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## **The in vitro interaction of *Trypanosoma cruzi* bloodstream forms and mouse peritoneal macrophages**

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### **Summary**

The uptake and further development of bloodstream forms from *T. cruzi* *Y* and *CL* strains in mouse peritoneal macrophages have been investigated. Parasites from the *Y* strain (which present predominance of slender forms) are 20 to 30-fold more infective to macrophages than those from *CL* strain in which stout forms highly predominate. A complete amastigote-trypomastigote cycle is observed in normal or thioglycollate-induced macrophages infected with parasites from both strains. – Opsonization significantly increases the uptake by normal macrophages of parasites from both strains. The fate of the opsonized parasites is, however, different: the *Y* trypomastigotes present a normal cycle which culminates with the release of newly formed trypomastigotes whereas *CL* parasites are extensively destroyed by normal macrophages. – The differences in the uptake and fate displayed by both *T. cruzi* populations are not well understood. They are apparently related to parasite membrane components or macrophage receptors differences, which are probably influencing endocytosis and the further intracellular development of the parasites.

*Key words:* macrophages; *Trypanosoma cruzi*; opsonization; differences in parasites strains.

### **Introduction**

Macrophages from the mononuclear phagocytic system (M. P. S.) play an important role in *T. cruzi* life-cycle in the vertebrate either as regular host cells or effector elements of the cell mediated immunity (Taliaferro and Pizzi, 1955). The existence of “reticulotropic” *T. cruzi* strains exhibiting marked preference

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for cells of the M.P.S. as well as strains whose parasites are able to prevent their uptake by those phagocytic cells (Taliaferro and Pizzi, 1955; Melo and Brener, 1978), demonstrates the importance of investigating in vitro the interaction between macrophages and bloodstream forms from different *T. cruzi* populations. Culture forms often used in such studies (Dvorak and Schmunis, 1972; Hoff, 1975; Kress et al., 1975; Nogueira and Cohn, 1976) may not represent the most adequate stages for investigating this aspect of *T. cruzi* life-cycle. They do not present some important biological characteristics displayed by bloodstream trypomastigotes which are likely to influence the host-parasite relationship at the cellular level, such as the polymorphism (reviewed by Brener, 1973) or differences in antibody-binding capability (Krettli and Brener, 1976). In the present paper the uptake and development of bloodstream forms from two different *T. cruzi* strains (*Y* and *CL*) were investigated using mouse macrophage cultures.

## Material and methods

*Animals.* DBA mice kindly supplied by Dr. Ricardo Ribeiro dos Santos from Ribeirão Preto, Brazil and outbred albino mice from the Centro de Pesquisas René Rachou were used. In some experiments the mice, two days prior to the infection with *T. cruzi*, were irradiated with 650 r through a RT Mueller-250 apparatus (250 Kv, 15 MA).

*Parasites.* Bloodstream trypomastigotes were obtained from animals inoculated with *Y* (Silva and Nussenzweig, 1953) and *CL* (Brener and Chiari, 1963) *T. cruzi* strains. Parasites were collected by bleeding mice from the orbital sinus on the 7th and 12–14th days of infection for, respectively, *Y* and *CL* strains. The blood was defibrinated with glass beads and centrifuged at 100 g for 8 min at 4° C; the supernatant was then centrifuged at 1,000 g for 15 min at 4° C. The sediment containing the parasites was suspended in tissue culture medium 199 (M199) plus 20% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin); the number of motile trypomastigotes was counted in a hemocytometer, being accordingly diluted in M199-FCS.

*Normal macrophages.* Peritoneal macrophages were collected from DBA and outbred albino mice according to Cohn and Benson (1965). The macrophages were suspended in the medium (M199-2% FCS, 10 units/ml of heparin and antibiotics) and centrifuged at 100 g for 5 min; the pellet was then resuspended in the same medium without heparin to a concentration of  $4 \times 10^6$  cells/ml. About 0.10 ml of this cell suspension was deposited in  $8 \times 16$  mm glass cover-slips placed in glass or plastic Falcon Petri dishes (Baltimore Biological Laboratories, Md.) which were incubated at 37° C for 15 min in a 5% CO<sub>2</sub> atmosphere. The preparations were then repeatedly washed with Hanks' solution to remove non-adherent cells, and again incubated with 199 medium-20% FCS in the same conditions.

*Thioglycollate-induced macrophages.* The mice were intraperitoneally inoculated with 1 ml of a 4% aqueous solution of Brewer thioglycollate medium (Difco Laboratories, Detroit, Mich.). Macrophages were collected and cultivated as described above, four days after the inoculation of thioglycollate.

*Treatment of parasites by immune-sera.* Specific anti-*T. cruzi* immune-sera were obtained as previously described by Krettli and Brener (1976). The bloodstream trypomastigotes were incubated with non-agglutinating concentrations of homologous inactivated immune-sera (1:20 for anti-*Y* and 1:5 for anti-*CL*) for 30 min at 37° C. The parasite suspension was then added to the macrophages and accordingly incubated. Normal untreated parasites used as controls were suspended in medium containing the same concentration of inactivated normal mice sera (NMS). When anti-*Y* immune-sera was used, FCS or NMS was added to a final concentration of 20%.

*Macrophages infection and results evaluation.* After 18–24 h of cultivation the macrophages were infected using a parasite/cell ratio of 5:1 to 10:1. Occasionally, mostly with bloodstream forms from *CL* strain, higher ratios of 20:1 or 30:1 were used. The parasites were maintained in contact with the cells for variable periods of time (usually 2–4 h) at 37° C in 5% CO<sub>2</sub>; the preparations were then repeatedly washed to remove extracellular parasites and again incubated with M199-20% FCS and antibiotics, which was changed every 24 h. The cover-slips were fixed with Bouin, washed in water and stained in Giemsa for 18–24 h. The preparations were then differentiated in collophonium or 70% ethanol and mounted in synthetic resin. The percentage of initially infected macrophages was determined by examining 500 random cells under high magnification ( $\times 1,000$ ). The mean number of intracellular parasites for 100 macrophages was determined in a similar way. The number of macrophages in the preparations was estimated by counting 10 microscopic fields, each field corresponding to 41,500  $\mu\text{m}^2$ . The mean number of macrophages was 1,200–1,500/mm<sup>2</sup>.

## Results

*Infection of normal macrophages by trypomastigotes from Y and CL strains.* Extreme differences in the uptake of parasites from both strains were observed in paired experiments using macrophages from the same batch. The percentages of infection obtained with macrophages after 3 h exposure to bloodstream forms with ratio parasites/cell of 5:1 were the following:  $Y = 8.73 \pm 4.14$  and  $CL = 0.23 \pm 0.15$  ( $p < 0.001$ ). The stout trypomastigotes from the *CL* strain which constitute 95–98% of the inocula are apparently poorly equipped to enter the macrophages; no significant increase in the uptake was observed even when parasite/cell ratios of 30:1 or exposure time of 24 h were used. The *Y* strain parasites are in general 20 to 30-fold more infective to the macrophages than the *CL* strain. With the *Y* strain, within the range of 2–24 h of contact with the cells and ratios of 5–30 parasites/cell, the percentage of infection was directly related to time of exposure and number of parasites. Nevertheless, in practically all experiments, regardless of time and inoculum, only a very small percentage of bloodstream forms was ingested by the macrophages and most parasites remained extracellular and motile. No significant differences in the percentage of infection with *Y* strain were observed between macrophages from outbred and DBA mice. The variation in the percentage of infected macrophages in replicated experiments was rather low but large differences could be detected in experiments performed on several occasions.

*Infection of normal macrophages by trypomastigotes from X-irradiated mice.* Bloodstream forms of the *Y* strain collected from lethally irradiated animals are infective to macrophages. The percentages of infection of normal macrophages exposed during 4 h to parasites from normal and X-irradiated mice were, respectively,  $23.2 \pm 10.9$  and  $9.5 \pm 7.2$ . The difference is not statistically significant ( $p > 0.05$ ). Ratio parasites/cell was 10:1. The bloodstream forms collected from the animals immunosuppressed by lethal doses of X-rays were not lysed by human complement, conversely to what occurs with parasites from non-irradiated mice (Krettli and Nussenzweig, 1977), a demonstration that antibody binding was not occurring in the parasites from irradiated animals. Our findings

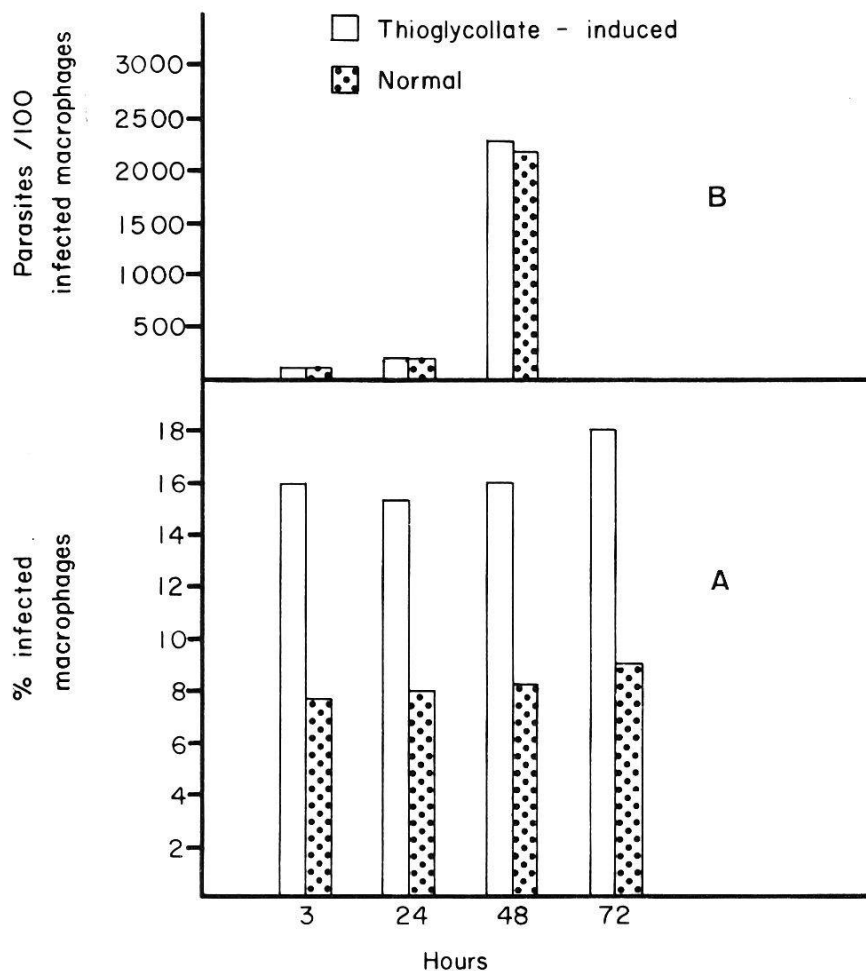


Fig. 1. Activation by thioglycollate is not influencing the fate of *Y* strain bloodstream forms in macrophages. A: The percentage of initially infected macrophages (determined after 3 h exposure) was kept constant within the 48-h period of observation showing that intracellular destruction was not occurring in normal and activated cells. B: The number of intracellular parasites showed a similar increase rate in both normal and activated macrophages.

suggest that immunological phagocytosis is not the only factor involved on the uptake of *T. cruzi* bloodstream forms from the *Y* strain.

*Infection of thyoglicollate-induced macrophages by trypomastigotes from the Y strain.* Macrophage induction by thyoglicollate increases the parasite uptake. The percentage of infection in thyoglicollate-induced macrophages ( $15.9 \pm 0.59$ ) was significantly higher than in normal macrophages ( $7.8 \pm 1.37$ ) ( $p < 0.01$ ). The ratio parasites/cell was 5:1. This finding is in accordance with data with *T. cruzi* culture forms reported by Nogueira and Cohn (1976).

*The fate of bloodstream trypomastigotes in normal and thyoglicollate induced macrophages.* The percentage of infected cells was constant within the first 24–48 h after inoculation. After 72 h the differentiation of amastigote stages into trypomastigotes already occurs in a variable percentage of infected cells and the uptake of newly released parasites produces a slight increase in the number of infected macrophages (Fig. 1A); on the other hand, the number of intracellular stages by 100 infected macrophages steadily increases within 48 h

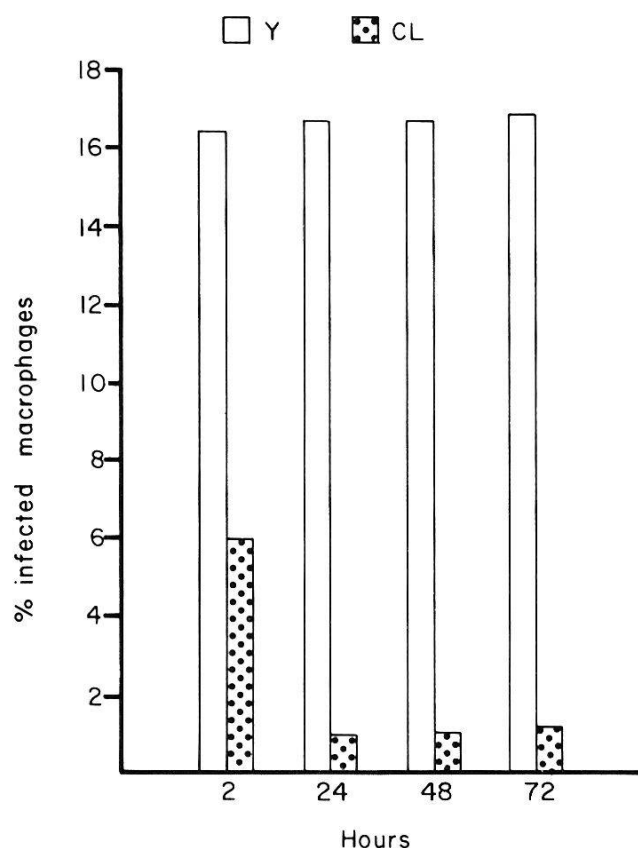


Fig. 2. The fate of opsonized bloodstream forms from *Y* and *CL* strains in normal macrophages is different. The percentage of initially infected macrophages (determined after 2 h exposure) was kept constant with *Y* strain, with *CL* strain a marked decline occurred as a consequence of the early intracellular destruction.

(Fig. 1B). This is a clear demonstration that most normal and thioglycollate-induced macrophages are neither inhibiting growth nor destroying intracellular stages of *T. cruzi*. The same sequence of events was observed in the small number of macrophages which had been infected with *CL* strain bloodstream forms.

*Effects of opsonization on the uptake and fate of bloodstream trypomastigotes from Y and CL strains.* Opsonization significantly increase the uptake of bloodstream parasites from *Y* strain. The percentage of infection in macrophages exposed for 2 h to parasites plus specific immune-sera was  $16.4 \pm 4.14$  whereas incubation with parasites plus NMS infected  $7.40 \pm 1.14$  of the macrophages ( $p < 0.05$ ). Much more impressive are the results obtained with immune-sera treated *CL* parasites, showing a 20 to 30-fold increase in the uptake by normal macrophages in relation to untreated trypomastigotes. Percentages of infection after exposure of normal macrophages for 2 h to *CL* bloodstream forms incubated with specific immune-sera and NMS were, respectively,  $6.03 \pm 1.54$  and  $0.26 \pm 0.10$  ( $p < 0.001$ ). Ratio parasites: cell was 5:1. Opsonization has not interfered with the intracellular development of the *Y* parasites, which keep multiplying in the macrophages as normal untreated parasites (Figs 2 and 1). The bloodstream forms from *CL* strain, however, are extensively destroyed after their uptake, as demonstrated by the marked decline of the



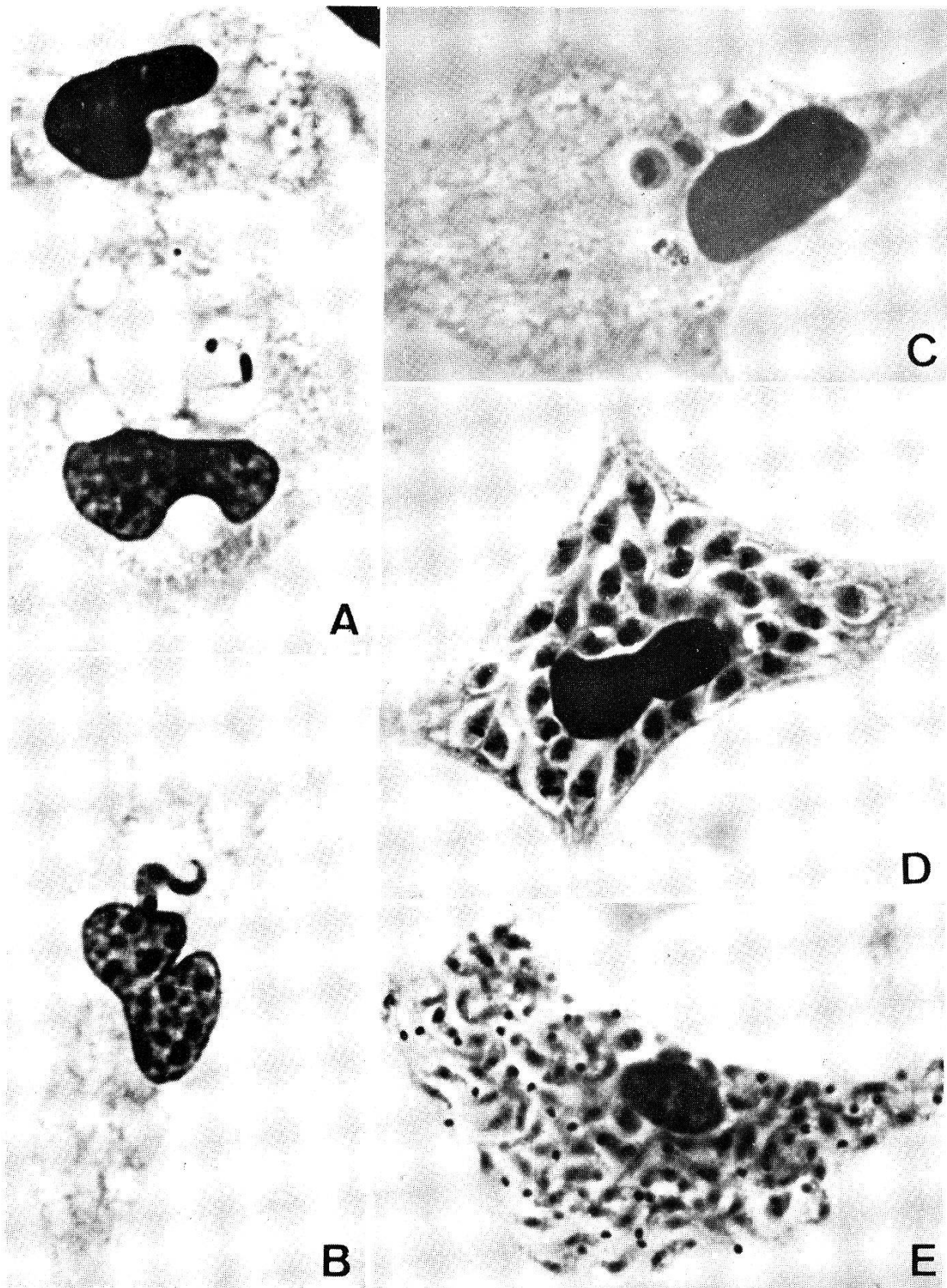


Fig. 3. Different aspects of the development of *T. cruzi* bloodstream forms in normal macrophages. A: Slender form of Y strain. B: Opsonized stout form of CL strain. C, D, E: Development of Y strain parasites.

percentage of infected macrophages which occurs in the first 24 h after inoculation (Fig. 2). A small percentage of the parasite population, however, evades the killing effect of the macrophages and normally develop in the host cells accomplishing the full intracellular cycle.

Fig. 3 shows different aspects of the development of *T. cruzi* bloodstream forms in macrophages.

## Discussion

Strains *Y* and *CL* were selected to investigate the interaction between *T. cruzi* and mouse peritoneal macrophages on grounds of their different characteristics: a) The *Y* strain presents a predominance of slender trypomastigote bloodstream forms whereas in the *CL* strain most parasites are stout forms. The biological significance of this polymorphism has been reviewed by Brener (1973); b) trypomastigotes from the *Y* strain are agglutinated by specific immune-sera whereas *CL* bloodstream forms are not affected by the antibodies (Krettli and Brener, 1976); c) *Y* strain bloodstream forms collected during the acute phase are lysed by human complement; the *CL* parasites, on the other hand, are not lysed by complement unless they are preincubated with specific immune-sera (Krettli and Nussenzweig, 1977); d) The *Y* strain show in the vertebrate a marked tropism for cells of the MPS whereas the parasites from the *CL* strain are able to prevent their uptake by phagocytic cells and are predominantly myotropic (Melo and Brener, 1978). Some of those intraspecific variations seem to be related to differences in the antibody-binding and/or antigen modulation of the parasites from both strains (Krettli and Nussenzweig, 1977).

We demonstrated in this paper that bloodstream trypomastigotes from the *CL* strain were 20 to 30-fold less infective to macrophages than those from the *Y* strain. The following possibilities could be suggested to explain the phenomenon: a) Bloodstream forms from some *T. cruzi* strains might bind host serum proteins which would interfere with the recognition and binding by macrophages; the presence of such "host antigens" has been already reported in *Trypanosoma vivax* (Ketteridge, 1970); b) the presence of protease-sensitive receptors which mediate the ingestion of *T. cruzi* culture forms by macrophages has been described (Nogueira and Cohn, 1976); a possibility exists that macrophages may lack specific receptors for parasites from certain strains such as *CL*; c) recent investigations strongly suggest that most intracellular protozoa enter macrophages and other cells by phagocytosis (Jones et al., 1972). The concept of "induced or stimulated phagocytosis" implies a mechanism of entry in which the parasite also plays an active role in the endocytosis (Jones et al., 1975). There is evidence that stout trypomastigotes from the *CL* strain, which constitute over 90% of the population at the peak of parasitemia, display a peculiar behaviour as regards interaction with host cells. Previous observations (Brener, 1969; Howells and Chiari, 1975) demonstrated, for instance, that a certain



percentage of *T. cruzi* stout forms inoculated intravenously into mice remain circulating in the bloodstream without penetrating the host cells, whereas slender forms very soon disappear from the blood to accomplish the intracellular cycle. When the circulating stout forms are removed by exsanguination of the host, 24 and 48 h after inoculation, they are less infective for the mouse than slender forms but highly infective for the vector (Howells and Chiari, 1975). Those forms are apparently more adapted for the development in the vector and may have lost the capability of inducing phagocytosis by changes in their membrane components or receptors.

The percentage of macrophage infection by *T. cruzi* Y strain has been directly related to ratio parasites: cell and exposure time. This peculiarity of the interaction between living microorganisms and macrophages in which infection increases with time exposure has been observed in other systems (Mackanness, 1960). As described in *Toxoplasma*, many factors, such as the ratio parasites: cell, volume of the inocula, conditions of the monolayer, etc. may influence the uptake (Jones et al., 1972). As regards *T. cruzi* Y strain, besides those factors, the variable number of parasites coated with antibodies might also affect the rate of infection. Parasites from the Y strain collected from lethally irradiated mice and surely uncoated by antibodies (as demonstrated by immunolysis) are readily ingested by macrophages, suggesting that immunological phagocytosis is not the only factor involved in the uptake of *T. cruzi* bloodstream forms. Nevertheless, the fact that opsonized trypomastigotes are significantly more infective to macrophages than normal parasites is an evidence that the final infection rates may be affected by the presence of a variable and increasing number of parasites coated with antibodies from the 7th day of infection on (Krettli and Nussenzweig, 1977).

The uptake of Y strain trypomastigotes by thioglycollate-induced macrophages is significantly higher than normal macrophages. Such macrophages increase their uptake of IgG coated particles (Bianco et al., 1975). A possibility exists that the increase in the parasite uptake is caused by specific immunoglobulins and C<sub>3b</sub> bound to trypomastigotes surface.

Evidence provided by fine structure shows that trypomastigotes from acellular cultures (Nogueira and Cohn, 1976) and bloodstream forms (Milder et al., 1973) are found inside phagocytic vacuoles, soon after being ingested by macrophages. The parasites' fate seems to depend on their ability to escape from the phagocytic vacuole and develop into the cytoplasmic matrix. Multiplying intracellular stages of *T. cruzi* observed in different cells are never found encircled by vacuoles plasma membranes (Tafari, 1970). The development of *T. cruzi* Y strain in normal and thioglycollate-induced macrophages suggests that the parasites are regularly escaping from the phagocytic vacuoles to accomplish their intracellular cycle, confirming recent observations of Milder et al. (1977). More impressive is the normal development of opsonized trypomastigotes from the Y strain in macrophages. Despite the effectiveness of antibody coating

(demonstrated by the significant increase of the uptake and occurrence of complement mediated lysis), parasites are evading the microbicidal action of macrophages. This behaviour contrasts with other systems in which opsonization strongly affects the fate of the microorganism in macrophages. Vaccinia virus, for instance, are ingested by endocytosis, escape from the phagocytic vacuole and multiply into the cell cytoplasm; opsonized viruses, however, are unable to escape and are destroyed inside phagolysosomes (Silverstein, 1975). Normal toxoplasms develop in the phagocytic vacuoles and prevent the formation of phagolysosomes; antibody exposed parasites, however, are not preventing the fusion of vacuoles with lysosomes and are destroyed (Jones et al., 1975). The *Y* strain opsonized trypomastigotes are apparently able to disrupt the vacuolar membrane and escape into the cytoplasm or then, resist to the lysosomal active components as described for *Leishmania* (Alexander and Vickerman, 1975). Trypomastigotes from *CL* strain, however, are apparently not adapted to the intracellular parasitism and thereby devoided of the mechanisms indispensable for the survival of amastigote stages into the phagocytic vacuole or for the escape from this organelle.

Milder et al. (1977) demonstrated that *Y* strain bloodstream forms survive and multiply into normal hamster peritoneal macrophages cultivated "in vitro" whereas the broad trypomastigotes from the *F* strain are rapidly destroyed. According to the authors, the destruction might be related to the presence of antibodies bound to these forms during the more prolonged infection caused by the *F* strain. Actually, the parasites had been collected at the peaks of parasitemia, which were at the 7th and 30–40 days of infection for, respectively, the *Y* and *F* strains. The observations reported by Milder et al. (1977) with the *F* strain and our findings with the *CL* strain strongly suggest that in some *T. cruzi* strains, parasites opsonized "in vivo" during the ongoing infection might be destroyed by macrophages. At this stage it is difficult to interpret the data of Kierszenbaum et al. (1974) who, working with a strain which like the *Y* strain is highly reticulotropic, reported an early destruction of bloodstream forms by normal macrophages both "in vivo" and "in vitro". Our data with the *Y* strain are not consistent with significant intracellular destruction by normal macrophages. As shown in Figs 1A and 1B the number of parasites per 100 infected macrophages demonstrates that practically all macrophages had been infected by single trypomastigotes and that the steady increase of the number of parasites corresponds to a free intracellular replication of the parasites; furthermore, the percentage of initially infected macrophages has been maintained stable within the first 48 h of observation, a demonstration that no destruction of the parasites nor reinfection of macrophages has occurred in this period.

Destruction of tissue culture derived trypomastigotes by normal macrophages was also described by Dvorak and Schmunis (1972). The authors suggested that this kind of interaction could be characteristic of specific strains of

host and parasite, being different the findings in highly adapted reticulotropic strains of the parasite.

Some of our "in vitro" findings parallels the events which occur in the intact host inoculated with *CL* and *Y* strains. This is the case of the *Y* strain, which shows in animals a selective and intense parasitism of the macrophages from the spleen, liver and bone marrow; with the *CL* strain the parasitism of the macrophages from MPS was negligible but the intracellular stages were highly prevalent in muscle cells (Melo and Brener, 1978). This is in agreement with previous observations of authors who described the occurrence of "reticulotropic" *T. cruzi* strains with marked preference for phagocytic cells of the "reticulo endothelial system" (Taliaferro and Pizzi, 1955). A further implication of this phenomenon is the possibility that the massive parasitism of the host macrophages produces a functional blocking of the MPS which would affect the immune response, a mechanism already suggested for malaria (Loose et al., 1972). Immunosuppression in experimental Chagas' disease has been already reported by Clinton et al. (1974), Lima Pereira (1976) and Schmunis et al. (1977).

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