Why does GM1 induce a potent beneficial response to experimental Chagas disease?

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(Received 30 April 2008; accepted 10 December 2008; published online 21 January 2009)

Being one of the world's neglected diseases, Chagas has neither a vaccine nor a satisfactory therapy. Inoculation of murine models with the ganglioside GM1 has shown a strikingly nonlinear effect, leading to a strong decrease in parasite load at low doses but reverting to a load increase at high doses. Cardiocyte destruction concomitant with the disease is also significantly reduced by a moderate application of GM1. A mathematical model for the interaction between the parasite and the immune system is shown to explain these effects and is used to predict an optimal dosage that maximizes parasite removal with minimal cardiocyte destruction. [DOI: 10.2976/1.3067922]

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Chagas disease has been endemic in parts of Latin America for about 9,000 years. Recent research indicates that the sylvatic life cycle of *Trypanosoma cruzi* was probably well established when humans first peopled the coasts of northern Chile and southern Peru and began acting as parasite hosts (Aufderheide *et al.*, 2004). Today, Chagas remains the most important vector-borne disease in the Americas (Franco-Paredes *et al.*, 2006), although it has also been identified in Europe and North America, due mainly to blood transmission. Vector-borne cases of Chagas disease have been noted in the southern United States (CDC, 2007). The disease develops after infection with the unicellular flagellate *T. cruzi*, a parasite first described by the Brazilian physician Carlos Chagas in 1909 (Chagas, 1909). The World Health Organization estimates that 90–100 million people are at risk of infection and that 10–20 million are actually infected, with an annual incidence of 560,000. About 50,000 annual deaths result from chronic cardiomyopathy. Surveys indicate that in certain regions of Brazil up to 30% of adults die of *T. cruzi* infection (Rosas, 2000; Schmidt and Roberts, 2000; WHO, 2002). Sadly, almost a century after the discovery and characterization of its causative agent, there is neither an

effective vaccination nor a suitable therapy for the disease. As recently as 2006, The Lancet stated that Chagas is a “pressing, highly prevalent danger endemic to Latin America which the world research community continues to ignore” (Lancet, 2006). Auspiciously, the world health community seems to be setting a global strategy to eliminate this hitherto neglected disease (WHO, 2007), whose eradication will require a continuous and concentrated effort on prevention, and the development of an effective treatment.

The course of the disease can be divided into three stages according to clinical and biochemical examination: (1) Acute phase: This initial phase is characterized by anemia, loss of strength, muscle and bone pain, and nervous disorders. This stage is scarcely evident and, when detected, heart disorders usually remain hidden. The acute phase is more often detected in children under three years old (Rosas, 2000; WHO, 2002). This phase rarely results in the host's death. (2) Undetermined phase: After the acute period, although most patients appear to be cured, many remain infected, but with a generally undetectable parasitemia. Clinical symptoms are absent but the cardiac damage progresses slowly and unremittingly in about 60% of the originally infected population

(Rosas, 2000). (3) Chronic phase: After an undetermined phase of 15–20 years, the condition of 30–40% of the originally infected people worsens, and they undergo autonomous nervous dysfunctions that may lead to the loss of almost 80% of the cardiac ganglion cells. The heart muscle becomes flabby and greatly enlarged (Schmidt and Roberts, 2000), with low tone, ending in a severe dilated cardiomyopathy (Rosas, 2000). A particular feature of Chagas disease is that tissue damage seems too extensive considering the observed parasitemia, which suggests that deregulation of immune functions is relevant to pathogenesis.

The life cycle of *T. cruzi* presents four main morphological stages. Epimastigotes, which divide in the proximal gut of the insect vector, differentiate into metacyclic trypomastigotes in the distal gut. In the host, metacyclic trypomastigotes released in bug faeces invade cells at bite wound sites, where they differentiate into intracellular amastigotes. These amastigotes multiply by binary fission, filling the cell. Then they differentiate and burst out of the cell, being released into the bloodstream as circulating trypomastigotes. Therefore, an intracellular reproductive form (amastigote) and a blood-circulating infective form (trypomastigote) are the two main forms identified in the vertebrate host. The circulating parasites can infect cells in a variety of tissues (with marked preference for cardiocytes and smooth muscle) and start replicating at new infection sites. Replication resumes when the trypomastigotes invade another cell or are ingested by another vector, the main vector being *Triatoma infestans* (Reduviidae). Therefore, circulating trypomastigotes are the main form implicated in both tissue colonization and insect vector infection. Here we wish only to remark that, in experimental Chagas disease, Brener and Kretzli showed that specific antibodies control the infection, and that humoral specific response is the reason for parasite clearance ending the acute phase (Brener, 1980; Kretzli and Brener, 1976, 1982).

Numerous parasiticide drugs have been assayed in therapeutic protocols against Chagas disease. Nitroimidazole derivatives have been used for the treatment of either acute or recent asymptomatic chronic infections (Andrade *et al.*, 1996). These antiparasitic drugs generate free radicals (Docampo and Moreno, 1986; Maya *et al.*, 2007) that explain both their parasitocidal and undesirable side effects. The side effects are a major drawback for their use, frequently forcing the physician to stop treatment (Castro *et al.*, 2006). Among these drugs, benznidazole was the most used and the one that has been applied until recently, in spite of its low levels of efficacy. Such treatment is thought to prevent pathology during the late chronic stages of Chagas. Chemotherapy for human Chagas disease requires long-term administration, is toxic (Castro *et al.*, 2003), and does not eliminate the possibility of natural drug resistance in parasite populations without previous exposure to the nitroimidazole derivatives (Docampo and Moreno, 1985; Filardi and Brener, 1987; Murta *et al.*, 1998; Wilkinson *et al.*, 2008). A consid-

erable percentage of patients treated with benznidazole during the acute phase of *T. cruzi* infection are diagnosed to be cured on the basis of negative hemocultures, xenodiagnoses, and serological analyses (Galvão *et al.*, 1992; Kretzli *et al.*, 1984). In experimental murine infections, the results of chemotherapy vary with parasite and mice strains (Filardi and Brener, 1987).

In the search for new therapeutic approaches, Luján and collaborators suggested that ganglioside treatment inhibits the parasite's phospholipase A2 enzymes (PLA2, then an unreported enzyme), which they supposed to be involved in membrane destabilization. Survival at day 30 was 96% for mice treated with a mixture of bovine brain gangliosides. Hearts from untreated animals showed acute chagasic myocarditis, while those from mice treated with gangliosides presented only minor mononuclear infiltration (Luján *et al.*, 1993). However, since total brain gangliosides were not toxic to the parasite, either in xenic or axenic cultures, it seems unlikely that their action *in vivo* relates to their inhibition of PLA2 (Bronia *et al.*, 1999). Cossy Isasi and co-workers (Cossy Isasi *et al.*, 1999) focused on the identification of the effective molecule in ganglioside mixture. They reported that the monosialoganglioside GM1 was effective to control parasitemia and enhance survival in mice infected with a lethal amount of parasites. Intramuscular treatment with 0.1 mg of monosialoganglioside GM1 during the acute phase reduced parasitemia to undetectable levels, restored normal electrocardiographic records, and led to infected mice surviving for one year more than the uninfected untreated controls. Histological examination of different organs failed to show amastigote nests after treatment.

In order to formulate more precisely the relevant questions and to make predictions about the disease dynamics, we developed a model that describes the competitive interaction between *T. cruzi* and the immune system (Cossy Isasi *et al.*, 2001). The model accurately predicts all possible outcomes of the disease: healing, death, and chronic infection, with stationary or quasi-cyclical populations (Condat *et al.*, 2003). It also reproduces experimental data and permits us to estimate the damage generated by direct parasitic action (Sibona *et al.*, 2007). This model was used to investigate diverse situations of interest, such as the action of parasite-released decoys, the influence of immune reaction delays, and the effects of an increase in the antibody efficiency (Sibona *et al.*, 2005). Since the mechanisms of GM1 action have not yet been elucidated, we now challenged our model with data from the experiments in Cossy Isasi *et al.* (1999), in order to uncover clues to their nature. Therefore, in this paper we extend our model of *T. cruzi* infection to describe observations of mice recovery from acute lethal infection when treated with GM1 ganglioside. One characteristic of the treatment is that GM1 at low doses drives the system towards parasite clearance whereas, at higher doses, the parasitemia increases to values above those lethal in nontreated mice. One possible

explanation may be that parasite and immune cells share a common molecular target for GM1. At low concentrations, GM1 is more accessible to circulating parasites and growth is impaired. Such molecular target could be calmodulin, an enzyme that is present in both parasites and host cells. There is evidence that gangliosides bind to calmodulin in the cytosol, thus preventing its action as enzyme regulator (Higashi and Yamagata, 1992; Higashi *et al.*, 1992). The inhibition of parasite calmodulin impairs the invasion of host cardiocytes (Yakubu *et al.*, 1994). Host calmodulin is essential to the process of lymphocyte activation (Ishiguro *et al.*, 2006; Pan *et al.*, 2005) and to cytotoxic function (Lin *et al.*, 2005; Poggi *et al.*, 2002). Since different gangliosides enhance or inhibit the calmodulin-dependent cascade at the same concentration (Zhao *et al.*, 2004; Duan *et al.*, 2006; Chen *et al.*, 2003; Slevin *et al.*, 1999), the same concentration could inhibit the *T. cruzi* enzyme while activating the isoform in host cells.

THE MODEL

Trypanosoma cruzi presents characteristics common to extracellular and intracellular parasites. From a schematic perspective, we hypothesize that the population of circulating parasites is controlled mainly by antibodies, noting that Krettli and Brener showed that specific antibodies control the infection, and that humoral specific response is the reason for parasite clearance ending the acute phase (Krettli and Brener, 1982). Specific antibodies are induced by circulating parasites, so the population of antibodies evolves following the evolution of parasite density in plasma. Immunoglobulins bind to the parasite surface and mediate parasite removal through lysis when antibodies are recognized by complement or through phagocytosis when antibodies form a shield on the parasite surface (opsonization) and are recognized by phagocytic cells. Since blood parasites are the infective forms, antibodies become a central part of the host defense against *T. cruzi*. Our basic model is built upon the following assumptions:

- (A1) A parasite batch invades, or is inoculated into the mammalian host.
- (A2) Trypomastigotes invade healthy cells, where they mutate into amastigotes and reproduce.
- (A3) Newborn parasites break out of the cell and restart the invasive process.
- (A4) Parasites activate antibody generation
- (A5) One or more antibody species mediate extracellular parasite removal.

These assumptions are embodied in a set of first-order equations that describe the evolution of the various populations (Sibona *et al.*, 2005; Sibona *et al.*, 2007). If N antibody species are present, the equation for the total number of circulating trypomastigotes, $n(t)$, is

$$\frac{dn(t)}{dt} = \eta N_r r(t) - n(t) \sum_{i=1}^N \alpha_i(t) A_i(t) / Z - \zeta n(t). \quad (1)$$

The first term on the right-hand side accounts for parasite generation; there η is the *cytotoxicity*, i.e., the probability per unit time that an infected cell will burst due to the large number of parasites in its interior; N_r is the mean number of trypomastigotes that emerge from the ruptured cell, and $r(t)$ is the number of infected cells. It is important to note that N_r is determined by the interaction between the invaded cell and the parasites within and is independent of parasite infectivity and immune efficiency. The middle terms correspond to antibody-mediated parasite killing, with Z being the average number of single molecules that are removed by a single parasite, A_i the number of antibody molecules of the i th species, and α_i the removal efficiency of these antibodies. The last term corresponds to parasites leaving the bloodstream to infect target cells; there ζ is the *infectivity*, the rate at which circulating parasites penetrate host cells. The source of blood parasites is the population of infected cells, whose time dependence is controlled by the competition between the infection and rupture rates,

$$\frac{dr(t)}{dt} = \zeta n(t) - \eta r(t). \quad (2)$$

The parameters Ω and N_r can be related to the mean amastigote duplication time ω by considering a Malthusian model for the amastigote replication dynamics (Sibona *et al.*, 2005; Sibona *et al.*, 2007). A simple calculation yields

$$\Omega = (1/\eta)(\ln 2/\ln N_r). \quad (3)$$

The equation for the variation in the antibody number is

$$\frac{dA_i}{dt} = \frac{1}{\tau_i} [A_{i0} - A_i(t)] + G_i n(t) - \alpha_i A_i n(t). \quad (4a)$$

As discussed in Cossi Isasi *et al.* (2001), it is convenient to rescale the antibody populations by writing $a_i = A_i/Z$. The equation for the rescaled number of antibody molecules is then

$$\frac{da_i}{dt} = \frac{1}{\tau_i} [a_{i0} - a_i(t)] + \gamma_i n(t) - \alpha_i(t) a_i(t) n(t), \quad (4)$$

where a_{i0} represents a continuous source exactly compensating for the spontaneously inactivated antibody molecules in the absence of parasites. Antibodies with a lifetime τ_i [the first term in Eq. (4)] are produced by B cells whose receptors have bound the antigen with their receptors and have been activated by T cells. This occurs at a rate γ_i , is induced by circulating parasites $n(t)$, and continues until the parasites have been completely removed, unless the antibodies cross-react with host molecules. In fact, a Chagas patient is said to be cured of the infection only when specific antibodies are undetectable. The source term must depend on the parasite number because (i) a constant source cannot control the

parasites and (ii) when the parasite numbers decrease, antibody generation must decrease, too (El Bouhdidi *et al.*, 1994). We have chosen for this term the simplest form consistent with the available data. The third term in Eq. (4) describes the sequestration of antibody molecules as a function of their efficiency α_i , which accounts for complement fixation, opsonization, and antibody-dependent cellular cytotoxicity. Since antibody avidity augments with time, α_i is a smoothly increasing function of time,

$$\alpha_i = \alpha_{Ai} + \alpha_{Bi}[1 - \exp(-t/T_i)]. \quad (5)$$

This equation describes a “learning” process, a continuous increasing avidity of IgG antibodies for their antigen as the infection takes course, from an initial value α_{Ai} to a saturating value $\alpha_{Ai} + \alpha_{Bi}$, over a learning time T_i . The learning times were obtained in Cossi Isasi *et al.* (2001) from fits to the experimental results of El Bouhdidi *et al.* (1994).

NUMERICAL ANALYSIS

Numerical solutions to the set of equations giving the time dependence of the parasite, antibody, and infected cell populations were obtained following Euler’s method. Some outstanding features are (Sibona *et al.*, 2005 and Sibona *et al.*, 2007):

- (1) The cytotoxicity η determines the severity of the symptoms leading to congestive heart failure as η increases.
- (2) The relevance of the initial inoculation size can be evaluated once a second antibody species is introduced.
- (3) The short-time system evolution is not necessarily a good predictor of the final result: Host death can occur after a short dominance of antibodies or host healing can occur after parasite dominance.
- (4) We can now explain why apparently less aggressive parasite strains that induce lower parasitemias may become more harmful. As the infection progresses, these strains cause more direct damage due to higher infectivity, with the immediate consequence of immune evasion and more cells infected and destroyed by parasite bursting. They also cause indirect damage since infected cells induce cellular cytotoxic immunity with inherent tissue destruction. We note that disrupted cells become antigens driving the immune response towards self-antigens. Our results are in accordance with experimental observations, particularly those of Andersson, Örn, and Sunnemark, who showed that both the parasite and the host strains are important in determining the outcome of Chagas’ disease (Andersson *et al.*, 2003). (They used Tulahuen and CA-1 parasites and CBA/J and BALB/C mice.) Our model reproduces the evolution of the parasite populations and predicts the importance of the cell damage qualitatively observed by these authors.
- (5) Another important feature of the model is that it reveals the contribution of the latency period after cell entrance and the onset of replication. Different strains or one

Table I. Fluorescence anisotropy (ρ) of *T. cruzi* plasma membrane after addition of gangliosides to culture medium. GM1 was added and ρ determined simultaneously. In the cases marked with an asterisk (*), ρ was determined after 18 h of GM1 preincubation. The microviscosity parameter $\rho_{DPH} = (\rho_0/\rho - 1)^{-1}$ is calculated from fluorescence depolarization measurement using diphenyl-hexatriene as a fluorescent probe.

Treatment	ρ	ρ_0/ρ	ρ_{DPH}
Control	0.1199±0.002	2.2527	0.4439
100 μ M	0.1245±0.002	2.1325	0.4689
200 μ M	0.1236±0.002	2.1553	0.4639
300 μ M	0.1326±0.0003	1.9411	0.5151
Control*	0.1070±0.001	3.6392	0.3789
300 μ M*	0.1422±0.0007	1.7426	0.5738

strain in different tissues may initiate intracellular replication immediately after cell entrance or may be arrested for a variable period of time. When latency periods are larger than antibody lifetime, those parasites generate more persistent infections.

- (6) The values of the antibody parameters were obtained in Cossi Isasi *et al.* (2001) from fits to the data of El Bouhdidi and co-workers (El Bouhdidi *et al.*, 1994). Since these fits indicate that three antibody species (IgG1, IgG2a, and IgM) are needed to reproduce the parasite evolution data, we have used three antibody species in our simulations.

GM1 action

The initial experiments with GM1 reveal a therapeutical threshold dose of 0.1 mg/mouse (Cossi Isasi *et al.*, 1999). Doses of less than 0.1 mg lowered parasitemia and doses over 0.1 mg increased parasitemia, although it was observed that treated mice died at parasitemia values two or three times higher than those registered for untreated mice.

We have performed fluorescence experiments to test the influence of GM1 on parasite and myocardiocyte membrane fluidity. Student’s “t” test was applied to the data analysis, the significance level being set at $p < 0.05$. Table I lists the fluorescence anisotropies of DPH [diphenylhexatriene] of living parasites’ plasma membranes immediately after the addition of GM1 and after 18 hours of incubation with GM1. The addition of increasing GM1 amounts results in moderate but significant increments in fluorescence anisotropy. When the parasites were pre-incubated with GM1 for 18 hours, the increment of fluorescence anisotropy was more pronounced. It is important to note that the observed values were obtained in the absence of GM1 in the assay buffer and that parasites had been previously thoroughly washed with PBS [phosphate buffered saline]–Na CL to remove externally bound gangliosides. The possibility that GM1 behaved as detergent was

Table II. Microviscosity variation in mice cardiomyocytes plasma membranes as result of *T. cruzi* acute lethal infection and/or GM1 treatment. ρ_{DPH} : fluorescence anisotropy parameter, microviscosity. Results are expressed as mean $\pm$ SE.

Groups	Sample size	ρ	ρ_0/ρ	ρ_{DPH}
Infected	25	0.13 $\pm$ 0.0002	2.27846	0.56
Uninfected	25	0.17 $\pm$ 0.00003	2.1294	0.88
Infected+GM1	25	0.11 $\pm$ 0.0002	3.2909	0.43
Uninfected+GM1	25	0.12 $\pm$ 0.00004	3.0166	0.49

discarded because treatment with 64 μ M of Triton X-100 diminished anisotropy and higher concentrations caused total dispersion of the plasma membrane.

Table II describes the microviscosity variation in cardiomyocyte plasma membranes obtained from mice that were either infected or not infected, and treated with 0.15 mg GM1 daily or left untreated. Cardiomyocyte membranes become more fluid after infection. GM1 treatment increases membrane fluidity both in infected and noninfected cardiocytes. It should be noted that GM1 was not added directly to pure membranes or cardiocytes during the determinations in these experiments. The values reflect the modifications in the parameter caused by GM1 injected in living mice.

Modeling GM1 action

Next we modify our model to account for the effects of GM1 on the parasite population. We add two new assumptions:

- (A6) *T. cruzi* penetration ability is impaired by GM1 addition to the parasite membrane.
- (A7) Cardiac cell resilience is increased by GM1 addition to the cell membrane.

If parasite penetration is impaired, the infection term $\zeta n(t)$ in Eqs. (1) and (2) must be corrected:

$$\zeta n(t) \rightarrow \zeta_0[1 - f([GM1])]n(t), \quad (6)$$

where ζ_0 is the infectivity in the absence of GM1, and f is a nonnegative function of the GM1 concentration that describes the reduction in the infectivity. It seems reasonable to assume that higher cell resilience will lead to a moderate reduction in the cytotoxicity which, according to Eq. (3), leads to an increased number of parasites released in each cell burst if the duplication time is not modified. We thus propose to modify the parasite generation term in Eq. (1) as follows:

$$\eta N_r r(t) \rightarrow \eta_0[1 - g([GM1])]N_r(\eta_0, [GM1])r(t), \quad (7)$$

where η_0 is the cytotoxicity in the absence of GM1, g is a nonnegative function of the drug concentration, and the number of trypomastigotes released per cell burst is

$$N_r = 2^{1/[\Omega \eta_0(1-g)]}. \quad (8)$$

With Eqs. (6) and (7), the equation describing the trypomastigote dynamics becomes

$$\frac{dn(t)}{dt} = \eta_0[1 - g([GM1])]N_r r(t) - n(t) \sum_{i=1}^N \alpha_i(t) a_i(t) - \zeta_0[1 - f([GM1])]n(t). \quad (9)$$

The solution of this equation depends on the detailed form of the functions f and g . We postulate that these are smoothly increasing functions of GM1 concentration.

If we further assume that GM1 concentration is a linear function of the applied dose c , $[GM1] \sim c$, the simplest forms for the infectivity and cytotoxicity are obtained by taking $f = \lambda_1 c / (1 + \lambda_1 c)$ and $g = \lambda_2 c / (1 + \lambda_2 c)$, which leads to

$$\zeta = \zeta_0 / (1 + \lambda_1 c) \quad (10a)$$

and

$$\eta = \eta_0 / (1 + \lambda_2 c). \quad (10b)$$

Here λ_1 and λ_2 are constants with the dimensions of $(\text{mg})^{-1}$. The evolution of the parasite population will depend on the values of the parameters λ_1 and λ_2 in a nonlinear fashion, but it can be understood if we examine the case $\lambda_2 \ll \lambda_1$, which turns out to be the one realized in the experiments. In this case, the infectivity will decay much more rapidly than the cytotoxicity at small GM1 doses, leading to fewer cells being infected, while the number of trypomastigotes released per burst remains more or less constant. This leads to a decrease in the number of circulating parasites, as observed in the experiments. At larger doses, the cytotoxicity is also strongly reduced, leading to a larger brood being hatched at each cell rupture and, consequently, to a much larger quantity of released parasites, which is likewise observed in the experiment. We emphasize that assumptions A6 and A7 are both necessary. It is impossible to reproduce the strongly nonlinear effect of the drug on the parasite number by letting either only ζ or only η decrease with the drug concentration. It is precisely the simultaneous decrease of both functions that leads to the observed effect.

RESULTS

To compare the results of our model with the experimental data of Cossy Isasi *et al.* (1999), we solved numerically (with a standard Euler method) the set of Eqs. (1), (2), and (4), with the dependence of the infectivity and toxicity on GM1 concentration given by Eqs. (10a) and (10b). In Fig. 1 we represent the total parasite populations obtained for various GM1 doses and the model solutions with parameters that were chosen to attain the closest visual agreement between experimental data and model predictions (see Table III). We also obtained $\lambda_1 = 19.5 (\text{mg})^{-1}$ and $\lambda_2 = 2.9 (\text{mg})^{-1}$, $\eta_0 = 0.33 (\text{dpi})^{-1}$, $\zeta_0 = 0.17 (\text{dpi})^{-1}$, and $N_r = 9.9227$ (dpi stands for days post-infection). Note the strong decrease in the

Figure 1. Parasite populations as a function of time for the indicated GM1 doses. For each value of the dose we present the experimental data (Cossy Isasi *et al.*, 1999) and model fits. The lines joining the experimental points are for eye guidance only. Note that the [GM1]=0.6 mg dose kills all the mice. The model describes what would occur to a putative immortal mouse, for which the parasite number becomes extremely high before being brought under control.

parasite number for intermediate doses (0.15 mg), an effect that is reversed for larger doses. For the highest dose, no mouse survives beyond 12 dpi. We remark that, although the highest dose, [GM1]=0.6 mg, kills the mice, a death criterion has not been implemented in the model. Therefore, the obvious overshooting of the model prediction for [GM1]=0.6 mg does not represent a bad fit; it would correspond to an immortal mouse whose parasitemy would eventually be brought under control. The model can therefore properly describe the most striking effects of GM1 on parasite population.

Our model predicts the damage to cardiocytes due to parasitic action. From Fig. 2, we see that damage is strongly reduced by the application of moderate GM1 amounts, but that it increases again for the largest dose, although in this case cell destruction is minimal during the first 10 dpi. These predictions are in complete agreement with the experimental results (Cossy Isasi *et al.*, 1999). These researchers had found that mice treated with GM1 doses of 0.1 and 0.3 mg survived much higher parasitemias than untreated mice. This is clearly related to the strong reduction in cell damage resulting from GM1 action, as described in Fig. 2. In the case

Figure 2. Number of cells destroyed by parasite action as a function of time after inoculation, for the indicated GM1 doses. Intermediate doses strongly reduce cell damage at all times, while the largest dose strongly reduces the initial damage but has almost no effect at long times.

of the 0.6 mg dose, the mice also survived an initially much higher parasitemia than their untreated counterparts, but eventually died (by day 14 post infection). This last result fits perfectly well with the delay in the onset of cell damage observed in the 0.6 mg curve of Fig. 2, and can be taken as a strong indication of the validity of our hypotheses.

The asymptotic value of the total cell loss for various GM1 doses is shown in Fig. 3. Cell destruction is minimized for $c \approx 0.15$ mg, but it stays relatively dose-independent for c in the range between 0.10 and 0.25 mg. This is important because the effect of dose optimization is robust. From Figs. 1 and 2 we can estimate a therapeutic range by finding the total cell loss for the $c=0.6$ mg dose at the last time (12 days) the hosts are found to be alive. This corresponds approximately to 5×10^5 destroyed cells. The window of effective therapeutic range emerges as a result of the combined effect of a decrease in the infectivity, which is mostly responsible for the initial decay in cell loss with increasing GM1 dose, and a decrease in the cytotoxicity, which is mostly responsible for the increase in cell damage at large GM1 doses.

DISCUSSION

The results presented in this work are important because, with the help of mathematical modeling and experimental

Table III. Antibody parameters for Fig. 1.

Antibody	a_0	α_A (dpi) ⁻¹	α_B (dpi) ⁻¹	γ (dpi) ⁻¹	T (dpi)	τ (dpi)	θ (dpi)
IgM	6×10^{15}	3.3×10^{-20}	2.4×10^{-20}	9.5×10^8	16.8	58.1	0.35
IgG1	2.5×10^{17}	0	8.6×10^{-17}	3.8×10^{10}	42.7	49.7	3.5
IgG2a	1.2×10^{18}	0	2.9×10^{-18}	1.2×10^{11}	49.7	49.7	3.5

Figure 3. Asymptotic value of the predicted total cell loss as a function of GM1 dose (dots). The dashed line corresponds to the maximum cell loss consistent with host survival. The range between the vertical solid lines is then the putative therapeutic dose range.

data on membrane fluidity, we have been able to characterize the influence of GM1 on the acute phase of experimental Chagas disease. GM1 is a ganglioside that has a high potential as a therapeutic agent for this disease. As seen from Fig. 1, our model can suitably describe the main effect of GM1 on parasite population: reducing it at low doses, for which the decrease in the infectivity is the controlling influence, and increasing it at higher doses, for which the decrease in the cytotoxicity becomes the dominant effect [note that the variation in the cytotoxicity appears in the exponent of Eq. (8)].

A satisfactory model must generate biologically reasonable values for the calculated parameters. Using Eq. (3) with our values for η (0.33 dpi⁻¹) and N_r (9.92), we obtain the very reasonable duplication time of 21.7 hours (Engel *et al.*, 1985). η itself corresponds to an intracellular time of three days, which is consistent with the practice of waiting five days to collect second generation parasites *in vitro*. N_r is not far from the result we previously estimated ($N_r = 13.8$) for the experiment of El Bouhdidi *et al.* (1994) with BALB/c mice infected with the Tehuantepec strain of *T. cruzi* (Sibona *et al.*, 2007) and it is consistent with the results ($N_r \approx 30$) of a recent *in vitro* experiment using the CL strain (Coimbra *et al.*, 2007). We remark that a higher *in vitro* value of N_r is not surprising because of the better feeding of (and weaker constraints on) cells in a monolayer culture. Although we know of no direct measurements of the penetration time, we can compare ζ (0.17 dpi⁻¹) with our previous results for other mice/parasite strains (Sibona *et al.*, 2005). For instance, for BALBc/Tulahuen we obtained $\zeta = 0.33$ dpi⁻¹. Finally, $(\lambda_1)^{-1} \approx 0.05$ mg and $(\lambda_2)^{-1} \approx 0.34$ mg are the GM1 concentrations at which the infectivity and cytotoxicity,

respectively, undergo substantial reductions. They agree very well with the concentration range used in the experiments (Cossy Isasi *et al.*, 1999).

Here we propose that GM1 modulates the physical boundary involved in the processes of host cell invasion and emergence after parasite multiplication. This physical boundary is formed by the parasite and host cell plasma membranes. During GM1 treatment of infected mice, plasma GM1 interacts with both membranes. Since we had previously found that GM1 does not alter parasite adhesion to the cell plasma membrane (Cossy Isasi *et al.*, 2003), we performed determinations of membrane DPH fluorescence polarization anisotropy to detect modifications in the plasma membrane upon interaction with GM1. Our results show that GM1 increased parasite membrane microviscosity in a dose-dependent manner immediately after being added to a suspension of parasites. This effect was not transient because parasites pretreated 18 hours with GM1 rendered similar values. When the same parameter was measured in mice cardiocyte plasma membranes, *in vivo* treatment with GM1 resulted in diminished microviscosity. Our viscosity measurements support the view that GM1 induces changes in the membrane structure that may be responsible for the processes described in assumptions A6 and A7.

Although we cannot identify the precise mechanism responsible for the relation between the structural membrane changes and the modifications in the cytotoxicity and infectivity, we can reasonably assume that the presence of GM1 modifies these variables in a dose-dependent manner. At the cellular level, the physical modifications of the plasma membranes are transduced to enzymatic activities, so we may speculate that GM1 modulates the same enzyme activity in the parasite and the host. This should be a signal transduction enzyme involved in the penetration process. Calmodulin is an enzymatic activity present in the parasite and immune cells that activates many other enzymes, among them PLA2 [phospholipase A2]. In mammals, calmodulin inhibition is accompanied by decreased intracellular calcium and less ordered membranes (Iqbal and Sze, 1994). The inhibition of parasite calmodulin impairs penetration (Yakubu *et al.*, 1994; Moreno *et al.*, 1994). Both calmodulin and PLA2 are relevant in the activation of different immune cells. It is well known that gangliosides are inhibitors of PLA2 (Farooqui *et al.*, 1999; Maggio *et al.*, 1994) and activators or inhibitors of calmodulin as a function of their concentration (Higashi and Yamagata, 1992; Higashi *et al.*, 1992). The puzzle of infection control and recruitment at increasing concentrations of GM1 may be pictured as follows.

At low GM1 concentrations the infection is controlled. The inhibition of the parasite enzymes is the determining event. This may be due to gangliosides being more accessible to circulating parasites than to cardiac cells, resulting in parasite calmodulin inactivation. As the concentration of GM1 is increased, the interaction with host cells becomes

dominant. Host calmodulin is inhibited leading to more parasites being released by each infected cell, due to reduced cytotoxicity. In heart tissue, calmodulin activates calmodulin-dependent kinase II, an enzyme directly involved in myocardial hypertrophy and arrhythmogenesis (Zhang *et al.*, 2004). Calmodulin inactivation is a way to protect cardiocytes in the acute phase and to avoid the pathologic process of chronic infection. It is important to note that calmodulin inhibition results in detrimental or beneficial effects depending on the affected tissue. In immune cells it is a signal to suppression or anergy (LeGrue and Munn, 1986). Thus, when GM1 concentrations are very high, parasite population grows faster because immune response is impaired and cell loss increases due to excessively high parasitemias (here infectivity becomes more important) leading to massive cardiac destruction in spite of calmodulin inhibition.

For simplicity, the direct effect of GM1 on the immune system has not been explicitly included in the model. At low concentrations GM1 is known to activate immune cells (Dozmorov *et al.*, 1997). As the GM1 concentration is increased, humoral response is no longer enhanced by GM1 and fewer parasites are removed by immune control. When GM1 concentrations are very high, the host cytotoxic response becomes inhibited by GM1 and parasite immunosuppressor molecules. This contributes to faster parasite population growth. It is clear that including these processes in the model would enhance its predictions in all cases. To describe precisely the GM1-induced modification to the parameters related to the immune response more experimental work is required.

To shed light on the mechanisms of GM1 action, we suggest some simple experiments: (a) To measure the dose dependent effect of GM1 on monolayers of heart cells alone and after infection. This would allow us to test the validity of hypotheses A6 and A7 and, in particular, of Eqs. (10a) and (10b). (b) To directly evaluate the amastigote content per cell under various GM1 concentrations (Coimbra *et al.*, 2007). (c) To repeat the *in vivo* experiments of Cossy Isasi (1999), and those *in vitro* just proposed, using other gangliosides, such as GM3, Gd1a, or GT1b, or related molecules, such as digoxin or sphingomyelin. This would help us to ascertain the precise nature of the relation between the lipid molecule structure and the membrane modifications.

In summary, we have used a mathematical model for the parasite-immune system interaction to characterize the influence of GM1 on the acute phase of the experimental Chagas disease. Our work validates our hypotheses, suggesting that the key ingredients for a description of GM1 action are its effects on the parasite virulence and infectivity.

MATERIALS AND METHODS

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised

1996). All the experimental procedures were approved by the Bioethics Board of the Medical Sciences School of the National University of Córdoba (Argentina).

Parasites: culture and treatment

Epimastigotes of *T. cruzi*, Tulahuen strain, were grown in BHI (brain-heart infusion) supplemented with 10% fetal calf serum, at 28 °C (Bronia *et al.*, 1986). Parasite populations develop presenting four phases: initial, middle, logarithmic, and declining. For this reason, parasites from different cultures were harvested at days 4, 7, 14, and 21 of the growth curve, washed twice with isotonic phosphate buffer 0.01 M (PBS), pH 7.3, and resuspended in Dulbecco's modified medium without serum. After they were separated in control and treated lots, their media were supplemented with saline (controls) or 300 μ M of GM1 for 18 hours. Then they were again harvested and washed twice. In fluorometric experiments, immediate and surplus changes in membrane DPH fluorescence emission anisotropy were recorded on parasites resuspended in PBS, magnetically stirred and treated with ganglioside during fluorescence recording, and compared with parasites pretreated with GM1 for 18 hours. Parasite mobility was checked by optical microscopy after each harvesting and before ganglioside treatment and was found to be greater than 90%. Determinations for each phase of the growth curve were made on separately initiated cultures. GM1 300 μ M treatment of trypomastigotes from infected monolayers in cell free medium began on day 5 post infection, when the first parasites burst out of the monolayer, and finished on day seven to allow maximum interaction of parasites with GM1.

Cardiocyte membrane purification

Mice were divided into four groups: (a) non-infected; (b) non-infected and treated with GM1 0.1 mg/day by intramuscular injection, for 30 days; (c) infected with *T. cruzi*; (d) infected with *T. cruzi* and treated with GM1 0.1 mg/day by intramuscular injection for 30 days. A pool of two ventricles was homogenized in ten volumes of ice-cold homogenization buffer (mM composition: sucrose 250; MgCl_2 1 and TRIS [trishydroxymethylaminomethane] HCl 20; pH 7.4). Homogenates were centrifuged at $2000 \times g$ for 10 min. Pellets were rehomogenized, centrifuged at $40,000 \times g$ for 30 min and washed twice with KCl 0.6 M in homogenization buffer only. The final pellet was suspended in an incubation buffer (mM composition: MgCl_2 12.5; EDTA [ethylenediaminetetraacetic acid] 1.5; TRIS HCl 75; pH 7.65) in a volume of 1 ml/g of wet tissue. This procedure renders pure cardiomyocyte membranes.

Fluorescence anisotropy measurement

The optical fluorescence anisotropy measurements were done essentially according to the method described by Lakowicz (Lakowicz, 1984). Their purpose was to determine

the microviscosity of the parasite and cardiocyte membranes. DPH fluorescence emission anisotropy (1,6-diphenyl 1,3,5-hexatriene) was determined using a SLM 4800 C spectrofluorometer controlled by a T format microprocessor. The light beam was polarized using Glan-Thompson calcite prism polarizers and made incident on the sample cuvette ($10 \times 10 \times 45$ mm) housed in a variable temperature unit. The fluorescence was collected at 90° to the incident beam. Fluorescence intensities were measured parallel and perpendicular to the vertically polarized incident beam. The G factor was determined before studying each sample. This ensured reliable data as any optical component could bring about its own optical effects. Anisotropy was calculated using the following relationship:

$$\rho = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}), \quad \text{where } G = I_{HV} / I_{HH}. \quad (11)$$

Here I_{VV} represents the fluorescence intensity parallel to the vertically polarized excitation beam and I_{VH} is that of the perpendicular component. I_{HV} and I_{HH} are the perpendicular and parallel intensities when the incident light is horizontally polarized. The equipment was checked with a concentrated solution of glycogen and ρ was found to be equal to 0.998. The samples were excited at 360 nm and the emission was measured at 420 nm. In all determinations a $10 \times 10 \times 45$ mm quartz cuvette was used. The cuvette was kept at a constant temperature. The parameter $\rho_{DPH} = (\rho_0 / \rho - 1)^{-1}$ measures the degree of impediment to the rotation of DPH molecules inserted in the membranes and varies inversely with the relative fluidity of these membranes. The term “membrane microviscosity” was used to refer to the structural and dynamic properties that determined the order and relative movements of the lipids in the membrane (Schachter and Shinitzky, 1986).

Fluorescence anisotropy measurement on living parasites

The effect of ganglioside treatment on the membrane fluidity of *T. cruzi* epimastigotes was probed with DPH. Parasites harvested as stated above and resuspended in PBS were labeled with DPH by adding a small quantity (1% of the final volume of incubation) of the probe in chloroformic solution (9 mg/ml) and incubating for 2 h at 28°C . After washing, the cells were resuspended and kept in isotonic PBS. Results are averages of ten determinations made on each of six assay repetitions on separately initiated cultures.

Fluorescence anisotropy measurement on cardiocyte membranes

The cardiac membranes suspension was diluted in 1000 μl of incubation buffer (pH: 7.65) to which 10 μl DPH solution was added (dissolved in chloroform) at the rate of 1% of the final volume of the incubation solution which, after being shaken, was let to settle for an hour at 37°C .

ACKNOWLEDGMENTS

This work was supported in part by SECyT-UNC (projects 05/B354 and 05/H188), ANPCyT (PICT 2205/33675), and CONICET (PIP 6311/05).

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