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Enzymatic markers of heart lesion in mice infected with *Trypanosoma cruzi* and submitted to benznidazole chemotherapy

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Abstract Creatine kinase (CK total and CK-MB) were studied as markers of lesion progression induced by *Trypanosoma cruzi* infection. After 3 weeks mice infected with 10^4 parasites showed an increase in both enzyme levels and in their frequency distribution. A trend to increase was already detected in the 2nd week. A short duration per os treatment with benznidazole (Bz) prevented the occurrence of tissue lesions, since no changes were observed in enzymes. However, in the 4th week, about 40% of Bz-treated mice showed an increase in CK-MB, as did those that survived until the 8th week. Long-term treatment with Bz in drinking water of mice infected with 10^2 parasites showed, after 32 weeks, a partial reversion of the occurrence of high CK-MB levels from 85.7% to 50%. We found a positive correlation between inflammatory infiltrates and CK-MB levels, indicating that this marker could be useful to monitor the occurrence of experimental chagasic myocarditis.

Introduction

Chagas' disease, caused by *Trypanosoma cruzi* infection, affects about 17 million people and is the most important cause of myocardial diseases among parasitic infections in Latin America (WHO 1997). Acute myocarditis occurs in about 8% of infected individuals, with intense parasitism and frequent lesions of heart muscle cells (reviewed in Dias and Coura 1997). Most of the non-treated acute cases evolve to the intermediate phase, characterized by the absence of symptoms and electrocardiographic or radiological alterations. Development of the chronic phase occurs in about 2–3% cases/year, mainly 10–20 years after the primary infection. About 27% of these patients present progressive cardiac damage due to destruction of myocardial cells and cells from the conductive system, resulting in cardiac insufficiency, rhythm and electric alterations, enlargement of the heart, and sudden death.

Following cell lesion, membrane integrity is lost, and macromolecules diffuse to extracellular spaces and drain into blood vessels. Creatine kinase (CK, E.C. 2.7.3.2.) and its cardiac CK-MB isoenzyme (Tietz 1980; Lott and Stang 1980; Ellis 1991) are useful enzymes serving as indicators of tissue lesion. CK-MB determination is one of the markers of myocardial injury, employed for the confirmation of acute myocardial infarction (Adam et al. 1992). CK is a key molecule involved in energy processes, representing a system of several isoenzymes compartmentalized where energy is produced and used: CK-MB in heart muscle, CK-MM in skeletal muscle, CK-BB in the brain and CK-Mi in mitochondria. It catalyses the reversible transfer of a phosphoryl group from phosphocreatine to ADP (Wallimann and Hemmer 1994). In experimental *T. cruzi* infection, lactate dehydrogenase and all CK isoenzyme activities increase during the acute infection (Mercado 1976; Mercado and Garbus 1979), and a parallel histopathological analysis associated these changes with severe heart and skeletal muscle damage. Depending on the parasite strain studied, total CK levels increased 4 and 70 times, with macrophagotropic and myotropic strains of *T. cruzi*, respectively (Mercado and Garbus 1979). The release of CK was also used as a marker of cardiomyocyte lesion in lymphocyte antibody-mediated cytotoxicity assays (Laguens et al. 1988).

In the present work we support the use of CK and CK-MB as markers of lesion progression induced by *T. cruzi* infection, and demonstrate a correlation between CK-MB levels and tissue inflammation in acute

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The authors dedicate this paper to the centenary of the Oswaldo Cruz Institute, founded on 25 May 1900.

myocarditis. We also analyse the effect of different schedules of treatment with benznidazole on CK and CK-MB levels in acute and chronically infected animals.

Materials and methods

Infection and treatment of the animals

Swiss mice weighing 18–20 g were used. The animals were divided into the following groups: infected and treated (GIBz), infected and non-treated (GI), non-infected and non-treated (GN). In some experiments, a non-infected and treated control group was included (GNBz). The animals were maintained in our animal facility in stable conditions of temperature and light with 12-h cycles of light/dark, controlled automatically. Bloodstream forms of the Y strain of *T. cruzi* (Silva and Nussenszweig 1953) were obtained from the blood of Swiss mice, purified by differential centrifugation and re-suspended in phosphate buffered saline. Benznidazole (*N*-benzyl-2-nitroimidazoyl acetamide, Rochagan, Roche) (Richle 1973) was prepared by trituration of the tablet, suspension in distilled water containing 3% Tween 80, followed by sonication for 15 min. Afterwards, the final concentration of the drug was adjusted with water. The following therapeutic schemes with benznidazole (Bz) were used: (a) intraperitoneal infection (i.p.) with 10^4 bloodstream forms of *T. cruzi* plus treatment per os with 100 mg Bz/kg of body weight for 9 consecutive days (scheme a) (Pereira et al. 1998); (b) i.p. infection with 10^2 parasites plus administration of Bz in the drinking water (0.25 mg/ml) for 50 days (scheme b). Both schemes started immediately after infection. In the latter scheme, the daily volume consumed by the animals was monitored for calculation of the amount of drug ingested, corresponding to 62.5 mg Bz/kg per day.

Parasitological parameters

Parasites were counted to check the level of parasitaemia using the Pizzi-Brener method (Brener 1962). The mortality was noted daily and the following indices were calculated: percentage of cumulative mortality (%CM) 40 days after infection, survival time (ST) for succumbing mice expressed in days, and the day when mortality attained 50% (M_{50}). Data were analysed at different days or weeks post-infection (dpi and wpi, respectively).

Plasma samples

Approximately 30 µl of blood was collected in heparinized micro-capillaries from the tail of each mouse prior to, and after different times during infection up to animal death or to the 40th day post-infection. Plasma was obtained after centrifugation of blood in a micro-haematocrit centrifuge. Samples were frozen at −20 °C until analysis.

CK and CK-MB assays

The activities of CK and CK-MB were measured weekly before and after infection, using commercial kits CK-NAC and CK-MB Granutest (Merck, Darmstadt, Germany). Incubation of plasma or sera samples with the substrate led to a net increase in NADPH concentration that is directly proportional to enzyme activity in the samples. The assay was adapted for reading in a microplate spectrophotometer (Spectramax 250 Molecular Device, USA), to allow the study of low volumes of mouse plasma. After preliminary standardization assays, the volume of plasma chosen was 5 µl, and for maintenance of the proportion recommended by the manufacturer, 125 µl of reagent (substrate) was used, and the experiments performed in triplicate in 96-well microplates (bottom U). After 3 and 5 min of reaction (for CK and CK-MB, respectively), the absorbance was read at each minute using a 340 nm filter . The results were expressed in ΔOD/min. Mean ΔOD of the first three and six readings was applied for the determination of CK and CK-MB, respectively. The normal cut-off for enzyme ΔOD was calculated corresponding to the 95th percentile: 0.025 for CK ($n = 219$) and 0.032 for CK-MB ($n = 217$). Successive analysis of positive samples after 1–6 cycles of freeze and thawing showed no significant decrease of enzyme activity.

Histopathological analysis

Four animals of GN and eight each of GI and GIBz were sacrificed at 0, 9 and 15 dpi. The heart tissue was processed in paraffin-embedded sections stained with haematoxylin and eosin. Quantification of the histopathological alterations was performed under an optical microscope by observation of 30 fields (objective ×40) counting per area (mm^2) the number of: (1) nests of parasites (cells with amastigotes); (2) foci of inflammatory infiltrate (containing at least 10 mononuclear cells).

Statistical tests

Statistical significance ($P < 0.05$) was evaluated using Student's *t*-test or ANOVA test for the log of parasitaemia levels, the log Rank (Mantel-Cox) test for survival analysis. Pearson Product-Moment was used to test the correlation between enzyme levels and the inflammatory process.

Results

Parasitological parameters of infected mice submitted or not to benznidazole treatment

Mice infected with 10^4 parasites (GI) presented high parasitaemia (4.7×10^6 parasites/ml), peaking at the 8th dpi. Cumulative mortality was 100% in the 3rd wpi, with $M_{50} = 16$ days and mean survival time 15.0 ± 0.8 days. With a lower inoculum (10^2), parasitaemia was lower (0.9×10^6 parasites/ml) and the peak occurred only at the 11th dpi. Survival time was higher (%CM = 25% in the 3rd wpi, reaching only 37.5% in the 4th wpi; ST = 21.3 ± 3.5 days), and the chronic phase was established. Both schemes of treatment with Bz (groups GIBz), per os and administration in the drinking water, prevented the detection of parasites in the blood and the animals did not die (%CM = 0%) until 40 dpi.

Enzyme detection in infected mice

Figure 1 shows the kinetics for CK and CK-MB activities up to 15 min, determined in 10 normal (Fig. 1a, b) and 16 infected mice (Fig. 1c, d), after 8, 14 and 21 dpi with 10^4 parasites. The two enzyme activities evolved slightly differently: in samples with high activity, a plateau was attained after 6 min for CK, and 13–14 min for CK-MB. As expected, wells containing only the substrate gave a horizontal line (blank). Normal plasma, as well as most of the infected mice, commonly presented low readings, with a linear pattern of activity evolution (Fig. 1a, b). Very high enzyme activity was detected only in samples from mice after 2 and 3 weeks of infection (Fig. 1c, d). Some samples showed high CK-MB levels (e.g., Fig. 1d,

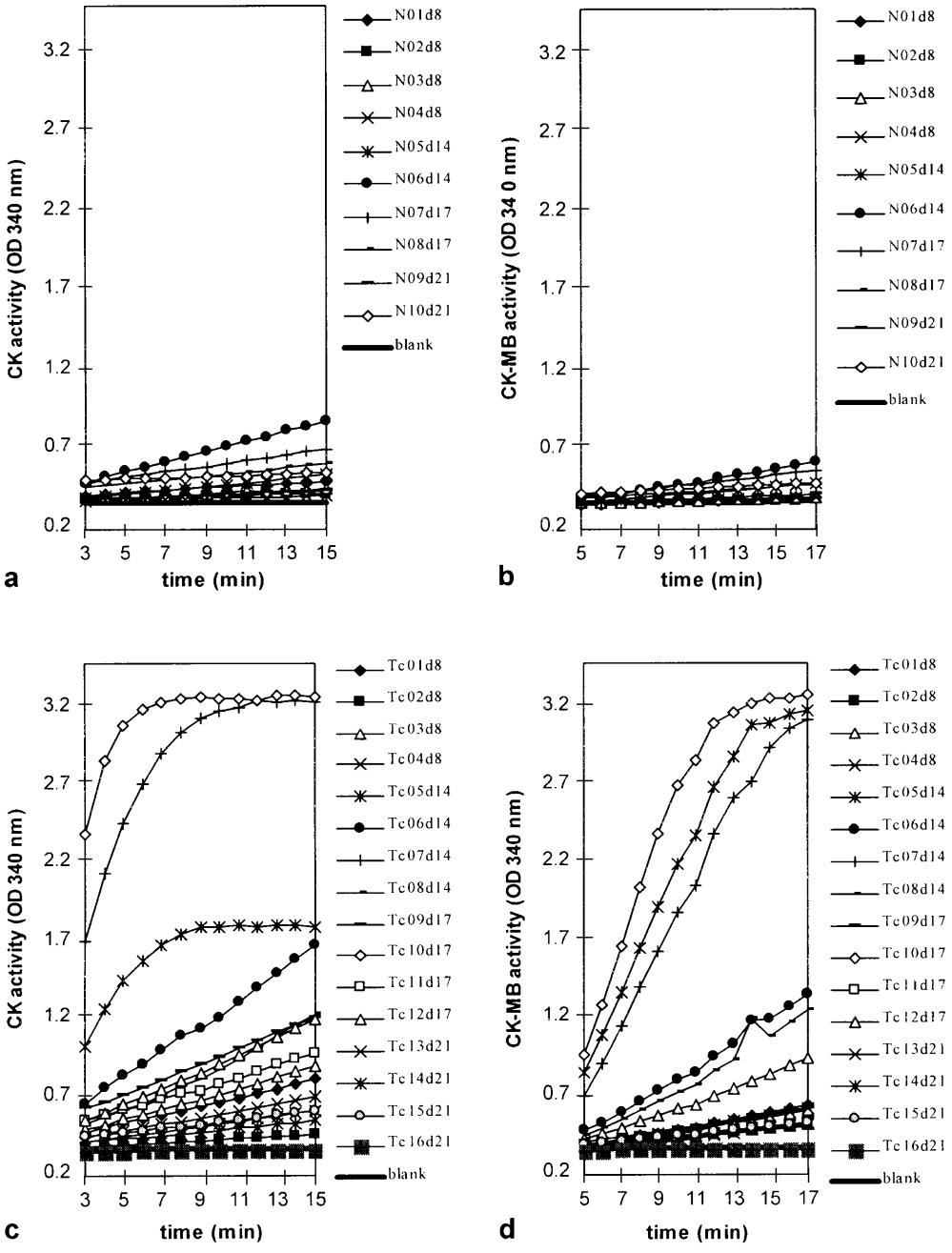

Fig. 1 Kinetics of CK (**a**, **c**) and CK-MB (**b**, **d**) activities for 15 min in plasma of non-infected (*N*) (**a**, **b**) and *Trypanosoma cruzi*-infected (*Tc*) (**c**, **d**) mice

Tc10d17, Tc07d14 and Tc05d14), concomitant with the increase in CK. Since CK activity in some samples evolved very quickly, with a linear pattern only in the first three measurements (Fig. 1b, Tc10d17, Tc07d14), we used for this enzyme only the three first readings to calculate ΔOD values, while for CK-MB the first six readings were used. Since we intended to follow up individual animals for long periods, we checked for changes and assert that during 32 weeks the levels of both enzymes did not change significantly in non-infected animals (data not shown). The median ΔOD values obtained for each time point were never higher than the cut-off level established for each enzyme (0.025 for CK and 0.032 for CK-MB). This indicated that the maintenance period in the animal facility did not influence the enzymatic levels that we intended to study. We also tested if Bz administration alone could induce any changes in both enzymes. Results obtained in the plasma of 24 non-infected and Bz-treated (GNBz) mice collected during 2–4 weeks were similar to those obtained with non-infected (GN) mice (data not shown).

CK and CK-MB analysis in normal, infected (10^4 parasites) and Bz-treated per os mice (scheme a)

The median value of ΔOD/min indicated a trend to increase both in CK and CK-MB levels in GI in the 2nd

and 3rd wpi (Table 1), which was reversed by Bz per os treatment. After the 3rd wpi, only GIBz animals survived and their enzyme activities were still increasing in relation to non-infected mice. The increase in enzyme levels was clearly observed by the values expressed in the 75th percentile (Table 1). Given the variations found in CK and CK-MB levels, we performed a frequency distribution study of enzyme activities (Fig. 2a–d). For CK, a significant proportion of 20% and 38.5% of the samples from GI were higher than the cut-off value, respectively, for the 2nd (Fig. 2a) and 3rd wpi (Fig. 2c). Ten percent of the samples attained $\Delta OD/min = 0.07$ in the 2nd wpi. For CK-MB, this percentage was 10% and 38.9% of the mice, respectively, for the 2nd (Fig. 2b) and 3rd (Fig. 2d) wpi, in GI. In conclusion, we observed that the infection with 10^4 parasites led to an increase in CK and CK-MB in about 40% of the animals.

In Bz-treated mice (GIBz), an important percentage of the surviving mice with negative parasitaemia had high enzyme levels in the 4th wpi (Table 1, Fig. 2e, f). Indeed, the frequency distribution analysis indicated that a percentage of mice in this group had increased CK (Fig. 2e) and CK-MB (Fig. 2f) levels throughout the period of infection. The two mice analysed in the 8th wpi both had high CK levels, in relation to CK-MB; one had a borderline value and the second had a very high enzyme level (Table 1, Fig. 2e,f). These results indicated that this therapeutic scheme controls parasite growth, favouring survival, but did not prevent the occurrence of muscle lesions.

CK-MB analysis in normal, infected (10^2 parasites) and mice administered Bz in drinking water (scheme b)

As CK and CK-MB increases were always associated in the experimental model with low inoculum (10^2 trypomastigotes), we analysed just the specific myocardial isoenzyme CK-MB for the study of chronic chagasic cardiomyopathy. As expected, CK-MB levels did not change in GI during the acute (2–4 wpi) phase and in the transition to the chronic phase (8th wpi, Table 2). At the 32nd wpi a very high median of 0.033 was observed in this group, with a mouse attaining the maximum level of 0.240, an increase of ×60 in relation to the median of GN. Eighteen out of 21 GI animals (85.7%) presented CK-MB levels higher than the 95th percentile obtained in this experiment in the 32nd wpi (Table 2), indicating that even with a low inoculum of the macrophagotropic Y strain, heart lesions in the chronic phase could be detected by the enzyme assay.

In the Bz-treated mice, at the 32nd wpi the median value was half the value found in GI, despite remaining fourfold higher than GN. Half of the surviving mice in GIBz still had high CK-MB levels. These results indicated that Bz treatment prevents an increase in CK-MB levels in the acute phase and reduces the intensity and the frequency of heart damage in the chronic phase. Interestingly, in the 8th wpi Bz-treated mice had a median CK-MB level of 0.011, which was higher ($P = 0.007$) than the value of 0.004 found in GN, but when compared to GI this increase was not statistically significant ($P = 0.06$) (Table 2). The hypothesis of a direct effect of Bz on the animals was tested by including the control group GNBz, which also showed a twofold increase in CK-MB levels compared to GN ($P = 0.014$). Statistical comparison between GNBz and GIBz at this time confirmed that they did not differ (Table 2), indicating a delayed toxic effect of the drug at the 8th wpi.

Correlation between heart histopathology and CK-MB plasma levels

Given that CK-MB levels did not increase in all the animals, we investigated if enzyme levels could be directly correlated with histopathological lesions. We

Table 1 CK and CK-MB activities in normal, *Trypanosoma cruzi*-infected and infected + treated mice (scheme a) (*GN* non-infected mice; *GI* infected mice; *GIBz* infected + Bz-treated mice)

wpi	GN	GI			GIBz				
		T. cruzi 10^4			*T. cruzi* 10^4 + Bz 100 mg/kg 0–9 dpi				
		1	2	3	1	2	3	4	8
CK median[a]	0.010	0.013	0.018[c]	0.018[c]	0.014	0.012	0.012	0.018[c]	0.063
*P*25	0.007	0.010	0.011	0.011	0.011	0.010	0.007	0.017	0.049
*P*75	0.017	0.016	0.023	0.029	0.016	0.027	0.016	0.032	0.077
n =	99	17	11	14	20	13	27	20	2
%[b]	5.1	0	20	38.5	5.0	30.8	11.1	35.0	100
CK-MB median	0.014	0.018	0.016	0.024[c]	0.017	0.010	0.015	0.025[c]	0.040
*P*25	0.010	0.012	0.013	0.012	0.016	0.009	0.012	0.014	0.035
*P*75	0.021	0.021	0.023	0.048	0.022	0.014	0.019	0.034	0.045
n =	119	18	11	19	20	12	26	19	2
%[b]	7.6	0	10	38.9	5.0	16.7	11.5	42.1	50

[a] Median values expressed in ΔOD/min
[b] Percentage of samples with enzyme levels higher than the cut-off (0.025 for CK and 0.032 for CK-MB)
[c] $P < 0.05$ when compared with GN

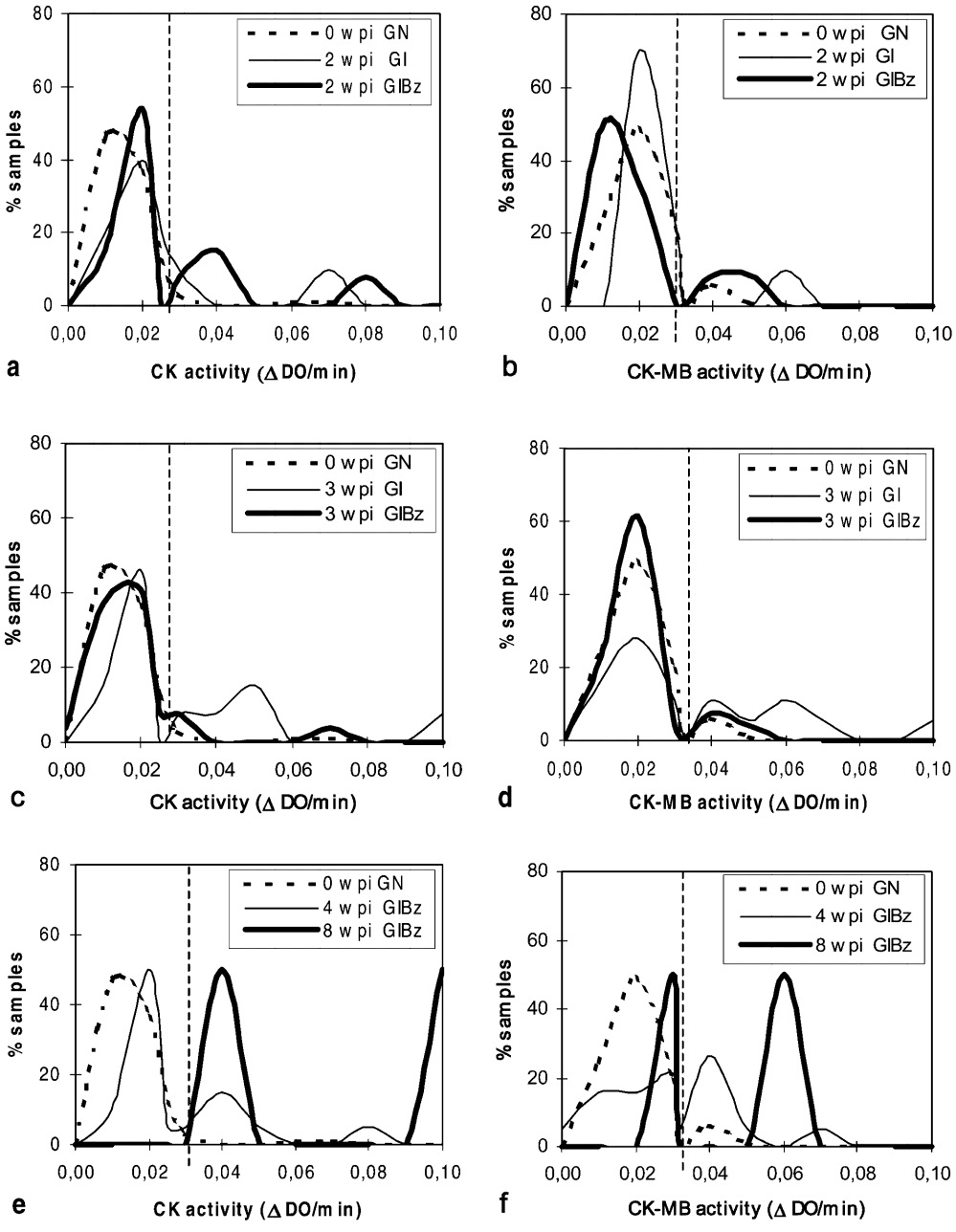

Fig. 2 Frequency distribution of CK (**a**, **c**, **e**) and CK-MB (**b**, **d**, **f**) levels in non-infected mice (*GN*), mice infected with 10^4 parasites (*GI*) and infected with 10^4 parasites and treated with Bz per os (*GIBz*), showing the results after 2 wpi (**a**, **b**), 3 wpi (**c**, **d**) and 4 and 8 wpi (**e**, **f**). *Vertical dotted lines* indicate the cut-off level for each enzyme

analysed individual CK-MB levels in a large number of mice and sacrificed for histopathology four non-infected mice (GN), eight infected (GI) and eight infected and Bz-treated mice (GIBz), with CK-MB levels below and above the cut-off (Table 3). In the 9th dpi, no differences were detected among the three groups. At 15 dpi the inflammatory process in the heart was very intense (Fig. 3b), with diffuse foci of mononuclear cells all over the tissue in GI. The presence of cell debris and a feeble faint in relation to normal fibres detected necrosis. Amastigote nests were clearly detected (Fig. 3b). This pattern was reversed by Bz treatment (Fig. 3c). When quantitative analysis of the inflammatory foci and the parasite nests were performed, differences were clearly detected (Table 3). In the 15th dpi, the infected animals presented statistically significant differences from GN and GIBz in all the parameters tested.

Comparison of CK-MB levels with histopathological alterations showed a significant positive correlation with the number of total inflammatory foci (Fig. 4a, $r = 0.788$, $P < 0.01$), but no correlation between enzyme with parasite nests (Fig. 4b, $r = 0.4265$). The correlation with foci in the periphery of the organ was even higher ($r = 0.8529$, $P < 0.00003$). The significant correlations of CK-MB and inflammatory foci were sustained even when the analysed sample was reduced to 12 mice, by excluding GIBz animals. In this case, correlation indices attaining $r = 0.8929$ for foci in the centre ($P = 0.000092$), or $r = 0.8467$ for total foci ($P < 0.00003$), were highly significant.

Table 2 CK-MB activities in normal, *Trypanosoma cruzi*-infected and infected + treated mice (scheme b) (*GN* non-infected mice; *GI* infected mice; *GIBz* infected + Bz-treated mice)

wpi	GN	GI				GIBz				GNBz	
		T. cruzi 10^2				*T. cruzi* 10^2 + Bz 62.5 mg/kg 0–25 dpi				Bz 62.5 mg/kg 0–25 days	
	0	2	4	8	32	2	4	8	32	8	2–8
CK-MB median[a]	0.004	0.002	0.007	0.004	0.033[c]	0.003	0.006	0.011[c]	0.016[c,d]	0.008[c]	0.005
*P*25	0.002	0.002	0.006	0.004	0.024	0.002	0.005	0.006	0.012	0.003	0.003
*P*75	0.010	0.003	0.009	0.007	0.041	0.004	0.007	0.030	0.035	0.023	0.008
n=	25	10	8	9	21	12	13	22	37	15	39
% > 0.015[b]	7.4	0	12.5	22.5	85.7	0	0	40.9	50.0	33.0	12.8
	n = 2		*n* = 1	*n* = 2	*n* = 18			*n* = 9	*n* = 19	*n* = 5	*n* = 52
% > 0.032	0	0	0	0	52.4	0	0	0	22.7	6.6	2.5
					n = 11				*n* = 5	*n* = 1	*n* = 1

[a] Median values expressed in ΔOD/min
[b] Percentage of samples with enzyme levels higher than the cut-off (95th percentile = 0.015 for GN of this experiment and 0.032 for all the 119 non-infected mice)
[c] $P < 0.05$ when compared with GN
[d] $P < 0.05$ when compared with GI

Table 3 Individual data of animals from non-infected, *Trypanosoma cruzi*-infected and infected + Bz-treated groups (scheme a) for correlation between CK-MB plasma levels and heart histopathological alterations (*GN* non-infected mice; *GI* infected mice; *GIBz* infected + Bz-treated mice)

Groups		cdg/dpi	CK-MB	Parasite nests[a]		Inflammatory foci[b]		Necrosis
			ΔOD/min[c]	Centre	Border	Centre	Border	
GN	d9	N1d9	0.008	0	0	3	15	–
	d15	N2d15	0.009	0	0	2	11	–
		N3d15	0.009	0	0	2	5	–
		N4d15	0.008	0	0	3	8	–
GI	d9	Tc1d9	0.011	0	0	8	10	–
		Tc2d9	0.016	1	0	1	8	–
		Tc3d9	0.020	0	0	0	8	–
		Tc4d9	0.023	2	0	6	13	–
	d15[d]	Tc5d15	0.039	9	12	14	14	+
		Tc6d15	0.044	0	0	27	36	+
		Tc7d15	0.139	9	19	37	32	+
		Tc8d15	0.053	34	86	23	19	+
GIBz	d9	Bz1d9	0.008	0	0	0	3	–
		Bz2d9	0.009	0	0	2	0	–
		Bz3d9	0.038	0	0	2	3	–
		Bz4d15	0.007	0	0	3	5	–
		Bz5d15	0.010	0	0	2	2	–
		Bz6d15	0.020	0	0	3	9	–
		Bz7d15	0.017	0	0	3	5	–
		Bz8d15	0.034	0	0	0	0	–

[a] Number of nests/6 mm^2, counting 15 fields at the periphery and 15 at the centre
[b] Number of foci with more than 10 mononuclear cells/6 mm^2, counting 15 fields at the periphery and 15 at the centre
[c] Median values expressed in ΔOD/min
[d] $P < 0.05$ for GI × GN and GI × GIBz (Mann–Whitney *U*-test)

An association was also noted between high CK-MB levels and the presence of tissue necrosis (Fig. 4d), since necrosis was also associated with the high inflammatory process (Fig. 4c). The four infected mice that presented necrosis at the 15th dpi had higher CK-MB levels (Table 3, Fig. 4d), but only three had detectable parasite nests (Fig. 4e).

Discussion

In the present work, we confirmed that the plasma levels of CK and CK-MB increase in an important percentage of mice infected with *T. cruzi*, and showed for the first time a positive correlation between CK-MB activity and the degree of heart inflammatory process detected histopathologically in the animals.

Our first step was to use commercial kits for both enzymes, to work with small volumes and to allow monitoring of individual animals during the course of infection. The next step was the definition of the range of normal values for each enzyme in non-infected mice. Analysing more than 280 animals, as both enzyme levels showed no Gaussian distribution, we expressed the results in median and percentiles, using the 95th percentile as the cut-off value for CK and CK-MB. Another

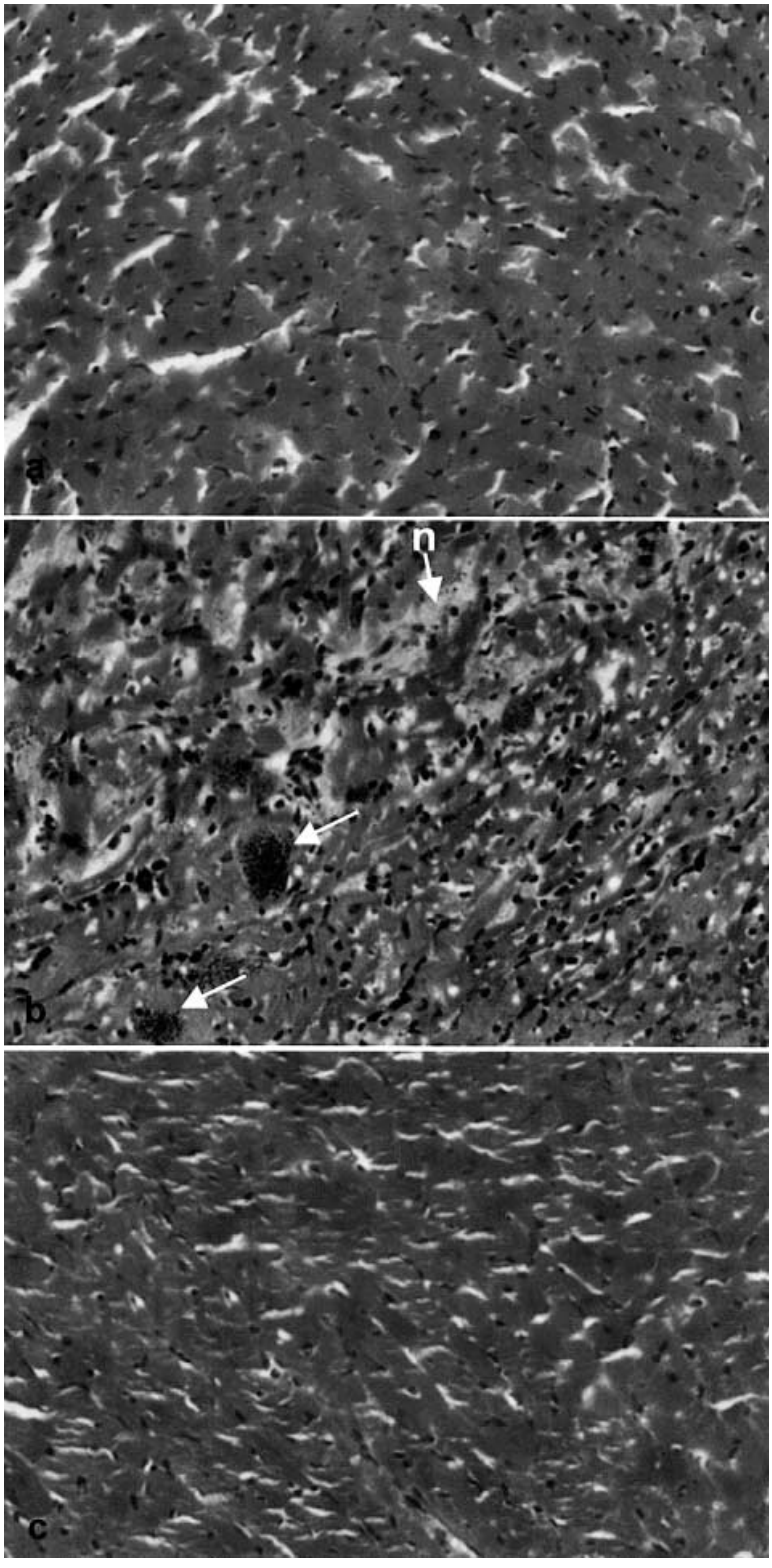

Fig. 3 Histological patterns in heart sections of non-infected mice (**a**), mice infected after 15 dpi (**b**) and infected and Bz-treated (**c**) mice . Note the intensity of the inflammatory process (**b**) with mononuclear cell infiltrates, necrosis (*n*, *arrow*). Note also the presence of parasite nests (*arrows*). Magnification ×920

important point was the evidence that neither enzyme level suffered any significant influence by maintenance of the mice for more than 32 weeks in the animal facility.

The increase in CK and CK-MB levels was monitored by: (1) the kinetics of individual animals, which showed an increase in relation to the cut-off in the 3rd wpi; (2) observation of median and percentiles values that showed a trend to increase by the 2nd wpi; (3) frequency distribution of the enzymes levels after different times of infection. The two inocula used were low, compared to those commonly used by other experimenters to induce acute myocarditis. For example,

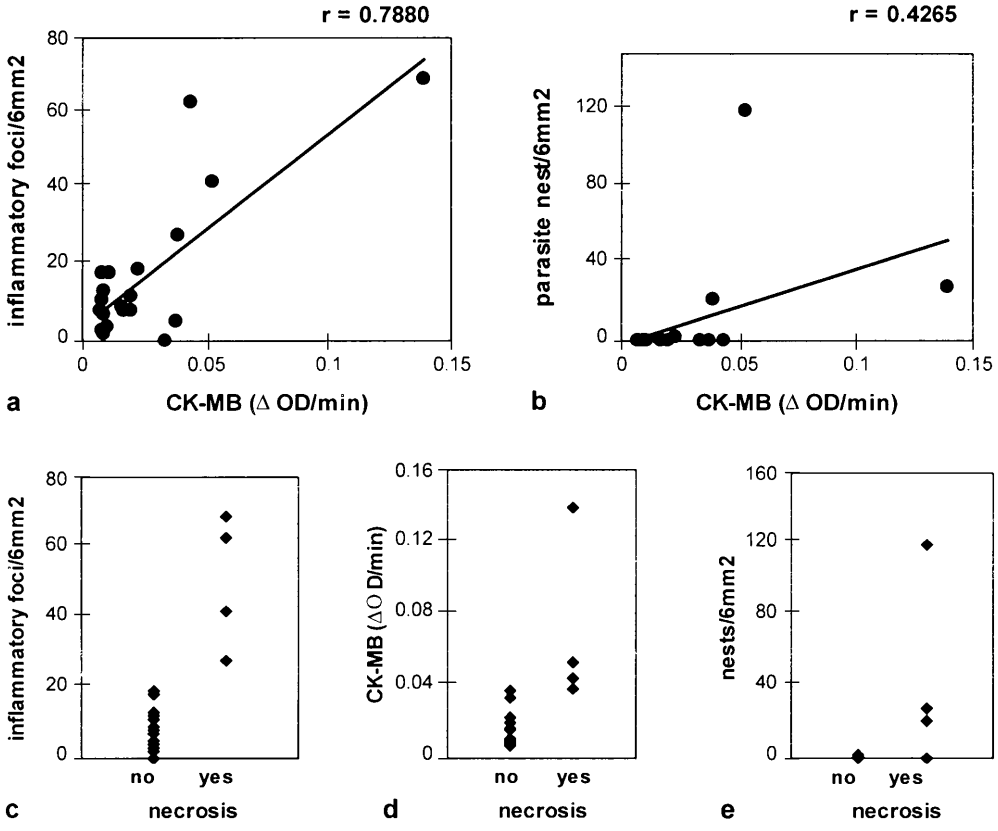

Fig. 4a–e Correlation of CK-MB activity and the inflammatory and parasitological parameters obtained from histopathological analysis of the heart of animals from groups *GN* ($n = 4$), *GI* ($n = 8$), *GIBz* ($n = 8$). Correlation of CK-MB with the number of inflammatory foci (**a**) and with the number of parasite nests (**b**). Association of the presence of necrosis and inflammatory foci (**c**), CK-MB levels (**d**) and parasite nests (**e**)

Andrade and coworkers (1985), employing an inoculum of 10^5 trypomastigotes of the Y strain, observed myocardial damage in the 2nd wpi. This fact, as well as the macrophagotropic characteristics of the Y strain (Melo and Brener 1978), could explain why mice infected with 10^2 parasites did not present changes in CK-MB levels and why just a relatively low percentage (40%) of mice infected with 10^4 parasites did present biochemically detectable myocarditis in the acute phase. Mercado and Garbus (1979) reported that total CK increased about 70 times in animals infected with myotropic strains, while the increase was only 4 times for a macrophagotropic strain. They clearly showed that myotropic strains induced extensive damage to the myocardium, expressed by much higher CK levels, than a macrophagotropic strain. Similar to our results, the increase in CK levels began in the 2nd wpi. These data reinforce the concept that tissue lesions are induced by the parasites from different populations presenting different characteristics (Vago et al. 1996; Andrade et al. 1999).

When we followed animals submitted to treatment with Bz, we observed that both administration per os and in the drinking water were effective in maintaining sub-patent parasitaemia levels and preventing animal death. Using the per os treatment (scheme a), no changes in CK and CK-MB levels on the 3rd wpi were observed, suggesting that Bz prevented the occurrence of lesions on cardiac and on other muscular tissues. This fact was confirmed by histopathological analysis. However, in the 4th wpi, about 40% of the Bz-treated animals showed an increase in CK and in CK-MB, as well as those that survived until the 8th wpi. As the per os treatment was of short duration (0–8th dpi), it is possible that a microscopically undetectable parasite load could induce heart inflammation at the 4th wpi and cause tissue damage, reflected in the increase in plasma levels of both enzymes. Alternatively, a later toxic effect of Bz could also account for these results. This interpretation was reinforced by results showing that normal non-infected mice (GN) had levels half those of normal Bz-treated mice (GNBz) after 8 weeks, when scheme b (see below) was employed.

The long-term treatment with Bz in the drinking water (scheme b: 0–25 dpi) of animals infected with a low inoculum of *T. cruzi* allowed us to monitor the animals until the chronic phase. This scheme induced no biochemically detected myocarditis in the acute phase of the infection. Despite the reduction in mortality and parasitaemia at the 32nd wpi, 85.7% of the infected and non-treated group (GI) presented high levels of CK-MB, while the treatment with Bz (GIBz) partially reduced the incidence of high enzyme levels to 50%. This treatment did not prevent cardiac muscle lesions in the chronic phase. This fact emphasizes the need for a more thorough investigation of animals submitted to therapy with new drugs, combined with serological and parasitological criteria for cure, as well as biochemical and functional markers of myocarditis.

Comparison of the low and high inoculum models used in the present work points to the important role of

the parasite load on the subsequent development of heart lesions. This result confirms recent data from Marinho and coworkers (1999), showing that the intensity of parasitism in the acute phase is a determinant in the development of chronic myocarditis.

The existence of animals with high CK-MB levels encouraged us to perform a quantitative histopathological analysis of the heart inflammatory process. We found a positive correlation between inflammatory infiltrates and the plasma levels of the cardiac enzyme. It is important to point out that no correlation was established between enzyme levels and the number of parasite nests, indicating that the myocardial tissue damage was mainly associated with the inflammatory response and not with a direct effect of *T. cruzi*.

Previous reports on chagasic myocarditis point to the importance of the presence of mononuclear cells in the formation and development of the inflammatory response. In a kinetics study of the inflammatory infiltrate in CBA/J mice infected with 10^5 trypomastigotes from the Tulahuen strain, Molina and Kierszenbaum (1988) found myocardial regions with incipient inflammation and preserved tissue. These regions presented a low number of inflammatory cells, composed of 96–100% mononuclear cells, showing their contribution to the inflammatory response. These authors showed that the higher the degree of this response, the greater the tissue destruction. The number of mononuclear cells was even higher in necrotic areas, and thus explains the presently found association between CK-MB levels and necrosis, and the correlation with inflammatory foci. None of the publications on this topic (Packchanian and Robinson 1958; Abelman 1969; Kumar et al. 1969; Molina and Kierszenbaum 1988) correlated the intensity of this process with any biochemical marker of tissue lesion. The association of the biochemical determination of CK-MB with the several parameters used nowadays in the follow-up of chagasic patients could be valuable in monitoring both the evolution of the carditis and the aetiological treatment of Chagas' disease. We are presently testing this biochemical marker in other experimental models of *T. cruzi* infection and in patients with different forms of Chagas' disease.

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