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# Comparative Study on High and Low Virulent Trypomastigotes of *Trypanosoma cruzi* : Infectivity to Mouse Macrophages, L-cells and Newly Cultured Fibroblasts from Mouse Heart

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**Abstract**: Trypomastigotes of *Trypanosoma cruzi* seem to play the most important role in infection to mammals. In the present experiment, trypomastigotes of different virulence were comparatively studied in terms of their infectivity to mouse macrophages, newly cultured fibroblasts from mouse heart and established fibroblasts (L-cells). Virulent trypomastigotes showed more rapid development both in macrophages and newly cultured fibroblasts than those of low virulence. On the other hand, trypomastigotes of low virulence grew more rapidly in established fibroblasts than highly virulent ones, though both of them showed slower development in established fibroblasts than in newly cultured fibroblasts. Trypomastigotes of low virulence could not induce apparent infection in normal mice but could induce gradually progressive infection in nude mice. These results show that virulence of *T. cruzi* in mice relates to the speed of parasite growth not only in macrophages but also in newly cultured fibroblasts and enable us to make the following suggestion that the age of cultured cells to be infected must be taken into consideration when examining the virulence of *T. cruzi* in cell culture systems.

*Key words*: *Trypanosoma cruzi*, Trypomastigote, Virulence, Fibroblast, Macrophage

## INTRODUCTION

In general, virulence of *Trypanosoma cruzi* has tendency to decrease corresponding to the length of time the parasite is maintained in culture. Strains attenuated by long passage in culture or strains of naturally low virulence have been used for immunological

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experiments to induce protective immunity to challenge with highly virulent strains (Kagan and Norman, 1962; Norman and Kagan, 1960; Basombrio and Besuschio, 1982). The reasons for the difference in virulence, however, have not been studied extensively yet. According to Taliaferro and Pizzi (1955), culture forms of low virulence were actively phagocytized and destroyed by local macrophages six hours after subcutaneous injection and thereafter, hematogenous macrophages became increasingly important in removing parasites. On the other hand, most of the virulent blood forms developed normally in macrophages.

The trypomastigote is considered to be the most highly differentiated among the three developmental forms of *T. cruzi*. It is also recognized as the most important in completing infection to mammals. Therefore a comparative study on trypomastigotes of high and low virulence may explain the factors responsible for virulence. In the present experiment, L-cell culture derived trypomastigotes of high and low virulence were compared in terms of their penetrative and developmental ability in different cells. Trypomastigotes used were of the same strain.

## Materials and Methods

*Source of parasites*

The Tulahuen strain of *T. cruzi*, obtained in 1971 from the National Institute of Health, USA through Keio University, Japan and subsequently maintained in mice and tube culture, was used.

*Preparation of parasites*

1) Virulent trypomastigotes from L-cell culture

Blood trypomastigotes from heavily infected mice were collected by centrifugation on a Ficoll-Hypaque column and then transferred into L-cell cultures. The overlay medium was removed and fresh medium (MEM with 10% calf serum) was added every 3–4 days. Around one month later, numerous trypomastigotes appeared in the overlay medium of the heavily infected L-cells. Pure trypomastigotes were collected by passing through a CM-cellulose column according to the method of Kanbara and Nakabayashi (1983b) except for the use of phosphate-saline-glucose buffer (PSG), pH 7.2, as eluent. The trypomastigotes mixture was also suspended first in the overlay medium before being applied to the column. To insure virulence of trypomastigotes used, only those that were cultivated in L-cells within three months or less after transfer from mouse were used. The dose of $5\times10^5$ virulent trypomastigotes induced fatal infection in 4–6 weeks old mice (DD-Y) within 14 days after inoculation (Kanbara, 1975).

2) L-cell culture derived trypomastigotes of low virulence

*T. cruzi* maintained in culture since 1971, consisting of more than 99% epimastigotes were transferred into L-cell cultures. As in the case of the virulent trypomastigotes above, numerous trypomastigotes appeared in the overlay medium about one month later.

These were collected and purified through a CM-cellulose column.

*Preparation of host cells*

1 ) L-cell

L-cells were kindly supplied by Dr Ikuta of the Department of Pathology, Research Institute for Microbial Diseases, Osaka University.

2 ) Macrophages

Ten weeks-old DDY mice were intraperitoneally injected with 2 ml of a 3 % thioglycollate medium to stimulate macrophage production, and then killed three days later by cervical dislocation. The peritoneal cavity was exposed and rinsed twice with 5 ml of MEM containing penicillin (100 U/ml), streptomycin (100 $\mu$g/ml) and heparin sodium (10 U/ml). The pooled fluid was centrifuged at 4 °C and the precipitate was suspended in MEM with 10% fetal bovine serum, dispensed into culture dishes (100×20mm, Falcon 3003) and incubated for 2 hr at 37°C in a 5 % $CO_2$-incubator. After 2 hr of incubation, most of the macrophages have attached to the bottom of the dish so that white blood cells and other cell debris can be removed by a medium change.

3 ) Newly cultured cells from mouse heart

Three days old mice were killed and the heart was isolated into dishes, rinsed in MEM with 10U/ml heparin sodium, minced with scissors, suspended in MEM with 20% fetal bovine serum and then incubated in a 5 % $CO_2$-incubator at 37°C. After 2 - 3 days unattached minced tissue pieces were removed by changing the overlay medium. The attached pieces from which new cells, suspected to consist chiefly of fibroblasts, developed to surrounding areas were maintained by changing the medium (MEM with 20% fetal bovine serum) every 3 - 4 days and transferring a quarter of cells into a new dish once a week. The cells were used for the experiment within three months after the initial cultivation.

*Experimental infection to different host cells*

L-cells, macrophages and cells newly derived from heart were cultured in tissue culture dishes (100×20mm). A certain number of trypomastigotes of different virulence were inoculated into each culture and then the number of trypomastigotes in the overlay were counted at specified intervals. Twenty-four hr after inoculation, the overlay medium was removed and culture dish was gently washed twice with fresh medium and overlaid with new medium. The culture was maintained at 37°C in a 5 % $CO_2$-incubator. The resultant development of parasite was determined by counting the number of newly released trypomastigotes in the medium every 24 hr except for every 2 - 3 days in the experiment with L-cells. Medium change was done concurrently.

*Experimental infection of nude mice (nu/nu) with trypomastigotes of low virulence*

In the above experiment (Fig. 1 ), it was shown that trypomastigotes of low virulence can grow in macrophages though to a lesser extent compared with virulent ones. A

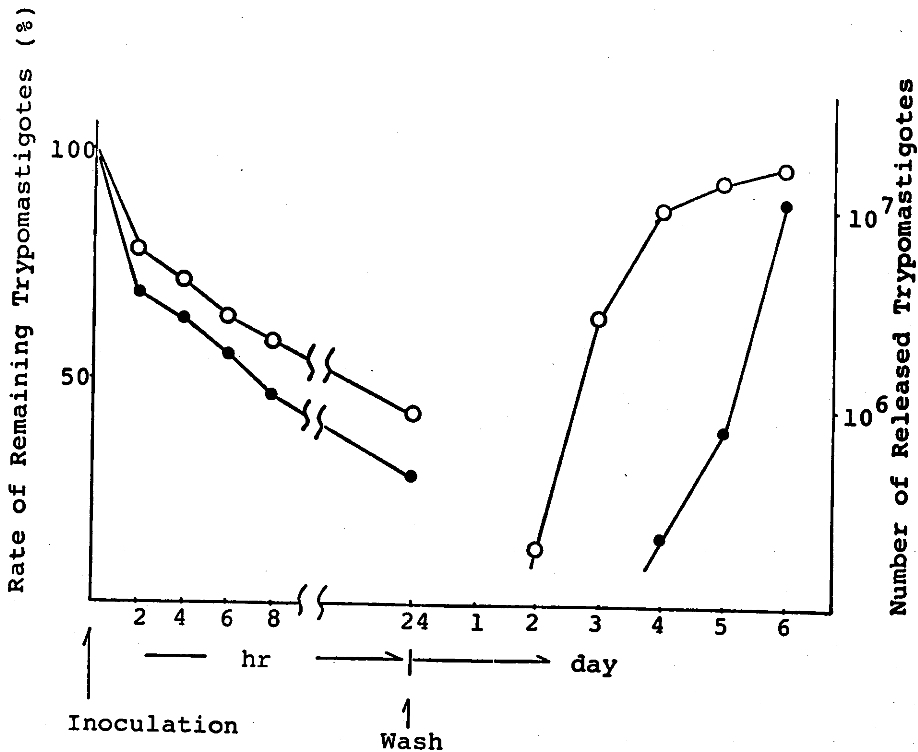


Fig. 1. Experimental infection to mouse macrophages. $10^6$ of high (○——○) and low (●——●) virulent trypomastigotes were inoculated into separate macrophage cultures and incubated at 37°C in a 5% $CO_2$-incubator. Relative rate of trypomastigote entry was measured by countng the number of trypomastigotes remaining in the overlay medium at specified intervals up to 24hr of incubation. Subsequent development was measured by changing the medium and counting the number of newly released trypomastigotes every 24hr.

dose of $3 \times 10^6$ trypomastigotes of low virulence collected from macrophage cultures through a CM-cellulose column was inoculated intraperitoneally into each of nude mice (nu/nu) and normal control mice (DD-Y, 5 weeks old). The parasitaemia was examined at proper intervals. On the 17th and 20th days of infection, nude mice were killed and the parasite density in various organs was examined by the method of Kanbara and Nakabayasi (1983a).

## RESULTS

### 1. *Experimental infection to mouse macrophages*

The relative rate of phagocytosis of trypomastigotes by macrophages was measured by counting the number of trypomastigotes remaining in the overlay medium at specified intervals until 24 hr after inoculation (Fig. 1). Trypomastigotes of low virulence have a tendency of being phagocytized more rapidly than virulent ones. However, the subsequent

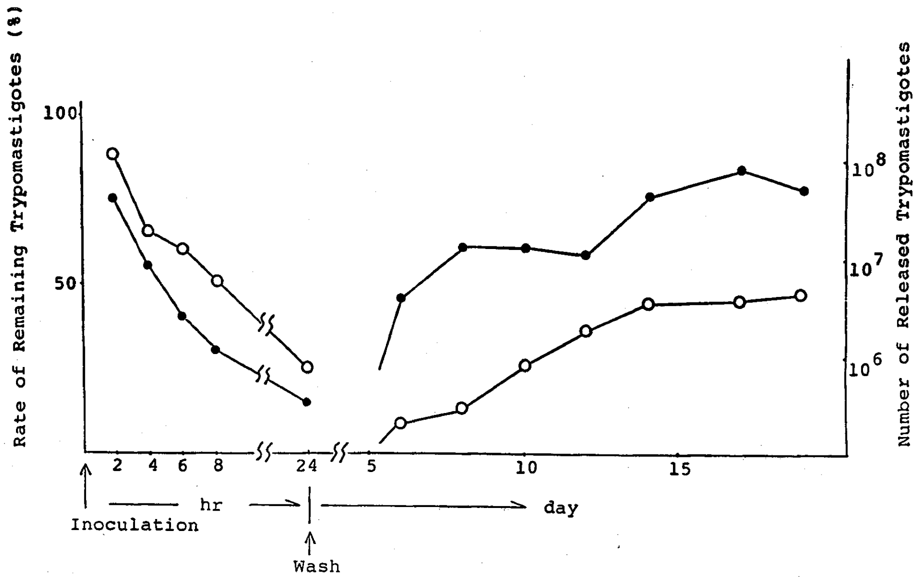


Fig. 2. Experimental infection to L-cells. $1.5\times10^7$ of high (○——○) and low (●——●) virulent trypomastigotes were inoculated into separate L-cell cultures. Following steps are the same as those in experimental infection to mouse macrophages except that counting and medium changing were done at intervals of 2–3 days.

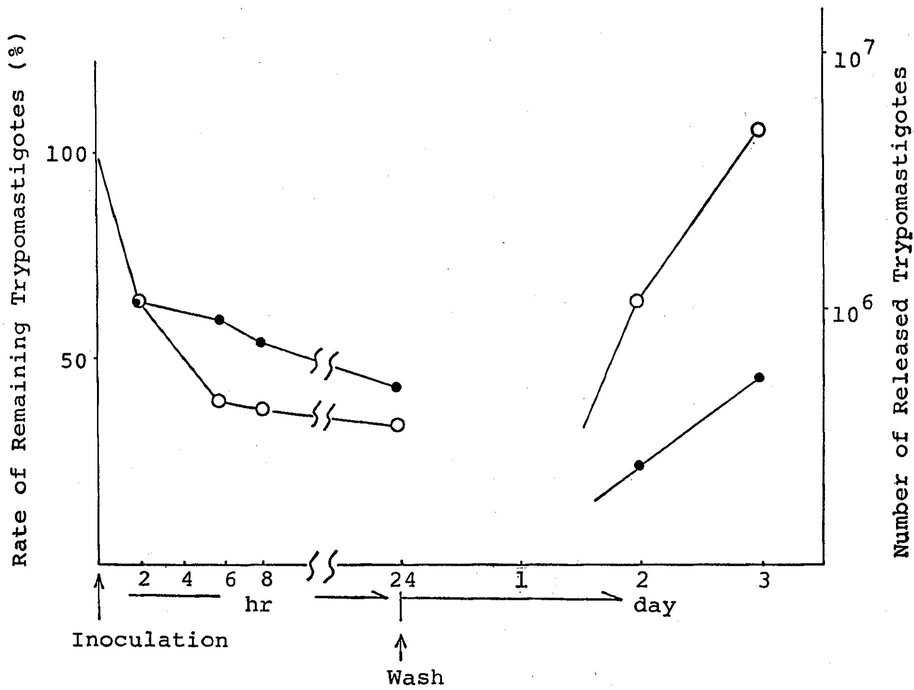


Fig. 3. Experimental infection to newly cultured cells from hearts of newborn mice. $2.5\times10^6$ of high (○——○) and low (●——●) virulent trypomastigotes were inoculated into separate cultures of new fibroblasts. Following steps are the same as those in experimental infection to mouse macrophages.

growth and development of low virulent ones are less than those of high virulent ones judging from the number of newly developed trypomastigotes in the overlay medium 2 to 6 days later.

2 . *Experimental infection to L-cells*

Results show that trypomastigotes of low virulence penetrated and developed in L-cells more rapidly than virulent ones (Fig. 2).

3 . *Experimental infection to newly cultured cells from heart of newborn mice*

Results here were opposite from those obtained in the previous experiments with L-cells. Virulent trypomastigotes tended to invade heart-derived cells more rapidly than those of low virulence. Moreover, the subsequent development of trypomastigotes of high virulence in the overlay medium was more rapid and pronounced (Fig. 3). Compared with growth in L-cells both parasites of high and low virulence showed better growth in new cells.

TABLE 1 . Experimental infection to nude mice with trypomastigotes of low virulence of the Tulahuen stain of *Trypanosoma cruzi*

| Days after inoculation | Parasitaemia | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Nude mice | | | | | Control | | | | |
| | A | B | C | D | E | A | B | C | D | E |
| 7 | + | + | + | + | + | + | + | + | + | + |
| 9 | + | + | + | + | − | + | + | − | − | − |
| 12 | ++ | ++ | ++ | + | − | + | − | − | − | − |
| 14 | ++ | ++ | ++ | ++ | ++ | − | − | − | − | − |
| 15 | ++ | ++ | ++ | ++ | ++ | − | − | − | − | − |
| 17 | +++* | ++* | ++* | ++ | ++ | − | − | − | − | − |
| 20 | | | | +++* | +++* | − | − | − | − | − |

$3 \times 10^6$ trypomastigotes of low virulence from macrophage cultures were inoculated intraperitoneally into each of 5 nude mice and 5 control normal mice. Parasitaemia was examined at specified time intervals.
(−): no parasites observed: (+): low parasitaemia;
(++), (+++): progressively higher parasitaemias.
(*): nude mice klled and the parasite densities in various organs examined(see Table 2).

TABLE 2 . Comparison of parasite densities in various organs of nude mice infected 17 and 20 days previously with trypomastigotes of low virulence of *Trypanosoma cruzi*

| Organ | Number of parasites | |
|---|---|---|
| | 17 days after infection | 20 days after infection |
| Heart | 176 | 12 |
| Liver | 400 | 134 |
| Spleen | 621 | 635 |
| Muscle | 3433 | 2360 |

4 . *Experimental infection of nude mice with trypomastigotes of low virulence.*

One week after infection, both nude and control mice showed very low parasitaemia. Later, however, parasitaemia in control mice decreased up to a point when no more parasitaemia was observed 12 days after infection (Table 1). On the other hand, parasitaemia in nude mice gradually increased. Three nude mice killed on the 17th day of infection showed that parasite density, consisting mainly of amastigote forms, was highest in muscle tissue followed by spleen, liver, and heart (Table 2). One of the three mice showed very high parasitaemia and conspicuous ascites containing trypomastigotes. A dose of $4 \times 10^6$ trypomastigotes from the ascitic fluid was inoculated into each of three normal mice (DDY, 5 weeks old). Low parasitaemia was observed on the 6th and 9th day after infection but disappearing thereafter. Two nude mice killed on the 20th day also showed same tendency of parasite density.

## DISCUSSION

The present experiment showed that growth of low virulent trypomastigotes in peritoneal macrophages was more suppressed in spite of more rapid interiorization compared with virulent ones. This result corresponds with the observation by Taliaferro and Pizzi (1955) of active phagocytosis by macrophages in mice infected with low virulent strain of *T. cruzi*, although they used culture form (mainly epimastigotes). Kanbara (1975) reported that avirulent trypomastigotes could not complete the conversion of surface antigens accompanying the transformation from amastigotes to trypomastigotes judging from the fact that specific surface antigens of amastigotes remained on the surface of trypomastigotes in a speckled pattern. This meant that the functional development of trypomastigotes of low virulence was incomplete. Low virulent trypomastigotes were able to develop in macrophages though much slower than virulent ones. They were also able of develop in nude mice in spite of growth suppression. Newly developed trypomastigotes in macrophages and in nude mice did not show recovery of virulence since they were not able to induce apparent infection in normal mice. Experimental infection to nude mice and normal mice showed that complete suppression of low virulent trypomastigotes would be achieved by involving sensitized T-cell populations as Rodriguez et al. (1983), Kierszenbaum and Pienkowski (1979), Nogueira et al. (1977), and Nogueira and Cohn, (1978) described, because low virulent trypomastigote infection in nude mice became progressively severe with prolonged course of infection. This result might correspond with the observations in normal mice by Mero and Brener (1978) that sudden and massive destruction of parasites and cells occured in the spleen within 10 days after having been infected with some virulent strains of *T. cruzi*, although progressive proliferation of parasites continued in other organs such as heart, skeletal muscles and intestines. The progressive proliferation of parasite in skeletal muscles and heart even after becoming incapable of living in macrophages was also

observed in mice inoculated with a small number of blood form trypomastigotes (less than $10^5$) of the Tulahuen strain (unpublished data). This phenomenon may be correlated with our finding that virulent trypomastigotes showed higher affinity to newly isolated cells from heart than low virulent trypomastigotes do. This may explain why low virulent forms could not develop infection even in tissue other than lymphoreticular tissue. The present results suggest that comparative studies on the differences between low and high virulent trypomastigotes may be helpful in understanding factors that may be important to virulence.

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クルーズトリパノソーマ Trypomastigote の病原性とマクロファージ，新しい線維芽細胞，および株化線維芽細胞（L-cell）に対する感染性との関連

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*Trypanooma cruzi* はその発育環において代表的に三つの形態を示すが，このうち Trypomastigote from が最も感染に重要な位置を占め機能的にも分化している．そこで私達は同一株由来の強毒および弱毒の Trypomastigote についてその感染性，増殖性をマウス由来のマクロファージ，心より新しく培養した線維芽細胞，株化線維芽細胞（L-cell）を用いて比較した．その結果マクロファージおよび新しい線維芽細胞において強毒 Trypomastigote は早い増殖性を示した．一方 L-cell に対しては両者共新しい細胞に比し増殖が遅かったが特に強毒株において遅かった．ヌードマウスを用いた感染実験では弱毒株といえども経過が長いが除々に重症化する感染を示す．これらのことから *T. cruzi* が一定の毒力を示すためにはマクロファージに対する抵抗性だけでなく他の細胞内での増殖の速さが重要な要素となり，この速度は L-cell のような株化細胞では測定できないことが明らかとなった．

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