Parasite-specific antibody responses of ruminants infected with *Trypanosoma vivax*

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SUMMARY

Sera from goats and cattle that were infected with two *Trypanosoma vivax* clones (ILDat 1.2 and ILDat 2.1) derived from different stocks were analysed for antibody activity against the variable surface glycoproteins (VSGs) of the infecting clones by enzyme-linked immune assays (ELISA) and immune lysis. To obtain purified VSG, lysed trypanosomes were separated on dodecyl sulphate–polyacrylamide gels. The gels were copper stained and the VSG protein band was excised from the gel. After destaining, the proteins were electroeluted from the gel slices and used as antigens in ELISA. High titres of IgM and IgG_1 antibodies and lytic antibodies against the VSG of the infecting clone were detected. The IgG_1 response appeared about 4 days later than the IgM response. IgG_2 antibodies were only detected in goats and cattle that were infected with ILDat 1.2. Two goats and two calves that were infected with ILDat 1.2 showed recurrent peaks in lytic activity and of IgM and IgG_1 antibody activity to the VSG of the infecting variable antigenic type (VAT). Two goats that were infected with ILDat 2.1 showed a similar pattern, but in two other goats there was a recurrent peak only in the IgM class. Recurrent peaks of antibody activity to the VSG of ILDat 1.2 and ILDat 2.1 were not detected in the sera of goats that had been inoculated with irradiated trypanosomes or that had been infected with an unrelated *T. vivax* clone. The recurrence of antibody peaks against the VSG of infecting VATs suggests that trypanosomes with completely or partially identical surface determinants reappear during *T. vivax* infection of ruminants.

Key words: *Trypanosoma vivax*, variable surface glycoproteins, antigens, antibody response.

INTRODUCTION

The African trypanosomiases are diseases of medical and veterinary importance, the latter constituting a major constraint to livestock production in areas of Africa that are infested by the tsetse fly (Morrison, Murray & McIntyre, 1981). Infections are characterized by fluctuating parasitaemias and, during the infection, the trypanosomes demonstrate a capacity to express different surface antigens (Vickerman, 1974; Gray & Luckins, 1976; Doyle, 1977). These antigens are glycoproteins that cover the surface of the trypanosome (reviewed by Turner, 1982).

During infection, an unknown number of immunologically different variable surface glycoproteins (VSGs) can be expressed, giving rise to trypanosome populations of different variable antigen types (VATs). The antibody responses that can contribute to protection of the infected host are thought to be directed against the exposed determinants of the VSG (Murray & Urquhart, 1977). The ability of antibodies to invariant antigens to protect against trypanosome infection has been suggested only in a single instance (Olenick *et al.* 1988). Antigenic variation, therefore, enables the parasite to evade the host's immune response and allows the infection to persist.

Studies of antigenic variation and the consequent identification, isolation and biochemical descriptions of trypanosome VSG have been concentrated on *Trypanosoma brucei*. Antigenic variation of the more important parasite of livestock, *T. vivax*, has been described in ruminants (Clarkson & Awan, 1969; Jones & Clarkson, 1971; Dar, 1972) and mice (de Gee, Shah & Doyle, 1979, 1981; Barry, 1986; Barry & Gathuo, 1984; Gardiner *et al.* 1986). The antigenic instability (Barry & Gathuo, 1984) and fragility (Gardiner *et al.* 1987) of *T. vivax* and the refractoriness of rodent hosts to infection with the parasite have made it impossible to obtain large numbers of VAT homogeneous trypanosomes for the purification of VSG. However, more recently, techniques have been devised for the characterization and isolation of *T. vivax* VSG from relatively small amounts of parasite material (Gardiner *et al.* 1987). The general characteristics of the VSG of *T. vivax* were shown to be similar to those described for other trypanosome species but the molecular weight was smaller than those reported for *T. brucei* VSGs. The antibody responses to the surface antigens of *T. vivax* have so far been measured by the immune lysis test that employs live trypanosomes. In this study, the VSG-specific antibody response of infected ruminants was further analysed using purified VSG in enzyme-linked immunosorbent assays (ELISA). To obtain sufficient purified VSG as antigen, a method of eluting proteins from copper-

stained sodium dodecyl sulphate–polyacrylamide gels (Lee, Levin & Branton, 1987) was applied to *T. vivax* VSGs.

MATERIALS AND METHODS

Experimental animals

Adult male East African cross-bred goats and male Boran cattle, 6 months of age, were obtained from areas in Kenya known to be free of trypanosomiasis and were kept in fly-proof isolation facilities. Adult male and female C3H/He mice and male Swiss mice were obtained from the ILRAD breeding colony. Mice were sublethally irradiated by exposure to 650 rad. of gamma-irradiation from a ^{137}Caesium source.

Trypanosomes

T. vivax ILDat 1.2 (Barry & Gathuo, 1984) is a clone derived from *T. vivax* Y486, isolated from an infected Zebu cow in Zaria, Nigeria and which is naturally infective for rodents (Leeflang, Buys & Blotkamp, 1976). ILDat 2.1 (Gardiner & Clarke, 1987) is a clone from IL 2006, a derivative of EATRO 1262, isolated in Lugala, Uganda and has been adapted to rodent passage (Gathuo, Nantulya & Gardiner, 1987). *T. vivax* IL 2969 is a clone derived in culture from the stock IL 2133, originally isolated from a steer at Kilifi, Kenya, as has previously been described (Vos *et al.* 1988) and is confined to infection of ruminants. C3H/He mice were infected to obtain parasites for infection of goats and for use in the immune lysis test, and Swiss mice to obtain parasites for the purification of VSG. The antigenic homogeneity of the trypanosomes was checked by immune lysis using reference antisera (Barry & Gathuo, 1984). ILDat 1.2 was 97–100% homogeneous and ILDat 2.1 was 100%. In some experiments trypanosomes were irradiated by exposure to 60000 rad. of gamma irradiation (Sendashonga & Black, 1982).

Experimental design

Goats and cattle were infected by intravenous inoculation with either of the 2 clones of *T. vivax* as follows: 2 goats and 2 steers were inoculated with 10^6 bloodstream forms of ILDat 1.2. Four goats were inoculated with 10^6 bloodstream forms of ILDat 2.1. Two goats were inoculated with 10^8 irradiated ILDat 1.2. Blood samples were collected daily from the jugular vein in EDTA and examined for the presence of trypanosomes using the haematocrit centrifugation technique (Woo, 1970). Parasitaemias of lower than 10^5 trypanosomes/ml were estimated by the method of Paris, Murray & McOdimba (1982); higher parasitaemias were accurately counted in a haemocytometer. Sera were collected twice a week, stored at −60 °C, and later tested for antibody activity by ELISA and immune lysis. Trypanosomes from each parasitaemic wave in animals originally infected with ILDat 1.2, were analysed for the presence of the infecting clone by immune lysis with reference sera (Barry & Gathuo, 1984), provided that the parasitaemia was greater than 5×10^5 parasites/ml. Sera of goats that had been infected by tsetse with IL 2969 were included as a control for the specificity of the response to the purified VSGs.

Preparation of T. vivax *variable surface glycoproteins*

Trypanosomes were isolated from infected mouse blood by anion-exchange chromatography using DEAE 52 (Lanham & Godfrey, 1970), solubilized at 4×10^8 trypanosomes/ml in buffer containing 2% (w/v) sodium dodecyl sulphate (SDS) and 5% (v/v) 2-mercaptoethanol and then boiled. The proteins were separated on SDS–polyacrylamide gels (SDS–PAGE) employing a 5–15% acrylamide gradient (Laemmli, 1971). The gels were stained with 0·3 M copper chloride according to a method described by Lee *et al.* (1987). The surface molecules of ILDat 1.2 and ILDat 2.1 have molecular weights between 43000 and 46000 (Gardiner *et al.* 1987) and 38000 and 40000 (unpublished results), respectively.

Slices of acrylamide containing VSG which had been excised from copper stained gels (32 cm × 20 cm × 2 mm), were destained in three changes of 0·25 M EDTA/0·25 M Tris–HCl, pH 9·0, and electroeluted in a buffer containing 50 mM ammonium bicarbonate, 1 mM dithiothreitol and 0·1% SDS. Electroelution was performed in an electroelutor/concentrator (model no. ECU 040, CBS Scientific Co., Del Mar, California) for 17 h at 60 mA at room temperature. The eluted proteins were dialysed overnight at room temperature in distilled water containing 0·02% sodium azide, the SDS was removed by extractigel (Pierce, Illinois) and the proteins were stored at −60 °C until further use in ELISA

Test for purity of electroeluted VSG

To ensure that the proteins purified by electroelution (see above) from excised bands of polyacrylamide gels were indeed VSGs, proteins purified from surface-biotinylated clones (Hurley, Finkelstein & Holst, 1985) were subsequently electrophoresed in the same gels as whole lysates of the same clones prepared after biotinylation. Exact correspondences in molecular weight of biotinylated proteins were seen when purified proteins and whole lysates were compared (Fig. 1). Further, eluted proteins were run on SDS–PAGE and compared with protein patterns resulting from the electrophoresis of whole solu-

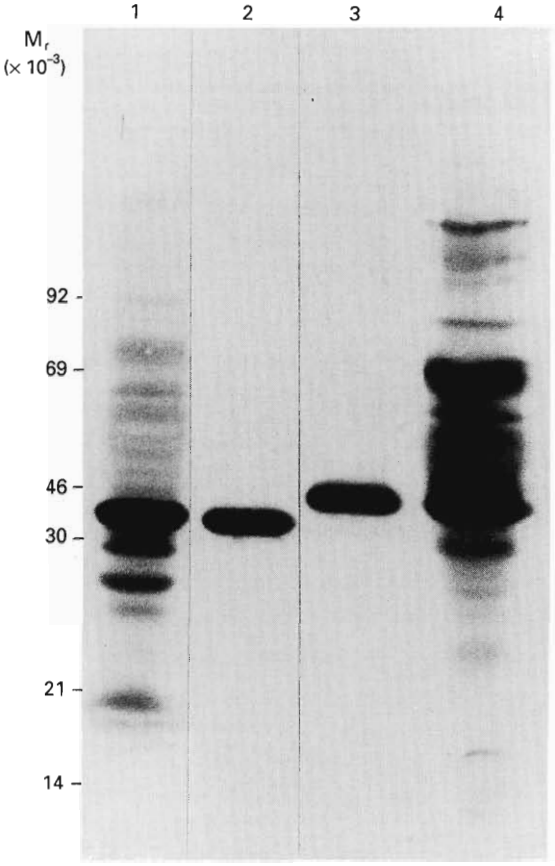

Fig. 1. Comparison of the proteins of whole, biotinylated *Trypanosoma vivax* clones and their respective, purified VSGs after SDS–PAGE. Lane 1, whole, biotinylated ILDat 2.1; Lane 2, excised and electro-eluted VSG of biotinylated ILDat 2.1; Lane 3, excised and electro-eluted VSG of ILDat 1.2; Lane 4, whole biotinylated ILDat 1.2.

bulized trypanosomes and with molecular weight markers. The gels were stained with Coomassie brilliant blue R-250 and destained in 20 % ethanol or transferred to nitrocellulose. The nitrocellulose blots were incubated with antisera that had been raised in rabbits to the excised protein bands (Gardiner *et al.* 1987). After incubation with 10 μg of biotinylated protein A (Vector Labs, California), the bands were visualized as described (Hurley *et al.* 1985; Gardiner *et al.* 1987).

Enzyme-linked immunosorbent assay (ELISA)

Flat-bottomed microelisa plates (Immulon, Dynatech, Alexandria, VA, USA) were coated with 7 μg/ml VSG in 0·01 M phosphate-buffered saline (PBS), pH 7·2, for 2 h at 37 °C, and then overnight at 4 °C. The antibody activity of sera from infected animals to purified VSG and trypanosome lysate (10 μg/ml PBS) was tested by ELISA as previously described (Voller, Bidwell & Bartlett, 1976; Vos *et al.* 1988).

IgG_1 and IgG_2 were isolated from sera of uninfected goats as described by McGuire, Musoke & Kurtti (1979). Further purification was obtained by chromatography on protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in 0·1 M phosphate buffer, pH 9·1 (Delacroix & Vaerman, 1979). The euglobulin fraction of uninfected goats was processed as described by Fey *et al.* (1976) and separated on Sepharose 6B (Pharmacia, Uppsala, Sweden) to obtain IgM. The IgM fraction was further purified by isoelectric focusing (McGuire *et al.* 1979). Antisera to purified goat IgG_1, IgG_2 and IgM were prepared in rabbits as described by McGuire *et al.* (1979) and labelled with horseradish peroxidase according to the method described by Wilson & Nakane (1978). The peroxidase-conjugated antibodies against goat IgG_1 and IgG_2 were used at a dilution of 1:2000 in PBS and against IgM at 1:1000. Monoclonal antibodies against bovine IgG_1, IgG_2 and IgM (kindly donated by Dr D. Williams, ILRAD) were diluted 1:1000. Peroxidase-conjugated goat anti-mouse Ig (Amersham, UK) was used at a dilution of 1:2500.

Immune lysis test (ILT)

Antibodies against surface antigens of *T. vivax* were measured in the immune lysis test as described by Barry & Gathuo (1984). The starting dilution of serum was 1:5 and the last serum dilution which yielded greater than 50 % parasite lysis was recorded as the endpoint.

RESULTS

Preparation of T. vivax *variable surface glycoproteins*

In Fig. 1 it is shown that the bands that were excised from gels after copper staining represented the major surface protein when compared with surface biotinylated trypanosomes. In these experiments, development of the biotinylated protein samples to completion revealed a greater number of labelled proteins than earlier experiments designed to label the VSGs alone (Gardiner *et al.* 1987). The eluted proteins migrated as single bands upon electrophoresis and were recognized by antisera that had been raised against the excised VSGs (results not shown).

Variant-specific antibody response to T. vivax

Both goats and cattle which were infected with ILDat 1.2 showed a very similar pattern in their antibody responses to the VSG of the infecting clone. Representative examples are presented in Figs 2 and 3. From 9 days after infection IgG_1, IgG_2 and IgM antibodies could be detected. The IgM antibody titre generally peaked (1:1600) on day 9 whilst

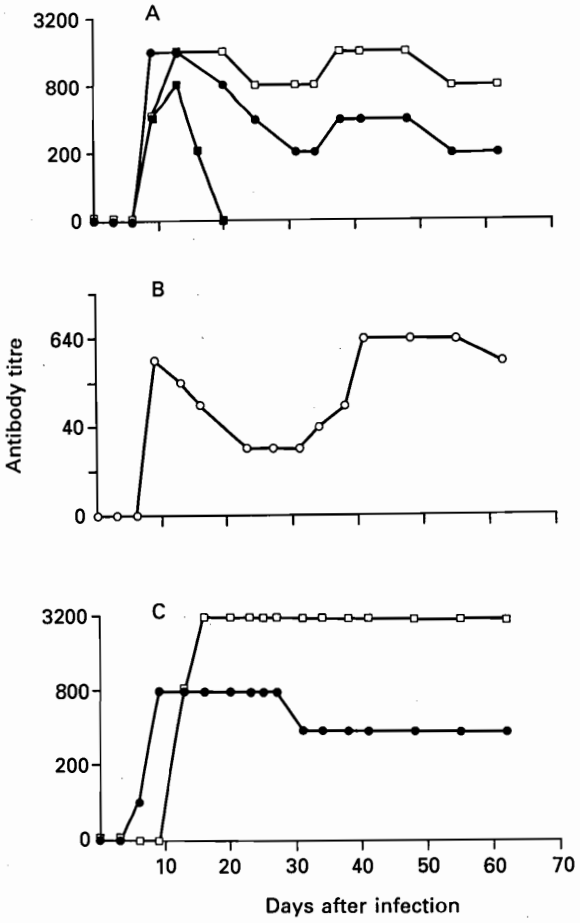

Fig. 2. Antibody responses of goat O150 after infection with *Trypanosoma vivax* ILDat 1.2. Reciprocal antibody titres (ELISA) to (A) purified VSG and (C) trypanosome lysate. (B) Reciprocal lytic titre to live ILDat 1.2. (□) IgG_1; (●) IgM; (■) IgG_2.

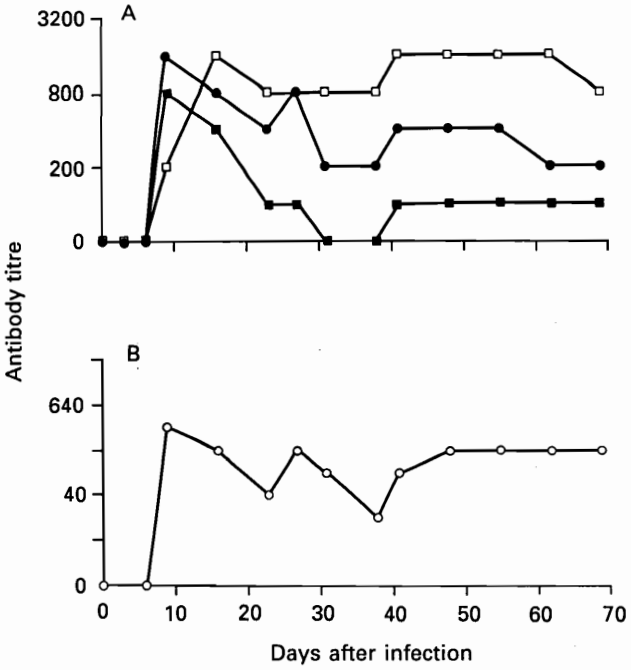

Fig. 3. Antibody titre of steer D 239 after infection with *Trypanosoma vivax* ILDat 1.2. (A) Reciprocal antibody titre (ELISA) to purified ILDat 1.2 VSG. (B) Reciprocal lytic titre to live ILDat 1.2. (□) IgG_1; (●) IgM; (■) IgG_2.

the IgG_1 antibody titre reached a peak of similar titre in all animals on day 13, 4 days later than the IgM response. After this initial peak, the antibody titres of both immunoglobulin classes dropped. The lowest IgM titres were reached between day 31 and day 34 in goats, and between day 31 and day 38 in the two cattle. After this drop the antibody titres rose again to a second peak. The IgG_1 antibody titre of the second peak reached the same height as the first peak in the 2 goats and 1 steer but was lower (1:800) in the other steer. The IgM titre of the second peak was lower (1:400) than the first peak (1:1600). In steer D 239 there was an extra IgM peak on day 27 after infection (Fig. 3). IgG_2 antibodies were found in all animals but these antibodies disappeared after day 20–23 after infection, except in steer D 239 where antibodies followed a pattern similar to that described for the IgG_1 and IgM antibodies but demonstrated only a very low antibody titre (1:100) in the second 'peak'.

The lytic antibody titres were measured in the same sera that were analysed by ELISA. There was a high lytic antibody titre to the infecting clones on day 9 after infection. These titres dropped to 1:20 by day 23 in the goats, and by day 31 in cattle, in a manner similar to the fall in the anti-VSG IgG_1 and IgM titres. This was followed by a rise, on day 34 in goats and day 38 in cattle, to an antibody titre of the same magnitude as during the first peak. In steer D 239 there was also an extra peak on day 27 after infection.

Five days after infection of goats and cattle $92{\cdot}5 \pm 1{\cdot}3$ % of the parasites consisted of ILDat 1.2. The percentage had decreased to $11{\cdot}2 \pm 1{\cdot}5$ % on day 7 and on day 9 no parasite was ILDat 1.2. ILDat 1.2 was not identified further during the infection.

The antibody response of goats to the other infecting clone (ILDat 2.1) was detected from day 6 after infection (Fig. 4). The IgM response showed a peak on day 9 after infection. The IgG_1 response reached peak titres 9–13 days after infection. All goats then showed an initial decrease followed by an increase in IgM antibody titre and 2 goats (A 162 and A 216) had a similar pattern in the IgG_1 antibody titre. In the other 2 goats (A 201 and A 184) the IgG_1 antibody titre increased during infection. IgG_2 antibodies were not detected.

The lytic antibody titre to this infecting clone showed a similar pattern to that described for ILDat 1.2 (Fig. 4). There was a peak of antibody response from day 9 to day 13 in 2 goats (A 162 and A 216) followed by a decline and a second rise.

Goats that were inoculated with irradiated ILDat 1.2 exhibited an IgG_1, IgM and lytic antibody

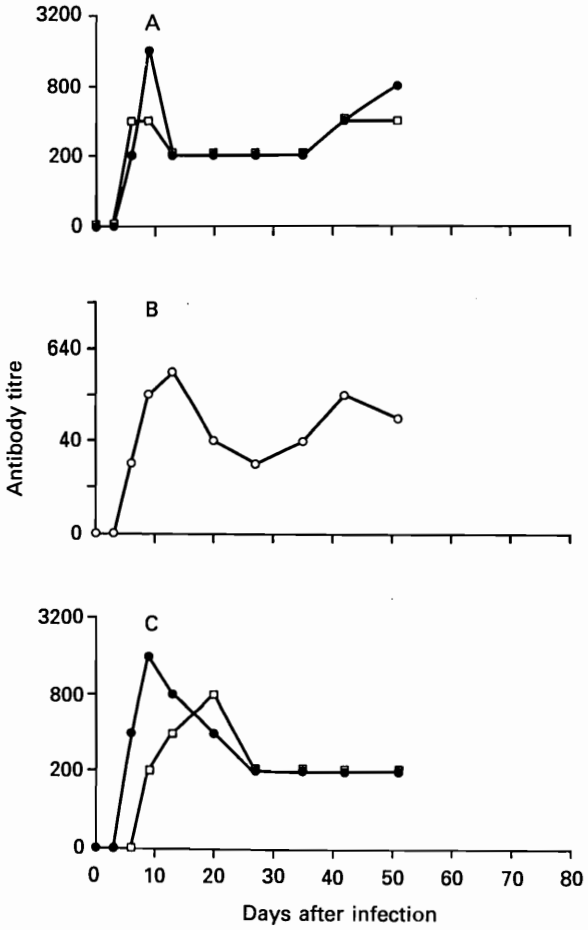

Fig. 4. Antibody responses of goat A 162 after infection with *Trypanosoma vivax* ILDat 2.1. Reciprocal antibody titres (ELISA) to (A) purified VSG and (C) trypanosome lysate. (B) Reciprocal lytic titre to live ILDat 2.1. (□) IgG_1; (●) IgM.

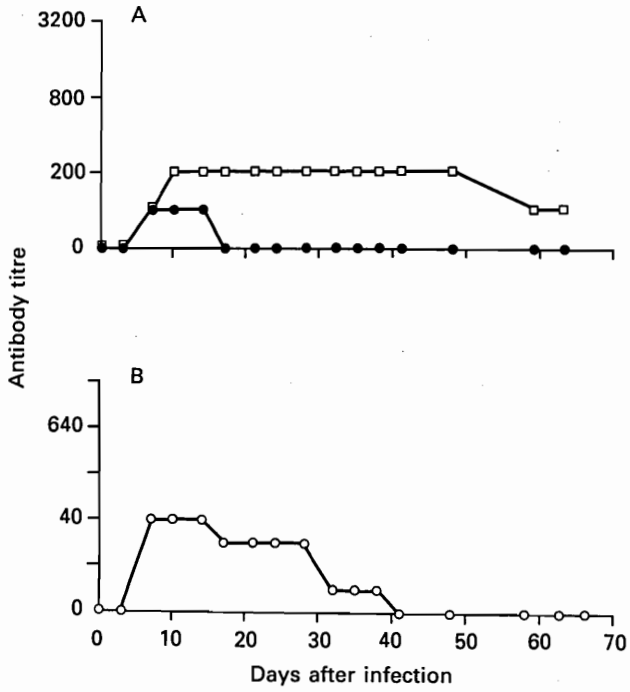

Fig. 5. Antibody responses of goat A 217 after inoculation with 10^8 irradiated *Trypanosoma vivax* ILDat 1.2. (A) Reciprocal antibody titre (ELISA) to purified VSG. (B) Reciprocal lytic antibody titre to live ILDat 1.2. (□) IgG_1; (●) IgM.

response that was lower than that of goats infected with live trypanosomes (Fig. 5). The IgM antibodies disappeared very rapidly and the IgG_1 antibody titre decreased but remained above background during the period of the experiment. The lytic antibody titre returned to pre-infection values between 40 and 50 days after infection. There were no recurrent peaks of antibody activity.

Recurrent peaks of antibody activity to purified VSG of ILDat 1.2 and ILDat 2.1 were also not detected in the sera of goats that were infected with an unrelated clone, IL 2969 (data not shown). In these goats IgG_1 antibody activity to both VSGs was detected from day 24 after infection but no IgM activity was found.

Antibody response to trypanosome lysate

Antibodies to lysates of the infecting clones were detected by ELISA and appeared at the same time of infection as antibodies to the purified VSG. IgG_1 and IgM antibodies were routinely detected, but IgG_2 in only one goat (A 184) and then later in infection. The IgG_1 titre rose during infection whereas the IgM titre declined after a first peak. Both IgM and IgG_1 titres, however, remained elevated during infection. There were no recurrent peaks of antibody activity to trypanosome lysates during infection and the antibody responses to homologous and heterologous *T. vivax* lysates were similar.

DISCUSSION

The antibody response of goats and cattle to the VSG of 2 clones of *T. vivax* was detected by immune lysis and ELISA and demonstrated at least 2 peaks of antibody activity to the infecting VAT and its VSG. The immune lysis test detects the antibody response to the exposed surface epitopes of the parasite since live trypanosomes are used in this test. Purified VSG was used to measure the antibody response by ELISA so that this test probably also measures responses to epitopes that are hidden in live trypanosomes.

Since it is not possible to obtain the large numbers of VAT homogeneous *T. vivax* organisms that would be necessary for the purification of VSG according to Cross (1975), VSGs were purified from copper-stained and destained polyacrylamide gel slices. Copper staining proved a very rapid method for visualizing the electrophoretically separated proteins of trypanosomes and the recovery of protein after electroelution from copper-stained gels has been

suggested to be as high as 90% (Lee *et al.* 1987). The single bands that were excised from polyacrylamide gels were shown to represent the VSGs of the *T. vivax* clones as they contained the major surface biotinylated protein of the clones, as demonstrated previously for clones of a Nigerian *T. vivax* (Gardiner *et al.* 1987).

Goats and cattle produced IgM and IgG antibodies to the surface and internal antigens of the infecting clone. Antibodies against the VSG consisted of IgM and IgG_1. IgG_2 antibodies were only detected in goats and cattle that were infected with ILDat 1.2 (Nigeria) but not with ILDat 2.1 (Uganda). Musoke *et al.* (1981) detected only IgG_1 and IgM antibodies to the VSG of two clones of *T. brucei* but Masake, Musoke & Nantulya (1983) detected antibodies of all three immunoglobulin types against the VSGs of three *T. congolense* clones. The induction of the IgG_2 response could therefore be dependent upon the infecting clone.

During the course of infection with the two *T. vivax* clones there were at least 2 peaks of lytic antibody activity and of IgM and IgG_1 antibodies against the infecting clone. The peaks of antibody activity in the IgM and IgG_1 response detected by the VSG ELISA coincided with peaks of lytic antibody activity, thus confirming that this response was directed against antigens on the surface of the parasite. The purified VSG preparation probably included epitopes that are not accessible to antibodies on live organisms but which might be shared since sera from goats that had been infected with an unrelated clone reacted with the purified VSG but not the infecting, live organisms in immune lysis tests (Vos, unpublished observations). This response appeared later in infection (day 24), only in the IgG_1 class, and recurrent peaks of antibody activity were not detected.

These results were similar to the observations of Nantulya *et al.* (1979) who recorded recurrent peaks of antibody activity to the infecting VATs of *T. brucei* within 7 weeks of infection of cattle. Recurrent peaks of antibody activity to the infecting VAT was also described for *T. congolense* infections in cattle (Masake *et al.* 1983). The second antibody peak against the infecting VAT was explained as a secondary antibody response against a completely or partially identical VAT that (re)appeared during infection (Nantulya *et al.* 1979; Masake *et al.* 1983). Barbet, Davis & McGuire (1982) have indeed identified two trypanosome populations originating from a single *T. brucei* parasite that were not identical but had partially homologous VSGs and shared similar exposed epitopes causing them to be serologically cross-reactive.

In the present experiments, ILDat 1.2 parasites, following their initial decline upon inoculation, were not again detected prior to the second antibody peak. This is in agreement with observations made with *T. brucei* (Nantulya *et al.* 1979). It could be that these parasites only constituted a small percentage of the population, were reduced in number by immune elimination, or were not detected because the parasitaemia was too low for use in the immune lysis test.

The primary response to the *T. vivax* VSGs consisted of an IgM peak that preceded the IgG response. The secondary peak was mainly IgG_1 in combination with a lower IgM response. The biological activity of the two peaks was not determined. Musoke *et al.* (1981), however, found that the neutralizing activity of IgM was the same in both peaks but the activity of IgG_1 had greatly increased in the second peak. Luckins (1976) detected most of the neutralizing activity against a *T. congolense* stock in the IgM fraction of sera of infected cattle. IgG, in contrast, had only slight neutralizing activity. The sera for the isolation of antibodies in this author's experiments, however, were probably collected before a secondary response had appeared since the peak of neutralizing activity was between days 17 and 22 and the sera were analysed only until day 35.

Antibodies against *T. vivax* lysates, in which common or invariant antigens predominate, appeared at the same time in infection and involved the same immunoglobulin classes as specific antibodies against the VSG. The response was directed against common *T. vivax* antigens since it reacted with lysates from homologous or heterologous clones. The value of antibodies against invariant antigens for the protection of the host is not known but is generally thought to be negligible (Murray & Urquhart, 1977). However, since the response to common antigens occurred in parallel to the VSG-specific response, the detection of such antibodies could be used as an indication of the capacity of the infected host to mount an immune response to this parasite. This would be useful for those *T. vivax* infections that cause very low parasitaemias when it is difficult to obtain sufficient parasites for VSG purification or even immune lysis tests.

The specific antibody responses against the VSG of *T. vivax* are very similar to the responses against VSGs of *T. brucei* and *T. congolense*. It would be interesting therefore to identify populations of *T. vivax* arising later in infections, which are cross-reactive with the initiating VAT and to determine whether they express partially or completely identical VSGs. This study provides indirect evidence for the existence of such populations.

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