Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 39 (1982)
Heft: 2

Artikel: "Trypanosoma cruzi" : the fate of bloodstream trypomastigote, amastigote, metacyclic trypomastigote and epimastigote forms in the peritoneal macrophages of immune and non-immune mice in vivo

Autor: Burger, E. / Lay, W.H. / Hypolito, L.V.R.

DOI: https://doi.org/10.5169/seals-312968

Acta Tropica *39*, 111–122 (1982)

[1] Departamento de Microbiologia e Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Brasil
[2] Instituto de Medicina Tropical de São Paulo, Departamento de Medicina Preventiva, Universidade de São Paulo, Brasil
[3] Departamento de Bioquímica, Instituto de Química; Universidade de São Paulo, Brasil

Trypanosoma cruzi: the fate of bloodstream trypomastigote, amastigote, metacyclic trypomastigote and epimastigote forms in the peritoneal macrophages of immune and non-immune mice in vivo

E. BURGER[1], W. H. LAY[2], L. V. R. HYPOLITO[3], J. F. FERNANDES[3]

Summary

Experiments were done in vivo in order to compare the ability of *Trypanosoma cruzi* bloodstream trypomastigotes (BTr), amastigotes (Am), metacyclic trypomastigotes (MTr) and epimastigotes (Ep) to proliferate in the peritoneal macrophages of the following groups of A/Sn mice: actively immunized with increasing doses of living BTr (AIM); mice passively immunized with homologous immune serum (ISTM); mice passively transfused with normal homologous serum (NSTM) and normal mice (NM). Parasite load was evaluated by counting the number of infected peritoneal macrophages harvested on the 4th day after i.p. challenge with one of the forms. All four parasite stages infected NM. AIM seldom showed parasitized macrophages after challenge with any of the forms. ISTM always harboured numerous parasitized macrophages. The only form susceptible to humoral immunity was the Am.

Key words: *Trypanosoma cruzi;* peritoneal macrophages; in vivo experiments; immunity.

Introduction

The aim of the present study was to test the susceptibility of the various stages in the life cycle of *T. cruzi* to the vertebrate's immune mechanisms using as our measure the number of infected peritoneal macrophages vs. the number

Correspondence: Dr. Eva Burger, Depto. Microbiologia e Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, C. P. 4365, CEP 05508, São Paulo, Brasil

of uninfected ones at the end of the first intracellular reproductive cycle. The quantification of amastigote loaded macrophages to evaluate infection was chosen instead of the customary parasitemia curves used by others because previous unpublished observations showed us that in experimental Chagas' disease the absence of free circulating parasites does not always correlate with the absence of infection. A marked discrepancy exists between cellular parasitism and bloodstream parasitemia, particularly in serum transfer experiments.

The present study included groups of actively immunized mice, which are known to exhibit both cellular and humoral immunity, and groups of immune serum transfused mice, in which only humoral immunity was responsible for specific protection against *T. cruzi*. Our results with actively immunized mice showed no evidence for the existence of stage specific immunity in chronically infected animals. In passively immunized mice a higher susceptibility of Am to humoral immunity was observed.

Materials and Methods

Animals. Female mice of the A/Sn strain, bred in our laboratory, weighing 20–25 g, were used in the experimental groups described below. The blood of Wistar rats was used as our source of complement for the purification process of MTr.

Immunization procedure. Immunization of A/Sn mice was performed inoculating 10, 100, 1,000 and 10,000 BTr i.p. with intervals of approximately one month between each dose. These animals, resistant to lethal doses of BTr were used for two purposes: as actively immunized mice (one month after the last dose) and as the source of immune serum (7 days after the last dose). The immune sera were pooled and titrated, giving a titre of 160 with the indirect fluorescence test (Camargo, 1966); 640 with the passive haemagglutination test (Camargo et al., 1973) and a moderate amount of precipitate with the flocculation test (Hoshino-Shimizu et al., 1975). Pooled sera were stored in 8 ml aliquots and kept at –20° C.

Culture cell line. LLC-MK2 cells were maintained in Hank's solution containing 0.5% hydrolysed lactoalbumin (Difco), 20% fetal calf serum inactivated at 56° C for 30 min, 1 U/ml of penicillin, 100 μg/ml of streptomycin and 0.2% phenol red.

Parasites. The Y strain of *T. cruzi* (Silva and Nussenzweig, 1953) was used in all experiments. The parasites were maintained in the laboratory by serial passages in outbred mice or cultivation in media. *BTr stage:* A/Sn mice were bled on the 7th day after infection, their blood was collected in 3.8% sodium citrate, centrifuged at 400–500 *g* for 10–15 min and kept at room temperature for 30 min to allow active migration of the parasites to the plasma. This plasma containing the parasites was centrifuged at 700 *g* for 20 min. The sediment was resuspended in 0.85% NaCl and the parasites were counted in an haemocytometer. *Ep stage:* Blood from mice on the 7th day after infection with *T. cruzi* was used to inoculate sterile flasks containing LIT medium (Castellani et al., 1967). This culture was kept at 28° C, and the volume doubled 7 days later by adding fresh medium. Parasites were used for experiments on the 14th day, when only Ep were present, as confirmed by careful observation under phase contrast microscopy. The cultures were centrifuged in plastic tubes at 400 *g* for 8 min, the sediment was washed and resuspended in 0.85% NaCl and filtered through nylon wool. Parasites were counted in an haemocytometer. The resulting suspension used in our experiment contained only Ep. *MTr stage:* Ep of *T. cruzi* which had been kept for several weeks in LIT medium were seeded on modified NNN medium (blood-agar covered with a small volume of LIT medium) where they eventually differentiated into MTr forms. Cultures containing 50% or more of these forms were purified as described by Kimura et al. (1978). This process is based on selective lysis of the Ep by the alternative complement pathway (Nogueira et al., 1975). The MTr obtained

were resuspended in 0.85% NaCl, carefully observed under phase contrast microscopy and counted in an haemocytometer. The resulting suspension used in our experiments contained only MTr. *Am stage:* One day old LLC-MK2 cell cultures were infected with *T. cruzi* grown in LIT medium and harvested at the stationary phase of growth, when a high proportion of MTr were present. After 24 h the parasites remaining outside the cells were eliminated by careful washing with buffered saline solution. Washing was repeated on the 3rd day after infection. The cells were then removed from the flasks by treatment with 0.25% trypsin for 10 min, centrifuged at 800 *g* for 5 min and disrupted using a homogenizer in order to liberate the Am. The suspension was centrifuged for 1 min at 50 *g* to sediment cellular debris. This procedure was repeated several times. The resulting suspension of Am was centrifuged at 450 *g* for 15 min, the parasites were resuspended in 0.85% NaCl, filtered through nylon wool, carefully observed under phase contrast microscopy and counted in an haemocytometer. The resulting suspension used in our experiment contained approximately 1 Ep/1,000 Am, and no trypomastigotes.

Quantitative evaluation of the parasitism of mice. In order to minimize contamination of peritoneal cells with blood leucocytes, the mice used in these experiments were anesthetized by ether inhalation and bled out by sectioning the axillary plexus prior to sacrifice. Five ml of cold Hank's solution were introduced into the peritoneal cavity and after gentle massage this liquid was withdrawn. This procedure was repeated. Smears with excellent cellular morphology were obtained as follows: a strip of filter paper with a central hole 1 cm in diameter was placed onto a glass slide, and covered with a thick plastic piece with a coinciding central hole. Thus a well of 0.5 cm in height was formed. These components were held together by two clamps. The well was filled repeatedly with the suspension of peritoneal exudate cells, and the liquid was slowly absorbed by the filter paper, allowing gradual deposition of cells. Three slides were obtained with the cells collected from each experimental animal, fixed in cold Bouin solution and stained with Giemsa. All cells were observed for the presence of intracellular parasites but only macrophages, identified by morphological criteria, were included in the quantitative evaluation because other peritoneal exudate cells never showed parasites. A wide variation in the number of macrophages was observed from one slide to another.

Experimental design. A minimum of 16 female mice were used for each experiment: 4 actively immunized mice (AIM), 4 normal mice (NM), 4 immune serum transfused mice (ISTM) and 4 normal serum transfused mice (NSTM). In one experiment a larger than normal volume of challenge parasites in suspension allowed the inclusion of 5 more animals, and in 2 other experiments one animal was lost for technical reasons (peritoneal bleeding). AIM were obtained inoculating BTr as already described, and NM controls received only 0.85% NaCl on corresponding days. ISTM were injected with two doses of immune serum: 0.3 ml by the i.p. route 10 to 20 min prior to infection and 0.2 ml i.v. into the rethroorbital plexus on the 3rd day after infection. NSTM controls received identical amounts of normal serum on the same days via the same route. The parasites used for challenge were inoculated i.p. in doses which differed from one experiment to another according to the infectivity of the various stages of *T. cruzi*. The parasite burden of peritoneal macrophages of all groups was evaluated on the 4th day after infection, when the peak of the 1st intracellular reproductive cycle is reached and the most pronounced differences between experimental and control groups are observed (Burger and Lay, unpublished). The experiments were performed with each one of the following stages of *T. cruzi:* BTr, Am, MTr, Ep.

Statistical analysis. To unify the analysis of comparisons among treatments (AIM, NM, ISTM, NSTM) we used the analysis of categorical data by linear models introduced by Grizzle et al. (1969). This technique takes into account the different number of macrophages counted in each treatment (the number varied from one animal to another) and the larger number of animals employed in one experiment (Table 1). The number of macrophages counted per animal was sufficiently high (usually >500), and was the basis for the observed proportions of parasitized macrophages. The statistical analysis was made using these proportions. The proposed technique, besides unifying comparisons among treatments, can also be used when there is a column of zeros. The value zero is then replaced by the value 0.5, a procedure fully justified by Grizzle et al.

Thus all the comparisons among different treatments were done according to the following proposed model: $Y = XB + \varepsilon$, where:

Y is the vector of the observations, that is, the proportion of infected macrophages in the groups,

X is the design matrix (that relates each animal to each treatment),

B is the vector of the parameter (that measures the treatment effects),

ε is the error vector, and by assumption, ε is normally distributed with zero mean and variance-covariance V.

The GENCAT (Generalized Categorical Data UNC Chapel Hill, North Carolina) program was used to analyse the data. The χ^2 distribution was used to compare the treatment within groups. The 1% level of significance was adopted.

Results

Parasitism of peritoneal cells by bloodstream trypomastigotes. The 4 groups of mice were infected i.p. with 6×10^6 BTr. On the 4th day, the peritoneal cells were collected and the parasitized proportion evaluated. The results are given in Table 1. Analysis of individual mice clearly showed that those actively immunized with BTr (AIM) had no parasitised cells. Parasitism of NM cells was significantly lower than those in NSTM. On the other hand, no differences in parasitism were observed between ISTM and NSTM suggesting that BTr infectivity was not altered by immune serum.

Parasitism of peritoneal cells by amastigotes. The 4 groups of mice were infected i.p. with 2.8×10^6 Am. On the 4th day, the peritoneal cells were collected and parasitism evaluated. The results are given in Table 2. Analysis of individual mice clearly showed that Am were destroyed efficiently in AIM and were highly infective for NM. Significantly lower infections were obtained in ISTM when compared to NSTM, thus indicating that humoral immunity can effectively reduce infection by Am.

Parasitism of peritoneal cells by metacyclic trypomastigotes: The 4 groups of mice were infected i.p. with 4×10^6 MTr. On the 4th day, the peritoneal cells were collected and parasitism evaluated. The results are given in Table 3. Analysis of individual mice clearly showed that actively immunized mice (AIM) were able to efficiently destroy the inoculated MTr. The MTr were infective, as demonstrated by the parasite burden found in NM, in spite of the purification process which they had to undergo. Humoral immunity was not harmful to those parasites, as suggested by the fact that the parasitism of ISTM was not significantly lower than that of NSTM.

Parasitism of peritoneal cells by epimastigotes. The 4 groups of mice were infected i.p. with 3.8×10^7 Ep. On the 4th day, the peritoneal cells were collected and parasitism evaluated. The results are given in Table 4. The parasitism found in NM showed that Ep are infective for the A/Sn strain of mice, although Ep are effectively destroyed in AIM. No significant difference in parasitism was observed between ISTM and NSTM cells. Remarkably, the parasitism of NM was significantly higher than that of ISTM and NSTM, indicating that Ep are

Table 1. Evaluation of the parasitism of peritoneal macrophages on the 4th day after i. p. inoculation of 6×10^6 bloodstream trypomastigotes of *Trypanosoma cruzi* in individual mice, actively or passively immunized, and their controls

Immune status of the mice							
Actively immunized mice							
No. of parasitized macrophages	0	0	0	0			
No. of macrophages counted	2585	772	856	850			
Percentage of parasitized macrophages	0	0	0	0			
Normal mice							
No. of parasitized macrophages	0	0	0	0			
No. of macrophages counted	1517	1409	498	67			
Percentage of parasitized macrophages	0	0	0	0			
Immune serum transfused mice							
No. of parasitized macrophages	1	11	5	1	2	12	
No. of macrophages counted	611	1487	1715	186	126	441	
Percentage of parasitized macrophages	0.16	0.74	0.29	0.54	1.59	2.72	
Normal serum transfused mice							
No. of parasitized macrophages	0	0	3	1	24	16	4
No. of macrophages counted	882	502	1425	119	570	488	78
Percentage of parasitized macrophages	0	0	0.21	0.84	4.21	3.28	5.13

Statistical analysis of these results indicate that the following comparisons showed significant differences:
NSTM $\times$ NM ($\chi^2 = 45.71$)
ISTM $\times$ AIM ($\chi^2 = 30.92$)
ISTM $\times$ NM ($\chi^2 = 30.11$)
NSTM $\times$ AIM ($\chi^2 = 46.41$)
The following comparisons were not significantly different:
NM $\times$ AIM ($\chi^2 = 0.03$)
NSTM $\times$ ISTM ($\chi^2 = 4.77$)

Table 2. Evaluation of the parasitism of peritoneal macrophages on the 4th day after i. p. inoculation of 2.8×10^6 amastigotes of *Trypanosoma cruzi* in individual mice, actively or passively immunized, and their controls

Immune status of the mice				
Actively immunized mice				
No. of parasitized macrophages	0	1	2	0
No. of macrophages counted	1470	1955	804	1223
Percentage of parasitized macrophages	0	0.05	0.25	0
Normal mice				
No. of parasitized macrophages	57	57	57	62
No. of macrophages counted	1555	1105	1131	1076
Percentage of parasitized macrophages	3.66	5.16	5.04	5.76
Immune serum transfused mice				
No. of parasitized macrophages	47	36	24	12
No. of macrophages counted	2677	1565	1707	1364
Percentage of parasitized macrophages	1.75	2.30	1.41	0.88
Normal serum transfused mice				
No. of parasitized macrophages	84	79	12	
No. of macrophages counted	1312	1030	647	
Percentage of parasitized macrophages	6.40	7.67	1.85	

Statistical analysis of these results indicate that the following comparisons showed significant differences:
ISTM $\times$ AIM ($\chi^2 = 100.03$)
NM $\times$ AIM ($\chi^2 = 231.27$)
NSTM $\times$ ISTM ($\chi^2 = 86.63$)
NM $\times$ ISTM ($\chi^2 = 86.42$)
NSTM $\times$ AIM ($\chi^2 = 178.47$)
The following comparison was not significantly different:
NSTM $\times$ NM ($\chi^2 = 4.09$)

susceptible to an unspecific effect of the non-inactivated serum introduced into the peritoneal cavity shortly before the challenge.

Comparison of the infectivity of different stages in the life cycle of T. cruzi. Our data on NM can be used to calculate the average proportion of infected peritoneal macrophages for a given number of parasites (10^7) but only for those stages which do not escape from the peritoneal cavity, as follows:

$$\text{Am:} \quad \frac{0.0479}{2.8 \times 10^6} \times 10^7 = 0.17$$

$$\text{MTr:} \quad \frac{0.020101}{4 \times 10^6} \times 10^7 = 0.05$$

$$\text{Ep:} \quad \frac{0.04844}{3.8 \times 10^7} \times 10^7 = 0.012$$

The following decreasing order of infectivity was obtained: Am > MTR > Ep.

Table 3. Evaluation of the parasitism of peritoneal macrophages on the 4th day after i. p. inoculation of 4×10^6 metacyclic trypomastigotes of *Trypanosoma cruzi* in individual mice, actively or passively immunized, and their controls

Immune status of the mice				
Actively immunized mice				
No. of parasitized macrophages	0	0	0	0
No. of macrophages counted	573	877	1508	862
Percentage of parasitized macrophages	0	0	0	0
Normal mice				
No. of parasitized macrophages	5	3	3	17
No. of macrophages counted	161	74	228	930
Percentage of parasitized macrophages	3.11	4.05	1.31	1.83
Immune serum transfused mice				
No. of parasitized macrophages	13	30	9	24
No. of macrophages counted	804	672	126	343
Percentage of parasitized macrophages	1.62	4.46	7.14	7.00
Normal serum transfused mice				
No. of parasitized macrophages	19	18	36	23
No. of macrophages counted	311	998	755	711
Percentage of parasitized macrophages	6.11	1.80	4.77	3.23

Statistical analysis of these results indicate that the following comparisons showed significant differences:
ISTM $\times$ AIM ($\chi^2 = 76.55$)
NM $\times$ AIM ($\chi^2 = 27.03$)
NSTM $\times$ AIM ($\chi^2 = 95.22$)
The following comparisons were not significantly different:
NSTM $\times$ NM ($\chi^2 = 8.02$)
NSTM $\times$ ISTM ($\chi^2 = 0.64$)
ISTM $\times$ NM ($\chi^2 = 10.76$)

Discussion

Immunological research on another protozoan disease, malaria, has already established that immunity against parasites with different developmental stages in their life cycle may be stage specific. The possibility that a similar stage specificity exists in experimental Chagas' disease, led to the present investigation.

The fact that stage specific protection in malaria is easily transferred by serum (Nussenzweig et al., 1972) suggests that antibodies possess the discriminating ability to recognize different developmental stages of the parasite. In experimental Chagas' disease only BTr have been used for challenge in immune serum transfer experiments, and evaluation of the protective effect of antibo-

Table 4. Evaluation of the parasitism of peritoneal macrophages on the 4th day after i. p. inoculation of 3.8×10^7 epimastigotes of *Trypanosoma cruzi* in individual mice, actively or passively immunized, and their controls

Immune status of the mice				
Actively immunized mice				
No. of parasitized macrophages	4	0	0	0
No. of macrophages counted	2196	1181	1418	2013
Percentage of parasitized macrophages	0.18	0	0	0
Normal mice				
No. of parasitized macrophages	71	27	68	36
No. of macrophages counted	1372	632	1264	902
Percentage of parasitized macrophages	5.17	4.27	5.38	3.99
Immune serum transfused mice				
No. of parasitized macrophages	33	35	12	31
No. of macrophages counted	1037	1251	883	883
Percentage of parasitized macrophages	3.18	2.80	1.36	3.51
Normal serum transfused mice				
No. of parasitized macrophages	31	25	55	
No. of macrophages counted	2917	1539	1537	
Percentage of parasitized macrophages	1.06	1.62	3.58	

Statistical analysis of these results indicate that the following comparisons showed significant differences:
NM × NSTM ($\chi^2 = 63.54$)
ISTM × AIM ($\chi^2 = 107.83$)
NM × AIM ($\chi^2 = 205.52$)
NM × ISTM ($\chi^2 = 25.17$)
NSTM × AIM ($\chi^2 = 103.05$)
The following comparison was not significantly different:
NSTM × ISTM ($\chi^2 = 8.17$)

dies has always been based on the comparison of long lasting parasitemia curves and mortality data (Culbertson and Kolodny, 1938; Krettli and Brener, 1976; Kierszenbaum and Howard, 1976). These approaches provide information on the overall evolution of the acute disease, a period in which the parasite constantly changes from free circulating BTr to intracellular Am and vice-versa. Moreover, the interference of a possible active immunization of the host cannot be excluded. Our approach of interrupting the first intracellular reproductive cycle in ISTM challenged with a given stage of the parasite allows a better evaluation of the antibodies' effect on the inoculum itself.

In unpublished serum transfer experiments with periodical evaluation of the infection, we have found that, in spite of the very low bloodstream parasitemia in ISTM, it was often possible to detect intracellular parasite load of the

same magnitude as that found in the control animals (NSTM). The present data also show no significant difference between the parasite load of peritoneal macrophages of ISTM and of NSTM challenged with BTr. This does not necessarily contradict data of Krettli and Brener (1976) who described marked differences between the parasitemia curves and the mortality rates of ISTM and of NSTM challenged with BTr of the Y strain of *T. cruzi*. The low number of parasites found by these authors in the bloodstream of ISTM may merely reflect an opsonic effect of antibodies on BTr, not affecting their subsequent intracellular evolution. The lower mortality of the ISTM, however, points to a higher susceptibility to humoral immunity of a certain stage of the parasite in its later development, perhaps only of those forms recently released from infected cells.

We have also used the macrophage assay system to assess the effect of humoral immunity on MTr, Ep and Am of *T. cruzi*, a hitherto unresearched topic. Am was the only form which produced significantly lower infection in ISTM when compared to NSTM, and therefore, the only form which showed some susceptibility to humoral immunity. Our hypothesis is that antigenic determinants on Am could be more accessible to antibodies; since Am are normally intracellular parasites, they do not need to develop special protective mechanism against antibodies.

In AIM we have found virtually no parasitism in the peritoneal cells, whatever the stage used for challenge. It has been repeatedly shown that activated macrophages play a prominent role in the development of resistance against *T. cruzi* (Taliaferro and Pizzi, 1955; Goble and Boyd, 1962; Kierszenbaum et al., 1974). The broad range of activity of these cells, which may even destroy parasites unrelated to the activating infection (Mackaness, 1964), could explain the absence of stage specificity in the immunity developed against *T. cruzi* in the chronically infected animals (AIM) of our experiment. Hoff (1976), however, stresses that probably not only activated macrophages function to produce the strong resistance to challenge in mice that have recovered from primary *T. cruzi* infection; specifically sensitized lymphocytes and antibodies are also involved. In vivo experiments, however, do not allow one to measure the exact extent of contribution by each of these elements.

Besides establishing the absence of stage specificity in the protection developed against *T. cruzi*, our experiments also provided other interesting information.

The presence of immune or normal serum in the peritoneal cavity results in a greater parasitism of peritoneal macrophages in normal mice after challenge with BTr. Therefore, a localizing or attracting effect of serum factors can be suggested to act on BTr, which usually escape from the peritoneal cavity and are found in the bloodstream shortly after their i.p. inoculation (Brener et al., 1974).

Our experiment with Ep, on the contrary, showed higher parasitism of peritoneal macrophages in normal mice when compared to serum transfused

mice. It is known that the activation of the complement system by the alternative pathway lyses these forms of *T. cruzi* (Nogueira et al., 1975), but A/Sn mice are deficient in C5 (Cinader et al., 1964; Erickson et al., 1964). Nevertheless, the C3b mediated immune adherence enhancement of phagocytosis can operate in these animals, and this has been shown to be a much more efficient defense mechanism than the lysis of microorganisms itself. It explains the significantly lower peritoneal parasitism that we found in both normal and immune serum transfused mice challenged with Ep.

We have shown that all 4 stages are infective for A/Sn mice, a strain highly susceptible to *T. cruzi*, although there is an evident difference in the degree of infectivity among them. BTr and MTr are widely recognized as infective, Ep are considered harmless, and we do not know of any reference concerning the infectivity of Am isolated from infected cells.

The infectivity of Ep, denied by many authors, was observed by us using Ep obtained from cultures during exponential growth. Very careful examination under phase contrast microscopy excluded the presence of MTr in the suspension. The parasitism found in the challenged mice was much higher than that expected from a contamination of the inoculum by a minimal undetectable number of MTr. This shows that at least part of the Ep population was able to survive and proliferate in A/Sn mice, but the possibility that only those parasites already prone to differentiate towards MTr would be infective cannot be excluded.

Our experiments with Am were done with a parasite suspension obtained by disrupting *T. cruzi* infected tissue cells, 3 days after culture. Careful examination of this suspension proved that differentiation to trypomastigotes had not taken place, but a low contamination of the Am with Ep was detected (approximately 1 Ep/1,000 Am). These were remnants of the infecting inoculum. This number of Ep, however, could not be responsible for the high parasitism found in the challenged animals. Not only were the macrophages infected, but a larger number of parasites per cell was observed on the 4th day, suggesting that the inoculated Am, having no need to go through a process of differentiation, initiate their multiplication process immediately after being phagocytosed.

Since the present work deals only with the evaluation of the parasitism of peritoneal cells, it was not possible, with our data, to compare the infectivity of BTr with the infectivity of the other forms of *T. cruzi*, because part of the BTr escape from the peritoneal cavity shortly after their inoculation, and thus, the real parasite: peritoneal macrophage proportion cannot be determined. However, the well known fact that much higher doses of the other stages are necessary to cause the same parasitism and mortality as small doses of BTr demonstrates that BTr are indeed the most infective form of *T. cruzi*. For the other forms our data showed the following decreasing order of infectivity: Am $>$MTr $>$Ep.

Acknowledgments

This investigation received financial support from the Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant Nr. 72/888, and from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grants Nr. SIP/08/050, 4575/75 and 2222.8.053/78. We are grateful to Dr. A. W. P. Canton, from the Instituto de Matemática e Estatística – U.S.P. for having made the statistical analysis of the data.

Brener Z., Chiari E., Alvarenga N. J.: Observations on *Trypanosoma cruzi* strains maintained over an 8-year period in experimentally inoculated mice. Rev. Inst. Med. trop. S. Paulo *16*, 39–46 (1974).

Camargo M. E.: Fluorescent antibody test for the diagnosis of American trypanosomiasis. Technical modification employing preserved culture forms of *Trypanosoma cruzi* in a slide test. Rev. Inst. Med. trop. S. Paulo *8*, 227–234 (1966).

Camargo M. E., Hoshino S., Siqueira G. R. V.: Hemagglutination with preserved, sensitized cells, a practical test for routine serologic diagnosis of American trypanosomiasis. Rev. Inst. Med. trop. S. Paulo *15*, 81–85 (1973).

Castellani O., Ribeiro L. V., Fernandes J. F.: Differentiation of *Trypanosoma cruzi* in culture. J. Protozool. *14*, 447–451 (1967).

Cinader B., Dubiski S., Wardlaw A.: Distribution, inheritance and properties of an antigen Mu β_1, and its relation to hemolytic complement. J. exp. Med. *120*, 897–924 (1964).

Culbertson J. T., Kolodny M. H.: Acquired immunity in rats against *Trypanosoma cruzi*. J. Parasit. *24*, 83–90 (1938).

Erickson R. P., Tachibana D. K., Herzenberg L. A., Rosenberg L. T.: A single gene controlling hemolytic complement and a serum antigen in the mouse. J. Immunol. *92*, 611–615 (1964).

Goble F. C., Boyd J. L.: Reticulo-endothelial blockade in experimental Chagas' disease. J. Parasit. *48*, 223–228 (1962).

Grizzle J. E., Stamer C. F., Koch G. G.: Analysis of categorical data by linear models. Biometrics *25*, 489–504 (1969).

Hoff R.: Recent advances in cell-mediated immunity to *Trypanosoma cruzi*. Pan Amer. Hlth Org. Sci. Publ. *318*, 162–166 (1976).

Hoshino-Shimizu S., Camargo M. E., Umezawa E. S.: A rapid slide flocculation test for the diagnosis of American Trypanosomiasis using *Trypanosoma cruzi* fragments preserved by lyophilization. Comparison with hemagglutination, immunofluorescence, and complement fixation tests. Amer. J. trop. Med. Hyg. *24*, 586–589 (1975).

Kierszenbaum F., Knecht E., Budzko D. B., Pizzimenti M. C.: Phagocytosis: a defense mechanism against infection with *Trypanosoma cruzi*. J. Immunol. *112*, 1839–1843 (1974).

Kierszenbaum F., Howard J. G.: Mechanisms of resistance against experimental *Trypanosoma cruzi* infection: the importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. J. Immunol. *116*, 1208–1211 (1976).

Kimura E., Lay W. H., Fernandes J. F.: Extracellular in vitro evolution of metacyclic trypomastigotes isolated from *Trypanosoma cruzi* cultures. Rev. Inst. Med. trop. S. Paulo *20*, 133–138 (1978).

Krettli A. U., Brener Z.: Protective effects of specific antibodies in *Trypanosoma cruzi* infections. J. Immunol. *116*, 755–760 (1976).

Mackaness G. B.: The immunological basis of acquired cellular resistance. J. exp. Med. *120*, 105–120 (1964).

Nogueira N., Bianco C., Cohn Z.: Studies on the selective lysis and purification of *Trypanosoma cruzi*. J. exp. Med. *142*, 224–229 (1975).

Nussenzweig R. S., Vanderberg J. P., Sanabria Y., Most H.: *Plasmodium berghei:* accelerated clearance of sporozoites from blood as part of immune-mechanism in mice. Exp. Parasit. *31*, 88–97 (1972).

Silva L. H. P., Nussenzweig V.: Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. Folia clin. biol. (S. Paulo) *20*, 191–208 (1953).

Taliaferro W. H., Pizzi T.: Connective tissue reactions in normal and immunized mice to a reticulotropic strain of *Trypanosoma cruzi.* J. infect. Dis. *96*, 199–226 (1955).