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IgG subclasses responsible for immune clearance in mice infected with *Trypanosoma cruzi*

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Summary To examine the role of different immunoglobulin subclasses in the immune clearance of *Trypanosoma cruzi*, mice containing bloodstream trypomastigotes were injected intravenously with immune serum, IgG-depleted serum, or with the IgG1 or IgG2 fractions and the rate of removal of the parasites from circulation was determined. Using IgG concentrations similar to those found in the immune serum, the rate of clearance mediated by IgG2 was six-fold higher than that obtained with IgG1. This difference did not appear to be due to differences in antibody specificity, as Western blotting showed that each isotype recognized a similar set of antigens extracted from the parasite. However, the *T. cruzi* specific antibody content of the IgG2 was approximately five-fold higher than IgG1. When the dose of IgG was adjusted to equalize the antibody content, the clearance ability of the IgG1 and IgG2 was very similar. It is concluded that the two subclasses have a similar clearance ability.

INTRODUCTION

The mechanism of immunity responsible for acquired resistance against *Trypanosoma cruzi* is not fully understood. During the chronic phase of *T. cruzi* infection in both humans and mice, there is a strong humoral response as shown by circulating specific antibodies. The participation of these antibodies in defence mechanisms has been suggested by three different approaches: (i) the passive transfer of protection with immune serum (1); (ii) the correlation between resistance to *T. cruzi* and the ability to produce antibodies (2); and (iii) the enhancement of *T. cruzi* infection by suppression of antibody production (3). Using mouse serum obtained in the chronic phase of the infection, Takehara *et al.* showed that protective antibodies are located in the IgG2 isotype (4). However, the exact mechanism through which antibodies protect the infected host is not known. One conspicuous biological activity of these antibodies is their ability to induce clearance of *T. cruzi* bloodstream trypomastigotes (Btrys) when infected mice are injected with these parasites (5). Recently, Umekita *et al.* have shown that the clearance of Btrys is dependent on the Fc region of the antibodies and suggested that the clearance is mediated mainly by opsonization rather than by lysis of the parasites (6).

In this report we characterize the IgG subclasses responsible for the immune clearance and discuss their possible role in the serum protective ability. A brief report of this work has already been published (7).

Abbreviations used in this paper: Btrys, bloodstream trypomastigotes; IMS, immune mouse serum; i.p. intraperitoneal; i.v., intravenous; NMS, normal mouse serum; PBS, phosphate-buffered saline.

MATERIAL AND METHODS

Animals

Inbred A/Sn mice weighing 20–23 g were used throughout.

Maintenance of the parasites

The Y strain of *T. cruzi* (8), maintained in mice by weekly passage, was used in all experiments.

Immune mouse sera

Normal mice were injected intraperitoneally (i.p.) with an infective dose of 10 *T. cruzi* Btrys, and to prevent death induced by the acute infection they were treated during the first 21 days of infection with nifurtimox (3-methyl-4-[(5′nitrofurfurylidene) amino]-tetrahydro-4H-1,4-thiazine 1,1-dioxide) (Bayer do Brasil, SA, São Paulo, Brazil) dissolved in drinking

water (0·5 mg/mL). This schedule was used since a shorter treatment with the drug was much less efficient in protecting the animals. At 10–15 days after infection the animals were bled to determine whether parasites were present in their blood. Animals not showing parasites were eliminated, whilst those showing parasites were kept for 45 days, and challenged i.p. three times (1 week apart) with 100 Btrys, then bled 7 days after the last infectant dose. The serum obtained at this time was very rich in antibodies as determined by complement fixation, haemagglutination and immunofluorescence.

Isolation of IgG by affinity chromatography with protein A

Protein A bound to Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was prepared as directed by the manufacturers, resuspended in 0·14 mol/L phosphate buffer, pH 8·0 and mounted in a 1×10 cm column. An aliquot (1 mL) immune mouse serum (IMS) was loaded on to the column and the non-adsorbed proteins washed off with 0·14 mol/L phosphate buffer, pH 8·0. The resulting IgG-free serum was then concentrated to the original serum volume by positive pressure filtration using an ultrafiltration cell (Amicon Corporation, Cambridge, MA, USA). The adsorbed proteins were then eluted in a stepwise way using buffers of different pH as described by Ey *et al.* (9). IgG1 was eluted with 0·1 mol/L citrate buffer, pH 6.0, whereas IgG2 was eluted with citrate buffer, pH 3·0. Samples (3 mL) were collected, fractionation was monitored by measurements of the optical density at 280 nm. The eluates of each protein peak were pooled separately, dyalized against phosphate-buffered saline (PBS) 0·15 mol/L pH 7·2, and concentrated by positive pressure filtration to the original serum volume. The final protein concentration of the fractions was determined by spectrophotometry at 280 nm using an extinction coefficient (1% w/v solution) of 16 (10). The absence of IgG in the IgG-free serum was confirmed by agar gel immunodiffusion using rabbit anti-mouse gamma chain and the purity of IgG1 and IgG2 preparations was checked in the same way with isotype-specific antisera. When tested by immunoelectrophoresis against a pool of mouse normal sera these antisera produced a single sharp line indicating their monospecificity.

Preparation of antibody-free trypomastigotes

In order to suppress antibody production, mice were treated i.p. with cyclophosphamide (350 mg/kg) 72 h after having received a dose of 3×10^4 Btrys i.p. Seven days after infection the animals were bled from the brachial plexus under slight ether anaesthesia and the blood immediately collected in an equal volume of a sterile 3·8% sodium citrate solution. To exclude the presence of membrane bound antibodies, the trypomastigotes were incubated with complement (fresh human serum) for 30 min at 37°C. Only suspensions of parasites not lysed in the presence of complement and unable to induce clearance were considered free of antibodies. The Btrys were isolated as follows: the parasites containing blood were centrifuged at 200 *g* for 10 min. The pellet containing the parasites was resuspended in PBS, containing 10% normal mouse serum (NMS) and the parasites washed twice by centrifugation at 1000 *g* for 20 min with the same buffer. The parasites were then resuspended in 1 mL of PBS, centrifuged at 500 *g* for 10 min and allowed to rest at 37°C for 15 min. During this step the motile trypomastigotes rose to the top of the centrifuge tube, leaving the contaminating cells at the bottom. The supernatant was then collected gently, and the number of parasites determined and adjusted with a Neubauer chamber.

Determination of immune clearance

In order to measure the rate of disappearance of the Btrys from the peripheral blood, normal mice were injected via the tail vein with 0·2 mL 0·15 mol/L NaCl containing 2×10^6 antibody-free Btrys. Fifteen minutes later, the animals were bled by puncture of the ophthalmic plexus and the number of parasites in the circulation was determined (time 0). At that time the number of Btrys recovered from the blood was practically 2×10^6 and was considered to represent 100% of the parasites. Thereafter, the animals were injected i.v. with 0·25 mL of immune serum or immunoglobulin preparation. Samples of blood were collected at different times by puncture of the ophthalmic plexus and the parasites counted.

Parasite enumeration

Blood (5 µL) was dispersed on a glass with a 22×22 mm coverslip and the parasites enumerated with the aid of a microscope using a 400X magnification.

IgG1 and IgG2 antibody titration

The anti-*T. cruzi* IgG1 and IgG2 antibody content of the immune sera was determined by ELISA using an extract of cultured epimastigotes as antigen (11), anti-mouse IgG (gamma chain specific) antibodies labelled with horse peroxidase (Sigma Chemical Company, St Louis, USA) as enzyme conjugate and *o*-phenylenediamine/H_2O_2 as substrate. The absorbance of the wells was determined at 492 nm with an ELISA plate reader (Uniskan, Eflab, Finland).

Antibody specificity

The antibody specificity of IgG1, IgG2 and immune serum was determined by the Western blotting technique using Btrys isolated by carboxymethyl cellulose (12) as antigen. Parasites (2×10^8) were solubilized in 0·5 mL Tris buffer containing 2·5% (w/v) SDS, 2·5% (v/v) 2-mercaptoethanol and protease inhibitors (phenylmethylsulfonylfluoride, Tosyl. lysine chloromethyl ketone and ethylenediaminetetra acetic acid, 1 mmol/L), boiled for 3 min and applied to a 10 cm polyacrylamide gel 10% (w/v). The electrophoresis was performed as described by Laemmli (13); the separated antigens were transferred to nitrocellulose membranes according to Towbin *et al.* (14). The antigens were detected by the sequential addition of IgG1, IgG2 or IMS, then peroxidase-labelled anti-mouse IgG

and finally H_2O_2 plus 4-chloro-1-naphthol as enzyme substrate.

RESULTS

Clearance of trypomastigotes induced by passive transfer of immune mouse sera

To find out the clearance ability of IMS, mice containing a known number of parasites in the circulation (time 0), were injected with different dilutions of immune serum and the number of circulating parasites determined at different times after the injections. The results of these experiments, depicted in Fig. 1, showed that 15 min after the injection of 0·25 mL of undiluted immune serum, the parasites had been removed completely from the circulation and that even 0·25 mL of a 1:5 or 1:10 dilution of the immune serum were still efficient at inducing clearance of the parasites. IMS (twenty times diluted) and NMS (0·25 mL) were unable to induce clearance of the parasites.

Participation of IgG fraction in the clearance of Btrys

In order to verify that the clearance induced by IMS was due to IgG antibodies, mice containing a known number of Btrys were injected with either the fraction that bound to Protein A (IgG) or the IgG-free serum (non-bound proteins). As can be seen in Fig. 2, the IgG fraction was as efficient as IMS in inducing clearance of parasites, whereas the IgG-free serum behaved like NMS.

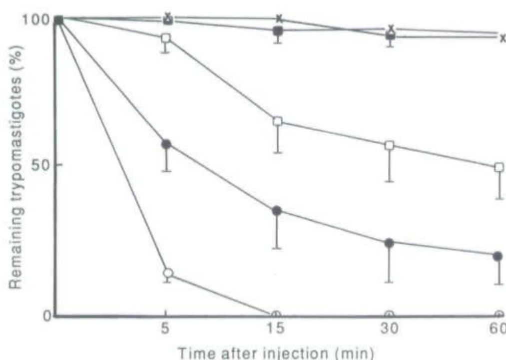

Fig. 1. Clearance of Btrys in mice induced by passive transfer of IMS. The number of circulating parasites was determined 5, 15, 30 and 60 min. after injecting 0·25 mL total IMS (○), its 1:5 (●), 1:10 (□) or 1:20 dilution (■). Remaining Btrys were calculated as a percentage of parasite numbers in control mice injected with NMS (x). $\bar{x} \pm$ s.d.; $n=6$ mice per group.

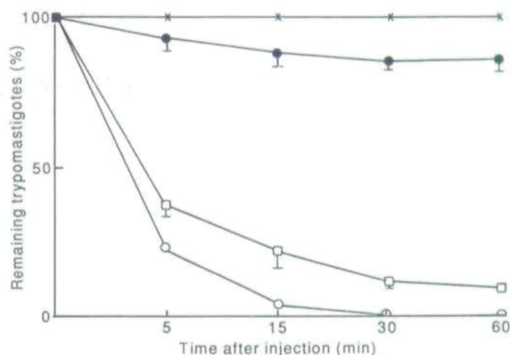

Fig. 2. Clearance of Btrys in mice induced by passive transfer of IMS (○), IgG-free serum (●), IgG fraction (□) or NMS (x). $\bar{x} \pm$ s.d.; $n=4$ mice per group.

Clearance ability of IgG subclasses

Since the clearance ability was located mostly in the IgG fraction, it seemed of interest to compare the clearance ability of IgG1 and IgG2. IgG1 and IgG2 were purified as described, and 0·2 mL of each was injected i.v. in mice to assess their clearance ability. As can be seen in Fig. 3, IgG2 and IMS did not differ significantly in their ability to induce clearance of the parasites, whereas IgG1 induced the same results as NMS, that is, both were unable to promote the removal of parasites from circulation.

IgG1 and IgG2 antibody specificity

In order to verify whether differences in the clearance ability of the IgG1 and IgG2 fractions could be due to different specificities, the Western blotting technique was used to determine which Btrys antigens were recognized by

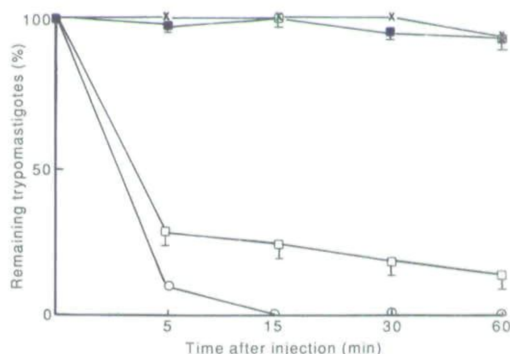

Fig. 3. Clearance of Btrys in mice induced by transfer of IMS (○), IgG1 fraction (■), IgG2 fraction (□) or NMS (x). $\bar{x} \pm$ s.d.; $n=4$ mice per group.

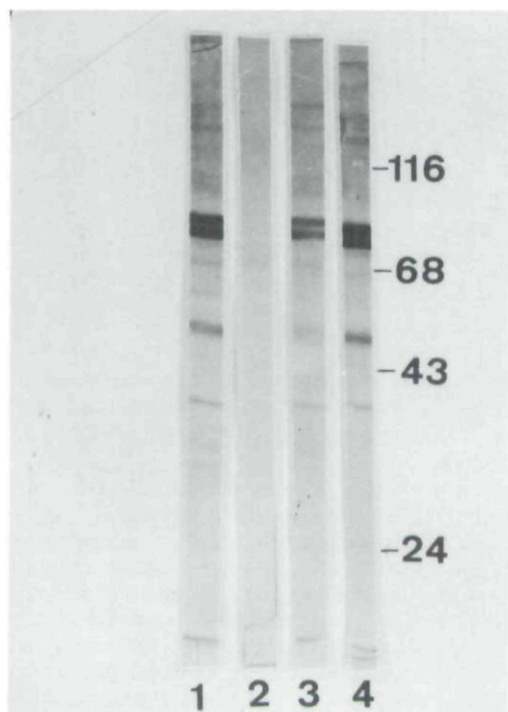

Fig. 4. Identification of Btrys antigens by Western blotting using IMS (1), IgG-free serum (2), IgG1 (3) and IgG2 (4) fractions as source of antibodies. Markers of molecular weight: β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and trypsinogen (24 kDa).

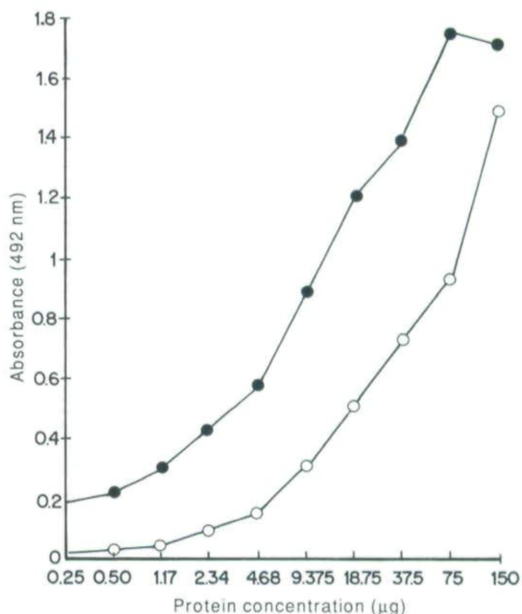

Fig. 5. Antibody content of IgG1 (○) and IgG2 (●) determined by ELISA.

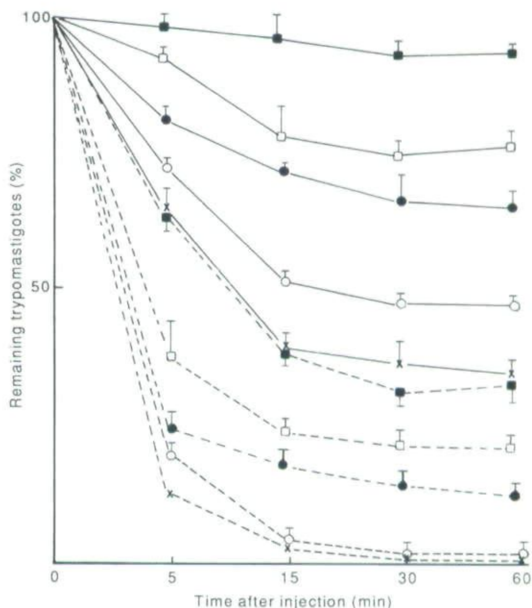

Fig. 6. Clearance of Btrys induced by increasing amounts of IgG1 and IgG2. 0·5 mg (■) 0·75 mg (□), 1 mg (●), 2 mg (○), 3 mg (x). Solid lines, IgG1 fraction; dashed lines, IgG2 fraction. $\bar{x} \pm$ s.d.; $n = 4$ mice per group.

each subclass. As can be seen in Fig. 4, the IMS revealed a series of bands with a variable intensity of staining, with molecular weight ranging between 150 and 25 kDa. The major bands were located in the region of 72 and 76 kDa. The IgG1 and IgG2 fractions revealed almost the same antigens, differing only in relation to the intensity of staining. Thus, IgG1 and IgG2 did not differ in relation to antigen specificity.

Quantitative parameters related to IgG1 and IgG2 anti T. cruzi *antibodies*

Since the specificity of IgG1 and IgG2 anti-*T. cruzi* antibodies seemed to be the same, the specific antibodies present in both fractions were titrated to find out whether a quantitative difference could explain the higher efficiency of the IgG2 to induce clearance. Figure 5 shows that specific anti-*T. cruzi* antibodies were approxi-

mately five-fold higher in the IgG2 fraction than in the IgG1 on a weight basis.

To examine more precisely the clearance ability of these subclasses, the dose–response curve of each subclass was determined, taking into consideration their antibody content. To this end, groups of mice containing known numbers of Btrys in circulation were injected with different amounts of either IgG1 or IgG2, and the clearance determined. As can be seen in Fig. 6, the IgG2 fraction was very efficient in inducing clearance of the parasites, even when small amounts were used. However, when higher amounts of IgG1 were used, it was possible to induce a significant decrease in the parasite number. In fact, it was necessary to inject six times more IgG1 than IgG2 in order to induce a similar rate of clearance. This quantitative difference corresponded closely to the difference in their specific antibody content, suggesting that antibodies of both subclasses have a similar ability to induce clearance.

DISCUSSION

Our results showing that IMS free of IgG antibodies is unable to induce immune clearance of *T. cruzi* suggest that neither IgM nor any other of the immunoglobulins present in IgG-free serum is responsible for this phenomenon, which is due mostly to IgG antibodies. Takehara *et al.* (4) showed that protective antibodies against infection by *T. cruzi* are located mostly in IgG2 isotype and our present findings which show that the antibodies responsible for the clearance are also located mostly in the same subclass, suggest a correlation between these two phenomena. However, as shown here, there were not many qualitative differences between the antibody specificity of IgG1 and IgG2 fractions of IMS, that is, antibodies present in IgG1 fraction recognized the same proteins of Btrys as detected by IgG2. These findings agree with previous results of Araujo *et al.* who, working with epimastigotes and amastigotes, detected a similar antigenic pattern with different isotypes of immunoglobulins and suggested that both subclasses have the same ability to bind to the parasite (15). Another aspect shown here was the remarkable difference between IgG1 and IgG2 fractions related to the amount of specific antibodies present in each subclass. In this sense, we showed that the clearance ability of IgG1 and IgG2 is very similar provided the amount of specific antibodies of each subclass are equalized. In this regard, it is pertinent to quote the report of Takehara *et al.* who have observed that sera collected from the acute phase of the infection are also able to protect the animals against a fatal challenge provided that antibody content is equalized to that of serum collected in the chronic phase of the infection (16). Thus, the disagreement between the protective ability of anti-*T. cruzi* sera collected in the acute or in the chronic phase of infection (5) seems to be due to a quantitative rather than a qualitative difference in their content. The lack of protective ability of IgG1 observed by Takehara *et al.* (4) may be due similarly to the low content of specific antibody in this subclass.

It is interesting to observe that infection by *T. cruzi* results in a remarkable increase of IgG2 as already reported by Scott and Goss-Sampson (17) who showed that IgG2 was the predominant antibody isotype in mice chronically infected with *T. cruzi*, whereas IgG1 and IgG3 were absent or present at very low levels. This isotype restriction is also detected at cellular level as D'Império Lima *et al.* (18) reported a predominance of IgG2 B cells in mice infected with *T. cruzi*. There are other examples of similar isotype restriction, mainly in responses elicited by parasites. For instance, infection of rats with *Nippostrongylus brasiliensis* is associated with increased levels of serum IgE (19) and infection of mice with *Mesocestoides corti* results in an increase in serum IgG1 levels (20). This kind of response may be associated with activation of specific subpopulations of T helper (Th) cells. These subpopulations are distinguished by a different pattern of lymphokine expression when activated (21). Thus, the response of mice to many helminths probably reflects the activation on Th2, with the predominance of IgE and IgG1 responses, due to preferential IL-4 production. Recently, Locksley (22) and Scott *et al.* (23) showed that the activation of Th1 or Th2 in mice infected with *Leishmania major* could be related to the resistance or susceptibility of the strain of mice, suggesting that parasite antigens can stimulate either Th1 or Th2 depending on the host/parasite interrelationship. In this way, the predominance of IgG2 isotype in chronic infection by *T. cruzi* may be due to preferential activation of Th1 subpopulation.

REFERENCES

1. Krettli, A. & Brener, Z. (1976). Protective effects of specific antibodies in *Trypanosoma cruzi* infection. *J. Immunol.* **166**: 755–760.
2. Kierszenbaum, F. & Howard, J. G. (1976) Mechanisms of resistance against experimental *Trypanosoma cruzi* infection: The importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. *J. Immunol.* **116**: 1208–1211.
3. Rodriguez, A. M., Santoro, F., Afchain, O., Bazin, H. & Capron, A. (1981) *Trypanosoma cruzi* infection in B cell deficient rats. *Infect. Immunol.* **31**: 524–528.
4. Takehara, H. A., Perini, A., Silva, M. H. & Mota, I. (1981) Role of different antibody classes in protection against infection in the mouse. *Exp. Parasitol.* **52**: 137–146.
5. Krettli A. U. (1984) Protective antibodies in *Trypanosoma cruzi* infections: Detection, functional activity and possible mechanisms of trypomastigotes killing *in vivo* and *in vitro*. *Mem. Inst. Oswaldo Cruz.* **79**: 59–66.
6. Umekita, L. F., Takehara, H. A. & Mota, I. (1988) Role of the antibody Fc in the immune clearance of *Trypanosoma cruzi*. *Immunol. Lett.* **17**: 83–89.
7. Brodskyn, C. I., Silva, A. M. M., Takehara, H. A. & Mota, I. (1988) Characterization of antibody isotype responsible for immune clearance in mice infected *Trypanosoma cruzi*. *Immunol. Lett.* **18**: 255–258.
8. Silva, L. H. P. & Nussenzweig, V. (1953) Sobre una cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia Clin. Biol.* **20**: 191–207.
9. Ey, P. L. S., Prowse, S. J. & Jenkin, C. R. (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Immunochem.* **15**: 429–436.
10. Little, J. R. & Donahue, H. S. (1968) Spectral properties of proteins and small molecules of immunological interest. In *Methods in Immunology and Immunochemistry*. G. A. Williams and M. W. Chase, (eds), Vol. 2, pp. 343–364. Academic Press, New York.
11. Hoshino-Shimizu, S., Camargo, M. E., Shimizu, T. & Nagase, T. K. (1982) A study on the reproducibility of a stable, lyophilized reagent for the Chagas' disease haemagglutination test: Proposals for quality control analysis. *Rev. Inst. Med. Trop.* **24**: 63–68.
12. Silva, A. M. M., Costa, H. H., Takehara, H. A. & Mota, I. (1988) *Trypanosoma cruzi*: Advantages of isolating bloodstream trypomastigotes by the carboxyl-methyl-cellulose method. *Trans. Roy. Soc. Trop. Med. Hyg.* **82**: 715–718.
13. Laemmli, U. K. (1970) Clearance of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
14. Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**: 4350–4354.
15. Araujo, F. G., Heilman, B. & Tighe, L. (1984) Antigens of *Trypanosoma cruzi* detected by different classes and subclasses of antibodies. *Trans. Roy. Soc. Trop. Med. Hyg.* **78**: 672–677.
16. Takehara, H. A., Silva, A. M. M., Brodskyn, C. I. & Mota, I. (1988) Properties of mouse anti-*Trypanosoma cruzi* antibodies obtained in the course of infection. *Mem. Inst. Oswaldo Cruz* **83**: 132.
17. Scott, M. T. & Goss-Sampson, M. (1984) Restricted IgG isotype profiles in *Trypanosoma cruzi* infected mice and Chagas' disease patients. *Clin. Exp. Immunol.* **58**: 372–379.
18. D'Império Lima, M. R., Joskowicz, M., Coutinho, A., Kipnis, T. & Eisen, H. (1985) Very large and isotopically atypical polyclonal plaque-forming cell responses in mice with *Trypanosoma cruzi*. *Eur. J. Immunol.* **15**: 201–203.
19. Olgivie, B. M. (1967) Reagin-like antibodies in rats infected with the nematode parasite *Nippostrongylus brasiliensis*. *Immunology* **12**: 113–131.
20. Mitchell, G., Marchalonis J., Smith, P., Nicholas, W. L. & Warner, N. L. (1977) Studies on immune response to larval cestodes in mice. Immunoglobulins associated with larvae of *Mesocestoides corti*. *Aust. J. Exp. Biol. Med.* **55**: 187–211.
21. Mosmann, T. R. & Coffman, R. L. (1987) Two types of mouse helper T-cell clones. *Immunol. Today* **8**: 223–227.
22. Locksley, R. M., Heinzel, F. P., Sadick, M. D., Holaday, B. J. & Gardner, K. D. (1987) Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets. *Ann. Inst. Pasteur* **138**: 744–749.
23. Scott, P., Natovitz, P., Coffman, R. L., Pearce, E. & Sher, A. (1988) $CD4^+$ T cell subsets in experimental cutaneous leishmaniasis. *Mem. Inst. Oswaldo Cruz* **83**: 256–259.

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