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ISOLATION AND CHARACTERIZATION OF TOTAL RIBONUCLEIC ACIDS FROM DIFFERENT SPECIES OF TRYPANOSOMATIDAE

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As a first step in the study of gene expression in Trypanosomatidae, total RNA was isolated from *Trypanosoma cruzi, Crithidia fasciculata, C. oncopelti, Blastocrithidia culicis, Leptomonas dysdercus, Herpetomonas megasaliae, Leptomonas tarentolae,* and *Herpetomonas sp* and characterized by polyacrylamide gel electrophoresis (PAGE). Cells were lysed by 1% sodium (or lithium) dodecyl sulfate and the RNA extracted by one of the following methods: phenol-cresol [3], phenol-chloroform [1], sodium dodecyl sulphate-diethylpyrocarbonate [6] or CsCl ultracentrifugation [2].

When the RNA isolated by any of these methods was analysed by PAGE in non-aqueous solvents (formamide [7] or gel scanning in the ultraviolet [5]) seven fractions could be resolved. The molecular weights and the sedimentation coefficients (S_E) estimated by electrophoresis together with 4, 5, 16 and 23S *E. coli* RNA markers were the following for *C. fasciculata*:

RNA Fraction	P.M. (daltons)	S_E
A	2.6×10^4	4.0
B	3.6×10^4	5.0
C	5.2×10^4	5.8
D	6.1×10^4	6.4
E	5.0×10^5	15.5
F	6.2×10^5	16.5
G	7.3×10^5	18.0

We could never observe in formamide gels a RNA fraction heavier than peak G. This fact suggested that the ribosomal RNA of the big subunit was either abnormaly unstable or selectively lost during extraction.

The RNA analysis in aqueous gels [14] demonstrated the existence of variable amounts of a 26 S_E peak. Recent experiments demonstrated that this RNA is the big subunit RNA which is highly unstable at temperatures above 0°C. Degradation of this RNA is already detectable after 10 min. incubation at 5°C; incubation at 25°C during 10 minutes can degrade more than 90% of this 26 S_E peak and at 37°C degradation is complete.

The disappearance of this RNA is accompanied by the appearance of RNA peaks similar to the peaks *E* and *F* (above).

These results demonstrate the existence of 'hidden breaks' in the ribosomal RNA of the big subunit of trypanosomatidae.

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INTERMEDIARY METABOLISM, GROWTH AND DIFFERENTIATION OF *TRYPANOSOMA CRUZI*

J. F. FERNANDES and Odécio CÁCERES

Trypanosoma cruzi quickly exhausts the glucose of the culture medium. Succinic and malic acids are the predominant organic acids that accumulate in the culture during the exponential growth phase, resulting in a decrease of the pH of the medium. The oxidation of the acids accumulated and consequent rise in pH of the medium run closely parallel to the rate of differentiation of the epimastigotes into metacyclic trypomastigotes when the medium and culture conditions are appropriated. If glucose concentration is maintained by daily additions into the culture medium, the differentiation is inhibited, the medium pH decreases progressively to toxic levels and growth is slightly slower. Anaerobiosis inhibits growth and differentiation when the initial pH of the medium is 6.7. Glucose oxidation through the pentose phosphate pathway is very pronounced and Krebs cycle does operate at limited speed.

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STRUCTURAL AND ENZYMATIC MEMBRANE MARKERS IN *TRYPANOSOMA CRUZI*

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We have been dealing with the extraction and purification of total glycopeptides from *T. cruzi* since their ability to inhibit the con A-induced agglutination of epimastigotes suggests a membrane location for these substances [1]. Sonicated cells are extracted with phenol and the water phase, after diethylether extraction, is precipitated with ethanol. The precipitate is dissolved in H_2O (3.3% w/v) and an insoluble matter (mostly nucleic acids) is removed by filtration. To the soluble portion ethanol is slowly added to a final concentration of 50% (v/v). A precipitate is formed which contains 89.5% band D, 6.45% band A and 4.04% band C. Band D can be further purified by column chromatography [2]. Pure band D, after hydrolysis with 1 N H_2SO_4 revealed the following sugar content: mannose (35) : galactose (22) : glucose (1).

In order to demonstrate the membrane location of these glycopeptides, intact cells were labeled with ^{131}I in the presence of lactoperoxidase and H_2O_2. Polyacrilamide gel electrophoresis of the 105,000 x g precipitate showed that many proteins were labeled. However, when the glycopeptides were isolated from the cells, no label co-migrated with these substances. Treatment of cells with trypsin or lipase were ineffective to promote specific labeling of glycopeptides. Treatment of the isolated glycoprotein complex with labeled iodine resulted in poor labeling of the 4 glycopeptides. This result suggests that the peptide moieties of these substances are poor in tyrosine residues. However, other possibilities like the localization of the glycopeptides in structures other than the membrane or the burial of the peptide residues in membrane compartments unaccessible to the lactoperoxidase, cannot be ruled out.

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In many cell systems, adenylate cyclase activity has been shown to be a property of plasma membranes. In *T. cruzi* this activity is restricted to the 105,000 X g pellet of a sonicated extract of cells. The activity is highly dependent on Mn^{2+} but not Mg^{2+}. Kinetic experiments suggest that the true substrate is the binary complex Mn^{2+}—ATP rather than free ATP which seems to be inhibitory. Preincubation of the membranes or rupture of the cells in the presence of Mn^{2+} or Mg^{2+} produce a membrane aggregation and result in a 3-fold activation of adenylate cyclase activity. This effect is reversed by washing the membranes with 1 mM EDTA. These results suggest that these ions, in some manner, activate the adenylate cyclase through the maintenance of membrane integrity.

During growth of the epimastigote forms the adenylate cyclase is highly active 3 hours after the cells are transferred to a fresh medium. This activity falls during the first day, raises again on the 4th day of growth (beginning of the stationary phase) and then falls steadily, reaching very low levels on the 9th day of growth. cAMP levels respond to this prompt increase in cyclase activity reaching its maximum in the lst day of growth (22 pmoles/ 10^8 cells). Probably the response is not immediate because the soluble cAMP phosphodiesterase is also very active in the beginning of growth but rapidly decays to lower levels.

It is our belief that the glycopeptides and the adenylate cyclase system are two suitable markers of plasma membrane in *T. cruzi*. Since this organelle is the first barrier for nutrients and signals of the external environment which could be influencing growth and differentiation, the study of these two systems, their mutual relationships and their possible role in growth and in the epimastigote-trypomastigote transformation might shed some light in the understanding of the behaviour of *T. cruzi* in culture.

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AMINOACID TRANSPORT BY «Y» AND «MR» STRAINS OF *TRYPANOSOMA CRUZI*

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Differences in membrane specific sites may explain differences in the ability of cells to respond to stimuli that induce normal differentiation [2]. Differences in epi- and trypomastigote membrane for *Con A* binding has been reported in Y strain of *T. cruzi* by ALVES & COLLI [1]. In the present study permeability changes for arginine and lysine transport during differentiation of *Y* and *MR* strains has been investigated employing kinetic technique. Our results may be summarized as follows:

1) The uptake of both aminoacids was 2-3 times greater in 3-day than 10-day old cultures; 2) The rate of uptake for arginine and lysine is greater for the *MR* strain; 3) For bothstrains arginine is taken up at a rate 1.5 times faster; 4) Flagellates treated in a DEAE-cellulose column present a consistently increased uptake for arginine and lysine; 5) The kinetic behaviour of transport was modified by passage of the flagellates through DEAE-cellulose column: the non-saturable component and the anticompetitive inhibition of arginine transport by lysine were eliminated; 6) Methionine inhibited uncompetitively the uptake of lysine and arginine for *MR* and *Y* strains. Lysine inhibited the uptake of arginine and viceversa in both strains by a mixed mechanism. In all experiments at high levels of inhibitor concentration the inhibition disappeared; 7) The results presented here suggest the presence of recognition sites in the flagellates subject to a modulation by effectors and qualitative and/or/quantitative modification of transport sites for arginine and lysine during differentiation.

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GROWTH OF *TRYPANOSOMA CRUZI* WITH LIPID FRACTIONS EXTRACTED WITH N-PENTANE FROM DIFFERENT BIOLOGICAL MATERIALS

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The culture medium 199 was enriched with lipid fractions from Tryptose (Difco), Liver infusion (Difco), and calf serum extracted with n-pentane. This solvent was reported to extract ubiquinones very efficiently (BARRET & DAWSON [2]). These substances apparently acted as growth factors for *T. brucei* (CROSS & MANNING [3]) and *T. cruzi* (ANDERSON & KRASSNER [1]). The extracts in pentane were evaporated to dryness under nitrogen stream. The calf serum was freeze-dried prior to organic solvent extraction. The residue was suspended in phosphate buffer, pH 7.2, and sonicated. The inoculum for the growth experiments was 1 ml of 7-days cultures growing in LIT's medium, at 28°C. Growth was measured in a Neubauer chamber.

Preliminary results showed that the serum lipid fraction stimulated the growth of *T. cruzi* more efficently than the other pentane extracts. After four transfers, growth obtained with the serum pentane fractions was comparable to that in the LIT's medium and definitely more pronounced than growth in the unsupplemented 199 medium.

Experiments are under study to determine the active substance in the pentane extracts.

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METABOLISM OF *TRYPANOSOMA CRUZI*

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Two aspects of the metabolism of *T. cruzi* have been examined: the biosynthesis of purines and the insensitivity to cyanide of oxygen consumption.

The cultures were maintained in exponential growth in LIT medium [1] and transferred to Boné and Parent's medium [1] for production of a large cell mass. The labelling experiments were carried out in a defined medium, No. 199, with known levels of glycine, purines and derivatives using cells at the middle of log phase (0.8-1.0 x 10^8 cells/ml). Adenine-^{14}C and guanine-^{14}C were incorporated in nucleic acids (DNA and RNA). Glycine-^{14}C was not incorporated into purines when the conditions described by FERNANDES [2] were used.

Cultures of the middle and end of the log phase were used for the studies of cyanide insensitivity. In the former case, cyanide and salicylhydroxamate (both at 10^{-3}M) did not inhibit completely the oxygen consumption; however, in the latter cultures 100% inhibition was obtained in the "mitochondrial fraction". The levels of peroxidase and catalase of both preparations are presently being studied.

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ULTRASTRUCTURE OF *TRYPANOSOMA CRUZI* MORPHOGENESIS

Regina MILDER and Maria P. DEANE

The complex life cycle of trypanosomes involves many morphological and physiological stages which can be correlated up to a certain point, with ultrastructural modifications.

We studied the electron microscopic aspects of trypanosomes in differentiation from bloodstream forms to cellular and acellular culture forms as well as bloodstream trypomastigotes.

During morphogenesis the modifications of the ultrastructure of *Trypanosoma cruzi* are connected with the acquisition or alteration of various organelles.

When transformation from bloodstream to culture forms takes place we often found concentric whorled membranes associated with the mitochondrial or kinetoplast envelope. These whorls were also observed in the transforming trypanosomes in *T. brucei* [1] and there is some evidence that these whorled membranes could represent different steps in the mitochondriogenesis [3].

The rough endoplasmic reticulum is well developed in the bloodstream forms particularly in stout forms and is seen frequently around the Golgi apparatus. We could sometimes see in bloodstream forms the so called subtending granular reticulum like the one observed in *T. congolense* [5], and suggesting the same kind of relationship described in the secretory cells. The smooth endoplasmic reticulum in all stages of *T. cruzi* is, as a rule, very scarce. When transformation takes place in culture from trypomastigote to epimastigote, there is a well developed smooth reticulum that is frequently seen close to mitochondria. The particular development of these smooth membranes at this stage of morphogenesis in unknown.

The Golgi complex is well developed, with a varying number of membranes and vesicles. An electron dense material could sometimes be detected alongside the Golgi membranes which may be related with secretion substances.

A great variety of vesicular and inclusion bodies scattered throughout the flagellates body are seen in certain stages of the life cycle of trypanosomes. In bloodstream forms we found the bacilliform bodies similar to those observed in *T. congolense* [5]. The presence of electron dense bodies inside specific organelles was found in the flagellar pocket of culture forms [2] and in the kinetoplast of bloodstream forms of the same strain.

The origin and function of the microtubules (sub-pellicular, flagellar and in the dividing nucleus) is still controversial, specially when morphogenesis takes place. The cytostome is observed in certain stages — amastigotes and epimastigote forms of *T. cruzi*. The appearance of the cytostome could be detected early in the development from trypomastigote to epimastigote in acellular culture as well as in tissue culture forms.

The bloodstream stages of *T. cruzi* have an uneven filamentous coat and in *T. cruzi Y* strain the filopodium process is present [4]. We have seen a fuzzy coat in bloodstream stages of *T. cruzi*, specially in a strain with predominance of stout forms and in the first 48 hours of a primary culture of *T. cruzi* (with predominance of slender forms) we observed the filopodium-like process. The existence of *T. cruzi* exoantigens as well as their possible correlation with these structures is still not well established.

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CYTOCHEMISTRY AND FREEZE-FRACTURE OF THE CELL MEMBRANE OF *TRYPANOSOMA CRUZI*

Wanderley de SOUZA, A. MARTINEZ-PALOMO and A. GONZALEZ-ROBLES

A regional specialization of the cell surface of *T. cruzi* culture forms was found at the cytostome as a localized thick surface coat rich in carbohydrate-containing components. The prominent surface coat was located over a region of the plasma membrane devoid of intramembranous particles. In turn, the particle free region is related to specialized submembrane fibrils not present under other regions of the plasma membrane. The cytostome region provides a striking example of a stable regional differentiation of the plasma membrane; the specialization is evident at the outer surface, at the hydrophonic membrane domain and at the submembrane cytoplasmic region. In addition, independence of concanavalin A receptors, colloidal iron binding sites and ruthenium red stainable surface components from membrane particles was demonstrated at the flagellar membrane.

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FINE STRUCTURE OF EPIMASTIGOTE FORMS OF *TRYPANOSOMA CRUZI* FROM AN ACELLULAR CULTURE MEDIUM

Wanderley de SOUZA, Noemia GRYNBERG and F. NERY-GUIMARÃES

The fine structure of the epimastigote forms of *Trypanosoma cruzi* maintained in an acellular culture medium is described. During division the nucleus shows a homogenous structure owing to the dispersion of its chromatin and nucleolar material. Microtubules similar to those of a mitotic spindle in metazoan cells appear, running from one pole to the other. During the whole process of division the nuclear membrane remains intact.

The cytoplasm shows vacuoles. With the cytochemical method of periodic acid thiosemicarbazide-silver proteinate, a polysaccharide surface coat is observed on the whole surface membrane system of *T. cruzi*. A positive reaction is also observed in the membrane of some vacuoles in the cytoplasm of the parasites.

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ANTIGENIC VARIATION IN BLOODSTREAM FORMS OF *TRYPANOSOMA CRUZI* FROM DIFFERENT STRAINS

A. U. KRETTLI and Z. BRENER

Agglutination and absorption tests of *T. cruzi* culture forms show the presence of three immunological types (NUSSENZWEIG et al. [2]). More recently, antigenic differences have been demonstrated between amastigotes, epimastigotes and bloodstream trypomastigotes of *T. cruzi*, by indirect immunofluorescence tests (KLOETZEL et al. [1]). Although there is increasing evidence that *T. cruzi* is a pool of populations inducing different clinical forms, which might be related with regional differences in the pathology of Chagas Disea-

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se, there are no means of grouping bloodstream trypomastigotes by immune reactions. In an attempt to detect immunological differences in bloodstream trypomastigotes we have studied several strains of *T. cruzi* isolated from patients (Y, B and J strains) and from naturally infected triatomids (CL and FL). All the strains have been maintained in laboratory by serial blood passages in mice, for different numbers of years.

The action of immune sera from animals with chronic Chagas Disease (anti-Y and anti-CL sera) on the bloodstream trypomastigotes was investigated. Parasites isolated in plasma of acutely infected mice were incubated at 33°C for 1 h with homologous or heterologous immune sera and then observed in fresh or stained preparations. The presence of clumps and the number of free parasites was determined and compared with that of parasites which were incubated with normal mouse serum. Strains could be easily divided in three groups as follows: a) those whose parasites were strongly agglutinated and lised by homologous or heterologous immune sera (Y and B strains), the highest titer of agglutination being 1:64 with both antisera; b) parasites which were not agglutinated by immune sera (CL and J), in presence or absence of complement; c) trypomastigotes which were only slighly agglutinated (FL strain) by the immune sera, forming a few clumps with small numbers of trypomastigotes.

The infectivity of trypomastigotes from two strains (Y and CL) was tested after their "in vitro" incubation with either homologous or heterologous immune sera. Y strain parasites had their infectivity significantly decreased while those from CL strain had their infectivity unaffected by the immune sera. In addition, we observed that sera from three patients with chronic Chagas Disease clearly agglutinated trypomastigotes of Y strain, two of these sera decreasing their infectivity.

It has been suggested that the variant-specific antigen(s) in African trypanosomiasis are located on the surface of the trypomastigotes and are involved in protection and agglutination (SEED [3]). Whether the present results reflect antigenic variation or only different antigenic types of *T. cruzi* is still a matter for investigation. However, bloodstream trypomastigotes might represent a more suitable model for the studies of intra-specific variation and immunological typing of *T. cruzi*.

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MECHANISMS OF IMMUNITY IN CHAGAS DISEASE

A. U. KRETTLI and Z. BRENER

Malaria has an immunosuppressive effect on the host, which is believed to be related to the severe depletion of B lymphocytes (KRETTLI & NUSSENZWEIG). In an attempt to investigate the possible interference of malaria with the mechanism(s) which control the immune response in the chronic phase of Chagas Disease, *Plasmodium berghei* malaria has been inoculated in *T. cruzi* chronically infected mice. The chronic infection in the experimentally infected mice was developed either spontaneously, by treating them with nitrofurans, or after exposing infected mice to high temperatures (35°C). Animals inoculated with Y and CL *T. cruzi* strains 2 to 12 months previously, were injected i.p. with 1-2 x 10^5 red blood cells infected with *P. berghei*. Daily fresh blood preparations were then made to determine the number of trypomastigotes after the malaria infection. We observed that 12 out of 29 (41%) mice chronically infected with CL strain developed a new acute phase with high parasitemias. However, after several experiments, a total of

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44 mice which had been inoculated with Y strain remained in the chronic phase after the malaria infection and the number of bloodstream trypomastigotes was not affected. Similarly, the use of strong immunosuppressive agents such as gamma rays and cyclophosphamide interferes with the steady balance between host and CL parasites but has no effect on the chronic phase of Y-inoculated animals (BRENER & CHIARI [1]). On the other hand, bloodstream forms from these two strains have different susceptibility to antibodies (see our previous abstract).

It has been shown that malaria immunosuppression affects humoral immunity but does not interfere with the host cellular immune mechanisms (GREENWOOD [2]). Thus, our results suggest that apparently, different mechanisms control the immune response in the chronic phase of Chagas Disease produced by different strains. This immunity is: a) very strong with strains like Y, and cannot be suppressed either by X-rays, immunosuppressive drugs or by diseases like malaria, which mostly interferes with humoral immunity; b) apparently very weak with CL strain, new outbreaks of acute parasitemia being observed in a high percentage of animals chronically infected and submitted to the action of those three immunosuppressive agents.

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ANTIGENIC STRUCTURE AND TYPING OF *TRYPANOSOMA CRUZI* PATHOGENIC MECHANISMS INVOLVED IN THE PRODUCTION OF LESIONS

Antonio R. L. TEIXEIRA

Subcellular fractions of *T. cruzi* have been tested for antigenic properties. TEIXEIRA & SANTOS BUCH [3] used trypomastigotes and amastigotes derived from tissue culture which were fragmented in a high rotation tissue homogenizer. Subcellular fractions were obtained by differential centrifugation and their antigenicity was characterized in rabbits. In general, the soluble supernate of the homogenates, the cytosol, was able to induce high titers of humoral antibodies as well as an immediate type of skin response. In contrast, the particulate antigens in the subcellular microsomal fractions reacted with lymphocytes from rabbits with chronic Chagas Disease eliciting strong delayed hypersensitivity reactions. These experiments were carried out with the Ernestina strain of *T. cruzi* and further studies with other strains are needed.

In acute Chagas Disease many lesions seen in various organs and tissues are probably related to the presence of parasites (TEIXEIRA et al. [5]). However, some lesions of acute Chagas Disease might not be so straightforward and directly produced by the parasites. For instance, there are quiescent parasitisms of tissue cells contrasting with inflammatory lesions in which parasites are not seen. It is very likely, therefore, that the latter type of lesion is produced by immunological means, like those described in the chronic phase of the disease.

In chronic Chagas Disease the lesions seen in the heart and in the digestive tract are characterized by diffuse lymphocyte infiltrates in the absence of parasites *in situ* (TEIXEIRA et al. [4]). Many Authors have looked upon the-

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se lesions and considered them as produced by an altered "allergic" state of the host. Recently, Cossio et al. [1] reported on the presence of striated muscle fibers and endothelial cells in ninety-five per cent of patients with Chagas' heart disease. By absorption experiments they suggest that there is an antigenic system present in both mammalian tissues and in *T. cruzi*. Yet, the role of these antibodies in the pathogenesis of the disease is not clear. Santos Buch & Teixeira [2] cultivated foetal rabbit heart cells in the presence of rabbit anti-*T. cruzi* serum and did not observe any toxic effect to the cultured heart cells. Furthermore, when *T. cruzi* sensitized-lymphocytes derived from rabbits with chronic Chagas' disease were incubated with allogenic foetal rabbit heart cells, destruction of the normal heart cells was observed. The latter Authors demonstrated the presence of an antigenic determinant common to both, heart cells and *T. cruzi*. Santos Buch & Teixeira [2] believe that the recognition of a cross-reactive antigen of host cell with *T. cruzi*-sensitized lymphocytes leading to target cell destruction might be the pathogenic basis for subsequent host cell injury in chronic Chagas Disease.

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INTERACTION OF PERITONEAL MACROPHAGES AND *TRYPANOSOMA CRUZI*

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Peptone-stimulated mouse peritoneal cells were cultured on coverslips in medium 199 containing 30% (v/v) calf serum at a 5% CO_2 atmosphere at 37°, during 24 hours. Colchicine dissolved in PBS and diluted in medium 199 was added into the cultures in order to give a final concentration of 3 x 10^{-4} M.

Equal numbers of *T. cruzi* strains "Y" and "F" were used. Epimastigotes were harvested from stationary phase in LIT medium whereas trypomastigote forms were obtained from VERO cultures. As a control 0.1% EA (sheep erythrocytes x antibodies) was used. Cultured macrophages treated or non treated with colchicine were inoculated with epimastigotes, trypomastigotes or EA during 1 h at 37°. After this period the monolayed macrophages were fixed in Bouin and stained with H. E. Both forms of *T. cruzi* were found attached to and inside the cells, even in the colchicine treated cells. EA, on the other hand, was found attached and engulfed only by untreated macrophages. These experiments suggest that those forms penetrate the macrophages by a mechanism different from that working in phagocytosis.

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STUDY OF THE HOST-PARASITE RELATIONSHIP AT PHAGOSOME-LYSOSOME LEVEL

A. OLIVEIRA LIMA and M. QUEIROZ JAVIERRE

There are several mechanisms by which parasites can evade the vacuolar system of the phagocytic cell and survive in the cytoplasm: 1) preventing the attachment to macrophage membrane; 2) avoiding triggering the ingestion process; 3) preventing the phagosomes-lysosomes fusion; 4) resisting destruction by lysosomal hydrolases; 5) inducing lysis of phagolysosome membranes. The mechanism by which *T. cruzi* escape from the vacuolar system of macrophages is not yet understood. Apparently the parasite is easily ingested by macrophages, but how it can induce phagolysosome lysis and survive in the cytoplasm is not known. When ingested by "activated" macrophage, in the presence of specific antibodies, great percentage of parasites are killed, but the organisms found outside intracellular vacuoles may escape destruction.

Studies now in progress on the kinetic of phagosome-lysosome fusion after staining the lysosomes with acridine orange, following the fluorescence technique described by HART & YONG [1] might help clarifying some points on the mechanism by which parasites can evade the vacuolar apparatus of macrophages. Preliminary *in vitro* assays using *T. cruzi* and peritoneal mouse macrophages led us to recommend this new method as a valuable tool for studying the host-parasite relationship at phagosome lysosome level.

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CIRCULATING ANTIGENS IN MICE ACUTELY INFECTED WITH *TRYPANOSOMA CRUZI*

Fausto G. ARAÚJO

Circulating antigens in plasma of mice acutely infected with *Trypanosoma cruzi* strains "Y" and "FL" were detected through counterimmunoelectrophoresis, thus confirming previous reports [1, 2]. The antigens were detected using serum of mice or rats chronically infected with the strain Y of the parasite as source of antibody. Plasma of mice acutely infected with the FL strain of *T. cruzi* consistently gave 2 precipitin lines when reacting against serum of animals chronically infected, whereas plasma of mice infected with the Y strain yielded only one precipitin line when reacted against the same chronic serum. Circulating antigens were present only during the acute phase of the infection when parasitemia averaged 3×10^4 trypomastigotes per 5 cmm. The antigens were first detected 5 days after infection of mice with 1×10^5 blood forms of any of the strains employed. The amount of antigens appeared to increase up to day 12 when all animals died due to the infection. Circulating antigens were not detected in plasma of mice chronically infected with *T. cruzi*. Absorption of chronic serum with live blood trypomastigotes or culture epimastigotes completely removed the antibodies which react with circulating antigens. Of 36 samples of human sera from patients known to be parasitized with *T. cruzi*, 19 reacted against plasma Y, 10 against plasma FL, 8 against both Y and FL, and 15 did not react. An interesting finding was that some of the human sera had an immunofluorescent titer for Chagas Disease of 1:512 but did not react in the counterimmunoelectrophoresis test with either Y or FL plasmas.

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INTERACTION OF PERITONEAL MACROPHAGES AND *TRYPANOSOMA CRUZI*

Regina MILDER, JUDITH KLOETZEL and Maria P. DEANE

The interaction between macrophages and bloodstream and culture forms of two trypanosome strains was observed by optical and electron microscopy.

Unstimulated hamster or mouse peritoneal cells were cultured in medium 199 containing 10% inactivated calf serum. We studied bloodstream forms of *Trypanosoma cruzi Y* strain (with a predominance of slender forms) and *T. cruzi F* strain (with a predominance of stout forms). The culture forms were from *T. cruzi Y* strain containing approximately 90% epimastigotes and 10% trypomastigotes.

Macrophages were infected by overlaying the cell monolayer with trypanosome suspension. After 1 hour contact, macrophage cultures were repeatedly washed and reincubated with fresh medium. The material was then either fixed immediately or after 12 to 72 hours of incubation.

After one hour of contact with bloodstream forms a striking difference between the two strains was observed. In this initial stage, *T. cruzi F* strain trypomastigotes, which have immunoglobulin on their membrane, show a diversity of aspects. Most of them are enclosed within large vacuoles presenting various degrees of degeneration and the ones which survive and multiply are probably the few remaining slender forms. After trypanosome destruction the macrophage recovers completely showing a normal aspect.

The intracellular forms of *T. cruzi Y* strain at this early stage do not suffer any appreciable transformation and are enclosed within a phagosome membrane.

During the subsequent hours trypanosomes from *T. cruzi Y* strain undergo the process of differentiation into epimastigotes or amastigotes and start active multiplication eventually filling the whole host cell. At this stage phagosome membrane can no longer be visualized and parasites are localized within the host cell cytoplasm.

With respect to culture forms, the parasites initially seem to be immersed within the host cell cytoplasm. After longer periods these culture forms degenerate outside or inside phagosome vacuoles. These last ones probably come from the free flagellates which are ulteriorly phagocytised.

Some parasites, immersed within the host cytoplasm are able to survive and to differentiate; they probably originate from the few metacyclic forms present in cultures.

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IMMUNOGLOBULINS ON THE SURFACE OF TRYPOMASTIGOTES

Judith KLOETZEL and Maria P. DEANE

Bloodstream trypomastigotes of *Trypanosoma cruzi F* strain were collected from mice at the peak of infection around the 25th-30th day, when they are predominantly of the broad form, washed 3 times in PBS, and fixed with formalin. When treated with fluorescein conjugated anti-mouse rabbit gamma globulins they show patchy staining.

The mouse blood containing these trypanosomes was seeded in LIT medium and incubated at 26°C. Daily samples were collected and treated as above. Initial differentiating forms — amastigotes and short epimastigotes — have immunofluorescence concentrated at one point, at the anterior pole. When they have evolved to epimastigotes they stain uniformely with intense bright fluorescence. This suggests that either antigenic determinants these forms have in common with trypomastigotes may have a distinct localization; or else the initial differentiating forms may be able to cap antibody-antigen-complement complexes, while epimastigotes are unable to do so. This point is being investigated.

Slender and intermediary forms of Y and Brasil strains, collected up to the 14th day of infection, showed no immunofluorescence when submitted to the same procedure.

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IMMUNOGENICITY OF RIBOSOMAL FRACTION AND RNA FROM *CRITHIDIA FASCICULATA* AND *TRYPANOSOMA CRUZI* AGAINST *T. CRUZI* INFECTION

Noema GRYNBERG, F. N. GUIMARÃES [1], F. T. de CASTRO and A. Oliveira LIMA

The immunogenicity of the ribosomal and RNA fractions isolated from *C. fasciculata* or *T. cruzi* and emulsified in complete Freund adjuvant was assayed in rabbits. All the ribosomal preparations tested induced humoral and cellular hypersensitivity. The RNA fractions did not show immunogenic properties in rabbits.

Immunization of mice with the same preparations was carried out with two subcutaneous doses of the immunogen (100 μg of RNA). Seven days after the last injection the mice were challenged with a suspension of *T. cruzi* (Y strain), containing 5 x 10^4 or 10^3 blood trypomastigotes.

The parasitemia which was manifested 5 days after the challenge was always significantly lower in pre-injected animals than in controls; there was no difference in survival time between control animals and animals injected with *C. fasciculata* fractions; all the animals died within 10-14 days of infection. 30% Of the mice injected with *T. cruzi* ribosomes or RNA survived 30 days after the challenge.

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TENTATIVE FOR GROUPING DIFFERENT *TRYPANOSOMA CRUZI* STRAINS IN SOME *TYPES*

Sônia G. ANDRADE

Parasite strains may be an important factor in the pathobiology of Chagas Disease. In one comparative study of different strains of *Trypanosoma cruzi*, it has been shown that when several parameters are considered together, the various strains can be well characterized in a few types [2]. The parameters were: evolution in mice, curves of parasitemia, morphology of blood forms, tissue tropism and histopathological lesions in the experimental animal. Those characteristics seem to be inter-related. There was, for example, a positive correlation between early and rapid multiplication of the parasite and the predominance of slender forms in the peripheral blood, as well as reticulotropism. On the contrary, the slow and progressive multiplication observed in some strains coincides with the predominance of broad forms and myotropism. Based on those observations we suggest that the parasite strains can be grouped into different types or standard patterns according to their behaviour. Based on the study of several strains of *T. cruzi*, three types were established: *Type I* — (Y and Peruvian strains): rapid multiplication, high and early peaks of parasitemia and mortality, predominance of slender forms and reticulotropism in the initial phase of the infection; *Type II* — (16 strains isolated in Bahia Sta-

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te — Recôncavo Baiano) : slow multiplication, irregular peaks of parasitemia between 12 and 20 days, predominance of broad forms throughout the infection but with a small number of slender forms in the initial phase, myocardiotropism and myorcardial lesions predominating; *Type III* — (Colombian strain) with slow multiplication, high peaks of parasitemia between 20 and 30 days of infection, low rates of mortality from the 50th day of infection, predominance of broad forms throughout the course of infection and skeletal muscle involvement predominating.

With a few groups of standard strains, it seemed possible to perform future comparative studies to investigate the importance of this factor in human infection. That would not be feasible if one considers as different strains every time trypanosomes are isolated from every human case or naturally infected animal or insect vector.

With this approach, we studied several isolates of *Trypanosoma cruzi* from a single and endemic area of Chagas Disease (Recôncavo Baiano) [1] and concluded that strains of this area fit in a single general pattern (*Type II*) whose members in experimental animals and autopsied patients, have a clear tropism for miocardium. It seems interesting to note that cardiac involvement is a predominant manifestation of Chagas Disease in this region.

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ON THE POSSIBILITY OF INCORPORATION OF HOST ANTIGENS BY BLOOD FORMS OF *TRYPANOSOMA CRUZI*

Sônia G. ANDRADE

The possibility of *Trypanosoma cruzi* incorporating host-antigens and thus evade the host immunological defenses was investigated by two methods: 1) by looking for the presence of host antigens on the surface of trypomastigote forms by immunofluorescence; 2) investigating the resistance to infection of mice previously sensitized with proteins from the host from which the infecting trypomastigotes were obtained (guinea-pig).

In the first experiment, trypomastigote forms from the Peruvian strain of *T. cruzi* were isolated from guinea pig blood by centrifugation and washed in pH 7.2 phosphate buffered solution, dry fixed and treated with an anti-guinea-pig serum produced in rabbits and revealed by a fluoresceinate anti-rabbit globulin. Results were negative.

In the second experiment, mice sensitized with guinea-pig antigen (intramuscular injections in complete Freund's adjuvant) were afterwards injected with *T. cruzi* trypomastigotes obtained from guinea pig and previously washed. No evidence of resistance was observed in the sensitized mice when compared with intact controls and controls sensitized with rat antigens. The presence of antibodies in the mouse sera, against guinea-pig antigen, was demonstrated by immunodiffusion (Ouchterlony technique).

These experiments fail to demonstrate the presence of host antigens incorporated on the surface of the blood forms of *T. cruzi* Peruvian strain.

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IMMUNOLOGICAL TYPING OF *TRYPANOSOMA CRUZI*

Judith KLOETZEL and Mário E. CAMARGO

The serum obtained from a rabbit immunized repeatedly with dead and live cultures of *T. cruzi, Y* strain, gave the following results in indirect immunofluorescence test, with epimastigotes and bloodstream trypomastigotes of the Y and Brasil strain, before and after absorption with Brasil strain epimastigotes:

Antigen	Titer	Titer after absorption with Brasil strain epimastigotes
Y epimastigotes	1:1.280	1:320
B epimastigotes	1:320	0
Y Bloodstream trypomastigotes	1:320	1:320
B bloodstream trypomastigotes	1:320	0

Thus, antigenic differences between these strains, which are A and B respectively, by NUSSENZWEIG & GOBLE [1] classification, were detected, and this difference is observed both in epimastigotes and trypomastigotes.

The same serum was reacted with epimastigotes of other strains giving the following results:

Epimastigotes antigen with strains	Titer	Titer after absorption with Brasil strain epimastigotes
Y	1:1.280	1:320
Brasil	1:320	0
Lazaro	1:640	1:40
Rio	1:640	1:160
S.M.J.	1:640	1:320
Montagut	1:320	1:160

Further strain classification studies are being carried out.

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DIFFERENTIATION OF *TRYPANOSOMA CRUZI* IN TISSUE CULTURES

Maria de Oliveira MUSACCHIO

With hanging-drop method of tissue culture for cultivation of *Trypanosoma cruzi* it was possible to observe all the stages of the intracellular cycle in the living cells, confirming histopathological findings. One trypanosome penetrates into one cell and the continuous observation of such cells showed the transformation into the epimastigote and eventually into the amastigote form. Repeated binary division of this form (every 12 hours) continued until the cell was filled and only then began the transformation into the trypomastigote which is completed in 24 hours. Then the cell ruptures, releasing the trypano-

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somes. The duration of the entire cycle is 4-5 days at 38-39°, but at room temperature it is somewhat delayed. Some cells rupture before the cycle is completed freeing immature forms which degenerate after a while. The number of the parasites formed in one cycle depends on the size of the host-cell. Several strains of *Trypanosoma cruzi* were used, all with the same results.

The ultrastructure of the amastigote forms of cell cultures showed a short flagellum so that the name "amastigote" proposed by HOARE & WALLACE [1] for these forms does not correspond to their real structure. They were called temporarily "spheromastigotes" [2].

Since chicks are refractory to the infection with *T. cruzi* [4] cultures of trypsinized heart cells of a 7-day chick were infected with trypomastigotes from the supernate of the blood of a mouse, infected with the Y strain. No parasitized cells were found after 5 days of cultivation. Cultures of macrophages from a 15 day chick, infected simultaneously with the same material showed intracellular forms after 5 days of cultivation but no intra-or extracellular trypomastigotes [3].

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CULTIVATION OF *TRYPANOSOMA CRUZI* IN DEFINED MEDIUM

Maria Hermelinda MUNDIN, H. P. AZEVEDO, C. ROITMAN, Maria I. C. GAMA, A. C. MANAIA, J. O. PREVIATO and I. ROITMAN

Attempts to grow the "Y" strain of *Trypanosoma cruzi* in a defined medium based on a culture medium developed to grow *T. brucei* [2] are being carried out.

In such medium the growth of the Costa Rica strain of *T. cruzi* [1] was obtained.

A good growth up to the 7 th transfer was obtained in a medium similar to one described by CROSS & MANNING [2], but without Coenzimes Q_6 and Q_{10}.

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BIOLOGY OF *TRYPANOSOMA CRUZI* IN CULTURE

Egler CHIARI and Anibal S. A. PEREIRA

The possibility for growing *T. cruzi* in HX 25 [1], a chemically defined medium developed by CROSS & MANNING [2] for *T. brucei* opened up new possibilities for biological and biochemical studies of this protozoan.

At present we have undertaken the use of this for *T. cruzi* culture *in vitro*. This research is aimed at developing the essential conditions for this culture: a) determination of minimum medium required; b) osmolarity; c) temperature; d) maintenance in serial passages. Once fundamental parameters have been established, the research work in biology, physiology and biochemistry will be much easier.

We have used "Y", "MR", "GILMAR", and "TD" strains (originally used by ANDERSON & KRASSNER [1] and kindly given to us by Dr. R. Zeledon from Costa Rica). A month later we had cultures in the 6th successive passage. We observed quantitative differences in the behaviour of the different strains as to growth and differentiation, compared with cultures kept in LIT and Warren medium.

The *T. cruzi* previously isolated in LIT medium by hemocultures from human Chagas Disease patients and then immediately transferred to HX25 medium showed a good growth rate. New isolations performed directly from bloodstream of *T. cruzi* infected mice in HX 25 were done at least for "Y" and "MR" strains and already allowed for 3 successive passages.

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TAXONOMIC POSITION OF *"LEPTOMONAS" PESSOAI*

I. ROITMAN, Z. BRENER, C. ROITMAN and E. W. KITAJIMA

Leptomonas pessoai [1], a trypanosomatid which protects mice from challenge with *T. cruzi*, was cloned by plating in a complex medium. The growth and morphology of the original strain and clones were studied in two different media (liver-infusion-tryptose and a defined medium [3]) at 28, 35 and 37°C. The original strain and the clones were able to differentiate from promastigote to epistomastigote forms and such differentiation rate was higher at 35-37°C than at 28°C. It was suggested to change the taxonomic position of *L. pessoai to Herpetomonas* sp. from *Zelus leucogramus* [2].

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INTRASPECIFIC VARIATIONS IN *TRYPANOSOMA CRUZI*

Z. BRENER

T. cruzi strains isolated from human patients by xenodiagnosis or direct inoculation of bloodstream forms (from acute cases) present different behaviour as regards their early adaptation to laboratory mice. Some strains readily adapt themselves to the vertebrate and induce in the first passages high parasitemia and mortality rates; other strains present very low parasitemia and the animals usually survive the infection regardless of the inocula size and number of blood passages in laboratory; finally, some strains produce high parasitemia only in new-born mice or animals submitted to x-ray immunosuppressive dosis, and are apparently unable to induce virulent infections in normal hosts. The meaning of those inherent characteristics has been discussed.

Strains maintained over a long period of time in the laboratory and well adapted to the vertebrate present, when examined under different criteria exhibited steady characteristics which provide to some of the populations specific "profiles". A detailed study of strains *Y* and *CL*, for instance, showed that they present different patterns as regards morphology of bloodstream forms, curves of parasitemia, mortality rates, growth in liquid media, susceptibility to chemotherapeutic agents, sensitivity of bloodstream forms to humoral antibodies, and sensitivity to immunosuppressive agents.

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POLYMORPHISM OF *TRYPANOSOMA CRUZI*

Maria P. DEANE, Judith KLOETZEL and Regina MILDER

The physiologic significance of morphologically distinct forms observed during *T. cruzi* infection has been difficult to determine due to technical problems involved in separating these forms. We have now succeeded in obtaining pure slender and pure broad populations using *Y*, *Brasil*, *F* and *MR* strains.

Slender populations are obtained by seeding bloodstream trypomastigotes in culture tubes containing LIT medium, leaving them undisturbed for 3 days at about 26°C. At that time all broad and intermediary forms have differentiated and divide while slender forms continue as such. These slender forms survive for another few days in LIT at 26°C, and then degenerate; they are able to penetrate cultured Vero and Hela cells, and evolve therein, at 35°C; they degenerate rapidly in medium 199 without cells, at 35°C; they probably penetrate macrophages and complete their cycle within them; most probably they are unable to infect triatomids and apparently bear no host immunoglobulins on their membranes.

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Pure populations of broad forms are obtained by seeding Vero or Hela tissue culture cells with bloodstream trypomastigotes. After 3 days at 35° C the supernatant contains only broad forms. These are unable to penetrate tissue cultures when transferred to new monolayers; however they are viable, differentiating in LIT medium at 26°C; they bear host immunoglobulins on their surface, as shown by direct immunofluorescence; they are rapidly destroyed by peritoneal macrophages *in vitro;* platelets adhere to them and at the same time trypanosomes are lysed rapidly. It seems that this lysis is mediated by antigen-antibody-complement complexes on the trypanosome surfaces, and the mechanism of this phenomenon is under investigation. In LIT medium at 26°C, broad trypomastigotes shorten within 3 hours, and their kinetoplast starts migrating; within 48 hours they form masses of roundish epimastigotes with a short flagellum, which then evolve to normal epimastigotes.

It is impossible to determine morphologically when intermediary forms have differentiated to a point where they are committed to become broad forms. They are able to penetrate and evolve in tissue cultures at 35°C; they are able to survive for a few days in medium 199 at 35°C, without cells, a few of them undergoing division; they are taken up by macrophages and evolve within them. When seeding a mixed population in LIT medium at 26°C, the slender forms behave as decribed. Two kinds of differentiation can be observed side by side; one as described for broad forms, the other, which would correspond to intermediary forms, results in amastigotes apparently arising from trypomastigotes folding upon themselves, forming small masses of amastigotes, different in aspect and size from masses evolving from broad forms. After 48 hours trypomastigotes are seen to emerge from these masses. Apparently they are able to give rise to another generation of amastigotes, before evolving into epimastigotes. It thus would seem that intermediary forms are facultatively intra or extracellular.

INFLUENCE OF TEMPERATURE ON THE DIFFERENTIATION OF *TRYPANOSOMA CRUZI* IN TISSUE CULTURE

Maria Sônia BERTELLI, R. GOLGHER and Z. BRENER

"Vero" cells grown in monolayer with 199 medium *plus* 1% sheep serum, were infected with culture forms from 7 different *T. cruzi* strains kept in "LIT" medium (liver infusion tryptose). The infected tissue cultures in floating cover-slips were maintained at 33° and 37°C and then removed on the 5th and 7th days of infection, stained by Giemsa and the percentage of cells harbouring amastigotes and trypomastigotes determined microscopically. With three strains (CL, Berenice and Buriti) a significant decrease of the percentage of trypomastigote-harbouring cells was observed at 37°C when compared with cultures kept at 33°C. The inhibition of the intracellular differentiation at the higher temperature seems to be a strain-dependent characteristic since the other four *T. cruzi* strains (Y, MR, JAT and Gilmar) readily developed into intracellular trypomastigotes at both temperatures.

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PROTEASES AMONG TRYPANOSOMATIDAE

SHIDUCA ITOW and E. PLESSMANN CAMARGO

The cultural forms of all genera of Trypanosomatidae have been screened for the detection of proteolytic activities. It was found that all genera present similar enzymes.

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PROTEASES IN CELL EXTRACTS OF *TRYPANOSOMA CRUZI*

E. PLESSMANN CAMARGO and SHIDUCA ITOW

At least four different proteolytic activities have been disclosed in homogenates of epimastigotes cultural forms of *T. cruzi*. Some of their physical and chemical properties have been studied.

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