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A 24 000 MW *Trypanosoma cruzi* antigen is a B-cell activator

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SUMMARY

Trypanosoma cruzi, the causative agent of Chagas' disease, is a protozoan parasite that infects humans and other mammals in Central and Latin America. Several alterations of the immune response after infection have been described, such as severe immunosuppression of both cellular and humoral responses and massive polyclonal B- and T-cell activation, including the expansion of self-reactive clones. We have investigated the effects of the intraperitoneal injection of a recombinant 24 000 MW *T. cruzi*-specific antigen (rTc24) on the immune response of normal and deficient strains of mice. We analysed the *in vivo* and *ex vivo* levels of lymphocyte activation and the proliferative responses to rTc24 by determining the expression of CD69 activation marker and the levels of thymidine incorporation by spleen cells. The numbers of antibody-producing cells were determined by ELISPOT and the levels of immunoglobulin in the sera by isotype-specific enzyme-linked immunosorbent assay. We observed an increased [^{3}H]thymidine ([^{3}H]TdR) incorporation by spleen cells after rTc24 stimulation *in vivo* and *in vitro*. This proliferative activity induced by rTc24 was independent of the mouse strain used in the experiments (including C3H/HeJ mice) and ruled out the possibility that rTc24 preparations were contaminated by lipopolysaccharide. The injection of rTc24 protein induced preferentially the activation of B cells, as determined by the increased expression of CD69 molecules on IgM$^+$ spleen cells. Considerable increases of IgM-secreting B cells were determined in both athymic and euthymic BALB/c mice. Mice that are deficient in B cells (BALB.Xid) responded to rTc24 but to a lesser extent. These increases in IgM B-cell numbers were accompanied by elevated levels of IgM immunoglobulins in the sera of injected animals. Our results suggest a role for rTc24 in B-cell activation.

INTRODUCTION

The interaction between *Trypanosoma cruzi*, the causative agent of Chagas' disease, and its host is characterized by multiple disturbances of the immune system. In the early stages of infection the number of immunoglobulin-secreting cells in the spleen and peripheral lymph nodes is very high and the majority of activated B cells secrete antibodies that are non-specific for parasite antigens.[1] The immunosuppression and the autoimmunity associated with *T. cruzi* chronic infection[2–4] could be consequences of the intense B- and T-cell polyclonal activations observed during the early stages of infection.[5] These responses lead to the expansion of anti-self clones that may be responsible for the destruction of parasitized and non-parasitized cells.[5,6]

In the same way as other micro-organisms, *T. cruzi* displays mitogenic-like activities that activate a large percentage of lymphocytes in a non-specific manner. The isolation of lipopolysaccharide (LPS)-like substances from *T. cruzi* has been reported.[7] The products released *in vivo* and *in vitro* by *T. cruzi* have not been well studied. We and others have described that affinity-purified Trypomastigote Excretory/Secretory Antigen (ESA)[8,9] induces a significant decrease in parasitaemia levels during the acute phase of experimental Chagas' disease, if injected in the presence of BpAl (*Bordetella pertussis* and Alum) 8 days before infection, with a regular boost every 7 days.[10] The 24 000 MW protein (Tc24), is one of the ESA products released by *T. cruzi*. This protein is mainly localized in the membrane of all evolutive stages of the parasite, particularly in the flagella pocket of epimastigote and trypomastigote forms.[11] The gene sequence of Tc24 shows similarities with the sequences of several proteins that bind calcium.[12] The sequence of the recombinant form (rTc24) has been well studied and experiments with synthetic peptides derived from its sequence showed that the peptide 109–124 (containing a putative T-cell epitope) represents the most protective epitope, inducing 30–50% of protection against mortality during acute

¶In memoriam.

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infection.[10] Thus, a similar protocol of immunization of BALB/c mice as above, consisting of three injections at 2-week intervals of the 109–124 peptide coupled to the carrier ovalbumin, showed that this peptide derived from the primary sequence of rTc24 has the capacity to induce significant levels of protection in BALB/c mice against a lethal *T. cruzi* challenge.[10] Similarly, the injection of mice with Tc24 fusion protein showed that the protein has the capacity to induce a partial degree of protection, and this following protocols requiring hyperimmunization and adjuvants.[13]

In the present report, we demonstrate that although rTc24 possesses putative T-cell epitopes in its sequence, it is a potent B-cell activator in the presence or absence of T cells. Moreover, a single injection of rTc24 might result in polyclonal, non-specific B-cell responses.

MATERIALS AND METHODS

Mice

BALB.Xid mice were obtained from our laboratory animal facilities. BALB/c mice were obtained from Charles River (Saint-Aubin-les Elbeuf, France) and from the Gulbenkian Institute of Science (Oeiras, Portugal). BALB/c athymic (nude) mice were purchased from the Gulbenkian Institute of Science and from IFFACREDO (L'Arbresle, France).

Subcloning and purification of rTc24. A *T. cruzi* trypomastigote cDNA insert encoding a 24 000 MW protein (Tc24)[10,13] was purified and subcloned into the high-expression vector, pGEX-2 plasmid (Pharmacia P-L-Biochemicals Inc., Milwaukee, WI). This vector directs the synthesis of foreign polypeptides in *Escherichia coli* as a fusion peptide with the 26 000 MW *Schistosoma japonicum* gluthatione-S-transferase (Sj26). Tc24-positive clones expressed in *E. coli* were selected by expression conditions and the fusion (rTc24) protein was purified using gluthatione agarose beads as described.[14] The purity of the recombinant protein was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein content was determined using the Folin procedure.[15]

Mouse injections. Six- to seven-week-old mice per group were injected intraperitoneally (i.p.) with 50 μg of rTc24. Control mice were injected with 25 μg of Sj26 or 10 μg of LPS, 2, 4, 7, or 15 days before the assay, as described in individual experiments.

Immunofluorescence and flow cytometry

Spleen cells from the different groups of mice were obtained 7 days after rTc24 injection and stained for immunofluorescence with direct-labelled antibodies or with biotin-labelled antibodies followed by a further incubation with fluorescein isothiocyanate (FITC)–avidin. The following purified monoclonal antibodies (mAb) were used in immunofluorescence staining: fluorescein-conjugated anti-CD4, clone GK1.5[16] and anti-CD8, clone 53-6.17[17] were from Becton-Dickinson (Rungis, France) and Caltag (South San Francisco, CA), respectively; phycoerythrin-conjugated anti-CD69 from PharMingen (San Diego, CA) and biotinylated anti-μ, clone 53-6.72.[17] Flow cytofluorometric analysis was done in a fluorescence-activated cell sorter (FACS) Scan (Becton Dickinson). Dead cells were excluded from all samples by propidium iodide labelling, as a light scatter forward scatter/side scatter combined gate.

Cell culture and proliferation assays

Spleen cells from mice injected with rTc24 and respective controls were adjusted to 2×10^6/well in RPMI-1640 culture medium (Flow Laboratories, Asnières, France), supplemented with 2 mM glutamine (Flow Laboratories), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; Sigma Chemical CO, St. Louis, MO), 0·05 mM 2-mercaptoethanol (2-ME), 20 mM HEPES (Flow Laboratories) and 10% FCS (Flow Laboratories). Spleen cells ($2{\cdot}5 \times 10^5$) were added to each well of 96-well flat bottom plates in a final volume of 200 μl. Protein rTc24 (50 μg/ml), Sj26 (25 μg/ml) and LPS (10 μg/ml; Difco, Detroit, MI) were added to the medium. On days 1, 2 and 3 of culture the wells were pulsed with 1 μCi of [^{3}H]TdR (Amersham Corp, Arlington, IL) for 8 hr. Assays were done in triplicate and the stimulatory index was calculated by dividing the arithmetic mean of counts per minute (c.p.m.) obtained from stimulated cultures by the arithmethic mean of c.p.m. obtained from control cultures without stimulation.

Enzyme-linked immunosorbent assay (ELISA) for total immunoglobulins

Flat-bottomed plates (Nunc, Immune Plate Maxisorp, France) were coated with unlabelled goat anti-mouse immunoglobulin (5 μg/ml), or with rTc24 (5 μg/ml) or total *T. cruzi* (10 μg/ml) in carbonate buffer, pH 8·5, overnight at 4°. Coated plates were washed with phosphate-buffered saline (PBS) containing 0·1% Tween-20 (PBS-T; Merk, Darmstadt, Germany) and blocked with PBS 1% gelatin (200 μl/well) for 1 hr at room temperature. The plates were then incubated with serial dilution of each serum for 2 hr at room temperature. After washing with PBS-T, the plates were incubated for 1 hr at room temperature with peroxidase-labelled goat anti-mouse immunoglobulin isotypes (anti-IgM, anti-IgG3, anti-IgG1, anti-IG2a and anti-IG2b) and developed with adequate peroxidase substrate. Unlabelled purified isotypes were used in serial dilution as standards.

Complement-dependent killing with mAb

Spleen cells (10^7) from normal, non-injected mice were treated with appropriate dilution of monoclonal anti-CD4 (IgG, clone GK-1.5) plus anti-CD8 (IgM, clone 53-6.72) antibodies for 30 min at 4°. After two washes, low-tox rabbit complement (Cedarlane Laboratories, Hornby, Ontario) was added and incubation was followed for 30 min at 37°. The cells were then washed twice in medium and their viability was assessed by trypan blue exclusion. Control of T-cell depletion was determined by FACS analysis.

ELISPOT

An ELISPOT assay was done according to Czerkinky *et al.*[18] with some modifications. Flat-bottomed plates (Nunc, Immune Plate Maxisorp) coated with unlabelled goat anti-mouse immunoglobulin (5 μg/ml; Southern Biotechnologies, Birmingham, AL) in PBS pH 8·5 overnight at 4°, and washed with PBS-T, were incubated for saturation with PBS containing 1% gelatin (200 μl/well) for 1 hr at room temperature. Serial dilutions of spleen cells in medium (5×10^5–5×10^2) were added to each well and cultured for 6–8 hr at 37° in 5% CO_2.

The cells were then lysed with 0·05% Tween-20 and washed three times with PBS. IgM-secreting cells were detected with biotin-labelled goat anti-mouse IgM. This complex was bound to avidin–alkaline phosphatase for 2 hr at 37° (Southern Biotechnologies). The reaction was developed with 5-bromo-4-chloro-3-indol phosphate (5-BCIP, Sigma) substrate in 2-amino-2-methyl-1-propanol buffer (AMP, Sigma Chemical). The plates were incubated for 2 hr at 37° and the number of spots per well was calculated. The assays were done in triplicate.

Statistical analysis

The Student's *t*-test was used for the mean comparisons of all experiments when applied. Probability (P) $< 0{\cdot}01$ was considered as significant with 99% confidence.

RESULTS

In vitro proliferation of spleen cells from different strains of mice is induced by rTc24

Spleen cells from different strains of mice (BALB/c, BALB.Xid and BALB.nude) were cultured with rTc24 for 24 and 48 hr. The results presented in Table 1 show that spleen cells from mice of all mouse strains studied here respond to rTc24 *in vitro*, as revealed by the corresponding stimulation index (SI). Cells from BALB/c and BALB.Xid mice cultured with rTc24 showed SI twofold greater than those obtained with cells cultured in the presence of the control Sj26. Spleen cells from BALB.Xid mice (B-cell-immunodeficient mice that are low responders to T-cell-independent antigens), showed a lower thymidine incorporation after 24 and 72 hr (data not shown) of rTc24 stimulation. Moreover, spleen cells from athymic nude mice were significantly stimulated by rTc24 if compared to the results obtained with euthymic BALB/c and B-cell-immunodeficient BALB.Xid mice.

Table 1. Stimulation index of spleen cells from BALB/c, BALB.nude and BALB.Xid mice stimulated with rTc24

	BALB/c	BALB.nude	BALB.Xid	24hr	48hr
LPS	+	−	−	7·6	9·8
	−	+	−	24·4	18·2
	−	−	+	ND	ND
rTc24	+	−	−	2·1	2·0
	−	+	−	4·8	2·9
	−	−	+	1·6	2·2
Sj26	+	−	−	1·2	0·9
	−	+	−	2·3	1·3
	−	−	+	1·2	1·7

Spleen cells from BALB/c, BALB.nude and BALB.Xid mice were cultured at a density of $2{\cdot}5 \times 10^5$/well. The cultures were stimulated with LPS (10 μg/ml), rTc24 (50 μg/ml) or SJ26 (25 μg/ml), for 24 and 48 hr. [^{3}H]TdR was added for the last 8 hr of culture and incorporation was determined in harvested cells using a β-plate. SD for mean incorporations were always < 10%. The data present a mean of triplicate cultures and are representative for two to five experiments. The stimulation index was calculated by dividing the c.p.m. arithmetic means from stimulated cultures by the c.p.m. arithmetic means obtained from control cultures with medium alone.

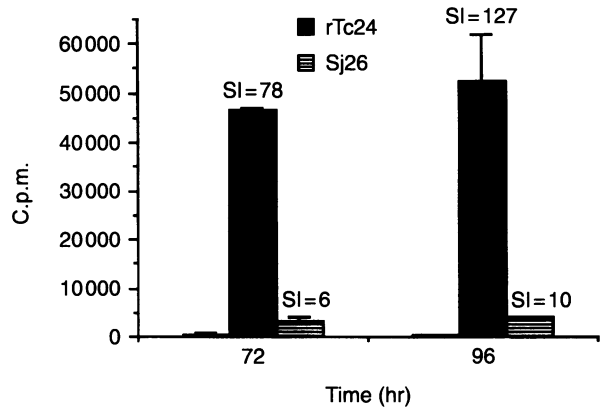

Figure 1. Lymphocyte proliferation of C3H/HeJ mouse spleen cells induced by rTc24. Spleen ($2{\cdot}5 \times 10^5$) cells from C3H/HeJ mice were stimulated with rTc24 (black bars), Sj26 (striped bars) or medium (open bars). After 72 and 96 hr of culture, the cells were pulsed with [^{3}H]TdR for the last 8 hr. Data (c.p.m. counts) represent the mean of triplicate cultures + standard deviations. SI = stimulation index.

In order to rule out the possibility that LPS contaminating rTc24 preparation was reponsible for cell stimulation, spleen cells of the C3H/HeJ mice (LPS non-responder) were stimulated with rTc24 and Sj26. As shown in Fig. 1, the proliferation of spleen cells induced by rTc24 was markedly increased compared to the control, indicating therefore that stimulatory activity is truly due to rTc24.

B cells, but not T cells, express CD69 in response to rTc24

To identify the cell populations responding to rTc24, we searched for the membrane expression of an early activation marker (CD69) in CD4$^+$, CD8$^+$ and B-cell populations. The FACS analysis showed a high expression of CD69 in B cells after 20 hr of *in vitro* stimulation with rTc24 (Fig. 2). In both BALB/c and BALB.Xid strains, we detected 21% and 12%, of the B cells expressing CD69, respectively. The percentage of CD69 in T cells (CD4$^+$ and CD8$^+$) after rTc24 stimulation and after Sj26 stimulation was not significantly different. To elucidate whether the stimulation of B cells by rTc24 requires the participation of T-cell dependent activities, we treated spleen cell suspensions with T-cell-depleting antibodies plus complement before their *in vitro* culture with rTc24. The effectiveness of T-cell depletions was assessed by staining splenocytes from normal and T-cell-depleted spleens with anti-CD4$^+$ and anti-CD8$^+$ fluoresceinated mAb and further flow cytometry analysis. As can be seen in Table 2, normal untreated animals, positive cells accounted for 38% and 47% in BALB/c mice, and 35% and 23% in BALB.Xid mice for T and B cells, respectively. After T-cell depletions only 1% and 2% of spleen cells of BALB/c and BALB.Xid mice, respectively, were positively stained with the mAb to T-cell surface marker, thus confirming the efficiency of the treatment. In these circumstances, the percentage of B cells increased to 80% and to 45% in spleens of BALB/c and BALB.Xid animals, respectively. Furthermore, mice depleted of T cells showed significant increases in CD69 expression on B cells after rTc24 *in vitro* stimulation. Indeed, the expression of CD69 in B cells increased up to 28% and 23%, respectively, in BALB/c and BALB.Xid mice. The expression of CD69 molecule after spleen cell

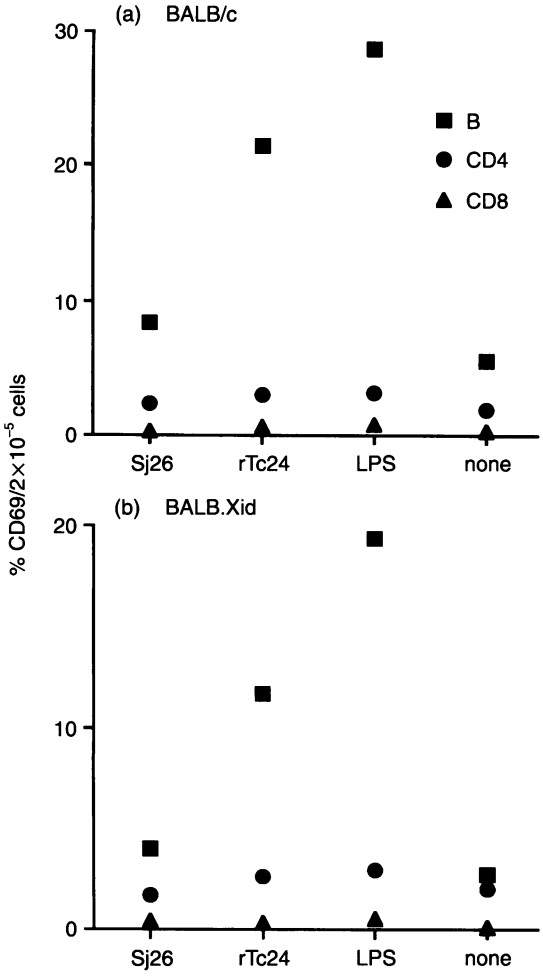

Figure 2. rTc24 induces the expression of CD69 activation molecule on spleen cells from BALB/c and BALB.Xid mice. Spleen cells ($2{\cdot}5 \times 10^5$) per well from BALB/c (a) or BALB.Xid (b), were cultured with rTc24 (50 μg/ml), Sj26 (25 μg/ml), LPS (10 μg/ml) or without antigen. After 20 hr the expression of CD69 was determined by double labelling using flow cytometry and the percentages of CD69$^+$ B cells (■), CD4 (●) and CD8 (▲) were determined.

stimulation with Sj26 was as low as the level recorded with non-stimulated cells: 16% and 10% of spleen cells from BALB/c and BALB.Xid mice, respectively (Table 2). B-cell expression of CD69 after culture with LPS was around 50% and 34% in BALB/c and BALB.Xid mice, respectively. It is reasonable to assume that rTc24 could act as an LPS-like mitogen that stimulates B-cell populations.

In vivo assessment of B-cell responses to rTc24

In order further to dissect the effects of *in vivo* administration of rTc24 in total humoral B-cell responses, we determined the levels of different isotypes in the sera of treated and untreated mice in the first 7 days following injection. A significant increase of total non-specific IgM levels in the sera of BALB/c mice injected with rTc24 was observed 2 days after treatment ($P=0{\cdot}004$) when compared to sera from Sj26-injected mice (Fig. 3a). In comparison, this isotype only significantly increased by days 4 ($P=0{\cdot}004$) and 7 ($P=0{\cdot}0021$) in the sera

Table 2. Expression of CD69 activation marker (%) by spleen cells from BALB/c and BALB.Xid mice after T-cell depletion and culture with rTc24

	BALB/c	BALB.Xid	T cells	B cells
Medium	+	−	−	14·82
	−	+	−	12·26
LPS	+	−	−	49·80
	−	+	−	34·46
rTc24	+	−	−	**28·55**
	−	+	−	**23·45**
Sj26	+	−	−	16·35
	−	+	−	10·52
			Control of killing*	
Before	+	−	37·81	46·85
	−	+	35·44	23·35
After	+	−	1·25	80·41
	−	+	2·37	44·74

Spleen cells from BALB/c and BALB.Xid mice were treated with anti-CD4$^+$ plus anti-CD8$^+$ mAb and complement. Depleted spleen cells of both animal models ($2{\cdot}5 \times 10^5$ per well). were incubated with rTc24 (50 μg/ml), Sj26 (25 μg/ml), LPS (10 μg/ml) or without antigen (medium) during 20 hr and the expression of CD69 (%) was determined by FACS analysis.

*Control of T-cell depletion. Determination of CD3$^+$ (T) and μ$^+$ (B) cells before and after T-cell killing. The depletion levels of T cells from BALB/c and BALB.Xid spleen cell suspensions were 96·7% and 93·3%, respectively.

from BALB.Xid mice. To appreciate better the activation of secretory B lymphocytes by rTc24 over the non-specific induction observed with the control Sj26 (and this before full maturation of B lymphocytes to plasma cells), the numbers of B cells secreting IgM in the spleen of BALB/c mice treated with rTc24 or Sj26 were determined by ELISPOT (Fig. 3b). After days 4 and 7 of rTc24 injection, BALB/c mice presented significantly higher levels of IgM-secreting spleen cells compared to the mice that were treated with Sj26 ($P=0{\cdot}01$ and $P=0{\cdot}007$, respectively). A lower, but not significant, increase of IgM-secreting spleen cells was also observed after 2 days. However, no increases of IgM-secreting B cell numbers was observed in spleens of BALB.Xid mice treated with rTc24 during the period analysed when compared to Sj26 treatment. No particular differences were recorded concerning the number of B cells secreting other immunoglobulin isotypes or levels of these antibodies in the sera of BALB/c and BALB.Xid mice treated with rTc24 or with Sj26, and the levels were consistently lower than the values obtained for IgM (data not shown).

IgM secretion by spleen cells is induced *in vitro* by rTc24

Acute and chronic Chagas' infection in mouse is characterized by polyclonal B-cell activation in lymphoid organs of several strains of mice.[19–21] Consequently, we attempted by the following experiments to examine the *in vitro* effect of rTc24, Sj26 and LPS on the numbers of IgM-secreting spleen cells of BALB/c and nude mice after 48 and 96 hr of culture with these antigens. As shown in Fig. 4, the numbers of spleen cells from BALB/c and nude mice that produce IgM were significantly increased by the incubation with rTc24. In contrast, stimulation of BALB/c and nude mice spleen cells with Sj26 did not lead to an increase in the number of B cells secreting

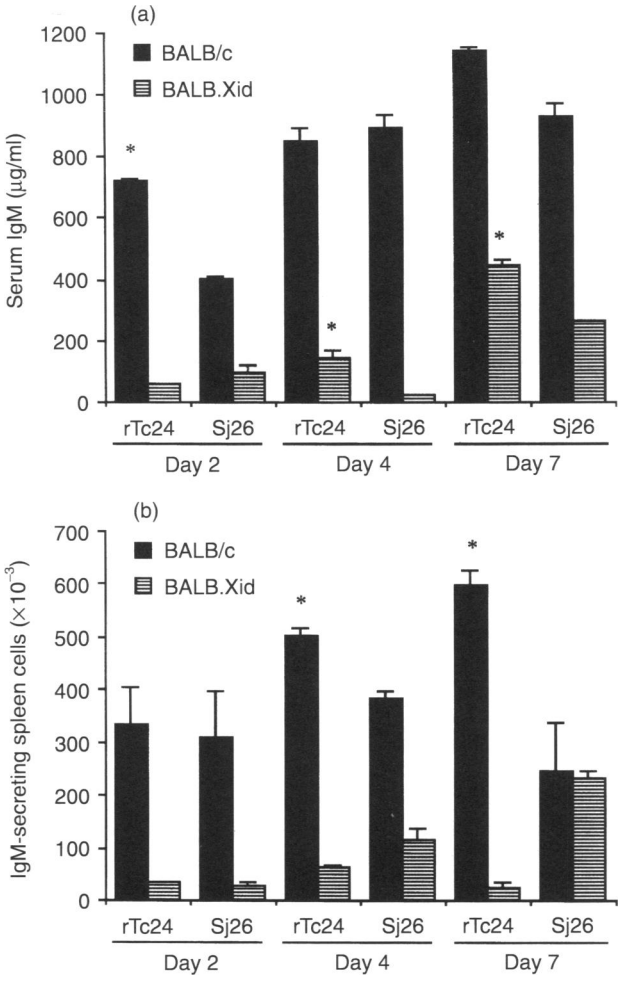

Figure 3. Levels of IgM in the sera and numbers of IgM-secreting cells in the spleen after *in vivo* administration of rTc24. (a) IgM levels in the sera of BALB/c and BALB.Xid mice injected intraperitoneally with rTc24 (50 μg/ml) or Sj26 (25 μg/ml). IgM antibody levels were quantified by ELISA at days 2, 4 and 7 in comparison to standard curves using purified mouse IgM. Data represent the means of triplicate samples ± standard deviations and are representative of two experiments. (b) Numbers of IgM secreting spleen cells in BALB/c and BALB.Xid mice injected i.p. with rTc24 (50 μg) or Sj26 (25 μg). Numbers of IgM-producing cells were determined by ELISPOT at days 2, 4 and 7 and represent the means ± SD of two independent experiments assayed with three animals individually.

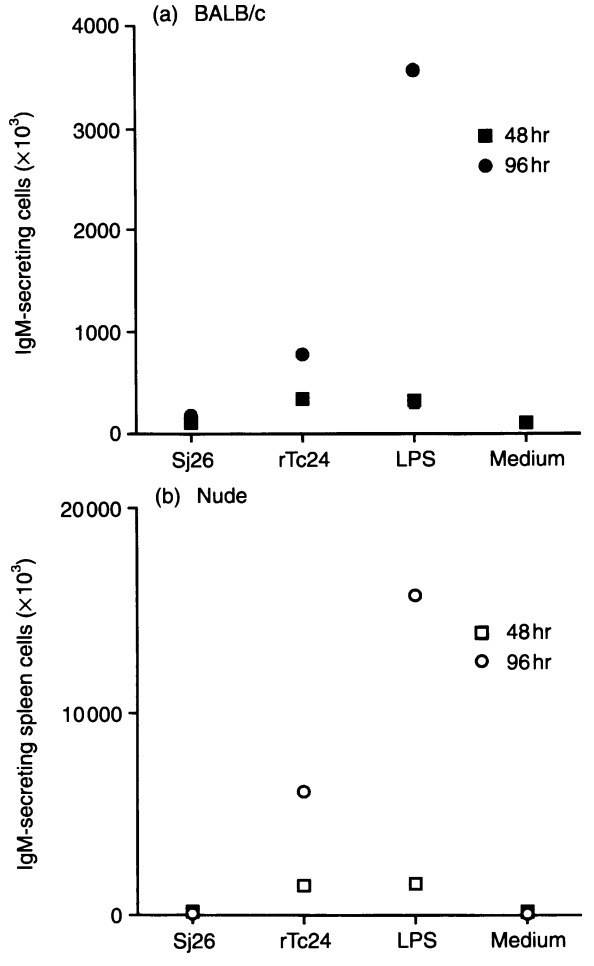

Figure 4. Numbers of IgM-secreting cells in the spleens of euthymic and athymic mice after *in vitro* stimulation with rTc24. Spleen cells from BALB/c (a) or BALB.nude (b) mice were incubated with rTc24 (50 μg/ml), Sj26 (25 μg/ml), or LPS (10 μg/ml) for 48 hr (●, ○) and 96 hr (■, □). The numbers of IgM-secreting cells were determined by ELISPOT and represent the means ± SD of three independent experiments.

IgM when compared to non-stimulated cells. After 48 hr of stimulation, rTc24 induced an increase in IgM-producing cells which was similar to the one induced by LPS. However, after 96 hr of culture with LPS a significantly higher increase of IgM-secreting cells was observed when compared to that induced by rTc24 stimulation. Higher levels of IgM-secreting cells were also recorded in spleens of nude mice compared to spleens of BALB/c mice stimulated with rTc24, suggesting that this protein is directly involved in T-cell-independent B-cell activation.

Non-specific polyclonal B-cell response induced by rTc24 *in vivo*

Our results, obtained by measurements of secreted antibodies and immunoglobulin-secreting cells *in vivo*, gave us indications that rTc24 induces proliferation of B cells and differentiation to high-rate immunoglobulin secretion. As proliferating B cells can already secrete sufficient amounts of antibodies to be detected as secretory, even before achieving full maturation to plasmocytes, we assessed the ability of rTc24 to induce a specific B-cell proliferation and terminal differentiation *in vivo*, analysing B-cell responses 14 days after injection. Thus, we injected BALB/c and nude mice with this protein and analysed total and specific serum responses to rTc24 after 14 days of injection. The rTc24 did not induce significantly detectable IgG levels in sera of BALB/c and nude mice (not shown). However, as can be observed in Table 3, a significant increase was observed in the IgM secretion in euthymic or athymic mice injected with rTc24 in comparison to controls injected with Sj26 ($P = 0{\cdot}0077$ and $0{\cdot}0073$, respectively). Although total titres of IgM antibodies were enhanced after rTc24 injection (1/4500 and 1/12 500 for BALB/c and Nude mice, respectively), specific recognition of total parasite extract or rTc24 was irrelevant in both BALB/c and nude mice and titres obtained against rTc24 and total parasite extracts (1/100 for both

Table 3. Total serum immunoglobulins of BALB/c and Nude mice after 14 days of injection with rTc24

		Total serum Ig (mg/ml)	
		IgM	IgG
BALB/c	Sj26	1·750 ± 0·0071	0·040 ± 0·010
	rTc24	2·533 ± 0·058	0·050 ± 0·005
		$P = 0·0077$	$P = 0·0960$
Nude	Sj26	1·303 ± 0·039	0·150 ± 0·060
	rTc24	3·100 ± 0·058	0·250 ± 0·080
		$P = 0·0073$	$P = 0·1090$

groups) were not different from those following Sj26 injection (1/100) (Fig. 5a,b). It is worth noting that 1/100 dilution is the minimum required serum dilution to avoid non-specific binding of antibodies to any coated antigens under study. These results showed that the specific serum recognition of rTc24 and other parasite antigens after injection of rTc24 protein was substantially irrelevant or negative if compared to the levels of non-specific polyclonal IgM induction.

DISCUSSION

The 24 000 MW protein of *T. cruzi* is present among the trypomastigote ESA[10,13,23] and is expressed by all developmental stages of the parasite. The rTc24 fusion protein is recognized by chronic-phase sera of both susceptible BALB/c and resistant BALB.Xid infected mice. Similar results were obtained using chagasic human sera.[13,22] To identify the rTc24 domains that are specific to the parasite, Taibi *et al.* used antibodies previously produced against a 20–40 amino acid synthetic peptide present in the N-terminal region of rTc24.[10,23] In contrast to other *T. cruzi* proteins, this synthetic peptide is specific for all parasite strains tested.[13] Monoclonal antibodies specifically recognized this peptide and Northern and Southern blot analyses revealed that its sequence is homologous in all studied *T. cruzi* strains.[13,23]

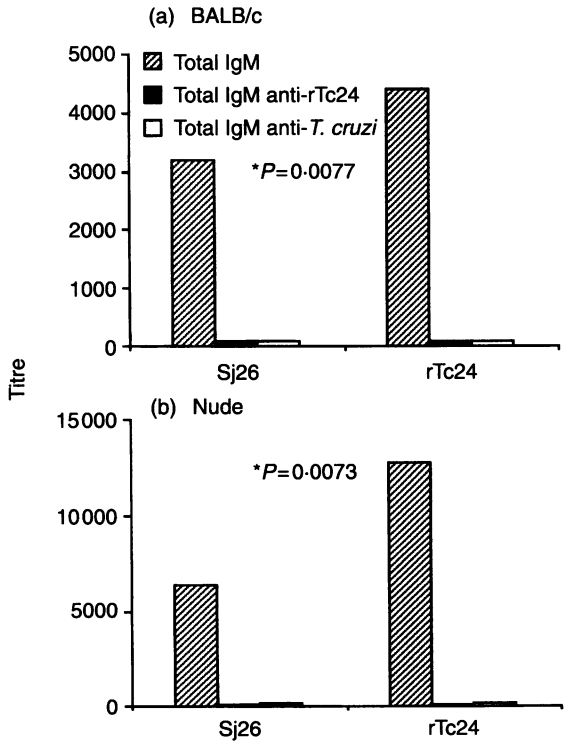

Figure 5. Levels of total and specific IgM antibodies in the serum of euthymic and athymic mice injected with rTc24. Titres of IgM antibodies in the sera of BALB/c (a) or BALB.nude (b) mice after 14 days of rTc24 injection were determined by ELISA. Total IgM, total IgM anti-rTc24 and total IgM anti-*T. cruzi* extracts. The results are the arithmetic means of three mice injected with 25 μg of Sj26 or 50 μg of rTc24 and are representative of three different experiments.

It is well known that the characteristics of a vaccine candidate include the non-induction of polyclonal activation that is usually accompanied by the secretion of non-specific immunoglobulins and immunosuppression.[24] Previous hyperimmunization studies suggested the important role of rTc24 in displaying protection to *T. cruzi* infection.[10,13] However, the acute phase of experimental infection in mouse was described as inducing an extensive polyclonal B-cell activation accompanied by high levels of antibodies that do not recognize parasite antigens.[1,19,21] Additionally, Minoprio *et al.*[25] have shown that the polyclonal B-cell activation induced by *T. cruzi* infection was mainly dependent on $CD4^+$ T cells, since treatment of mice with GK 1·5 mAb (anti-$CD4^+$ mAb) at the time of the infection reduced the plaque-forming cell numbers to normal levels. We decided thereby to study the cell populations activated by the rTc24 protein *in vivo* as well as the state of activation of the immune system following the injection of this protein. In the present report, we describe that this *T. cruzi*-excreted protein is a potent B-cell activator. Thus, the *in vivo* treatment with the recombinant rTc24 protein induces a response that is manifested by an immediate increase in the numbers of B cells secreting immunoglobulins, predominantly of the IgM isotype. The IgM response is mostly unrelated to the antigens present in total parasite extracts or to the protein itself. This polyclonal activation of the immune system could facilitate the parasite evasion of specific immune responses.

It is well known that T-cell-independent antigens, or mitogens such as LPS, mainly stimulate the production of IgM and IgG3 antibodies.[26] IgM has structural features that enhance complement fixation,[27] which could easily promote *T. cruzi* phagocytosis by CR1 bearing effector cells[28] or contribute to complement-mediated lysis of parasites.[24] However, in the sensitive BALB/c mouse model of *T. cruzi* infection the preferential increase of $CD5^+$ B cells, responsible for the majority of the IgM secretion, is at the origin of the polyclonal B-cell/immunoglobulin responses observed. In contrast, in the resistant BALB.Xid mouse model, the absence of this cell population correlates with a lower B-cell response and with the control of the acute phases of infection.[29] Interestingly, this lower B-cell response observed in the BALB.Xid model revealed also low levels of specific recognition of parasite antigens.

The lack of specificity of antibodies after the infection with *T. cruzi* or after the injection of purified parasite antigens suggests that specific B-cell responses play a minor role in the mechanisms leading to resistance. Therefore, the present studies using rTc24 and mouse models of resistance and susceptibility to infection indicate that this protein is not a good vaccine candidate if used in a single dose injection and in the absence

of adjuvants. In fact, the isotype selectivity of antibodies can be highly determined by the structure and functional properties of the carrier molecules and adjuvants. Nevertheless, polyclonal non-specific responses are not an exclusive result of direct stimulation of B-cell lymphocytes by mitogenic factors but also of T-cell participation which may lead to the release of cytokines enhancing and/or modulating B cell differentiation and immunoglobulin secretion. The pattern of isotype distribution of *T. cruzi*-infected mice is different from that reported for other agents which stimulate polyclonal B-cell response *in vivo* either by helper cell dependent 'or' by independent mechanisms.[19,30] D' Império Lima *et al.* described that mice infected with *T. cruzi* present a B-cell-secreting isotypic pattern that is unique in both the extent of stimulation and in the distribution of isotypes where IgG2a > IgG2b > IgG1 > IgM.[30] This isotypic pattern is atypical with components of both helper T-cell-dependent and T-cell-independent stimulations. Similarly, in animals immune protected against malaria, the displayed immunoglobulin isotypic pattern is completely different from that occurring during primary infection. Thus, IgG2a- and IgG2b-secreting cells represent the majority of the B response following infection with *Plasmodium chabaudi chabaudi*.[31] Reinfection of protected animals with *P. chabaudi*, however, induces a strong dose-dependent stimulation of immunoglobulin-secreting cells of all classes with selective emergence of IgG1 and IgM isotypes that is dependent on particular T-cell activities.[31,32]

On the other hand, our present results using euthymic and athymic mice are suggestive of a direct T-cell-independent mitogenic activity of rTc24 exoantigen of *T. cruzi* on B cells. However, we have recently reported that a hyperimmunization scheme of BALB/c mice with rTc24 protein plus adjuvant lead to a significant level of protection against a lethal *T. cruzi* challenge.[13] Moreover, spleen cells from rTc24 hyperimmunized mice secreted significant amounts of interferon-γ (IFN-γ) and interleukin-2 (IL-2) upon *in vitro* stimulation with rTc24. Likewise, in the case of rTc24 hyperimmunized mice, the isotypic profile of antibodies targetted against this antigen (IgG1 > IgG2b > IgG2a) was different from that observed in the case of acutely or chronically infected mice (see above and refs 13,19,20).

Finally, our present results show that rTc24 induces a strong B-lymphocyte response using *in vitro* and *in vivo* assays, indicating that the nature of the B-cell responses following rTc24 injection is indeed the polyclonal expansion of non-specific, non-parasite-directed B-cell clones, similarly to other mitogen stimulations. The single injection of rTc24 without any adjuvants induces the production of immunoglobulins that are not specific for parasite antigens, as described for other mitogenic products released by several micro-organisms responsible for bacterial, viral, or parasitic infections.[5,33,34] A classical example of these findings is the polyclonal B-cell activation with absent specific recognition observed after injection of Lipid A.[30,35,36] More importantly, preliminary results suggest that previous injection of rTc24 protein to naive mice is responsible for low lymphocyte responses to heterologous stimulation with sheep red blood cells (SRBC) or with KLH antigens *in vivo* and to concanavalin A (Con A) proliferation *in vitro* (data not shown). These results are compatible with a mitogenic induction of the system accompanied by a state of immunosuppression to non-related antigens. Besides, the quality of the activating stimuli can direct the type of switches occurring in the responding cell population.[30] Taken together our results call the attention for the utilization of immunodominant parasite antigens to the development of vaccines.

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