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# The Natural Heterogeneity of Trypanosoma cruzi: Biological and Medical Implications

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Trypanosoma cruzi is a heterogeneous group of parasites. The imposition of natural or artificial pressures can result in the selection of subsets of the population with concomitant changes in characteristics used to evaluate the group. In order to ascertain the extent of heterogeneity, stocks of single-cell clones were prepared from various sources. Selected cell biological, biochemical, immunochemical, parasitological, and histopathological parameters of these clones have been studied. A ten-fold difference in the rate of growth of the epimastigote stage of T cruzi clones has been observed. The extracellular growth rates of the clones correlate with the rate of growth of the obligate intracellular amastigote stage and consequently, the length of intracellular cycle of the parasite. A 40% difference in the amount of total DNA/parasite has been found between clones. Although the amount of DNA/kinetoplast and nucleus varies between clones, the major contribution to the differences in total DNA/parasite appears to be the nucleus. From 16 to 35 antigens have been demonstrated in the T cruzi clones assayed to date. Five to seven of these antigens are common to all of the stocks assayed. However, both isolate- and clone-specific antigens have also been demonstrated. The susceptibility of inbred strains of mice to T cruzi clones varies with the clone of the parasite. These data imply that the genetics of the parasite as well as the host modulate both the course and outcome of a T cruzi infection. The influence of monosaccharides on the receptor-mediated infection of vertebrate cells by trypomastigotes of T cruzi also varies between clones. The implications of these findings upon our concept and understanding of present and future problems in Chagas disease are discussed.

**Key words: Chagas disease, Trypanosoma cruzi, clones, characterization, biochemistry, cell biology**

In 1909, Carlos Chagas, a relatively unknown Brazilian scientist, described a zoonosis that occurred in the interior of Brazil [1]. Since that time it has become apparent that the zoonosis is widely distributed in South America, directly affecting some 20 million people with perhaps 200 million more people at risk to infection [2].

It is a tragedy that the brilliant work of Carlos Chagas has not been translated into English for two basic reasons: First, essentially all of the fundamental problems associated with attempts to understand Chagas disease were identified by Carlos

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Chagas. Second, most of the phenomena currently under study were identified by Carlos Chagas, albeit in a more primitive format. The "wheel" has been rediscovered in Chagas disease research many times.

One of the basic problems in Chagas disease is that it can present in a variety of forms from an acute fulminant infection to an inapparent chronic infection. In order to explain this diveristy, Lambrecht in 1965 [3] advanced the premise that the causative agent, Trypanosoma cruzi may be composed of a genetically heterogenous population of organisms and that this genetic heterogeneity may be *one* of the factors that modulates the disease process. A logical extension of this argument is that Chagas disease per se as a single entity does not exist. Chagas disease is a disease complex with certain common attributes. I shall attempt to defend and amplify this proposition.

With the objective in mind of testing the premise of parasite genetic heterogeneity, some years ago we began to develop stocks of single-cell isolates of T cruzi. I will summarize several studies of these clones, most particularly those bearing upon the extent of diversity of this species, the expression of this diversity in terms that may be detrimental to both the definitive host *and* the ego of the investigator attempting to understand the infectious and/or disease process, and finally, the implications of these data for future studies of the Chagas disease problem.

The classical microbiological definition of a strain, although used commonly with T cruzi, probably does not strictly apply. Hoare [4] concluded that in protozoa in which there is no refreshing of the genome through sexual process, each member of the population is, in fact, a clone. Therefore, the immediate first problem was to isolate these clones. There are two basic approaches that can be employed: micromanipulation and limiting dilution. Limiting dilution, the more popular of the two techniques, is a statistical method. It is assumed that the dispersion of organisms in a diluent can be represented as a Poisson distribution, which in fact may not be true. For example, clumping and/or settling of the organisms in the diluent would alter the distribution. However, as shown in Table I, if the organisms to be cloned are diluted to an average concentration of one organism/sample, the percent of samples with at least one organism/sample is 63.2%, whereas only 58.2% of these samples will have *exactly* one organism/sample. Increasing the dilution to an average of 0.05 organisms/sample improves but does not solve this problem. In addition, a new problem arises in that 95.1% of the samples will not contain any organisms. Although markedly more difficult and time consuming, the micromanipulation method [5] does not suffer from this problem and is, therefore, the method of choice for the production of T cruzi clones. All of the clones studied in my laboratory were produced by micromanipulator isolation.

Once isolation had been accomplished, the first step in biological characterization was a study of the kinetics of growth of epimastigotes in liquid medium. We established that individual T cruzi clones have stable growth rates and the range in doubling times was from about 22 h to over 200 h [6]. One obvious question arises. Is there any correlation between the rate of growth of epimastigotes in liquid culture and the rate of growth of intracellular amastigotes? Or are the differences observed due to the presence of nutritional mutants that are expressed when the epimastigotes are placed in what may be a suboptimal growth medium?

This question was answered elegantly by a quantitative study of the extra- and intracellular growth kinetics of a number of T cruzi clones [Engel et al, unpublished data]. The results are summarized in Table II. A positive correlation exists between

**TABLE I. Calculation of Probability Statistics for Limiting Dilution Technique**

| $\bar{X}$ Organisms/sample | Expected % of samples w/ | | Expected % of positive samples with exactly one organism |
|---|---|---|---|
| | No organisms | At least one organism | |
| 0.05 | 95.1 | 4.9 | 97.5 |
| 0.10 | 90.5 | 9.5 | 95.1 |
| 0.50 | 60.7 | 39.3 | 77.1 |
| 1.00 | 36.8 | 63.2 | 58.2 |

**TABLE II. Relation Between Growth of Epimastigotes and Intracellular Amastigotes**

| Clone | Doubling time (hr)[a] | | | |
|---|---|---|---|---|
| | | Cell culture | | |
| | Epimastigotes[b] | Amastigotes | Prerepro lag (hr) | Total cycle[c] (hr) |
| CA-I/72 | 31.8 ± 1.1[c] | 10.3 ± 0.3 | 22.2 ± 0.4 | 108 |
| CA-I/73 | 36.5 ± 1.6 | 12.0 ± 0.2 | 21.7 ± 0.5 | 120 |
| Miranda/78 | 46.6 ± 2.2 | 13.3 ± 0.3 | 26.3 ± 0.3 | 165 |
| Miranda/80 | 58.4 ± 1.5 | 21.5 ± 0.5 | 34.2 ± 0.8 | 215 |
| Sylvio-X10/5 | 22.5 ± 0.8 | 8.6 ± 0.1 | 18.2 ± 0.1 | 96 |

[a]Mean ± standard error.
[b]Grown in liver infusion tryptose (LIT) liquid medium.
[c]Elapsed time from infection of a vertebrate cell to release of mature trypomastigotes.

the intra- and extracellular growth rates of cloned stocks. Consequently, growth rate is an intrinsic parameter of a T cruzi clone. Subsequently, T cruzi clones have been isolated and identified that have an intracellular cycle that lasts for 4 1/2 days, and other clones that have an intracellular cycle that lasts for 3 mo [Dvorak, unpublished data].

Flow cytometry was employed as a tool to study the DNA synthetic cycle of the clones [7] in an attempt to understand the basis of the tremendous diversity of the organisms. The fluorochrome chosen to label the DNA is mithramycin, a large molecular weight antibiotic that binds selectively and stoichiometrically to guanine [8]. Examples of epimastigotes and trypomastigotes prepared for flow cytometry and stained with mithramycin are shown in Figure 1.

Marked interclonal differences in the amplitude of the $G_1$ peak were observed. An example of DNA histograms of two clones with the position of an appropriate standard is shown in Figure 2. The difference in the distance between the $G_1$ peak and standard amplitude is not due to instrumentation or protocol problems. It is an intrinsic property of the particular clone and represents a difference in the total DNA/organism between the clones. In addition, the position of the $G_1$ peak is independent of the culture density of the parasite population (Fig. 3). A scattergram showing the total DNA/organism in arbitrary units for 33 T cruzi clones is shown in Figure 4. Note that a difference in total DNA/organism in excess of 40% exists between the clones.

Is the difference in total DNA/organism between T cruzi clones due to differences in the amount of DNA in the nucleus or kinetoplast? As shown in Figure 1, the kinetoplast and nucleus of a trypomastigote are spacially well separated, whereas these organelles are not well separated in the epimastigote forms. In addition,

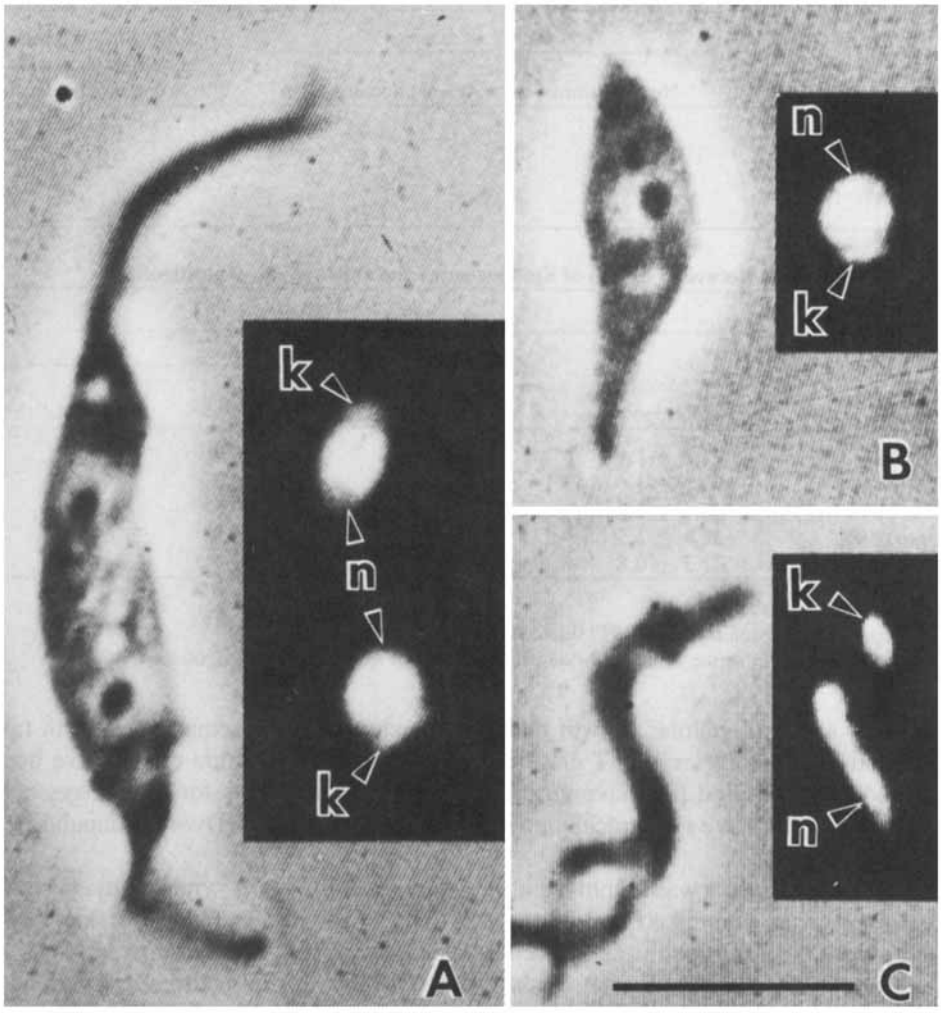


Fig. 1. Low-light-level video phase contrast and fluorescence photomicrographs of mithramycin-stained Trypanosoma cruzi. Inserts show the fluorescence image of the mithramycin-stained nucleus and kinetoplast of the organism shown in phase contrast. A) Dividing epimastigote. B) Single epimastigote. C) Trypomastigote. Note the close proximity of the nucleus (n) and kinetoplast (k) in the epimastigote and the spacial separation of these structures in the trypomastigote. Bar in C = 5 $\mu$m.

trypomastigotes are $G_0$ cells [9]. Therefore, the amount of DNA per nucleus or kinetoplast should be constant. This is not the case with epimastigotes, where the position of the organism in the DNA synthetic cycle is unknown, and consequently, the amount of DNA/organelle may vary from one organism to the next. Microspectrofluorometry was used to quantify the amount of DNA/nucleus and kinetoplast of the trypomastigote stage of several T cruzi clones. Representative examples of the results of these studies are shown in Figure 5. These data confirm the flow cytometry data and demonstrate that both the kinetoplast and nucleus vary between clones.

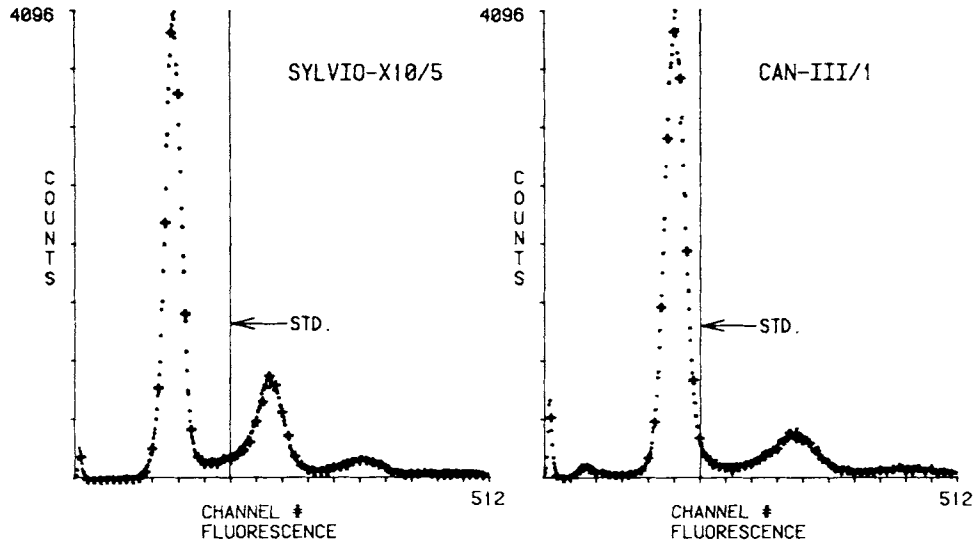


Fig. 2. DNA histograms obtained by flow cytometry of stationary-phase epimastigote cultures of T cruzi clones Sylvio-X10/5 and CAN III/1. The channel location of the singlets peak of a standard is depicted as a vertical line at the right of the $G_1$ peak. Note the difference in amplitude between the standard and the two $G_1$ peaks, which reflects a difference in total DNA/organism between the two clones.

However, the major contribution to the differences in DNA/organism is due to the nucleus.

One obvious criticism of these data concerns the use of mithramycin as the reagent to label the DNA. Are the observed differences really differences in total DNA/organism or are they simply a reflection of different guanine levels in the DNA molecule? In order to answer this question, the study was repeated using propidium iodide, an analogue of ethidium bromide that has a high quantum efficiency, to stain the DNA. The results were identical to those shown for mithramycin [Dvorak, unpublished data]. Consequently, it was concluded that the differences demonstrated are really due to differences in the total DNA/organism between the T cruzi clones.

The relative proportions of epimastigotes in the $G_1$, S, and $G_2$ phases of the DNA synthetic cycle during exponential growth were also determined by flow-cytometric analyses of organisms labeled with mithramycin. The results are shown in Figures 6–8. There is an inverse correlation between growth rate and the percentage of the parasite population in $G_1$ and a direct relationship between growth rate and the percentage of the parasite population in S or $G_2$. These data are what one would intuitively expect [10]. However, this is the first clear demonstration of the phenomenon, as there has never been described previously such a broad range in growth rates within a single species.

It seems reasonable to ask what are the practical implications of these data for the Chagas-disease problem. One implication in the area of chemotherapy comes to mind immediately. It has been demonstrated, most particularly through biochemical studies, that the epimastigote stage of T cruzi can be used in a screen for pharmacologically active compounds [11]. By knowing the % $G_1$, S, and $G_2$ organisms within

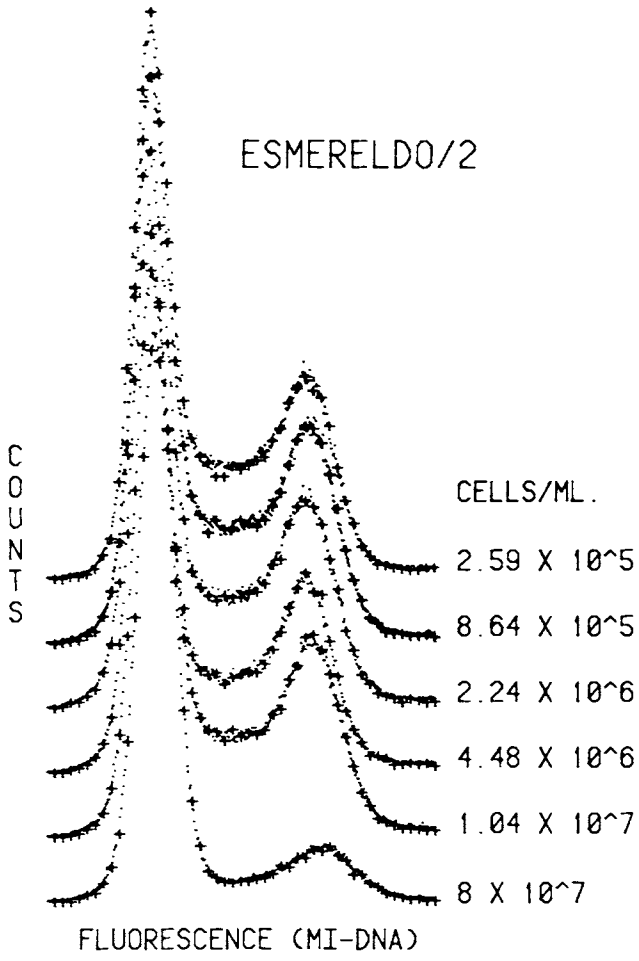


Fig. 3. DNA histograms obtained by flow cytometry of an epimastigote culture of T cruzi clone Esmereldo/2 sampled at various times during growth. Note that the amplitude of the $G_1$ peak remains constant.

a population and the rate of growth of the organisms, it is possible to calculate the transit time of the organisms through the various phases. An example of the results of these calculations for two clones with markedly different doubling times is shown in Figure 9. The S phase, in which DNA synthesis occurs, has been set off as a shaded sector. Assume that the compound being considered affects DNA synthesis. The Sylvio-X10/7 organisms are spending, disproportionately more of the DNA synthetic cycle time in S phase than the CAN-III/2 organisms and are cycling through S phase more often than the CAN-III/2 organisms within the same time period. These organisms are vulnerable to and will probably be killed by a drug that interferes with DNA synthesis. It is probable that the CAN-III/2 organisms will escape from the effects of the drug, as it may not be possible to attain or sustain a level of drug for sufficient time to kill all organisms as they pass through S phase. Therefore, it may

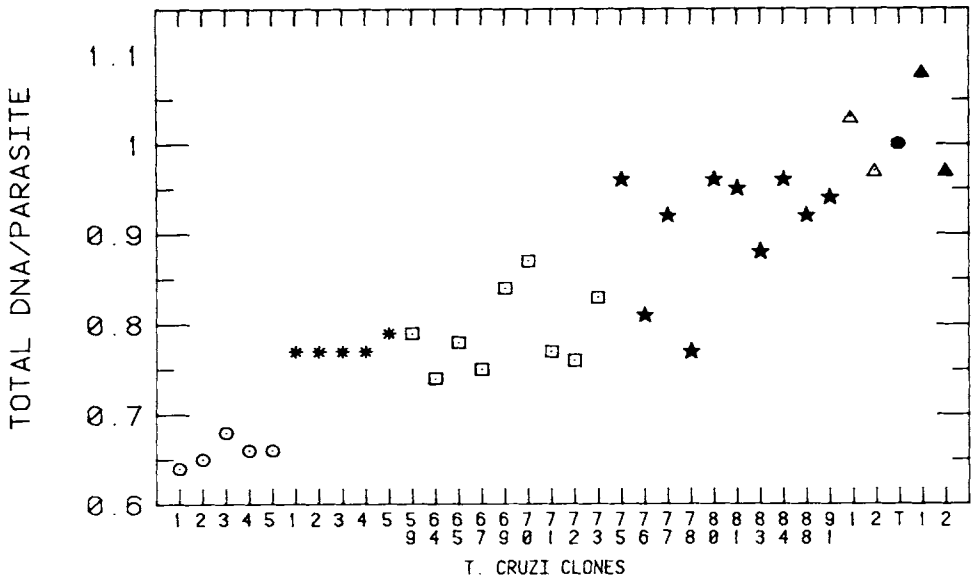


Fig. 4. Graphical representation of the total DNA/organism obtained by flow cytometry for 33 T cruzi-cloned stocks and one strain. Note that both intraisolate and intraclonal differences in total DNA/organism exist. ⊙(1–5) = Sylvio-X10, clones 4, 6, 7, 8, 9; * (1–5) = WA-250, clones 1, 2, 5, 10, 13; ⊡ = CA-I clones; ★ = Miranda clones; △(1–2) = Esmereldo, clones, 2, 3; ● = Tulahuen strain; ▲ = CAN-III, clones.

be worth considering the cell biological consequences of the diversity of T cruzi in chemotherapy studies.

I would like to mention briefly two other studies concerned with the heterogeneity of T cruzi clones to reemphasize the magnitude of the problem.

Vera Bongertz has demonstrated that *antigenic* heterogeneity exists between T cruzi clones [12]. Of the 16 to 35 antigens identified to date, both isolate- and clone-specific antigens have been demonstrated. Although we do not know the functional role of these antigens, it is possible that the presence or absence of some of these antigens may influence the course of the immune response both qualitatively and quantitatively. In addition, the development and utilization of monoclonal antibodies for diagnosis and therapy is becoming an increasingly more popular research area [13]. It is important in screening these monoclonal antibodies that a sufficiently large panel of known antigenically different clones be studied to justify any generalizations made for the monoclonal antibodies. This problem is already beginning to be acute in monoclonal antibody research in Chagas disease [14].

Miriam Postan has studied the course of infection of inbred strains of mice with T cruzi clones. In one of these studies [15], two clones isolated from an acute T cruzi infection in man produced two markedly different disease courses (acute fatal and chronic) in C3H/HeN mice. These data imply that the genetics of the parasite are as important as the genetics of the host in influencing the course of a T cruzi infection.

As mentioned previously, trypomastigotes are $G_0$ cells. Following the infection of a vertebrate cell, a trypomastigote undergoes a morphological transition to an amastigote, the reproductive intracellular stage of the parasite. During the course of

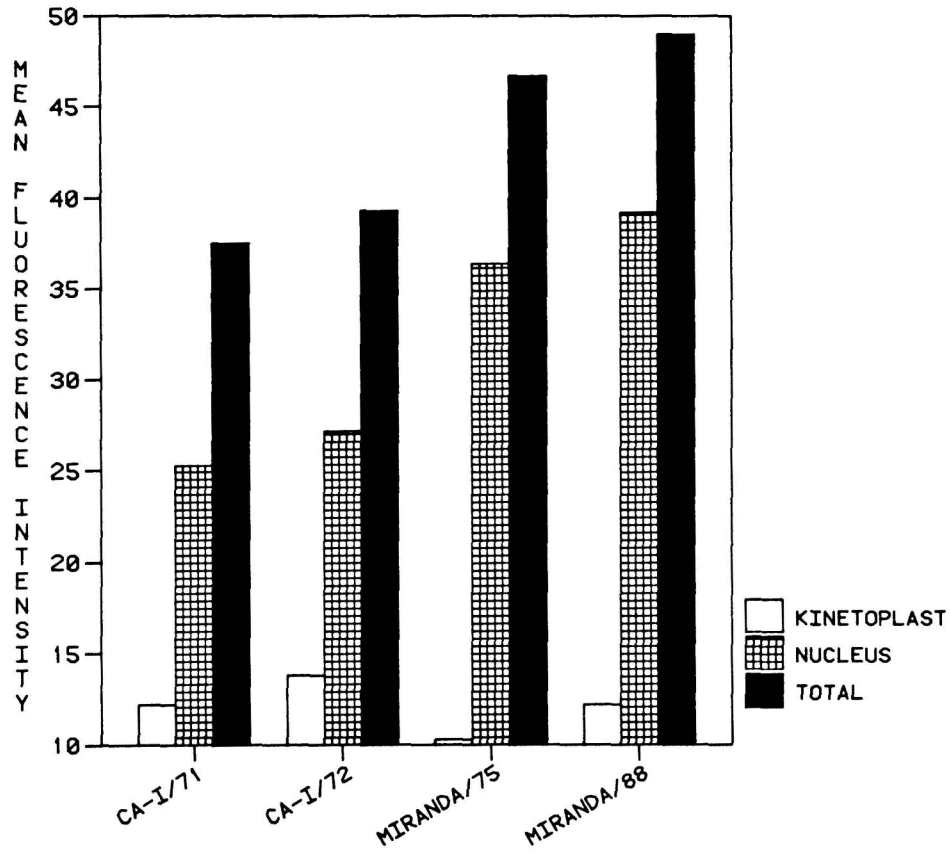


Fig. 5. Histogram of the mean fluorescence intensity obtained by microspectrofluorometry of the nucleus, kinetoplast, and total DNA/trypomastigote of four T cruzi clones stained with mithramycin.

autoradiographic studies designed to elucidate the onset and kinetics of DNA synthesis by T cruzi in vertebrate cells [9] a curious phenomenon was noted. A disproportionately large number of the infected host cell nuclei were labeled with $^3$H-thymidine, indicating that these host cells were undergoing DNA synthesis at the time of exposure to parasites. It has been amply demonstrated that the appearance and disappearance of many substances on vertebrate cell surfaces are cell-cycle dependent [16]. It was postulated that a substance recognized and utilized by the parasite as a receptor for infection of the vertebrate cells appeared and/or changed as the vertebrate cell passed through its growth cycle. HeLa cells were synchronized by mitotic shakeoff [17], exposed to trypomastigotes in the presence of $^3$H-thymidine, and assayed. The ability of trypomastigotes to infect HeLa cells increased as the HeLa cells proceeded from the $G_1$ phase to S phase and decreased as the HeLa cells entered $G_2$-M phase [18].

These data imply that a putative receptor for T cruzi exists on the vertebrate cell surface. With the thought that the receptor could be a glycoprotein, Mark Crane studied the influence on the infectious process of those monosaccharides found commonly in vertebrate cell surface glycoproteins [19]. The results of one of these studies is shown in Figure 10. The infection of vertebrate cells by Ernestina- or

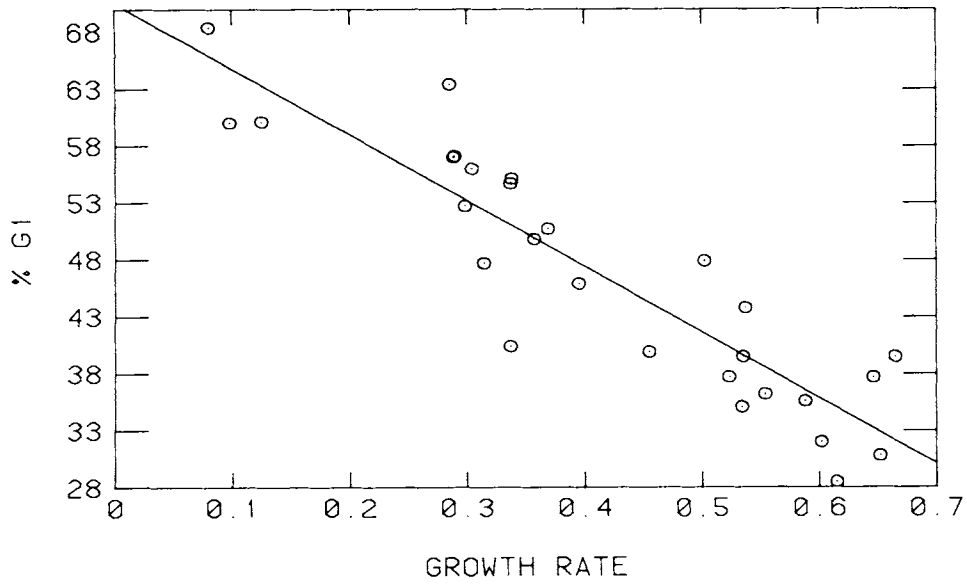


Fig. 6. Graphical representation of the inverse relationship between the exponential rate of growth of T cruzi clones and the percentage of $G_1$ phase organisms in the population.

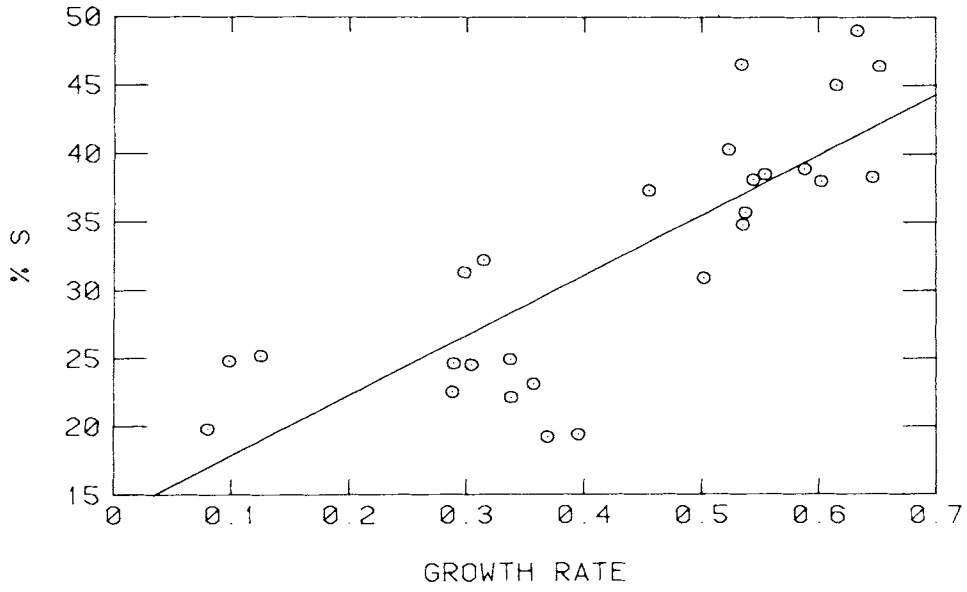


Fig. 7. Graphical representation of the direct relationship between the exponential rate of growth of T cruzi clones and the percentage of S phase organisms in the population.

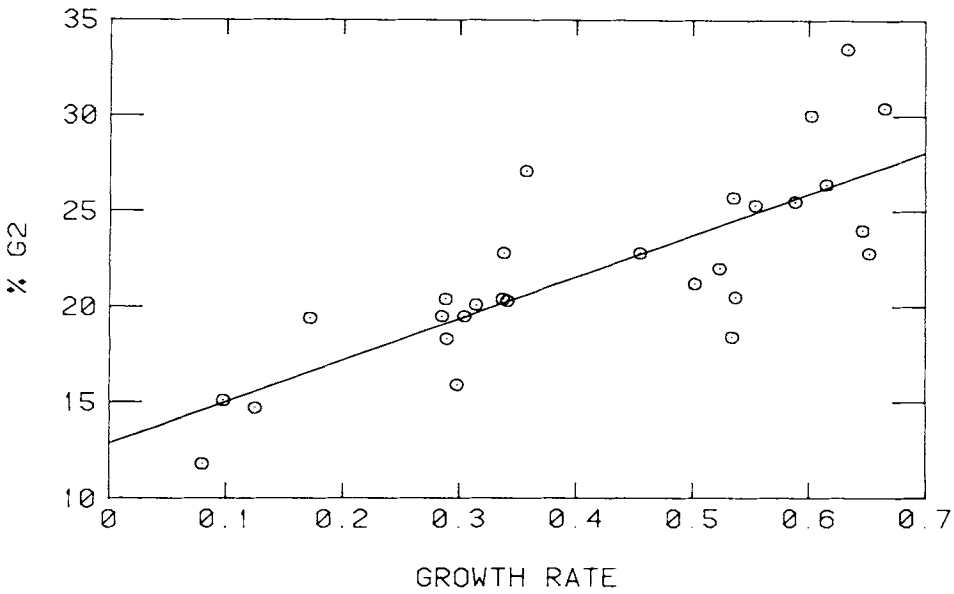


Fig. 8. Graphical representation of the direct relationship between the exponential rate of growth of T cruzi clones and the percentage of $G_2$ phase organisms in the population.

Y-strain trypomastigotes was severely inhibited by low concentrations of N-acetyl glucosamine. No significant effect was observed with other monosaccharides. Wash-out experiments (Fig. 11), in which vertebrate cells were preincubated with the monosaccharides, washed, and then combined with trypomastigotes, indicated that the penetration indices returned to control values. However, after preincubation of trypomastigotes with each monosaccharide, except for N-acetyl glucosamine, the penetration index returned to control values. These data imply that T cruzi trypomastigotes attach to N-acetyl glucosamine-containing receptors on the vertebrate cell surface. The inhibition observed upon preincubation of trypomastigotes with N-acetyl glucosamine implies that these parasites have a wheat germ agglutinlike lectin on their surface that interacts with the N-acetyl glucosamine moiety on the vertebrate cell surface.

During the course of this work, the infection of vertebrate cells by T cruzi clones was being quantified [20]. The results of some of these studies are shown in Figure 12. Both interclone and interisolate differences were observed. The CA-I/71 clone is the most infectious clone thus far encountered. In the belief that this phenomenon may be due to quantitative differences in the wheat germ agglutinlike receptor on this parasite—and, therefore, the CA-I/71 parasite would be a good candidate for receptor isolation and characterization studies—the N-acetyl glucosamine experiments were repeated using this clone. The results are shown in Figure 13. In contrast to what was observed with the Ernestina and Y strains, N-acetyl glucosamine did not inhibit the infection of vertebrate cells by clone CA-I/71 trypomastigotes. In addition, as shown in Figure 14, *none* of the monosaccharides found as common components of vertebrate cell surface glycoproteins have any effect on the infection of vertebrate cells by this parasite. Consequently, it appears that clone-dependent differences in the receptors recognized by the parasite in the infectious process also

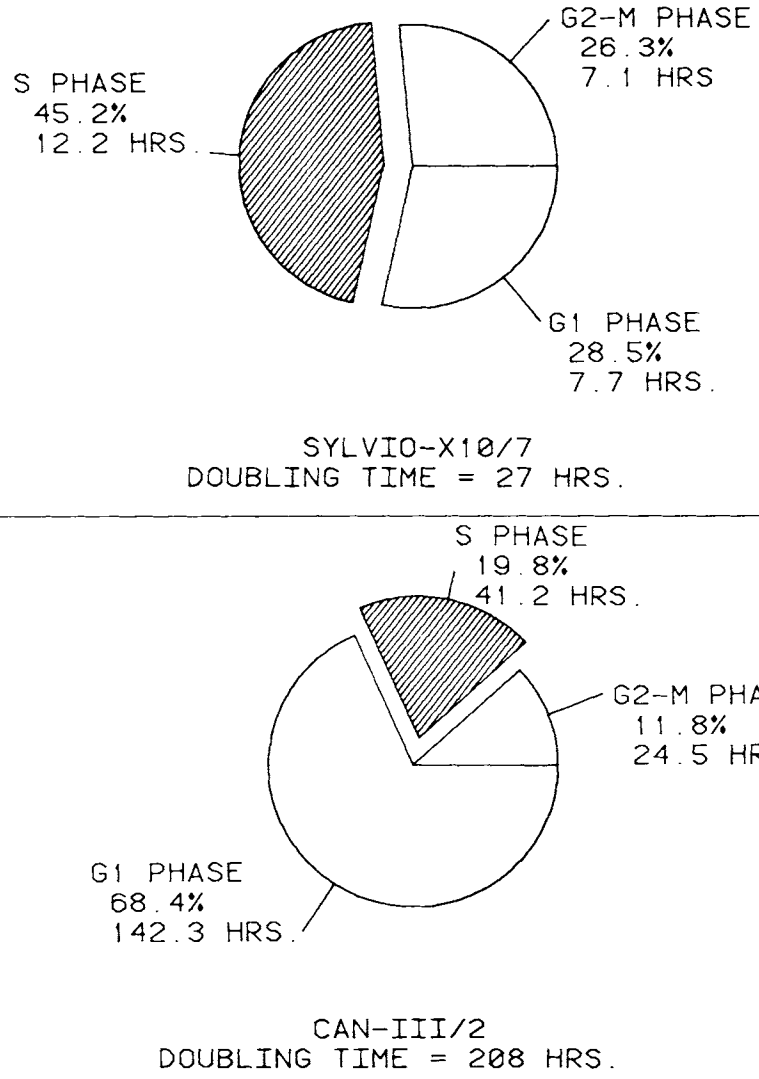


Fig. 9. Graphical representation of the $G_1$, S, and $G_2$ transit times of two clones of T cruzi having markedly different growth rates.

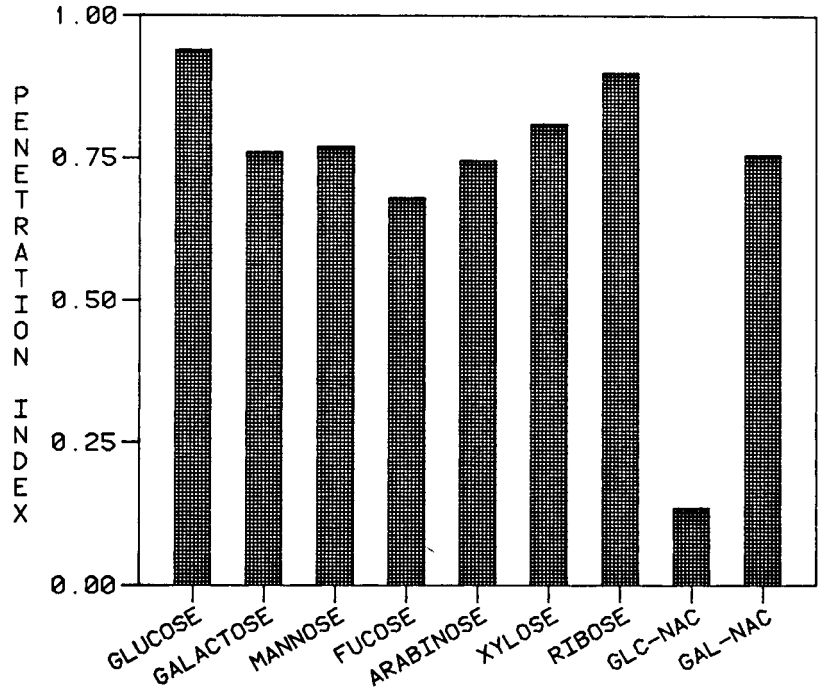


Fig. 10. Histogram showing the effect of 20 mM monosaccharide on the infection of bovine embryo skeletal muscle cells by Ernestina strain T cruzi trypomastigotes. Penetration index = E/C where E = $\overline{X}$ parasites/cell (experimental) and C = $\overline{X}$ parasites/cell (control).

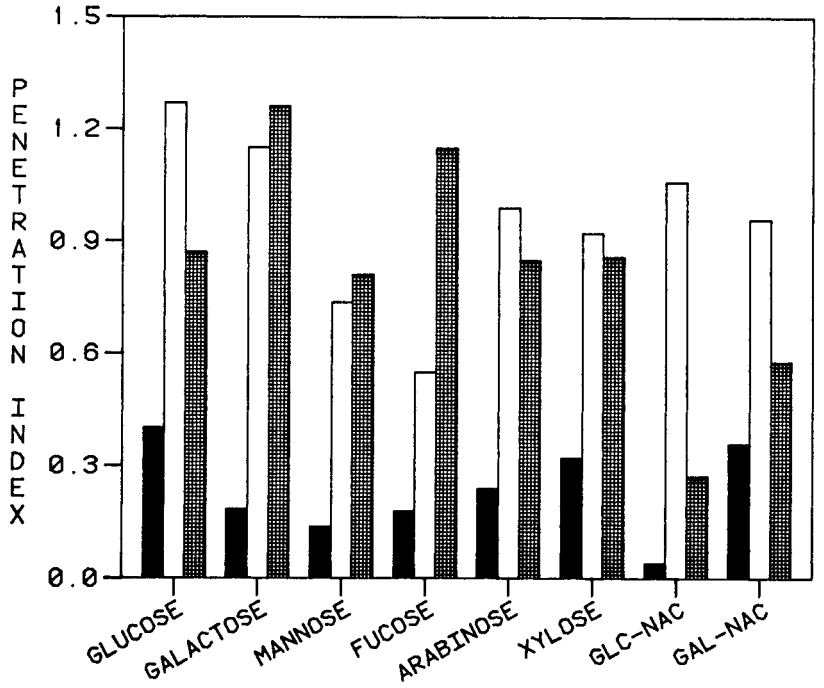


Fig. 11

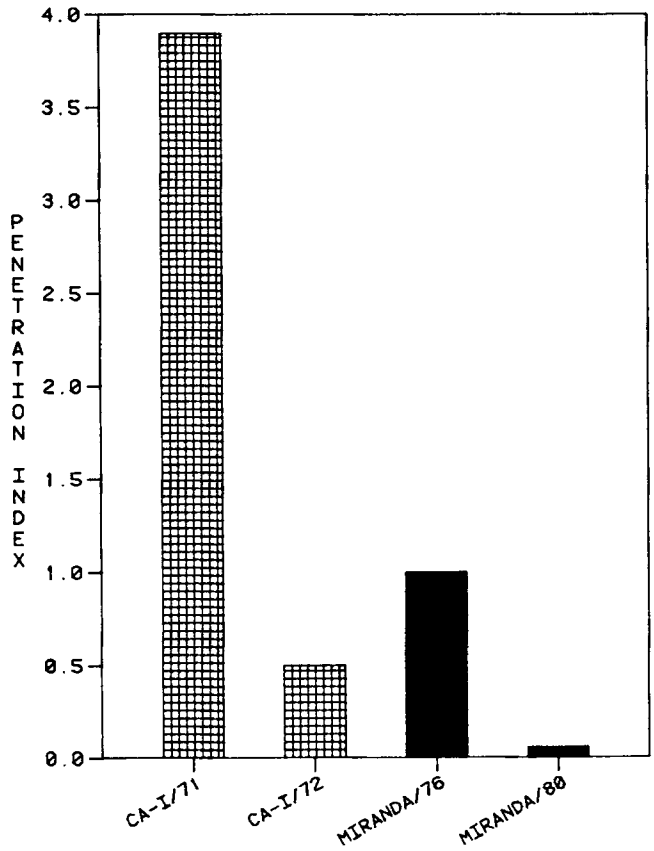


Fig. 12. Histogram showing intraisolate and interclonal differences in the ability of T cruzi trypomastigotes to infect bovine embryo skeletal muscle cells. The PI was calculated as shown in the legend for Figure 10.

exist. Intraspecific diversity at such a fundamental level has profound implications on receptor-modulated interference of infection as a means to control Chagas disease.

This concludes a very brief summary of some of the studies that have been carried out in my laboratory using T cruzi clones. A fundamental question remains. How and why did this tremendous heterogeneity arise in T cruzi? Is this group of organisms evolving toward increased or decreased heterogeneity, *or* is the group relatively stable?

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Fig. 11. Histogram showing the effect of 50 mM monosaccharide on the infection of bovine embryo skeletal muscle (BESM) cells by Ernestina strain T cruzi trypomastigotes. Filled bar represents the Penetration index (PI) when BESM cells were exposed to parasites in the presence of the monosaccharide; open bar represents the PI when BESM cells were preincubated with the monosaccharide, washed and exposed to parasites; cross-hatched bar represents the PI when parasites were preincubated with the monosaccharide, washed, and used to infect BESM cells. The PI was calculated as shown in the legend for Figure 10.

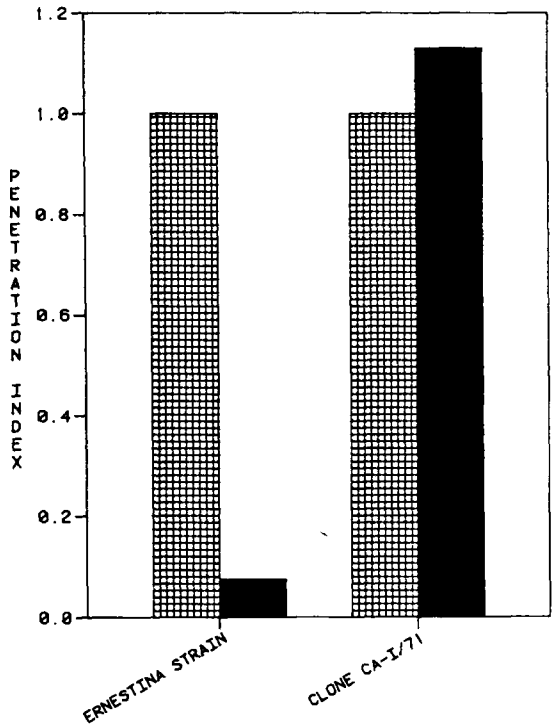


Fig. 13. Histogram showing the difference in response of the Ernestina strain and CA-I/71 clone of T cruzi to N-acetyl glucosamine-modulation of the infection of bovine embryo skeletal muscle cells by trypomastigotes. The PI was calculated as shown in the legend for Figure 10.

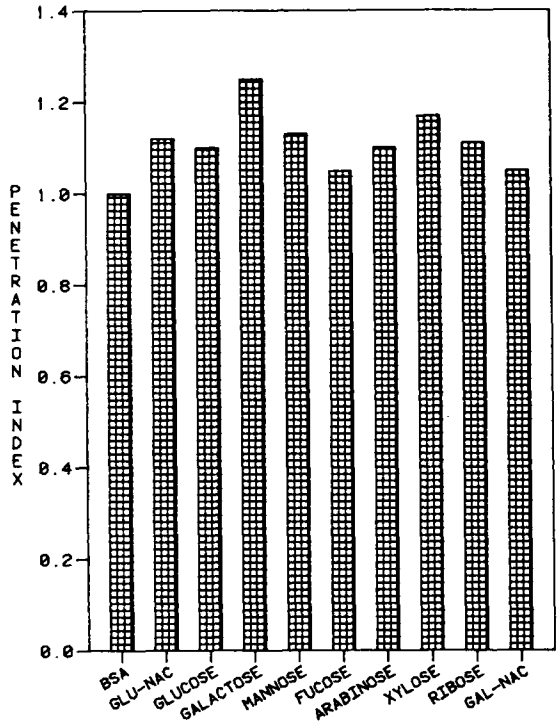


Fig. 14. Histogram showing the lack of influence of nine monosaccharides on the ability of CA-I/71 trypomastigotes to infect bovine embryo skeletal muscle cells. The PI was calculated as shown in the legend for Figure 10.

Although it is obviously an overly simplistic view to ascribe all observed phenomena to be the consequence of parasite heterogeneity, one can develop testable models using T cruzi clones that may help to elucidate the importance of this diversity in Chagas disease.

## ACKNOWLEDGMENTS

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