Studies on the Virulence and Attenuation of *Trypanosoma cruzi* Using Immunodeficient Animals

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Tissue invasion and pathology by Trypanosoma cruzi *result from an interaction between parasite virulence and host immunity. Successive* in vivo *generations of the parasite select populations with increasing ability to invade the host. Conversely, prolonged* in vitro *selection of the parasite produces attenuated sublines with low infectivity for mammals. One such subline (TCC clone) has been extensively used in our laboratory as experimental vaccine and tested in comparative experiments with its virulent ancestor (TUL). The experiments here reviewed aimed at the use of immunodeficient mice for testing the infectivity of TCC parasites. It has not been possible to obtain virulent, revertant sublines by prolonged passaged in such mice.*

Key words*: Trypanosoma cruzi* - immunosuppression - athymic mice - attenuation

Clinical observations on the progress and modalities of *Trypanosoma cruzi* infection in immunosuppressed patients have substantially contributed to elucidate pathogenic mechanisms in Chagas disease (Andrade et al. 1987, Kier-zenbaum & Sztein 1994, Sartori et al. 1995) and to establish norms for follow up and treatment in immunocompromised patients (González Cappa & Barousse 1988). While the conclusions of clinical work result mostly from retrospective analysis, animal models of disease and immunosuppression have allowed a fertile field of prospective experimentation. Mice and rats have been mostly used for this purpose and the experimental systems have involved differential immunocompetence associated to various mouse and rat strains, to age of the host (Revelli et al. 1993), to drug induced immunosuppression (Andrade et al. 1987, Gonçalves da Costa et al. 1991) or to specific immunologic defects in mutant mouse strains (Calabrese et al. 1991, Tarleton et al. 1992). These systems have mostly been applied to analyse pathogenic mechanims (Calabrese et al. 1991, Tarleton et al. 1992, Kierzeenbaum 1994) or to facilitate the parasite isolation (Britto et al. 1996) and production (Gómez et al. 1996).

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The questions addressed by our group, using immunosuppressed mice as experimental tool, refer to the stability or possible modulation of virulence and attenuation as inherited traits of *T. cruzi* strains.

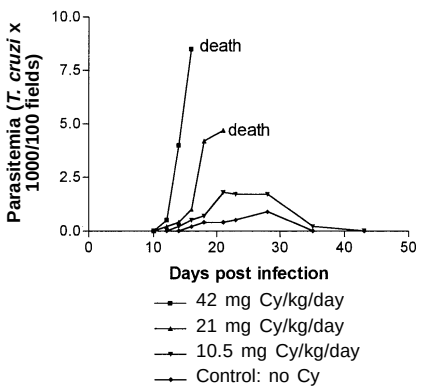
The Tulahuen (TUL) strain of *T. cruzi*, isolated in northern Chile, has been kept by our group in two sublines. A mouse passage (TUL) subline of high virulence, and a culture subline (TCC) maintained by uninterrupted *in vitro* culture and cloned twice since 1977. This clone is unable to infect in immunocompetent animals and was used as experimental vaccine in mice (Basombrío & Besuschio 1982) and in field trials against natural *T. cruzi* infection in guinea pigs (Basombrío 1990) and dogs (Basombrío et al. 1993). The stability or reversibility of attenuation in this "vaccine stain" was an issue of concern, considering the long term maintenance of virulence in other *T. cruzi* cultures (Chiari 1974) and, particularly, the reported reversion to virulence induced in an attenuated *T. cruzi* culture by passage in athymic mice (Leguizamón et al. 1993). Our attempts at selecting virulent revertants from the TCC clone by passage through immunologically incompetent mice are summarized in the next sections.

TCC cultures were kept in LIT medium. Harvests of these cultures consisted mostly of epimastigotes (non infective stage). However, the only stages taken into account in inoculation experiments were the transformed metacyclic trypomastigotes (infective stages) arising spontaneously or induced by adding triatome gut filtrates to the culture. For diagnosis of infection in mice, the main methods used were fresh blood mounts (FBM), xenodignosis (X) with 10 *Triatoma infestans* nymphs, hemocul-

ture (HC) and histopathological examination of tissue sections. The Reed-Muench method (Reed & Muench 1938) was used for estimating the 50% infective (ID 50) and lethal (LD 50) doses of trypomastigotes. To test the effects of immunodeficiency, we have used three methods: treatment with cyclophosphamide, use of newborn BALB mice and use of athymic mice.

Cyclophosphamide (Cy) - Administration of Cy at different dosages has been a practical way to obtain different degrees of immunosuppression for experimental purposes. There is a direct, proportional correlation between Cy dose and infectivity of the virulent TUL strain, as exemplified in the Figure: the higher the dose, the higher infectivity becomes. Mortality also increased in steps, from 0 to 100%. When these immunosuppressive regimes were tested comparatively, using virulent TUL (10^3 trypomastigotes) and attenuated TCC inocula (10^6 epimastigotes + 10^3 trypomastigotes), a sharp contrast in invasion was observed at all levels of immunosuppression. The percentage of positive findings, at each level (0, 10.5, 21 and 42 mg/kg/day of Cy) for TUL versus TCC were FBM: 100 vs 0 at all levels; MH: 100 vs 0 at all levels; X: 100 vs 0, 100 vs 29, 100 vs 50 and 100 vs 60; mortality: 0 vs 0, 20 vs 0, 100 vs 14 and 100 vs 29. Additional experiments indicated that the mortality found in the TCC inoculated mice with highest immunosuppression was due to intercurrent infections not related to *T. cruzi* and that all positive findings with this strain disappeared after day 30.

Newborn, BALB mice - Inoculation of newborn mice, in which the immune system has not reached full development, has been successfully used to isolate organisms of low infectivity (Gross 1950). We have observed that TUL parasites are highly invasive and lethal in this system, doses of 10^2 trypomastigotes being all infective and lethal.



Effect of Cyclophosphamide (Cy) on *Trypanosoma cruzi* infection in mice

Testing the TCC strain in this system, we have observed that parasitemias are not detected by FBM, but infection with 10^3 or more trypomastigotes can be detected by HC applied on days 10-20 post infection and not later. This allowed serial zig-zag passages of TCC between culture and mice.

Athymic mice - Homozygous mice, carrying both alleles of the mutant gene (Nu/Nu) are highly susceptible to exogenous organisms and easily acquire pathogenic infections when housed in conventional animal facilities. In our laboratory, the average lifespan of these animas is 4-6 months.

We have inoculated Nu/Nu mice and heterozygous controls with the attenuated and infective strains, injecting stepwise increasing numbers of trypomastigotes in order to calculate the 50% infective dose. We soon observed that 50% infective (as detected by parasitemia in FBM) and lethal doses were very high for TCC and very few parasites could be seen in blood (Table). However, negative FBM search in athymic mice did not indicate lack of infectivity, since in several FBM (-) athymic mice inoculated with the attenuated strain, tissue invasion by amastigotes could be detected after 40 days.

Effects of immunodeficiency on tissue parasitism - A close correlation exists, in virulent *T. cruzi* infections, between the number of trypomastigotes in blood and amastigotes in tissues (Laguens et al. 1980). When TUL trypomastigotes were inoculated into athymic mice these animals died within 25 days. Those that were autopsied and studied histologically had very high parasitemias and countless amastigotes in urinary bladder, heart, liver, spleen and muscle. Brain and lungs displayed much lower parasitism. Knowing that TCC parasites reached very low concentrations in blood of athymic mice and wishing to recover them for serial passage, a systematic quantitative search for amastigotes was done in several organs on days 5, 10, 15, 20, 26, 31 and 50 after inoculating of 53,000 TCC trypomastigotes. Parasites were hardly seen in blood at any time, or in tissues of the athymic animals up to day 15. From day 20 on, quantitative estimates (amastigote nests per mm^2 of tissue section) indicated a progressive increase, from 1 up to 47 nests. Spleen, brain or lung had a much lower parasite load than heart, urinary bladder and liver.

Effects of serial passage through immuno-deficient mice on the infectivity of attenuated, TCC strain, T. cruzi - *In vivo* serial passage of these parasites is technically difficult because they can hardly be recovered for further transfer generations. However, the above mentioned experiments indicated two ways in which this might be attempted: (a) zig-zag passage between culture and

TABLE

Fifty percent infective[a] and lethal doses (1D 50 and LD 50) of virulent (TUL) and attenuated (TCC) trypomastigotes in athymic, heterozigous, newborn BALB and adult Swiss mice

Parameter (attenuated)	Mice	TUL (virulent)	TCC
ID 50 (parasitemia)			
	Athymic (Nu/Nu)	19	85×10^3
	Heterozygous (Nu/Nu)	$< 10^2$	$> 10^6$
	Newborn BALB	$< 10^2$	$2{,}2 \times 10^6$
	Adult Swiss	$< 10^2$	$> 10^7$
LD 50 (mortality)			
	Athymic (Nu/Nu)	19	10^5
	Heterozygous (Nu/Nu)	ND[b]	ND
	Newborn BALB	$< 10^2$	10^6
	Adult Swiss	5×10^4	$> 10^7$

a: infectivity detected by fresh blood mounts. Does not correspond to results of parasite search with HC or histology; *b*: not done.

newborn BALB mice with recovery from the animals by hemoculture on day 15 and transformation of the culture to metacyclic stages before the next infection; (b) passage from one athymic mouse to the next using homogenates of amastigote-rich heart and liver after day 20 of infection. These two modalities of passage were systematically attempted in our laboratory.

Modality (a) allowed the development of a TCC subline (TCCR) passed in mice through eight generations. No differences were found between TCC and TCCR. Infectivity for mice, development in *T. infestans*, ability to invade heart and sekeletal muscle or ability to induce lytic antibodies remained as low or absent in TCCR as they had been in TCC. Moreover, the electrophoretic patterns of isoenzymes and the growth pattern in axenic medium showed no differences between the culture or the mouse-passaged sublines. In order to test if this method was efficacious to select virulent variants of *T. cruzi*, a 10^7: 10 mixture of TCC and TUL trypomastigotes was inoculated into mice. The first hemoculture generation developed highly virulent parasites, indicating the high selectivity of this system for virulent progenies. Modality (b) of the TCC passage was attempted in four lines (A, B, C and D) checking in histologic sections, obtained 30-40 days post infection, the success of each transfer. Line A was extinguished at the first transfer. Lines B and D succeeded for three generations and the fourth was negative. Line C extinguished on the second generation. Tissue amastigote numbers apparently diminished at each transfer. Conversely, Giemsa-stained blood smears and FBM from these animals indicated that, along with the amastigotes, bacteria were apparently transferred. This bacterial infection increased toward later generations and resulted in lethal septicemia in some animals, an observation consistent with the findings of Calabrese et al. (1991).

Genomic and biochemical analysis of *T. cruzi* is revealing a multiplicity of genes and functions which independently contribute to virulence (Pereyra 1996, Ajioka & Swindle 1996, Basombrío et al. 1996, Cortes et al. 1998). Very likely, several subfunctions which are necessary for infectivity may be non-essential for *in vitro* growth. *T. cruzi*, having a remarkably plastic genome and being subjected to long periods of growth in culture may suffer temporary reversible attenuation (Chiari 1974, Leguizamón 1993) as well as irreversible loss of virulence in selected cultures (Basombrío 1982, Rowland & Ritter 1984, Figueiredo et al. 1996), a possibility supported by experiments in immunosuppressed animals.

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