In conclusion, a certain intra and interspecific variability in the heat shock response has been found at the puffing level. This does not agree with the conservative character of the response to temperature. Future molecular analysis will probably explain the unexpected heat shock puff differences found between both species.

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0014-4754/87/11/121225-03$1.50 + 0.20/0

Circulating *Trypanosoma cruzi* from the same cloned population show differences in the ability to infect cells and to cause lethal infection in mice[1]

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Summary. Two subpopulations of circulating parasites displaying different abilities to infect mammalian cells and to cause lethal infection when inoculated into normal mice were demonstrated in the blood of mice acutely infected with *T. cruzi*. Parasites of one subpopulation rapidly penetrated mouse fibroblasts and were readily phagocytized by normal mouse peritoneal macrophages whereas parasites of the other subpopulation showed little ability to invade non-phagocytic cells and resisted phagocytosis. Inoculation of organisms of this latter population into mice resulted in infections with lower parasitemias and longer time to death as compared to controls inoculated with organisms from a population containing both types of parasites. When a population of parasites containing both types of trypanosomes was cultured in acellular medium at 28 °C a decrease in the number of parasites was noted to occur in the initial days of culture. This decrease was not noted when parasites of the subpopulation of trypanosomes resistant to phagocytosis were cultured similarly.
Key words. Trypanosoma cruzi; Chagas' disease; macrophages; mouse fibroblasts.

Heterogeneity in *Trypanosoma cruzi*, the causative agent of Chagas' disease or American trypanosomiasis, was first reported in the original description of the parasite as morphologically distinct 'broad' and 'slender' bloodstream forms[4]. Since then, heterogenous populations of *T. cruzi* have been demonstrated in regard to biological, pathological, and antigenic make-up[5-7]. Because of the suggestion that differences in infectivity might be associated with morphological variations[6], a number of studies have been conducted using parasites from isolates with predominance of either 'broad' or 'slender' forms[8-11]. These studies have been conducted with organisms from cell cultures submitted to different conditions of temperature, culture medium, and incubation periods[8,9] or with trypanosomes obtained by centrifugation on density gradients after passage of the organisms through an ion exchange column[10], a technique which has been shown to selectively deplete subpopulations of parasites[11]. In addition, organisms from cell cultures have been shown to be only partially equivalent to bloodstream trypanosomes[12].

In this work we used a biological system to separate subpopulations of blood trypanosomes from the same cloned population and examined them for their capacity to invade cultured cells, to cause lethal infection in mice and to multiply in acellular cultures.

Methods and results. The Y strain of *Trypanosoma cruzi*, isolated in 1953 from a human case of Chagas' disease[13], was obtained from Dr Z. Brener (Centro de Pesquisas Rene Rachou, Belo Horizonte, State of Minas Gerais, Brasil) and cloned in our laboratory according to the technique described by Goldberg and Chiari[14]. Parasites derived from a single clone were obtained from the blood of adult, male, outbred, Swiss-Webster mice (Simonsen Laboratories, Gilroy, CA) at the peak of parasitemia (7 days after i.p. infection of the mice with 10^5 blood-form trypanosomes). The organisms were purified by centrifugation in 3% dextran as previously described[15], pelleted and resuspended at a concentration of 2×10^6/ml in RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated (60 °C) fetal bovine serum. One half of this trypanosome suspension was kept at 37 °C for 18 h under tissue culture conditions (original population). The other half was distributed over monolayers of murine peritoneal macrophages or monolayers of mouse fibroblasts (L929 cells, ATCC CCLI) on tissue culture slides (Lab-Tek Products, Napperville, Ill.) and cultured as previously described[15]. The organisms present in the supernatant of these cultures after 18 h of incubation (non entering subpopulation) were pelleted and resuspended in tissue culture medium as above. At least 80% of the organisms dispensed were recovered. The cell monolayers were washed, fixed with absolute methanol, stained with Giemsa stain and examined microscopically for intracellular parasites[15].

The in vitro and in vivo infectivity of the parasites of the 'non entering subpopulation' was compared with that of the same number of organisms of the 'original population' which had been kept at 37 °C for 18 h. For the in vitro assay, monolayers of peritoneal macrophages or of L929 cells were infected with trypanosomes of the 'original population' or of the 'non

Infection of normal mouse peritoneal macrophages and L929 mouse fibroblasts with different subpopulations of blood trypanosomes of *T. cruzi*

Parasites*	Percent infected	
	M	L929
Fresh	49 ± 3	18 ± 5
Original population	37 ± 3	16 ± 8
Non entering subpopulation	9 ± 1**	< 0.5

* Fresh = Parasites used just after harvesting from infected mice; original population = after storage at 37 °C, non entering subpopulation = after exposure to cultured cell monolayers. See text for details. Expressed as mean ± SE. ** $p < 0.001$ as compared with both fresh and original population trypanosomes.

entering subpopulation'. The results (table) revealed that trypanosomes from the 'non entering subpopulation' were significantly ($p < 0.001$) less infective than parasites of the 'original population' to both macrophages and L929 cells. Differences in infectivity were not related to age of cultured host cells since similar results were obtained with freshly prepared or with 24-h-old monolayers (data not shown).

To further examine infectivity, blood trypanosomes were incubated with monolayers of L929 cells for 24 h at a ratio of 100 parasites/cell. Thereafter, the 'non entering' organisms were recovered and added to fresh L929 cell monolayers. This procedure was repeated twice and at the end of the third incubation period the recovered parasites were layered over a monolayer of murine peritoneal macrophages and incubated for an additional 24 h. After this period of time slides were fixed, stained and examined for intracellular parasites. The results revealed that less than 1% of L929 cells of the second and third monolayers had intracellular parasites, and less than 5% of the cells of the last monolayer of murine peritoneal macrophages were parasitized. The rate of multiplication of the interiorized parasites, however, was equal to the rate of multiplication of freshly isolated organisms (data not shown) suggesting that trypanosomes maintained extracellularly for a prolonged period of time (over 72 h in these experiments) retained the ability to transform into amastigotes and multiply upon becoming intracellular.

For the in vivo experiments, the same inoculum of trypanosomes of the 'non entering subpopulation' or of the 'original

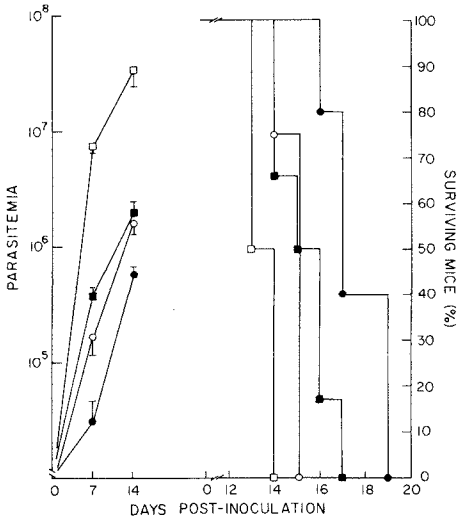

Figure 1. Parasitemia and survival in mice infected with 5×10^3 (○, ●) and 5×10^4 (□, ■) trypanosomes recovered from the supernatant of cultured cell monolayers of normal mouse peritoneal macrophages (non entering subpopulation, ●, ■, n = 6) or incubated in tissue culture medium for 18 h at 37 °C (original population, ○, □, n = 4). Mean parasitemia ± SEM were calculated on the survivors at each time point.

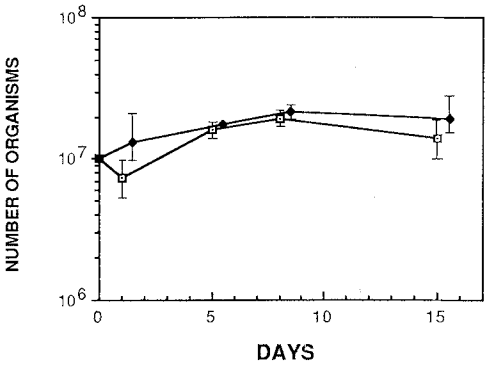

Figure 2. Multiplication of *T. cruzi* in acellular culture medium. 10^7 trypanosomes of the original population (□) or of the 'non entering subpopulation' (◆) were seeded into each of 3 tubes containing acellular growth medium. The multiplying organisms were counted at different periods of time and the bars indicate the range of the numbers in the 3 tubes.

population' was injected i.p. in 6- and 4.4-week-old male mice, respectively. The results revealed that inoculation of 5×10^3 organisms from the 'non entering subpopulation' resulted in significantly lower parasitemias ($p < 0.01$ at day 7 p.i., $p < 0.005$ at day 14 p.i.; Student's t-test) and significantly longer survival times ($p < 0.005$; Student's t-test) than inoculation of the same number of trypanosomes from the 'original population'. Significant differences in the survival time ($p < 0.01$) were also noted after infection with 5×10^4 parasites of the 'non entering subpopulation' (fig. 1). Because of the above results, it was considered of interest to determine whether trypanosomes of the 'non entering subpopulation' would show different rates of multiplication and differentiation into epimastigote forms, as compared with organisms of the 'original population', when cultured in acellular medium at 28 °C. Thus, trypanosomes, collected from mice and exposed to normal mouse macrophages or incubated at 37 °C as described above, were seeded into triplicate tubes containing epimastigote medium[17] and cultured at 28 °C. At different periods of time, beginning 24 h after initiation of the cultures, an aliquot of each triplicate tube was removed and motile organisms with intact morphology were counted in a hemocytometer. Stained smears were also prepared to determine morphological changes. The results are shown in figure 2. A decrease in the number of organisms of the 'original population' but not of the 'non entering subpopulation' was noted 24 h after the initiation of the cultures. Transformation of the trypanosomes into round or oval forms was noted to occur after 12 h of culture. These forms became elongated and started to divide around 24 h. By 72–96 h most of the organisms had an epimastigote morphology. After 5 days of culture, organisms from both populations multiplied at the same rate. Multiplication, however, was not very active possibly because of an early depletion of essential nutrients from the culture or lack of a multiplication-enhancing factor in the culture medium employed. We have noticed that active differentiation and multiplication occurs when, at least, 10% of blood is added to the culture medium. In this case, however, a lot of clumping of multiplying organisms occur and the counting is made difficult.

Discussion. Our results revealed that, by using a cell culture system, bloodstream trypanosomes derived from a single cloned organism of the Y strain of *T. cruzi* could be separated into two subpopulations of parasites expressing different biological characteristics. Organisms from a small subpopulation were able to penetrate non phagocytic L929 cells and were interiorized by phagocytes, whereas trypanosomes from another relatively large subpopulation ('non entering' trypanosomes) did not penetrate L929 cells and apparently

avoided phagocytosis by normal murine macrophages. Organisms of this latter subpopulation remained in the supernatant of cultures of L929 cells or macrophages for prolonged periods of time and when inoculated into mice produced infections with parasitemias and survival times that were significantly lower and longer than those noted in mice inoculated with preparations containing trypanosomes of both subpopulations. This observation is in agreement with the report by Howells and Chiari[17] who showed that mice inoculated with trypanosomes recovered from the blood of other mice inoculated with the organisms two days before had longer prepatent parasitemias and increased survival times than controls. In addition, when trypanosomes of the 'non entering' population were added to acellular medium and cultured at 28 °C they immediately started multiplying and a slight increase in the number of organisms could be noted as early as 36 h of culture. In contrast, when a control preparation containing organisms of both populations was similarly cultured a decrease in the number of organisms was noted at 36 h of culture. The decrease in the number of organisms was not statistically significant but may have indicated death of those trypanosomes that were unable to differentiate into epimastigotes and multiply as such, possibly because they had been biologically programed to invade cells and maintain the infection in the mammalian host. So far, attempts to identify a particular subpopulation of parasites responsible for maintenance of the infection in mammalian hosts have not been successfull. The clonal approach has revealed the existence of differences among individual parasites in regard to invasiveness and pathogenicity[5]. Comparison of trypanosomes obtained from insect vectors, from bloodstream of mammalian hosts, from cell cultures or from axenic cultures have also demonstrated differences in invasiveness and in lethality[11, 17–19].

Long term maintenance of parasite strains in cultures or in laboratory mammalian hosts selects organisms with the highest ability to adapt to the culture conditions or to the experimental host but do not change the genetic make-up of individual organisms[20, 21]. Heterogeneity, however, has to be intrinsic to each organism and its expression genetically regulated since differences present in isolates kept in the laboratory for long periods of time were also demonstrated in parasites derived from a single clone.

At the moment, lectins are being used in our laboratory to examine the changes that occur on the cell membrane surface of trypanosomes following their retrieval from mammalian hosts and culturing until their final differentiation into epimastigotes.

1 This work was supported by a Research Training grant from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases to E. H. de Titto.
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Asymmetric suction feeding in primitive salamanders

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Summary. During suction feeding, the primitive aquatic salamanders *Cryptobranchus alleganiensis* and *Andrias japonicus* frequently display asymmetric movements of the lower jaw and hyoid apparatus that have some similarity to working and balancing side kinematics of mammalian mastication.
Key words. Salamander; feeding; asymmetry.

Previous detailed studies of suction feeding in vertebrates have demonstrated, or assumed, that the right and left visceral arch elements of the head (jaws, hyoid and branchial arches) move symmetrically[1–6]. Suction feeding usually entails very rapid movements of the feeding apparatus[7], even in salamanders[5, 6], and is characterized by marked overlap in the activity of antagonistic muscles[1, 5, 8, 9]. We were therefore surprised to discover asymmetries in jaw and hyoid movements during rapid suction feeding in the large, primitive aquatic salamanders *Cryptobranchus alleganiensis* and *Andrias japonicus*.

Cryptobranchus alleganiensis lives in unpolluted streams and rivers of the Appalachian and Ozark regions of the United States and feeds on a variety of aquatic invertebrates and small vertebrates[10, 11]. *Andrias japonicus* inhabits montane streams of the Japanese island of Hondo and exhibits food and habitat requirements similar to those of *Cryptobranchus*[12]. We here report the nature of asymmetric movements of the feeding apparatus in these two species, the structural basis of these movements in *Cryptobranchus*, and the potential relevance of our findings to ideas concerning the evolution of vertebrate feeding mechanics.