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Buza, Joram

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CD5⁺ B lymphocytes are the main source of antibodies reactive with non-parasite antigens in *Trypanosoma congolense*-infected cattle

J. BUZA, M. SILEGHEM*, P. GWAKISA† & J. NAESSENS *International Livestock Research Institute, PO Box 30 709, Nairobi, Kenya*

SUMMARY

Mice infected with African trypanosomes produce exceptionally large amounts of serum IgM, a major part of which binds to non-trypanosome antigens such as trinitrophenol and single-strand DNA. In this paper, we describe that in cattle infected with *Trypanosoma congolense* and *T. vivax*, similar antibodies are found, although they bind mainly to protein antigens, such as β -galactosidase, ovalbumin and ferritin. The parasite non-specific IgM antibodies appear around the same time as the parasite-specific antibodies, but their origin and function are not clear. We tested the hypothesis that CD5⁺ B cells (or B-1 cells), which increase during trypanosome infections in cattle, are responsible for production of antibodies to non-trypanosome antigens. Splenic CD5⁺ and CD5⁻ B cells from infected cattle were sorted and tested in a single cell blot assay. The numbers of immunoglobulin-secreting cells were similar in both B-cell populations. However, antibodies with reactivity for non-trypanosome antigens were significantly more prevalent in the CD5⁺ B-cell fraction and were exclusively IgM. The preference for production of these antibodies by CD5⁺ B cells and the expansion of this subpopulation during infections in cattle, strongly suggest that CD5⁺ B cells are the main source of trypanosome non-specific antibodies. We propose that these antibodies are natural, polyreactive antibodies that are predominantly secreted by CD5⁺ B cells. Since B-1 cells are up-regulated in many states of immune insufficiency, the immunosuppression associated with trypanosome infections may be responsible for the increase of this subset and the concomitant increase in trypanosome non-specific antibodies.

INTRODUCTION

African trypanosomiasis is one of the major disease constraints to livestock production in sub-Saharan Africa.¹ While the pathology of the disease has been well documented, surprisingly little is known about the mechanisms that cause disease. An unusual feature associated with the immune response during trypanosome infections in man and laboratory animals is that a large fraction of the antibodies produced are IgM and bind to antigens of non-parasite origin including autologous antigens.^{2–8} Despite the occurrence of autoreactive anti-

bodies, the pathology of trypanosomiasis is not consistent with an autoimmune disease. In contrast, parasite non-specific and autoantibodies which develop during chronic *Trypanosoma cruzi* infections contribute greatly to pathology in cardiac and neuronal tissues.⁹

Whether these antibodies develop in infected cattle remains to be resolved. Although increases in serum IgM have been consistently observed in many trypanosome infections of cattle, the appearance of trypanosome non-specific antibodies was not a consistent finding.^{10–12} Antibodies reactive to trinitrophenol-bovine serum albumin (TNP-BSA), one of the strongest parasite non-specific responses in infected mice,^{7,8} could not be detected in serum from *T. congolense*-infected cattle.¹² However, other studies in cattle infected with *T. vivax* have demonstrated antibodies binding autologous erythrocytes and platelets¹³ and cattle infected with *T. congolense* produced antibodies reactive with a number of non-trypanosome antigens, including the *Escherichia coli* enzyme β -galactosidase (β -gal).¹⁴

The mechanisms leading to the development of trypanosome non-specific antibodies and their subsequent role in infection and pathology are still unknown. They have been found in infected, athymic (*nu/nu*) mice,^{8,15} suggesting that they are produced by a T cell-independent mechanism, possibly

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Abbreviations: β -gal, β -galactosidase; BSA, bovine serum albumin; mAb, monoclonal antibody; SIG, silver immunogold assay; TNP, trinitrophenol; VSG, variant surface glycoprotein.

*Present address: I.W.T., Bisschofsheimlaan 25, 1000 Brussels, Belgium.

†Present address: Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture, PO Box 3019, Morogoro, Tanzania.

Correspondence: Dr J. Naessens, International Livestock Research Institute, PO Box 30 709, Nairobi, Kenya.

involving the polyclonal activation of B cells by a mitogen-like molecule from the parasite.^{7,8} However, searches for this mitogen-like molecule have so far been unsuccessful.^{16,17} Evidence of polyclonal activation of B cells could not be found in cattle.^{18,19} Other workers attributed the non-trypanosome specificities to cross-reactive antibodies that were generated against the various variant surface glycoproteins that appeared during infection or against other parasite molecules.^{10,12,18,19} Recently, following the observation that numbers of CD5⁺ B cells increased in blood and spleen concomitantly with an increase in serum IgM during *T. congolense* infection of cattle, it was suggested that non-trypanosome and autospecificities may be the result of antibodies secreted by the CD5⁺ B-cell subpopulation.²⁰ CD5⁺ B cells form a distinct B-cell lineage (B-1), and spontaneously produce IgM with the features of natural antibodies in normal individuals.²¹ They have also been shown to secrete 'polyreactive' antibodies with reactivity to multiple antigens, including self epitopes.²¹⁻²³ CD5⁺ B cells have also been implicated in the production of parasite non-specific antibodies during infection with *T. cruzi*.^{24,25} In this study we confirm the existence of antibodies to non-trypanosome antigens in cattle and support the hypothesis that during infection most of these antibodies are secreted by CD5⁺ B cells.

MATERIALS AND METHODS

Animals and parasites

Boran and N'Dama cattle used in this study were raised at Kapiti plains Ranch, Athi River, Kenya, an area known to be free from trypanosomiasis. Animals were infected with trypanosomes by tsetse fly (*Glossina morsitans centralis*) bite on their flanks as described.²⁶

The first experiment involved six Boran and six N'Dama cattle, (6 months old and sex-matched). These animals were infected with *T. congolense* IL 1180 as described previously.²⁷ The second experiment involved four naive Boran bulls aged 3 years which were infected with *T. vivax* IL 2337.²⁸ In the final experiment, seven Boran calves aged 6 months were infected with *T. congolense* IL 1180.

Preparation of trypanosome antigens

Trypanosomes were isolated from the blood of infected rats as described previously.²⁹ Fifteen sublethally irradiated rats were infected with a stabilate of *T. congolense* IL 1180 and blood parasitaemia was monitored daily. At peak parasitaemia, blood was collected in heparinized phosphate saline glucose buffer. Trypanosomes were isolated on diethylaminoethyl (DEAE)-cellulose and washed several times in phosphate saline glucose buffer before lysis.

A trypanosome lysate was prepared from 10⁹ trypanosomes by three cycles of freezing and thawing in the presence of protease inhibitors (10 µg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, 0.2 mM *N*-tosyl-L-phenylalanine chloromethyl ketone and 0.05 mM *N*-alpha-*p*-tosyl-lysine chloromethyl ketone) using liquid nitrogen as described previously.³⁰

Enzyme-linked immunosorbent assay (ELISA) for bovine antibodies

Serum was prepared from blood samples collected sequentially over a 5-week period during trypanosome infections and immediately stored at -70° until use.

Each well of a 96-well Dynatech ELISA plate (Dynatech, Plochingen, Germany) was coated with 100 µl of a 40 µg/ml solution of trypanosome lysate in coating buffer (0.05 M bicarbonate buffer, pH 9.6) and the plates were incubated overnight at 4°. The plates were then washed three times with washing buffer (phosphate-buffered saline pH 7.4, containing 0.1% Tween 20). Thereafter, 100 µl of serum diluted 1/100 in washing buffer were added in duplicate wells and incubated for 2 hr at 37°. The plates were then washed and 100 µl of horseradish peroxidase-conjugated monoclonal antibodies (mAb) IL-A2, specific for bovine IgG,³¹ or mAb IL-A30, specific for IgM,³¹ were added for 1 hr at room temperature. After washing, 100 µl of substrate K-blue (ELISA Technologies, Lexington, KY) was added to each well and the optical density determined at 650 nm on a Titertek Multiskan MCC/340 ELISA plate reader (Flow, Oy, Finland) following a 30-min incubation. For non-trypanosome antigens, microtitre ELISA plates were coated with 1 µg/ml solution of the following antigens: β-gal, calf thymus single-strand (ss) DNA, keyhole limpet haemocyanin (KLH), ovalbumin, TNP and trypsin (all from Sigma, St Louis, MO), dextran, aldolase, catalase, ferritin and thyroglobulin (all from Pharmacia, Uppsala, Sweden), cytochrome C and myoglobin (from Schwarz/Mann, NY) and lysozyme (from Polysciences Inc., Worthington, PA).

Preparation of spleen mononuclear cells

Seven calves infected with *T. congolense* were slaughtered between 31 and 51 days. The spleens were individually disrupted in Alsever's solution and mononuclear cells were obtained by flotation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) at 1000g for 25 min. The cells were washed three times in Alsever's solution by centrifugation at 200g for 10 min. Contaminating red blood cells were lysed by incubation with a lysis buffer containing 0.017 M Tris, 0.14 M ammonium chloride, pH 7.2, for 5 min at 37°. Cells were washed and resuspended in complete RPMI-1640 + HEPES (containing 10% normal rabbit serum, 1% penicillin-streptomycin and 1% L-glutamine).

Fluorescent staining and flow cytometry

Staining of B cells for CD5 was done using a mixture of mAb IL-A50 (IgG2a, anti-bovine IgM), IL-A58 (IgG2a, anti-bovine immunoglobulin) and IL-A67 (IgG1, anti CD5) as described previously.²⁰ Fluorescein-conjugated anti-mouse IgG1 (Sigma, Poole, UK) and phycoerythrin-conjugated anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) were used as second-step reagents. Populations of CD5⁺ and CD5⁻ B cells were obtained by sorting using a flow-cytometer (FACStar, Becton Dickinson, Sunnyvale, CA). The concentration of purified B-cell subpopulations was adjusted to 10⁶/ml in complete RPMI-1640.

Silver Immuno Gold (SIG) blot assay

To determine the numbers of cells secreting antibody of a given specificity, purified B-cell fractions were tested in a SIG blot assay as described previously.³² Briefly, equal numbers (10⁵) of CD5⁺ and CD5⁻ B cells were plated on wells precoated with β-gal or a mAb to bovine immunoglobulin light chain, IL-A58³¹ and incubated in a humidified atmosphere for 2 hr at 37°. The plates were washed with Tris-HCl,

pH 9.6 and specific antibodies localized by addition of biotinylated mAb IL-A2 (anti-bovine IgG) or IL-A30 (anti-bovine IgM). The blots were visualized by addition of silver initiator and enhancer reagents (Amersham International, Buckinghamshire) and counted using an inverted microscope at $\times 40$ magnification.

Statistical analysis

Significant changes in mean levels of antibodies reacting with various non-trypanosome as well as trypanosome antigens over the infection period [0–35 days postinfection (p.i.)] was determined by subjecting optical density values obtained for each serum to one-way analysis of variance (ANOVA). Probability of $p < 0.05$ were considered significant. Where a significant change was detected, the mean optical densities were subjected to comparisons of means using the least significant difference method at a rejection level of $p = 0.05$.

The significance of variations between CD5⁺ and CD5⁻ B cells in the number of antibody-secreting cells was determined using a *t*-test.

RESULTS

Antibodies to non-trypanosome antigens

Twelve cattle (six Boran and six N'Dama) were infected with *T. congolense* IL 1180. Serum was collected at weekly intervals and tested in ELISA for antibody-binding activity to trypanosome and non-trypanosome antigens (see the Materials and Methods for the list). Since preinfection sera showed some binding of IgM to both trypanosome and non-trypanosome antigens, the absorbance of the preinfection sera was subtracted. The means and standard deviations of the changes in anti- β -gal (Fig. 1), anti-ferritin (Fig. 2) and anti-trypanosome lysate (Fig. 3) antibody levels in six Boran and six N'Dama cattle are represented in Figs. 1–3. Significant differences between optical density values from pre- and post-infection sera (up to 35 days p.i.) obtained for the various non-trypanosome antigens were analysed by one-way analysis of variance for each cattle breed.

In Boran cattle, significant differences in optical density values over 35 days of infection were detected for IgM reacting with β -gal ($P < 0.01$) (Fig. 1a), ferritin ($P < 0.01$) (Fig. 2a), cytochrome ($P < 0.01$) and thyroglobulin ($P = 0.07$) (not shown). There was no significant change in IgM levels reacting with other non-trypanosome antigens including aldolase, KLH, myoglobin, ssDNA and TNP over the infection period ($P > 0.5$). IgM reacting with cytochrome, ferritin and β -gal were significantly increased between 21 and 35 days p.i. ($P < 0.05$). However, IgM reacting with thyroglobulin was only increased on day 31 p.i. ($P < 0.05$) and had declined by day 35 p.i.

In N'Dama cattle, a significant change in levels of IgM reacting with β -gal ($P < 0.01$) (Fig. 1b), cytochrome ($P < 0.01$), ferritin ($P = 0.02$) (Fig. 2b), ovalbumin ($P = 0.018$) and lysozyme ($P < 0.01$) was detected over 35 days of infection. IgM reacting with cytochrome was significantly increased between 21 and 31 days p.i. ($P < 0.05$), and by day 35 p.i., the levels had declined. The β -gal- and lysozyme-binding IgM were significantly increased between 21 and 35 days p.i. ($P < 0.05$). However, antibodies reacting with ferritin and ovalbumin were only increased on day 21 p.i. ($P < 0.05$). There was no signifi-

cant change in IgM reacting with thyroglobulin, TNP, ssDNA, myoglobin, KLH and aldolase over the infection period ($P < 0.05$).

No significant increases ($P > 0.05$) in the binding of IgG to non-trypanosome antigens were measured in any of the samples from Boran (Figs. 2c, 3c) and N'Dama (Figs. 2d, 3d) cattle.

Antibodies to trypanosome antigens

Analysis of variance for trypanosome-specific IgM levels in Boran and N'Dama cattle revealed significant changes in levels during the 35 days of infection ($P < 0.01$) (Fig. 3). In Boran, trypanosome-specific IgM levels were increased between 14 and 21 days p.i. ($P < 0.05$) and subsequently decreased, while in N'Dama, the levels were significantly increased between 14 and 31 days p.i. ($P < 0.05$) and declined by day 35 p.i.

In contrast to trypanosome non-specific IgG antibodies, the levels of trypanosome-specific IgG significantly changed ($P < 0.01$) over the infection period in both Boran and N'Dama cattle. In Boran cattle, trypanosome-specific IgG was significantly increased between 14 and 35 days p.i. ($P < 0.05$). In N'Dama cattle, trypanosome-specific IgG was significantly increased by day 14 p.i. ($P < 0.05$), thereafter, the levels increased further and were at the highest level between 21 and 35 days p.i. ($P < 0.05$).

Other trypanosome species

To test whether another trypanosome species induced trypanosome non-specific antibodies, four Boran cattle were infected with *T. vivax*. Antibodies to β -gal were detected after 2 weeks of infection (Fig. 4).

Immunoglobulin-secreting cells in spleen

CD5⁺ and CD5⁻ B-cell populations were prepared from spleen cells from seven infected cattle between 31 and 51 days p.i. When stained for CD5 antigen, CD5⁺ and CD5⁻ populations from blood are distinctly separated,²⁰ but splenic CD5⁺ and CD5⁻ B cells overlap (Fig. 5). Those B cells that were most positive and most negative for fluorescence with the CD5 antibody were therefore sorted. Together, the two populations constituted between 75% and 90% of the total B cells. After a re-run of the sorted populations, only 0.5% of the sorted CD5⁺ B cells were CD5⁻, but 3% of the sorted CD5⁻ B cells were CD5⁺ and over half expressed a low amount of CD5 (Fig. 5).

Equal numbers (10^5 cells) of sorted CD5⁺ and CD5⁻ B cells were tested in the SIG blot assay for comparison of the percentage of cells secreting IgM and IgG, and antibodies reactive with β -gal (Table 1). The proportion of cells secreting IgM in both CD5⁺ and CD5⁻ B cells ranged from around 400 up to 9000 per 10^5 cells, while the number of IgG-secreting cells was about tenfold lower in both populations (between 14 and 600 per 10^5 cells). There were no significant differences ($P > 0.05$) between the number of IgM- or IgG-secreting cells in CD5⁺ and CD5⁻ B-cell subpopulations.

Cells secreting anti- β -gal IgM were measured (Table 1, columns 5 and 6) and ranged from 4 to 212 per 10^5 sorted cells. No cells secreting anti- β -gal IgG were found. Significantly

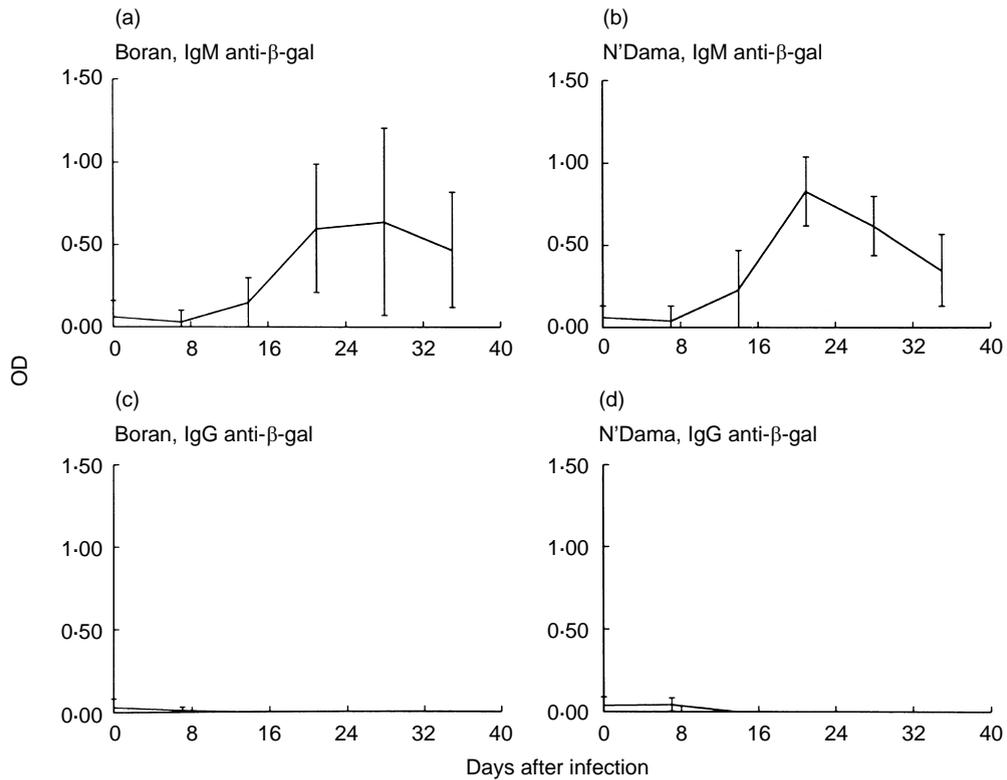


Figure 1. Changes in the levels of IgM (a, b) and IgG (c, d) reactive with β-gal in sera from Boran (a, c) and N'Dama (b, d) cattle after infection with *T. congolense* (means and standard deviations of six cattle).

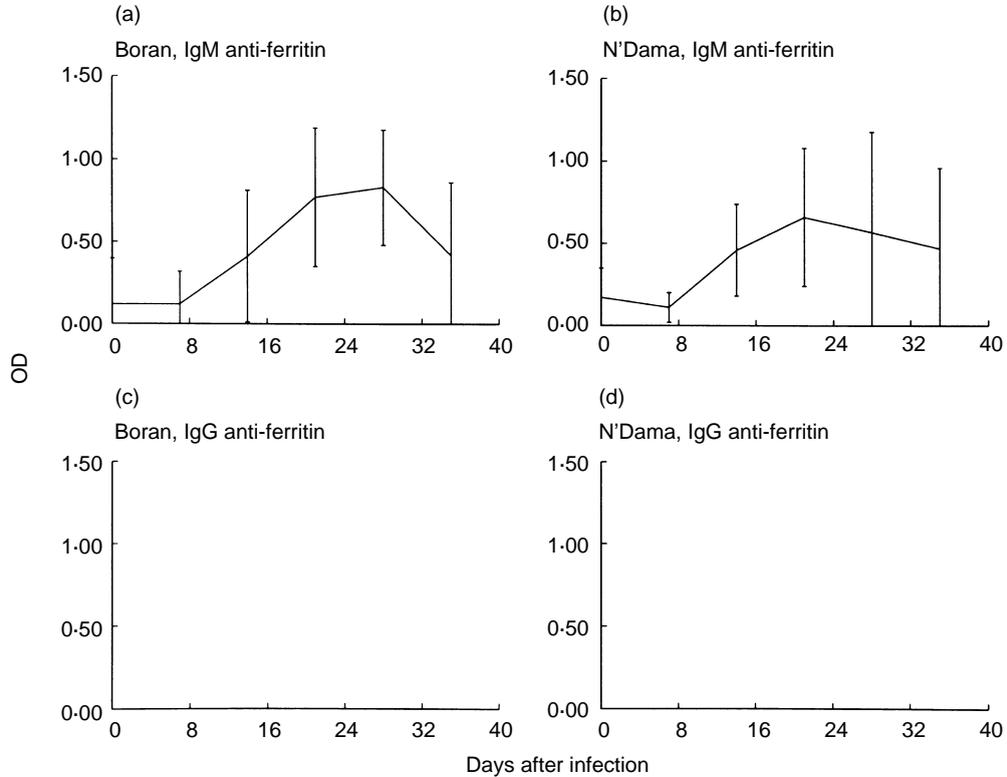


Figure 2. Changes in the levels of IgM (a, b) and IgG (c, d) reactive with β-ferritin in sera from Boran (a, c) and N'Dama (b, d) cattle after infection with *T. congolense* (means and standard deviations of six cattle).

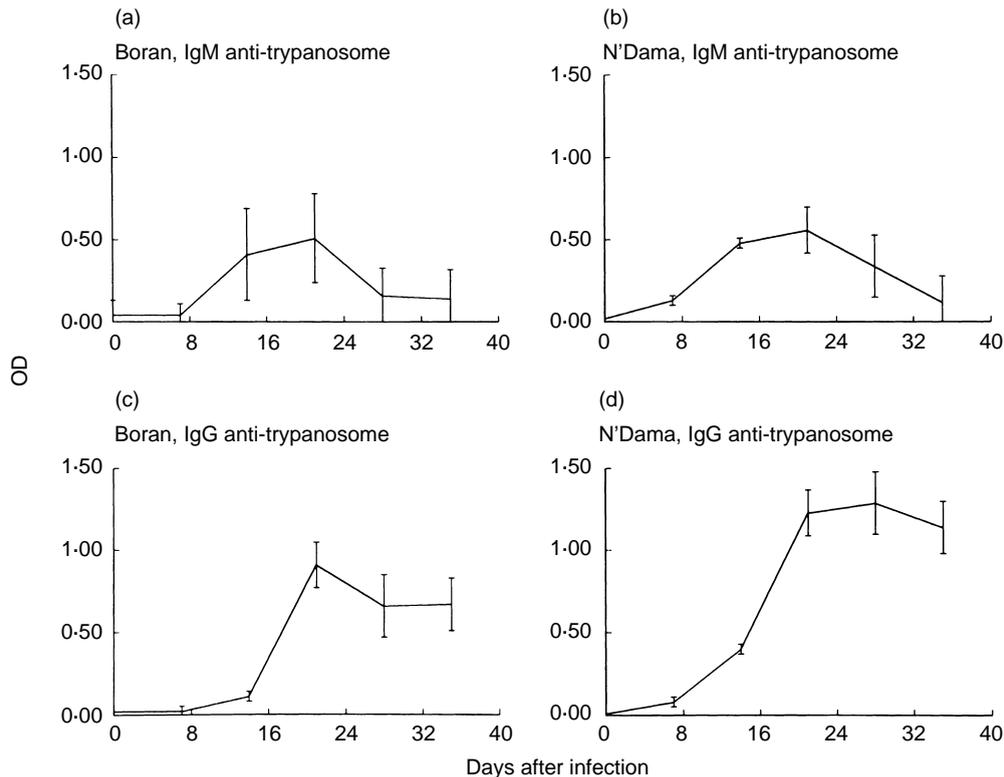


Figure 3. Changes in the levels of IgM (a, b) and IgG (c, d) reactive with a whole trypanosome lysate in serum of Boran (a, c) and N'Dama (b, d) cattle after infection with *T. congolense* (means and standard deviations of six cattle).

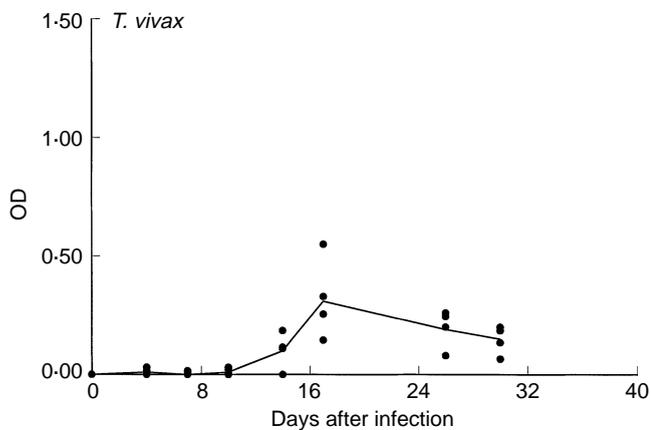


Figure 4. Changes in levels of IgM reactive with β -gal after a primary infection with *T. vivax* in four Boran cattle. Values for the four individual animals are represented by filled circles; the averages are shown by a continuous line.

($P < 0.05$) more cells secreting β -gal-reactive antibodies were found in the sorted $CD5^+$ B cells than in an equal number of $CD5^-$ B cells (Table 1). When calculated as a proportion of the total IgM-secreting cells, anti- β -gal-secreting cells were still significantly ($P < 0.05$) higher in the $CD5^+$ B cells than in the $CD5^-$ B cells (Table 1, last two columns).

DISCUSSION

Cattle infected with *T. congolense* and *T. vivax* developed antibodies, exclusively of the IgM class, which bound to a

number of non-parasite-derived antigens. The production of such trypanosome non-specific antibodies was not dependent on the trypanosome strain or species. These results extend the observation of a wide range of non-trypanosome specificities in antisera from trypanosome-infected mice, monkeys and man. In a previous study, failure to detect antibodies binding to TNP-BSA in cattle led to the conclusion that non-parasite-specific antibodies did not develop in this species.¹² In our assay, antibody reactivity to TNP was also weak, but more activity was measured for other non-trypanosome antigens such as β -gal, ferritin and cytochrome C.

There have been contrasting opinions regarding the development of trypanosome non-specific antibodies in infected cattle. Some investigators concluded that all antibodies which develop in infected cattle are specific for the infecting trypanosomes and that the apparent trypanosome non-specific reactivity observed is due to cross-reactive epitopes.¹⁰ This view could not be corroborated in our studies, since our results strongly indicate that the two antibody types (trypanosome-specific and trypanosome non-specific) comprise different antibody populations. First, antibodies against trypanosome antigens were of both IgM and IgG classes, while antibodies reacting with non-trypanosome antigens were exclusively of the IgM class. In this study, IgG activity to non-trypanosome antigens could not be demonstrated in serum or in spleen B cells. If the antibody activity to β -gal was due to cross-reactive epitopes shared with trypanosome antigens, we would have expected it to be present in the IgG subclass. Secondly, the levels of trypanosome-specific antibodies were highest between days 14 and 21, similar to previous observations,^{14,30} while the levels

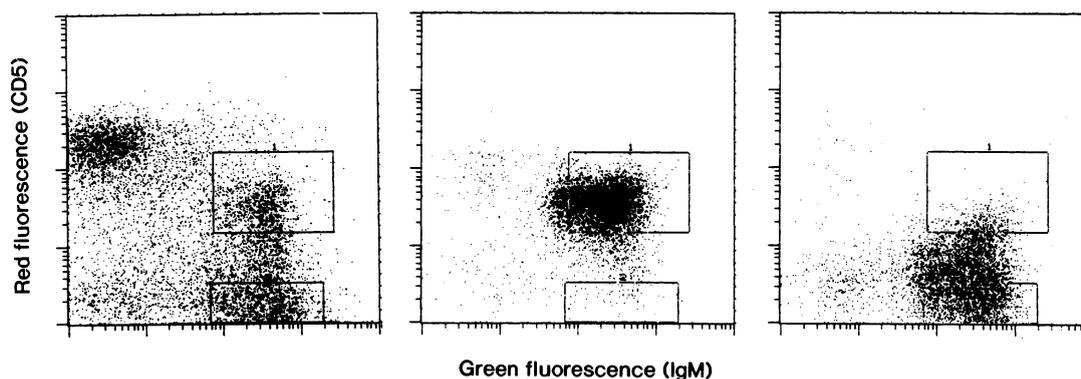


Figure 5. Two-colour fluorescence profiles of whole spleen cells stained for CD5 (y-axis) and IgM (x-axis), with the windows used to sort CD5⁺ and CD5⁻ B cells (left). After sorting, the two populations were rerun and checked against the same windows (middle and right). The fraction of CD5⁻ B cells contained cells that stain weakly for CD5 (right).

Table 1. Number of cells secreting IgG, IgM and anti- β -gal IgM per 100 000 CD5⁺ or CD5⁻ B lymphocytes

Animal	Total IgM		Total IgG		IgM anti- β -gal		Anti- β -gal as % of total IgM	
	CD5 ⁺	CD5 ⁻	CD5 ⁺	CD5 ⁻	CD5 ⁺	CD5 ⁻	CD5 ⁺	CD5 ⁻
BL209	9455	4929	364	103	182	23	1.9	0.5
BL351	1617	1562	35	24	80	23	4.9	1.5
BM218	594	970	125	370	29	4	4.9	0.4
BM219	912	1050	387	634	26	19	2.9	1.8
BM221	1710	746	400	191	28	4	1.6	0.5
BM222	550	400	280	150	212	87	38.4	21.8
BM326	2070	2455	90	144	204	144	9.9	5.9

of parasite non-specific antibodies reached their maximum a week later. These differences strongly suggest that trypanosome-specific and non-specific antibodies make up different sets.

It is therefore likely that the two sets of antibodies are generated through a different mechanism or by different B-cell subpopulations. A previous study suggested that bovine CD5⁺ B cells might be directly responsible for the high titres of IgM and for the production of auto- and trypanosome non-specific antibodies.²⁰ We used the blot assay described by Taylor *et al.*³² to monitor immunoglobulin-secreting splenic B cells. Our data showed that IgM-secreting cells occurred in both B-cell populations, without preference for either one. However, production of antibodies to non-trypanosome antigens was always higher in the CD5⁺ B-cell population. We believe that these differences may even be greater, as we suspect that the sorted CD5⁻ fractions still contain some B cells expressing low levels of membrane CD5. The bias for production of trypanosome non-specific antibodies by CD5⁺ B cells, together with the fact that CD5⁺ B cells constitute the largest B-cell population in blood and spleen of infected cattle,²⁰ indicates that this B-cell subset is the main source of IgM antibodies to non-trypanosome antigens in infected cattle. CD5⁺ B cells have also been shown to be involved in the production of parasite non-specific antibodies in *T. cruzi* infections in mice.^{24,25}

Three features of trypanosome non-specific antibodies,

restriction to the IgM isotype, polyreactivity¹⁴ and predominant secretion by CD5⁺ B cells, strongly suggest that they are part of the 'natural' antibodies. Murine and human CD5⁺ B cells have been shown to be the main source of natural antibodies. These natural antibodies are predominantly IgM and are polyreactive, binding to several antigens, including self-antigens, with low affinities.³³ Elevated numbers of CD5⁺ B cells are found in patients with autoimmune diseases, cancer, AIDS, transplants and in early life (reviewed in refs. 34 and 35). However, up-regulation of CD5⁺ B cells is not always associated with pathogenic autoantibodies or disease activity.^{33,34} According to one hypothesis, the anti-self and/or polyreactive antibodies produced by the CD5⁺ population, which make up most of the 'natural' antibodies found in the normal individual, are positively selected by a set of dominant autoantigens, called 'immunological homunculus'.³⁶ A stable network of low-affinity autoantibodies and this set of autoantigens act as a controlling mechanism to prevent production of more dangerous anti-self antibodies following activation of CD5⁻ B cells by cross-reactive epitopes on foreign antigens.

We therefore suggest that the trypanosome non-specific antibodies detected in trypanosome-infected cattle are derived from natural antibodies that are predominantly secreted by the CD5⁺ B cells at low levels in a normal healthy state. Since the repertoire of natural antibodies has been found to be species-specific,³⁷ it is not surprising that parasite non-specific antibodies in cattle and mice react with different antigens, as discussed before. Natural antibodies occur in the absence of any known antigen stimulation,²¹ and this may explain why trypanosome non-specific antibodies are exclusively IgM. In contrast, production of trypanosome-specific antibodies is antigen-driven and can therefore be expected to undergo an isotype switch to IgG. An impairment in isotype switching has been suggested to occur in infected Boran cattle to explain lower IgG1 levels to certain trypanosome antigens when compared to N'Dama.¹⁴ However, since trypanosome non-specific antibodies were restricted to the IgM isotype in both breeds, their occurrence cannot be simply explained by the lesion in isotype switching described above. The same mechanism that up-regulates CD5⁺ B cells in immunodeficient states in humans may be responsible for their up-regulation in infected cattle²⁰ and for the subsequent increase in trypanosome non-specific antibodies. Severe immunosuppression is

characteristic of trypanosome infections (reviewed in refs. 1 and 38).

Parasite non-specific antibodies have been reported in both *T. cruzi* (Chagas' disease) and African trypanosomiasis infections, but autoimmunity and pathology have only been observed in chronic Chagas' disease.⁹ The biological significance of the trypanosome non-specific antibodies in African trypanosomiasis remains unknown. Like natural antibodies, they might play an immunoregulatory role, preventing the induction of autoimmune responses by parasite-epitopes that cross-react with self antigens.³⁶ Failure to do so could trigger pathogenic autoimmune responses, such as those seen in chronic *T. cruzi* infections.³⁹

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