloodstream forms of *Trypanosoma brucei* group can be grown successfully in various mammalian feeder layer cells (Hirumi *et al.*, 1977a & b; Hill *et al.*, 1978a; Brun *et al.*, 1981; Mhando *et al.*, 1987b). Although these feeder layer cells (FLC) have been the subject of numerous metabolic and nutritional studies in supporting and promoting continuous growth of trypanosomes, mechanisms by which these cells do so are still far from clear (Tanner, 1980; Mhando *et al.*, 1987a).

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The predilection of trypanosomes for adherence on the surface of the cells or formation of clusters between the FLC has proved to be essential for a continuous cultivation (Hirumi *et al.*, 1977a & b; Hill *et al.*, 1978a; Tanner, 1980; Yabu and Takayanagi, 1986). Our previous data on two feeder layer system indicated that there is indeed a high degree of specificity involved in the adherence of bloodstream forms of *Trypanosoma brucei gambiense* (Wellcome strain) [*T. b. gambiense* (W)] to brain cells (Mhando *et al.*, 1987a). Furthermore, trypanosome adherence on FLC vary widely from various mammalian cells (Brun *et al.*, 1984) even in organs derived from the same litter of mice (Mhando *et al.*, 1986). Despite the wide spread adherence of trypanosomes on various mammalian cells, remarkably little is known about the components and mechanisms involved.

It is well accepted that mammalian cells contain various glycoproteins on the surface, which are implicated as receptors either on cell-cell or cell-parasite interactions (Theodor, 1970; Hughes, 1975; Ashwell and Morell, 1977; Keusch, 1979; Vickerman, 1969; Henriquez *et al.*, 1981). Taken together, it is logical to assume that some surface structures of *T. b. gambiense* (W) might have recognized some components on the surface of these cells. Some researchers have shown that attachment of parasites to cells can be inhibited by sugars that constitute the surface of the former (Ögmundsdottir and Weir, 1976; Ofek *et al.*, 1977). Thus, the purpose of the present study was to examine the effect of various monosaccharides and anti-mouse brain cell rabbit serum on the growth of *T. b. gambiense* (W) on mammalian feeder layer system as tools for the search of surface components which might have been involved in the adherence phenomenon.

Materials and Methods

Parasites:

Trypanosoma brucei gambiense (Wellcome strain) *T. b. gambiense* (W). The history of this isolate has been described elsewhere (Mhando *et al.*, 1986).

Trypanosoma brucei gambiense (TH−1/78E) *T. b. gambiense* (TH−1/78E). Was received from Tropical Institute, Basel Switzerland. It was isolated from an infected tsetse midgut. The isolate was passaged two times in mountain vole (*Microtus montanus*), once in BALB/c mice and once through *Glossina morsitans*. Procyclics were adapted in SDM-79 supplemented with 10% FBS and cryopreserved as STIB 733.

Compounds tested for inhibitory activity

Monosaccharides: 6-carbon sugars, α-D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, α-D-(+) fucose; 5-carbon sugars, D-(−)-ribose, D-(+)-xylose, D-(−)-arabinose and amino sugars, N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-galactosamine (GalNAc).

Mannan: Yeast mannan (*Saccharomyces cerevisiae*).

Lectin: Concanavalin A (Con A) type IV highly purified lyophilized and substantially free of carbohydrates. The above compounds were purchased from Sigma Chemical Company.

Feeder layer cells (FLC), culture medium and cultivation of trypanosomes: New born mouse cells obtained from brain (NMBC) were prepared and maintained as described earlier (Mhando *et al.*, 1986).

Eagle's Minimum Essential Medium (MEM) with Earle's salt (GIBCO) containing penicillin 200 U/ml and streptomycin 100 μg/ml was supplemented with a mixture of 5% new born bovine serum and 5% foetal bovine serum and used for experiments.

Most experiments were carried out in 24-well tissue culture clusters (NUNCLON DELTA SI-ROSKILDE, DENMARK). Forty eight hours prior to trypanosomes inoculation, each well was seeded with 1×10^5/ml NMBC and incubated at 37℃ in 5% CO_2 incubator. Trypanosomes from infected mouse blood were separated from blood components by chromatography method (Lanham and Godfrey, 1970). Trypanosomes were once washed, resuspended in culture medium and counted. Volumes of 0.1 ml parasite suspension (4.5×10^5 trypanosomes) were put into each tissue culture well containing 1.4 ml culture medium and incubated in the CO_2 incubator at 37℃.

Initiation of the cultures was carried out either by centrifuging the supernatant fluid and return all the pelleted trypanosomes into culture wells or by replacing half of the old medium by fresh one once every day until trypanosomes showed propagation or completely disappeared from the cultures. In those cultures where growth of trypanosomes became established in FLC. whole medium was changed every day and daily trypanosome density was estimated from the culture supernatant fluid.

Inhibition assay by monosaccharides: Tissue culture wells were seeded with NMBC and just before introduction of the parasites into the wells with confluent monolayer cells, 1.4 ml aliquots of culture medium containing different concentrations (5, 10, 25, 50 mM) of each monosaccharide except glucose which had different concentrations (15, 20, 25, 50 mM) (due to contents of 11 mM glucose in MEM) were placed into culture wells and cultivation of parasites was carried out as described above.

Effect of mannose on trypanosomes in various conditions:

1. *Different concentrations of mannose*: To determine minimum inhibition dose of mannose, culture medium was supplemented with different concentrations of mannose (5, 2.5, 1.25, 0.625, 0.313 mM) and was used for cultivation of *T. b. gambiense* (W) either at the inoculation of the parasites or after the formation of clusters.

2. *The time of addition of mannose into cultures*: Preparation of FLC and inoculation of *T. b. gambiense* (W) were performed as above, except that culture medium deprived of monosaccharides was used for cultivation until trypanosomes formed clusters between FLC. Thereafter (3−5 days post parasite inoculation) 1.5 ml aliquots of culture medium containing 10 mM D-(+)-mannose were used for cultivation and mannose was discontinued just before the parasites disappeared from the cultures. In another experiment 1.5 ml portions of culture medium containing different concentrations of one of the followings: D-(+)-

mannose (10 mM), mannan (5 mg/ml) and Con A (0.25 mg/ml) were used for cultivation after cluster formation.

3. *Feeder layer free culture*: To check the direct effect of mannose on parasites, cultures were initiated in 24-well tissue culture clusters without FLC. Volumes of 1.4 ml of culture medium containing mannose (5, 10, 25, 50 mM) and culture medium without mannose (as control) were distributed into culture wells and 0.1 ml of trypanosome suspension were inoculated to each well to give a final concentration of 3×10^5/ml trypanosomes. The cultures were incubated at 37℃ in a 5% CO_2 air-atmosphere.

4. *Procyclic forms*: Procyclics were cultured in T-25 tissue culture flasks (25 cm^2-3013-Falcon) with/without FLC. 5 ml portions of culture medium containing different concentrations of mannose (5, 10, 25, 50 mM) or without mannose (control) were dispensed into tissue culture flasks. Procyclics from culture were used as inoculum. A density of 3×10^5 /ml trypanosomes were inoculated into each tissue culture flask and the cultures were incubated at 27℃.

Uptake inhibition test: To determine interaction between glucose and mannose the method of Ruff and Read (1974) was used with modifications. Briefly, different concentrations of glucose (15, 20, 40 mM) (inhibitor) and 1 mM mannose (substrate) were mixed with culture medium and used for cultivation of *T. b. gambiense* (W).

Preparation of antiserum to feeder layer cells: Feeder layer cells (NMBC) were harvested from the cultures, washed 3 times with phosphate buffered saline [PBS (−)] and finally resuspended in the same buffer at a concentration of 1×10^7 cells /ml. Cell suspension was mixed well with an equal volume of Freund complete adjuvant. Rabbits (2,000 g body wt) were subcutaneously inoculated with 2 ml of the mixture at four sites of the back. The same volume of booster injections, which contained Freund incomplete adjuvant instead of complete one were carried out 3 times at an interval of two weeks. A fortnight after the last inoculation, rabbits were bled from either the ear vein or carotid artery. Antiserum titre of 1:6,000 was determined by indirect immunofluorescence method. Antiserum (AMBRS) and normal rabbit serum (NRS) were then heat-inactivated (56℃ for 30 min), filter-sterilized through a membrane filter with a pore size of 0.22 μm and stored in 10 ml aliquots at −20℃ until use.

Cultivation of T. b. gambiense (W) with antiserum: Various dilutions of antiserum and normal rabbit serum (1:50, 1:100, 1:200 and 1:400) were prepared by mixing with culture medium and used for cultivation of *T. b. gambiense* (W) in various conditions: 1. In concurrence with parasites inoculation. 2. Incubation of FLC with antiserum culture medium for 2 h at 37℃ before parasite inoculation. 3 After cluster formation.

RESULTS

Effect of monosaccharides on the growth of Trypanosomes: When bloodstream forms of *T. b. gambiense* (W) were cultured on FLC in medium supplemented with various concentrations of each of the monosaccharides, remarkable growth inhibition of trypanosomes was observed with mannose at a concentration of 5 mM and above (Fig. 1). This inhibiting effect

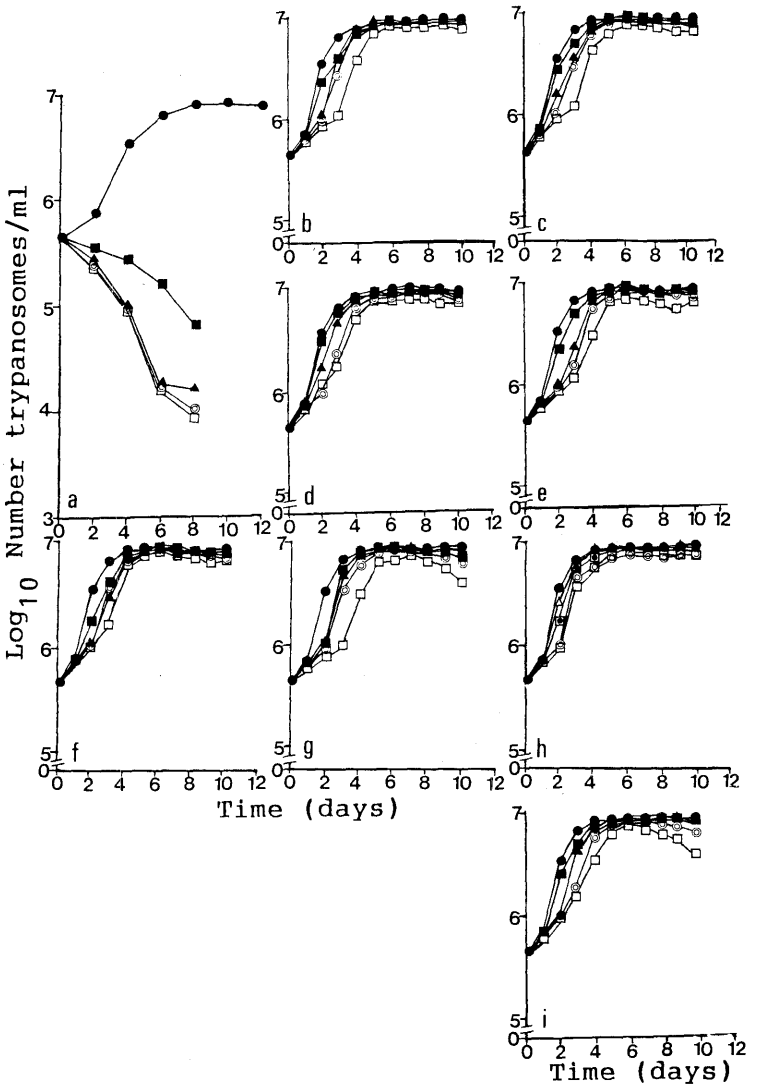

Fig. 1. The effect of monosaccharides on *T. b. gambiense* (W) growth over NMBC. The parasites were cultured in complete medium in the presence of various concentrations of monosaccharides: 5 mM (■), 10 mM (▲), 15 mM (△), 20 mM (◉), 25 mM (◎), 50 mM (□) and normal culture medium (●). a: mannose, b: galactose, c: fucose, d: xylose, e: arabinose, f: GlcNAc, g: GalNAc, h: glucose and i: ribose. Each point represents the mean log number of parasites in quadruplicates.

became apparent 24 h after incubation with mannose. At a concentration of 100 mM or greater, the rest of monosaccharides tested inhibited the growth of the trypanosomes. Inhibitory effect of high concentrations of monosaccharides is probably due to increased osmotic pressure in the culture medium rather than biochemical effect of sugars.

Effect of various concentrations of mannose on T. b. gambiense (W) growth: The effect of different concentrations of D-(+)-mannose on the growth of trypanosomes is shown in Fig. 2. When mannose was introduced into culture after cluster formation, growth curves indicate as if the parasites decreased faster than when mannose was used during the initiation period of trypanosomes. This inclination difference of growth curves was probably due to localization of trypanosomes. In the former case, parasites adhered or formed clusters between FLC, while in the latter most of trypanosomes were in the culture medium. The decrease of trypanosomes depended on the dose of mannose used but statistically there was no significant difference on the decrease of parasites between 25 mM and 50 mM. ($P<0.05$ by Scheff's Test).

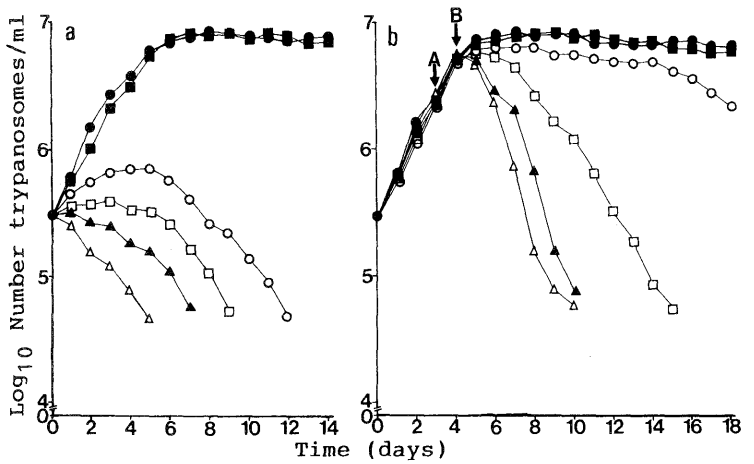

Fig. 2. The effect of various concentrations of D-(+)-mannose 0.313 mM (■), 0.625 mM (○), 1.250 mM (□), 2.50 mM (▲), 5.0 mM (△) and culture medium (●) on the growth of *T. b. gambiense* (W) on NMBC. a: mannose introduced into the cultures from the beginning of cultivation, b: mannose introduced after cluster formation. Arrows indicate A: formation of clusters, B: introduction of mannose into the cultures. Each point represents the log number of parasites from four replicates.

Effect of Con A and mannan on established T. b. gambiense (W) in culture: After cluster formation, normal culture medium was replaced by culture medium supplemented with one of the followings: 10 mM mannose, 5 mg/ml mannan and 0.25 mg/ml Con A. Parasites tremendously decreased in number in those cultures where mannose was used, whereas, no inhibitory effect was detected in cultures with mannan (Fig. 3), likewise, Con A had neither inhibitory nor agglutinative effect on trypanosomes but after 4 days there was destruction of FLC and formation of precipitate, hence, it was abondaned.

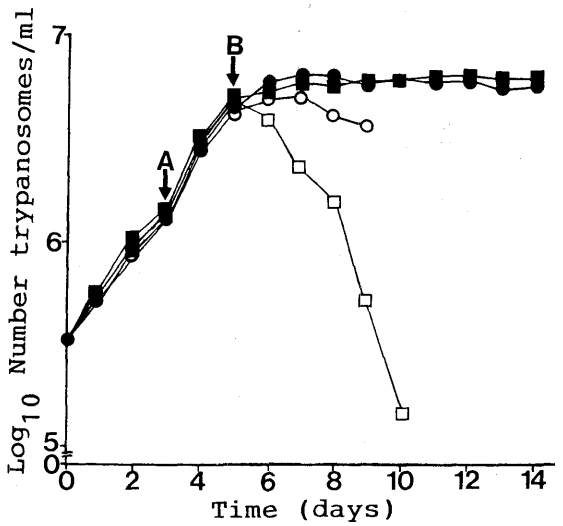

Fig. 3. Effect of 10 mM D-(+)-mannose (□), 5 mg/ml mannan (■), 0.25 mg/ml Con A (○) and normal culture medium (●) on the growth of *T. b. gambiense* (W) over NMBC after cluster formation. Arrow A indicates cluster formation and B shows time of introduction of mannose, mannan and Con A into the cultures. Each point represents mean long number of parasites from four replicates.

Effect of mannose on FLC: To cross-check whether the inhibitory effect of mannose was due to an effect on FLC, culture medium containing 10 mM mannose was introduced into cultures with established trypanosomes. Before the parasites completely disappeared from the cultures, mannose was removed and then parasites and feeder cells were cultured again using normal culture medium (Fig. 4). There was neither interference on cell-cell (NMBC) interaction nor morphological change of monolayer cells after the addition of 10 mM mannose. The lag-phase after discontinuation of mannose was probably due to small number of trypanosomes in the cultures or temporary, if any remaining effect of mannose on the FLC. However, on day twenty of cultivation there was no difference of parasite density between cultures in which normal culture medium was used and in those cultures in which mannose was discontinued, indicating that there was no severe damage on FLC.

Effect of mannose on bloodstream and procyclic forms on feeder layer free cultures: To determine the effect of mannose on parasites, bloodstream and procyclic forms were incubated with mannose (5−50 mM). Bloodstream forms disappeared after 2−3 days of cultivation in both control and test cultures (data not shown). No difference on trypanosome growth was detected between control and test cultures of procyclic forms (Fig. 5).

Competitive inhibition effect: Various concentrations of glucose even at a ratio of 1:40 (substrate : inhibitor) could not eliminate growth inhibitory effect of mannose (Fig. 6).

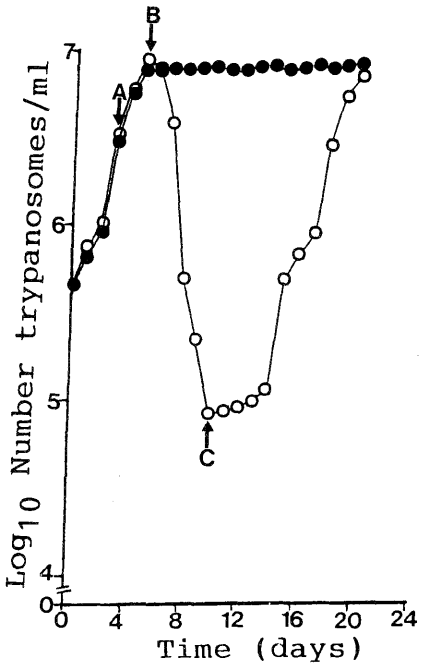

Fig. 4. Trypanosome growth on feeder layer cells before, during and after introduction of culture medium supplemented with 10 mM D-(+)-mannose from day 5 to day 9 (B−C) (○), normal culture medium (●). Arrows A, B, C indicate cluster formation, time of introduction of mannose into cultures and time of discontinuation of mannose respectively. Each point represents mean log number of parasites in quadruplicates.

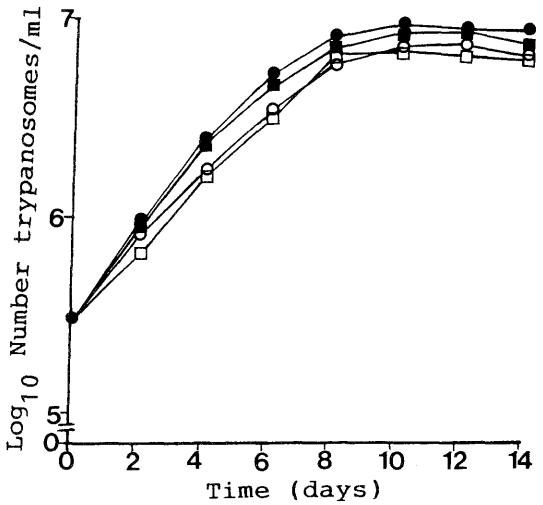

Fig. 5. Procyclic growth on feeder layer cultures with normal culture medium (●), culture medium plus 50 mM mannose (■) and feeder layer free cultures with normal culture medium (○), culture medium plus 50 mM mannose (□). Each point represents the mean log number of parasites in quadruplicates.

Effect of antiserum on the growth of T. b. gambiense (W): When different dilutions of antiserum (1:50 and greater) were used for cultivation of bloodstream forms in various conditions no inhibitory growth effect on parasites was detected (Fig. 7). Nevertheless, at a dilution of 1:25, the parasites decreased in number due to the destruction of FLC.

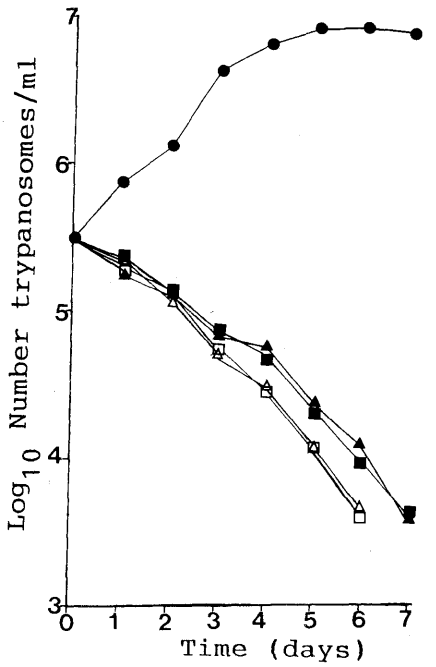

Fig. 6. Competitive effect of glucose and mannose in various concentrations on *T. b. gambiense* (W) over NMBC. Culture medium is supplemented with 15 mM (□), 20 mM (▲), 40 mM (■) glucose against 1 mM D-(+)-mannose. Normal culture medium (●) and culture medium plus 1 mM D-(+)-mannose (△) were used as controls. Each point represents mean log number of parasites in quadruplicates.

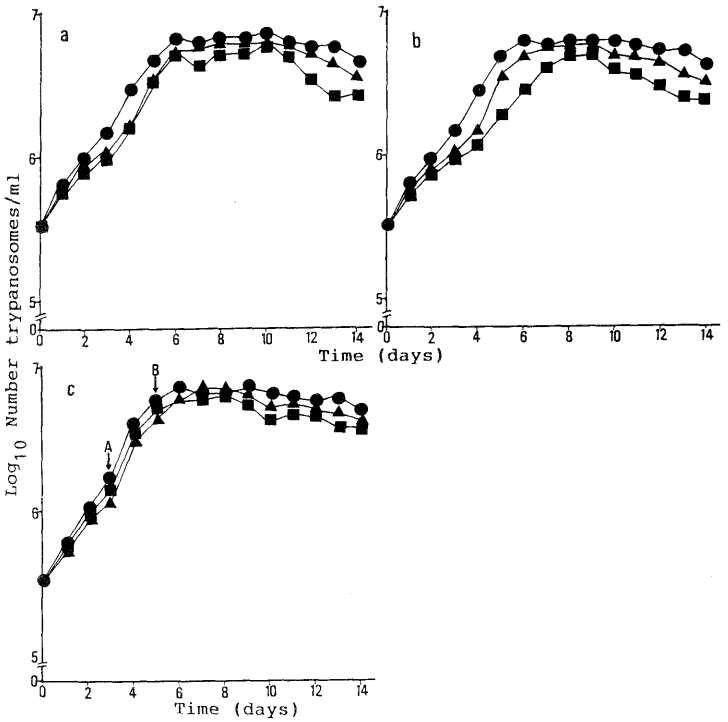

Fig. 7. The effect of AMBRS on *T. b. gambiense* (W) growth on various conditions, a; AMBRS or NRS was introduced during inoculation of parasites, b; FLC were incubated with AMBRS or NRS for 2 h at 37℃ prior parasite inoculation, c; AMBRS or NRS was introduced on day 5 (B) after cluster formation on day 3 (A). The parasites were cultured in normal culture medium (●) and culture medium plus 1:50 NRS (▲) or plus 1:50 AMBRS (■). Each point represents mean log number of parasites in quadruplicates.

DISCUSSION

In previous studies we demonstrated that trypanosome adherence on the surface of NMBC and formation of clusters between these FLC were essential for a continuous cultivation. The importance of this close association between FLC and intercellularly localized parasites has also been reported by other researchers (Hirumi *et al.*, 1977a; Tanner, 1980; Brun *et al.*, 1979; Agda *et al.*, 1985; Curtis, 1972). The mode of attachment of bloodstream forms of trypanosomes to FLC and mechanisms by which these FLC support and promote continuous growth of trypanosomes have not been well clarified.

Of all the carbohydrates tested in our present study mannose was the only monosaccharide which inhibited trypanosome adherence and cluster formation on the surface of FLC and consequently, lead to inhibition of trypanosome growth. Several studies have shown that adherence between cell-cell or cell-parasite can be inhibited by lectins (Ofek *et al.*, 1977; Chiari *et al.*, 1978; Nicolson, 1974; Weir, 1980) or monosaccharides that con-

stitute cell/parasite surface carbohydrates (Weir *et al.*, 1979; Warr, 1980). However, the experiment using mannan in the cultivation of trypanosomes ruled out the idea that some components on the surface of *T. b. gambiense* (W) might have acted on mannose-containing receptors on the surface of FLC or vice-versa, because no inhibition of cluster formation or adherence of parasites on the FLC was observed. Further confirmation came from the finding that Con A, a lectin which has the ability to bind to cell surface carbohydrates with α-D-glucopyranosyl, α-D-mannopyranosyl, β-D-furanosyl or α-D-arabinofuranosyl residues and identify cell surface accessible sugars (Sharon and Lis, 1972; Goldstein *et al.*, 1973; Lis and Sharon, 1973), could not inhibit the adherence phenomenon. Destruction of FLC might have been caused by the cytotoxic effect of Con A (Briles and Kornfeld, 1978). The anti-NMBC rabbit serum was used on the notion that antigenic molecules on the surface of FLC might have been implicated in the adherence phenomenon. However, from the present results it is less likely that NMBC possess such components.

Studies on transport and metabolism of mannose and other monosaccharides in trypanosomes have been reported (Ryley, 1956; Von Brand *et al.*, 1967; Southworth and Read, 1969). Early studies showed that glucose and mannose share the same transport locus and that one can act as a competitive inhibitor of the other depending on the concentration used (Ruff and Read, 1974; Southworth and Read, 1970; Seed *et al.*, 1965). It therefore appears in our present study that mannose might have inhibited the uptake of glucose by trypanosomes, as a result the parasites disappeared from the cultures. However, when competitive inhibition test was carried out by using various concentrations of glucose against 1 mM mannose, no improvement of trypanosome growth was observed. The failure of high concentrations of glucose to eliminate the ability of mannose inhibiting trypanosome growth indicate that inhibitory activity of mannose was not solely due to the uptake-mechanisms and therefore, cannot be used as an argument infavour of inhibitory mechanisms. This was further confirmed from the results obtained when the growth of procyclic forms was not affected by 50 mM mannose.

When bloodstream forms of trypanosomes were cultured for 2−3 days with culture medium or culture medium plus 20−50 mM mannose in feeder layer free system, parasites equally decreased from the cultures. Furthermore, incubation of trypanosomes with 20−50 mM mannose for 6 h could neither affect the normal motility nor infectivity of the parasites (data not shown). In addition, there was no inhibitory effect of mannose on the growth of *T. b. gambiense* (W) when feeder layer cells were incubated with mannose for 24 h before parasite inoculation or when mannose was removed from cultivation just before the parasites disappeared from the cultures. It appears that probably mannose is not directly toxic for either parasites or feeder layer cells or more time is required for this effect to occur. In fact, the observation of clustering trypanosomes at intervals of 2 h for 12 h after mannose introduction into the cultures, showed no obvious separation of trypanosomes from a cluster which could have been expected if mannose either masked receptors on FLC or directly produced toxic effects to these cells. Since feeder-cells are not affected by mannose, it is most likely that mannose may act on bloodstream forms in

culture through slow but unknown metabolic processes. In the present study, the inhibitory effect of mannose was also detected in the cultivation of other species of bloodstream forms of *T. b. gambiense* (ILRAD 1582) and *T. b. rhodesiense* (ILRAD 1501) suggesting that results obtained in this study are not due to a peculiarity of a single strain or species of trypanosomes.

At present, inhibitory effect of mannose on *T. b. gambiense* (W) growth over NMBC is not clear. It is proposed that an intensive study using labelled mannose and derivatives of mannose may be helpful in elucidating the exact growth inhibition mechanism(s). Nevertheless, it is worth pointing out that mannose can be useful in eliminating bloodstream forms of trypanosomes in those culture systems in which procyclic forms are selectively grown.

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培養 *Trypanosoma brucei gambiense* の増殖に対する単糖類の影響

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Trypanosoma brucei gambiense の血流型原虫 (TGBSF) を培養するにあたって，適した侍養細胞 (feeder cell) を用いると，原虫は細胞に付着あるいは細胞間隙に巣を形成して増殖し，培養液中でも増えてくる．原虫が細胞と接してそのような巣を形成できないと原虫増殖は維持できず，結果としてその細胞は侍養細胞として不適である．この現象が原虫も含めて細胞の表面構造に関連していると想定して，単糖類および侍養細胞として用いた新生仔マウス脳細胞に対する家兎抗血清の TBGSF 増殖に対する影響を検討した．

9種類の単糖類α-D-(+)-グルコース，D-(+)-ガラクトース，D-(+)-マンノース，α-D-(+)-フコース，D-(−)-リボース，D-(+)-キシロース，D-(−)-アラビノース，N-アセチル-D-グルコミサンそしてN-アセチル-D-ガラクトサミンを種々の濃度に加えみたところマンノースのみ 1.25 mM 以上の濃度で TGBSF の増殖を阻害した．しかしプロサイクリック型に対してマンノースの影響はなかった．又侍養細胞に対する家兎抗血清は TGBSF の培養を阻害しなかった．用いた糖でマンノース以外の糖でも TGBSF の増殖阻害が認められたが，それは 100 mM 以上の濃度でないと現れなかった．以上，その作用機序は不明ながら，マンノースが特異的に TGBSF の増殖を阻害することを見出した．

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