body inhibits transformation from epimastigotes to trypomastigotes[35]. Control of differentiation might be mediated by interaction between GP72 and lectins in the triatomine alimentary tract[35,36].

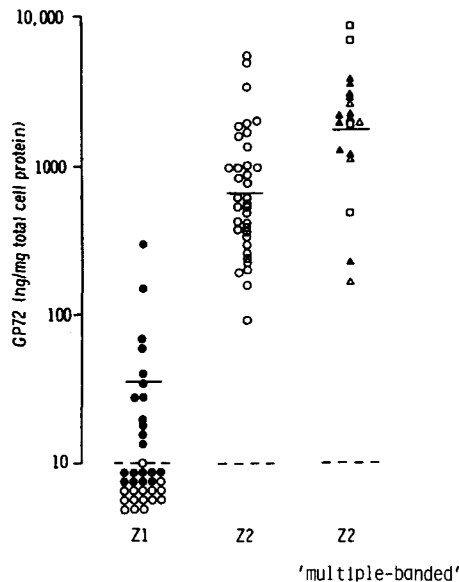

Fig. 3. Zymodeme associated expression of the GP72 carbohydrate epitope[28].

Stability, Amplification and Selection

The main features of *T. cruzi* isozyme profiles remain stable over long periods in clonal populations[23,37]. There are minor but consistent differences between different stages of the life cycle (epimastigote, trypomastigote, amastigote)[38]. Differences in GPI band intensities have also been noted when clones are grown at different temperatures (G. Widmer *et al.*, unpublished). It is therefore essential that the same life cycle stages and growth conditions are used when *T. cruzi* stocks are prepared for isozyme comparisons. The only exception to the evidence that isozymes provide stable markers of intrinsic genetic differences is a report by A. Tanuri and D.F. de Almeida (unpublished) who observed radical changes in isozyme profiles when clones were grown on enriched or depleted culture media. Cloning by colony selection on agar was used, however, and the results might be explained as selection of sub-populations.

Laboratory strains of *T. cruzi* or stocks newly isolated from single mammals or vectors may consist of heterogeneous mixtures of zymodemes. Some *T. cruzi* stocks show mixed isozyme profiles (Refs 31, 32 and J. Alencar and M.A. Miles, unpublished), and repeated isolation from the same host can yield stocks with different profiles[32]. Clones derived from a single stock can also have different profiles[25,37]. Zymodeme growth rates can differ markedly[16] and selection of sub-populations readily occurs *in vitro* or *in vivo* as stocks are amplified. Rapid clonal selection during growth of mixed populations can be predicted from computer models of growth rates (J.A. Dvorak, unpublished). Thus any method for characterizing *T. cruzi* that requires prior amplification of populations will fail to detect the full range of heterogeneity. This has been well illustrated by schizodeme analysis which provides a more sensitive means of detecting mixed populations in amplified stocks (see below). Nevertheless, during surveys the same isozyme profile has generally arisen independently of isolation procedure[3] and the consistency of the geographical and host associations described above suggest that isozyme data reflect phenomena of fundamental importance.

The decision as to whether *T. cruzi* is a species-complex or a single polytypic species will benefit from a more complete understanding of *T. cruzi* genomic structure and function. This will emerge from molecular karyotyping by pulsed field gradient gel electrophoresis and recombinant DNA techniques. It is already known that *T. cruzi* has around 20 chromosomes, with no detectable mini-chromosomes: gene distribution and copy number indicate that the organism is diploid for at least some genes (W.C. Gibson and M.A. Miles, unpublished). Zymodeme analysis of *T. cruzi* has achieved much, and its discriminate use has a future role, but its precise role will only become apparent once the *T. cruzi* genome is understood in more detail.

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The Complexity of *Trypanosoma cruzi* Populations revealed by Schizodeme Analysis

C. M. Morel*, M. P. Deane** and A. M. Gonçalves*

Department of Biochemistry and Molecular Biology* and Department of Protozoology** Instituto Oswaldo Cruz – FIOCRUZ PO Box 926, Rio de Janeiro, RJ, Brazil

Stocks, strains and clones of the haemoflagellate parasite *Trypanosoma cruzi* can be characterized at the genotype level by means of schizodeme analysis[25]. This

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technique is based on the electrophoretic separation of restriction-endonuclease generated fragments of kinetoplast DNA – the bizarre mitochondrial DNA that characterizes the Kinetoplastida. The electrophoretic profiles, also known as 'restriction fingerprints', are stable biochemical markers which can be efficiently used to differentiate closely related parasite populations[25,40–44].

Fig. 4 shows the schizodeme analysis of several *T. cruzi* strains, on a gel stained by ethidium bromide (left) or silver (right). Each profile is formed by sharp bands which differ in molecular weight as well as in intensity. These bands are derived mostly from the minicircles of kinetoplast DNA. As these molecules are present in large amounts and have some degree of heterogeneity, fragments are abundant and not in stoichiometric amounts. Therefore the restriction profiles are complex and highly informative, allowing the discrimination of closely related organisms.

These characteristics of schizodeme analysis allow us to investigate more efficiently the complexity and the dynamics of *T. cruzi* populations. Our results demonstrate that *T. cruzi* is a very complex collection of organisms which frequently exist as heterogeneous populations in a dynamic equilibrium.

How homogeneous are the isolates of *T. cruzi* obtained from the vertebrate and invertebrate hosts? Schizodeme analysis of cloned cultures has previously shown that one laboratory strain (CL) isolated originally from an invertebrate host, was really a mixture of at least two subpopulations of quite distinct biological behaviour[25]. Fig. 5 (left) and also our previous data[41] show that mixtures are sometimes found in isolates from human chagasic patients. Other isolates seem to be quite homogeneous, all the clones displaying absolutely identical profiles even after different schemes of passages (not shown). In Fig. 5 (right) we see another commonly observed pattern: the clones display restriction fingerprints that are quite similar but present minor differences among them.

Thus *T. cruzi* appears to circulate in nature as heterogeneous populations of varying complexity. How can this affect our laboratory 'strains'? What are the consequences in clinical, diagnostic and epidemiological studies?

In order to see what occurs when a given 'strain' is maintained for long periods in the laboratory, we investigated what had happened with one of the most frequently used *T. cruzi* strains – the Y-strain isolated in 1953[45]. For this purpose we asked for samples of this strain from several laboratories and analysed them side by side. Our partial results are shown in Fig. 6 (Gonçalves *et al.*, unpublished). Although most of these samples displayed the profile shown at the extreme left (the 'canonical' Y-strain restriction fingerprint), we detected at least four other different profiles. This heterogeneity could be due either to selection of subpopulations or to laboratory mix ups. (Errors due to mishandling or mislabelling of trypanosomatid strains are probably not uncommon. One example has been reported in the literature[46] and another is illustrated in Fig. 6 (right) where we received two cultures, one labelled *Leishmania mexicana*, the other *Leishmania*

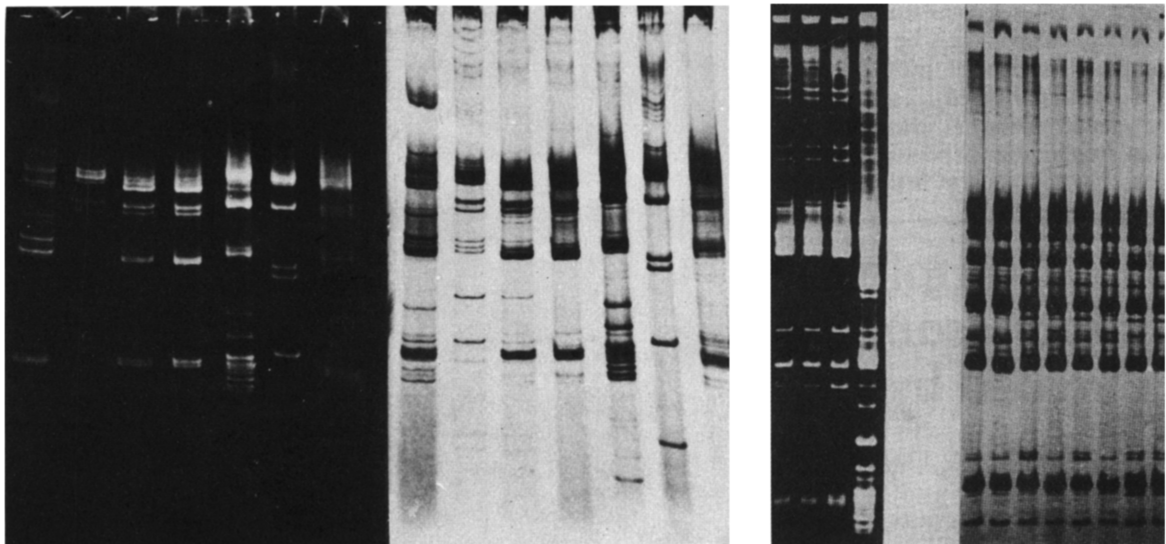

Fig. 4. Schizodeme analysis of several strains of T. cruzi. The kinetoplast DNA from several strains of T. cruzi was isolated, digested with the restriction endonuclease EcoRI and analysed by high resolution polyacrylamide gradient mini-slab gel as previously decribed[42]. The same gel was stained with ethidium bromide (left) or silver (right).

Fig. 5. Schizodeme analysis of cloned T. cruzi cultures. Cloned cultures were prepared from strains isolated from human chagasic patients by limiting dilution and plating in agar[51]. Schizodeme analysis was done with EcoRI nuclease. Left: clones from one patient infected with two very different schizodemes, ethidium bromide staining. Right: clones from another patient infected with a much more homogeneous parasite population, silver staining.

braziliensis, but schizodeme analysis showed both to be *L. mexicana*.)

Heterogeneous Populations

We have previously studied the behaviour of heterogeneous populations of *T. cruzi* in the laboratory using both natural and artificial mixtures of parasites[41,44,47]. Dvorak and collaborators have also studied this subject using different approaches, including computer simulation of the behaviour of mixtures of different strains[15,48]. Fig. 7 shows some of our experiments. The two lanes at the extreme left display the profiles of the two frequently used strains (Y and F). The next four lanes represent the analysis of the populations present in an opossum at different intervals after infection with a mixture of the two strains. The Y-strain, distinguished by a characteristic band, was only detectable at the second time point.

Fig. 7 (right) shows what can happen when *T. cruzi* populations are isolated from human patients and brought to the laboratory. In this experiment, strains isolated by haemoculture were inoculated into mice and followed during two years by schizodeme analysis. The first two lanes show an example where we could not detect any modification of the strain, but the next two lanes show that another isolate underwent a complete change in the restriction profile, indicating that the passages in mice had selected a subpopulation of the parasite.

These results illustrate the complexity of natural populations of *T. cruzi* and how dynamic is the equilibrium among the various types of organisms in any given sample. The study of *T. cruzi* relies on methods for the amplification of the initial parasite population, but these methods can induce selection of subpopulations. We cannot always be sure that the strain isolated from a human patient is responsible for the clinical form, and not a minor component of the parasite population that was over-amplified during isolation. Thus, how can we preserve the original complexity of the isolated populations during laboratory manipulations?

We still do not have answers to these problems. *T. cruzi* seems to be a universe of organisms with quite different biological characteristics which often co-exist in mixed populations. The development of new methods for parasite characterization, in particular those using DNA probes[48–50], will probably bring new light in the study of these problems, once the need for multiple steps for population amplification has been minimized. But we should never forget that the real complexity and dynamics of *T. cruzi* in nature will always be much richer than those we have inside the laboratory.

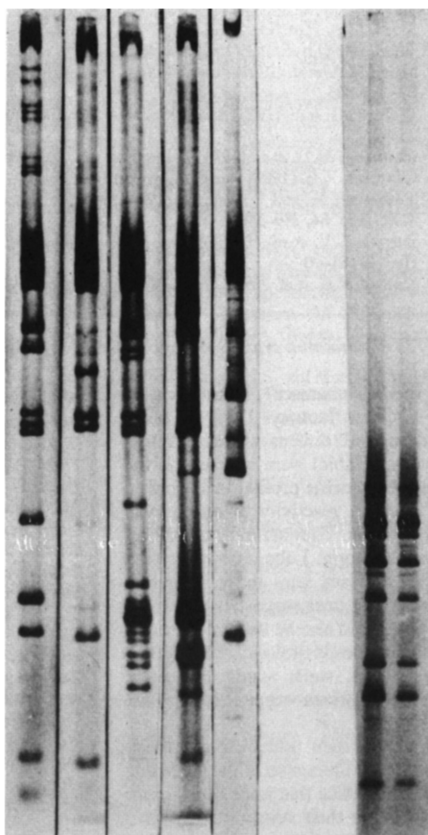

Fig. 6. Monitoring of laboratory strains of T. cruzi. Left: samples from the Y-strain of T. cruzi were obtained from different research laboratories and analysed by EcoRI schizodeme analysis. The first lane from the left represents the most abundant, canonical, profile; the next four lanes differ considerably from the standard restriction fingerprint, indicating that subpopulation selection or strain mix-up might have occurred. Right: schizodeme analysis of samples received as L. mexicana and L. braziliensis showing that both were in fact L. mexicana.

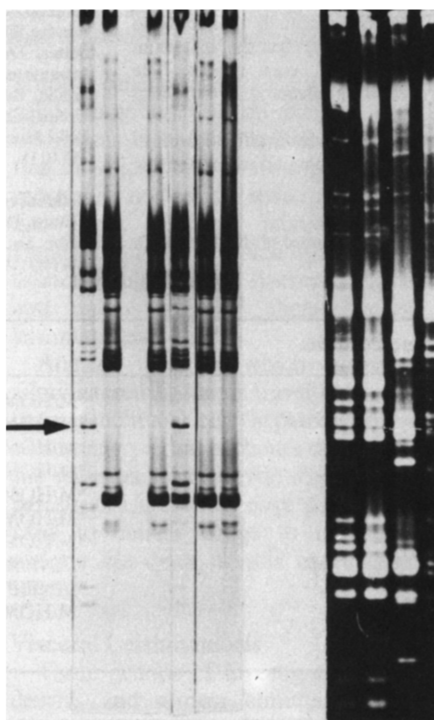

Fig. 7. Monitoring of natural and artificial heterogeneous populations of T. cruzi. Left (silver staining): an opossum (Didelphis marsupialis) was infected with a mixture of the Y- and F-strains of T. cruzi (first and second lanes, respectively). The next four lanes are samples isolated at different time points from the animal. The Y-strain can only be detected in one of the isolates. Right (ethidium bromide staining): stocks isolated from human patients were kept in laboratory mice for two years and isolates at the beginning and at the end of this period were analysed by EcoRI schizodeme analysis. In one case, there was no difference between the isolates (first two lanes); in another, the new isolate was completely different from the initial one, indicating subpopulation selection by the mice (lanes at the right).

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Trypanosoma cruzi Reference Strains and Characterization Methods

Following the example of the 'leishmaniacs'[a], researchers on *T. cruzi* met in Panama City in January 1985 to select international reference strains and discuss characterization methods. Reference strains (see Table) were selected on the basis of their zymodeme and schizodeme profile, behaviour in mice, growth rate, DNA content, reactivity to monoclonal antibodies, or susceptibility to drugs. They are to be assembled and amplified at the Gorgas Memorial Laboratory (Panama) and will be distributed, as stabilate sets, to collaborating laboratories (13 national centres have been suggested, see right). A data sheet was designed that could also be used as a request form for the characterization of newly isolated strains. The coding system and terms (isolate, stock, clone etc.) were adopted that are in use for African trypanosomes and *Leishmania*.

It is notable that 13 of the *T. cruzi* reference strains are derived from clonal populations. As illustrated in the preceding article it was emphasised at the meeting that uncloned *T. cruzi* populations could radically change their composition during isolation or subsequent growth *in vitro* or *in vivo*. The uncloned reference strains might therefore be unstable in their behaviour. In this context, the latest progress with characterization methods was considered, encompassing morphological, behavioural, biochemical, antigenic and lectin binding properties. Papers presented on these topics are published as a special issue of the *Revista da Sociedade Brasileira de Medicine Tropical*[b].

Unlike the leishmanias, *T. cruzi* is not formally separated into distinct taxa. Nevertheless, it is abundantly clear that there is an extraordinary heterogeneity among *T. cruzi* strains. The Panama meeting and report[c] are a significant contribution to uniting the *T. cruzi* research effort.

They will encourage researchers to tackle the difficult areas of strain-related research with new vigor in a sound and systematic fashion.

†With profound regret, we have recently learned of the death of Dr Hugo Lumbreras in Peru.

List of Collaborating Laboratories

Argentina: Dr E.L. Segura, Instituto 'Dr M. Fatala Chaben' (INDIECH), Av. Paseo Colon 568, 7° piso, Buenos Aires 1063. Tel: 33-7732; 33-2330. Dr A.C.C. Frasch, Fundacion Campomar, Antonio Machado 151, 1405 Buenos Aires. Tel: 884013. Telex: 18694 IBUBA-AR. **Bolivia:** Dr C. La Fuente, Centro Nacional de Enfermedades Tropicales (CENETROP), Casilla 2974, Santa Cruz. Tel: 39101. **Brazil:** Dr C.M. Morel, Biochemistry and Molecular Biology Department, Instituto Oswaldo Cruz, Avenida Brasil 4365 – Manguinhos, Cep. 21040, Rio de Janeiro, RJ. Tel: (021) 2907549; (021) 2702946. Telex: (021) 23239 FUOC BR. **Chile:** Dr A. Solari, Depto. de Biologia Celular y Genetica, Facultad de Medicina-Norte, Universidad de Chile, Casilla 6556, Santiago-7. Tel: 370081/776560 (Ext. 5214). **Columbia:** Dr N. Saravia, Centro Internacional de Investigaciones Médicas (CIDEIM), Apartado Aéreo No. 5390, Cali. **Costa Rica:** Dr R. Zeledon, Escuela de Medicina Veterinaria, Universidad Nacional, Apartado 86, Heredia. **Honduras:** Dr H. Cosenza, Departamento de Microbiologia, Universidad Nacional Autonoma de Honduras, Tegucigalpa DC. Dr C. Ponce, Ministerio de Salud, Laboratorio Central, Centro de Salud 'Dr Alonso Suazo', 3° piso, Tegucigalpa DC. Tel: 32 11 35. **Mexico:** Dr L. Zarate, Centro de Investigaciones Ecologicas del Sureste (CIES), Calle Real de Guadalupe 55, San Cristobal de las Casas, Chiapas. **Peru:** Dr H. Lumbreras†, Instituto de Medicina Tropical 'Alexander von Humboldt', Universidad Cayetano Heredia, Calle Honorio Delgado No. 932, Ap. 5045, Lima. **Republic of Panama:** Dr O.E. Sousa, Laboratorio Conmemorativo Gorgas, (Gorgas Memorial Laboratory), Depto. de Parasitologia, Apartado Postal 6991, Panama City 5. Tel: 27-4111. Telex: 3433. **United Kingdom:** Dr M.A. Miles, London School of Hygiene and Tropical Medicine, Keppel Street (Gower Street), London WC1E 7HT. Tel: (01) 636-8636 (399). Telex: 8953474. **United States:** Dr J. Dvorak, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205. Tel: (301) 4964880. **Venezuela:** Dr J. Urbina, Centro de Biologia Experimental, Calle Suapure – Colinas de Bello Monte, Universidad Central de Venezuela, Caracas 106. Tel: 751.0111.

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Table: *Trypanosoma cruzi* reference strains

M/HOM/PE/00/Peru	*M/HOM/BR/82/Dm 28c
M/HOM/BR/00/12 SF	*M/HOM/BR/78?/Sylvio-X10-CL1
M/HOM/CO/00/Colombia	*M/HOM/BR/78/Sylvio-X10-CL4
M/HOM/BR/00/Y strain	*M/HOM/BR/77/Esmeraldo CL3
M/HOM/BR/00/CL strain	*M/HOM/BR/68/CAN III CL1
M/HOM/CH/00/Tulahuen	*M/HOM/BR/68/CAN III CL2
M/HOM/AR/74/CA-I	*M/HOM/BO/80/CNT/92: 80 CL1
*M/HOM/AR/74/CA-I/72	*I/INF/BO/80/SC43 CL1
*M/HOM/AR/00/CA-I/78	*I/INF/PY/81/P63 CL
*M/HOM/AR/00/Miranda 83	M/HOM/AR/78/RA
*M/HOM/AR/00/Miranda 88	

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Liposomes as Drug Carriers in Leishmaniasis and Malaria

REVIEW

C.R. Alving

Department of Membrane Biochemistry
Walter Reed Army Institute of Research
Washington, DC 20307-5100 USA

Experimental studies suggest that liposomes could substantially improve the performance of anti-leishmanial drugs in the chemotherapy of visceral leishmaniasis. In this article, Carl Alving discusses the potential for overcoming resistance to antimonial drugs; for 'buffering' the toxicity of drugs; and for drug delivery under conditions where hospitalization is impossible or inconvenient. Liposomes can also be used experimentally to reduce the toxicity and increase the efficacy of parenterally-administered primaquine in the treatment of sporozoite-induced murine malaria.

Liposomes are synthetic lipid spheres that can encapsulate a wide variety of water-soluble or water-insoluble drugs (see Box 1); they have been proposed as drug carriers in a broad range of clinical disorders, including visceral leishmaniasis and malaria[1–4]. The ability to synthesize liposomes according to specific requirements has led to several 'targeting' strategies to increase the precision of liposomal drug delivery[4].

For most types of drug targeting it is desirable to concentrate a drug in a particular body compartment, to enter a compartment normally forbidden to the drug, or to avoid a particular body compartment[4]. Few, if any, drugs or drug carriers completely satisfy all these requirements. In the case of liposomes (or other particles) containing antitumour drugs, a major difficulty has been the propensity of parenterally administered particles to concentrate in reticuloendothelial (RE) cells, particularly Kupffer cells, rather than being delivered to solid tumours[4,5]. But in the case of leishmaniasis, the RE cell is the very location in which the parasite organism spends most of its normal life span in the mammalian host. Several laboratories have exploited this to show that liposome-encapsulated drugs can be delivered efficiently and safely in the chemotherapy of leishmaniasis.

Another cell to which parenterally-administered lipsomes travel in the liver is the parenchymal cell (hepatocyte)[6–9] – the same cell type that harbours the hepatic tissue stage (exoerythrocytic stage) of malaria parasites. Liposomes have therefore been used to deliver drugs to interrupt the malaria life cycle at this exoerythrocytic stage.

Liposomes tend to concentrate in reticuloendothelial cells, where Leishmania parasites accumulate, and in hepatocytes where exoerythrocytic stages of Plasmodium are harboured. This property makes liposomes potentially useful in targeting drugs to these parasites.

Visceral Leishmaniasis

Three groups of investigators independently, and almost simultaneously, re-

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