INFECTION AND IMMUNITY, July 1996, p. 2861–2866 Vol. 64, No. 7
0019-9567/96/$04.00+0

Chronic Experimental Chagas' Disease: Functional Syngeneic T-B-Cell Cooperation In Vitro in the Absence of an Exogenous Stimulus

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Received 18 January 1996/Returned for modification 20 February 1996/Accepted 24 April 1996

We have investigated $CD4^+$ T-cell autoreactivity to normal syngeneic B cells in vitro in chronic experimental Chagas' disease. Resting B cells induced an intense proliferative response and lymphokine secretion by splenic $CD4^+$ T cells from *Trypanosoma cruzi*-infected (8 months or more of infection) donors, compared to much lower responses by uninfected controls. On the other hand, lipopolysaccharide-activated B cells induced syngeneic $CD4^+$ T-cell activation in both control and infected groups. The observed syngeneic T-B-cell cooperation was bidirectional. In the absence of any exogenous stimulus, $CD4^+$ T cells from *T. cruzi*-infected animals induced much higher production of all tested immunoglobulin (Ig) isotypes (IgM, IgG1, IgG2a, IgG2b, IgG3) by syngeneic B cells, compared to T cells from uninfected donors. When lipopolysaccharide-treated B cells were used, $CD4^+$ T cells from either control or infected donors enhanced IgG1 and IgG3 production, but only $CD4^+$ T cells of infected origin induced IgG2a production in this system without addition of exogenous gamma interferon. Enhanced T-cell proliferation and Ig production were also observed with highly purified $CD4^+$ T cells and in serum-free medium. Both proliferation and Ig production could be blocked with anti-major histocompatibility complex class II monoclonal antibodies. Enhanced reactivity and help for Ig production were seen only in response to syngeneic BALB B cells and not in response to allogeneic B10 B cells. These results indicate that chronic infection with *T. cruzi* results in increased $CD4^+$ T-cell reactivity towards syngeneic B cells, which leads to spontaneous Ig production. These autoreactive T cells might play a role in polyclonal autoantibody production in chronic Chagas' disease.

Autoimmune phenomena have been described in the chronic stage of both human (2, 9, 15) and experimental (14, 29) Chagas' disease (American trypanosomiasis) and are considered a potential cause for the progressive cardiac tissue damage seen in chronically infected hosts (24). Both molecular mimicry (24, 31) and repertoire change (20) models have been proposed to explain the link between *Trypanosoma cruzi* infection and autoimmune attack, although responses to parasites persisting in the heart at the chronic stage (11) cannot be ruled out. In the murine model of *T. cruzi* infection, enhanced and persistent B-cell activation and immunoglobulin (Ig) production have been described, even after parasitemia resolves (3). On the other hand, autoreactive $CD4^+$ T-cell clones have been previously described as capable of polyclonally activating Ig production by B cells in both major histocompatibility complex (MHC)-restricted and noncognate fashions (7).

In the present study, we investigated whether enhanced B-cell Ig production in chronic experimental Chagas' disease could be caused by enhanced autoreactive $CD4^+$ T-cell activity. We present evidence that splenic $CD4^+$ T cells from chronically infected mice have markedly enhanced autoreactivity towards normal syngeneic, but not allogeneic, resting B cells. As a consequence, high levels of Ig production can be detected in B-cell cultures containing $CD4^+$ T cells from infected donors in the absence of any exogenous stimulus. Thus, enhanced $CD4^+$ T-cell autoreactivity can be a potential cause of persistent B-cell activation and autoantibody production in chronic Chagas' disease.

Male BALB/c or C57BL10 (B10) mice (6 to 8 weeks old) were obtained from the Institute of Microbiology, Federal University of Rio de Janeiro. Mice were infected subcutaneously (10^5 parasites per 0.1 ml per mouse) with chemically induced metacyclic trypomastigotes from the *T. cruzi* clone Dm28c (1), as described previously (17). Uninfected littermates were used as controls. Mice were left until 8 months or more of infection before being used in experiments. No parasitemia could be detected in these animals by microscopic examination of blood smears or in splenocyte suspensions. Primary T-cell-enriched populations were obtained from control and infected donors by nylon wool filtration of splenic cell suspensions after erythrocyte depletion by treatment with Tris-buffered ammonium chloride. In order to obtain $CD4^+$ T cells, nonadherent cells (20×10^6 to 50×10^6/ml) were treated with a saturating dose (10 μg/ml) of anti-CD8 monoclonal antibody (MAb) 53.6.7 for 30 min at 4°C, washed, and treated with anti-rat Ig MAb MAR 18.5 (1% ascites) plus 10% low-toxicity rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) for 45 min at 37°C. Alternatively, in order to obtain highly purified $CD4^+$ T cells, nylon-passed splenic T cells were subjected to magnetic cell sorting as described previously (18), using a mixture of saturating doses of anti-CD8, anti-B220, anti-MHC class II, and anti-MAC-1 (CD11b/CD18) MAbs. Cells were washed and treated with anti-rat-Ig-coated magnetic beads (Biomag, Advanced Magnetics, Cambridge, Mass.). Negatively selected cells were obtained after three cycles of repeated magnetic separation. Flow cytometry analysis indicated that T cells were >99% $CD4^+$. In order to obtain normal purified B cells, whole splenocyte suspensions were depleted of T cells by treatment with a mixture of anti-Thy1.2 MAb 30.H12 (1 μg/ml), anti-CD4 MAb GK 1.5 (2.5 μg/ml), and anti-CD8 MAb 53.6.7 (2.5 μg/ml) for 30 min at 4°C,

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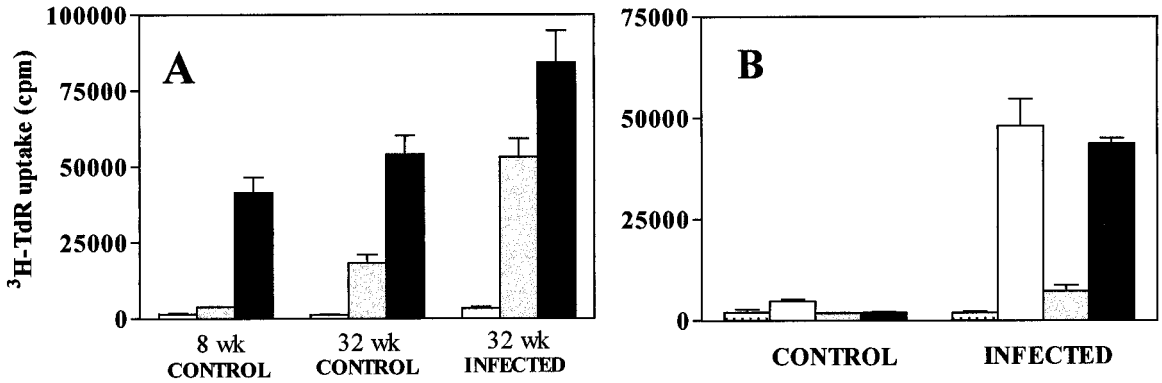

FIG. 1. Enhanced CD4$^+$ T-cell autoreactivity to syngeneic B cells in mice infected with *T. cruzi* (A) CD4$^+$ T cells (from either control or infected donors, as indicated) were cultured in medium only (□), with irradiated syngeneic resting B cells (▨), or with LPS-treated B cells (■). (B) Highly purified CD4$^+$ T cells from chronically infected or control littermates, were cultured, either alone (▨) or with irradiated syngeneic resting B cells from normal donors, in fetal calf serum-free medium containing 1% Nutridoma, in the absence (□) or in the presence (▨) of anti-MHC class II MAb (Anti-I-A+ I-E, IgG2a, 5 μg/ml) or isotype-matched anti-B220 MAb (5 μg/ml) (■). Proliferation was assessed by tritiated thymidine (^{3}H-TdR) uptake after 3 days in culture. Vertical bars indicate the standard errors of the means of triplicate cultures.

followed by an incubation with MAR 18.5 and low-toxicity complement for 45 min at 37°C. Cells were centrifuged over a Percoll (Pharmacia, Uppsala, Sweden) gradient, and resting (dense) B cells were collected at the 70% Percoll interface. Resting B cells were left overnight in culture medium alone or with lipopolysaccharide (LPS) (*Escherichia coli* O111:B4; DIFCO Inc., Detroit, Mich.) (20 μg/ml) at 37°C. Cells were washed, irradiated (1,200 rad) and added to 96-well, round-bottom microtiter plates (Corning Glass Works, Corning, N.Y.), at 5×10^4 per well. CD4$^+$ T cells at 10^5 per well were added in triplicate in a 0.2-ml final volume, and cultures were incubated at 37°C with 5% CO_2 for 3 days. Cultures were pulsed with 1.0 μCi of tritiated thymidine (20 Ci/mmol; Sigma) after 48 h, and the amount of radioactivity incorporated into DNA was assessed 18 h later, after harvesting (PHD cell harvester; Cambridge Technology, Watertown, Mass.), by liquid scintillation spectroscopy. Culture medium consisted of Dulbecco modified Eagle medium (Sigma Chemical Co., St. Louis, Mo.), supplemented with 10% fetal calf serum–2 mM L-glutamine–5×10^{-5} M 2-mercaptoethanol–10 μg of gentamicin per ml–sodium pyruvate–minimum essential medium (MEM) nonessential amino acids–10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine–*N*′-2-ethanesulfonic acid). In some experiments, 1% Nutridoma-SR (Boehringer Mannheim GmbH, Germany) was used instead of fetal calf serum. In order to measure Ig production in vitro, unirradiated normal or LPS-pulsed B cells (5×10^4) were cultured with CD4$^+$ T cells (1×10^5) in round-bottom microtiter plates for 3 to 7 days, in the presence or absence of additional lymphokines or MAbs. Supernatants were recovered, pooled from several wells, and stored frozen until use. The means and dispersions of two independent experiments are shown in the figures. The amount of Ig isotype produced was measured by enzyme-linked immunosorbent assay (ELISA) in Corning ELISA plates. Briefly, a color product was generated from cleavage of PNPP (*para*-nitrophenol phosphate; Sigma) by alkaline phosphatase-conjugated antibodies (from Southern Biotechnology Associates Inc., Birmingham, Ala.) against different murine Ig isotypes (IgM, IgG1, IgG2a, IgG2b, IgG3), or IgM a and b allotypes (Pharmingen, San Diego, Calif.). The developed indicator was measured on an ELISA microplate reader (Model 3550; BioRad, Melville, N.Y.), and optical density units were converted to Ig concentrations by means of standard curves determined in each assay by using purified myeloma proteins (ICN Biomedicals Inc., Costa Nera, Calif.) of known concentrations. Each assay system showed no significant cross-reactivity to the additional isotypes or allotypes present in the supernatants. Recombinant murine interleukin 4 (IL-4) (150 U/ml), IL-5 (400 U/ml) and gamma interferon (IFN-γ) (10 U/ml) were from Sigma. MAbs AMS-32.1 (anti-I-A^d), 2G9 (anti-I-A^d + I-E^d) and M1/70 (anti-MAC-1) were purchased from Pharmingen. Antibodies to mouse CD4 (GK 1.5), mouse CD8 (53.6.7), and rat Ig κ chain (MAR 18.5) were a gift from Ethan Shevach, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Anti-Thy1.2 MAb 30.H12 and anti-B220 MAb 6B2 were obtained from American Type Culture Collection, Rockville, Md.

Normal irradiated, resting or LPS-treated splenic B cells were used to stimulate syngeneic CD4$^+$ T cells from chronically infected or uninfected donors, in the absence of any additional stimulus. Round-bottom microtiter wells were used in order to increase cell interactions in vitro. Resting B cells failed to stimulate CD4$^+$ T cells from 8-week-old controls, stimulated to some extent CD4$^+$ T cells from 32-week-old controls, and induced a marked proliferative response in CD4$^+$ T cells obtained from 32-week-old, *T. cruzi*-infected donors (Fig. 1A). LPS-pulsed B cells, on the other hand, induced strong proliferative responses in CD4$^+$ T cells from all three groups (Fig. 1A). The assay was also performed with CD4$^+$ T cells extensively depleted of accessory cells and in the presence of 1% Nutridoma-SR instead of fetal calf serum. Again, a strong proliferative response was induced by syngeneic B cells in purified CD4$^+$ T cells from the infected donor (Fig. 1B). Control CD4$^+$ T cells gave much lower proliferative responses to syngeneic B cells. This proliferative response was blocked by the addition of anti-MHC class II I-A MAb (not shown) and more intensely by an anti-MHC class II MAb specific for I-A and I-E (Fig. 1B). An isotype-matched control MAb also reactive with B cells (anti-B220) failed to block CD4$^+$ T-cell proliferation (Fig. 1B). Anti-CD4 MAb also blocked T-cell proliferation by more than 90% (not shown). Lymphokine production was also measured on indicator cell lines, and both IL-2 and IL-4 (to a lesser extent) activities were increased in supernatants from infected groups compared with controls (data not shown). To investigate whether enhanced T-cell autoreactivity in *T. cruzi* infection also resulted in B-cell activation, nonirradiated B cells were cultured for 7 days with syngeneic CD4$^+$ T cells from different origins and the super-

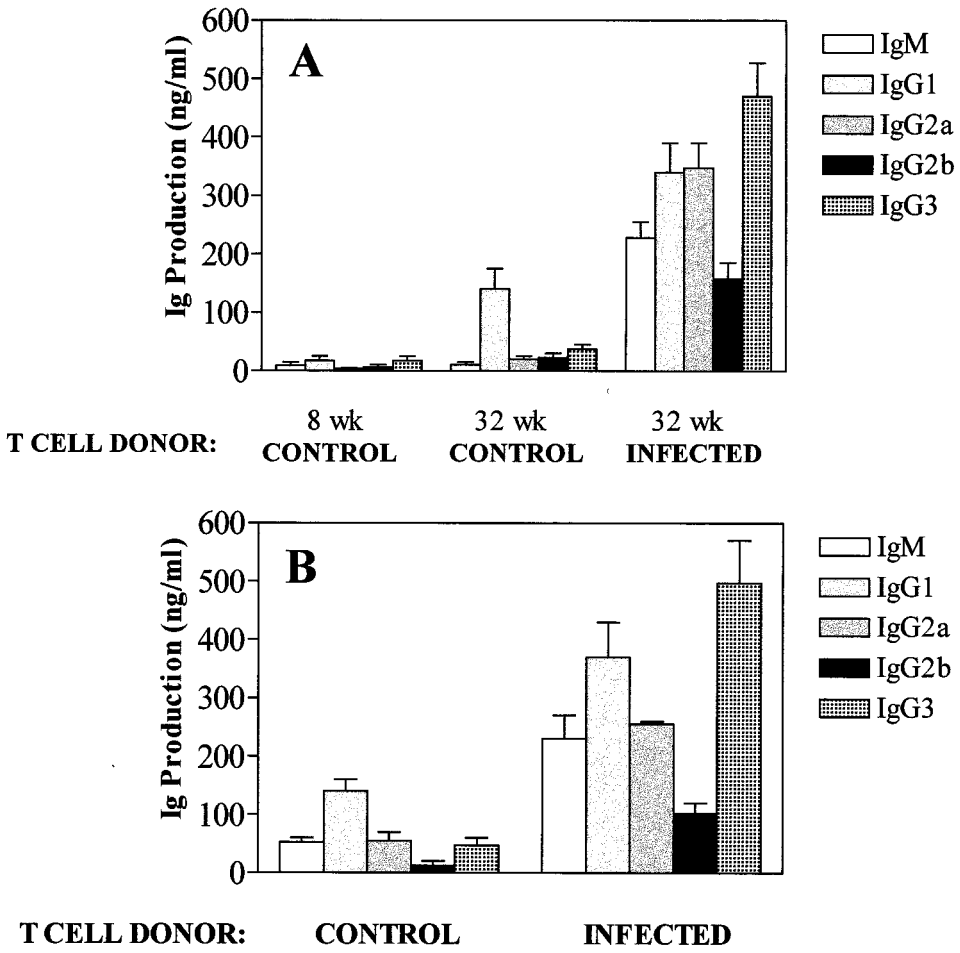

FIG. 2. Enhanced autoreactive $CD4^+$ T-cell help for immunoglobulin production in mice infected with *T. cruzi*. (A) $CD4^+$ T cells from the indicated control and infected donors were cultured with syngeneic resting B cells for 7 days. Supernatants were collected and assayed for the production of the indicated Ig isotypes. (B) A similar experiment, except that highly purified $CD4^+$ T cells from the indicated donors were used instead, and cultures were done in the absence of fetal calf serum but in the presence of 1% Nutridoma. Vertical bars indicate the standard errors of the means of two experiments.

natants were assayed for Ig content by using Ig isotype-specific ELISAs. Normal B cells produced significant amounts of IgM, IgG2a, IgG2b, and IgG3 upon coculture with $CD4^+$ T cells from infected donors, while the production of these same isotypes was near background levels with $CD4^+$ T cells from uninfected controls (Fig. 2A). One exception was IgG1, which was higher in 32-week-old donors than in 8-week-old controls. However, even in this case, IgG1 production by syngeneic B cells was threefold higher with helper $CD4^+$ T cells derived from chronically infected donors. To avoid potential carryover of parasite-derived antigens and effects of heterologous serum proteins, we tested Ig production induced by highly purified $CD4^+$ T cells and in the absence of serum (1% Nutridoma-SR was used as supplement). As can be seen (Fig. 2B), Ig production was three- to sevenfold higher, depending on the isotype tested, with $CD4^+$ T cells from chronic *T. cruzi* infection than with controls. Addition of either anti-MHC class II or anti-CD4 MAbs also blocked Ig production (data not shown). We also investigated whether $CD4^+$ T cells could enhance Ig production by LPS-treated B cells (Fig. 3). In this case, IgM production did not appreciably increase in the presence of $CD4^+$ T cells. Both IgG1 and IgG3 production, on the other hand, were potentiated in the presence of $CD4^+$ T cells from either uninfected or infected mice. Interestingly, IgG2a production could be induced at appreciable levels only with $CD4^+$ T cells from infected donors (Fig. 3). Addition of IFN-γ, however, resulted in IgG2a production in cultures containing either type of CD4 T cells (not shown). Overall, these results indicate that CD4 T cells from chronically infected mice have enhanced capacity to induce Ig synthesis of several isotypes by normal syngeneic B cells.

To investigate whether enhanced $CD4^+$ T-cell autoreactivity was genetically restricted or had resulted from enhanced noncognate costimulatory activity, normal resting B cells from syngeneic and allogeneic donors were used as stimulators (Fig. 4). Again, $CD4^+$ T cells from infected donors showed a marked response to syngeneic BALB/c B cells, compared to control $CD4^+$ T cells (Fig. 4). On the other hand, $CD4^+$ T-cell alloreactivity to B10 B cells was of much smaller amplitude and was comparable in control and infected T-cell donors (Fig. 4, inset). Ig production by syngeneic or allogeneic B cells was also compared, using allotype-specific ELISAs for IgM (Fig. 5). Day 3 in culture was chosen, both to avoid primary allogeneic B cell activation at day 6 and to test for a possible default and nonspecific helper activity by T cells from infected donors. IgM production in the presence of $CD4^+$ T cells from *T. cruzi* infection was detected in syngeneic (IgMa allotype) but not in allogeneic (IgMb allotype) B cells (Fig. 5). We also added a

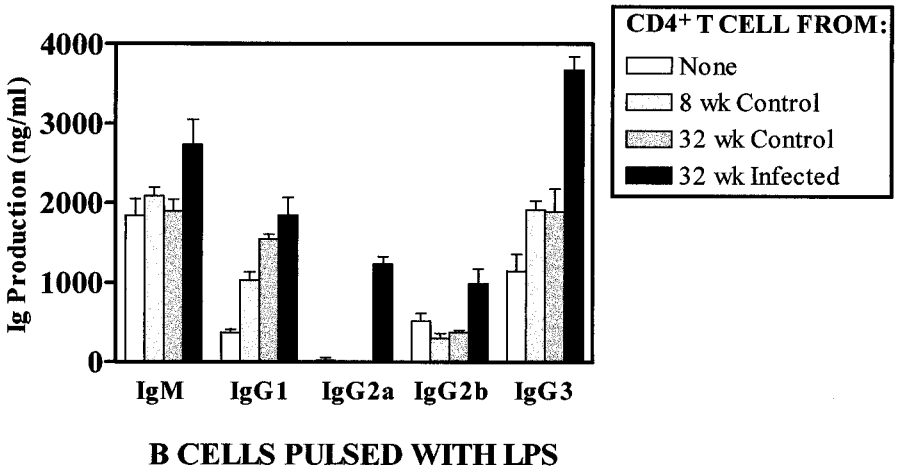

FIG. 3. Effect of coculture with $CD4^+$ T cells on LPS-induced B-cell Ig production. Normal B cells were pulsed overnight with LPS and cultured for 7 days, either alone or with $CD4^+$ T cells from the indicated donors. Supernatants were collected and assayed for the indicated Ig isotypes. Vertical bars indicate the standard errors of the means of two experiments.

mixture of syngeneic and allogeneic B cells to $CD4^+$ T cells. Interestingly, only in this case was enhancement of IgMb production by allogeneic B10 B cells observed, suggesting noncognate activation of allogeneic B cells initiated by syngeneic B-T-cell interactions (Fig. 5). A similar syngeneic preference in Ig production helped by $CD4^+$ T cells from infected donors was also seen after 6 days in culture, although T cells from control donors had already initiated allogeneic IgMb production by day 6 (not shown). Therefore, lack of allogeneic IgM production by day 3 in culture indicates a genetic restriction to syngeneic B-cell preference also operating at the Ig production level.

Autoantibody production directed to several self molecular constituents is a major characteristic of human chronic infection with *T. cruzi* (2, 15). Although T-cell-mediated immune responses are thought to be the primary factor involved in host heart tissue damage (24), humoral mechanisms of autoantibody reaction with target tissues have also been implicated, either as an amplifying factor of inflammatory damage (13, 21) or as protective mechanisms regulating parasite entry into host cells (8). In the murine model of experimental Chagas' disease, a marked polyclonal B-cell activation during the acute phase of infection has been described (4, 23), resulting in both parasite-specific and nonspecific Ig production. In vivo depletion of the $CD4^+$ T-cell subset suppresses polyclonal antibody production (19), increases parasite load in tissues, and reduces inflammatory lesions in the heart of infected mice (25), highlighting the importance of $CD4^+$ T-cell-mediated polyclonal B-cell activation in regulating the outcome of disease. The exact mechanisms by which $CD4^+$ T cells induce and/or assist polyclonal B-cell activation during infection with *T. cruzi* are unknown. In this report, we presented evidence that $CD4^+$ T cells from chronically infected mice contain an increased amount of autoreactive T-cell precursors, capable of proliferating in response to normal syngeneic B-cell stimulators and of inducing Ig production of several isotypes in vitro. There was a possibility that this autoreactivity resulted from enhanced nonspecific costimulatory activity for B cells. However, T cells from *T. cruzi* infection showed similar reactivity to allogeneic resting B cells, compared to controls. In contrast to syngeneic T-B collaboration, these T cells did not help Ig production by allogeneic B cells after 3 days in culture. Therefore, B-cell reactivity was genetically restricted to syngeneic stimulators, although definitive mapping to MHC was not done in the present work.

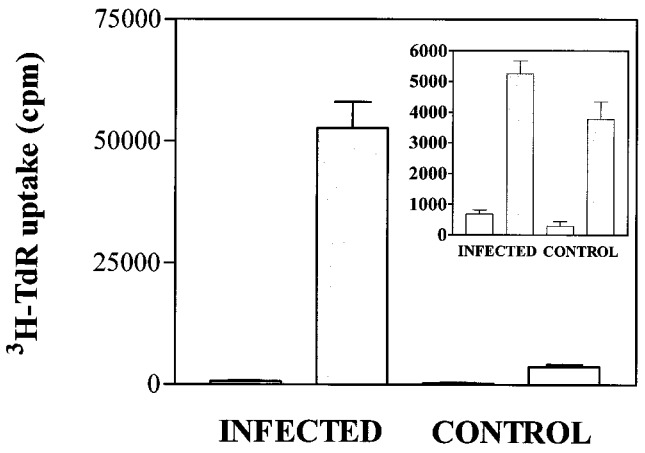

FIG. 4. $CD4^+$ T-cell reactivity is restricted to syngeneic B cells. Highly purified $CD4^+$ T cells from chronically infected or control littermates were cultured in the absence (open bars) or in the presence (gray bars) of resting B cells. Main graph: syngeneic BALB B cells as stimulators. Inset: allogeneic B10 B cells as stimulators. Proliferation assessed by thymidine uptake after 3 days in culture. Vertical bars indicate the standard errors of the means of triplicate cultures. ^{3}H-TdR

Autoreactive proliferating T cells have been described in several experimental conditions (7), including those following adjuvant-induced T-cell responses to conventional nonself antigens (5, 16), and responses to recall antigens in humans (30). Different cell types were identified as stimulators for T cells, including B lymphocytes in mice (7). In this case, autoreactive $CD4^+$ T-cell clones are able to proliferate to, and to mediate both MHC-restricted and noncognate B cell help, resulting in polyclonal Ig production in vitro (7). In our system with $CD4^+$ T cells from infection, the autoreactive response is blocked by either anti-CD4 or anti-MHC class II MAbs. We speculate that the stimulus for these autoreactive T cells are self peptides associated with MHC class II molecules on the B-cell surface. Induction of $CD4^+$ T-cell activation with resting B cells was not surprising, even though resting B cells are poor stimulators of primary T-cell proliferative responses (12). It is likely that autoreactive $CD4^+$ T cells from *T. cruzi*-infected mice are not

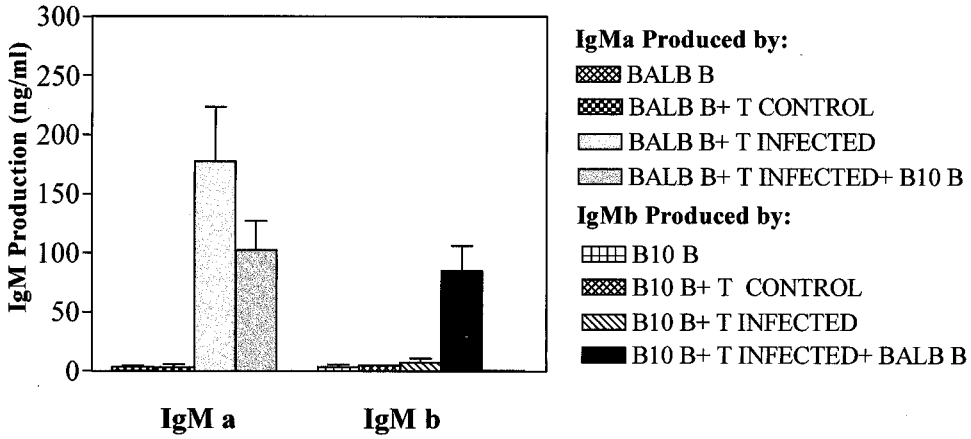

FIG. 5. $CD4^+$ T-cell help for Ig production is restricted to syngeneic B cells. Normal resting BALB B cells (IgM allotype a producers) or B10 B cells (IgM allotype b producers) were cultured either alone or in the presence of highly purified $CD4^+$ T cells from chronically infected or control BALB mice. In addition, some cultures received a mixture of both BALB and B10 B cells, as indicated. IgM production of the a and b allotypes was determined by ELISA after 3 days in culture. Vertical bars indicate the standard deviations of the means of two experiments.

naive and therefore obviate the need for a potent costimulatory function on B cells. The finding that human Chagas' disease patients have increased levels of circulating T cells with memory/activated phenotype (6) agrees with this possibility. On the other hand, it has been shown that resting B cells are competent to restimulate preactivated T cells (10).

As a consequence of syngeneic activation, autoreactive $CD4^+$ T cells from infected donors induced Ig production in B cells without exogenous stimulation. The isotype profile of secreted Ig, with production of both IgG1 and IgG2a, suggests no Th1 or Th2 bias. This finding agrees with the reported production of all Ig isotypes by *T. cruzi*-infected mouse spleen cells (4, 27). On the other hand, when we used LPS-pulsed normal B cells, only IgG2a production was selectively induced by CD4 T cells from infected but not control donors. Since IgG2a secretion by LPS-treated B cells is induced by IFN-γ (26), it is possible that $CD4^+$ T cells from chronically infected donors release IFN-γ upon interaction with syngeneic B cells. This possibility should be investigated in our system. It is noteworthy that there is an unusual in vivo production of high levels of IgG2a during *T. cruzi* infection (4, 27, 28). On the other hand, it is likely that our model will not reproduce some features of the disease, since we did not use B cells from infected donors. We wished to compare only $CD4^+$ T-cell activity and to avoid the possibility of residual parasite antigen carryover by B cells from infected mice.

In summary, we have developed an in vitro system to characterize autoreactive $CD4^+$ T cells circulating during chronic *T. cruzi* infection. These cells recognize syngeneic B cells in an exaggerated form but distinct from noncognate B-cell help, as shown by lack of enhanced responsivity to allogeneic B cells. As a consequence, these $CD4^+$ T cells proliferate and mediate help for Ig production in the absence of exogenous stimulation. The nature of the antigens recognized by the produced Igs was not addressed in the present study. Since this type of T-B collaboration may be of relevance in autoantibody production, future studies will be directed towards a comparative analysis of the autoantibody repertoire induced in vitro by autoreactive $CD4^+$ T cells from normal and infected donors, using a recently developed Western blot technique to quantitate and characterize global autoantibody repertoires (22).

This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR grants 930330 and 930125), Financing Agency for Studies and Projects (FINEP), and PADCT/CNPq. C. Freire-de-Lima is a fellow of FIOCRUZ-RJ.

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Editor: J. M. Mansfield