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Establishment of clones of *Trypanosoma cruzi* and their characterization *in vitro* and *in vivo**

S. CHIA-TUNG PAN[1]

An efficient technique for isolating clones of Trypanosoma cruzi *from cultures and from animals has been developed. It is based on the inoculation of one organism, obtained by serial dilutions of cultured epimastigotes or isolated blood stream trypomastigotes, into enriched NNN medium (NNN-F:93). The cloning efficiency (percentage of positive cultures over the number inoculated) was 70% for cultured epimastigotes and 30 – 40% for blood-stream trypomastigotes.* In vitro *cultural characteristics of 14 secondary clones of an avirulent strain indicated that 12 clones grew in the F-94 medium primarily as epimastigotes at 27 °C and exclusively as amastigotes at 37 °C; 2 clones grew in F-94 medium primarily as amastigotes regardless of incubation temperature (27 °C or 37 °C).* In vivo *characterization of 7 clones from 2 virulent strains indicated that the virulence of individual clones was low immediately after isolation in NNN-F:93 medium, but the virulence of some clones returned to the level of the parent strain after more than 8 serial passages in CD-1 mice.*

Chagas' disease is caused by *Trypanosoma cruzi* and occurs in the zone that extends from the southern United States of America to northern Argentina and Chile. It has been estimated that 35 – 50 million people are at risk of infection and that 20% or more may be infected (*12*, *17*, *28*).

Trypanosoma cruzi, a stercoralian trypanosome (*7*), has one of the most complex life cycles among the trypanosomes found in man (*2*, *14*, *20 – 22*). Although trypomastigotes circulate in the blood of the vertebrate host, the parasite divides intracellularly in the amastigote stage, and the attendant process may damage such vital organs as the heart and brain. Previous studies (*6*, *15*, *16*) indicate that *T. cruzi* may be separated into at least three major antigenic groups. These antigenic types may be mixed in isolates from vertebrates, and such a situation makes studies on antigenic variation *in vivo* during a natural course of infection extremely difficult. These difficulties can be eliminated or minimized by using clones of *T. cruzi*. At the same time genetic variability among or within strains may be eliminated or reduced. Cloned *T. cruzi* have not been used until recently in most experimental studies (*11*, *24*), although cloned strains of *T. brucei* are being used extensively in studies on the antigenic variation of that subgroup (*1*, *9*, *25 – 27*). Van Meirvenne et al. (*25*) employed combined techniques of dilution and microscopy to establish clones of *T. brucei*. Samuels (*23*) was able to obtain clones of *Trichomonas* spp. by inoculating nutrient agar plates with a diluted suspension of these organisms and growing discrete colonies from individual parasites. Recently Dwyer (*3*) established clones of *Leishmania donovani* by micromanipulation, using fine capillary pipettes and diluted cultures. Gillin & Diamond (*5*) also successfully cloned several species of *Entamoeba* in agar media. In this study, we have succeeded in establishing clones of *T. cruzi* maintained *in vitro* and *in vivo* and have determined the *in vitro* and *in vivo* characteristics of the cloned strains. The technique developed in this study could easily be adopted by other investigators for obtaining clones of *T. cruzi* for use in genetic and immunological studies on Chagas' disease as well as for zymodeme[a] determinations on newly isolated strains of *T. cruzi*. The technique may also be useful for experimental infection using a single parasite.

MATERIALS AND METHODS

Media

F:93 medium. This medium is a slight modification of F:29 (*19*) and has an osmotic pressure of 401 mOsmol. The composition of F:93 is given in Table 1. The medium was prepared without the

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[1] Professor, Tropical Public Health, Harvard School of Public Health, 655 Huntington Avenue, Boston, MA 02115, USA.

[a] Zymodemes = trypanosome populations that possess like forms of specified enzymes (T. V. BARRETT ET AL. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 74: 84 – 90 (1980).

Table 1. Composition of F:93 medium

Distilled water	58.4 ml
Glucose (100 g/litre)	2.0
Medium 199 (as 10-fold concentrate)	10.0
Trypticase (50 g/litre)	10.0
Hemin (0.5 g/litre of 0.01 mol/litre NaOH)	5.0
Penicillin G (10^5 IU/ml) and streptomycin (10^5 μg/ml)	0.1
Bovine fetal serum (inactivated at 56°C for 30 min)	10.0
$NaHCO_3$ (42 g/litre)	1.5
L-glutamine (200 mmol/litre)	3.0

sodium hydrogen carbonate ($NaHCO_3$) solution and stored at −20 °C. $NaHCO_3$ was added just before use.

F:94 medium. This is a modification of F:69 medium (*20*) containing 10 mmol of HEPES salt and 150 ml of inactivated bovine fetal serum per litre (Table 2). The osmotic pressure of F:94 is 366 mOsmol. This medium was used to determine the *in vitro* cultural characteristics of isolated cloned strains. The medium was prepared without the $NaHCO_3$ and nucleotide solutions and stored at −20 °C. These two components were added just before use. Full details of the method of preparation have been given previously (*20*).

Cloning of a strain of low pathogenicity

The Brazil strain of *T. cruzi* (*18–20*) has been maintained in a modified NNN medium (Bactoagar, 7.0 g; NaCl, 3.0 g; trypticase, 2.5 g; H_2O, 450 ml; heparinized normal rabbit blood, 50 ml) continuously since 1960, when it was isolated from an infected rat. This strain is still infective to CD-1 mice,[b] giving a low parasitaemia which can be detected only by cultivation in NNN medium (*20*). The strain, however, grows well in F:29 or F:69 medium (*19*, *20*) at 27 °C, primarily as epimastigotes, forming few rosettes.

In this study, the Brazil strain was first cultured in F:93 medium (see above) at 27 °C for several passages at intervals of 4–6 days. When the growth of organisms reached a stationary phase in the final passage, a sample of the culture (1 ml) was diluted with 4 ml of a solution of 100 ml of formalin per litre of phosphate-buffered saline (PBS) at pH 7.1 and counted in a haemocytometer at 400× magnification. After computation of the number of organisms per ml of medium, a sample of the culture was appropriately

[b] Charles River Breeding Laboratory, Wilmington, MA, USA.

Table 2. Composition of F:94 medium

Distilled water	36.9 ml
Glucose (100 g/litre)	2.0
Medium 199 (as 10-fold concentrate)	10.0
Trypticase (50 g/litre)	10.0
Sodium pyruvate (0.1 mol/litre)	3.0
Vitamin mixture (see below)	2.0
Biotin (20 mg/litre)	2.0
Folic acid (100 mg/litre)	5.0
Vitamin B_{12} (100 mg/litre)	2.0
Methyl cellulose (40 g/litre)	5.0
L-glutamine (200 mmol/litre)	3.0
Hemin (500 mg/litre of 0.01 mol/litre NaOH)	5.0
HEPES (1.0 mol/litre in an NaCl solution of 8.5 g/litre)	1.0
ATP, ADP, AMP solution (see below)	10.0
$NaHCO_3$ (42 g/litre)	3.0
Penicillin G (10^5 IU/ml) and streptomycin (10^5 μg/ml)	0.1
Bovine fetal serum (inactivated at 56°C for 30 min)	17.6

Composition of the vitamin mixture

4-Aminobenzoic acid	30 mg
D-calcium pantothenate	40
Choline chloride	30
Isoinositol	30
Nicotinamide	50
Nicotinic acid	20
Pyridoxal hydrochloride	20
Pyridoxine hydrochloride	20
Pyridoxamine 2-hydrochloride	20
Riboflavin 5′-phosphate sodium ($2H_2O$)	4
Thiamine hydrochloride	20
Distilled water	100 ml

Sterilized by filtration with Millipore filter (pore size, 0.45 μm)

Composition of ATP, ADP, and AMP solution

ATP (adenosine 5′-triphosphate)	200 mg
ADP (adenosine 5′-diphosphate)	100
AMP (adenosine 5′-monophosphate)	100
Glutathione (reduced)	20
L-cysteine hydrochloride	20
Ascorbic acid	20
Distilled water	100 ml

Sterilized by filtration with Millipore filter (pore size, 0.45 μm)

diluted serially with PBS containing 4 g of glucose per litre (PBS-G) to a concentration of 10 organisms per millilitre of PBS-G. Finally, by diluting 1 ml of this suspension (containing 10 organisms) with 9 ml of F:93 medium, a suspension of one organism per millilitre of F:93 was obtained. Aliquots of 1 ml of this suspension were inoculated into each of ten freshly prepared NNN tubes (hereafter designated NNN-F:93). The NNN-F:93 tubes were incubated at 27 ± 1 °C and examined microscopically once a week for six weeks. The clones of *T. cruzi* thus obtained were designated primary clones. To ensure the purity of the clones, secondary cloning was carried out using primary clones as the source of the material. These were designated secondary clones.

Cloning of pathogenic strains using bloodstream trypomastigotes as source material

Two pathogenic strains of *T. cruzi*, i.e., Col-A and H510 (*4*, *8*, *22*), have been maintained in CD-1 white mice for over six years in this laboratory. These parasite strains were highly pathogenic for CD-1 mice, producing a parasitaemia of over 10^7 trypomastigotes per millilitre of blood 3 – 4 weeks after intra-abdominal inoculation of 10^5 trypomastigotes. Usually, all mice died 3 – 6 weeks after inoculation. Although fresh isolates of these strains grew well in either F:93 or F:94 medium (see above), many rosettes of epimastigotes and clumps of amastigotes were usually present in these cultures. Thus, accurate counts and the subsequent dilutions of the suspension to obtain one organism per millilitre could not be done with confidence. Therefore, bloodstream trypomastigotes were used to establish clones in NNN medium and in CD-1 mice with aseptic procedures as follows. Several mice were inoculated with about 2×10^5 bloodstream trypomastigotes from a previous passage. They were sacrificed 2½ weeks post-infection by fracture of the cervical cord, and the cardiac blood was pooled. Heparin solution in PBS was used as anticoagulant at a final concentration of 10 IU/ml of blood. The heparinized blood was diluted with an equal volume of F:93 medium and centrifuged at 400 *g* for 40 min at 25 °C. After centrifugation, the blood was kept, without disturbance, at 37 °C for 60 min to let most of the trypomastigotes move into the diluted plasma. The plasma was then transferred to a sterile, conical, centrifuge tube and centrifuged at 750 *g* for 20 min at room temperature. After centrifugation, most of the supernatant was discarded leaving about 3.5 ml at the bottom.

After the trypomastigotes had been resuspended in the plasma with a sterile capillary pipette, 1 ml of the suspension was diluted with an equal volume of 10% formalin in PBS, and the trypanosomes were counted in a haemocytometer. After computation, 1 ml of the remaining suspension was used to obtain one organism per millilitre of F:93 medium by performing serial dilutions as described above. Ten tubes of NNN medium were each inoculated with 1 ml of trypanosome suspension in F:93 (containing one organism) and incubated at 27 ± 1 °C. Weekly microscopical examinations were made for 6 weeks. Also, 10 white mice (CD-1, 3-week-old, male) were each inoculated with 1 ml of F:93 containing 1 trypomastigote. The tail blood of each animal was examined microscopically once a week for 5 weeks, starting 3 weeks after inoculation. The mice were eventually sacrificed and 0.4 ml of the heparinized cardiac blood was inoculated into each of three NNN-F:93 cultures. Secondary cloning was carried out as above from mice inoculated with primary clones.

Procedures for characterization of clones

Isolated clones were normally maintained in NNN medium overlayed with 1 ml of F:93 plus 1 ml of phosphate-buffered saline (pH 7.2) containing 100 IU of penicillin G and 100 μg of streptomycin per millilitre. Cultures were regularly transferred at 3-week intervals. Characterization of clones was carried out when clones were in the second passage in the overlayered NNN cultures described above.

In vitro *cultural characteristics.* Large numbers of organisms (*c.* 2×10^7) in 1 ml of fluid from 1-week-old NNN-F:93 cultures were first inoculated into each of two tubes (16 × 125-mm, screw-cap) of F:94 medium (5 ml/tube) and incubated at 27 °C for 4 – 6 days. At the end of incubation, 1 ml of culture from each tube was inoculated into each of two fresh tubes of F:94. The remaining culture was used to make two smears which were stained with Wright-Giemsa as described previously (*20*). Five hundred organisms were counted (at 1000×) on each of two smears per passage and classified as to morphological type (*19*, *20*). Clones were serially cultured as described above for at least 6 passages before experiments were discontinued. Occasional counts of organisms indicated that most clones grew to a density of $2 - 5 \times 10^7$ organisms per millilitre of medium at the end of exponential growth.

In vivo *cultural characteristics.* Only the secondary clones of the pathogenic strains (Col-A and H510) were characterized in CD-1 white mice as described below. Clones in the second passage in NNN-F:93 medium were used to prepare inocula for mice (CD-1, 3 weeks old, male or female). Usually one tube (16 × 125-mm, screw-cap) containing 5 ml of F:94 was inoculated with 1 ml of fluid from a one-week-

old NNN-F:93 culture and incubated for 5 – 8 days at 27 °C. At the end of the incubation period, cultures in F:94 were used as inoculum to infect five mice, intraperitoneally, for each clone. Each clone in mice was thereafter passaged at 3 – 4 week intervals by inoculating intra-abdominally 0.4 ml of diluted blood (3 × dilution with a heparin solution containing 75 IU of heparin per ml of PBS) from a previous passage. Parasitaemia was determined semiquantitatively by covering a drop (*c.* 0.02 ml) of diluted blood (3 ×) with a 22 × 22-mm coverslip and counting 10 microscope fields (high-dry objective, 400 ×). The density of trypomastigotes was expressed as numbers of organisms in undiluted blood per microscope field (at 400 ×).

RESULTS

Cloning of the strain of low pathogenicity (Brazil)

Primary cloning and characterization in vitro. Two experiments were carried out three weeks apart with almost identical results. In both experiments 7 out of 10 inoculated NNN-F:93 cultures became positive microscopically 2 – 4 weeks after inoculation. At the time when organisms were first microscopically detected, 6 clones were growing entirely as amastigotes, 4 clones entirely as epimastigotes, and 4 clones had both amastigotes and epimastigotes in the cultures. All except clone C22 could be subcultured indefinitely in NNN-F:93 medium.

Characterization of three selected clones indicated that two of them (C15, C18) grew in F-94 medium at 27 °C primarily as epimastigotes (over 96%), and at 37 °C as amastigotes exclusively, when subcultured at 4 – 7 day intervals (for detailed technique, see Pan (*20*). One clone (C13) grew exclusively as amastigotes (over 99%) regardless of incubation temperature (27 °C or 37 °C) after first passage in F:94 medium, although epimastigotes were present in NNN-F:93 culture.

Secondary cloning and characterization in vitro. Two experiments were carried out concurrently using two primary clones (C15 and C18). The results of these experiments were again almost identical and were comparable to those of primary clonings. In both experiments 7 out of 10 inoculated NNN-F:93 cultures became positive by the end of 3 weeks after inoculation, with one culture displaying microscopically detectable organisms as early as the end of the first week.

At the time when organisms were first microscopically detected, 5 secondary clones were growing exclusively as amastigotes, 1 exclusively as epimastigotes, and 8 as mixed amastigotes and epimastigotes.

Characterization of all 14 clones *in vitro* in F:94 medium was carried out for five serial passages. The results are summarized in Table 3. Two of the 14 secondary clones grew in F:94 primarily as amastigotes regardless of incubation temperature (27 °C or 37 °C). The other 12 secondary clones grew primarily as epimastigotes at 27 °C and as amastigotes at 37 °C.

Cloning of pathogenic strains (H510 and Col-A)

Primary cloning. One experiment was carried out for each of the two pathogenic strains of *T. cruzi*. For each strain 3 clones were isolated from the 10 inoculated NNN-F:93 cultures after two weeks of incubation. At the time when organisms were first microscopically detectable, all 3 clones of H510 strain were growing both as amastigotes and epimastigotes, while all 3 clones of Col-A strain were growing exclusively as amastigotes. None of the 10 mice inoculated with either strain was infected. Therefore, mouse inoculation was omitted from experiments for secondary cloning.

Secondary cloning and characterization of isolated clones in vitro *and* in vivo. One experiment was carried out with each of the two pathogenic strains, employing primary clones C8 of H510 and C6 of Col-A as cloning material. Three clones were isolated from 10 NNN-F:93 cultures inoculated with the H510 strain. All three cultures became positive by the end of the second week after inoculation, and all clones displayed both amastigotes and epimastigotes at the time when organisms became microscopically detectable. In the experiment with Col-A strain, 4 clones were obtained from 10 inoculated NNN-F:93 cultures, and at the time when organisms were microscopically detectable, 2 clones displayed only amastigotes in the culture, and 2 displayed both amastigotes and epimastigotes.

The results of characterization *in vitro* in F:94 medium are summarized in Table 4. In the first passage, all except secondary clone C6C9 (Col-A strain) displayed relatively abundant trypomastigotes and/or epimastigotes which disappeared by the eighth passage, when amastigotes predominated in all clones.

All 7 secondary clones were serially passed through CD-1 mice more than eight times at 3 – 4 week intervals. For each passage, observations were made routinely for as long as 10 weeks, after which any survivors were sacrificed to obtain antisera. In experiments with the Col-A strain, 2 secondary clones (C6C1 and C6C4) did not produce any mortality in mice during the observation period for eight passages; the parasitaemia in mice was low, ranging from 3 to 9

Table 3. Relative frequency of the 3 structural types of *Trypanosoma cruzi* in the first and fifth passages in F:94 medium at 27°C among 14 secondary clones of the Brazil strain

Clone	First passage			Fifth passage		
	Trypomastigote (%)	Epimastigote (%)	Amastigote (%)	Trypomastigote (%)	Epimastigote (%)	Amastigote (%)
C15C1	0.6	99.0	0.4	1.8	98.2	0.0
C15C2	2.9	96.6	0.5	3.5	96.3	0.2
C15C3	1.0	96.2	2.8	2.3	97.7	0.0
C15C4	0.7	96.7	2.6	0.5	99.5	0.0
C15C5	1.0	95.4	3.6	0.2	99.8	0.0
C15C6	0.0	0.0	100.0	0.0	0.0	100.0
C15C8	0.0	99.5	0.5	0.0	100.0	0.0
C18C1	4.8	94.9	0.3	0.0	100.0	0.0
C18C2	5.7	16.1	78.2	0.0	0.2	99.8
C18C3	2.1	94.3	3.6	0.2	99.8	0.0
C18C4	2.0	96.7	1.3	0.2	99.8	0.0
C18C6	5.1	23.1	71.8	6.2	93.6	0.2
C18C9	7.8	86.5	5.7	4.3	92.1	3.6
C18C10	1.9	94.3	3.8	0.4	99.6	0.0

Table 4. Relative frequency of the 3 structural types of *Trypanosoma cruzi* in the first and eighth passages in F:94 medium at 27°C among the secondary clones of H510 and Col-A strains

Strain	Clone	First passage			Eighth passage		
		Trypomastigote (%)	Epimastigote (%)	Amastigote (%)	Trypomastigote (%)	Epimastigote (%)	Amastigote (%)
H510	C8C3	28.0	9.2	62.8	0.0	0.9	99.1
	C8C4	4.8	12.2	83.0	0.0	0.5	99.5
	C8C5	9.2	21.2	69.6	0.0	4.6	95.4
Col-A	C6C1	7.2	21.6	71.2	0.0	0.0	100.0
	C6C4	8.0	29.0	63.0	0.0	0.0	100.0
	C6C5	3.0	20.0	77.0	0.0	0.0	100.0
	C6C9	3.0	1.0	96.0	0.0	0.0	100.0

per microscope field (0.4 mm in diameter). The clones C6C5 and C6C9 also did not produce mortality in mice for 6 passages and the parasitaemia was low, ranging from 3 to 5 per microscope field. However, the parasitaemia increased to 14 – 38 per microscope field and nearly 40% of mice died in the eighth passage; discernible oedema was present around the hind quarters in most animals.

During the first three passages, the 3 clones of H510 strain (C8C3, C8C4, C8C5) did not produce mortality or marked signs of disease in CD-1 mice, but parasitaemia was moderate in all 3 clones by the third passage, ranging from 9 to 39 per microscope field. Heavy parasitaemia and severe signs of disease (general oedema) began to appear by the fifth passage. In the eighth passage in CD-1 mice, over 90% of the animals died in 5 weeks and parasitaemia became heavy, ranging from 140 to 280 per microscope field. The heavy parasitaemia observed in the clones of the H510 strain may have been due, in part, to the presence of numerous amastigote nests in skeletal muscles as observed in histological sections, whereas in the Col-A strain amastigote nests were essentially confined to the heart.

DISCUSSION

Although there are many advantages in using cloned strains of *T. cruzi* for research, they have in fact been used only recently (*11*, *24*). There is little doubt that genetic studies on *T. cruzi* and other trypanosomes will require cloned strains, as they have in other microorganisms (*5*, *11*, *23*).

The efficiency of our cloning technique as reported here was 70% (7 clones from 10 NNN-F:93 tubes inoculated) for a culture-adapted (Brazil) strain and 30–40% for pathogenic mouse-adapted (Col-A and H510) strains. Our results were consistent, and the techniques were relatively simple. Our technique appears to be more efficient than that used by Miles (1974) who obtained 7% and 12.5% isolation efficiencies, respectively, for avirulent and virulent strains of *T. cruzi*. This difference may be due to the fact that our modified NNN medium was overlaid with 1 ml of F:93 medium (NNN-F:93), thus making the combination more suitable (richer) for the growth of *T. cruzi*. Neal & Miles (*13*) reported that 100–200 trypomastigotes of *T. cruzi* may have to be ingested by each *Rhodnius prolixus* in order to obtain a consistently positive xenodiagnosis. In contrast, we obtained 30–40% positive cultures when each of 10 NNN-F:93 tubes was inoculated with only one bloodstream trypomastigote; and in another unrelated experiment, all 10 inoculated cultures (which each received 20 bloodstream trypomastigotes) became positive within 3 weeks of incubation. It appears that enriched NNN cultures such as those we used (NNN-F:93) may prove to be as or more efficient than the classical xenodiagnosis for detecting infection with *T. cruzi*. Inoculation of CD-1 mice with one trypomastigote was not sensitive enough to isolate clones.

In vitro cultural characterization of the 14 secondary clones of the avirulent Brazil strain revealed that 2 clones (C15C6 and C18C2) grew primarily as amastigotes regardless of incubation temperature (27 °C or 37 °C), while the rest grew primarily as epimastigotes at 27 °C and as amastigotes at 37 °C. Although such *in vitro* cultural characteristics cannot yet be used as genetic markers for clones, they may be useful for selecting clones for the study of genetic markers and other biological properties. Similar *in vitro* experiments carried out with 7 secondary clones of pathogenic strains (Col-A and H510) indicated that the *in vitro* cultural characteristics of clones from the same strain were not markedly different. Since both strains had been passaged continuously in CD-1 mice for over 6 years, they may be relatively uniform genetically and thus it may be necessary to isolate more clones before any with different cultural characteristics are found. *In vivo* characterization of the 7 clones of the pathogenic strains did not indicate any noticeable difference among clones of either strain. However, it should be noted that individual clones of the H510 strain required 4–5 passages after isolation and those of Col-A more than 8 passages in CD-1 mice before the individual clones became pathogenic for the mice, causing low (under 50%) mortality, in spite of the fact that each clone was passaged only twice in NNN-F:93 medium and once in F:94. In contrast, the parent stock strains regularly caused over 90% mortality in CD-1 mice within 10 weeks after infection. The results suggest that either the individual clones used in the experiments were, genetically, not highly pathogenic or that organisms of variable genetic composition in the parent complex may act synergistically in producing high pathogenicity in CD-1 mice. It would be extremely important to find out in the future whether highly pathogenic clones coexist with less pathogenic clones in natural isolates from patients or animals.

RÉSUMÉ

ÉTABLISSEMENT DE CLONES DE *TRYPANOSOMA CRUZI* ET LEUR CARACTÉRISATION *IN VITRO* ET *IN VIVO*

Trypanosoma cruzi peut être divisé en trois groupes antigéniques principaux au moins. Ces types antigéniques peuvent être mélangés dans des isolements provenant d'hôtes vertébrés, ce qui rend difficiles diverses études sur ce parasite, notamment sur la variation antigénique ou sur la génétique. Une telle difficulté peut être écartée grâce à l'utilisation de souches clonées. Nous avons mis au point une technique relativement efficace de clonage de *T. cruzi* par isolement dans un milieu NNN enrichi.

Le milieu NNN enrichi (NNN-F:93) est préparé par addition de 1 ml de milieu F:93 en surcouche à chaque gélose au sang inclinée NNN, contenant, par litre, 100 ml de sang normal hépariné de lapin. Une souche avirulente et deux souches virulentes de *T. cruzi* ont été utilisées dans huit expériences. Dans le cas de la souche avirulente, des épimastigotes cultivés dans du milieu F:93 ont été dilués en série avec du soluté salin tamponné au phosphate contenant 4 g de glucose par litre (PBS-G) et un microorganisme était inoculé dans chaque tube contenant le milieu NNN-F:93. En ce qui concerne les souches virulentes, des trypomastigotes du courant sanguin ont été d'abord séparés de la majorité des cellules sanguines puis dilués en série avec du PBS-G pour être inoculés dans du NNN-F:93. L'efficacité moyenne de clonage (pourcentage de cultures positives par rapport au nombre de cultures inoculées) était de 70% pour la souche avirulente et de 35% pour les souches virulentes. Ces résul-

tats étaient constants pour les expériences effectuées avec chaque souche.

Les caractéristiques de culture *in vitro* des 14 clones secondaires de la souche avirulente révélaient que 12 clones croissaient en milieu F:94 principalement sous forme d'épimastigotes (plus de 96%) à 27 °C et exclusivement sous forme d'amastigotes à 37 °C; deux clones croissaient en milieu F:94 principalement sous forme d'amastigotes (plus de 99%) indépendamment des températures d'incubation (27 °C ou 37 °C). La caractérisation *in vivo* de sept clones provenant des deux souches virulentes a montré que la virulence de clones individuels était faible tout de suite après isolement en milieu NNN-F:93 mais que, pour certains clones, elle rattrapait celle des souches parentales après plus de huit passages en série chez les souris CD-1.

REFERENCES

1. BARRY, J. D. ET AL. Detection of multiple variable antigen types in metacyclic populations of *Trypanosoma brucei. Transactions of the Royal Society of Tropical Medicine and Hygiene*, **73**: 205 – 208 (1979).
2. BRENER, Z. Biology of *Trypanosoma cruzi. Annual review of microbiology*, **27**: 347 – 382 (1973).
3. DWYER, D. M. *Leishmania donovani:* surface membrane carbohydrates of promastigotes. *Experimental parasitology*, **41**: 341 – 358 (1977).
4. FEDERICI, E. E. ET AL. Chronic and progressive myocarditis and myositis in C3H mice infected with *Trypanosoma cruzi. American journal of tropical medicine and hygiene*, **13**: 272 – 280 (1964).
5. GILLIN, F. D. & DIAMOND, L. S. Clonal growth of *Entamoeba histolytica* and other species of *Entamoeba* in agar. *Journal of protozoology*, **25**: 539 – 543 (1978).
6. GONZÁLEZ-CAPPA, S. & KAGAN, I. G. Agar gel and immunoelectrophoretic analysis of several strains of *Trypanosoma cruzi. Experimental parasitology*, **25**: 50 – 57 (1969).
7. HOARE, C. A. *The trypanosomes of mammals.* Oxford and Edinburgh, Blackwell Scientific Publishers, 1972, 749 pp.
8. HOFF, R. Killing *in vitro* of *Trypanosoma cruzi* by macrophages from mice immunized with *T. cruzi* or BCG, and absence of cross-immunity on challenge *in vivo. Journal of experimental medicine*, **142**: 299 – 311 (1975).
9. LE RAY, D. ET AL. Antigenic heterogeneity of metacyclic forms of *Trypanosoma brucei. Nature (London)*, **273**: 300 – 302 (1978).
10. MILES, M. A. Cloning *Trypanosoma cruzi. Transactions of the Royal Society of Tropical Medicine and Hygiene*, **68**: 256 (1974).
11. MILES, M. A. ET AL. Further enzymic characters of *Trypanosoma cruzi* and their evaluation for strain identification. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **74**: 221 – 237 (1980).
12. MOTT, K. E. ET AL. The epidemiology and household distribution of seropositivity to *Trypanosoma cruzi* in a rural community in northeast Brazil. *American journal of tropical medicine and hygiene*, **25**: 552 – 562 (1976).
13. NEAL, R. A. & MILES, M. A. The number of trypomastigotes of *Trypanosoma cruzi* required to infect *Rhodnius prolixus. Revista instituto medicina tropical, São Paulo*, **19**: 177 – 181 (1977).
14. NOBLE, E. R. The morphology and life cycles of trypanosomes. *Quarterly review of biology*, **30**:1 – 28 (1955).
15. NUSSENZWEIG, V. ET AL. Differences in antigenic constitution of strains of *Trypanosoma cruzi. Experimental parasitology*, **14**: 221 – 232 (1963).
16. NUSSENZWEIG, V. & GOBLE, F. C. Further studies on the antigenic constitution of strains of *Trypanosoma (Schizotrypanum) cruzi. Experimental parasitology*, **18**: 224 – 230 (1966).
17. *Health conditions in the Americas 1969 – 1972.* Washington, DC, Pan American Health Organization, 1974, p. 34. (Scientific Publication No. 287).
18. PAN, C. Cultivation of the leishmaniform stage of *Trypanosoma cruzi* in cell-free media at different temperatures. *American journal of tropical medicine and hygiene*, **17**: 823 – 832(1968).
19. PAN, C. Cultivation and morphogenesis of *Trypanosoma cruzi* in improved liquid media. *Journal of protozoology*, **18**: 556 – 560 (1971).
20. PAN, S. C. *Trypanosoma cruzi*: Intracellular stages grown in a cell-free medium at 37 °C. *Experimental parasitology*, **45**: 215 – 224 (1978).
21. PAN, S. C. *Trypanosoma cruzi: In vitro* interactions between cultured amastigotes and human skin–muscle cells. *Experimental parasitology*, **45**: 274 – 286 (1979).
22. PAN, S. C. *Trypanosoma cruzi:* Ultrastructure of morphogenesis *in vitro* and *in vivo. Experimental parasitology*, **46**: 92 – 107 (1978).
23. SAMUELS, R. Agar techniques for colonizing and cloning trichomonads. *Journal of protozoology*, **9**: 103 – 107 (1962).
24. SNARY, D. *Trypanosome cruzi:* Antigenic invariance of the cell surface glycoprotein. *Experimental parasitology*, **49**: 68 – 77 (1980).
25. VAN MEIRVENNE, N. ET AL. Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei.* I. Rationalization of the experimental approach. *Annales de la Société belge de médecine tropicale*, **55**: 1 – 23 (1975).
26. VAN MEIRVENNE, N. ET AL. Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei.* II Comparative studies on two antigenic-type collections. *Annales de la Société belge de médecine tropicale*, **55**: 25 – 30 (1975).
27. VICKERMAN, K. Antigenic variation in trypanosomes. *Nature (London)*, **273**: 613 – 617 (1978).
28. WOODY, N. C. & WOODY, H. B. American trypanosomiasis. I. Clinical and epidemiologic background of Chagas' disease in the United States. *Journal of pediatrics*, **58**: 568 – 580 (1961).