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Studies of Cells Derived from Brain and Muscles of New Born Mouse in Supporting the Growth of Bloodstream Forms of Trypanosomes

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Abstract: Factors which support and promote the growth of bloodstream forms of *Trypanosoma brucei gambiense (Wellcome)* over new born mouse brain cells (NMBC) and muscle cells (NMMC) were analysed. *T.b. gambiense* were cultured in a two feeder layer system in which half of a tissue culture dish was seeded with NMBC or NMMC and the other half with one of the feeder layer cells (new born mouse kidney cells–NMKC, established mouse brain cells–EMBC, established mouse muscle cells–EMMC) which did not support the growth of trypanosomes. No trypanosomal growth effect was detected on the side of the latter feeder layer cells, likewise, no trypanocidal effect was observed on the side of NMBC or NMMC. Some investigations have shown that rapidly growing cells could not support continuous growth of trypanosomes. Thus, to suppress the growth of these feeder layer cells, NMBC, NMMC, new born mouse skin cells, NMKC, EMBC, EMMC, new born mouse heart cells and L–cells were exposed to different doses of X–irradiation two days prior to parasite inoculation. Nevertheless, no improvement for the growth of trypanosomes was noticed on feeder layer cells which did not support the growth before, while NMBC and NMMC retained the ability to support and promote the growth. Trypanosomes adhered on the surface of cells and formed clusters between the feeder layer cells when they grew successfully. This close association between feeder layer cells and intercellularly localized trypanosomes might have influenced continuous growth of *T.b. gambiense (Wellcome)*.

Key Words: *Trypanosoma brucei gambiense, in vitro* cultivation, feeder layer, new born mouse, growth factor.

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INTRODUCTION

Insect forms of African trypanosomes can easily be cultured in a variety of axenic media (Taylor and Baker, 1978) but many infective bloodstream forms have achieved little success. Le Page (1967) partially succeded to grow bloodstream forms of *Trypanosoma brucei* at 37℃ in the presence of mammalian feeder layer cells, but the parasites lost their infectivity for mammals after a week of *in vitro* cultivation. Continuous cultivation of animal infective bloodstream forms of *T. brucei brucei* stock 427 at 37℃ in a system consisting of bovine fibroblast−like cells in HEPES−buffered Roswell Park Memorial Institute (RPMI) 1640 supplemented with 20% heat inactivated foetal bovine serum was successfully achieved by Hirumi *et al.* (1977a, b). Hill *et al.* (1978a, b) propagated the same stock of *T.b. brucei* and *T.b. rhodesiense* in a system consisting of Chinese Hamster and buffalo lung tissue culture cells. Brun *et al.* (1979, 1981, 1984) used fibroblast−like cells isolated from embryos of New Zealand white rabbit, mountain vole (*Microtus montanus*) human lung cells and fibroblast cells from different organs of African wild bovidae. Mhando *et al.* (1986) succeded to cultivate *T.b. gambiense* Wellcome strain in a system consisting of new born mouse brain cells (NMBC) and new born mouse muscle cells (NMMC) in MEM with Earle's salt supplemented with a mixture of 5% foetal bovine serum and 5% calf serum.

Efforts have been made to determine the mechanisms of these feeder layer cells but, so far, no satisfactory function has been described. When RPMI 1640 supplemented with 20% foetal bovine serum was used without feeder layer cells, it did not support the growth of trypanosomes (Hirumi *et al.*, 1977a). Growth characteristics of bloodstream forms of trypanosomes varied considerably depending on growth characteristics of mammalian cells. Feeder layer cells which multiplied very rapidly could not support continuous growth of *T. brucei* (*T. brucei* TC221−3 and TC227−F) (Hirumi *et al.*, 1980). To inhibit multiplication of feeder layer cells Agda *et al.* (1985) exposed human fibroblast−like cells to a 5,000 R−gamma−irradiation before inoculation of *T. brucei* strain 336D.

Tanner (1980) checked trypanosomal growth supporting factors by separating feeder layer cells from trypanosomes by a millipore filter. Trypanosomes cultured in that system could not grow. We suspected that trypanosomal growth supporting factors could not pass through the millipore filter at a stationary condition. Hence, in our investigation we used two feeder layer system which could allow these factors pass or diffuse freely from one feeder layer area to another.

In the present study we have attempted to analyse the factors which might promote the growth of bloodstream forms of *T.b. gambiense* Wellcome strain on feeder layer cells derived from brain and muscles of a new born ICR mouse.

Materials and Methods

Parasites:

Trypanosoma brucei gambiense (Wellcome strain), was used in the experiments. The history of this stock was described earlier (Mhando *et al.*, 1986).

Feeder layer cells:

1. *Trypanosoma growth supporting cells:* New born mouse cells were obtained from brain (NMBC) and muscles (NMMC). These cells could be used for cultivation of trypanosomes up to four months after which they became established and were unable to support and promote the growth of parasites.

2. *Non−growth supporting cells:* New born mouse cells were obtained from heart (NMHC), kidneys (NMKC) and skin (NMSC). L−cells, established mouse cells derived from brain (EMBC) and muscles (EMMC) which showed rapid growth and have lost the ability to support the growth of trypanosomes were also used for experiments. These cells were prepared and maintained as previously described (Mhando *et al.*, 1986).

Preparation of feeder layer cells:

1. *Two feeder layer cells:* Two days prior to parasite inoculation, feeder layer cells were initiated by seeding one half of a tissue culture dish (Falcon 100×20 mm) with 2×10^5/ml NMBC or NMMC and the other half with the same density of NMKC, EMBC or EMMC. The feeder layer cells were separated by a silicon rubber sheet (SILICON 7025 SANPLATEC 87×17× 4 mm) and incubated at 37℃ CO_2 incubator for 3 h, and thereafter silicon rubber sheet was removed (Photo. 1).

2. *Irradiated cells:* The minimum level of radiation required for inhibiting cell division was determined in all new and established feeder layer cells used for experiments. Each of thirty two tissue culture dishes (Nunc 60×15 mm) was seeded with 2×10^5/ml cells derived from the same organ. The culture dishes were then incubated at 37℃ in 5% CO_2 incubator for 24 h to allow the formation of confluent monolayer. Thereafter, following the method previously described (Puck and Marcus, 1955), group of eight culture dishes were irradiated in X−ray source (Toshiba KXC−19−7 depth therapy generator) at one of the following doses: 1,000, 2,000, 3,000 and 5,000 rad. After every two days, two tissue culture dishes from each group were trypsinized and viable cells assessed by trypan blue exclusion, were counted in Neubauer haemocytometer. Inhibition effect of cells was observed at the level of 2,000 rad. and above (Fig. 1). At 5,000 rad feeder layer cells began sloughing off from the culture dish. Hence, in this study doses of 1,000, 2,000 and 3,000 rad

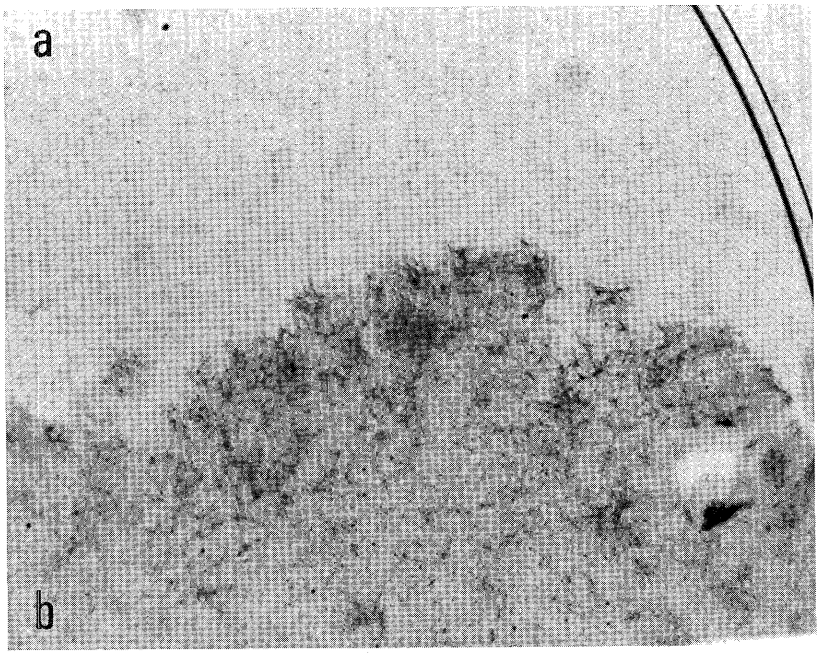

Photo. 1. Two feeder layer cells seeded in the same tissue culture dish.
a: New brain cells.
b: New kidney cells.

were chosen for irradiation of cells. Parasites were inoculated two days post irradiation of feeder layer cells.

Culture medium: MEM with Earle's salt (GIBCO) containing penicillin 200 U/ml, streptomycin 100 μg/ml and pH 7.4 was supplemented with a mixture of 5% foetal bovine serum and 5% calf serum (FCS) before use in propagation of trypanosomes.

Cultivation of T.b. gambiense: Blood from infected ICR mouse was obtained by cardiac puncture when parasitaemia reached 1×10^8 trypanosomes/ml of blood. Trypanosomes were separated from blood components following the method described by Lanham and Godfrey (1970). Trypanosomes were once washed and resuspended in culture medium. The volumes of 15 ml and 5 ml of parasite suspension (3×10^5/ml) were introduced into each culture dish with confluent monolayer cells of two feeder layer cells and irradiated cells respectively and incubated at 37℃ in 5% CO_2 incubator. Initiation and maintainance of the cultures were carried out as previously described. After every 24 h the trypanosome density was estimated from the culture supernatant fluid. But, in case of two feeder layer cultures population densities were determined from each half of a tissue culture dish after placing a silicon rubber sheet between two feeder layer cells.

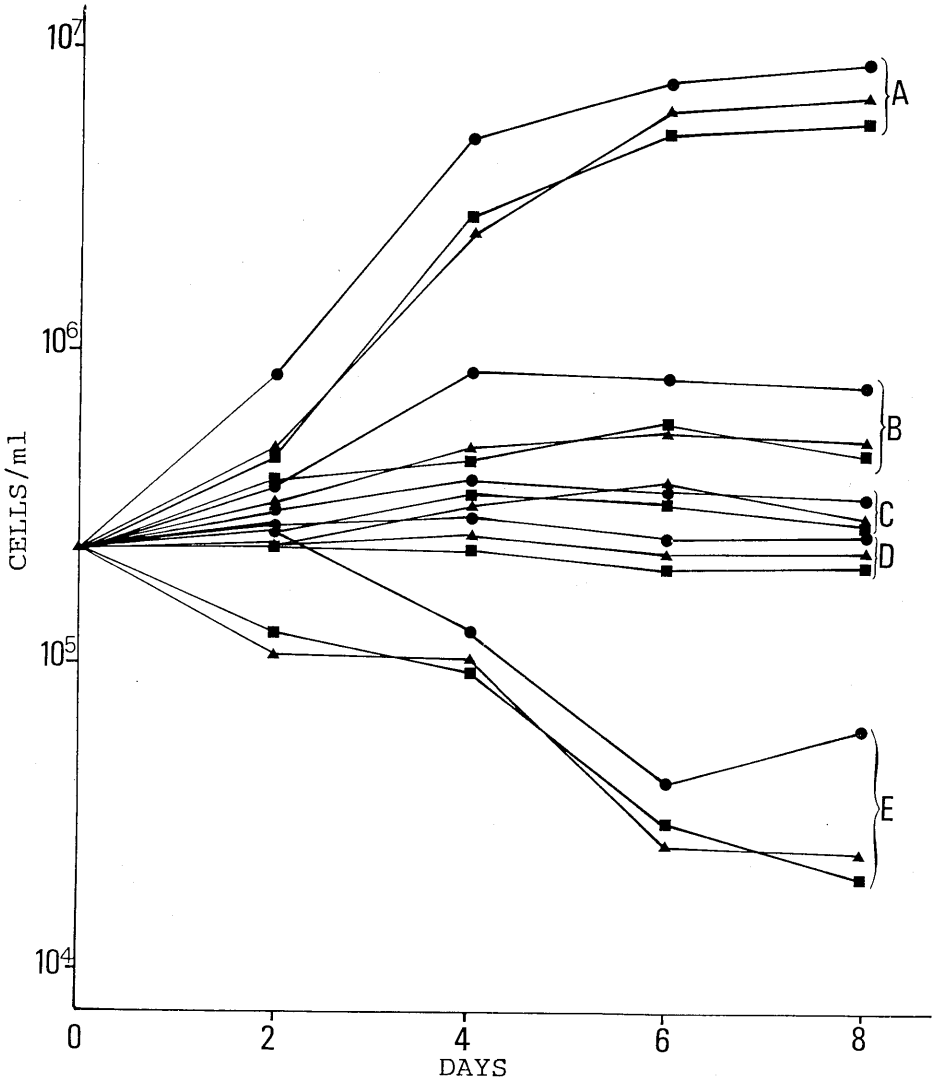

Fig. 1. Effect of different doses of X−irradiation on the growth of established muscle cells (●), new skin cells (▲) and new brain cells (■). A: 0 rad, B: 1,000 rad, C: 2,000 rad, D: 3,000 rad, E: 5,000 rad.

RESULTS

1. *Growth of T.b. gambiense in two feeder layer cells:* When trypanosomes were cultured in a dish seeded with two feeder layer cells, they grew differently on these feeder layers. Continuous growth of bloodstream forms of *T.b. gambiense* was observed only on the side of NMBC and NMMC, while on the side of NMKC, EMBC and EMMC trypanosomes gradually decreased and were uncountable after 7−9 days (Fig. 2.).

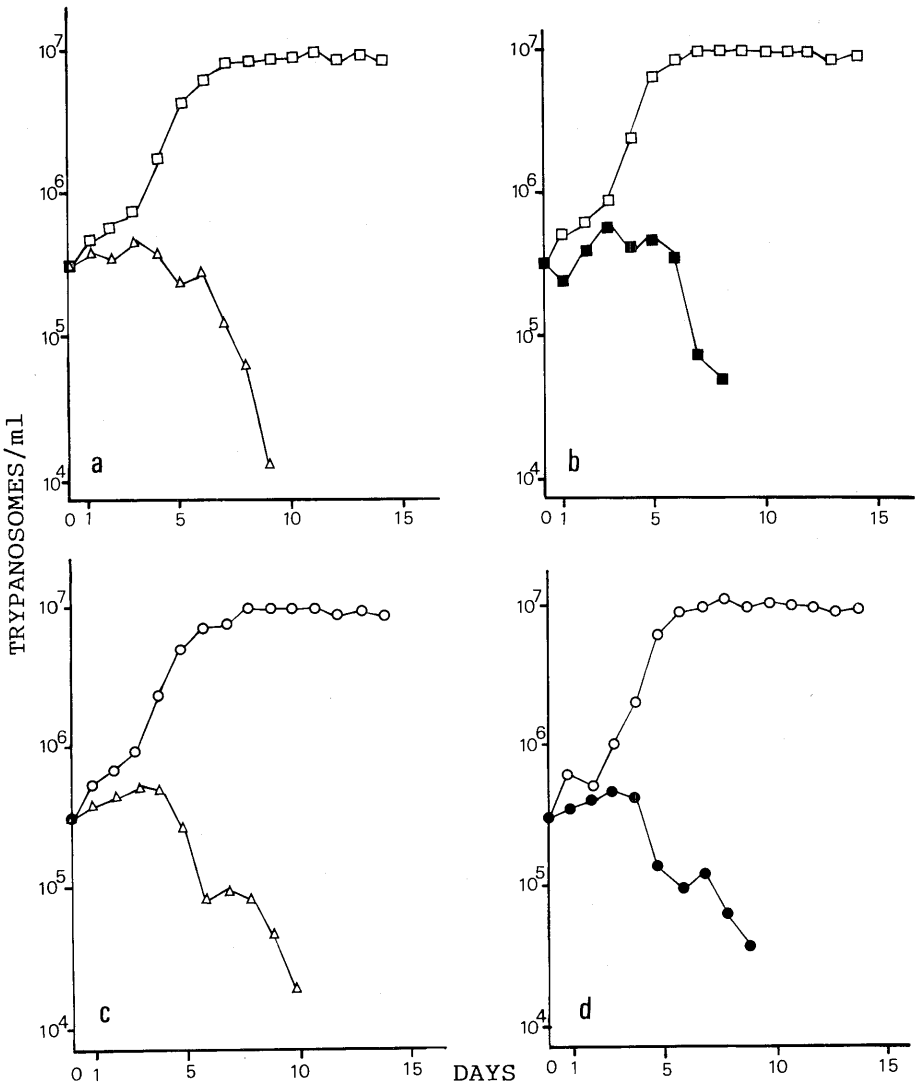

Fig. 2. Growth curves of *T.b. gambiense* in a tissue culture dish seeded with a: New brain cells (□) and new kidney cells (△) , b: New brain cells (□) and established brain cells (■), C: new muscle cells (○) and new kidney cells (△), d: New muscle cells (○) and established muscle cells (●). The mean number of trypanosomes counted every day in three experiments is represented by each point.

2. *Growth of T.b. gambiense on irradiated cells:* When EMMC, NMBC and NMSC were exposed to one of the following doses 1,000, 2,000 and 3,000 rad, growth of bloodstream forms was observed on NMBC only, which originally had the ability to support the growth of trypanosomes (Fig. 3). Furthermore, when the remaining feeder layer cells (L−cells, NMMC, NMKC, NMHC and EMBC) were irradiated with 3,000 rad only NMMC could support the growth of trypanosomes (data not shown).

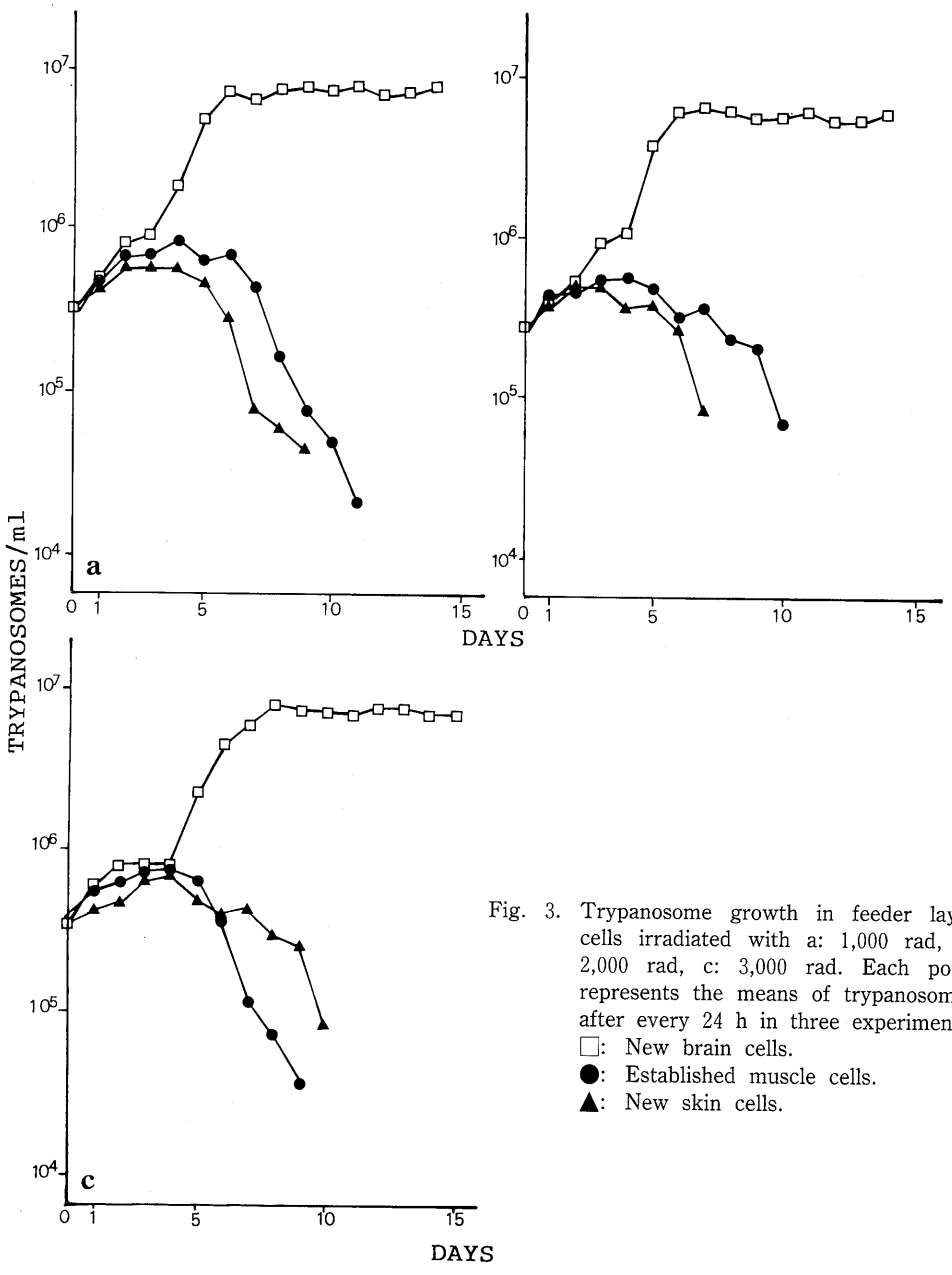

Fig. 3. Trypanosome growth in feeder layer cells irradiated with a: 1,000 rad, b: 2,000 rad, c: 3,000 rad. Each point represents the means of trypanosomes after every 24 h in three experiments.
□: New brain cells.
●: Established muscle cells.
▲: New skin cells.

DISCUSSION

In recent years there have been substential improvements in axenic cell culture media for the propagation of Trypanosomes (Baltz *et al.*, 1985, Duszenko *et al.*, 1985) but still feeder layer cells are extensively used, though mechanisms by which these cells support and promote the growth of bloodstream forms of trypanosomes are still obscure.

NMBC and NMMC proved to support continuous growth of bloodstream forms of *T.b. gambiense* Wellcome strain (Mhando *et al.*, 1986). In order to quantitate the growth supporting effects of these feeder layer cells, NMBC or NMMC were seeded in the same tissue culture dish with either new cells obtained from other organs of a new born mouse or established cells. Nevertheless, no special factors were transferred or diffused from either NMBC or NMMC to other feeder layer cells, subsequently, there was no trypanosome growth on those feeder layer cells which previously did not support the growth. Moreover, the formation of clusters in NMBC and NMMC just near the border with other cells denotes that no trypanocidal factors were produced by those cells which could not support the growth. In an established culture, trypanosomes adhered on the surface of cells or formed clusters between the feeder layer cells. Even after washing the cells, still trypanosomes adhered on the surface. It is unclear whether parasite adhesion on the surface of cells or formation of clusters between the cells is atributed by short−range diffusible factors or just self−recognition processes of a quasi immunological nature (Theodor, J., 1970).

Loss of adhesion between trypanosomes and feeder layer cells will often lead to elimination of the former. This close association might have influenced continuous growth of trypanosomes. Hirumi *et al.*, (1980) pointed out that slow−growing fibroblast cell could support the growth of trypanosomes more effectively than fast−growing ones.

Earlier studies have illustrated that irradiation suppressed cell division, but did not affect cell metabolism (Jessop and Hay, 1979). Using this approach, gamma−irradiated human fibroblast cell line (flow, F2000) supported continuous growth of *T. brucei* strain 336D for 3 months (Agda *et al.*,1985). In the present work NMBC and NMMC exposed to an irradiation dose of 3,000 rad lost the ability to divide but were capable of supporting the growth of bloodstream forms of trypanosomes. In the contrary, irradiated EMBC, EMMC, NMSC and NMKC also lost the ability to divide but could not supply conditioning factors for the growth of trypanosomes. We can therefore assume that continuous growth of *T.b. gambiense* Wellcome strain dose not depend merely on the growth rate of fibroblast cells isolated from ICR mouse.

Many reports have been published on isolation of growth stimulating factors from the culture media incubated with certain cells (Schodell, M., 1972; Bürk, R.R., 1973). Applying this technique to our experiments we found that when conditioned media obtained from NMBC and NMMC were used in full strength or diluted with fresh medium they could not support the growth of trypanosomes. This indicates that no trypanosomal growth factors were produced by NMBC and NMMC into culture media.

The above findings support Tanner's assumption (Tanner, 1980) that a short−range interaction between trypanosomes and feeder layer cells is necessary for the growth of trypanosomes. It still remains unclarified whether factors that might have led to adhesion of trypanosomes on the surface of feeder layer cells or formation of clusters between the cells are components of cell to cell adhesion apparatus per se or are components which influence cell metabolism.

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トリパノソーマ血流型の増殖をたすけるマウス新生仔の脳，筋由来細胞に関する研究

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Trypanosoma brucei gambiense (Tg) Wellcome 株の培養に，侍養細胞として ICR マウス新生仔由来細胞を用いると，新しく分離された脳及び筋由来細胞は Tg の増殖をたすけるが，分離後40日を経過し，増殖が確立した脳及び筋由来細胞は侍養細胞としての能力を失う．新しく分離された細胞でも腎由来細胞は Tg の増殖をたすけない．

Tg を侍養するか否かに関して増殖因子の有無について検討した．Tg を侍養する細胞を培養皿の半面に，他の半面に侍養しない細胞を播いて，その上で Tg を培養したところ，前者の側でのみ Tg は増殖した．増殖速度の速い細胞はトリパノソーマの侍養細胞として適してないという報告があるので，上記の細胞に，その増殖を抑制するに足る最少量のX線を照射してから，侍養細胞として用いてみたが，Tg の増殖をたすけることに関して変化は認められなかった．Tg が高率に増殖する系では，Tg は侍養細胞の上に，あるいは細胞間にはいって，極めて密に接触した状態で増殖する．以上のことにより，侍養細胞から増殖因子が出ているのではない（出ているとしても限局された近傍でのみ有効）と考えられ，ただ細胞の増殖速度が遅いことだけでなく，細胞と Tg との間に密な接触をもたらすことが Tg の増殖を推進するのに必要であると考えられる．

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