Description of inflammation and cytokine profile at the inoculation site and in heart tissue of mice re-infected with *Trypanosoma cruzi* vector derived-metacyclic trypomastigotes

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SUMMARY

We studied the role of *Trypanosoma cruzi* reinfection in regard to inflammatory and cytokine response at the inoculation site, lymph node and heart. We reinfected Balb/c mice intradermically into the hind foot-pad with natural infective metacyclic trypomastigotes. They were followed from 24 h to 30 days after the last reinfection. At the inoculation site 24 h after the last re-infection, the infiltrating inflammatory cells increased dramatically with respect to baseline inflammation, reaching maximum infiltrates for the third day. In contrast, parasite DNA was undetectable 24 h after inoculation, despite poor cytokine induction, only IFN-gamma, IL-12 and TGF-β were noticeable on days 7 and 15, whereas in the lymph nodes draining the inoculation site positive expression of IL-2, IL-4, IL-12 and TGF-beta were found to be induced as soon as 24 h after re-entry of parasite. In the heart, the inflammatory response increased immediately 24 h after re-entry of parasites, reaching its maximum on the 7th day and returning to baseline on day 30. In conclusion, although the inflammatory response is triggered in both compartments by re-entry of parasites, the inflammatory process returns almost to baseline after 30 days, leaving a persistent low-grade inflammation.

Key words: *Trypanosoma cruzi*, Chagas disease, re-infection, cytokines, inoculation site.

INTRODUCTION

Vertebrate hosts infected with *Trypanosoma cruzi* usually develop 2 distinct phases of Chagas disease: the acute phase with marked parasitaemia followed by the chronic phase with slight parasitaemia. During the chronic phase, a balance seems to be reached between parasite and host; there are no spontaneous outbreaks of parasitaemia, even after re-inoculation with large inocula (Krettli, 1984), unless immunosupression or concomitant infection with other parasites are present (Krettli & Lima Pereira, 1981). Recently published papers have shown that re-infection appears to be a factor in the development of the inflammatory process in hamsters and in murine models (Cabrine-Santos *et al*. 2001; Bustamante *et al*. 2002), but contradictory results were observed by others (Lauria-Pires & Teixeira A., 1997; Machado *et al*. 2001).

In endemic areas for Chagas disease the natural routes of infection are mucous membranes or small wounds in the skin contaminated by metacyclic trypomastigotes contained in the vector's faeces. In the literature there are few studies that resemble this natural condition. In pioneer works, characterization of the inflammatory infiltrate in the site of infection used culture derived- or insect derived-metacyclic trypomastigotes. However, Dias (1934) and Romaña (1943), using uncontrolled inoculae, and more recently Monteón *et al*. (1996) and Nascimento & Abrahamsohn (1987) have shown that the local immune response at the inoculation site in the first infection after *T. cruzi* invasion is characterized by polymorphonuclear leukocyte and mononuclear cell infiltration, which reach their maximal density at 1 h and 15 days, respectively after inoculation. However, *T. cruzi* parasites can survive despite the local inflammatory reaction induced by innate immune mechanisms (Monteón *et al*. 1996). Macrophages and natural killer (NK) cells are probably the most important cells involved in protection against *T. cruzi* parasites in the very early phase of infection (Rottenberg *et al*. 1988). Immune soluble mediators, such as IFN-γ and IL-12, have been identified as key cytokines involved in protection (Silva *et al*. 1992; Aliberti *et al*. 1996), but these cytokines are poorly induced at the inoculation site in newly-infected

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mice inoculated with metacyclic trypomastigotes (unpublished observations). Experimental infection of mice with *T. cruzi* leads to disturbances in the peripheral immune system, such as polyclonal lymphocyte activation, autoantibody production and host cardiac tissue damage (Petry & Eisen, 1989). Also, splenic CD4+ T cell apoptosis in a murine experimental model infected with metacyclic forms of *T. cruzi*, with the onset of activation-induced cell death that selectively ablates IFN-gamma production and up-regulates parasite replication in macrophages *in vitro*, has recently been reported (Lopes *et al*. 1995; Nunes *et al*. 1998). This pro-apoptotic activity is initiated in macrophages by IFN-gamma and a *T. cruzi* ceramide-containing glycolipid (Freire de Lima *et al*. 1998).

Our objective in the present study was to determine how re-entry of parasites may influence the inflammatory kinetic process, apoptosis and cytokine response at the site of inoculation and in nearby tissues, in animals re-infected with natural vector derived-metacyclic trypomastigotes mimicking a natural condition, prevalent in endemic zones.

MATERIALS AND METHODS

Source of parasites

Mexican *T. cruzi* isolate Ninoa, obtained from a human acute-phase patient, was used in this work. This isolate belongs to biodeme 3 (Monteón *et al*. 1996). It was maintained by sequential culture in LIT medium, *Triatoma pallidipenis* infection, and murine passage. First-instar larvae were fed *T. cruzi*-infected mice to obtain metacyclic trypomastigotes. When insects became adults, they were fed on rabbits, infectious urine was collected, and metacyclic trypomastigotes were counted and adjusted to 1×10^5 parasites/ml with sterile PBS before inoculation. Metacyclic trypomastigotes always represented up to 95% of the population.

Re-infections

Inbred female Balb/c mice (8–10 weeks old) were obtained from the Institute's animal house facility. Mice were inoculated by intradermal injection (1000 metacyclic trypomastigotes) into the hind foot-pad. Forty-five days after initial inoculation, mice were re-infected twice, at 45-day intervals. Control and experimental animals were sacrificed at 0, 1, 3, 7, 15 and 30 days after the last reinfection. (Time '0' means 1 day before the last re-infection.) Control animals received trypomastigote-free Triatoma's urine under similar conditions.

RNA isolation and cDNA synthesis

A pool of skin, from 3 hind foot-pads and lymph nodes of re-infected and control animals, were homogenized with Trizol (Life Technologies, Grand Island, NY) and total RNA was prepared following the manufacturer's recommendation. Then 1 μg of total RNA was reversed transcribed in 20 μl reaction containing 0·2 μg of oligo dT 12–18 mer (Life Technologies, Grand Island, NY), 100 U of Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Grand Island, NY), 0·5 mM each of dATP, dCTP, dTTP, and dGTP; 50 mM Tris-HCl (pH 8·3); 75 mM KCl; and 3 mM $MgCl_2$. The reaction was stopped after incubation for 1 h at 37 °C.

PCR for cytokines

Samples of cDNA (1 μl) were amplified in a 50 μl reaction containing 10 mM Tris-HCl (pH 8·3); 50 mM KCl; 1·5 mM $MgCl_2$; 0·2 mM each of dATP, dGTP, dCTP and dTTP; 100 ng each of the gene-specific upstream and downstream primers for IL-2, IL-4, IL-10, IL-12, TGF-β and β-actin (Clontech, Palo Alto Ca, USA); and IFN-γ (5′-CTG GCT GTT ACT GCC ACG GCA CAG TC-3′; 5′-TCG GAT GAG CTC ATT GAA TGC TTG GCG CT-3′); 1 U of *Taq* polymerase (Life-Technologies, Grand Island, NY) using a DNA Thermocycler (Whatman Biometra, Göttingen).

The amplification program consisted of an initial 3 min denaturation at 95 °C, followed by 30 repeated cycles of denaturation for 45 s at 95 °C, primer annealing for 45 s at 60 °C and extension for 45 s at 72 °C. A negative control lacking cDNA and a commercial positive control for murine IL-2, IL-4, IL-10, IL-12, and IFN-γ (Clontech, Palo Alto Ca, USA) were used to set up the procedure. PCR products were run on a 2% agarose gel and stained with ethidium bromide. Semi-quantification of PCR products was performed by comparison of signals for each cytokine under study with respect to β-actin. The stained gel was scanned for densitometrical analysis. Cytokine expression data are represented as the ratio of each cytokine signal to the corresponding β-actin signal.

Parasitaemia and parasitism

Parasitaemia was followed up by 2 procedures: direct observation and PCR.

Direct observation was performed as follows. A 5 μl blood sample from a group of 5 re-infected mice was obtained from the caudal vein. In the fresh preparation parasites were counted in 100 microscopic fields (magnification 400×) as described elsewhere (Brener, 1962).

PCR for *T. cruzi* detection was done as follows. A 20 μl blood sample from each re-infected mouse was used for DNA extraction with the phenol/chloroform method followed by precipitation with sodium acetate, pH 6·5, at a final concentration of 0·3 M and

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absolute ethanol. PCR was performed as reported previously (Monteón, Reyes & Rosales-Encina, 1994). DNA samples were incubated in a 50 μl volume with KNS1 and KNS2 primers designed from kinetoplast DNA minicircules of *T. cruzi*. A sample reaction was subject to 35 amplification cycles: each cycle was 92 °C for 1 min, 56 °C for 2 min and 72 °C for 1·5 min. Amplified products were analysed after electrophoresis in 1·5% agarose gels. Positive and negative controls were included in all experiments. (The amplified positive product has a size of 300 bp.)

In order to evaluate tissue parasitism, skin (inoculation site) and lymph nodes were digested in lysis buffer (Tris 100 mM, EDTA 1 mM, NP-40 and 1% SDS and 0·1 mg/ml proteinase K). DNA extraction, precipitation and PCR were performed as mentioned above. Our PCR assay shows a sensitivity around 10 fg of *T. cruzi* DNA detection (data not shown).

Evaluation of circulating cytokine

Murine serum cytokines IL-2, IL-4, IL-10, TNF-α and IFN-γ were measured in sera by specific 2-site enzyme-linked immunoabsorbent assay (ELISA) according to manufacturer's specifications (BD PharMingen, USA), using reference standard curves, and known amounts of the respective murine recombinant cytokines. All samples were processed individually and assayed in duplicate, plates being read at 405 nm in an ELISA reader.

Histology and in situ *cell death detection by TUNEL*

Tissues (skin and heart) were formalin-fixed (10%), embedded in paraffin, and processed by conventional techniques (haematoxylin-eosin stain) for histological studies. The inflammation grade was evaluated by 2 independent observers (A.A.F. and V.M.M.) in tissues from 3 re-infected mice, using at least 5 slides for each. Observation was made under a light microscope at 400×.

Frozen tissues sections were cut in a cryostat for TUNEL assay according to the manufacturer's instructions (Boehringer, Mannheim). In brief, 4 μm sections were fixed in paraformaldehyde (4% in PBS), permeabilized with 0·1% Triton X-100, and labelled with TUNEL reaction mixture. Sections were then developed for peroxidase enzyme with the appropriate substrate solution, mounted and analysed under a light microscope.

Controls were included in each experimental set up. The positive control consisted on peripheral blood mononuclear cells subject to DNase I (grade I, 1 mg/ml in 50 mM Tris-HCl, pH 7·5) digestion to induce DNA strand breaks. The negative control consisted of fixed and permeabilized tissue sections in labelling solution (without terminal transferase) instead of the TUNEL reaction. Endogenous peroxidase activity was blocked by inmersion in 0·3% H_2O_2 in methanol prior to cell permeabilization in all tested tissues.

Statistical analysis

The Mann-Whitney test was used in comparative analysis and a P value of $<0{\cdot}05$ was considered significant.

The protocol was approved by the Research and Bioethical Committee of the National Institute of Cardiology and according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, NIH publication (No. 85-23, revised 1996).

RESULTS

Parasitaemia and parasitism

Mice received 3 inoculae with 1000 metacyclic trypomastigotes. Forty-five days after the first inoculation, animals were re-infected twice at intervals of 45 days.

The level of parasitaemia was followed from day 9 to day 41 after the last re-infection. Direct observation failed to show circulating parasites. However, using PCR for detection of *T. cruzi* DNA, the persistence of circulating parasites over the whole experimental time-period was observed in the blood, demonstrating that parasites can persist at low levels even in multiply immunized animals (Fig. 1).

At the inoculation site, 24 h after the last re-inoculation, parasites were no longer detectable by PCR. This finding may suggest that parasites escape from the inoculation site or are eliminated *in situ* in re-infected animals. Evidence of *T. cruzi* DNA at the inoculation site only was observed very early after the last re-inoculation, i.e. from 1 to 12 h. After this time and throughout the experimental time-period parasite DNA was undetectable. In contrast, in lymph nodes, positive evidence of *T. cruzi* DNA persistence was corroborated, suggesting that parasites may be easily cleared from some tissues but not from others (data not shown).

Histopathological findings

In order to determine the magnitude and persistence of inflammation in the skin (inoculation site) and hearts of re-infected mice, tissues were excised 1 day before the last re-inoculation. Mononuclear cell infiltrate (MC) was observed in the dermis at very low intensity (1·7 MC/field). This dramatically increased 1 day after re-inoculation up to 15·2 MC/field, reaching maximum infiltrates on the third day (35·9/field). Rich plasmatic cell infiltrates were also seen at

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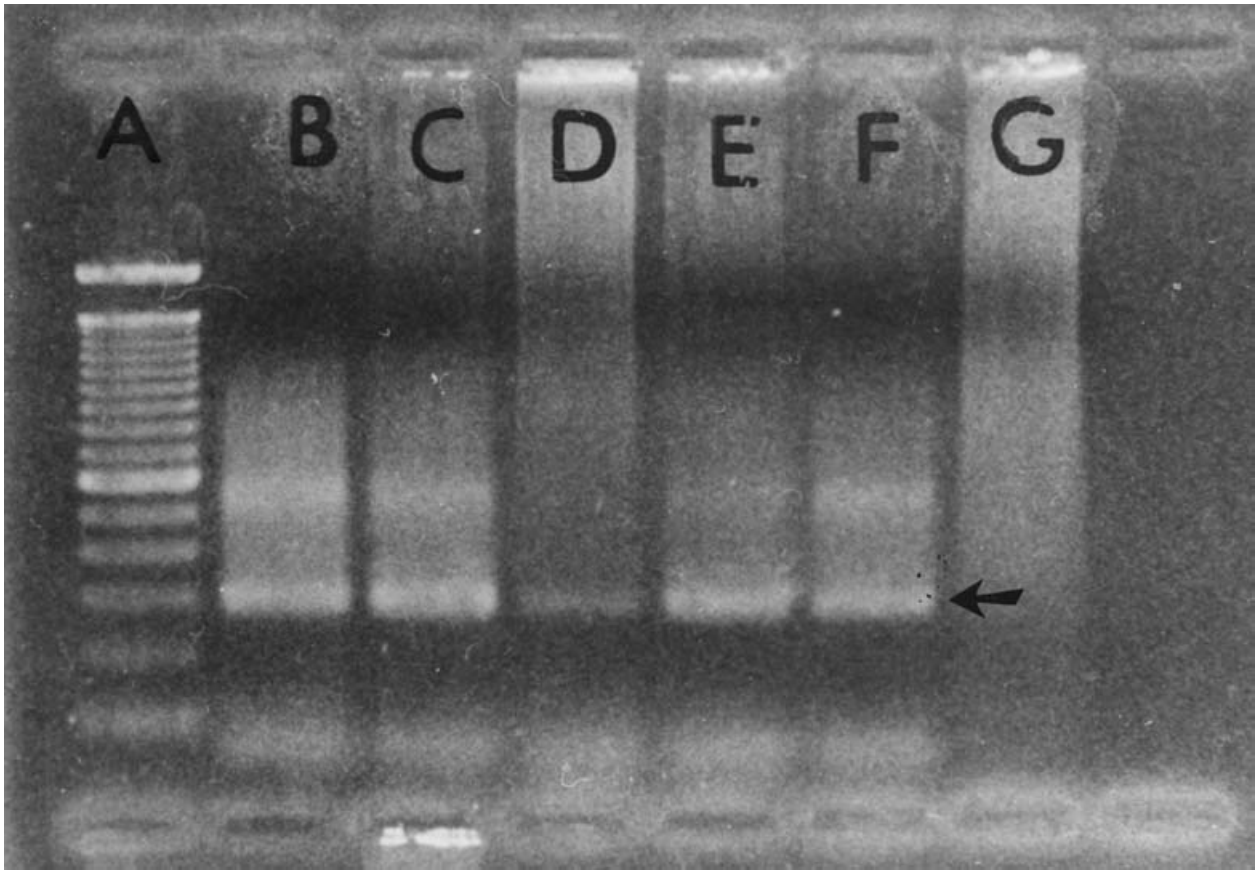

Fig. 1. PCR products amplified with specific primers of *Trypanosoma cruzi* KNS1 and KNS2. DNA was extracted from blood of re-infected mice at different times after the last reinfection. Lane A: molecular weight markers; Lanes B–E: blood DNA corresponding to day 5, 10, 15, and 30; Lane F: positive control (*T. cruzi* DNA); Lane G: negative control (mouse DNA).

Table 1. Inflammation at the inoculation site and heart in mice re-infected with metacyclic trypomastigotes of *Trypanosoma cruzi*

Days	Inoculation site (skin)	Heart
0†	1·7(+/−0·3)/field*§	0·24(+/−0·04)/field*
1	15·2(+/−0·7)/field*	4(+/−0·13)/field*
3	35·9(+/−0·48)/field	4·9(+/−0·1)/field
7	23·8(+/−0·6)/field	10(+/−0·2)/field
15	16·1(+/−0·5)/field	10(+/−0·08)/field
30	6·6(+/−0·4)/field*	0·53(+/−0·09)/field*

† One day prior to last re-infection, mice received 2 re-infections at 45-day intervals.
§ Mean and S.D. of mononuclear cells observed under light microscope at 400× of at least 5 slides from 3 re-infected animals.
* Statistical difference $P<0{\cdot}05$ was observed for days 0 *vs* 1, 0 *vs* 30 in skin and heart (Mann-Whitney test).

this point. A steady decreasing MC infiltrate was observed after day 7 until day 30 (Table 1, Fig. 2A).

The heart cell infiltrate consisted basically of mononuclear cells (Fig. 2B). There were changes in the inflammation that were time dependent, as observed for the inoculation site. One day before the last re-inoculation low-grade myocarditis persisted (0·21 MC/field), which increased up to 20-fold 24 h after parasite re-entry (4 MN/field). Maximum myocarditis was reached between the 7th and the 15th day (10 MN/field), decreasing after this point to reach values similar to those observed before re-entry of parasites (0·6 MN/field) on day 30 (Table 1).

Induction of mRNA cytokine in the site of infection and draining lymph node

At the inoculation site, on the day before the last re-infection, a negative signal for mRNA cytokine assayed was observed (IL-2, IL-4, IL-10, IL-12, TGF-β and IFN-γ). It was noticeable that the first cytokine induced was IFN-γ at day 7. On day 15 after re-infection, IFN-γ persisted, in addition to IL-12 and TGF-β (Fig. 3A). This experiment was done twice with identical results. The β-actin control was always positive.

In lymph nodes draining the inoculation site, on the day before the last re-infection, negative results were also obtained for all cytokines tested, except for

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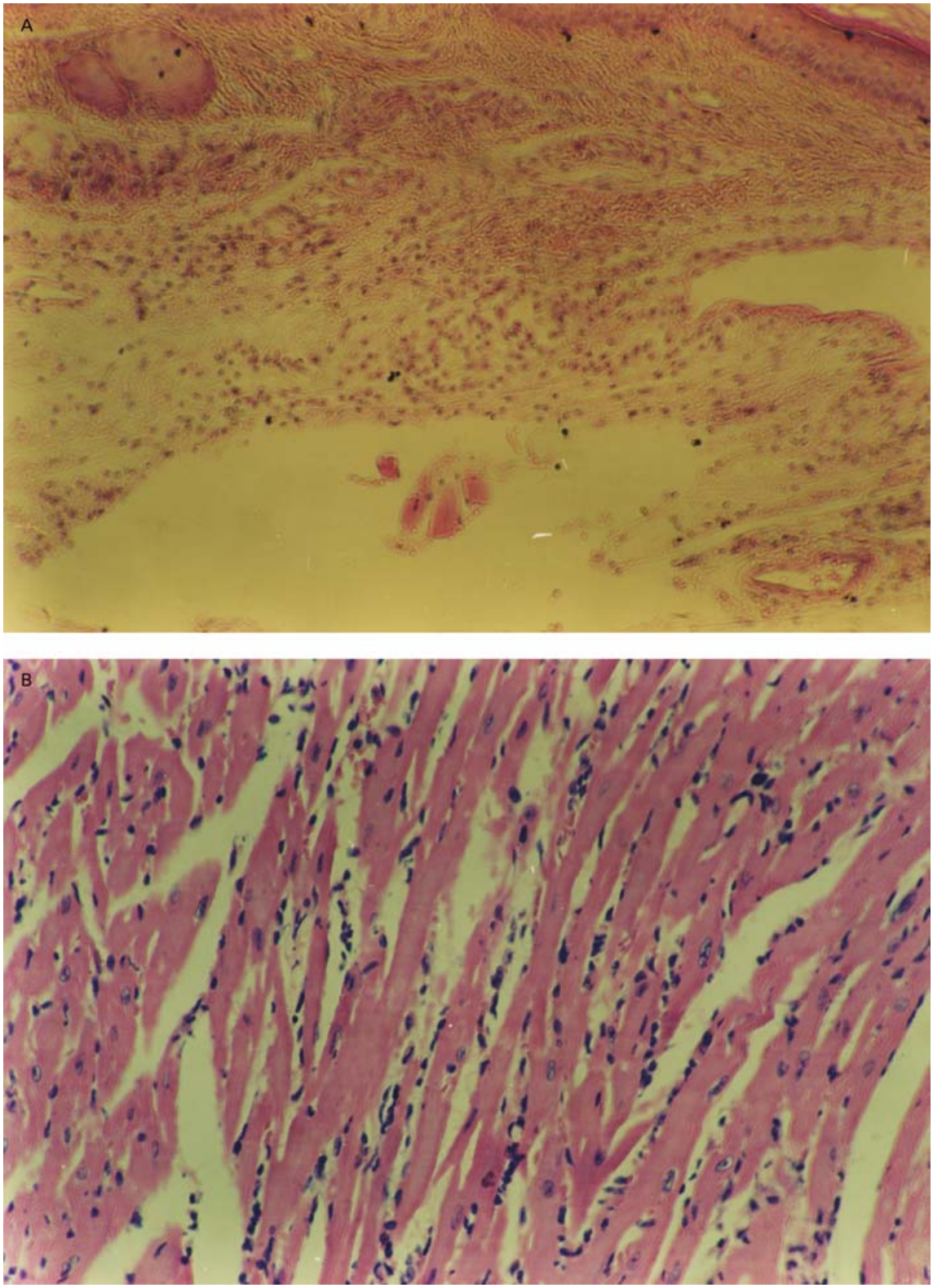

Fig. 2. (A) Skin from inoculation site of re-infected mice with 1000 metacyclic trypomastigotes 3 days after the last re-infection. Areas of cell infiltration can be seen (150×). (B) Hearts from mice re-infected with 1000 metacyclic trypomastigotes 30 days after the last reinfection. A low-grade myocarditis can be seen (250×).

constitutive mRNA β-actin. Twenty-four h after the last re-entry of parasites, positive expression of IL-2, IL-4, IL-12 and TGF-β was induced. A single cytokine, IL-12, persisted for 2 weeks, while IFN-γ was recognized only on day 7 (Fig. 3B).

Blood levels of cytokines

Blood cytokines, detected as protein by ELISA assay, showed high and persistent levels of IFN-γ and TNF-alpha from the first day after the last

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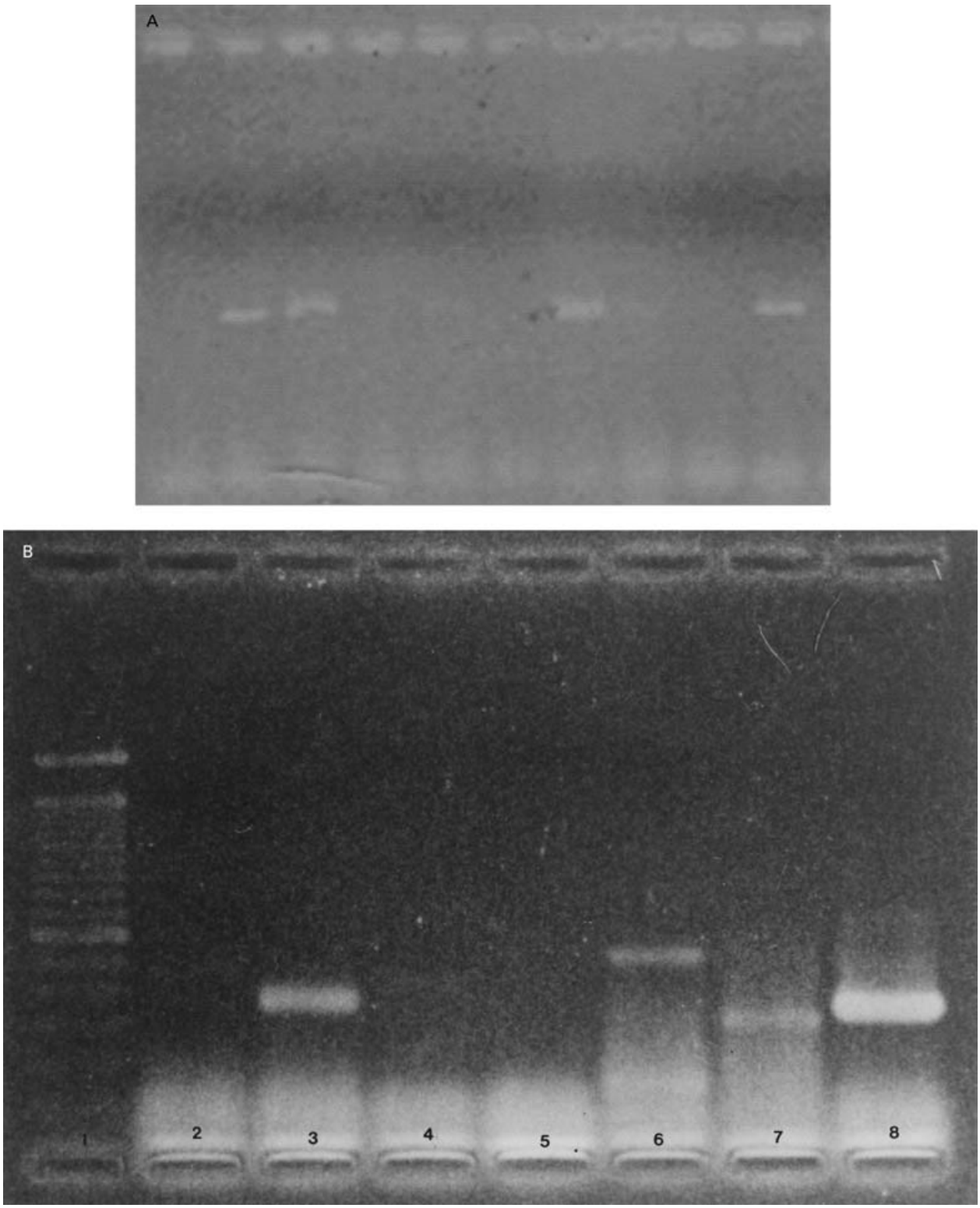

Fig. 3. (A) Cytokine profile at the inoculation site at day 15 after re-infection. RT-PCR was applied to RNA extracted from skin of mice re-infected with metacyclic trypomastigotes. Lane 1: blank; Lane 2: IFN-gamma; Lane 3: IL-12; Lane 4: negative; Lane 5: IL-2; Lane 6: IL-4; Lane 7: TGF-β Lane 8: IL-10; Lane 9: negative; Lane 10: control β-actin. (B) Cytokine profile in lymph nodes 24 h after re-infection. RT-PCR was applied to RNA extracted from lymph nodes of mice re-infected with metacyclic trypomastigotes. Lane 1: molecular weight markers; Lane 2: IL-10; Lane 3: IL-4; Lane 4: IL-2; Lane 5: IFN-gamma; Lane 6: TGF-β; Lane 7: IL-12; Lane 8: control β-actin.

reinfection, and throughout the experiment time without critical variation, except for IL-4 which reached its maximum peak on the 30th day. A discrete and constant amount of IL-10 was detected throughout the experiment. A notorious exception was IL-2, which had undetectable levels (Table2).

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Table 2. Level of cytokines in blood of mice re-infected with metacyclic trypomastigotes of *Trypanosoma cruzi*

(Values represent mean and S.D. of cytokine levels in a group of 5 animals.)

Days	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)	IFN-gamma (pg/ml)	TNF-alfa (pg/ml)
0†	0	4141+/−280*	148+/−19*	7030+/−290	3450+/−180
1	0	7098+/−229*	305+/−15*	6949+/−250	3228+/−160
2	0	6994+/−240	366+/−12	6452+/−245	3666+/−165
5	0	7180+/−110	396+/−8	8913+/−286	6228+/−180
10	0	2050+/−260	184+/−35	9000+/−279	3420+/−143
20	0	3280+/−222	744+/−22	8913+/−295	3764+/−152
30	0	4257+/−260	146+/−8	10 512+/−280	3500+/−160

† One day prior to last re-infection, mice received 2 re-infections at 45-day intervals.
* Statistical difference $P<0{\cdot}05$ between day 0 *vs* 1 was observed.

TUNEL technique in the site of infection and lymph node

TUNEL performed on the skin of re-infected animals stained around 20% of infiltrating mononuclear cells 1 day prior to the last re-infection, as well as 24 h after re-infection. The proportion of TUNEL-positive infiltrating cells at the dermis from the beginning and throughout the experiment remained constant. The same was true for lymph nodes where TUNEL-positive cells were around 2% (Fig. 4B and D). The control group, in which TdT was omitted from the reaction, showed negative stain (Fig. 4A and C). In control animals inoculated with urine-free parasites, TUNEL-positive cells were observed only in the uppermost layers of the skin, with negative signal in the dermis (data not shown).

DISCUSSION

Vectorial transmission of *T. cruzi* dominates in endemic areas. Thus, metacyclic trypomastigotes, the natural infective phase for mammals, are responsible for infection. Metacyclic trypomastigotes access the body across small wounds on the skin or via mucous membranes (Brumpt, 1912; Romaña, 1943; Soares & Marsden, 1986). Sequential description of inflammatory reaction at the inoculation site has shown polymorphonuclear cells (PMN) as early as 1 h after inoculation and peaks at 24 h. These PMN are gradually replaced by mononuclear cells (MN) that become predominant by day 15 after infection. The parasite can persist at the inoculation site from the first day through 15 days after inoculation in primo-infected animals (Monteón *et al*. 1996). In this work we found, in metacyclic trypomastigote re-infected animals, that the inflammatory reaction at the inoculation site is characterized by MN cells and plasmatic cell foci. Neither eosinophils nor basophils were observed, while the parasite persists briefly at the inoculation site for no more than 24 h.

An interesting finding was the rapid and intense inflammatory reaction that took place at the inoculation site. The amount of MN infiltrating cells increased up to 10-fold or more only 24 h after the last re-entry of parasites, in comparison with the baseline cell population on the previous day (1·7 MN cells/field). The maximum infiltrate peaked on the third day (35·9 MN cells/field), representing a 20-fold increase in comparison to the baseline cell population, moreover, at this time the largest plasmatic cell foci were evident. In the first week, an intense infiltration into the dermis was the hallmark; after this point and throughout the remaining duration of the experiment a marked decrease of inflammation and scarce cells (6·6 MN cells/field) were recognized. Although re-infection under natural conditions is unlikely to occur at the same sight, these data support the concept that re-exposure to the same agent generates a more intense response. In particular, *T. cruzi* is able to induce an immediate and strong inflammatory response at the site of infection, which is characterized essentially by mononuclear cells. Inoculated parasites were identified at the inoculation site by use of specific PCR, disclosing that the parasite does not persist beyond 24 h after re-infection. In contrast, in primo-infected mice, persistence of the parasite was apparent from 24 h up to 2 weeks after inoculation (Monteón *et al*. 1996). This finding may suggest that re-inoculation of parasites by the same route promoted their prompt elimination from the site of infection in immune re-infected, but not in primo-infected mice.

Although from our data it is difficult to demonstrate whether some parasites can escape from the inoculation site before being destroyed *in situ*, it is plausible to consider that some parasites may escape from the inoculation site through lymphatic or blood vessels and resist humoral and cellular immune mechanisms, based on previous data in pre-immune infected animals that showed that some parasites can escape from the inoculation site as rapidly as 5 min

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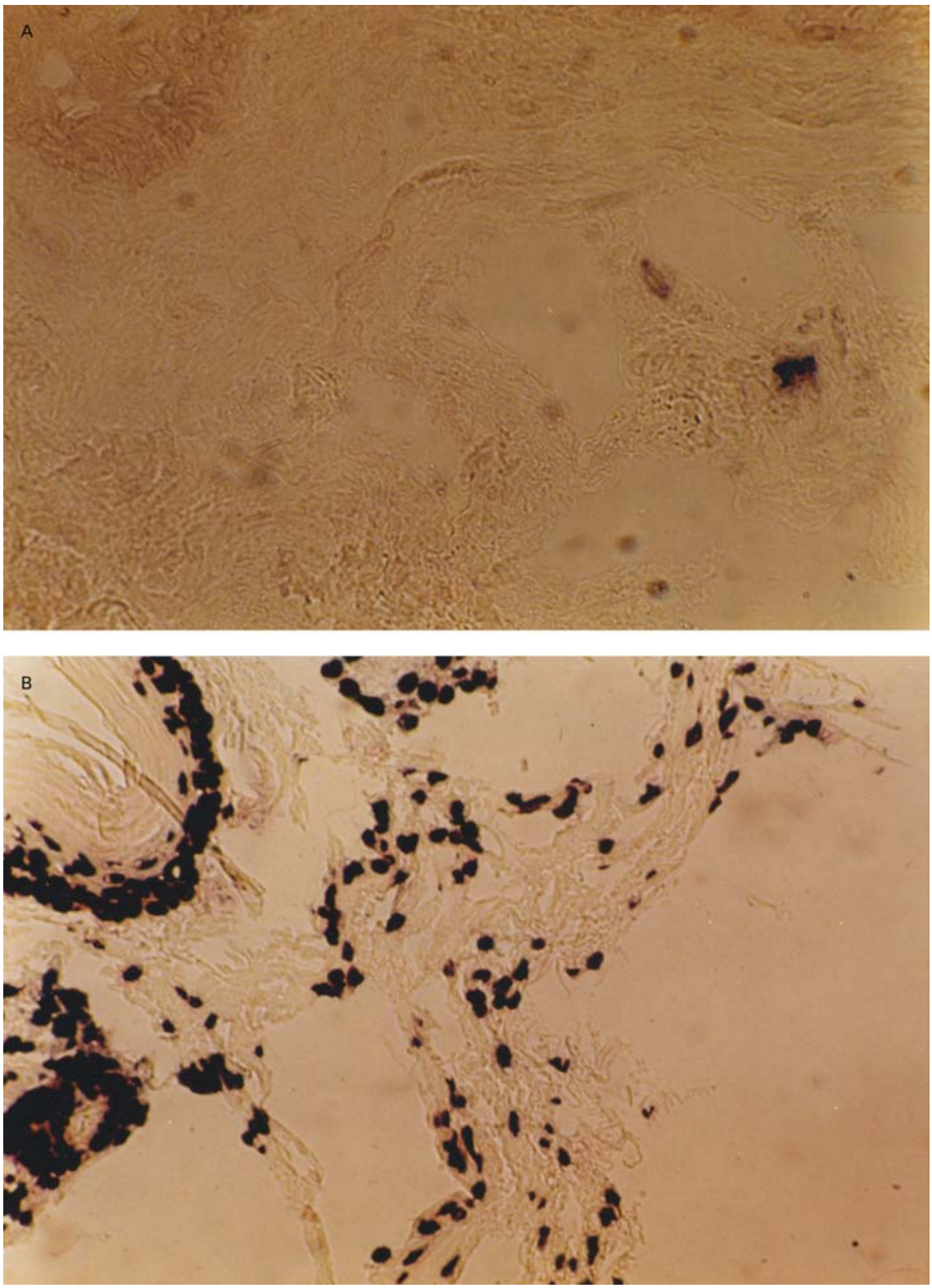

Fig. 4. (A) Negative control of terminal deoxynucleotidyl transferase-mediated DNA nick end labelling (TUNEL) staining on skin from mice re-infected with metacyclic trypomastigotes where terminal transferase was omitted (250×). (B) TUNEL staining on skin from mice re-infected with metacyclic trypomastigotes. Positive nuclear staining of MN infiltrating cells can be seen (250×). (C) Negative control of TUNEL staining on lymph nodes from mice re-infected with metacyclic trypomastigotes where terminal transferase was omitted (250×). (D) TUNEL staining on lymph nodes from mice re-infected with metacyclic trypomastigotes. Positive nuclear staining of MN cells can be seen (250×).

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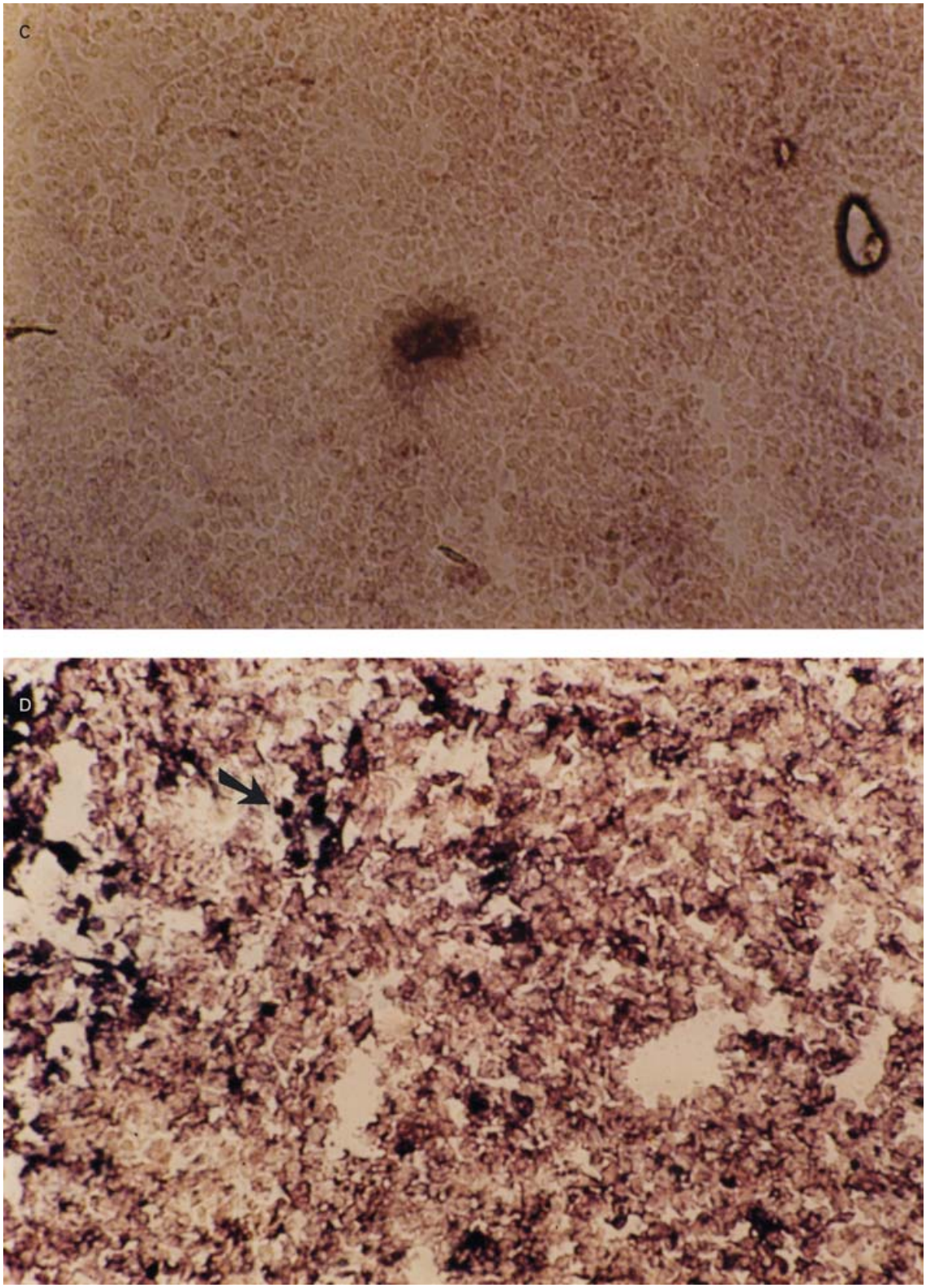

Fig. 4. (*Cont.*)

after its first contact (Schuster & Schaub, 2000). In unreported data, we observed that sera from re-infected mice are able to destroy only 98% of metacyclic trypomastigotes *in vitro* in a complement-mediated lysis assay (CoML). Thus, in immune re-infected animals, it is likely most of the inoculated parasites could be destroyed *in situ*.

The role of IL-12 and IFN-gamma as resistance factors to *T. cruzi* has previously been reported (Aliberti *et al*. 1996; Cardillo, Volterelli & Reed, 1996). It is known also that the parasitic phase can influence immune responses. Metacyclic trypomastigotes have the ability to impair IL-12 but not IL-1 release in trypomastigote-infected macrophages

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in vitro (De Diego *et al*. 1997). Furthermore, metacyclic trypomastigotes were unable to trigger synthesis of nitric oxide by macrophages primed with IFN-gamma (Camargo *et al*. 1997). Although a more complex response should occur in an animal model as compared to *in vitro* assays, a comparable response was observed in this *in vivo* model.

We explored cytokine induction throughout the first month after re-infection in order to measure early and late immune responses. For this propose we selected the inoculation site to ascertain whether re-entry of parasites is able to induce vigorous inflammation, together with protective cytokines, assuming that memory immune cells should be enriched in lymph node tissues draining nearby the inoculation site. We observed late IFN-gamma and IL-12 expression at the inoculation site on the 7th and 15th day, while in draining lymph nodes an immediate cytokine response was observed for IL-2, IL-4, IL-12 and TGF-β and a delayed response for IFN-gamma which did not appear until day 7 after the last reinfection. These data indicate that the profile of cytokines depends on the compartment analysed.

The poor induction of cytokines observed in our present model does not rule out the possibility that other cytokines or chemokines could be produced as recruitment or activating factors, or that minute amounts of cytokines, below the level of detection, could have been present.

In spite of an undetectable protective immune response mediated by IFN-gamma and IL-12, parasites were no longer detected at the inoculation site 24 h after re-entry. To explain this finding, we propose that antibody-mediated lysis may play a major role in controlling the inoculum at the inoculation site, because rich plasmatic foci were observed in the dermis. However, it is necessary to expand this preliminary analysis to test this hypothesis.

Experimental infection of mice with *T. cruzi* leads to disturbances in the peripheral immune system, such as polyclonal lymphocyte activation, autoantibody production and host cardiac tissue damage (Petry *et al*. 1989) Also, CD4+ T cell apoptosis in a murine experimental model infected with metacyclic forms of *T. cruzi*, and the onset of activation-induced cell death that selectively ablates IFN-gamma production and upregulates parasite replication in macrophages *in vitro* has recently been reported (Lopes *et al*. 1995; Nunes *et al*. 1998). This pro-apoptotic activity is turned on in macrophages by IFN-gamma and a *T. cruzi* ceramide-containing glycolipid (Freire de Lima *et al*. 1998).

In this work, we found, at the inoculation site, high levels of TUNEL-positive infiltrating cells (up to 23%) in the dermis, whereas in draining lymph nodes, TUNEL-positive cells were below 2·3%. These findings indicate that apoptosis in MNC may be influenced by their microenvironment, although our data do not allow us to propose a conclusive mechanism, although two explanations could be suggested. According to the data mentioned above, parasite components have pro-apoptotic activity that may lead to apoptosis of those susceptible MNC, including lymphocytes and macrophages (Freire de Lima *et al*. 1998). A second explanation came from early and recent studies on the fate of effectors cells which stated that activated T cells died or disappeared within 2–3 weeks after migration to lymphoid and non-lymphoid tissues, including homing to inflamed skin (Sprent & Miller, 1976; Masopust *et al*. 2001; Reinhardt *et al*. 2001). Therefore, apoptotic cells detected in the dermis, in this study, may be the result of parasite components or due to the natural physiological cycle of immune cells or both. Re-infection in animal models: mice, rats, hamsters and dogs shows contradictory results with regard to the development of more severe pathological lesions in the heart (Rivelli *et al*. 1990; Lauria-Pires & Teixeira, 1997). An association between re-infection and more severe cardiopathy was found in mice and hamsters (Bustamante *et al*. 2002; Cabrine-Santos *et al*. 2001) but other authors did not find any association between re-infection and more severe pathological lesions in dogs (Machado *et al*. 2001). These contradictory results obtained in animal models could be explained because of varying design protocols. For example, in re-infected dogs histopathological analysis was done after 10 months following the last re-inoculation, establishing no association between re-infection and more severe cardiopathy (Machado *et al*. 2001). In re-infected mice and hamsters, on the other hand, histopathological analysis was done after 1 and 2 months after the last re-inoculation and demonstrated a positive correlation between re-infection and more severe pathology. Moreover, blood-stream trypomastigotes were used instead of the natural infective phase to infect animals in all cases (Cabrine-Santos *et al*. 2001; Bustamante *et al*. 2002).

In the present work, using the natural infective trypomastigote metacyclic phase, we observed in a re-infection murine model that an inflammatory process in the heart was triggered by parasite re-entry as soon as 24 h and peaked on day 7 reaching up to 10 MC/field. However, this resulted in a partially reversible and controlled process within the first month. This observation is in contrast to a primary infection where slight inflammation starts on day 7 and peaks at day 30, reaching up to 5 MC/field (Monteón, 1994; Monteón *et al*. 1996). It seems that re-infection influences the degree and speed of inflammation, but it is not quantitatively different.

Although cytokines were measured in the heart tissue, only TGF-β, IL-10 and IFN-gamma were detected during the first 5 days after re-entry of parasites (data not shown).

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In conclusion, our data suggest that inflammatory reaction both in the heart and in the site of infection is triggered by parasite antigens and is subject to regulation. Furthermore, a poor and late induction of protective cytokines such as IL-12 and IFN-gamma were observed at the site of re-entry of parasites; however, parasites were undetectable 24 h after re-entry.

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