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African Trypanosome Antigens Recognized During the Course of Infection in N'dama and Zebu Cattle†

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The humoral immune responses to *Trypanosoma brucei* infection were examined in N'dama and in Zebu, two breeds of cattle recognized for their differing susceptibility to trypanosomiasis. Regardless of the clinical course, animals of both breeds produced antibodies to nonsurface trypanosome antigen(s) detectable by both immunodiffusion and immune fluorescence. As a new approach to assessment of the humoral response to trypanosome infection, protein antigens responded to were isolated by immune precipitation, and their molecular weights were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This allowed the detection of differences in the immune response which correlated with the clinical course of the disease. All cattle of both breeds which exhibited a capacity to control the disease recognized at least one of three specific antigens: proteins of 110,000, 150,000, and 300,000 daltons. The N'dama, which proved less susceptible to the disease, generally responded to more of the three identified trypanosome protein antigens than did the Zebu. Animals which died of trypanosomiasis failed to produce detectable antibodies to any of the three specific proteins, although they sometimes exhibited antibodies to another trypanosome antigen.

African trypanosomes are responsible for widespread disease of humans and domestic animals in large areas of Africa. A unique characteristic of these protozoan parasites is that each cell is ensheathed in a surface coat composed of a matrix of a single glycoprotein (9, 13, 35). The parasites have the ability to vary this surface glycoprotein. The potential number of different variable surface glycoproteins expressible by any given trypanosome is unknown; however, it is in excess of 100 (6). Upon infection the immune response of the host appears to be largely directed against these surface antigens (10, 13, 34). Therefore, variation of these antigens enables the parasite to evade the host immune response, allowing persistence of infection.

In humans and domestic animals, African trypanosomiasis, if left untreated, often leads to death (11, 30). However, certain breeds of cattle, sheep, and goats as well as some species of wild animals exhibit a significant degree of reduced susceptibility to the pathological effects of trypanosome infection and may even spontaneously recover (1, 14, 23). This characteristic has been termed trypanotolerance. The mechanism(s) of resistance to the disease in these cases is unknown. Factors of both innate and acquired resistance may contribute. Some species and breeds may be poor hosts for the trypanosome for physiological reasons such as the presence of deleterious substances (16, 31) or the absence of some unknown essential nutrients (12) in their bloodstream or tissues. Alternatively, some animals may successfully control the parasite by mounting a more effective immune response. A more efficient immunological response to the numerous variable surface antigens encountered might be the mechanism for such resistance. Also, recognition of subsurface antigen(s) common to trypanosomes of different variable surface type might contribute to mechanism(s) by which the infection is controlled.

Animals infected with African trypanosomes produce antibodies against both variable surface antigen and nonvariable, common antigens (10). If an immune response to some common trypanosome antigen(s) contributes to host defense against the parasite, then recognition of such common antigen(s) should correlate with the course of infection. To date, the common trypanosome antigens recognized during infection have not been well characterized, and no comparison has been made between their recognition and the clinical course of the disease. In the present study we have examined the humoral responses to trypanosome infections in N'dama, a breed of cattle recognized for its reduced susceptibility to trypanosomiasis, and in Zebu, a

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susceptible breed (23). We found that the recognition of certain common trypanosome protein antigens correlated closely with the clinical course of the disease.

MATERIALS AND METHODS

Cattle. The work upon which the experiments reported here is based was part of a large-scale investigation into the phenomenon of trypanotolerance in cattle. That study which involved N'dama and Zebu cattle was performed in The Gambia during 1976 and 1977 (23, 25). Zebu cattle were purchased in Northern Senegal from a ranch well beyond the northern limits of the tsetse fly belt. N'dama cattle were obtained from the Government Agricultural Experimental Station at Yundum, The Gambia, a ranch considered free from trypanosomiasis risk, although surrounded by areas infested with *Glossina palpalis gambiensis*. Sera from all animals were checked by immunofluorescence on fixed trypanosomes (*Trypanosoma brucei*, *T. congolense*, and *T. vivax*) and found to be negative for antibodies to common trypanosome antigen before experimental infection (22). All animals used were adults aged 3 to 4 years at the start of the experiment. All nine Zebu were male, whereas the N'dama consisted of six males and three females.

Trypanosomes. *T. brucei* from two separate field isolations were used. Cattle were infected with trypanosomes obtained from a rat passage of *T. brucei* GUTR22 isolated from a naturally infected N'dama in The Gambia. The trypanosome used for immune assays was *T. brucei* ILTat 1.3. This is a cloned line of homogenous variable surface antigen type derived from *T. brucei* UHEMBO/64/EATRO/795 isolated in 1964 from an ox in Kenya.

Trypanotolerance study. The N'dama and Zebu were inoculated subcutaneously with 2.6×10^8 bloodstream forms of *T. brucei* (GUTR 22). After 5 months (20 weeks) these animals came under a natural challenge from *G. palpalis gambiensis*; at that time a daily mean of 0.17 flies were caught per trap. Jugular vein blood samples for anemia assessment, detection of parasites, and serum were collected before challenge and thereafter at regular intervals until the experiment was terminated 13 months later (week 57). Packed erythrocyte volume percent of blood samples was used to estimate the degree of anemia. The blood buffy coat darkground phase-contrast microscopic technique was used to detect trypanosomes (24).

Double immunodiffusion. Antigen-antibody reactions were performed by the method of Ouchterlony (26) in 0.8% (wt/vol) agarose dissolved in 50 mM Tris-hydrochloride buffer (pH 7.2) made 100 mM in NaCl and 0.01% (wt/vol) in thiomersalate and also containing 3% (wt/vol) polyethylene glycol to enhance precipitation of antigen-antibody complexes (15). Sera were used undiluted. The antigen mixture contained approximately 5 mg of trypanosome protein per ml extracted in the same manner as for immune precipitation (see below) in a buffer containing 20 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, 100 mM NaCl, and 0.1% (vol/vol) Nonidet P-40. Reactions were allowed to develop for 48 h at room temperature in a humid chamber.

Immune fluorescence. The immunoglobulin G fraction of rabbit anti-bovine immunoglobulin serum (Miles Laboratories, Slough, United Kingdom) was conjugated with fluorescein isothiocyanate (Aldrich Chemical Co., Dorset, United Kingdom) by dialysis (7), and the products were separated on DEAE cellulose (DE52; Whatman, Kent, United Kingdom) (4). The fraction eluted with 0.3 M NaCl was used at a dilution of 1:40 in phosphate-buffered saline (20 mM sodium phosphate, pH 7.6, 150 mM NaCl) containing 0.01% (wt/vol) Evans blue.

Immune fluorescence was performed on live and acetone-fixed trypanosomes. Trypanosomes (*T. brucei* ILTat 1.3) were isolated from infected blood by chromatography on DEAE-cellulose (20) and collected by centrifugation. Cells were suspended at a concentration of 5×10^7/ml in PSG buffer (60 mM sodium phosphate [pH 8.0], 45 mM NaCl, 55 mM glucose) containing 10% (vol/vol) fetal bovine serum. These cells were either used immediately for immune fluorescence on live trypanosomes by the method of Barbet and McGuire (2) or applied to wells of a fluorescent antibody microscope slide, allowed to dry, and fixed by incubation of the slide in acetone for 15 min for use in the immune fluorescent procedure described by Van Meirvenne et al. (33). After mounting, the trypanosomes were examined using the ×40 fluorescent phase-contrast oil objective on a Leitz Orthoplan microscope equipped with a Ploemopak vertical fluorescence illuminator.

Immune lysis. Trypanolysis tests were performed by the method of Van Meirvenne et al. (33). Briefly, suspensions containing 10^5 trypanosomes (*T. brucei* ILTat 1.3) in 5 μl of guinea pig serum were mixed with 5 μl of twofold dilutions of test sera in guinea pig serum. Reactions were incubated at room temperature for 2 h and then examined under the ×25 objective of a phase-contrast microscope, and the percentage lysed cells was recorded. Monospecific antiserum against the purified variable surface antigen of ILTat 1.3 was the gift of A. F. Barbet, Washington State University. This antiserum had been prepared in a rabbit (2) by using antigen purified by the method of Cross (9).

Immune precipitation of radiolabeled trypanosome proteins. In vivo-radiolabeled trypanosome proteins were obtained by intravenous injection of a mixture of 1 mCi of [^{35}S]methionine (about 1,000 Ci/mmol; Amersham International, Amersham, United Kingdom) and 1 mCi of [^{35}S]cysteine (about 600 Ci/mmol; Amersham) into sublethally irradiated (600 rad) NMRI mice 3 days after infection with *T. brucei*, ILTat 1.3. At 2 h after injection of the isotope, infected blood was collected. Trypanosomes were isolated from the blood by DEAE-cellulose chromatography (20) and collected by centrifugation at 1,800 × *g* for 20 min at 4°C. An extract of trypanosome proteins was prepared by suspending the cells (2×10^9) in 10 ml of extraction buffer (5 mM Tris-hydrochloride (pH 9.2), 1 mM EDTA, 1% Nonidet P-40, 5 mM iodoacetamide, 0.5 mM phenylmethyl sulfonyl fluoride) and following the procedure previously described (29), with the exception that the final dialysis buffer was at pH 8.5. Extracts prepared in this manner contained greater than two thirds of the total incorporated radioactivity.

The immune precipitation technique used to characterize trypanosome antigens responded to by the host humoral immune system was a modification of the *Staphylococcus aureus* immunoadsorbent method of Kessler (18) as previously described (29). The choice

of this technique was dictated by the small amounts of sera available for testing. Although bovine immunoglobulins usually do not bind well to *S. aureus* protein A (19), it is possible to improve binding by raising the pH (A. J. Musoke, personal communication); therefore, these precipitations were performed at pH 8.5.

After immune precipitation and extensive washing antigen-antibody-bacteria complexes were suspended in 50 μl of electrophoresis sample buffer (62 mM Tris-HCl [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate, 5% [vol/vol] 2-mercaptoethanol, 7.5% [vol/vol] glycerol). The samples were heated for 5 min at 100°C followed by centrifugation for 20 min at 3,000 × *g* to remove bacteria. Electrophoresis was performed in a 7.5 to 17.5% gradient polyacrylamide slab gel in the presence of 0.1% sodium dodecyl sulfate (21). Autoradiography of the dried gel slab was performed with Ultrofilm ^{3}H (LKB-Produkter AB, Bromma, Sweden).

In these experiments specific immunoadsorbence was very low compared with non-immunospecific background adsorbance despite the better binding obtained at the elevated pH. The addition of rabbit anti-bovine immunoglobulin to create a tertiary complex increased the background without substantially improving specific precipitation (data not shown). Nevertheless, it is possible to differentiate specifically precipitated trypanosome antigens from nonimmunospecific background by careful comparison of proteins precipitated by serum collected from animals after infection with those precipitated by serum from the same animal taken before infection.

RESULTS

Susceptibility of N'dama and Zebu cattle to trypanosomiasis. The N'dama cattle proved much less susceptible to trypanosomiasis than the Zebu cattle after both needle challenge with *T. brucei* and exposure to natural challenge from *G. palpalis gambiensis*. The level of the first peak of parasitemia was significantly lower in the N'dama (25), and the prevalence of parasitemia over the first 20 weeks was less in N'dama than in Zebu (Fig. 1). The N'dama developed less severe anemia (Table 1) and suffered no deaths, whereas five of the nine Zebu died of trypanosomiasis. During exposure to *G. palpalis gambiensis*, three of the nine N'dama became transiently parasitemic, whereas seven of the eight remaining Zebu suffered reinfection (in both groups *T. vivax* and *T. congolense* were detected as well as *T. brucei*).

On the basis of prevalence of parasites during the first 20 weeks of the study, the Zebu cattle can be divided into two groups. By week 12 after infection with *T. brucei*, five Zebu (no. 1, 3, 7, 10, and 12) had eliminated or reduced their parasites to nondetectable levels as had the N'dama (Fig. 1). In the remaining group of four Zebu (no. 5, 6, 9, and 13), all remained parasitemic until death (Fig. 1). These differences in parasitemia between groups of Zebu were reflected in the levels of anemia (Table 1). After week 20 four of the five Zebu in the first group became demonstrably reinfected; only one animal (no. 1) subsequently died, whereas the remaining four animals in this group again demonstrated improvement.

Reactivity of cattle sera for trypanosome anti-

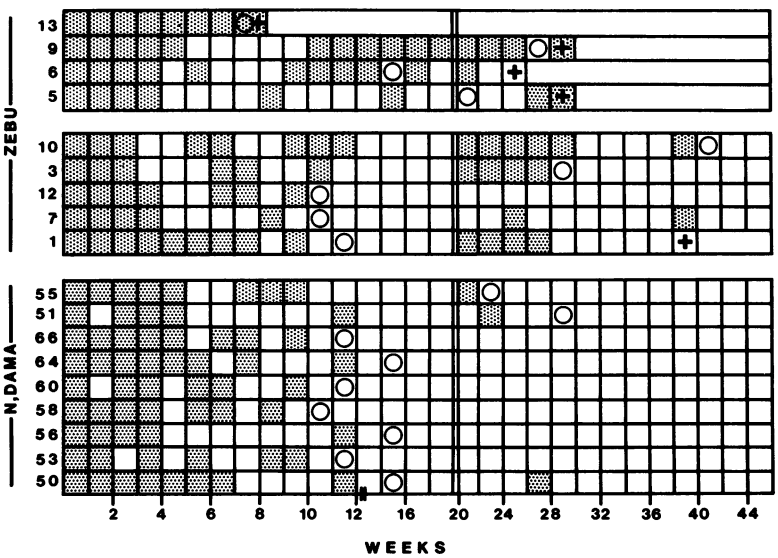

FIG. 1. Prevalence of trypanosomes. The detection of parasitemia is shown on a weekly basis up to week 12 and thereafter at 2-week intervals. The hatching indicates parasitemia was detected on at least one occasion during a 7 or a 14-day time span. The time of commencement of challenge with *G. palpalis gambiensis* is indicated by the vertical bars at week 20. The times when the test serum samples were collected (○) and the times of deaths (+) are also indicated.

TABLE 1. Association between the number recognized of a set of three trypanosome antigens[a] and the clinical course of the disease in N'dama and Zebu cattle

Breed	No. of cattle	Clinical course as judged by PCV%[b]				Clinical outcome	Total no. of antigens recognized		
		Preinfection	Lowest	Test[c]	Final		3	2	1
N'dama	9	35 ± 4	21 ± 4	25 ± 5	29 ± 5	Recovered	3	5	1
Zebu	5	37 ± 4	19 ± 4	26 ± 4	25 ± 6	Recovered[d]	0	4	1
Zebu	4	36 ± 6	16 ± 4	25 ± 8	17 ± 4	Death	0	0	0

[a] These antigens were the 300,000-, 150,000- and 110,000-dalton proteins discussed in the text.
[b] PCV%, Packed erythrocyte volume percent.
[c] Average PCV at the time the serum was tested.
[d] This group included Zebu no. 1, which recovered from the initial experimental *T. brucei* infection but eventually died after field reinfection.

gen. Preinfection sera and sera from recovering and dying animals were tested for reactivity against a trypanosome antigen extract by the method of double immunodiffusion. Serum samples for testing were selected on the basis of the absence of parasitemia (Fig. 1), where possible, to avoid the possibility that absorption by trypanosome antigens in vivo would prevent the detection of anti-trypanosome antibodies. Sera from all recovering N'dama and Zebu had developed the capacity to precipitate trypanosome antigens. Precipitin lines were also formed in immunodiffusion tests by sera from some Zebu which subsequently died.

Experiments were performed to investigate the location of the trypanosome antigen(s) recognized. First, reactivity of sera was examined by immune fluorescence. Demonstrated in Fig. 2 is the positive fluorescence on acetone-fixed trypanosomes detected by using a dilution of 1:50 of serum from N'dama no. 53. All trypanosomes showed positive fluorescence. The same serum was negative when tested at a dilution of 1:5 on live trypanosomes. Similar results were obtained with sera from other N'dama and Zebu which had recovered as well as with sera from some of the Zebu which died. Positive fluorescence on acetone-fixed trypanosomes, but negative results with live trypanosomes, indicated that these sera only contained antibodies against subsurface trypanosome antigens. Sera were also tested for their ability to lyse live trypanosomes as an assay for anti-surface antibodies. Antisera from N'dama no. 53 and 66 were unable to lyse ILTat 1.3 trypanosomes in contrast to a rabbit serum containing antibodies against the surface antigen of these trypanosomes.

Immune precipitation analysis of recognized trypanosome antigens. To characterize protein antigen(s) recognized by host antibodies sera were investigated by using *S. aureus*-mediated immune precipitation. Figure 3 shows a typical result in which radiolabeled trypanosome proteins were immune precipitated by sera from two N'dama (no. 58 and 60). Serum from animal 58 immunospecifically recognized a protein of 150,000 daltons and a protein of approximately 300,000 daltons (Fig. 3, lane B). Serum from animal No. 60 recognized these two proteins and also proteins of 110,000 and 20,000 daltons (Fig. 3, lane D). Figure 4 shows the trypanosome proteins immune precipitated by sera from two

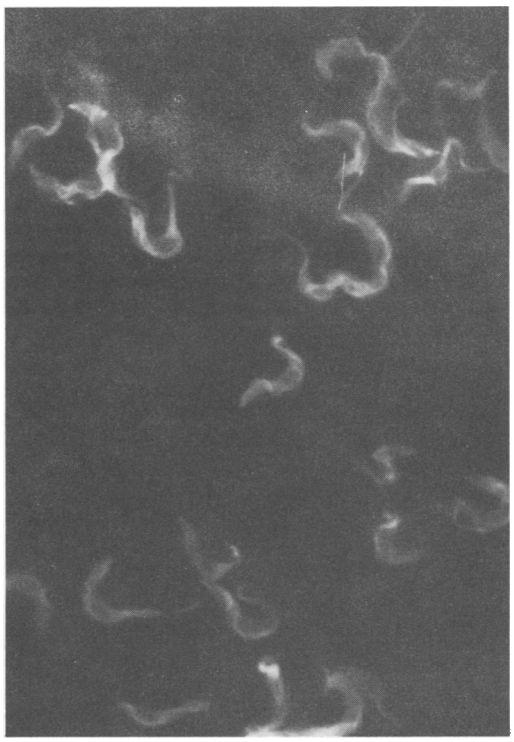

FIG. 2. Recognition of trypanosome antigen(s) by a recovered bovine serum demonstrated by the technique of immune fluorescence. Indirect immune fluorescence was performed on acetone-fixed *T. brucei* ILTat 1.3 by using serum from N'dama no. 53 after recovery at a dilution of 1:50 and FITC-conjugated rabbit anti-bovine immunoglobulin G.

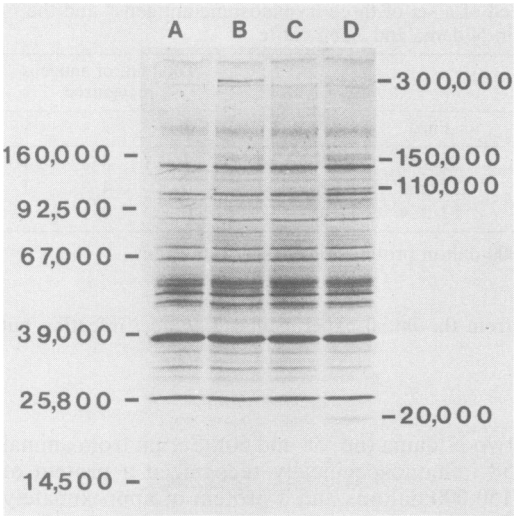

FIG. 3. Electrophoretic analysis of trypanosome antigens immune precipitated by recovered bovine sera. Proteins synthesized by *T. brucei* ILTat 1.3 were labeled by incubation with [^{35}S]methionine and [^{35}S]cysteine in vivo for 2 h. Trypanosomes were collected, and labeled proteins were extracted and immune precipitated with cow sera by a *S. aureus* protein A-mediated procedure (29). Precipitated proteins were resolved by sodium dodecyl sulfate-gel electrophoresis and examined by autoradiography. Demonstrated are radiolabeled polypeptides precipitated with: (A) preinfection serum from N'dama no. 58, (B) postrecovery serum from N'dama no. 58, (C) preinfection serum from N'dama no. 60, (D) postrecovery serum from N'dama no. 60. Marker protein molecular weights are indicated in the margin to the left of lane A: *E. coli* RNA polymerase, 160,000 and 39,000; phosphorylase α, 92,500; bovine serum albumin, 67,000; chymotrypsinogen, 25,800; lysozyme, 14,500. Recognized trypanosome antigens are indicated in the margin to the right of lane D.

Zebu (no. 5 and 6) which died of trypanosomiasis. Serum from animal no. 5 failed to immunospecifically precipitate any trypanosome proteins (Fig. 4, lane B). Serum from animal 6 did contain antibodies against a trypanosome protein of 40,000 daltons (Fig. 4, lane D).

The sera of all recovered cattle recognized at least one of a set of three proteins of 300,000, 150,000, and 110,000 daltons. Antigens of 180,000, 100,000, 45,000, 40,000, and 20,000 daltons were less consistently recognized. The results of immune precipitations with the sera samples taken at the times noted in Fig. 1 are summarized in Table 1. There was a tendency for the N-dama to produce antibodies against more of these proteins than did the Zebu; eight of the nine N'dama recognized two or more, whereas only four of the nine Zebu identified two of these proteins and not one Zebu recognized all three. All sera from animals which died, apart from Zebu no. 1, failed to recognize any of these three protein antigens, although they sometimes recognized another trypanosome protein. In addition to those serum samples we assayed by immune precipitation all serum samples collected throughout the course of the experiment from some animals (N'dama no. 51 and 60; Zebu no. 1, 3, 5, 6, 9, 12 and 13). The antibodies produced against the antigens of 300,000, 150,000, and 110,000 daltons appeared in sera taken from all recovering animals serially examined at least one month before apparent elimination of parasites from the bloodstream.

DISCUSSION

In the present investigation, we found a positive correlation between the capacity of the bovine host's immune system to recognize certain subsurface trypanosome proteins and the clinical course of the disease. It had previously been established that the trypanosome-infected host produces antibodies against nonsurface trypanosome antigens by complement fixation (28), indirect agglutination (3), and immune fluorescent techniques (27). However, these methods did not characterize the individual antigens involved, nor was recognition of specific antigens correlated with susceptibility to the disease.

Using an immune precipitation technique and

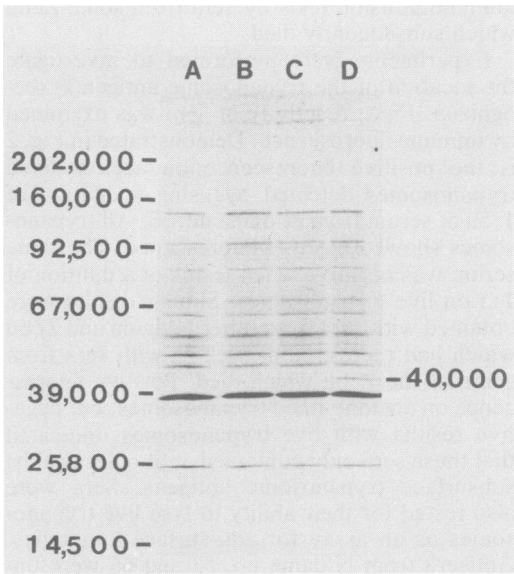

FIG. 4. Electrophoretic analysis of trypanosome antigens immune precipitated by sera from dying cattle. Demonstrated are radiolabeled proteins precipitated with: (A) preinfection serum from Zebu no. 5, (B) predeath serum from Zebu no. 5, (C) preinfection serum from Zebu no. 6, (D) predeath serum from Zebu no. 6. The position of myosin (202,000 daltons) along with the positions of the other markers listed in Fig. 3 are indicated in the margin to the left of lane A. Recognized trypanosome antigen is indicated in the margin to the right of lane D.

molecular weight analysis, we found that several trypanosome protein antigens were recognized by antibodies produced by trypanosome infected N'dama and Zebu. These antigens ranged in molecular weight from 20,000 to 300,000, but three in particular, with molecular weights of 300,000, 150,000 and 110,000, were more frequently recognized than others. That the same sera failed to detect any antigen on the surface of living trypanosomes by immune fluorescence and by immune lysis tests indicates that the proteins recognized are subsurface trypanosome antigens. Furthermore, the size and location of these antigens also precludes their being the trypanosome variable surface glycoprotein. It was observed that any animal with the capacity to recognize one, two, or all three of these antigens showed a significantly less severe anemia and a capacity to control parasitemia. Furthermore, in serial immune precipitations, antibodies against these antigens were detected in sera of recovering cattle even in samples collected before the animals had eliminated the parasites, but were never detected in sera of animals unable to control the infection.

The production of antibodies against these antigens therefore appeared to be a factor predictive of effective host control of trypanosome infection. The N'dama, a breed recognized for its superior resistance to trypanosomiasis, showed a greater capacity to recognize this set of three proteins than the Zebu, a susceptible breed. Nevertheless, those Zebu in the present study which showed greater resistance also recognized these antigens, although not quite to the same extent.

The role of immune response to the three trypanosome antigens, identified by this investigation, in host control of the disease awaits understanding. Many possibilities exist. It is difficult to envisage antibody penetrating the trypanosome surface coat to reach a target within the cell, but if these antigens are present in the uncoated membrane of the flagellar pocket, they might be accessible to direct antibody effects. Alternatively, an antibody response interfering with the action of any of the several pathogenic molecules generated by trypanosomes (8, 17, 32) might moderate the disease such that a less debilitated animal could more efficiently eliminate new waves of parasitemia. A third possibility is that three common antigens act as carriers promoting a primed immune response to the variable surface antigens; such a mechanism has been proposed in immunity to malaria (5). It must also be considered that the recognition of these three proteins might only be coincidental to some other mechanism of host resistance such as a superior innate immune responsiveness in some cattle allowing them to control trypanosome infection by an efficient antibody response to the variable surface antigens encountered. A drug-cured Zebu cow failed to produce antibodies against any trypanosome antigens (data not shown); therefore, it is unlikely that simple alleviation of the immunosuppression associated with trypanosomiasis (8, 10) in recovering animals could completely explain the antibody responses observed.

Additional experiments are required to characterize and ascertain the significance of the antibody response against these identified trypanosome protein antigens. Whether such antigens are present only in *T. brucei* or whether they also occur in *T. congolense* and *T. vivax* remains to be determined. Furthermore it is necessary to search for any non protein antigen(s) whose recognition may also correlate with reduced susceptibility to trypanosomiasis. Currently, studies similar to those described here are being performed with several species of African wild animals reputed to be very resistant to African trypanosomiasis with the intention of obtaining enough recovery sera to both characterize and purify recognized common antigens. Purification of recognized common antigens will permit further investigation into the roll of these antigens in host resistance to trypanosomiasis.

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