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Scanning Electron Microscopic Studies on Penetration of *Trypanosoma cruzi* into Fibroblast Cells

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Wood and Pipkin (1969) reported that epimastigote form appeared predominantly in the log phase of growth of *Trypanosoma cruzi in vitro*. Inoki *et al.* (1971) also demonstrated that transition from trypomastigote to epimastigote in the culture medium occurred within 90 hrs and *vice versa*. After the culture parasites were inoculated into Hela cell cultures, epimastigotes gradually changed into trypomastigotes and then this form penetrated into Hela Cells, which was demonstrated in electron micrographs by Sooksri and Inoki (1972). A recent report by Kongtong and Inoki (1975) revealed only the surface morphology of epimastigotes and trypomastigotes, but there are no reports on the penetration of these two different forms observed by scanning electron microscope. This paper is to describe the penetration method of trypomastigotes and epimastigotes into the fibroblast cells revealed by SEM.

Materials and Methods

A "Tulahuen" strain of *Trypanosoma cruzi* used in this work was obtained from NIH, Bethesda, Md., U.S.A., through the Department of Parasitology, Keio University Medical School, Tokyo. The media and procedures used for maintenance of the parasites and of Balb C Fibroblast cells were as described by Sooksri and Inoki (1972). The fibroblast cells were harvested by incubating their stock in EDTA for 5 minutes. Then the cells were washed twice by the MEM calf serum media and they were resuspended in this media. Cultivations of the cells were made on 12×35 mm glass cover slips immersed in the culture medium in 18×18×75 mm glass bottles. The cells were allowed to settle and attach to the cover slips for 2 days before inoculation of trypanosomes.

First series: The trypomastigote forms of *Trypanosoma cruzi* collected from infected culture Hela cells, were suspended in MEM calf serum media, and centrifuged at 1000 rpm for 5 min. A centrifugate which contained cell debris, intracellular forms and some trypomastigotes were discarded. A supernatant was recentrifuged at 2500 rpm for 8 min. The supernatant was discarded, and the centrifugate containing only trypomastigotes was retained. They were resuspended in a certain volume of MEM calf serum media to obtain the concentration of approximately $1.5\text{–}2\times10^6$ cells/ml. Three ml of this suspension were inoculated into each fibroblast cell culture bottle and incubated at 37°C. Sampling was made at the 1st, 2nd, 3rd, 4, 5, 6, 9, 12, 24th hour, 1st, 2nd, 3rd, 5, 7, 10, 13 and 16th day of incubation.

Second series: Approximately $1.5\text{–}2\times10^6$ cells of *T. cruzi* per ml of MEM calf serum media were inoculated into the fibroblast cell culture bottles as described in the first series. About 99% of the parasites used in this series were epimastigotes.

The infected fibroblast cells in both series still attached to cover slips were washed by

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cold Ringer's solution, fixed, dehydrated and coated with carbon and gold for SEM observation as described by Kongtong and Inoki (1975). The corresponding glass cover slips were stained with Giemsa's solution and subjected to the light microscopic observations.

Results

First series : The trypomastigotes strongly adhered to the surface of the fibroblast cells by their flagella from the 1st to the 24th hr after inoculation (Figs. 1, 2, 6, 7). They penetrated into the fibroblast cells within 2 hrs by their flagellar movement (Figs. 3, 4, 5). With aid of the filaments of the fibroblast cells holding the parasites, some trypomastigotes penetrated into the cells during the latter period of incubation time (Figs. 6, 7). Complete penetration into the fibroblast host cells occurred increasingly with an advancement of the incubation time. Multiple penetration into a single fibroblast cell could also be observed 5–6 hrs after inoculation (Figs. 8, 9). After complete penetration, the trypomastigotes still retained their active movement, and caused slight swelling of the fibroblast host cells (Fig. 8). A few hrs thereafter, the intracellular parasites began to divide (Fig. 9). The parasites rapidly multiplied and gradually developed to form elongate intracellular forms (Figs. 10, 11), and finally, trypomastigotes. The new trypomastigote forms occupied the cytoplasm of the fibroblast host cell when they reached their maximum development, 3 days after inoculation (Fig. 12). The intracellular form of various stages of growth and development, and the ruptured fibroblast host cells could be observed during the 3–7th day after inoculation (Figs. 12–16). The new trypomastigotes from the ruptured fibroblast host cells (Fig. 15), repenetrated into the intact fibroblast cells. Therefore, a number of early stages of development and total numder of infected fibroblast cells after the 3rd day incubation, were obviously increased (Table 1).

Second series : In the culture media, approximately 99% of the parasites were epimastigotes and their transition to trypomastigotes were very rare. The epimastigotes firmly attached to the surface of the fibroblast cells by their flagella, were odserved from the 1st hr through the early days of incubation (Fig. 18). During this period some epimastigotes were trapped by the filaments of the fibroblast cells (Fig. 19), but their penetrations into the cells were very rare (Fig. 20). On the 10th day after inoculation, a small number of new trypomastigotes emerged from the infected fibroblast cells, and the number of infected fibroblast

Table 1 Infectivity to the cultured Balb-C mouse fibroblast cells of trypomastigote forms of *T. cruzi* grown in the Balb-C mouse fibroblast cultured cells (Number of infected cells in 1,000 fibroblast cells)

Time after inoculation	Less than 9hrs	12hrs	1day	2days	3days	5days	7days
No. of fibroblasts with less than 10 parasites	35	66	105	311	47	25	38
No. of fibroblasts with 11–20 parasites	—	—	11	26	145	43	46
No. of fibroblasts with more than 21 parasites	—	—	2	5	134	162	134
No. of ruptured fibroblasts with parasites	—	—	—	—	30	155	30
Total	35	66	118	342	356	385	248

Table 2 Infectivity of cultured *T. cruzi* to cultured Balb-C mouse fibroblast cells (Number of infected cells in 10,000 fibroblast cells)

Time after inoculation	Less than 2days	3days	5days	7days	10days	13days	16days
No. of fibroblasts with less than 10 parasites	No clear trace of infection	6	8	7	2	21	37
No. of fibroblasts with 11–20 parasites		—	2	3	4	15	22
No. of fibroblasts with more than 21 parasites		—	—	1	2	4	24
No. of ruptured fibroblasts with parasites		—	—	—	1	4	18
total	—	6	10	11	9	44	101

cells through the 16 days of incubation was very low, although it increased during the latter periods (Table 2).

Discussion

From this experiment it was clearly shown that the flagellum plays an important role in the entry of the trypomastigote into the fibroblast cell. This result warrants the conclusion by Sooksri and Ionki (1972) that *T. cruzi* penetrated into the Hela cell by aid of its flagellum. The intracellular parasites multiplied, and developed until they filled the cytoplasm of the infected cells. Then, on the 3rd day of incubation, new trypomastigotes emerged. The infectivity increased on the latter days after inoculation indicating that newly emerged trypomastigotes repenetrated into the intact fibroblast cells (Table 1). It is known that after *T. cruzi* penetrated into the cell the divisible epimastigote form occurred intracellularly in the Hela cell (Sooksri & Inoki, 1972) as well as the amastigote and trypomastigote in cells maintained *in vitro* (Rodriquez and Marinkelle, 1970). Sanabria (1963), and Petana (1969) also noted that transformation of amastigote to trypomastigote occurs intracellularly in the vertebrate host. Behbehani (1973) reported that all forms of *T. cruzi* including the promastigote and the sphaeromastigote were found in three different cycles of development in mouse peritoneal macrophages. It is apparent that the intracellular forms complicately occur intracellularly in the vertebrate cells due to a certain environmental condition. Since the SEM allows rather limited information of the intracellular structure, it is hard to state exactly in what forms they appear in the fibroblast cells in this experiment.

It has not yet been determined what factors affect the morphogenetic process of *T. cruzi*. However, many investigators already reported that epimastigotes were capable of transforming directly into trypomastigotes *in vitro* (Pan 1971, Sooksri and Inoki 1972, Logan and Hanson 1974). From the previous reports including the low infectivity and long developmental period of the intracellular forms in the 2nd series experiment, it is suggested that epimastigote transformed to trypomastigote before the parasites penetrated into the fibroblast cells.

After 24 hr cultivation, the Balb-C fibroblast cells were completely spread and well flattened with their lamelloplasm adhered to the surface of the coverslip. Their general features including the elongated extensions and filaments were observed being similar to the Hela cells and many other L cells reported by Revel *et al.* (1974), and Rovensky

and Slavnaya (1974). Both trypomastigotes and epimastigotes were observed to be captured by the elongated extensions and filaments of the fibroblast cells. Therefore, the other method of entry into the fibroblast cell of *T. cruzi* is possible by phagocytosis similar to the phagocytosis of micro-organisms by Hela cells reported by Shepard (1960).

In the case of natural infection, epimastigotes firmly attach to the epithelial wall of the proboscis of the insect host by hemidesmosome formation (Vickerman, 1973). *In vitro*, the epimastigotes also showed these characteristics; they adhered to the surface of the fibroblast host cells for many days, and they retained their attachment through the flow of the daily changing culture media.

A very rare incident in which an epimastigote showed its capability to penetrate into the fibroblast cell with or without aid of phagocytosis was observed. However, it is probable that dying epimastigotes are phagocytosed by the fibroblast cells. In addition, there is no report on the development of the epimastigotes after this form has abnormally entered into the cells. Therefore, this dubious dilemma requires further study.

Summary

Trypomastigotes and epimastigotes of *Trypanosoma cruzi* (Tulahuen strain) were inoculated into Balb-C fibroblast cell cultures at 37°C and sampled at intervals in order to examine the method of entry into the host cells and the intracellular develpment by scanning electron microscopy. The trypomastigote penetrates into the fibroblast cell by flagellar movement. Intracellular forms can be observed within the infected cell beginning from the 9 hr after inoculation, then on the 3rd day, they emerged in trypomastigote form. Epimastigote seems to enter a fibroblast cell by aid of *phagocytosis*.

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Tryponosoma cruzi の線維芽細胞内への侵入に関する走査電顕学的研究

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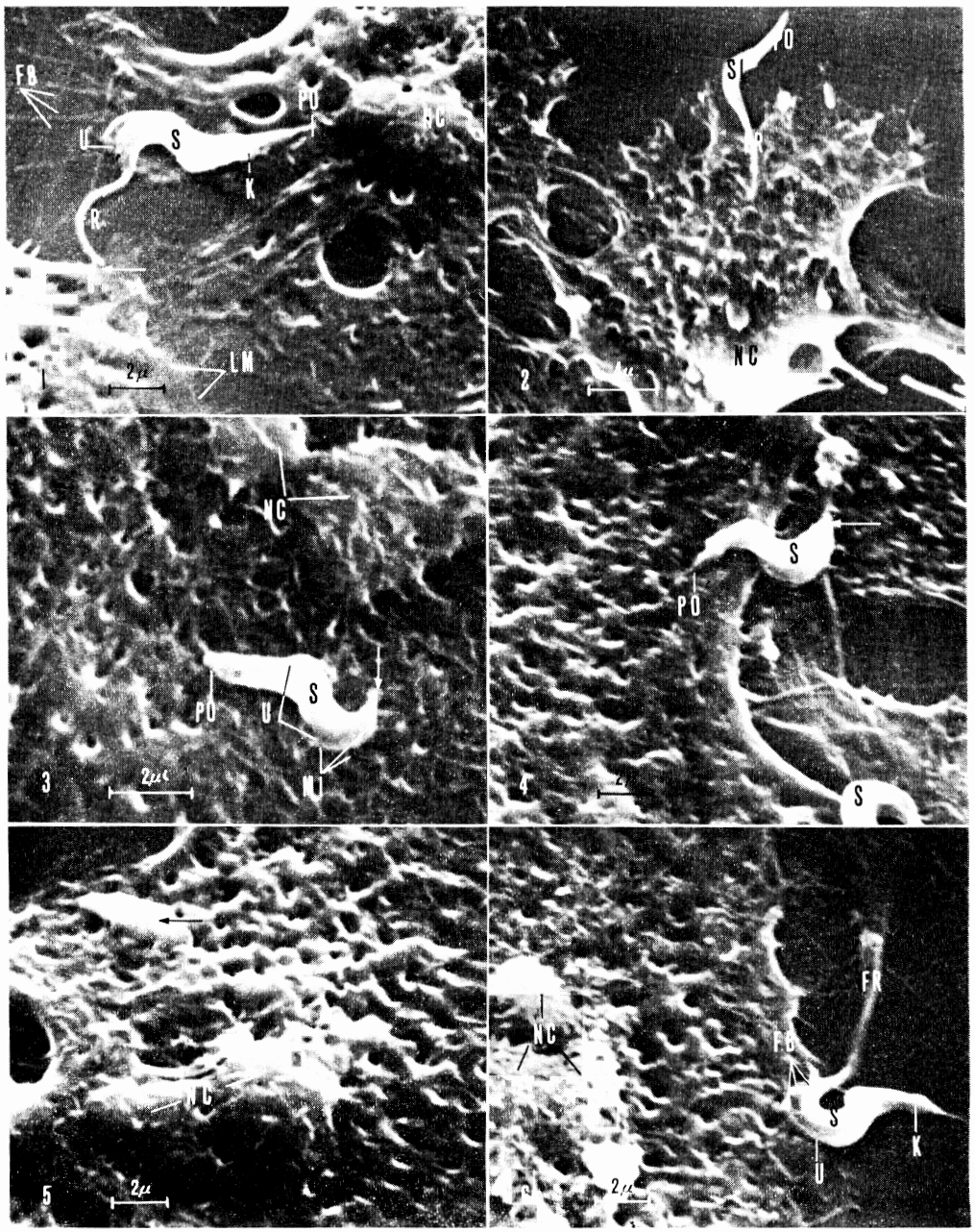
Trypanosoma cruzi（Tulahuen 株）の trypomastigote 型と epimastigote 型を 37°C で培養中の Balb-C fibroblast に加えて接種し，ときどき取り出し fibroblast 内への両型の侵入方法や侵入後の発育状況を走査型電顕をもつて観察した．

その結果，trypomastigote 型が鞭毛運動によつて fibroblast 内に侵入すること，接種後9時間目から fibroblast 内に原虫が見られ，3日目には trypomastigote 型が観察されること．また，epimastigote 型も fibroblast の喰菌作用によつて，その中に捕食される可能性などが明らかになつた．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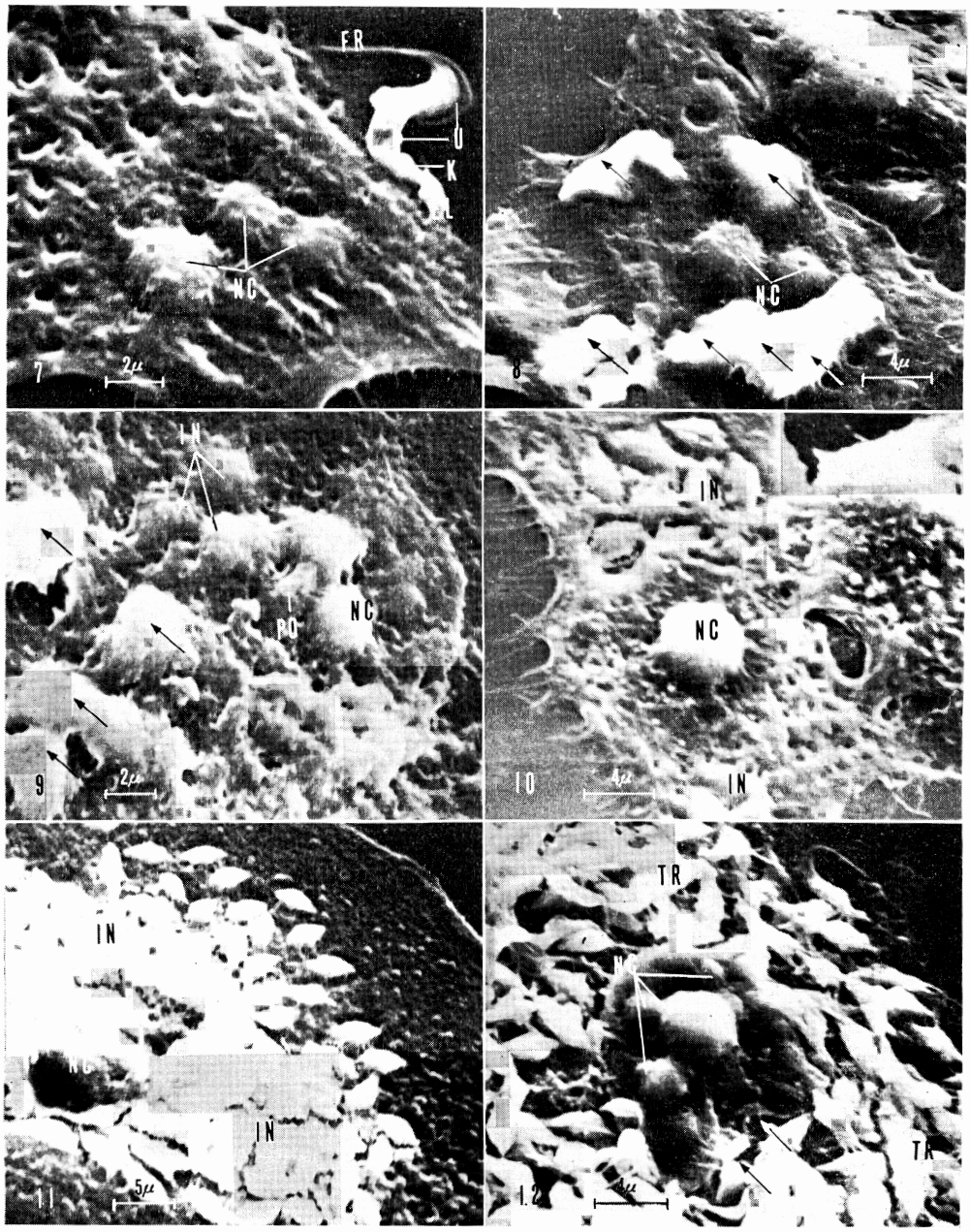
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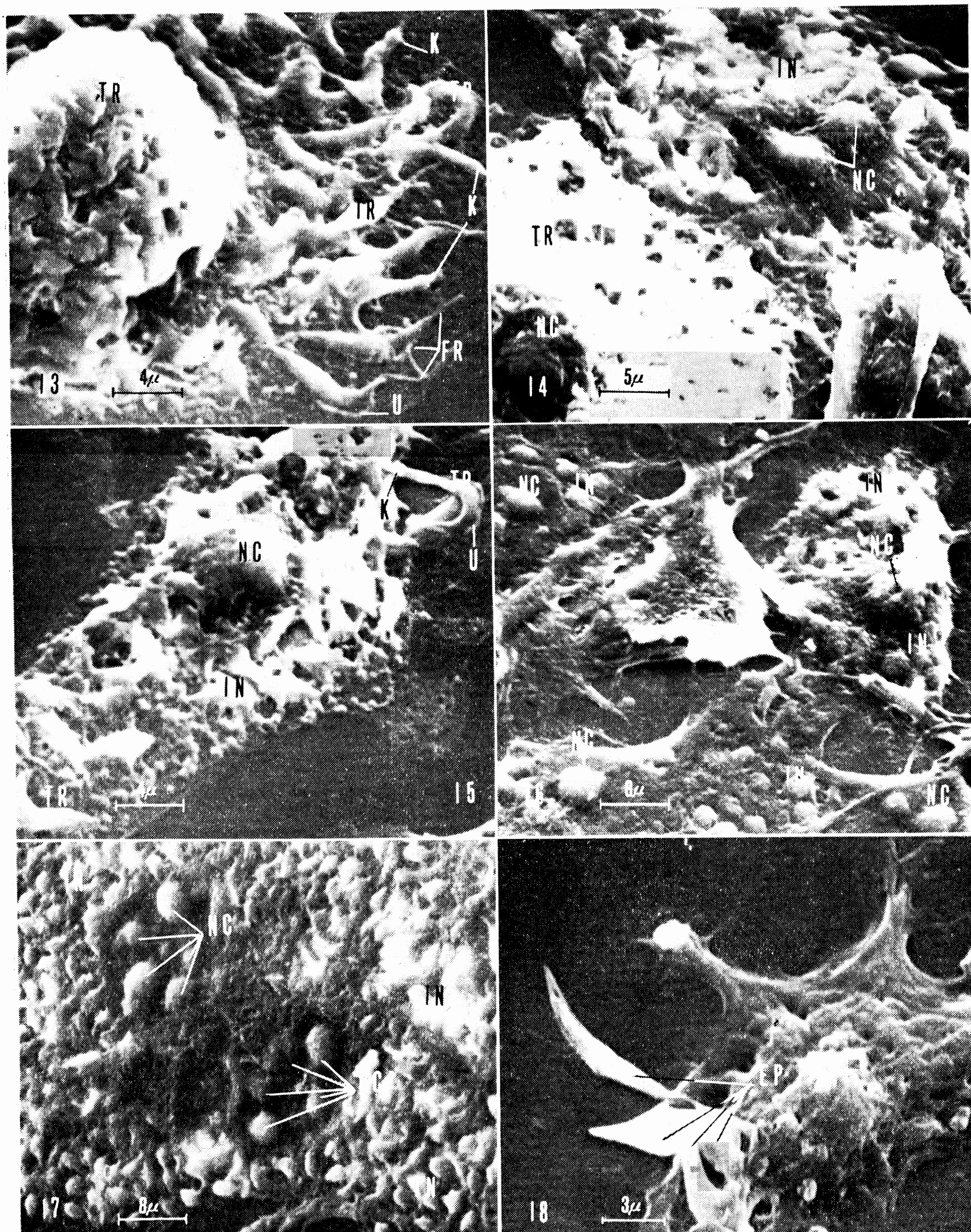
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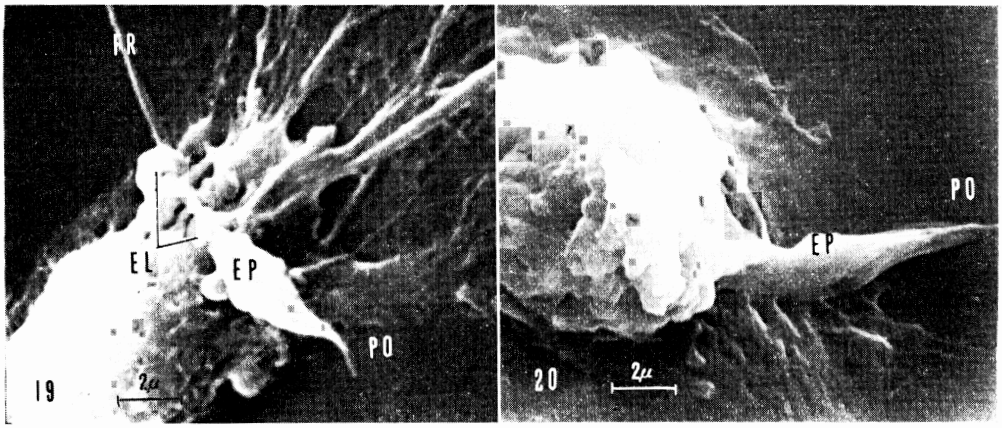
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Explanation of the figures

Figs. 1–5 2 hr after inoculation:

Fig. 1 A stumpy form of trypomastigote attachs to the surface of the fibroblast cell by its flagellum (arrow). The host cells firmly adheres to the substrate by its lamelloplasm and show numerous filamentous extensions.

Fig. 2 A slender form of trypomastigote adheres to the surface of the fibroblast cell, the first portion of its flagellum penetrates into the cell.

Figs. 3–4 A stumpy form of trypomastigote is on its halfway penetration into the fibroblast cell, the flagellum is entirely submerged (arrow).

Fig. 5 A complete penetration (arrow) without any swelling around the parasite suggests that it may penetrates into the fibroblast cell with in one hr after inoculation.

Figs. 6 and 7 3 hr after inoculation: A stumpy trypomastigote is captured by the filaments (fig. 6) and an elongated extension (fig. 7, arrows) of the fibroblast cell.

Figs. 8 and 9 Multiple infection with many parasites in a single fibroblast cell at the 5th hr (fig. 8) and the 6th hr (fig. 9) after inoculation (arrows). Slight swelling area around the parasites suggested that they retain active movement just after the penetration into the cell. The intracellular forms (fig. 9) lying close to the site of the possible incompleted penetration of another parasite which its posterior part (po) exposed on the edge of the fibroblast cell nucleus.

Fig. 10 24 hr after inoculation: 2 groups of the intracellular forms located around the border of the fibroblast nucleus. The parasites are spindle shaped and their sizes are approximately $2\times4\,\mu$.

Figs. 11–12 3 days after inoculation:

Fig. 11 The intracellular forms are small and more elongated when the number of parasites increases.

Fig. 12 Numerous fully developed trypomastigotes cause cytoplasmic rupture of the fibroblast host cell. The nucleus is partially damaged (arrows).

Figs. 13–15 5 days after inoculation:

Fig. 13 A fibroblast host cell contains numerous fully developed trypomastigotes.

Fig. 14 Two fibroblast host cells containing the fully developed trypomastigotes and the developing intracellular forms. Another adjacent cell remains intact.

Fig. 15 A ruptured fibroblast cell contains some intracellular forms and some fully developed trypomastigotes. Most of the fully developed trypomastigotes are released.

Fig. 16 7 days after inoculation: A ruptured fibroblast cell contains some developing intracellular form in a small remnant of cytoplasm around the nucleus (upper right cell). The neighboring cells contain few intracellular forms.

Fig. 17 7 days after inoculation: Two fibroblast host cells contain numerously multiplied intracellular forms. Since most of the infected cells containing trypomastigotes are ruptured on the 5th day, these developing intracellular forms possibly derived from reinfection.

Fig. 18 Four epimastigotes firmly attach to the surface of the fibroblast cell on the 12 hr after inoculation.

Fig. 19 24 hr after inoculation. An epimastigote is captured by 2 elongated extensions of a fibroblast cell.

Fig. 20 24hr after inoculation: An epimastigote penetrates into the fibroblast cell by its flagella, half of the body part and the posterior end remains outside of the cell.

Abbreviation: EL: elongated extension EP: epimastigote
FB: filament FR: free flagellum IN: intracellular form K: kinetoplast
NC: nuclear contents PO: posterior end S: stumpy trypomastigote SL: slender trypomastigote TR: trypomastigote