Experimental chemotherapy of *Trypanosoma cruzi* infection: persistence of parasite antigens and positive serology in parasitologically cured mice

S.G. Andrade,[1] L.A.R. Freitas,[2] S. Peyrol,[3] A.R. Pimentel,[4] & M. Sadigursky[1]

Mice infected with Trypanosoma cruzi, *but parasitologically cured after specific chemotherapy, continued to exhibit positive indirect immunofluorescence serological tests 3–6 months after the therapy. Treatment of trypanosome antigens with monospecific antisera produced in rabbits, and examination by immuno-electron-microscopy following peroxidase labelling disclosed the presence of membrane deposits in cell processes in the spleens of the mice. Similar deposits were observed in the external membranes of* T. cruzi *amastigotes in the spleens of acutely infected mice, but not in normal control mice. No reaction occurred in tissues not previously treated with the monospecific anti-*T. cruzi *serum. Positive cells in treated and cured mice, as well as in the not cured or untreated control mice, were located in germinal centres of the splenic white pulp and presented long and branching cytoplasmic processes, which are indicative of dendritic cells of the lymphoid follicles of the spleen.*

Infection of vertebrate hosts with *Trypanosoma cruzi* elicits production of specific antibodies that are useful for diagnosing Chagas disease. Experimental treatment of both acutely and chronically infected mice with different drugs results in parasitological cure in a variable proportion of cases, depending on the *T. cruzi* strain (*1–4*). However, confirmation of cure is often complicated by positive post-treatment serological tests. Previously we have reported that immunofluorescence tests remained positive for mice that exhibited negative parasitological tests 3–6 months after treatment (*1–4*). We hypothesized that this arose because of the persistence of *T. cruzi* antigens trapped in "memory cells" in the spleen, as has been described for other antigens (*5–8*).

Immunological memory is associated with the development of germinal centres in the follicles of the spleen, in particular with the generation of dendritic cells. Such cells can trap antigens and antibody complexes for long periods (*9, 10*).

To investigate our hypothesis, we looked for *T. cruzi* antigens in spleen cells using immunoelectron microscopy, with purified anti-*T. cruzi* antibodies labelled with peroxidase. As described previously, this method immunolabels the membrane of intracellular amastigotes and particulate antigens within macrophages in acutely infected mice (*11*). The procedure was also used to detect membrane antigens in the germinal centres of lymphoid follicles in the spleens of treated mice.

We describe here the results of studies to further investigate the sequestration of specific *T. cruzi* antigens in the spleens of mice, and to correlate positive indirect immunofluorescence tests 6 months after treatment with parasitological cure.

Materials and methods

A total of 30 Swiss mice were experimentally infected with either the 12 SF or Montalvania strain of *T. cruzi*, both of which are classified as type II (*12, 13*), by injecting each intraperitoneally with an inoculum of 1×10^5 blood forms. Of these mice, 23 were treated with the antitrypanosomal drug nifurtimox or benznidazole (*1*) during the acute phase of infection. Seven untreated mice were used as chronically infected controls. Table 1 shows the distribution of the mice into the various experimental groups.

As a second category of controls, five mice infected with the Peruvian strain of *T. cruzi* were killed during the acute phase to identify and immunolabel the parasites in the spleen. Five uninfected mice served as normal controls.

[1] Associate Professor, Universidade Federal da Bahia, Centro de Pesquisas Gonçalo Moniz, Rua Valdemar Falcão, 121, Brotas, 41.945 Salvador, Bahia, Brazil. Requests for reprints should be sent to Dr Andrade at this address.

[2] Assistant Professor, Universidade Federal da Bahia, Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, Brazil.

[3] Research Assistant, Institut Pasteur de Lyon–Centre de Microscopie électronique, Lyon, France.

[4] Biologist, Universidade Federal da Bahia, Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, Brazil.

Reprint No. **5162**

Table 1: **Distribution of the study mice infected with *Trypanosoma cruzi*, according to strain and treatment**

T. cruzi strain	Treated with benznidazole or nifurtimox: Yes		No	Total
	Cured	Uncured		
12 SF[a]	7	4	3	14
Montalvania[b]	12	0	4	16
Total	19	4	7	30

[a] 12 SF (type II strain) from an acute fatal case (São Felipe–Bahia–BR).
[b] Montalvania (22 Mont and 24 Mont type II strains) from acute cases (Montalvania–Goiás–BR).

Treatment schedules

Nifurtimox was administered in an initial dose of 200 mg per kg body weight for 4 days and then in daily doses of 50 mg per kg body weight for a total of 90 days. The dose of benznidazole was 100 mg per kg body weight 5 days a week for 90 days.

Tests for parasitological cure

Xenodiagnosis with 5th stage *Rhodnius prolixus* nymphs, subinoculation of blood into newborn mice (0.1 ml intraperitoneally), and haemoculture were performed 3–6 months after the end of treatment with both the treated mice and untreated controls.

Serological test

The indirect immunofluorescence (IIF) test was used with culture forms of *T. cruzi* as antigens, according to the method described by Camargo (*14*).

Immunoelectron-microscopy

The mice were anaesthetized using ether, their portal veins sectioned, and their spleens perfused by injecting phosphate-buffered saline (PBS) (pH 7.2, 4 °C) into the left ventricles.

Sections of spleen were fixed in 4% paraformaldehyde solution in 0.1 mol/l sodium cacodylate and 7.5 mol/l sucrose for 8–12 hours at 4 °C and washed with a mixture of 0.1 mol/l sodium cacodylate + 0.2 mol/l sucrose + 0.1 mol/l lysin. The sections were then cryopreserved, placed in Tissue Teck[a] and frozen in liquid nitrogen (*15*). Immunolabelling with peroxidase was performed using the technique described by Nakane & Pierce (*16*). Endogenous peroxidase was inhibited with hydrogen peroxide. Tissues were treated with bovine serum albumin[b] in PBS containing goat-anti-rabbit antibodies (1:50).

The sections were then treated with monospecific purified anti-*T. cruzi* antibodies raised in rabbits, followed by anti-rabbit immunoglobulin conjugated with peroxidase.[b] Reactions were developed using diaminobenzidine and hydrogen peroxide according to the method described by Graham & Karnovsky (*17*). The sections were then treated with osmium tetroxide, dehydrated in increasingly concentrated solutions of ethanol, and flat-embedded in Epon resin.[c] Ultrathin sections without contrast were examined by electron-microscopy (Zeiss EM 109) at 50 kV.

*Monospecific purified anti-*T. cruzi *rabbit antibodies*

Two rabbits were immunized with whole *T. cruzi* antigen in complete Freund's adjuvant. Serum examined using the indirect immunofluorescence test showed high titres of specific antibodies (1:312). Gammaglobulin was precipitated with ammonium sulfate, dialysed in PBS (pH 7.0), and purified a second time using Sephadex to separate IgG antibodies. A third purification was performed in cyanogen bromide activated-Sepharose with *T. cruzi* antigen. The eluate containing the monospecific antibodies was concentrated using polyethyleneglycol.

Controls

For each specimen that was immunolabelled, a control specimen was processed through all steps of the procedure, except for treatment with monospecific anti-*T. cruzi* antibody. Sections of the spleens of acutely infected mice that contained amastigote forms of the parasite were used as controls to test the specificity of the immunolabelling procedure. Sections of the spleens of normal intact control mice were always processed as controls for the reactions.

Results

Parasitological cure was demonstrated for 82.7% of the mice that were treated for 90 days with either benznidazole or nifurtimox. IIF tests were persistently positive for the cured mice, with titres that varied from 1:10 to 1:160 (Table 2). The uncured mice as well as the chronically infected, untreated controls (Table 3) had positive parasitological tests and high IIF titres (1:160 to 1:640).

[a] Wiles Diagnostic Division, Elkhart, IN, USA.

[b] Sigma Chemical Company, St. Louis, MO, USA.

[c] Shell Chemical Corporation.

Table 2: **General data on the mice that were infected with *Trypanosoma cruzi* and treated with benznidazole or nifurtimox and parasitologically cured**

Identification	*T. cruzi* strain	Drug[a]	Parasitological tests[b]	IIF test (titres)[c]	Post-treatment period (months)
A-7	12 SF	Benznidazole	−ve	1:80	3
A-9		"	−ve	1:10	3
A-10		"	−ve	1:160	3
A-11		"	−ve	1:160	3
A-14		"	−ve	1:160	3
A-17		"	−ve	1:20	4
A-18		"	−ve	1:160	4
A-21	24 Mont	Nifurtimox	−ve	1:40	6
A-22		"	−ve	1:40	6
A-23		Benznidazole	−ve	1:80	6
A-24		"	−ve	1:40	6
A-25		"	−ve	1:40	6
A-26		"	−ve	1:40	6
A-29	22 Mont	Nifurtimox	−ve	1:80	6
A-30		"	−ve	1:40	6
A-31		"	−ve	1:10	6
A-32		"	−ve	1:80	6
A-33		Benznidazole	−ve	1:10	6
A-36		Nifurtimox	−ve	1:40	6

[a] Benznidazole (RO 7.1051); nifurtimox (Bay 2502).
[b] Xenodiagnosis, blood inoculation into suckling mice, and haemoculture.
[c] IIF = indirect immunofluorescence.

During the acute phase of the infection, the positive controls developed high levels of parasitaemia 7–10 days after infection and were killed when parasitaemia was at its maximum.

Immunoelectron-microscopy

Normal controls. Cells in the germinal centre of the lymphoid follicles of the spleen exhibited no immunolabelling with peroxidase, irrespective of whether or not they were treated with specific anti-*T. cruzi* antibody.

Positive controls (acute phase). In the sections of spleen treated with anti-*T. cruzi* antibody, the intracellular amastigote forms of *T. cruzi* were specifically immunolabelled with peroxidase on the parasite surface membrane, in the intracellular vacuole membrane, and in disintegrated fragments

Table 3: **General data on mice infected with *Trypanosoma cruzi* (treated, but not cured and untreated controls)**

Identification	*T. cruzi* strain	Drugs	Parasitological tests	IIF test (titres)[a]	Post-treatment period (months)
A-6	12 SF	Nifurtimox	+ve	1:160	3
A-8		Benznidazole	+ve	1:640	3
A-13		Nifurtimox	+ve	1:640	3
A-16		Benznidazole	+ve	1:160	3
A-12		Untreated control	+ve	1:160	3
A-19		"	+ve	1:160	4
A-20		"	+ve	1:640	4
A-27	24 Mont	"	+ve	1:280	6
A-28		"	+ve	1:280	6
A-34		"	+ve	1:640	6
A-35		"	+ve	1:640	6

[a] IIF = indirect immunofluorescence.

in the interior of macrophages (Fig. 1a). The external membrane of parasitized macrophages and their cell projections exhibited positive immunolabelling, which was also observed on apparently nonparasitized splenic cells (Fig. 1b, c). The amastigotes of control sections, which were not treated with specific anti-*T. cruzi* antibody, did not exhibit immunolabelling.

Treated mice. Sections of spleen treated with specific anti-*T. cruzi* antibody exhibited immunolabelling with peroxidase on the external membrane of dendritic cell processes in the germinal centres of lymphoid follicles. These processes are broad, appear as lightly shaded areas in the electron-microscope, without phagosomes, and sometimes contain granular material (Fig. 2a–c). The labelling on the membrane appeared as fine granules (Fig. 2c). There were no parasite forms in the sections of spleen. Immunolabelling occurred both among the parasitologically cured mice as well as those that were treated but not cured. In the sections of spleen that were not pretreated with specific anti-*T. cruzi* antibody, immunolabelling did not occur and they were similar to those described for the normal controls (Fig. 3).

Untreated mice. The same pattern of immunolabelling described for the treated mice was observed also in sections of spleen previously treated with anti-*T. cruzi* antibody but not in sections that had not received this treatment.

Discussion

Conflicting reports about the efficacy of treatment for human Chagas disease may arise because of differences in the parasite strains, the use of different chemotherapeutic regimens, or different cure criteria (*18–22*). Experimental studies of mice have established the influence of *T. cruzi* strains on the response to chemotherapy and identified sensitive and resistant strains (*23–25*). It has been claimed that cure can be documented only if serological tests (IIF, indirect haemagglutination, and lytic antibody tests) are negative (*22*). With this criterion, patients who exhibit repeatedly negative xenodiagnosis are considered to be treatment failures if they have positive immunofluorescence tests, although currently the lytic antibody test is probably the most reliable method of assessing cure (*26*).

Experimental studies of the chemotherapy of Chagas disease have established no correlation between parasitological cure and reversion of serological test results from positive to negative, at least

Fig. 1. **Noncontrasted ultrathin sections of the spleen of a mouse with acute *Trypanosoma cruzi* infection, immunolabelled with peroxidase after treatment with anti-*T. cruzi* specific antibodies.** *a*) *T. cruzi* amastigote, showing specific immunolabelling on the surface membrane (long arrow), internal parasite structure (short arrow), and disintegrated fragments (× 12 000). *b*) Two amastigote forms (long arrows) showing positive immunolabelling—also present on the membrane of nonparasitized cells and the cell processes of macrophages (short arrows) (× 4400). *c*) Detailed view showing immunolabelling of the macrophage projections (arrow) (× 12 000).

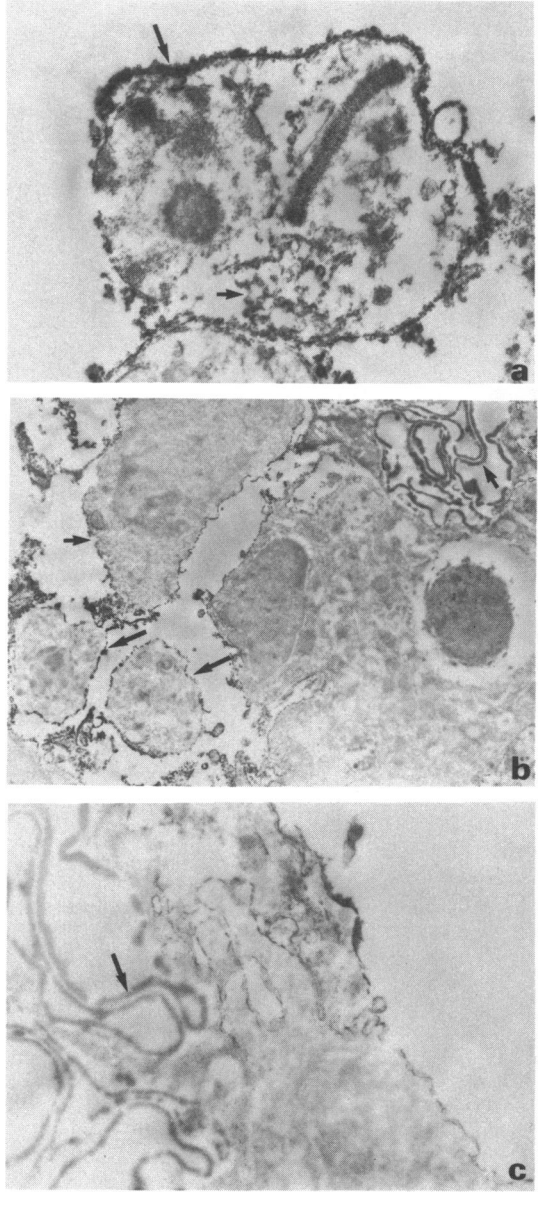


Fig. 2. **Sections of the spleen of a mouse infected with *Trypanosoma cruzi* and parasitologically cured after specific chemotherapy, but with persistently positive serological tests (indirect immunofluorescence) (the sections were treated with anti-*T. cruzi* antibodies and examined using immunoelectron-microscopy).** *a*) and *b*) Positive specific immunolabelling with peroxidase is shown as dark deposits on the membranes of the cellular processes of dendritic cells (arrows)—other splenic cells are not labelled (× 12 000). *c*) Membrane labelling is shown as fine granules (× 12 000).

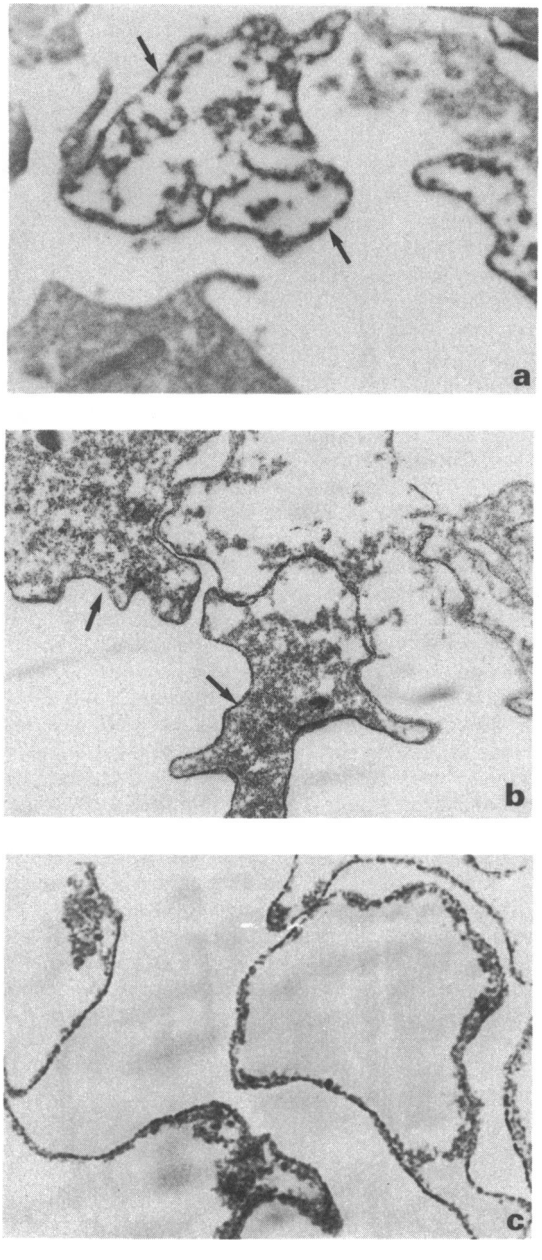
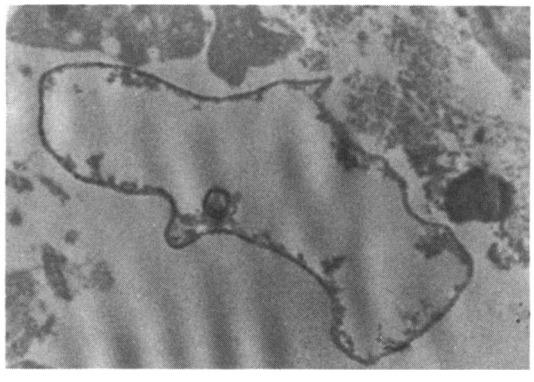


Fig. 3. **Spleen of a normal control mouse (sections were treated with anti-*Trypanosoma cruzi* antibodies and examined by immunoelectron-microscopy).** The response was negative and no specific immunolabelling was visible on the membrane of cellular processes. This same aspect occurs in sections from positive mice that were not treated with the specific antiserum (× 12 000).



within 6 months of treatment (*1, 2*). However, other reports have shown that in mice IIF tests become negative 10–12 months after treatment (*27*). In the present study, the occurrence of antigens bound to the cytoplasmic membrane of cellular processes in the germinal centres of splenic lymphoid follicles was detected using the immunoperoxidase technique. This finding supports the hypothesis that immunological memory is maintained by certain cells in the spleen. The morphological characteristics of the cells that were immunolabelled and their location (*5*) strongly suggest that these are dendritic cells. Dendritic cells in the germinal centres can trap antigens and antigen–antibody complexes (*10*). Using rats that had been injected intravenously with iodine-labelled salmonella flagella, Nossal et al. showed that the antigens were located in a large extracellular space in the follicles, close to or on the surface membrane of dendritic cell processes (*6*).

Ultrastructural studies of ^{125}I-labelled antigens in germinal centres of mouse spleen have been carried out by Hanna & Szakal (*5*). They described the presence of antigens in extensive cytoplasmic cellular processes and the disorganization of the membrane infoldings 3 days after secondary injection. In an autoradiographic study of the binding of labelled antigens and immune complexes to macrophages and dendritic cells, van Rooijen showed that, irrespective of whether or not germinal centres were present, the radiolabel was always located in the central part of the follicle, i.e., in the dendritic cell area (*8*). According to Klaus, follicular dendritic cells can trap antigens and antigen–antibody complexes for prolonged periods (*10*). Also Tew &

Mandel have shown that antigens retained in lymphoid follicles remain active for long periods and that they stimulate synthesis of antibodies whenever the concentration of the latter declines (*9*). According to these investigators, the antigens may persist in rabbits for 12.5 months and for 7 months in mice.

Of importance for the persistence of serological tests in Chagas disease after cure is whether *T. cruzi* antigens disappear after an as yet undetermined interval. We are currently investigating this phenomenon in mice spleens. According to Krettli et al., the complement-mediated lysis test is the only serological reaction that can correlate with parasitological cure (*26*). The persistence of a positive reaction in this test implies the presence of viable *T. cruzi* in the host. By means of the technique of immunolabelling with peroxidase, it should be possible to investigate at the ultrastructural level the significance of the complement-mediated lysis test and its relationship to the persistence of antigens bound to the lymphoid follicular cells of the spleen.

Acknowledgements

This investigation was supported financially by the UNPD/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, the Brazilian National Council for Scientific and Technological Development (CNPq), and the Smith–Kline Foundation (Brazil). We are also indebted to the INSERM/CNPq Cooperative Programme for providing collaborating facilities at the Institut Pasteur de Lyon, France.

Résumé

Chimiothérapie expérimentale d'infections par *Trypanosoma cruzi*: persistance d'antigènes parasitaires et d'une sérologie positive chez des souris guéries parasitologiquement

Des tests sérologiques régulièrement positifs ont été enregistrés en même temps que des xénodiagnostics négatifs chez des malades atteints de maladie de Chagas qui avaient reçu une chimiothérapie spécifique. Le traitement d'animaux infectés par *Trypanosoma cruzi* avec différents médicaments a entraîné une guérison parasitologique chez une certaine proportion d'entre eux; cependant, chez la plupart, les tests spécifiques d'immunofluorescence indirecte sont restés positifs.

La persistance d'antigènes de *T. cruzi* séquestrés dans des "cellules à mémoire" de la rate a été avancée pour expliquer ces observations. Pour vérifier cette hypothèse, nous avons effectué une étude par microscopie immunoélectronique pour rechercher des antigènes de *T. cruzi* dans les cellules dendritiques de la rate de souris, en utilisant des anticorps anti-*T. cruzi* purifiés, monospécifiques, marqués à la peroxydase. Pour cette recherche, 23 souris Swiss qui avaient été infectées expérimentalement avec les souches 12 SF ou Montalvania de *T. cruzi*, ont été traitées par du benznidazole (100 mg/kg de poids corporel par jour pendant 90 jours) ou par du nifurtimox (4 doses de 200 mg/kg de poids corporel, suivies par des doses quotidiennes de 50 mg/kg de poids corporel pour un total de 90 jours). Sept souris non traitées ont été gardées à titre de témoins porteurs d'une infection chronique. Comme second type de témoins infectés, cinq souris inoculées avec la souche péruvienne de *T. cruzi* ont été sacrifiées pendant la phase aiguë de l'infection pour identification et immunomarquage des parasites dans la rate. Cinq souris non infectées servaient de témoins intacts.

Les animaux étaient anesthésiés à l'éther, et après perfusion de la rate avec du sérum physiologique tamponné au phosphate (PBS; pH 7,2), des coupes ont été fixées dans le paraformaldéhyde et cryoconservées dans de l'azote liquide. Des coupes au cryostat ont été immunomarquées à la peroxydase après traitement par des anticorps anti-*T. cruzi* purifiés, monospécifiques, obtenus chez des lapins, puis par une immunoglobuline anti-lapin conjuguée à la peroxydase. Après inclusion dans de la résine Epon, des coupes ultraminces sans contraste ont été examinées au microscope électronique. Les témoins normaux n'avaient pas de réaction positive avec la peroxydase. Chez les témoins de phase aiguë, les formes amastigotes intracellulaires de *T. cruzi* étaient immunomarquées de façon spécifique par la peroxydase sur la membrane externe du parasite, dans la membrane des vacuoles intracellulaires et dans les fragments désintégrés à l'intérieur des macrophages. Les amastigotes des coupes témoins, qui n'avaient pas été traitées par des anticorps anti-*T. cruzi* spécifiques, ne montraient pas d'immunomarquage. Chez les souris traitées, aussi bien celles qui étaient parasitologiquement guéries, avec des tests d'immunofluorescence indirecte positifs, que celles qui n'avaient pas été guéries, un immunomarquage par la peroxydase a été observé sur la membrane externe des processus cellulaires dendritiques des centres germinaux des follicules lymphoïdes. Les souris non traitées montraient le même type d'immunomarquage. L'immunomarquage n'a pas eu lieu dans les coupes de rate non prétraitées par des anticorps anti-*T. cruzi* spécifiques.

Ces observations sont en faveur de l'hypothèse suivant laquelle une mémoire immunologique est maintenue par des "cellules à mémoire" de la

rate. Leurs caractéristiques morphologiques et leur localisation laissent fortement penser qu'il s'agit des cellules dendritiques de la rate.

References

1. **Andrade, S.G. et al.** Evaluation of chemotherapy with benznidazole and nifurtimox in mice infected with *Trypanosoma cruzi* strains of different types. *Bulletin of the World Health Organization*, **63**: 721–726 (1985).
2. **Andrade, S.G. et al.** Therapeutic action of MK 436 (2,5-nitroimidazole) on *Trypanosoma cruzi* infections in mice: a parasitological, serological, histopathological, and ultrastructural study. *Bulletin of the World Health Organization*, **65**: 625–633 (1987).
3. **Andrade, S.G. et al.** Treatment of chronic experimental *Trypanosoma cruzi* infections in mice with MK-436, a 2-substituted 5-nitroimidazole. *Bulletin of the World Health Organization*, **67**: 509–514 (1989).
4. **Andrade, S.G. et al.** [Therapy for the chronic phase of experimental infection with *Trypanosoma cruzi* using benznidazole and/or nifurtimox.] *Revista da Sociedade Brasileira de Medicina Tropical*, **22**: 113–118 (1989) (in Portuguese).
5. **Hanna, M.G. Jr, & Szakal, A. K.** Localization of ^{125}I-labelled antigen in germinal centers of mouse spleen: histologic and ultrastructural autoradiographic studies of the secondary immune reactions. *Journal of immunology*, **101**: 949–962 (1968).
6. **Nossal, G.J.V. et al.** Antigens in immunity. XV. Ultrastructural features and antigens capture in primary and secondary lymphoid follicles. *Journal of experimental medicine*, **127**: 277–289 (1968).
7. **Brown, J.C. et al.** Lymphocyte-mediated transport of aggregated human gammaglobulin into germinal centre areas of normal mouse spleen. *Nature*, **228**: 367–369 (1970).
8. **Rooijen van, N.** Binding of labelled antigens and immune complexes to macrophages and dendritic cells in cryostat sections of normal mouse spleen. *Acta morphologica Neerlando–Scandinavica*, **16**: 121–127 (1978).
9. **Tew, J.C. & Mandel, T.** The maintenance and regulation of serum antibody levels: evidence indicating a role for antigen retained in lymphoid follicles. *Journal of immunology*, **120**: 1063–1069 (1978).
10. **Klaus, G.G.B.** Mechanisms in the generation of immunological memory. In: Torrigiani, G. & Bell, R., ed. *Immunological recognition and effector mechanisms in infectious disease*. Basle, Schwabe, 1981.
11. **Andrade, S.G. et al.** *Trypanosoma cruzi* antigens detected by immunoelectron microscopy in the spleen of mice serologically positive but parasitologically cured by chemotherapy. *Revista da Sociedade Brasileira de Medicina Tropical*, **21**: 41–42 (1988).
12. **Andrade, S.G.** Morphological and behavioural characterization of *Trypanosoma cruzi* strains. *Revista da Sociedade Brasileira de Medicina Tropical*, **18**(suppl.): 39–46 (1985).
13. Report of the WHO Steering Committees. Research Activities of the Scientific Working Group on Chagas' disease. *Memorias do Instituto Oswaldo Cruz*, **81**(suppl.): 181–244 (1986).
14. **Camargo, M.E.** Fluorescent antibody test for the serodiagnosis of American trypanosomiasis. Technical modification employing culture forms of *Trypanosoma cruzi* in a slide test. *Revista do Instituto de Medicina Tropical de São Paulo*, **8**: 227–234 (1966).
15. **McLean, I.W. & Nakane, P.K.** Periodate–lysin–paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *Journal of histochemistry and cytochemistry*, **22**: 1077–1083 (1974).
16. **Nakane, P.K. & Pierce Jr., G.B.** Enzyme-labelled antibodies: preparation and application for the localization of antigens. *Journal of histochemistry and cytochemistry*, **14**: 929–931 (1966).
17. **Graham, R.C. & Karnovsky, M.J.** The early stages of absorption of horseradish-peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *Journal of histochemistry and cytochemistry*, **14**: 291–302 (1966).
18. **Schenone, H. et al.** [Treatment of chronic infection with Chagas disease: experience in Chile]. In: *Proceedings of the International Symposium on Chagas Disease, Buenos Aires, 26 November–2 December 1972*. Buenos Aires, Division Imprenta de la Secretaria de Salud Publica de la Nacion, 1972, pp. 287–291 (in Spanish).
19. **Prata, A. et al.** [Treatment of Chagas disease using nifurtimox (Bay 2502)]. *Revista da Sociedade Brasileira de Medicina Tropical*, **9**: 297–304 (1975) (in Portuguese).
20. **Rassi, A.** [Etiological treatment of Chagas disease]. *Arquivo Brasileiro de cardiologia*, **38**: 277 (1981) (in Portuguese).
21. **Apt. W.B.** [Treatment of Chagas disease]. *Revista médica del Chile*, **113**: 162–166 (1985) (in Spanish).
22. **Cançado, J.R.** [Specific treatment]. In: Cançado, J.R. & Chuster, M., ed. *Cardiopatia chagasica*. Belo Horizonte, Fundação Carlos Chagas, 1985, pp. 327–355 (in Portuguese).
23. **Andrade, S.G. et al.** [Influence of the strain of *Trypanosoma cruzi* on the reaction to experimental therapy with Bay 2502]. *Revista do Instituto de Medicina Tropical de São Paulo*, **17**: 380–389 (1975) (in Portuguese).
24. **Brener, Z. et al.** Differences in the susceptibility of *Trypanosoma cruzi* strains to active chemotherapeutic agents. *Revista do Instituto de Medicina Tropical de São Paulo*, **18**: 450–455 (1976).
25. **Neal, R.A. & van Bueren, J.** Comparative studies of drug susceptibility of five strains of *Trypanosoma cruzi in vivo* and *in vitro*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **82**: 709–714 (1988).
26. **Krettli, A.U. et al.** Effect of specific chemotherapy on the levels of lytic antibodies in Chagas' disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **76**: 334–340 (1982).
27. **Filardi, L.S. & Brenner, Z.** A nitroimidazole–thiazole derivative with curative action in experimental *Trypanosoma cruzi* infections. *Annals of tropical medicine and parasitology*, **76**: 293–297 (1982).