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Immunopathological mechanisms underlying the time-course of *Trichinella spiralis* cardiomyopathy in rats

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Abstract The present study shows that isolated, perfused hearts from rats orally infected with *Trichinella spiralis* have a reduced left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF). This reduction is considerably enhanced by a single bolus (100 pM) of PAF (platelet activating factor, an eosinophil activator), especially at 21 days post-infection (d.p.i.), which is the time of the maximum increase in blood and tissue eosinophilia. Helminthic DNA analysis shows that, from 21 d.p.i. onwards, the morphological and functional changes in the myocardium cannot be ascribed to the parasite's presence, whereas its antigens and the attendant immunopathological reactions might have a role in the induction of myocardial damage and dysfunction. Some perivascular inflammatory cells (eosinophils and mast cells) appear to undergo degranulation. All these data suggest a complex sequence of events, from acute myocarditis (21 d.p.i.) which may lead in time (48 d.p.i. onwards) to a dilating cardiomyopathy.

Key words Myocarditis · *Trichinella spiralis* · Eosinophils · PAF

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Introduction

Myocarditis is a relatively frequent complication of severe trichinellosis in humans and can be responsible for a lethal outcome [17]; the mechanisms underlying this process are not clear. For this reason, experimental infections in animals are useful as the may provide information that can shed some light on the matter.

The hearts of rats infected with *Trichinella spiralis*, retrogradely perfused accoding to Langendorff [19], show a markedly arrhythmic behaviour particularly at 21 days after oral infection (d.p.i.). This change is accompanied by histological modifications, such as perivascular mast-cell infiltration (with or without degranulation) and sparse foci of oedema and fibre disarray [19]. This pattern and the attendant functional alterations (including contractility and coronary flow changes) can also be observed at 48 d.p.i., when the myocardium begins to show a tendency to dilatation [18]. An immunopathological mechanism seems to be involved as well as possible vascular disorders (reduced arterial blood flow and/or lymph drainage), as reported in other parasitic diseases such as Chagas' disease [16, 20]; in fact IgG immunocomplexes become clearly evident from 30 d.p.i. in the absence of parasite antigens [19], in contrast to what has been reported in other parasite-induced models of myocarditis [3]. Moreover, the reported morphological and functional changes, at both 21 and 48 d.p.i., are concomitant with significant peaks in blood and tissue eosinophils.

This study was planned in order to verify whether migrant larvae are still present in the myocardial tissue when the rhythm disturbances are observed and to clarify the possible role of the parasite and its antigens in the induction of the observed myocardial changes. We also wished to evaluate whether the degranulation products of mast cells and eosinophils, such as platelet-activating factor (PAF), could enhance the myocardial dysfunction.

To this end the DNA of the newborn larvae (NBL) was sought in hearts, blood and diaphragm of infected animals at different times after infection (8, 15, 21, 30 and 48 days) by means of PCR analysis. Histological,

functional [left ventricular developed pressure (LVDP) heart rate (HR) and coronary flow (CR)] and biochemical [creatine phosphate kinase activity (CPK)] variables were monitored in hearts at 21 and 48 d.p.i., when blood and tissue eosinophils are prominent. Furthermore, since PAF is a powerful eosinophil activator, a comparison of the data obtained before and after PAF infusion was performed.

Materials and methods

An isolate of *Trichinella spiralis* has been maintained in our laboratory since 1970 by passage in mice suitable for this purpose, which are infected with 600–800 muscle larvae per os (reference code, MSUS/WG/65/ISS51). Newborn larvae (NBL) were obtained in vitro from cultured adult worms as previously described [2]. Briefly, worms were isolated according to the method of Dennis et al. [4] from rat intestines infected 6 days previously. Adult worms were cultured in Eagle's MEM with Earle's salts with 10% heat-inactivated fetal calf serum and antibiotics. After 3 h incubation at 37° C, NBL were separated, washed twice in saline medium and counted under the light microscope, then frozen and stored until use at –20° C. NBL (100,000) were suspended in 500 µl of 10 mM Tris-HCL, pH 7.5.

The experimental animals were maintained in groups of three or four in polystyrene cages. Food and water were provided ad libitum and a diurnal rhythm of light was maintained. Sprague-Dawley male rats (6–8 weeks old) were orally infected with 3,000 *Trichinella spiralis* infective larvae. Infected mice were sacrificed at least 30 days after infection; their carcases were minced and digested by agitation with a magnetic stirrer in an artificial gastric juice (1% HCl and pepsin). At the end of the digestion larvae were progressively concentrated by sedimentation at 1 g, counted and evaluated for their viability.

Blood eosinophilia was measured as previously described [18].

At times after infection (8, 15, 21, 30 and 48 days), samples from blood, diaphragm and myocardium were taken from infected and uninfected animals for PCR analysis. Hearts were quickly excised and washed in saline solution to remove any blood contamination. All samples were immediately frozen and stored until use. Two primers (TricF: CATTCTTGAGCCACACCATT; TricR: CTCCAGAGTTTGCATACCGA) were designed on the basis of a repetitive DNA sequence of *T. spiralis* [5] to amplify a 587-bp fragment by PCR. Two other primers, 18Sa (5′-CCTGGTTGAT CCTGCCAGT-3′) and 18Se (5′-ATGATCCTTCCGCAGGTT-CAC-3′), designed to amplify the gene encoding for the small subunit ribosomal rRNA (SSU rDNA) from virtually all the eukaryotes, were employed in separate PCR reactions to check the presence and accessibility of the DNA in the negative controls.

For DNA extraction haemoglobin was removed from the blood by osmotic shock. Heart and diaphragm samples were converted to fine powder in a mortar by cooling in liquid nitrogen. The powder was suspended into 500 µl of 10 mM Tris-HCl, pH 7.5. The samples (half heart in 500 µl of 10 mM Tris-HC, pH 7.5; 0.5 ml of blood; 100,000 NBL suspended in 500 µl of Tris-HCl, pH 7.5) were heated at 90° C for 10 min, resuspended in extraction buffer (50 mM Tris HCl, pH 7.5; EDTA 10 mM, pH 8; 1% SDS; 100 µg/ml proteinase K (10 vol for tissues; 2 vol for blood and newborn larvae), incubated at 55° C for 2 h, and heated at 90° C for 10 min. The DNA was extracted with phenol/chloroform, ethanol precipitated, resuspended in 50 µl TE [22], treated with RNAase (20 µg/ml; 37° C for 2 h), extracted again with phenol/chloroform, ethanol precipitated, and resuspended in 100 µl TE.

In PCR analysis 1 µl of each suspension of DNA obtained was used as a template in each PCR reaction. PCR conditions in a 10-µl volume were as follows: 1 µM each of the primers TricF and TricR, 0.2 mM for each of the dNTPs, 1.5 µM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C), 1% Triton X-100, and 0.5 U of Taq DNA polymerase (Promega, Madison, Wis.). PCR conditions were 94° C for 1.5 min, followed by 35 cycles at 94° C for 30 s; 55° C for 1 min; 72° C for 3 min. Then 5 µl of each amplification were loaded onto 2% agarose gel in TAE buffer containing 0.5 µg/ml ethidium bromide and run for 2 h at 50 V/cm. To determine the sensitivity of the PCR reaction in detecting NBL, primers Tric F and TricR were tested on the DNA extracted from isolated NBL, and from NBL mixed with diaphragm and heart samples. For the isolated NBL, PCR experiments were carried out on template DNAs corresponding to 100, 10, 1, 0.1 larvae (for DNA extraction, see above). In the cases of heart and diaphragm, 10,000, 1,000, 100 and 10 NBL were mixed with samples of half hearts or the entire diaphragms. Subsequently, the DNA was extracted as above, and resuspended in a 100-µl volume of TE. PCR experiments were performed on 1 µl of the above preparations, thus allowing analysis of samples of 1/200 heart, or of 1/100 diaphragm, containing 100, 10, 1, and 0.1 NBL.

Differences were considered significant at $P<0.01$, obtained with Student's *t*-test. The diaphragms of all infected animals were examined after heart excision; those in which no larvae were found were excluded from further experiments.

Three groups of hearts were perfused, from uninfected (control animals), and from rats infected 21 days and 48 days before. Under deep ether anaesthesia, the hearts were quickly removed from the chest, the aorta was retrogradely cannulated and perfused for at least 90 min using a Langendorff apparatus with nonrecirculating Tyrode solution at 37° C (gassed with 95% O_2+5% CO_2) at constant pressure 82–84 mmHg); the perfusion (aortic) pressure (PP) was measured by means of a pressure transducer applied to a side arm in the aortic cannula. LVDP and HR were monitored using a water-filled latex balloon placed in the left ventricle (contracting heart) and connected via a steel catheter to a separate pressure transducer (Statham P23 ID, Gould, Hato Rey, P.R.); the balloon volume was adjusted to bring LVEDP to 10–15 mmHg (depending on heart size). The ECG was recorded by means of bipolar wick electrodes. Signals from the PP transducer was shown on a display, while the other pressures and ECG were recorded by means of multichannel McIntosh software (Apple Computer, Cupertino, Calif.). CF was evaluated by collecting the perfusate directly every 10 min from the beginning of perfusion. The hearts were left beating spontaneously. After 50 min of stabilization (baseline conditions), hearts from each experimental group and control animals were exposed to a single bolus (100 pmol) of platelet-activating factor [(PAF) Sigma Chemical Co., Saint Louis, Mo], given via the aortic cannula. PAF was prepared previously by dissolving 120 µl stock solution in saline to give a final volume of 1 ml.

The perfusate was collected at intervals of 10–20 min to assay CPK activity using a diagnostic kit (CK10, Sigma Chemical Co.). The CPK method was based on enzymatic NADH formation, which was spectrophotometrically estimated at 340 nm.

After a simple washing with Tyrode solution, hearts from each experimental group and controls were quickly removed from the rat chest and allowed to stop beating spontaneously, fixed in formalin and then embedded in 58C Paramatt (GURR). The same procedure was followed for the infected and uninfected hearts at the end of the perfusion. Sections of 3 µm, obtained by a rotary microtome (Reichert-Jung 2055 autocut), were stained with haematoxylin-eosin for myocardial structure and infiltrate composition evaluation.

Sections were stained with haematoxilin and eosin to analyse the myocardium structure and with Mann-Dominici stain to evaluate eosinophilic infiltration. This latter staining method, suitable for the study of "extravasal blood", was used in the actual estimation of eosinophil number and density in the myocardium areas calculated with an image analyser. Furthermore, in addition to haematoxilin-eosin, Mann-Dominici staining was very useful in confirming the presence of activated eosinophils (degranulated) within inflammatory areas.

The image analysis system employed is based on a microcomputer running SEMPER 6 Plus an image analysis programming language (Synoptics, Cambridge, UK). This manipulates the images obtained from an Olympus microscope fitted with a computerized, motorized stage and a CCD-TV camera. A program has been

developed that enables the system to identify Dominici-stained eosinophils. The system makes it possible to scan the entire slide and count the total cell number and furthermore to distinguish the cells from similar-sized debris with an accuracy of 95% compared with examination by eye.

Results

PCR experiments on the DNA extracted from isolated NBL produced an amplification fragment of the expected length. Moreover, one additional fragment was observed (800 bp). These nonspecific amplifications could be due to the presence of further binding sites with partial homology on the various repeats of the target sequence [6]. The same amplification pattern was observed after the analysis of DNAs derived from 100, 10, and 1 NBL. These experiments were repeated five times for each number of NBL. Even when comparing the results obtained from 100 and 1 NBL, we did not observe consistent differences in the intensity of the PCR bands. PCR experiments on DNA samples corresponding to 0.1 NBL gave only sporadic amplifications. Similar results were obtained from heart and diaphragm samples to which 10,000, 1,000, 100 NBL were added (estimated final number of NBL for the examined samples: 100, 10, 1). No amplification was observed after analysis of heart and diaphragm samples to which 10 NBL had been added (estimated final number of NBL: 0.1). Diaphragm samples recovered from rats at 8, 15, 21, 30, and 48 d.p.i. gave amplifications corresponding to those obtained from the NBL DNA in each case. However, the analysis of heart and blood samples from the same rats gave the expected amplification only at 8 and 15 days, whereas no amplification was detected at 21, 30, and 48 d.p.i. (Fig. 1).

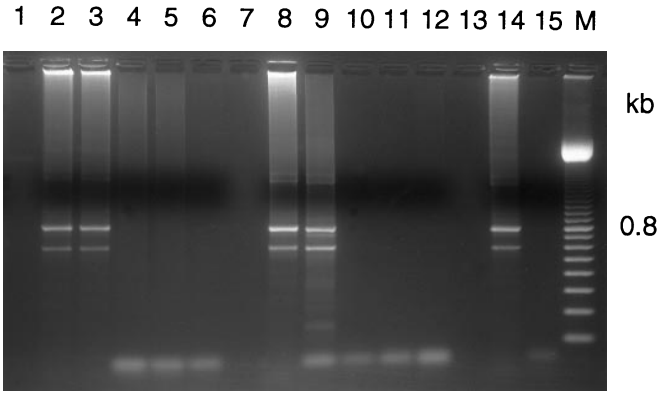

Fig. 1 PCR application to heart, blood and diphragm DNA of rats experimentally infected with *Trichinella spiralis*. PCR products from hearts at 8, 15, 30 and 48 days post infection (d.p.i.) were loaded in *wells 2, 3, 4, 5* and *6*, respectively. Those from blood samples at 8, 15, 30 and 48 d.p.i. were loaded in *wells 8, 9, 10, 11* and *12*. A diaphragm sample from a 48 d.p.i. rat was loaded in *well 14*. The negative control (DNA extracted from diaphragm of uninfected rat) was loaded in *well 15. Wells 1, 7* and *13* were not used. *M* indicates the molecular weight marker (100-bp Ladder, Pharmacia Biotech, Uppsala, Sweden)

No amplification was obtained from the negative controls with primers TricF and TricR; in this case, the expected amplification bands were observed only after using primers 18Sa and 18Se.

As shown in Table 1, PAF treatment produced a transient inotropic effect on both control and 48 d.p.i. hearts, whereas the contractility, already depressed in comparison with that in the other groups, was further reduced in 21 d.p.i. hearts, which did not undergo sudden fibrillation. Concerning HR, PAF administration led to a chronotropic positive effect of the same extent in both controls and 21 d.p.i. hearts, whereas the increment was smaller when PAF was infused in 48 d.p.i. preparations (data not shown). As with LVDP behaviour, 21 d.p.i. preparations treated with PAF showed an additional and more evident CF decrease than controls and 48 d.p.i. hearts. The CPK activity in the perfusate was dramatically increased after PAF treatment in 21 d.p.i., and a significant CPK release also occurred in 48 d.p.i. group (Table 1). It is noticeable that the PAF effects described above, with the exception of LVDP in both controls and 48 d.p.i., remained unmodified until the end of heart perfusion.

Haematoxylin-eosin staining revealed scattered inflammatory foci, particularly prominent at 21 d.p.i. (Fig. 2B), in which eosinophils and mononuclear cells were easily detectable and were accompanied by oedema, fibre disarray and necrotic areas (Fig. 2C). Eosinophil tissue evaluation (Table 2) showed that the peak level was reached at 21–30 d.p.i., when levels statistically different ($P<0.01$) from those of 15 and 48 d.p.i. were observed. Furthermore, nonperfused hearts from 48 d.p.i. animals

Table 1 Biochemical and functional differences in control and infected rat hearts before and after PAF treatment (*d.p.i.* days post infection, *PAF* platelet-activating factor, *CF* coronary flow, *CPK* creatine phosphate kinase)

Groups	Before PAF treatment			10 min after PAF treatment (100 pmol)		
	LVDP (mmHg)	CF (ml/min)	CPK (UI/l)	LVDP (mmHg)	CF (ml/min)	CPK (UI/l)
Controls	55±10*	21.4±0.8	7±4	80±12*	16.5±1.1*	14±8.0
21 d.p.i.	27±8	13.4±1.3	12±2	16±3 (n=5)	7.5±0.6* (n=5)	280±30* (n=5)
48 d.p.i.	50±6	21.5±0.8	8±4	67±7*	18±0.9*	20±5*

* Mean±SD. Significance was accepted at $P<0.01$ (difference in data obtained before and after PAF treatment)[a] n=7 for each group with the exception of 21 d.p.i.: 2 hearts in this group suddenly underwent fibrillation after PAF bolus (n=5)

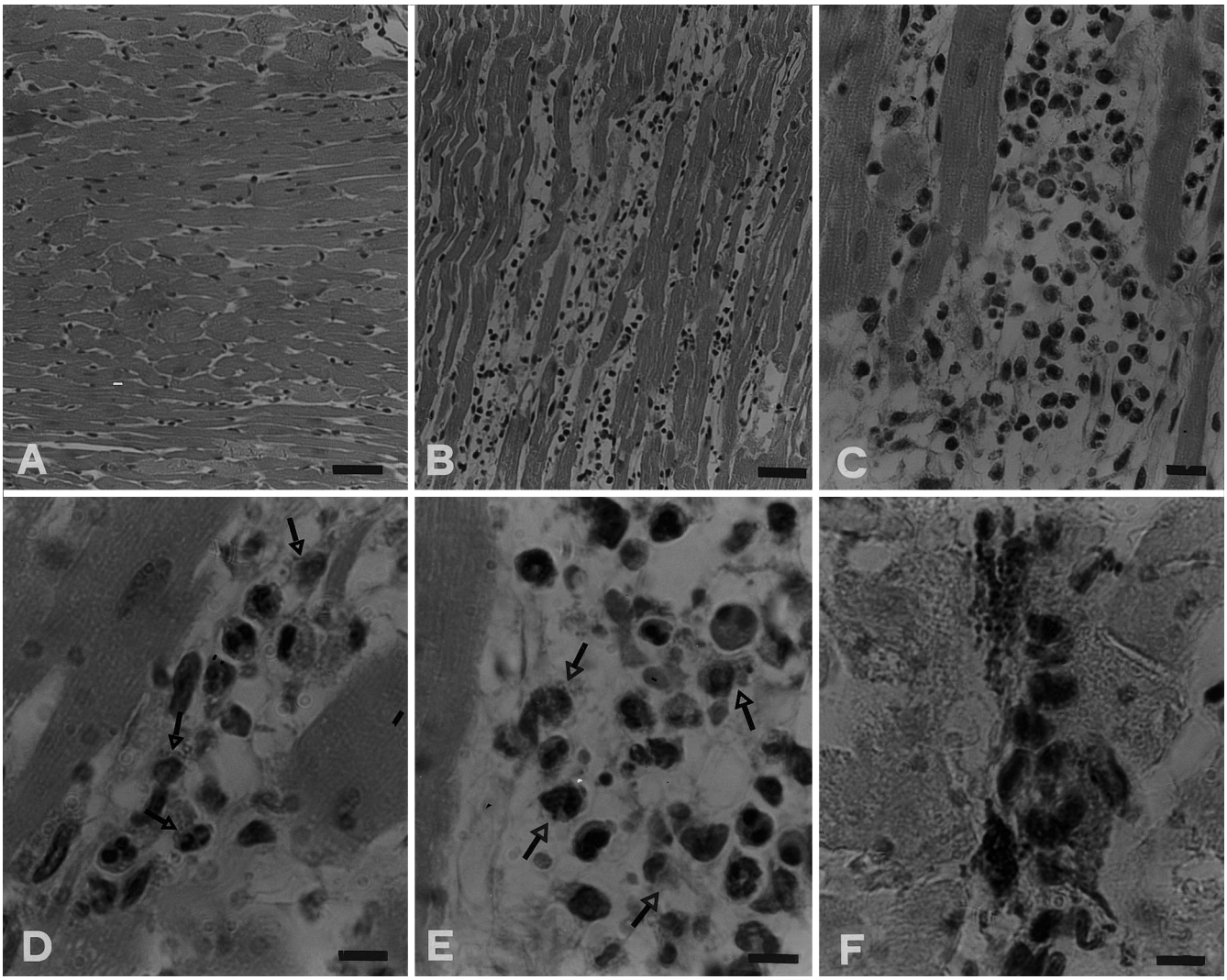

Fig. 2A–E Haematoxylin-eosin staining of myocardial tissue. **A** Control heart. *Bar* 42 μm. **B** Myocardial tissue at 21 d.p.i. show scattered inflammatory foci. *Bar* 42 μm. **C** Myocardial tissue at 21 d.p.i., where eosinophils and mononuclear cells are easily detectable in areas of myocardial necrosis. Bar 17 μm. **D, E** Myocardial tissue from 21 d.p.i. group after PAF infusion shows some degranulated eosinophils (*arrows*), accompanied by oedema and fibre disarrangement. Bar 7 μm. **F** Mann-Dominici staining of the same sections of **D** and **E**

showed thinning of ventricular walls (both right and left) compared with controls, as shown in Fig. 3.

After PAF infusion some markedly degranulated eosinophils were found in the inflammatory lesions (Fig. 2D–F).

Discussion

The host-parasite relationship in experimental infection with *T. spiralis* is peculiar, both because this helminth is intracellular and because it is able to infect a wide spectrum of host species in the phylogenetic scale (from mammals to birds, as in the case of *T. pseudospiralis*) [1]. In trichinellosis, since, with very rare exceptions [12], the parasite is unable to encyst in myocardial tissue, its presence is transient. This makes it difficult to understand how it can be directly responsible for myocardial damage. Since NBL larvae have long been regarded as responsible for the arrhythmias observed in the early infection phase owing to their passage through the

Table 2 Blood and tissue eosinophil levels during experimental trichinellosis in rats

Days post infection	Blood eosinophils/ml[a]	Tissue eosinophil density (No./mm^2)[a]
0	300±100	3.7±1.2
15	1500±200	2338.7±389.3
21	1800±200	4817.2±345.7*
30	1000±150	4580.6±376.8*
48	550±100	293.1±95.3

* $P<0.01$ (difference from 15 and 48 days)
[a] Mean±SEM

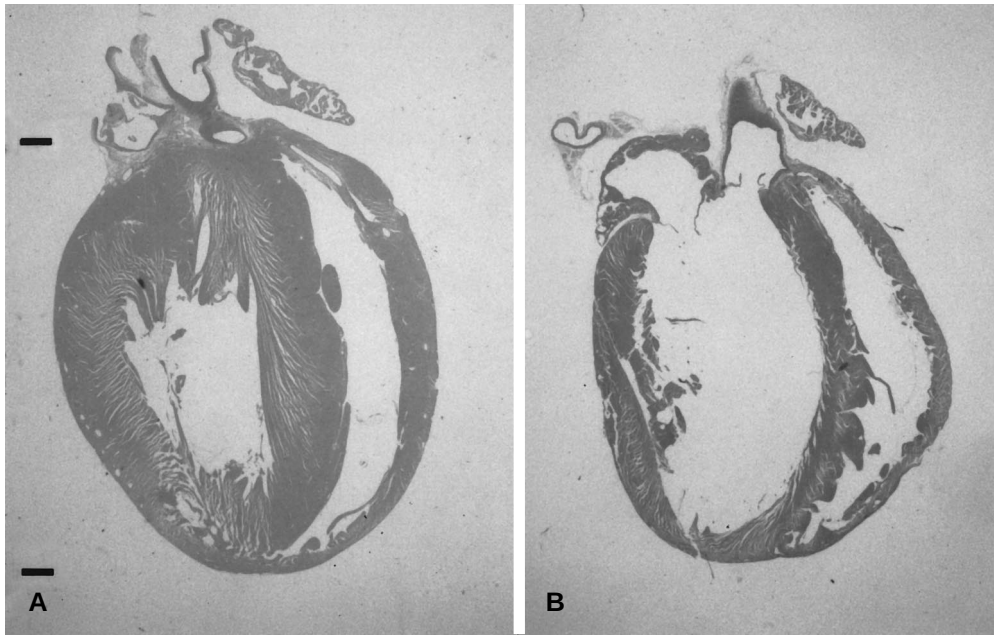

Fig. 3A, B Midsagittal sections of haematoxylin-eosin stained rat hearts (the space within the *bars* corresponds to 15 mm). **A** Control heart (uninfected and not perfused); **B** heart from 48 d.p.i. group (not perfused)

coronary vessels [9], we investigated the role of the migrant stage in inducing the myocardial changes observed during trichinellosis. Our results show that starting from 21 d.p.i., the DNA of NBL is undetectable in the myocardial tissue and also in the blood, confirming the previous data by Harley and Gallicchio [10], who were not able to find NBL in infected rat blood after 15 d.p.i. So, when irreversible rhythm disturbances are still present, particularly at 30 and 48 d.p.i. [18], NBL are no longer present in myocardial tissue. These data agree with previous observations in mice [13, 14], showing that NBL can be recovered from cardiac tissue until 14 d.p.i. Since our results show that NBL are detectable in both heart and blood only at 8 and 15 d.p.i., it is not clear whether migrant larvae are in the myocardium or simply passing through. Thus, at least in a subsequent infection phase, the role of NBL in inducing arrhythmias seems to be indirect. Whether NBL are really responsible for arrhythmias in the early phase remains to be demonstrated.

The parasite could play an indirect part in provoking the arrival and/or activation of inflammatory cells, such as eosinophils and mast cells. In fact, in experimentally infected rats at 21 d.p.i., the levels of tissue eosinophils, mast cell degranulation peak [19] and main peak of myocardium functional impairment occurred at the same time [18].

At 48 d.p.i. and later, when IgG-immunocomplexes are present at interstitial level, as previously described [19], the acute inflammatory response appears decreased. However, the damage is irreversible and a dilating cardiomyopathy follows, as shown by the thinning of the ventricular walls.

Trichinella spiralis infection, like other helminthiases, is also characterized by blood eosinophilia lasting several weeks, allowing the study of eosinophil-induced damage in different host tissues. In in vivo experiments, treatment with a monoclonal antibody specific for interleukin-5 completely suppresses the infection-induced eosinophilia, but infected mice treated in this way had the same worm burden as controls in both primary and secondary infections [11]. Although this result diminishes the importance of in vitro studies over the last 15 years, in which a cytotoxic effect of eosinophils against a great number of helminthic migrant stages (in the presence of antibodies) was reported [8], it shifts interest to the immunopathological effects of high levels of blood eosinophils which characterize helminthic infections. Among the different targets of the toxic effects caused by the release of activated eosinophil products the myocardium is one of the tissues most frequently affected during trichinellosis. It has been suggested that in *Trichinella*-infected rat eosinophils have a prominent role in the induction of myocardial [18]. In fact, the highest incidence of irreversible arrhythmias was observed in coincidence with a peak in blood eosinophilia. In *Toxocara canis* infection (also characterized by blood eosinophilia), experimentally infected mice show myocardial alterations characterized by focal hystiocyte formation and eosinophil infiltrates, which evolve into necrotic debris containing granulomata [3]. It has been demonstrated that the cytotoxic effect of eosinophil products are responsible for a decrease in cardiac performance (–25% cardiac work), simultaneously with increased eosinophilia [21]. Myocardial dysfunction was also observed after perfusing uninfected hearts with activated eosinophils; these hearts also presented important histological alterations that could explain the above dysfunctions. Our data suggest eosinophil involvement in myocardial functional impairment. In the first 2 weeks of infection, when the presence of NBL can be clearly demonstrated by PCR, these might attract cells by releasing chemotactic factors such as ECF-P (eosinophil chemotactic factor of parasitic origin)

and, in the subsequent phases, the inflammatory cells are recruited by mediators such as PAF and eosinophil chemotactic factor for anaphylaxis (ECF-A), which are released by degranulated mast cells activated by antigen binding to IgE. Among these mediators, PAF is one of the most relevant and studied [7]. We have observed a greater incidence of arrhythmia at 21 d.p.i. [18], when the mast cell number peaks and cells are more degranulated. At the same time, activated eosinophils are easily demonstrated in cardiac tissue, especially after PAF treatment, suggesting an enhancing cell activation effect induced by bolus PAF treatment. PAF could trigger anaphylaxis inducing arrhythmias, as shown in other models [15], in addition to inducing heart and vascular disease [7]. In our model PAf, by inducing eosinophil degranulation, may damage the cell membrane of myocardiocytes (shown by the dramatic increase of CPK activity in the perfusate after PAF treatment from hearts at 21 d.p.i. hearts) when myocardial eosinophil infiltration is at its peak. Kumagai et al. observed a higher eosinophil infiltration in the myocardium after a secondary infection than after a primary one in mice, suggesting an amplification of the inflammatory response [14]. At the moment we are not able to say what additional mechanisms could be involved in this model of experimental cardiomyopathy.

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