Effects of cannabinoid treatment on Chagas disease pathogenesis: balancing inhibition of parasite invasion and immunosuppression

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Summary

Trypanosoma cruzi invades heart cells via a calciumdependent, G protein-mediated mechanism, leading to severe cardiac inflammation considered by some to be autoimmune in nature. Cannabinoids inhibit calcium flux and G protein signalling; as potent immunosuppressive agents, they are effective in the treatment of autoimmune disease but contraindicated for the treatment of infections. We compared the action of the synthetic cannabinoid R(+)WIN55,212 and its inactive isomer S(-)WIN55,212 on cardiac myoblast invasion: R(+)WIN55,212 inhibited invasion by over 85%. We then tested for efficacy in modulating pathogenesis in mice by assaying parasite burden in heart and blood, cellular and humoral immunity to parasite and self antigens, and mortality. R(+)WIN55,212 significantly reduced cardiac inflammation but led to considerably increased parasitaemia. Cardiac parasitosis and mortality were not significantly different in treatment and control groups. We conclude that cannabinoids can block cardiac cell puncture repair mechanisms, thereby inhibiting trypanosome invasion as predicted by the mode of drug action, but, also inhibit immune cell effector functions, offsetting the benefit of inhibition parasite cell invasion. Refined use of cannabinoids may prove therapeutic in the future, but

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our results raise concern about the effect of cannabis use on those chronically infected by *T. cruzi* and on heart cell homeostasis generally.

Introduction

Chagas heart disease (CHD) is the world's most prevalent infectious cardiomyopathy (Tanowitz *et al.*, 2002). A total of 16–18 million people in 18 countries of Latin America are infected with the vector borne protozoan parasite *Try-panosoma cruzi*, with 300 000 new infections occurring annually and a disease burden of 649 000 disability-adjusted life-years, representing a huge economic cost to affected nations. CHD often presents years after infection with conduction abnormalities, arrhythmias, dilated congestive cardiomyopathy, thromboembolic phenomena, megaesophagus/megacolon and sudden death.

Trypanosoma cruzi is an intracellular parasite (Tyler and Engman, 2001) that invades cardiomyoctes by a novel mechanism (Burleigh and Woolsey, 2002; Yoshida, 2002). Cardiomyopathy results from parasite and immune mediated damage of cardiac tissue. The parasite invades cardiac cells, multiplies within a 'pseudocyst' and then erupts, lysing the host cell before invading new cells. In response, immune cells infiltrate infected areas causing severe inflammation; whether this infiltration is solely parasite directed or has a substantive autoimmune component is currently contentious (Leon and Engman, 2002).

Invasion stimulates calcium flux, driving cytoskeletal rearrangement and lysosome recruitment to the parasite synapse (Tardieux et al., 1992; Rodriguez et al., 1996). Signalling occurs via the synaptotagmin VII pathway (Caler et al., 2001), usurping the machinery of puncture wound repair for lysosome delivery to the cell surface juxtaposed to the parasite. Action of T. cruzi serine hydrolase, oligopeptidase B, upon an unknown substrate produces calcium agonist (Caler et al., 1998) that mobilizes intracellular calcium and elevates cAMP through the action of heterotrimeric G proteins. Correspondingly, activation of G_s has been shown to promote cell invasion while activation of G_i has been shown to inhibit it (Tardieux et al., 1994). Concordantly, a key receptor in T. cruzi invasion is the bradykinin B2 receptor, which has a widespread distribution and is itself associated with het-

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erotrimeric G protein signalling and cAMP elevation upon receptor ligation (Scharfstein *et al.*, 2000).

Cannabinoid receptor-1 (CB1) is another widely expressed G protein-coupled receptor. Signalling through CB1, G_{i/o} exerts an inhibitory effect on adenylate cyclase activity, lowering levels of cAMP. Furthermore, it is well established that CB1 signalling negatively regulates calcium currents through both N- and P/Q-type voltagesensitive calcium channels and activates G proteincoupled inwardly rectifying K⁺ channels (Caulfield and Brown, 1992; Mackie and Hille, 1992; Mackie *et al.*, 1993; 1995; Bayewitch *et al.*, 1995). Taken together these properties suggest that cannabinoid receptor ligation may be beneficial in inhibiting the calcium-dependent intracellular invasion of *T. cruzi*.

Current treatment for Chagas disease is unsatisfactory; the only drug currently manufactured for treatment of infected individuals is a probable carcinogen with severe side-effects, not considered safe for treatment of humans in the USA (Raasi and Luquetti, 2002). The invasion process is widely considered a potential target for chemotherapeutic intervention in the treatment of Chagas disease and this study represents the first attempt, to our knowledge, to derive therapeutic benefits from the inhibition of the process. Cannabinoids are currently being assessed as therapeutic agents in a variety of disorders, such as multiple sclerosis, neurodegeneration, Parkinson's disease, chronic neuropathic pain and chemotherapy-related emesis (reviewed in Croxford, 2003). Recent studies have also demonstrated that synthetic cannabinoids are therapeutic in a viral-induced, autoimmune model of multiple sclerosis (Arevalo-Martin et al., 2003; Croxford and Miller, 2003). Here we report the use of a synthetic cannabinoid, R(+)WIN55,212, for treatment in a mouse model of Chagas' disease.

Results

Cardiac expression of the CB1 receptor

To assess the potential of cannabinoids to attenuate CHD pathology, it was important to determine whether cardiac tissues express cannabinoid receptors. Using immunohistochemistry (Fig. 1A), and Western blot analysis of mouse heart lysates and the rat myoblast line used for invasion studies (Fig. 1B), we determined that CB1 was expressed, albeit heterogeneously, throughout the myocardium and in the myoblast cell line. It was not possible to discern the cell types expressing CB1; however, the level of expression varied among positive cells. We also sought to determine whether cannabinoid receptors might be present on the trypanosome itself but found no evidence of such either by Western blot analysis or by analysis of trypanosome genome databases.



Fig. 1. Expression of CB1 in mouse heart and cardiac myoblasts. A. Immunohistochemical analysis of CB1 expression in mouse heart using CB1-specific and isotype-matched control monoclonal antibodies. Cellular DNA is stained with DAPI.

B. Western blot analysis of *T. cruzi* trypomastigote (T), rat cardiac myoblast (C) and mouse heart (H) lysates using the antibodies specific for CB1 or β -tubulin. CB1 is expressed in both the heart and the myoblast cell line but not in the trypanosome.

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Β

S(–)WIN55

R(+)WIN55



Cannabinoid treatment inhibits the invasion of cardiac myoblasts by T. cruzi

The effect of cannabinoid treatment on *T. cruzi* invasion was tested using subconfluent rat cardiac myoblasts. The cannabinoid R(+)WIN55,212 and its inactive isomer S(-)WIN55,212 were used. A significant dose-dependent reduction in the number of parasites both attaching to and invading cells was apparent in response to R(+)WIN55,212 (Fig. 2 and Table 1). At 10 μ M drug, inhibition was well over 80%, although invasion was not abol-

ished completely even at very high drug concentrations. Interestingly, and unexpectedly, S(-)WIN55,212, which binds cannabinoid receptors but signals only weakly, also showed a less dramatic, dose-dependent inhibition of cell invasion that did not reach statistical significance.

The CB1 receptor is associated with the site of trypanosome cell contact

The slight reduction in *T. cruzi* invasion observed in response to S(-)WIN55,212 suggested the drug might

Table 1	Cannabinoid	treatment	inhibits	T. cr	<i>uzi</i> att	achment	to	and	invasion	of	cardiac	m۱	/00	/tes
Tuble I	ourniubiliolu	ucument	minuto	1.01	uzi un	aonnon	ιU	unu	11110001011	01	ouraido		,009	100

Number of parasi	tes per 100 my	voblasts ^a	Number of	Mode number	Mean number						
Treatment group Total I	nvading	Attached	with parasites	invaded cell	invaded cell						
Vehicle 271 (100) 2	211 (100)	60 (100)	58	3	3.6						
S(-)WIN55,212 (1 µM) 246 (90.7)	165 (78.2)	81 (135.0)	55	3	3.0						
R(+)WIN55,212 (1 µM) 132 (48.7)	53 (25.1)	79 (131.7)	36	1	1.5						
S(-)WIN55,212 (10 μM) 218 (80.4)	126 (59.7)	92 (153.3)	47	3	2.7						
R(+)WIN55,212 (10 µM) 43 (15.9)	30 (14.2)	13 (21.7)	21	1	1.4						

a. Figures in parentheses indicate the percentage of the vehicle group.

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Fig. 2. Cannabinoid treatment inhibits T. cruzi attachment and invasion of cardiac myoblasts. A. Invasion of cardiac myoblasts by tissue culture-derived trypomastigotes was allowed to proceed for 15 min prior to washing and 15 min after washing. Cells were fixed with paraformaldehyde and extracellular parasites were stained with chagasic human sera and Cy2labelled goat anti-human IgG (Ext T. cruzi, green). Cells were then permeabilized with digitonin and intracellular parasites were stained with parasite-specific hsp70 antiserum and Cy5-labelled goat anti-mouse IgG (Int T. cruzi, red). DAPI was included to stain host DNA. Assays included vehicle or low (1 µM) or high (10 µM) concentrations of R(+)WIN55,212 or S(-)WIN55,212 and invasion (striped bars), attachment (white bars) and the total number (black bars) of parasites associated with the myoblasts were quantified by immunocytochemical analysis of red fluorescent, green fluorescent and total parasites respectively. R(+)WIN55,212 significantly reduced attachment and invasion of the myoblasts by the parasite (*P < 0.05).

B. Representative immunocytochemistry fields are shown.

ABImage: A marked bound of the second of th

Fig. 3. Colocalization of CB1 and invading *T. cruzi* on mouse cardiac myoblasts. Cardiac myoblasts were incubated with *T. cruzi* trypomastigotes and stained with chronic chagasic human sera and Cy2-conjugated goat antihuman IgG (green) and CB1-specific monoclonal antibody and Cy5-conjugated goat antimouse IgG (red) and DNA was counterstained with DAPI (blue). Analysis of total fluorescence (A) or red fluorescence alone (B) demonstrates enrichment of the CB1 receptor at the region of the host cell membrane associated with the attached/invading parasite.

inhibit parasite attachment and invasion by steric inhibition rather than by dampening of the calcium flux or lowering of cAMP levels. To test the possibility of a direct interaction between *T. cruzi* and CB1, we used indirect immunofluorescence microscopy to determine the localization of the CB1 receptor during the process of invasion. Indeed, CB1 was concentrated at the parasite 'synapse' with the myoblast, indicating that it was being transported or sequestered there (Fig. 3).

Increased parasitaemia and trypomastigote morphological change in cannabinoid-treated mice may reflect a reduced capacity of the parasite to invade tissue

The effect of R(+)WIN55,212 on parasite invasion *in vitro* provided proof of principle that cardiac-derived cells, susceptible to invasion by *T. cruzi*, could be rendered refractory by the presence of cannabinoid. This led us to consider the potential of this compound for the treatment of a *T. cruzi*-infected mouse. Mice were infected with *T. cruzi* and then treated for the lifetime of the animal with low (5 mg kg⁻¹ day⁻¹) or high (20 mg kg⁻¹ day⁻¹) doses of R(+)WIN55,212 or S(-)WIN55,212. Parasitaemia in R(+)WIN55,212-treated mice at day 10 post infection was significantly higher (**P* < 0.001) than in untreated controls or in mice treated with S(-)WIN55,212 and this increase in parasitaemia was dose dependent (Fig. 4). Concomitantly, by 10 days post infection, mice treated with the

high dose of R(+)WIN55,212 appeared to be more affected by the infection, exhibiting weight loss and ruffled fur (data not shown). We considered that the increased parasitaemia might reflect a reduction in antiparasite immunity coupled to a reduced capacity to invade tissue and also noted that, at 10 days post infection, the morphology of the typical bloodstream trypomastigote in R(+)WIN55,212-treated animals was different from that in control animals, with the majority of these trypomastigotes exhibiting a 'stout' morphology rather than the normal, C-shaped 'slender' morphology. Six of 10 trypomastigotes in R(+)WIN55,212-treated mice were stout, compared with no stout forms in the control groups (Fig. 4B). We tested whether R(+)WIN55,212 affected trypanosome growth or differentiation in culture and found no discernable effect even at high concentrations (data not shown).

Antiparasite and antiself immune responses are suppressed by cannabinoid treatment

Cannabinoids are potent immunosuppressive agents. While useful for the treatment of autoimmune syndromes, this causes problems in treating infectious diseases. In CHD, however, much of the pathology is thought to be immune-mediated, possibly with some autoimmune component (Leon *et al.*, 2001). We therefore considered whether treatment of *T. cruzi*-infected mice with



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Fig. 4. Cannabinoid treatment increases parasitaemia in *T. cruzi*-infected mice. A. *T. cruzi*-infected mice were treated daily with low (5 mg kg⁻¹) or high (20 mg kg⁻¹) doses of R(+)WIN55,212 or S(-)WIN55,212 (or PBS) and parasitaemia was assessed at day 10 (black bars) and day 15 (striped bars) post infection (p.i.). Parasitaemia was significantly higher at day 10 in mice treated with high dose R(+)WIN55,212 than in controls (**P* < 0.001), but returned to the levels of other groups by day 15.

B. Morphology of bloodform trypomastigotes. Tubulin immunofluorescence microscopy on parasites isolated from the blood showed that those from R(+)WIN55,212-treated mice possesed a 'stout' morphology rather than the normal, C-shaped, 'slender' morphology of trypomastigotes found in S(-)WIN55,212-treated mice and control groups (not shown).



S(-)WIN55 high

R(+)WIN55 high

R(+)WIN55,212 would have an effect on the normal immune responses for both protective parasite-specific immunity and potentially damaging cardiac-specific autoimmunity. We tested the ability of T. cruzi-infected mice to mount normal cell-mediated immune responses by measuring delayed-type hypersensitivity (DTH) and humoral immune responses by enzyme-linked immunosorbent assay (ELISA) in mice treated with R(+)WIN55,212 or S(-)WIN55,212 (Fig. 5). Both assays were performed using cardiac myosin, a well-characterized heart-specific autoantigen associated with CHD, and parasite lysate. We found a significant reduction (*P < 0.005) in cell-mediated and humoral immunity to both antigens in animals treated with 20 mg kg⁻¹ R(+)WIN55,212 compared with controls, consistent with a general suppression of the immune response. Little difference was seen among the other groups.

Cardiac inflammation, but not parasitosis, was significantly reduced in T. cruzi-infected, cannabinoidtreated mice

To determine the effect of cannabinoid treatment on cardiac inflammation, we examined heart tissues to determine the extent of mononuclear cell infiltration and parasite burden (Fig. 6). We employed a double blind scoring system to establish histologic scores and values for the number of parasite pseudocysts and compared these scores among the treatment groups. We found significantly decreased (*P < 0.05) levels of mononuclear cell infiltration (Fig. 6D, white arrows, and Fig. 6E) in the hearts of mice treated with the high dose of R(+)WIN55,212, compared with control groups, consistent with the generalized immunosuppression observed in previous work with this agent described above. Surprisingly,



Fig. 5. Cannabinoid treatment inhibits parasite-specific and self-directed immune responses in *T. cruzi*-infected mice. *T. cruzi*-infected mice *T. cruzi*-infected mice were treated daily with low (5 mg kg⁻¹) or high (20 mg kg⁻¹) doses of R(+)WIN55,212 or S(-)WIN55,212 (or PBS). A. DTH responses to *T. cruzi* (striped bars) and cardiac myosin (black bars) were significantly reduced at 21 days post infection in mice treated with high dose R(+)WIN55,212 compared with other groups (**P* < 0.05). B. Production of myosin-specific ant *T. cruzi* specific antibodies was significantly inhibited in mice treated with high dose R(+)WIN55,212 compared with other groups (**P* < 0.05).

given this level of immunosuppression, we found little variation in cardiac parasitosis among treatment groups as evidenced by the number of pseudocysts observed (Fig. 6C and D, black arrows, and Fig. 6F).

Mortality of T. cruzi-infected mice is not significantly affected by cannabinoid treatment

Trypanosoma cruzi-infected mice treated daily with a high dose or a low dose of R(+)WIN55,212 or S(-)WIN55,212 were observed and the day of death was recorded for each animal. In spite of obvious differences in mouse size, weight, behaviour, and level of parasitaemia, the ultimate marker of pathology – lifespan – was not significantly affected by drug treatment, with all animals expiring between 18 and 24 days post infection (Fig. 7). This suggests that any benefits gained from inhibition of invasion and suppression of autoimmunity are balanced by opposing immunosuppressive effects on antiparasite immunity.

Discussion

Chagas heart disease is a chronic illness caused by *T. cruzi* infection. Current treatment is focused on antiparasite chemotherapy with benznidazole or nifurtimox. These drugs are most effective when administered soon after infection; however, side-effects of both drugs are considerable and potentially life-threatening. There is a need therefore for safe, novel therapeutic agents with which to treat Chagas disease. In this study we tested the inhibitory effect of the synthetic cannabinoid R(+)WIN55,212 on *T. cruzi* invasion of cardiac myoblasts *in vitro* and on the outcome of *T. cruzi* infection *in vivo*.

In vitro studies demonstrated that R(+)WIN55,212 effectively inhibited parasite attachment to and invasion of cardiac myoblasts. The inhibitory effects of R(+)WIN55,212 are likely to be mediated by CB1 expressed on the cardiac cells rather than any effect upon *T. cruzi* themselves as they do not express CB1. Likewise,



S(–)WIN55



Fig. 6. Cannabinoid treatment reduces cardiac inflammation but not parasitosis. *T. cruzi*-infected mice were treated daily with a high (20 mg kg⁻¹) dose of R(+)WIN55,212 or S(-)WIN55,212 (or PBS). Mice were sacrificed between 18 and 21 days post infection and hearts were examined by haematoxylin and eosin staining (A–D) and inflammation (E) and parasitosis (F) were quantified after examination of two sections from each of the five mice in each group. Infected mice treated with R(+)WIN55,212 exhibited significantly less mononuclear cell infiltration (white arrows in Panel D) compared with S(-)WIN55,212-treated mice (*P < 0.05). However, there was no difference observed in tissue parasitosis, as assessed by number of parasite pseudocysts (black arrows in Panels C and D).

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Fig. 7. Cannabinoid treatment has no effect on mortality in *T. cruzi*-infected mice. *T. cruzi*-infected mice were treated daily with low (5 mg kg⁻¹) or high (20 mg kg⁻¹) doses of R(+)WIN55 212 or S(-)WIN55 212 (or PBS). Mice were monitored daily until death.

it is unlikely that R(+)WIN55.212 or the vehicle compound is toxic for either myocytes or parasites, as parasite or cell viability was not affected by the presence of S(-)WIN55,212, a negative enantiomer of R(+)WIN55,212, which binds cannabinoid receptors with limited signalling capabilities. Interestingly, we observed that S(-)WIN55,212 also inhibited invasion, although to a lesser degree than did the active isomer. There are a number of potential explanations for this observation. There may be sufficient signalling from the control compound to impact the invasion process. Alternatively, S(-)WIN55,212 may actually antagonize endocannabinoids or parasite-derived molecules that signal through the CB1 receptor and are important to the invasion process. Finally, it is possible that the WIN55 ligand itself blocks a productive interaction between the parasite and host cell surface. We observed that CB1 expressed on cardiac myocytes appears to colocalize with T. cruzi on the cellular surface, lending support to the possibility of a direct interaction with the parasite. Interestingly, the cannabinoid receptor is a strong candidate for a lipid raft associated receptor as is another G protein coupled receptor shown to be strongly associated with the invasion process, Bradykinin B2 (Scharfstein et al., 2000). Lipid rafts are frequently associated with parasite-cell invasion (Manes et al., 2003) and these receptors may be drawn to the region of the parasite by virtue of raft association. In such cases, concentration of the receptors may be able to drive concerted signalling function without direct stimulation of the receptor by parasite or environment.

As the in vitro results using R(+)WIN55,212 as a low toxicity agent to inhibit T. cruzi-cell invasion were encouraging, we investigated the inhibitory effects of R(+)WIN55,212 on T. cruzi invasion in a mouse model of Chagas disease. Surprisingly, R(+)WIN55,212 treatment had no effect on mortality. Elevated early blood parasitaemia levels in high dose R(+)WIN55,212-treated mice suggested either an inhibition of T. cruzi invasion and/or a generalized increase in parasite burden, perhaps due to suppression of antiparasite immunity. Later parasitaemias were similar among all treatment groups; however, the trypanosome morphology in the high dose R(+)WIN55,212 group was affected, with the majority of trypanosomes exhibiting a 'stout' rather than 'slender' appearance, with retarded movement. 'Slender' forms which fail to invade cardiac cells differentiate to a 'stout' intermediate form, so the simplest interpretation of this finding is that the trypomastigotes are not able to invade with the normal facility. The possibility that R(+)WIN55,212 acts directly on the trypanosome was considered, but there was no effect of this drug on in vitro growth or differentiation. Furthermore, we found no cannabinoid receptor homologues in the trypanosome gene databases, and there is no evidence of G protein-coupled receptors of any sort in these organisms.

Two subtypes of cannabinoid receptor have been isolated and cloned to date (Arevalo-Martin et al., 2003; Croxford and Miller, 2003; Manes et al., 2003). While CB1 is expressed both in the central nervous system and peripheral organs, CB2 is expressed only in the periphery, particularly within lymphoid organs (Leon et al., 2001; Arevalo-Martin et al., 2003; Manes et al., 2003). R(+)WIN55,212, a CB1 and CB2 agonist, has been shown to have potent inhibitory effects upon the immune system when administered to mice (Mackie and Hille, 1992; Mackie et al., 1993). Consistent with this, infected mice treated with high dose R(+)WIN55,212 demonstrated reduced DTH responses to both T. cruzi and myosin. Histology of heart sections further supported this, with significant reduction in mononuclear cell infiltration. Cardiac parasitosis was not affected by treatment. Taken together, these results suggest that the presence of parasite pseudocysts in heart tissue is sufficient for pathology and mortality, and that the presence of host inflammation, whether parasite or myosin-directed, may be a secondary phenomenon of T. cruzi invasion and disease pathogenesis.

In this study we demonstrate that cannabinoids can inhibit *T. cruzi* invasion of cardiac myocytes *in vitro*, possibly by a calcium-dependent mechanism. It is believed that invasion may occur by multiple mechanisms and, particularly in the case of phagocytes, significant cell invasion may continue to occur in vivo by alternative mechanisms not blocked by cannabinoid agonists. Furthermore, the use of non-CB receptor specific cannabinoids may obscure this potentially therapeutic effect in vivo, by inhibiting the immune response to T. cruzi. Discrimination of the inhibitory effects on T. cruzi and the immune system may be difficult to achieve, because low doses of R(+)WIN55,212, which did not affect the immune system, also had little effect on T. cruzi invasion. At present, most cannabinoids are selective but not specific for cannabinoid receptors. It has been suggested that CB2 may be the main receptor responsible for the antiinflammatory effects of cannabinoids; therefore, the use of more CB1-selective compounds, localized cannabinoid administration, or employment of CB2 receptor knockout mice may allow a direct determination of the immunosuppressive effects of R(+)WIN55,212. Alternatively, the appropriate selection of a CB1-selective agonist and CB2-antagonist combination might provide a way forward to blocking invasion while minimizing immunosuppression. Nevertheless, this study provides data to suggest that the calcium-mediated pathway of T. cruzi invasion is a desirable therapeutic target and that future cannabinoid treatment with specific-receptor agonists, or other calcium-inhibiting compounds may be effective for treating Chagas disease.

Finally, it is of note that cannabis is widely used in regions where Chagas disease is endemic, for instance as maconha in the Northeastern states of Brazil. This study suggests that (ab)use of broad-spectrum cannabinoids may be detrimental to infected individuals. It is worth noting though, that the murine model we employed in this study, while it mimics some aspects of the human condition such as autoimmunity, is limited to the acute phase of infection. Therefore, following outcomes in a chronic model might yield additional insight into the ramifications of cannabis use during human disease pathogenesis. Currently, no epidemiological study has been published with which to correlate our outcome. However, additional studies are required to further explore the mechanisms involved in the dichotomy of cannabinoid treatment for Chagas disease. We have demonstrated that R(+)WIN55,212 has a significant inhibitory effect upon the adaptive immune response and can increase blood parasitaemia. Therefore, this study has important pathological implications for marijuana users infected with T. cruzi.

Experimental procedures

Cell culture

The Brazil strain of *T. cruzi*, used in all experiments, causes well documented pathology in mice and humans (Leon *et al.*, 2001; Tyler and Engman, 2001). Epimastigotes were maintained by

passage in LDNT medium as previously described (Tyler and Engman, 2000). Cultures of rat cardiac myoblasts (H9C2 cell line; American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's minimal essential medium (Life Technologies, Grand Island, NY) supplemented with 10% foetal calf serum.

Experimental animals and T. cruzi infections

Four- to six-week-old male A/J mice (The Jackson Laboratory, Bar Harbor, ME) were housed under specific pathogen-free conditions. Mice were infected by intraperitoneal injection of 1×10^4 *T. cruzi* trypomastigotes derived from infection H9C2 rat myoblasts. Parasitaemias were measured by haemocytometry from tail bleeds (three slides per mouse). Uninfected controls received an intraperitoneal injection of Dulbecco's PBS (Life Technologies, Grand Island, NY) of equal volume. The use and care of mice were conducted in accordance with the guidelines of the Center for Comparative Medicine at North-western University.

Administration of cannabinoids

R(+)WIN55,212 and S(-)WIN55,212 (Sigma-Aldrich, St. Louis, Missouri, USA) were dissolved in 10% Tween-80 (Sigma-Aldrich) and PBS to a final concentration of 4 mg ml⁻¹ or 1 mg ml⁻¹. The mixture was then vortexed and passed through a 24-gauge needle. Similar preparations without active compounds were used as vehicle controls. Mice were treated with suspensions (0.1 ml) injected intraperitoneally. Groups of *T. cruzi*-infected mice (n = 5) were treated with either a high dose (20 mg kg⁻¹) or a low dose (5 mg kg⁻¹) dose of WIN55 daily.

Trypanosome invasion of cardiac-derived myoblasts

Invasion assays were conducted by growth of myoblasts on coverslips until subconfluent. Tissue culture-derived trypomastigotes were added in a ratio of 10 trypomastigotes per myoblast, incubated 15 min for attachment washed three times with warm media and further incubated 15 min for invasion before fixation with freshly prepared 2% paraformaldehyde for 5 min. Attached parasites were stained with pooled sera from humans chronically infected with T. cruzi and preabsorbed Cy3-conjugated goat anti-human secondary antibody (Sigma-Aldrich). Cells were then permeabilized with 0.01% saponin and stained with mouse serum specific for T. cruzi cytoplasmic hsp70 and preabsorbed Cy5-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich). Primary antibodies were applied for 1 h in a humid chamber at room temperature. Samples were washed three times in PBS for 5 min per wash, secondary antibodies were applied, and the process was repeated. Finally, DNA was stained with 1 µg ml⁻¹ 4',6-diamidino-2-phenylindoledichydrochloride (Boehringer Mannheim, Indianapolis, IN) for 30 s and the sample was washed in distilled water before mounting under a coverslip using Permafluor (Immunotech, Marseille, France) and sealing with nail varnish. Microscopy was performed using an Olympus IX70 inverted system microscope (Olympus America, Melville, NY) and images were captured using the Delta Vision Image Restoration and Deconvolution System (Applied Precision, Issaquah, WA).

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Trypomastigote morphology

Blood smears were prepared from tail bleeds and processed for immunofluorescence microscopy as previously described (Tyler *et al.*, 1997). Smears were fixed for 5 min in 2% paraformaldehyde and permeabilized by immersion in methanol at -20° C overnight. Immunofluorescence assay for tubulin was performed essentially as described above. Trypanosomes were ascribed a slender or stout morphology as previously described (Tyler and Engman, 2001). For this analysis, three blood smears were analysed for each of the five mice per group, with at 10 cells scored for morphology per slide.

Western blotting

Trypanosomes were collected by centrifugation at 1000 *g* for 10 min, washing with PBS and pelleting again at 1000 *g*. Myoblasts were obtained by scraping of confluent cultured layers followed by similar washing and pelleting. Cardiac tissue homogenate was prepared from PBS-perfused and washed mouse hearts. Protein samples were then prepared by lysing the cells or homogenate in hot lysis buffer (1% SDS, 0.25 M sucrose, 0.125 m Tris-HCl, pH 6.8) containing protease inhibitors (50 mg ml⁻¹ chymostatin, 5 mg m⁻¹ pepstatin A, 50 mg ml⁻¹ leupeptin). Samples were centrifuged and the supernatant was decanted. Protein concentration was assayed using the Bradford assay (Bio-Rad protein assay). Extracts were then separated by SDS-PAGE, electroblotted on to nitrocellulose, incubated with antibodies and visualized as previously described (Tyler *et al.*, 1997).

Histopathology

Hearts were removed, perfused and rinsed with saline (PBS), and fixed for 24 h in 10% buffered formalin. Fixed hearts were paraffin embedded, sectioned in the atrial-apical axis, haematoxylin-eosin stained, and examined by light microscopy. Two sections were taken from each heart, one including both atria and the other both ventricles. Each section was examined for evidence of mononuclear and polynuclear cellular inflammation, and for *T. cruzi* pseudocysts. Sections were scored double blind with each investigator assigning a histologic score of between 0 (no involvement noted) and 4 (100% involvement), with 1, 2 and 3 representing 25%, 50% and 75% involvement of the histologic section (Leon *et al.*, 2003) for inflammation and absolute count for number of pseudocysts. In cases where scores varied, which was rare, both investigators re-examined the slide together (still blinded) and a score was agreed upon.

Immunohistochemistry

Mice were anaesthetized and (cold PBS) perfused. Hearts were removed and immediately frozen in OCT (Miles Laboratories; Elkhart, IN)/liquid nitrogen. Six micrometer thick cross-sections of the heart were cut on a Reichert-Jung Cryocut CM1850 cryotome (Leica, Deerfield, IL), mounted on Superfrost Plus slides (Fisher, Pittsburgh, PA), air dried, and stored at -80°C. Slides were stained using a Tyramide Signal Amplification (TSA) Direct kit (NEN, Boston, MA) according to the manufacturer's instructions. Heart sections were thawed, fixed in 2% paraformaldehyde at room temperature, and rinsed in 1× PBS. Non-specific staining was blocked using anti-CD16/CD32 (Fc γ III/IIR, 2.4G2; BD PharMingen), and an avidin/biotin blocking kit (Vector Laboratories) in addition to the blocking reagent provided by the TSA kit. Tissues were stained with biotin-conjugated anti-mouse CB1 (Calbiochem, MI, USA) and Cy5-conjugated streptavidin. Sections were counterstained with DAPI and mounted as described above.

Serologic analysis

Levels of cardiac myosin-specific and *T. cruzi*-specific immunoglobulin G (IgG) were analysed by ELISA. Briefly, Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl of antigen (2.5 µg ml⁻¹) in PBS. The plates were washed with PBS containing 0.05% (v/v) Tween 20 and then blocked with 2% bovine serum albumin (BSA) and 5% normal goat serum. The plates were then incubated with twofold serial serum dilutions (1:10 initial dilution for 16 dilutions) for 2 h at 37°C. After the washes, peroxidase-conjugated goat anti-mouse IgG (heavy and light chain) (0.25 µg ml⁻¹; KPL, Gaithersburg, MD), was added for 1 h at 37°C. The bound enzyme was detected with the 3,3',5,5'-tetramethylbenzidine substrate (KPL) and quantified by measurement of the optical density at 450 nm (OD₄₅₀) in a Kinetic MicroPlate Reader (Molecular Devices, Sunnyvale, CA).

Delayed-type hypersensitivity

Myosin DTH was quantified by a standard ear swelling assay. Prechallenge ear thickness was measured with a Mitutoyo model 7326 engineer's micrometer (Mitutoyo MTI Corporation, Aurora, IL). Myosin or *T. cruzi* antigen (10 μ g in 0.15 M K₂PO₄, 0.01 Na₄P₂O₇, 0.3 M KCl, pH 6.8) was injected intradermally into the dorsal surface of the ear with a 100 μ l Hamilton syringe fitted with a 30-gauge needle. BSA was injected in the opposite ear as an injection control. After 24 h, the net swelling of the control ear was subtracted from the net swelling of the challenge ear and expressed in units of 10⁻⁴ inch. Antigen-induced ear swelling was the result of mononuclear cell infiltration and exhibited typical DTH kinetics (i.e. minimal swelling at 4 h and maximal swelling at 24–48 h).

Statistical analysis

For parametric testing between multiple groups, one way ANOVA followed by two-tailed *t*-test was used; comparison of treated group with control was by two-tailed *t*-test for independent samples. For histopathology scores potentially reduced inflammation was tested using the non-parametric, one-tailed Mann–Whitney *U*-test. Scores of P < 0.05 were considered significant.

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