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Denervation and the immune response in mice infected with *Trypanosoma cruzi*

R. RIBEIRO DOS SANTOS* & L. HUDSON *Department of Immunology, St George's Hospital Medical School, London*

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SUMMARY

C57Bl mice were infected with *Trypanosoma cruzi* Y strain trypomastigotes and showed a peak of parasitaemia 9 days after infection. Virtually all mice survived the acute stage of infection and were aparasitaemic thereafter. Coincident with the peak of parasitaemia, there was a progressive loss of cardiac neurones (up to the 20th day after infection) and an appearance of *T. cruzi* antigen on myofibres. Anti-parasite immunity, evidenced by a significant inhibition of macrophage migration in the presence of *T. cruzi* antigens (MIF test) and the deposition of complement and immunoglobulin *in vivo* around the nests of parasites, developed between days 7–10 after infection. Immunity to 'self' components (MIF test using neurone and heart antigens) was not detected until 15–20 days after infection. Although the MIF test detected a progressive increase in anti-neurone immunity between 20–90 days after infection, there was no additional loss of cardiac neurones during this period. In contrast to current hypotheses, these data suggest that the immunity to heart and neuronal antigens commonly detected in animals infected with *T. cruzi* is the result rather than the cause, of host cell destruction.

INTRODUCTION

The pathology of chronic-stage Chagas' disease is characterized by the megasyndromes and cardiopathy (Köberle, 1963, 1968). Köberle & Alcântara (1960) suggested that the intense central and peripheral denervation seen in the acute stage of this disease, both in man and animals (Alcântara, 1961), might provide a rational basis on which to explain the development of these clinical findings. Although the relationship between the denervation and pathology has been studied extensively (reviewed by Köberle, 1970, 1974), there has been no satisfactory explanation for the initiation of neuronal destruction.

Neurones are only rarely parasitized in *T. cruzi* infections (Köberle & Alcântara, 1960) and so their destruction is unlikely to be a direct effect of parasite invasion. Other investigators have suggested that neuronal destruction might be produced by a non-specific inflammatory response by a neurotoxin-like substance (Köberle & Alcântara, 1960) or by an autoimmune response elicited by parasite antigens that cross-react with host tissue components (Teixeira, Teixeira & Santos-Busch, 1975). We believe, however, that our previous *in vitro* studies on the immune response to *T. cruzi* antigens might provide a more satisfactory basis on which to explain the observed temporal relationship between the destruction of uninfected host cells and the autoimmune response (Ribeiro dos Santos & Hudson, 1980b). We have shown that parasite antigens can be avidly

Correspondence: Dr Leslie Hudson, Department of Immunology, St George's Medical School, London, SW17 0RE.

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adsorbed onto mammalian cells thus rendering them potential targets for the immune response to *T. cruzi*.

In this paper, we have demonstrated that although mice show a progressive reduction in heart neurones, the reduction occurred in advance of the immune response against heart muscle and neuronal tissue.

MATERIALS AND METHODS

Infected mice. C57Bl mice were infected with *T. cruzi* organisms (Y strain) using either 10^4 blood trypomastigotes per gram body weight to produce a lethal high-level parasitaemia or 10^3 blood trypomastigotes per gram body weight to produce a chronic infection (greater than 90 days) with resolving parasitaemia.

Determination of cardiac neurones. The heart was excised and perfused by an intraventricular injection of warm (40°C) agar (2% w/v in phosphate-buffered saline; PBS). The expanded heart was fixed in formaldehyde solution (10% v/v in PBS), embedded in paraffin and sectioned at 7 μm. To ensure that each neurone was scored only once, counting was limited to each third serial section from the base of the auricles to the beginning of the ventricles.

Antisera. Rabbit anti-*T. cruzi* serum was prepared as described previously (Ribeiro dos Santos & Hudson, 1980a). This antiserum was absorbed with heart homogenate before use, and detected using a goat anti-rabbit immunoglobulin serum conjugated with fluorescein isothiocyanate (FITC). FITC-conjugated antisera to mouse immunoglobulin and C3 were purchased from Cappel Laboratories (Downington, Pennsylvania).

Immunofluorescent staining of neurones. Hearts were excised from *T. cruzi*-infected or normal C57Bl mice and perfused with PBS. After removing the ventricles, the cut surface of the heart was mounted on a microtome chuck in Tissue-Tek Compound OCT (Lab-Tek Products, Miles Labs Inc.) and frozen in liquid nitrogen prior to sectioning at 3 μm. Each section was examined for the presence of neuronal ganglia using a phase-contrast microscope, and where appropriate was stained by indirect or direct immunofluorescence for (a) *T. cruzi*, (b) mouse immunoglobulin or (c) mouse C3.

Parasite and host antigens. Epimastigotes were frozen and thawed three times and the supernatant recovered after centrifuging at 30,000 ***g*** for 60 min at 4°C (Teixeira & Santos-Busch, 1974). For the preparation of heart muscle antigens free of neuronal material, the innervated auricular area was sliced off and the neuronal-free ventricles homogenized in PBS using a Potter homogenizer. The homogenate was frozen and thawed three times and the supernatant recovered after centrifugation (30,000 ***g*** for 60 min at 4°C). Neurones were prepared from the central nervous system of newborn mice by sucrose-gradient centrifugation (Sellinger & Azcurra, 1975) and then frozen and thawed three times in PBS. Again, a 30,000 ***g*** supernatant was recovered for use.

Migration inhibition test

Preparation of lymphocytes. Cells were teased from the spleen of normal or *T. cruzi*-infected C57Bl mice and filtered through glass wool to remove adherent cells.

Lymphocytes were purified by density-gradient centrifugation using Ficoll/Hypaque ($\rho = 1{\cdot}076$) as described elsewhere (Ford & Hunt, 1973; Böyum, 1976).

Preparation of indicator cells. Normal C57Bl mice were injected intraperitoneally with 2% glycogen in PBS and the peritoneal exudate recovered 4 days later (Stuart, Habeshaw & Davidson, 1973).

Lymphocytes (10^7) were mixed with 2×10^7 peritoneal exudate cells and sealed into plastic capillaries for pelleting by centrifugation (George & Vaughan, 1962; David, Lawrence & Thomas, 1964). Capillaries were cut at the cell interface and incubated at 37°C in Sterilin migration chambers containing Eagle's minimal essential medium with 10% v/v fetal bovine serum and antigen at a final concentration of 100 μg protein $\cdot$ ml^{-1}. After 24 hr the plates were placed in a photographic enlarger and the area of migration traced onto paper. The traced migration area was then cut out, weighed and the inhibition of migration calculated as follows:

$$\% \text{ inhibition of migration} = 100 - \frac{\text{Weight of migration area with antigen}}{\text{Weight of migration area without antigen}} \times 100.$$

RESULTS

Normal C57Bl mice were found to have 1,625 ± 215 cardiac neurones (mean of 10 determinations ± s.e.). When mice of the same inbred strain were infected with *Trypanosoma cruzi*, Y strain, there was a decrease in cardiac neurones that corresponded closely with the course of parasite infection (Fig. 1). The greatest reduction in neurone numbers was found between 9–20 days after infection, when parasites were easily demonstrable in large numbers. Thus at day 20, infected mice had an average number of cardiac neurones of only 710 ± 140 (mean of five determinations ± s.e.). After day 20, when the infection had entered its chronic phase, neurone numbers showed no further significant decline.

The appearance of parasite antigens on neurones and myocardial fibres showed a similar relationship to the course of parasitaemia (Table 1). Using indirect immunofluorescence, parasite

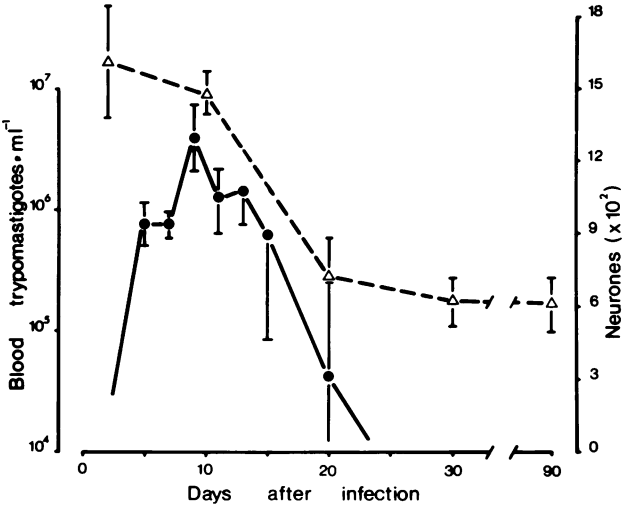

Fig. 1. Parasitaemia and neurone number in *T. cruzi*-infected mice. Counts of blood trypomastigotes (solid line) were determined by microscopic examination of fresh tail blood; data shown as mean of five determinations ± s.e. Neurone numbers (broken line) were initially determined in 10 control mice; thereafter, five infected mice were killed at each time point. Data shown as mean ± s.e.

Table 1. Detection of *T. cruzi* antigens, immunoglobulin and C3 on myocardial fibres of infected mice using indirect immunofluorescence

Days after infection	Immunofluorescent staining for:		
	T. cruzi antigen	Immunoglobulin	C3
0	–	–	–
7	+++	–	–
10	+	+++	+
15	–	+++	+++

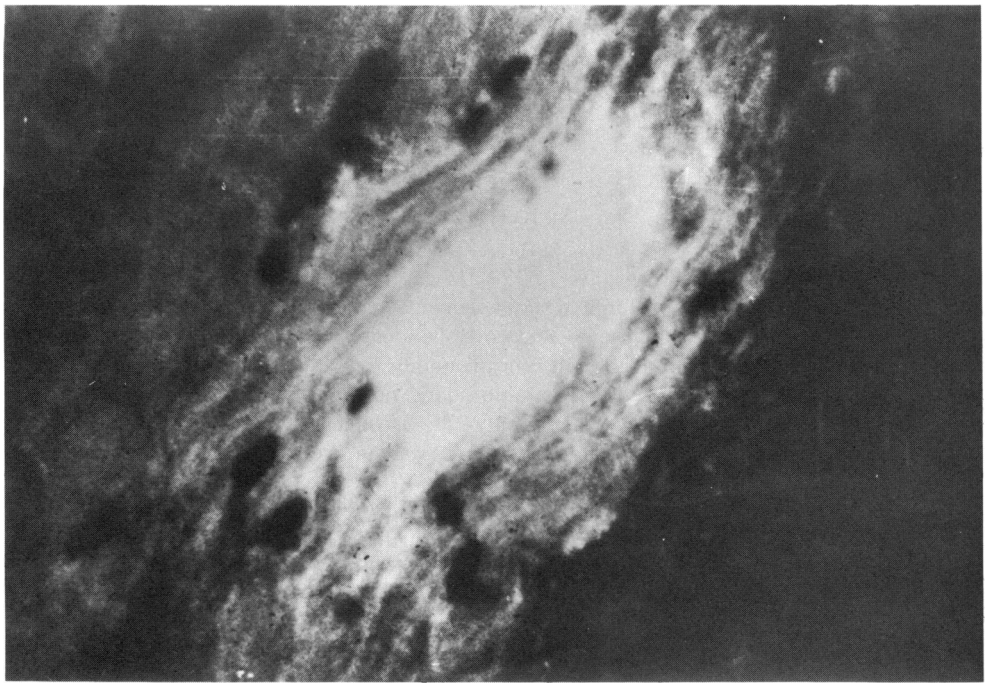

Fig. 2. Immunofluorescent demonstration of *T. cruzi* antigen surrounding a nest of parasites in heart muscle. Photograph of a cryostat section of heart muscle prepared from a mouse 5 days after infection with *T. cruzi*, Y strain, and stained with rabbit anti-*T. cruzi* serum. The antiserum was absorbed extensively with a homogenate of mouse heart before use.

antigens were first detected on the membranes of uninfected cells on the 7th day after infection (Fig. 2). No deposits of mouse immunoglobulin or C3 were demonstrable at this stage. The subsequent decline in anti-*T. cruzi* antigen immunofluorescence was accompanied by an increased binding of anti-immunoglobulin or anti-C3 sera (Table 1).

As an index of T lymphocyte-mediated immunity during the course of infection, lymphokine

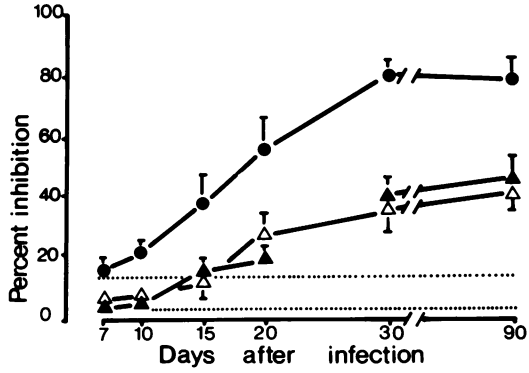

Fig. 3. Inhibition of migration of normal mouse peritoneal exudate cells by lymphocytes from *T. cruzi*-infected mice. The following antigen preparations were used to stimulate lymphocytes from *T. cruzi*-infected mice in the presence of normal syngeneic macrophages: (●——●) lysate of *T. cruzi* epimastigotes, (▲——▲) lysate of CNS neurones, and (△——△) homogenate of mouse heart ventricles. Data shown as mean of five determinations ± s.e. Area between horizontal broken lines represents the mean migration inhibition ± s.e. of the following controls: peritoneal exudate cells mixed with normal syngeneic lymphocytes either alone or in the presence of epimastigote, neurone or heart muscle antigens, or normal peritoneal exudate cells with syngeneic lymphocytes from *T. cruzi*-infected mice either alone or in the presence of kidney homogenate.

secretion was assayed by the inhibition of macrophage migration in the presence of *T. cruzi*, cardiac muscle, neuronal or kidney antigens (Fig. 3). Significant inhibition of macrophage migration was detected against *T. cruzi* antigens on the 10th day after infection. The intensity of migration inhibition increased to a plateau value on day 30 and was maintained until day 90 when the experiment was terminated. Although inhibition of macrophage migration against muscle and neurone antigens was not detected until the 20th day of infection, this anti-'self' reactivity increased in intensity until the end of the experiment. Inhibition of macrophage migration in the presence of kidney antigens was never greater than 16% of control values (normal peritoneal exudate cells alone) at any stage of the experiment.

DISCUSSION

The present data, derived from a mouse model, confirm previous human (Köberle, 1968, 1970, 1974) and rat (Alcântara, 1961) studies showing that heart muscle is denervated during infection with *Trypanosoma cruzi*. The close association between parasite numbers and the rate of neurone destruction is of particular interest (Fig. 1). The maximum reduction in neurone numbers occurred between 10–20 days after infection at which time parasites were easily detectable in the blood. After the 20th day of infection, when the parasitaemia had decreased to its chronic level, neurone numbers remained virtually constant even though a significant and progressive anti-neurone immune response developed (Fig. 3).

The *T. cruzi* antigens detected on neurones and myocardial fibres by indirect immunofluorescence probably represent parasite products released from pseudocysts and adsorbed onto the surface membranes of surrounding uninfected cells. It is likely that this passive adsorption of parasite antigens is analogous to the mechanism described previously in parasite–host cell *in vitro* culture systems (Ribeiro dos Santos & Hudson, 1980a, 1980b). In this context, it is important to stress that the anti-*T. cruzi* serum was absorbed with cardiac cells and did not show any detectable binding to sections of heart from normal, uninfected mice.

The apparent decrease in demonstrable antigen between 10–15 days after infection (Table 1), even though many tissue parasites were still present, is intriguing. This might be explained by the associated increase in mouse immunoglobulin and C3 bound to ininfected cells surrounding parasite pseudocysts. It is possible that this cell-bound immunoglobulin represents complement-fixing anti-trypanosome antibody produced in response to infection and which could compete with our immunofluorescent staining reaction for parasite antigens.

Of particular interest in these present data is the time relationship between the rate of neurone destruction and the appearance of anti-parasite and anti-'self' reactivity. There are two major findings: (a) neurone destruction preceded the development of an anti-neurone T lymphocyte-mediated immune response, whether measured by cytotoxicity (Ribeiro dos Santos & Hudson, 1980b) or lymphokine activity (Fig. 3); and (b) neurone destruction correlated closely with the anti-parasite response measured as antibody (Table 1), cytotoxic T cells (Ribeiro dos Santos & Hudson, 1980b) or lymphokine-secreting T cells (Fig. 3). The finding that neuronal destruction preceded the development of an anti-neurone immune response is not compatible with the previous suggestion that neuronal destruction is due to autoreactivity elicited by cross-reacting antigens shared by the parasite and host tissues (Santos-Busch & Teixeira, 1974; Teixeira *et al.*, 1975; Teixeira, 1979).

We believe that the present findings form an interconnected chain of events consistent with our suggested mechanism for the pathogenesis of acute-stage Chagas' disease, which was based on previous *in vitro* experiments (Ribeiro dos Santos & Hudson, 1980a, 1980b). Briefly, we suggest that when a nest of tissue parasites ruptures, antigen is released and can become associated with the surface membrane of surrounding uninfected cells. Any immune response leading to parasite lysis could serve to increase the amount of parasite antigen available for binding to host cells.

These parasite-modified host cells could then be killed by elements of the anti-*T. cruzi* immune response, as shown in previous *in vitro* experiments (Ribeiro dos Santos & Hudson, 1980b), thus releasing self-antigens. These self-antigens are probably immunogenic, as evidenced by the

development of inhibition of migration of macrophages in the presence of heart muscle or neurone antigens (Fig. 3) and the development of self-reactive cytotoxic T lymphocytes (Ribeiro dos Santos & Hudson, 1980b). We have not, however, been able to find evidence for a self-sustaining autoimmune destruction of host tissue, and so the contribution of this potential autoaggression to the secondary pathogenesis of the disease remains to be determined.

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