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Characterization, Quantitation, Regulation of Production
and Cellular Origin of the Trypanotoxin Present in Serum
From African Buffalo

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Executive Summary: The project goal was to elucidate the molecular basis for resistance to trypanosomiasis in cape buffalo. We established that cape buffalo have an innate capacity to curb parasitemia (Appendix 1). The animals also had a 150 kDa serum protein that killed all species of African trypanosomes in vitro (Appendix 1). The trypanocidal protein was isolated by sequential chromatography on hydroxylapatite, Protein A/G, Mono Q and Superose 12 and by immunoaffinity chromatography (Appendix 2). N-terminal amino acid sequences of 3 internal peptides of the protein showed that it was xanthine oxygen:oxidoreductase (EC 1.2.3.2) (Appendix 2) a bifunctional enzyme that catabolizes purine to uric acid generating NADH when exhibiting xanthine dehydrogenase activity and hydrogen peroxide and superoxide anion when exhibiting xanthine oxidase activity. Anti-trypanosome activity of cape buffalo serum, and of immunoaffinity-purified cape buffalo xanthine oxidase resulted from catabolism of purine that trypanosomes have to obtain from their environment and from the concomitant generation of trypanocidal hydrogen peroxide (Appendix 2). These data lead us to hypothesize; (i) that the sustained presence of xanthine oxidase in cape buffalo serum is responsible for their superior capacity to limit parasitemia and thus their innate resistance to African trypanosomiasis, and (ii) domestic cattle that are selected/developed to have similar plasma purine metabolism to cape buffalo will also have innate resistance to African trypanosomiasis. The project is continuing as a collaboration between my laboratory, the Kenya Agricultural Institute/Wildlife Disease Section and the International Livestock Research Institute. On-going research to clone the cape buffalo gene encoding xanthine oxidase is funded by an N.I.H. grant to the P.I. and may lead to a novel and sustainable method to control African trypanosomes in domestic animals.
**Research Objectives:** The overall goal of the project was to define the molecular basis for innate resistance to trypanosomiasis in cape buffalo. The specific aim was to isolate and identify a trypanocidal protein present in cape buffalo serum.

**Rationale:** Trypanosomiasis excludes cattle from the tsetse habitat, an area of 10 million square kilometers of Africa stretching from the southern aspect of the Saharan desert to a few degrees north of the Tropic of Capricorn. The wide distribution of African trypanosomes, the absence of conserved trypanosome antigens for use as vaccines, and the continuous requirement by susceptible hosts for chemotherapy while under trypanosome challenge combine to make trypanosomiasis the major constraint to cattle-based agriculture in the humid and semi-humid zones of Africa. Although trypanosomiasis excludes cattle from the tsetse habitat it has little or no effect on indigenous wild bovids amongst which cape buffalo are especially efficient at limiting the number of trypanosomes present in their bloodstream. The superior capacity of cape buffalo to curb trypanosome parasitemia is the result of an innate immune response. The animals also have a 150 kDa serum protein that kills all species of African trypanosomes *in vitro*, or limits their replication dependent on assay conditions (Appendix 1). Identification of the trypanocidal cape buffalo serum protein and elucidation of its mechanism of action may suggest novel and sustainable methods to control trypanosomiasis in domestic animals.

**Relationship to Research by other Scientists:** Our investigations show that the trypanocidal protein in cape buffalo serum is xanthine oxidase. The anti-trypanosome activity of xanthine oxidase is due to catabolism of purine, which
trypanosomes have to obtain from their environment, and to hydrogen peroxide which is produced as a byproduct of purine catabolism by xanthine oxidase. African trypanosomes cannot synthesize purine de novo [1] and thus are sensitive to a reduction in the purine content of their environment which is predicted to limit their ability to replicate, to transcribe genes and to make ATP. In addition, the organisms lack catalase [2] and consequently are highly vulnerable to hydrogen peroxide which inhibits glycolysis [3] and is expected to shut down trypanosome energy metabolism. In fact non-fatal exposure of trypanosomes to xanthine oxidase and purine leads to their accumulation in the first gap phase (G₁) of the cell division cycle (Hamilton and Black, unpublished), whilst fatal exposure is associated with a precipitous decline in trypanosome ATP and energy metabolism [4].

As a next step in development of the project we will focus on elucidating how cape buffalo maintain the stable presence of xanthine oxidase in their serum. Although cape buffalo xanthine oxidase has not been investigated, there is a rich literature on xanthine oxidase from other species eg: The structure and function of xanthine oxidase is known [5] as is the regulation of xanthine oxygen:oxidoreductase gene expression by inflammatory cytokines, activated neutrophils and interrupted blood circulation [6]. The enzyme is typically detected in the cytosol [7] of hepatocytes, vascular endothelium, skin and mammary epithelium and muscle [8]. It has not been detected in endoplasmic reticulum although paradoxically it is present on the lumenal face of vascular endothelium in association with proteoglycan [9]. We anticipate that our future investigations will contribute to understanding the origin and physiological role of extracellular xanthine oxidase.
Innovative Aspects and Organizational Support: The project area was novel; little work had been done on the mechanism of innate resistance to African trypanosomes in indigenous wild bovids. The work was possible because Dr. Grootenhuis in the Wildlife Disease Section of the Kenya Agricultural Research Institute (KARI) had established a breeding herd of cape buffalo that was maintained outside of the tsetse habitat and was free of trypanosome infection. Furthermore, animal technicians at KARI had developed a relationship with the captive bovids that allowed blood to be collected from the animals without anaesthesia. The magnitude of both of these accomplishments will be appreciated by those of you who have stood near a cape buffalo. In addition, Dr. Olubayo and Dr. Orinda of KARI established a working relationship with Dr. Murphy, Dr. Naessens and Dr. Dolan of the International Livestock Research Institute (ILRI) that made it possible to collect and store large quantities of cape buffalo serum for export from Kenya to my lab in U.S.A. I was fortunate that Dr. Murthy and Dr. Klontz of the U.S. Department of Agriculture, Animal and Plant Health Inspection Service facilitated importation of cape buffalo serum to U.S.A. first via their safety facility on Plum Island and later by direct import after inactivation at low pH. I was also fortunate that Dr. Rock and Dr. Torres of Plum Island volunteered to sort through hundreds of cape buffalo serum samples that were present in the first batch imported, to compile a representative batch for safety testing. I am indebted to Mr. Bugno of O.S.U. Research foundation who allowed some fairly inventive fund transfers between institutes to support the research, and to Dr. Wayne Ching who encouraged me to seek creative ways to transfer resources to my collaborators in Kenya. I want to thank Dr. Reeve, chair person of the Dept. of Microbiology, O.S.U, who supported my graduate students on student assistantships even after I
left O.S.U. I am grateful that the Dept. of Veterinary and Animal Sciences at the University of Massachusetts invited me to join its faculty thus providing start-up funds and making it possible to bring the project to its present state of development. Finally I want to acknowledge Dr. Gray, the director of ILRAD, Dr. Fitzhugh, the director of ILRI and Dr. Wafula, the associate director of KARI for allowing their scientists to participate in the project.

Methods and Results: These are presented in Appendix 1, 2, and 3 which jointly describe the studies supported by U.S.A.I.D. P.S.T.C 11.297. I will not reproduced figures and tables in this section. Briefly, blood was collected from male cape buffalo held in isolation in the Wildlife Disease Section of KARI, Kenya. Two batches of 10 liters, compiled as 50 ml aliquots collected over a 6 month period, were imported to my laboratory in U.S.A. where aliquots were fractionated by a variety of procedures including salting out with ammonium sulphate, preparative isoelectric focusing, chromatofocusing, gel chromatography on DEAE 52, Sephacryl S.300, Hydroxylapatite, Protein A/G, Mono Q, Mono S, Superose 12 and 6, Affigel Blue and eventually several immunoaffinity matrices prepared using antibodies that were raised against polypeptides present in the most enriched preparations of trypanocidal material. Each fraction was subjected to a short term bioassay to quantitate its trypanocidal activity. This entailed serial dilution in an assay buffer, addition of a standard number of healthy trypanosomes, incubation for 4 hours at 37°C and scoring of trypanosomes for motility by observation under an inverted tissue culture microscope. Purification of the trypanocidal component of cape buffalo serum proved to be very difficult due to the high protein content of serum, the great variety of proteins in serum, the low concentration of trypanocidal protein
in serum and the instability of isolated trypanocidal protein.... but we succeeded.

The next stage of the program namely, identification of the trypanocidal protein, was a nightmare. The putative active polypeptide was N-blocked and very unstable in solution. It took 5 months of constant immunoaffinity chromatography, SDS-PAGE and blotting onto PVDF membrane to collect enough material for cleavage and isolation and amino acid sequencing of peptide fragments. The work was done at the Wistar institute and funded by a small grant from U.Mass. The amino acid sequences were analyzed on the "Swissport Protein Sequencing Data Base". Observation of the results was a moment of great joy for myself, and joy tinged with relief for my Ph.D student Madhavi Muranjan who had stuck with the project through its many up and downs. There was a high degree of amino acid sequence homology between the peptide fragments of the putative trypanocidal protein and human, rat and mouse xanthine oxygen:oxidoreductase (EC. 1.2.3.2).

Knowledge that the putative trypanocidal protein was xanthine oxygen:oxidoreductase or was derived from that protein, suggested several straightforward studies to identify the active form of the molecule and its mechanism of action. We determined that: (i) cow's milk xanthine oxidase had the same trypanocidal activity as cape buffalo trypanocidal protein, (ii) antibodies raised against cow's milk xanthine oxidase precipitated trypanocidal protein from cape buffalo serum, (iii) allopurinol which inhibits xanthine oxidase inhibited the trypanocidal activity of cape buffalo serum and of purified trypanocidal protein, (iv) trypanocidal activity of cape buffalo serum and of purified trypanocidal protein required purine in the assay buffer and (v) trypanocidal activity resulted from
hydrogen peroxide that was generated as a byproducts of purine catabolism by xanthine oxidase and (vi) even when the amount of hydrogen peroxide generated was too low to kill trypanosomes the catabolism of purine in the medium limited their capacity to replicate.

In addition to the above, we: (i) developed baseline data on the capacity of different oxypurines, purine nucleobases and purine ribonucleosides to support trypanosome replication, (ii) examined the anti-trypanosome effects of cape buffalo serum and of purified cape buffalo xanthine oxidase when included in medium that contained combinations of the purines or single purine species, and (iii) assayed the capacity of cape buffalo serum, cape buffalo xanthine-oxidase, and of cape buffalo serum from which xanthine oxidase had been removed by immunoaffinity chromatography to catabolize the purines. The studies jointly lead us to hypothesize that the sustained presence of xanthine oxidase in cape buffalo serum is responsible for their superior capacity to limit trypanosome parasitemia and thus their innate resistance to African trypanosomiasis.

**Impact, Relevance and Technology Transfer:** The P.S.T.C 11.297 project has had a positive effect on research collaborations in Kenya, and between U.S.A. and Kenya, and may have a profound effect on agriculture development in sub-Saharan Africa.

If the hypothesis that xanthine oxidase is responsible for the innate resistance of African buffalo to trypanosomiasis proves correct it will lead to the development/selection of trypanosome resistant domestic animals and radical
change in the agricultural practices of sub-Saharan Africa. This exciting possibility lies at the core of the continuing collaboration between my lab, KARI and ILRI. At present a Ph.D student (Jun Wang) from my lab is located at ILRI where she is working with Dr. Orinda (KARI), Dr. Pele and Dr. Murphy (ILRI) to clone cape buffalo cDNA encoding xanthine oxidase. ILRI has also invited myself and 2 graduate students to work in Kenya during July and August 1997 to assay plasma purine catabolism in trypanosome infected cattle and possibly cape buffalo. Thus technology transfer is continuing to occur between U.S.A. and Kenya, and vice versa.

The project also had a positive (although indirect) impact on science in Kenya. It has stimulated similar research within the International Center of Insect Physiology and Entomology (ICIPE) and the Kenya Trypanosomiasis Research Institute (KETRI). It has strengthened the prospect for KARI to maintain funding from the Dutch Government to support the cape buffalo herd. It has also led to formal discussion between ILRI and KARI with regard to preserving the cape buffalo herd in the event that funding from the Dutch government is withdrawn, and it has encouraged Dr. Grootenhuis to persevere in his attempts to gain funds to establish a Wildlife Research Institute in East Africa.

**Project Activities/Outputs:** Two papers have been published (*Appendix 1 and 3*) and one submitted (*Appendix 2*). These are:


The project supported attendance at 3 meetings where poster and oral presentations were given. These were:

1. 1992 - American Society of Microbiology, Atlanta, Georgia. S.J. Black presented a poster on the trypanocidal component of African buffalo serum.

2. 1994 - Molecular Parasitology Meeting, Wood's Hole. M. Muranjan made an oral presentation on isolation of the trypanocidal cape buffalo protein. G. Morgan presented a poster on requirements for G₁ progression of *T. brucei*.

3. 1996 - Molecular Parasitology, Wood's Hole. S.J. Black will make an oral presentation on innate immunity to trypanosomiasis in cape buffalo. Q. Wang will present a poster entitled "Cape buffalo trypanocidal protein is xanthine oxidase". E.A. Hamilton will present a poster entitled "Tyrosine protein phosphorylation during G₁ progression of *T. brucei*.
The project contributed to training of 2 Ph.D students and partial training of a third. These were:


3. F.P. Otieno-Omondi joined the program and was trained for 2 years but unfortunately could not maintain the grade average required by O.S.U. graduate school.

The project also supported a visit by the P.I. (S.J. Black) to Kenya during June 1996 where he presented a seminar at ILRI, reviewed progress of Dr. Orinda (KARI) in examining the trypanocidal components of eland and human serum, and discussed future collaborations with ILRI and KARI.

**Project productivity:** We accomplished all of the project goals.

**Future work:** We will clone the cape buffalo gene encoding xanthine oxidase and determine whether the sustained presence of xanthine oxidase in cape buffalo serum reflects genotypic or environmental influences or both. We will develop monoclonal antibodies that inhibit cape buffalo xanthine oxidase, use these to deplete the enzyme from cape buffalo blood *in vivo*, and assay the effect of this treatment on
their innate resistance to trypanosomiasis. We will make similar studies in mice carrying the cape buffalo transgene encoding xanthine oxidase. If the investigations support the idea that sustained systemic xanthine oxidase is responsible for the innate trypanoresistance of cape buffalo, we will develop cattle with this property and examine their productivity in sub-Saharan Africa.

**Literature Cited.**


**African Buffalo Serum Contains Novel Trypanocidal Protein**

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**ABSTRACT.** The high ability of African buffalo, as compared to domestic cattle, to control infections with *Trypanosoma brucei* brucei ILTat 1.4 organisms did not correlate with the timing or magnitude of parasite surface coat-specific antibody responses and may have resulted from the constitutive presence in buffalo blood of a novel trypanocidal factor. Buffalo plasma and serum contained material that killed bloodstream stage *T. b. brucei, T. b. rhodesiense, T. b. gambiense, T. evansi, T. congolense, and T. vivax* organisms during four h of incubation at 37°C in vitro. Serum from eland was also trypanocidal whereas serum from oryx, waterbuck, yellow-back duiker, cattle, horse, sheep, goat, mouse, rat, and rabbit was not trypanocidal. The buffalo serum trypanocidal material was not lipoprotein, or IgG, and had the following properties: 1) a density of >1.24 g/ml determined by flotation ultracentrifugation; 2) insolubility in 50% saturated ammonium sulphate; 3) non-reactivity with anti-bovine IgM, and anti-bovine IgG; 4) non-reactivity with protein G, and protein A; 5) a relative molecular mass of 152 kDa determined by chromatography on Sephacryl S 300, and of 133 kDa determined by chromatography of the 50% SAS cut of IgG-depleted buffalo serum on Superose 12; 6) no associated cholesterol; and 7) inactivation by digestion with proteinase K that was immobilized on agarose.

**Supplementary key words.** Cape buffalo, trypanocidal serum protein, trypanosomiasis, VSG-specific antibody.

African trypanosomes cause fatal disease in man and domestic animals. In contrast, many wild animals co-exist with the tsetse-transmitted protozoa [2, 11, 14, 23, 30, 31]. African buffalo that are bred in captivity from trypanosome-free parents and maintained in a tsetse-free environment efficiently control experimental infections. In these animals, parasitemia is held at a low or undetected level and few or no pathologic signs of disease develop [12, 15, 27]. The infected buffalo produce neutralizing antibodies specific to the trypanosome variant surface glycoprotein (VSG) coat [12, 15, 27]. It is not known, however, whether these neutralizing antibodies are solely responsible for the superior ability of the buffalo to restrict the level of parasitemia. Here we show that production of VSG-specific antibodies does not correlate with the capacity of buffalo to control infection with a cloned *T. brucei*, and demonstrate the presence of a novel trypanocidal protein, or group of similar-sized proteins, in buffalo plasma and serum.

**MATERIALS AND METHODS**

**Trypanosomes.** Trypanosomes were grown in irradiated (650 rad) BALB/c mice, and purified [20] from blood that was collected during parasite exponential growth. These were: *T. b. brucei* ILTat 1.1, 1.2, 1.21, 1.23, 1.24, 1.25, 1.3 (provided by Dr. J. J. Doyle, ILRAD), and 1.4 [6]; *T. b. brucei* GUTat 3.1 [10], *T. b. brucei* Swiss Tropical Institute, Basel, Switzerland (STIB) 348T derived from STIB 247; *T. b. brucei* STIB 345B derived from EATRO 1529; *T. b. rhodesiense* 1292 [8, 9]; *T. b. rhodesiense* STIB 704 isolated in 1982 from a patient in Tanzania; *T. b. gambiense* TH-1/THE (031) isolated in 1978 from a patient in Daloa, Ivory Coast; *T. evansi* STIB 779 derived from KETRI 2443; *T. evansi* STIB 780A derived from CP 893 (a gift from Dr. E. Zweygarth); *T. congolense* STIB 68F [14]; *T. congolense* IL 1180 [15]; and *T. vivax* 1223B [21].

Exponentially growing *T. b. brucei* IL 3201 (derived from *T. b. brucei* TC221 Stock 427; 8) and IL 3202 (derived from *T. b. brucei* ILTat 1.4 Stock 667; 8, 10) were maintained in vitro as previously described [10].

**Animals.** BALB/c mice aged three mo were bred at ILRAD, or were purchased (Harlan Sprague Dawley Inc., Indianapolis, IN). Goats, rats and rabbits were bred at ILRAD or at the Swiss Tropical Institute and were aged between 3 and 12 mo. Boran and N'dama cattle were bred and maintained at ILRAD and were six mo to two yr of age.

African buffalo, eland, oryx and waterbuck were bred and maintained at the Wildlife Disease Section of The Kenya Agricultural Research Institute (KARI), Kabete, Nairobi, Kenya [12, 15] and were aged from 6 mo to 6 yr. Horses were reared at the Veterinary Laboratories of KARI. The yellow-back duiker (#864618) was maintained at the Columbus Zoo, Columbus, Ohio. Oryx and waterbuck, but none of the other animals, were immobilized by injection of 0.7 ml of a mixture of etorphine hydrochloride (2.45 mg/ml) and acepromazine (maleate 10 mg/ml) (Large Animal Immobilon, Reckitt and Colman Ltd., Hull, UK) plus 0.7 ml of xylazine (20 mg/ml) (Rompox, Bayer, Leverkusen, Germany) prior to blood collection.

The animals, with the exception of buffalo number 5641, had never been exposed to African trypanosomes. African buffalo 5641 had been infected with 10⁷ *T. b. brucei* GUTat 3.1 eight mo earlier, and with 2 × 10⁷ *T. b. brucei* GUTR 22 two mo earlier. Blood collected from buffalo 5641 one day prior to intravenous injection with *T. b. brucei* ILTat 1.4 (described below)
did not infect irradiated (650 rad) BALB/c mice (1.5 ml injected intraperitoneally (i.p.) into each of three mice), suggesting that the animal had cleared infection.

Infection with T. b. brucei IL Tat 1.4, and plasma/serum preparation. Cattle and African buffalo were infected via the jugular vein with 1.2 x 10^7 T. b. brucei IL Tat 1.4 purified from infected mouse blood and resuspended in phosphate saline glucose [20]. Blood for analysis of parasitemia, or preparation of plasma or serum, was collected from the jugular vein into vacutainers ±10 IU heparin/ml blood, with or without agents that reduce artifacts originating from blood collection [13], namely: final concentrations of 4 mM EDTA, 2 mM 2-mercaptoethanol (2-ME), 25 µg leupeptin/ml, 40 µM phenylmethyl-sulfonylfluoride (PMSF), and 10 µg aprotinin/ml, which is a kallikrein inhibitor (Sigma Chemical Co., St. Louis, MO). Parasitemia was quantitated using the darkfield buffy coat scoring system [28].

Analysis of T. brucei IL Tat 1.4 variant surface glycoprotein-specific antibody response. Glutaraldehyde-fixed bloodstream forms of T. b. brucei IL Tat 1.1, 1.2, 1.21, 1.23, 1.24, 1.25, 1.3 and 1.4 conjugated to a microtiter plate as previously described [32] were used as target antigen. Wells were washed with phosphate buffered saline (10 mM sodium phosphate in 150 mM NaCl, pH 7.2) (PBS). One hundred-µl aliquots of 1% rabbit serum in PBS were added to each well and incubated for 16 h at 4°C, and wells were washed with PBS. Aliquots (50 µl) of buffalo and cow serum diluted in PBS were added to each well, incubated for two h at room temperature, and unbound serum components were removed by washing with PBS. Bound antibodies were detected [34] using 125I-conjugated polyclonal goat anti-cow IgG (a gift from Dr. M. Murray), which cross-reacts with isotypic determinants on African buffalo IgG, and 125I-conjugated mouse monoclonal ILA2 (a gift from Dr. P. Wells), which reacts with isotypic determinants on cow IgG, and IgG2 [25, 38] and cross-reacts with buffalo IgG [26]. Antibodies were conjugated with 125I [37] while bound to cow or buffalo IgG or IgG (a gift from Dr. M. Murray) covalently linked to Sepharose 4B.

Trypanosone neutralization test in vivo. 5 x 10^7 T. b. brucei IL Tat 1.1, 1.2, 1.21, 1.23, 1.24, 1.25, 1.3, or 1.4 were suspended in 1 ml of PBS containing 1% glucose, and 25% v/v heat-inactivated (56°C, 30 min) cow serum, or African buffalo serum, or 0.5 mg affinity-purified buffalo IgM, or 2 mg affinity-purified buffalo IgG (described below). Mixtures were incubated at 37°C for one h and 0.2-ml aliquots injected i.p. into each of three irradiated (650 rad) BALB/c mice. Wet films of tail blood were examined microscopically at two-day intervals for the presence of trypanosomes.

In vitro assay of trypanocidal activity in plasma and serum preparations. Trypanosomes (10^5) were incubated in wells of a 96-well tissue culture tray (Costar, Cambridge, MA) in 100-µl aliquots of PBS supplemented with 1% glucose, 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS), and varying dilutions of plasma, or serum, or serum fractions, from the mammals listed above. When plasma was tested, PBS was supplemented with 10 IU heparin/ml. Incubations were for four h at 37°C in a humid atmosphere of 5% CO2 in air. Trypanosome motility was assessed at the end of each incubation by examining culture wells under phase contrast illumination using an inverted microscope.

Propagation of Theileria parva-transformed cow cells in vitro. Theileria parva-transformed bovine T-cells [4, 19] were resuspended at a density of 5 x 10^4/ml RPMI 1640 (Gibco BRL, Gaithersburg, MD) supplemented with 20% African buffalo serum, incubated at 37°C in a humid atmosphere of 5% CO2 in air, and counted in a hemocytometer each day.

Fractionation of serum. Ultracentrifugation. Serum density was adjusted to 1.25 g/ml with solid KBr, and the preparation was centrifuged at 200,000 g, for 48 h at 4°C using an L7 ultracentrifuge, and a 75.1 Ti rotor (Beckman Instruments, Fullerton, CA). Sequential 1.0-ml fractions were collected from the bottom to the top of the centrifuge tube using a calibrated Pasteur pipette. Each fraction was weighed, and dialyzed in a 25-kDa exclusion membrane (Spectrum Medical Industries, Inc., Los Angeles, CA) against three changes each of 500 ml PBS. The protein and total cholesterol content of dialyzed fractions were determined by Sigma kits (respectively 541-2, and 352-100).

Saturated ammonium sulphate. Saturated ammonium sulphate (SAS) (761 g/liter at 22°C) was added to a final volume of 50%. Precipitates were collected by centrifugation (1,000 g, 22°C), washed twice with 50% SAS, and dissolved in PBS. Supernatants remaining after precipitation with 50% SAS were concentrated over an Amicon YM 5 membrane. The solutions were dialyzed in 25-kDa exclusion membranes with stirring at 4°C against three changes of PBS (500 x vol), filtered (0.22 µm). Protein content was determined by OD280/1.4, and trypanocidal activity was assayed as described above.

Sephacyrl S 300. An XK 26/30 column (Pharmacia LKB Nuclear Inc., Gaithersburg, MD) packed with Sephacryl S 300 (Pharmacia) was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (BSA) (68 kDa) at flow rates of 0.25 g/BSA/min using an FPLC system (fast protein liquid chromatography, Pharmacia) in a 4°C chromatography box. Protein was detected at OD280 on a UV-M monitor (a discrete fixed-multiples-wavelength absorbance detector) set at 2 AU (Pharmacia) and recorded at 100 mV full deflection. Buffalo serum (0.3 ml) was chromatographed at 4°C using a flow rate of 0.25 ml PBS/min, and fractions of 0.5 ml were collected using a Frac 100 collector for bioassy of trypanocidal activity.

Superoxide 12. A prepacked 10/30 column of Superose 12 HR (Pharmacia) was calibrated with a polymer of serum amyloid protein (230 kDa, a gift from Dr. Mortensen, Ohio State University), trimer bovine serum albumin (204 kDa), mouse IgG (150 kDa), and BSA (68 kDa) at flow rates of 0.1 ml PBS/min, using FPLC at 4°C. Protein was detected at OD280 on a UV-M monitor set at 2 AU and recorded at 100 mV full deflection.

Affinity chromatography. Anti-buffalo Ig. Buffalo serum IgM was purified by affinity chromatography on monoclonal antibody ILAS0 conjugated to Sepharose 4B [25, 38]. Buffalo serum IgG was purified by affinity chromatography on monoclonal antibody ILA2 conjugated to Sepharose 4B [25, 38]. Protein G. Buffalo serum (1-ml aliquots) was diluted 1:3 with 0.1 M sodium phosphate pH 7, and chromatographed on agarose-Protein A/G-(5-ml gel, Schleicher and Schuell, Keene, NH) at a flow rate of 1 ml/min. Protein-G-binding material was eluted with 0.2 M glycine HCl pH 2.5 at a flow rate of 1 ml/min, and neutralized by addition of 20% v/v 1.0 M TRIS HCl pH 9. Protein in chromatographed material was detected by a LKB-Uvicord and recorded at 100 mV full deflection. Protein A. Buffalo serum (0.5-ml aliquots) was diluted 1:3 with 1.5 M glycine in 3 M sodium chloride pH 8.9, and chromatographed on agarose protein A/G as described above.

Sequential chromatography. Buffalo serum (5 ml) was depleted of IgG by chromatography of 1-ml aliquots on Protein G as described above. Serum components that did not bind to protein G under these conditions were mixed with an equal volume of SAS. The precipitate was collected at 1,000 g, dissolved in 50 ml PBS, concentrated to 2 ml by filtration over a YM 100 membrane (Amicon, Beverly, MA), and subsequently to 200 µl on a Centricon 30 (Amicon). The 200-µl sample was chromatographed on Superose 12 HR 10/30 at 0.2 ml PBS/min
antigenic variants at 13 days after infection (Table 1). Therefore, antibodies against the absence of detectable parasitemia was not due to the presence to establish a patent infection [34]. The parasites that grew in and was parasitemic for one day only. Buffalo 5641 did not exceeded 5 $\times$ 10^4 parasites/ml blood (500 g$^{-1}$), supernatant collected and assayed for trypanocidal activity as described above.

**Electrophoresis.** Isolated proteins were examined by SDS-PAGE in 7.5% homogeneous gels on a Phast System (Pharmacia) and were revealed by staining with Coomassie Blue, or by silver stain.

**RESULTS**

Parasitic profiles and antibody responses following infection of African buffalo, and cattle, with 1.2 $\times$ 10^4 T. b. brucei IL Tat 1.4 organisms. **Parasitemia.** Cattle developed several parasitic waves during 40 days. The first of these waves exceeded 5 $\times$ 10^4 parasites/ml blood (6 on score system, Fig. 1). African buffalo 5931 developed a first wave parasitemia of about 5 $\times$ 10^3 organisms/ml blood (5 on score system, Fig. 1). Buffalo 5931 became parasitic again on day 31 after infection with about 5 $\times$ 10^2 parasites/ml blood (1 on score system, Fig. 1) and was parasitic for one day only. Buffalo 5641 did not develop a detectable parasitic wave (Fig. 1). Blood collected 40 days after infection of both buffalo was injected into the peritoneal cavity of irradiated (650 rad) BALB/c mice (1 ml per mouse, three mice per blood sample). All of the mice became parasitic with a patent period of six or seven days, which is the same time taken for a single T. b. brucei IL Tat organism to establish a patent infection [34]. The parasites that grew in mice were monomorphic, consistent with the origin from the IL Tat serodeme [34].

**Antibody responses.** Buffalo 5641 had no detectable serum antibodies against T. b. brucei IL Tat 1.4 or other IL Tat 1.4 antigenic variants at 13 days after infection (Table 1). Therefore, the absence of detectable parasitemia was not due to the presence of VSG-specific antibodies. The animal developed very low-titer serum antibodies against T. b. brucei IL Tat 1.1, 1.2 and 1.3 by 20 days after infection, and against T. b. brucei IL Tat 1.21, 1.23, 1.24, 1.25, and 1.4 by 40 days after infection, consistent with a maintained low level infection with IL Tat organisms, that eventually stimulated antibody production.

Buffer 5931 generated low-titer serum IgM antibodies against the eight test IL Tat VAT between 13 and 20 days after infection, and developed low-titer serum IgG antibodies against the parasites between 30 and 40 days after infection. In contrast, cows 5939 and 5940 generated both IgM and IgG serum antibodies against the eight test T. b. brucei IL Tat VAT by 13 days after infection (Table 2). The IgM responses in both cattle were of higher titer than those of buffalo 5931, and both IgM and IgG responses of infected cattle increased in titer from day 13 to day 40 of infection. Thus, the magnitude of VSG-specific antibody responses that arose in infected cattle and African buffalo reflected the magnitude of parasitic waves, and presumably the amount of stimulatory antigen.

Incubation of 5 $\times$ 10^4 trypanosomes for one h at 37$^\circ$C in one ml buffer containing 25% v/v heat-inactivated pre-infection serum from either cow did not influence the infectivity of T. b. brucei IL Tat 1.4 for mice. Incubation with 25% v/v heat-inactivated day 13 post-infection serum from both cows completely neutralized infectivity of T. b. brucei IL Tat 1.4 but had little influence on infectivity of the other T. b. brucei VAT. Incubation with either pre-infection or day 13 post-infection serum from buffalo 5641 or 5931 completely neutralized the eight test T. b. brucei IL Tat VAT. Incubation, as above, at 37$^\circ$C with 0.5 mg IgM, or 2 mg IgG isolated from pre-infection serum of buffalo 5641, did not neutralize T. b. brucei IL Tat 1.4, suggesting that neutralization of IL Tat organisms by pre-infection buffalo serum was not due to IgM or IgG antibodies.

**African buffalo serum kills African trypanosomes in vitro.** Clones of salivarian trypanosomes from three subgenera (Duttonella, Nannomonas, Trypanozoon) and including the three subspecies of T. brucei (see Materials and Methods) were screened for sensitivity to African buffalo serum. The organisms were incubated at 37$^\circ$C in medium containing dilutions of heat-

<table>
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<tr>
<th>Serum donor</th>
<th>Target</th>
<th>IgM</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG</th>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>20</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<tr>
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<td>0</td>
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<td>0.6</td>
<td>0.7</td>
</tr>
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</tr>
<tr>
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<td>1.4</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
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<td>0.5</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
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<td>0</td>
</tr>
<tr>
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<td>0.3</td>
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<td>0.8</td>
<td>0.6</td>
<td>0.8</td>
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</tr>
<tr>
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<td>0.7</td>
<td>0.6</td>
<td>0.8</td>
<td>0.6</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
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<td>0.8</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>1</td>
</tr>
</tbody>
</table>

![Fig. 1. Growth of T. b. brucei parasites in cattle and African buffalo. The animals were inoculated in the jugular vein with 1.2 × 10^3 T. b. brucei IL Tat 1.4 collected during their exponential growth stage in BALB/c mice. Parasitemia was estimated by the buffy coat dark ground procedure.](image-url)
Table 2. Serum antibody responses of cattle infected with T. b. brucei IL-Tat 1.4. Titers are expressed as the differences between reciprocal log_{10} dilution of post-infection serum that gave 50% max binding minus reciprocal log_{10} dilution of pre-infection homologous serum that gave 50% max binding on indicated target parasite.

| Reciprocal log_{10} VSG-specific antibodies; day after infection |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Serum donor | IL-Tat | IgM | IgG | IgM | IgG | IgM | IgG |
| Cow 5939 | 13 | 2 | 1 | 2 | 1.5 | 2.5 | 1.5 | 1.5 | 3 | 1.5 |
| | 20 | 1 | 1 | 1.5 | 2 | 1 | 2.5 | 0.6 |
| | 30 | 1.2 | 0.5 | 1 | 1 | 0.5 | 2 | 1 | 2.5 |
| | 40 | 1.21 | 0.6 | 1 | 2 | 2 | 2 | 2 | 2 |
| | 1.23 | 1 | 0.5 | 2 | 1 | 1.5 | 2 | 2.5 | 3 |
| | 1.24 | 1 | 1 | 2 | 2 | 1.5 | 2 | 1 | 2 |
| | 1.25 | 1 | 1 | 2.5 | 2.5 | 2.9 | 2 | 2.7 |
| | 1.3 | 0.6 | 2 | 1 | 2 | 0.6 | 2 | 2 | 2 |
| | 1.1 | 0.5 | 0.2 | 0.6 | 0.4 | 1.5 | 0.4 | 1.9 |
| | 1.2 | 1 | 1 | 1.5 | 1.5 | 2.3 | 2 | 1.7 |
| | 1.21 | 1.5 | 1 | 2.6 | 1 | 3.4 | 2 | 2.6 | 2 |
| | 1.23 | 0.6 | 0.3 | 0.6 | 1 | 1 | 1.6 | 1.7 | 1.9 |
| | 1.24 | 0.7 | 0 | 0.5 | 0.1 | 1 | 1 | 1.8 |
| | 1.25 | 0.5 | 0.4 | 1.6 | 1.8 | 1.4 | 2 | 1.5 | 2 |
| | 1.3 | 1 | 1 | 2 | 1 | 1.3 | 2 | 1.4 | 2 |

inactivated pre-infection serum from African buffalo 5641, or cow 5940, and trypanosome motility was assessed at hourly intervals by observation on an inverted microscope. Similar results were obtained with all test parasites. During four-h incubation in 20% v/v African buffalo serum, the parasites became sluggish, and subsequently immobile. Immobile organisms were unable to infect irradiated (650 rad) BALB/c mice. Trypanosomes did not show reduced motility or loss of infectivity for mice after incubation for four h at 37°C in medium supplemented with ≤40% or ≤0.625% serum from buffalo 5641, or any concentration of serum from cow 5940. At a concentration of 20% v/v serum from buffalo 5641 and cow 5940 had a similar capacity to support replication of bovine T-cells that were infected and transformed by the intracellular schizont stage of the protozoan parasite Theileria parva, indicating that buffalo serum was not specifically toxic for eukaryotic cells. Doubling times during exponential growth were respectively 19.4 h and 20 h.

Host distribution of serum trypanocidal activity. Heat-inactivated serum samples from 20 African buffalo, 1 eland, 10 waterbuck, 5 oryx, 1 yellow-back duiker, 35 Boran cattle, 4 Ndama cattle, 5 horses, 10 goats, 10 sheep, 11 rabbits, and several inbred strains of mice were screened for trypanocidal activity. The serum samples were prepared on several occasions from each donor animal over a period of up to two yr. The T. b. brucei IL 3201 listed in Materials and Methods were used as target organisms. All of the African buffalo and eland sera were trypanocidal on all occasions tested. These sera killed all of the test trypanosomes. Trypanocidal activity was found in serum prepared from either male or female buffalo, and from buffalo of all ages from one mo up to six yr. No other serum preparations were observed to kill trypanosomes.

The inclusion during blood collection of heparin, EDTA, 2-ME, aprotinin, leupeptin, and PMSF did not influence the presence or titer of trypanocidal activity in African buffalo plasma, or the absence of this activity in plasma collected from the other donor species.

Table 3. Density gradient fractionation of African buffalo serum. African buffalo serum was adjusted to a density of 1.25 g/ml with solid KBr and 9.9 ml volumes were centrifuged at 200,000 g for 48 h at 4°C. Samples of 1.1 ml volume were collected from the bottom to the top of the tube using a calibrated pipette, and 1 ml volumes were processed.

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Weight (g/ml)</th>
<th>Protein content (mg/ml)</th>
<th>Total cholesterol (mg/ml)</th>
<th>Trypanocidal activity/mg protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.17</td>
<td>17.8</td>
<td>17.8</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.20</td>
<td>3.0</td>
<td>5.2</td>
<td>0</td>
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</tr>
<tr>
<td>5</td>
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<td>25.0</td>
<td>0.2</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
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<td>0.1</td>
<td>64</td>
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<tr>
<td>7</td>
<td>1.25</td>
<td>113.4</td>
<td>0.5</td>
<td>64.5</td>
</tr>
<tr>
<td>8</td>
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<td>62.25</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>1.30*</td>
<td>27.1</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

* Crystal of KBr formed at the bottom of the tube.
* Reciprocal end titer (buffalo serum has a reciprocal end titer of 64).
* Reciprocal end titer (mg protein/ml).

Fractionation of African buffalo serum. Trypanocidal activity was detected in fractions of density 1.24 g/ml to 1.25 g/ml obtained after density gradient ultracentrifugation at 4°C (Table 3). The trypanocidal material resided in a different part of the gradient to the majority of serum molecules that had associated cholesterol (Table 3), suggesting that trypanocidal activity was not associated with serum lipoproteins.

Ultracentrifugation and dialysis resulted in a loss of 68% of serum trypanocidal activity (Table 3). Buffalo serum that was adjusted to 1.25 g/ml with solid KBr and subsequently dialyzed against PBS to remove salt lost 70% of activity, whereas buffalo serum that was simply dialyzed against PBS at 4°C did not lose activity (data not shown). Thus, loss of trypanocidal activity during density gradient ultracentrifugation was due to exposure to KBr, and may have resulted from a salt-induced conformational change in the trypanocidal molecule(s), or a salt-induced loss of a required, low-M₅₀ co-factor.

Trypanocidal activity eluted with molecules of 152 kDa during chromatography on Sephacryl S 300 (Fig. 2), consistent with the possibility that it was associated with serum IgG. However, buffalo serum IgG isolated by immunoaffinity chromatography on monoclonal antibody IL-A2 (not shown), or on agarose-Protein G (Fig. 3, Table 4), had no trypanocidal activity. Passage of African buffalo serum over agarose-Protein G, and over agarose-Protein A, did not remove any trypanocidal activity (Fig. 3, Table 4).

Trypanocidal material present in buffalo serum that had been depleted of IgG by passage over agarose-Protein G precipitated at 50% SAS, with a loss of 65% of trypanocidal activity (Table 4). Loss of activity during salting out may be due to an irreversible change in conformation, or loss of a low molecular weight co-factor.

Concentration of the 50% SAS-cut of IgG-depleted serum over YM 100 and Centricon 30 membranes resulted in a further 50% loss of both protein and trypanocidal activity (Table 4). This loss in protein and activity probably resulted from binding to membranes, tubes, and pipettes during handling of the concentrated sample, and was not due to the removal of <100 kDa molecules during filtration. The filtrates contained little or no protein, and no detectable trypanocidal activity.

Trypanocidal material present in the concentrated >100 kDa fraction of IgG-depleted and SAS-precipitated buffalo serum
The high incidence of parasitism in cattle suggests that the wild bovids served as reservoir hosts of the organisms.

Control of parasitemia in infected buffalo was unlikely to be solely due to parasite surface coat-specific antibody responses. The conclusion is based on: a) the absence of antibodies against \textit{T. b. brucei} IL Tat VAT in buffalo 5641, which held parasitemia at a subliminal level, and b) the lack of correlation between control of parasitemia in buffalo 5931 and the kinetics of development, magnitude, and VAT specificity of antibody responses. Control of parasitemia is also unlikely to result from production by the buffalo of non-neutralizing growth-inhibitory antibodies. IgM and IgG purified from day 13 post-infection serum of buffalo 5641 had no influence on the growth or survival of \textit{T. b. brucei} IL 3202 during nine divisions in vitro (SJB & DJLW, unpubl.). \textit{Trypanosoma b. brucei} IL 3202 is derived from \textit{T. b. brucei} IL Tat 1.4 [10].

It has been recognized for some years that antibody-independent responses contribute to the control of parasitemia in animals infected with African trypanosomes [1, 3, 7-9, 36]. Seed and Sechelski [32] have demonstrated that serum prepared from infected mice contains an inhibitor of \textit{T. brucei} thymidine incorporation, suggesting that the infected mice may produce growth-inhibitory molecules. \textit{Trypanosoma congolense}-infected cattle acquire resistance to super-infection with other \textit{T. congolense} [22]. Some mammals also constitutively produce trypanocidal molecules. Cotton rat serum contains a 550-kDa com-
Fig. 3. Relative OD_{280} plots of buffalo serum fractionated on agarose-Protein A/G. A. A 1-ml aliquot of buffalo serum (end titer 1:64) was mixed with 2 ml of 0.1 M PO_{4} buffer pH 7 (Protein G-loading buffer) and loaded onto 5 ml agarose-Protein A/G gel at a flow rate of 1 ml/min. Unbound material was eluted with Protein G-loading buffer, and bound material was eluted with 0.2 M glycine HCl pH 2.5 into an equal volume of 1.0 M Tris-HCl pH 9. The ★ symbol indicates where elution was started. An aliquot of each fraction was dialyzed into PBS and assayed for trypanocidal activity. Activity (end titer 1:8) was associated only with the serum component that did not bind to Protein G, and this had a volume of 10 ml, indicating 125% recovery of input trypanocidal activity. B. Serum components that did not bind to Protein A/G, shown in the first peak in panel A, were re-chromatographed on Protein A/G as described for panel A. Protein G-binding material was not detected. Unbound material had a volume of 12 ml and an end trypanocidal titer of 1:8, indicating 120% recovery of input trypanocidal activity. C. A 5-ml aliquot of serum components that did not bind to Protein A/G in Protein G binding buffer (panel B) was mixed with 5 ml of 1.5 M glycine in 3 M NaCl pH 8.9 (Protein A-loading buffer) and loaded onto 5 ml agarose Protein A/G gel at a flow rate of 1 ml/min. Unbound material was eluted with Protein A-loading buffer, and bound material was eluted at pH 2.3 as described for panel A. Aliquots of each fraction were dialyzed into PBS and assayed for trypanocidal activity, indicated by the hatched area. Unbound material had a volume of 12 ml, and an end titer of 1:4, indicating 100% recovery of input activity.

Table 4. Sequential fractionation of buffalo serum by: affinity chromatography on Agarose-Protein G, SAS precipitation, N\_2 pressure dialysis on YM 100 and concentration on Centricon 30. African buffalo serum (5 ml in 1-ml aliquots) was depleted of IgG by passing over agarose Protein G as described in Figure 3, panel A. Material that bound to Protein G was eluted, neutralized, and pooled (line 2). Material that did not bind to Protein G was pooled (line 3). Material that did not bind to Protein G was adjusted to 50% SAS and centrifuged at 1,000 g. The supernatant was collected and dialyzed against PBS to remove salt (line 4). The precipitate was dissolved in PBS, and dialyzed against PBS to remove salt (line 5). The dissolved precipitate was adjusted to 50 ml with PBS, concentrated to 2 ml over a YM 100 membrane and subsequently to 200 µl using a Centricon 30 (line 6). This preparation was subsequently chromatographed on Superose 12 (Fig. 4). Protein concentrations were determined as OD_{280}/1.4 after dilution to give a value of 1.0 or less. Trypanocidal activities are the reciprocal end titers. The % protein recovery was determined as mg total protein/400 (5 ml buffalo serum contains 400 mg protein). The % trypanocidal activity recovered was determined as (reciprocal end titer x vol in mls)/320 (5 ml buffalo serum with a titer of 1:64 gives a value of 320).

<table>
<thead>
<tr>
<th>Component tested</th>
<th>mg protein/ml (Vol in ml)</th>
<th>Trypanocidal activity/mg protein</th>
<th>% Protein relative to 5 ml buffalo serum</th>
<th>% Trypanocidal activity relative to 5 ml buffalo serum</th>
</tr>
</thead>
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<td>Buffalo serum</td>
<td>80.0 (5)</td>
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<tr>
<td>Protein G-bound and eluted</td>
<td>2.13 (50)</td>
<td>0</td>
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<tr>
<td>Protein G-run through</td>
<td>6.06 (45)</td>
<td>1.32</td>
<td>68.2</td>
<td>112.5</td>
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<td>50% SAS-supernatant of Protein G-run through</td>
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<td>0</td>
<td>33.3</td>
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<tr>
<td>50% SAS-precipitate of Protein G-run through</td>
<td>23.8 (3.5)</td>
<td>1.34</td>
<td>20.0</td>
<td>34.9</td>
</tr>
<tr>
<td>YM 100 + Centricon 30 concentrate of 50% SAS-precipitate of Protein G-run through</td>
<td>168.0 (0.2)</td>
<td>1.90</td>
<td>8.4</td>
<td>19.9</td>
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ponent that kills *T. vivax* organisms [18, 35]. Human serum contains a component identified as a high density lipoprotein (HDL) [29], a very HDL [16], and a non-lipoprotein [5] that kills *T. b. brucei* organisms. Serum HDL from ground-dwelling primates also kills *T. b. brucei* [33].

Trypanocidal activity against trypanosomes of different species and variant antigenic types was detected in plasma and serum prepared from the blood of buffalo, but not in plasma or serum from highly trypanosusceptible domestic animals and laboratory animals. The precautionary use of anti-coagulant, a reducing agent, a chelating agent, protease inhibitors, and kalikrein inhibitor to avoid artifacts during blood collection [13] had no influence on the presence or level of trypanocidal activity detected in plasma, suggesting that the trypanocidal activity of buffalo plasma/serum was not a collection artifact.

Trypanocidal activity was also detected in eland serum. It was not detected in serum from trypanotolerant Nama and waterbuck. These animals are less able to control parasitemia than the African buffalo and eland but resist the development of anemia, which is a major pathogenic feature of bovine trypanosomiasis [24]. Trypanocidal activity was also not detected in the serum of oryx and a yellow-back duiker. Duikers have been shown to support low, sporadic parasitemic waves when infected with African trypanosomes [30], but have not been directly compared to buffalo or eland with respect to control of experimental infections.

The buffalo serum trypanocidal molecules differ in several respects from previously described trypanocidal serum components: a) They kill *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. evansi*, *T. congolense* and *T. vivax*, whereas other described trypanocidal serum components are specific for a single trypanosome species, or sub-species [29, 33, 36]. b) They have a density of >1.24 gm/ml, and do not contain cholesterol, features that distinguish them from *T. b. brucei*-lytic components of human serum [16]. c) Although they have a relative molecular mass of 152 kDa determined by chromatography on Sephacryl S 300, and of 133 kDa determined by chromatography on Superose 12, they are not IgGs. Purified buffalo serum IgG did not kill trypanosomes in vitro, and depletion of IgG from buffalo serum using mouse monoclonal ILA2 [24, a,b], or
agarose-Protein G and agarose-Protein A, did not reduce trypanocidal activity. d) They do not cause swelling around the parasite flagellar pocket region, a morphological characteristic of *T. b. brucei* lysis by human serum HDL [29]. Indeed, ongoing studies in our laboratory show that killing of *T. b. brucei* by buffalo serum is associated with a rapid decline in parasite ATP content, without loss of integrity of the parasite surface coat, plasma membrane, or organelle structure (M. M. & SJB, unpubl.).

High (40%) v/v concentrations of buffalo plasma and serum did not kill trypanosomes in vitro. This may result from the presence of a specific inhibitory molecule, or may result from formation of inactive complexes of the trypanocidal molecules at high serum concentration. Ongoing isolation studies support the former conclusion; trypanocidal proteins obtained by fractionation of buffalo serum on hydroxylapatite are active at high concentration (FPO-O & SJB, unpubl.). Lack of trypanocidal activity at high plasma concentration may account for survival of trypanosomes in buffalo. Infection-induced changes in the concentration of trypanocidal protein(s) or its inhibitors may account for rapid and non-specific control of trypanosome infections by African buffalo. It is also possible, however, that the trypanocidal protein is inactive in vivo and is not responsible for the high capacity of African buffalo to control trypanosomes. Whether this is the case or not, isolation of the molecule and elucidation of its mode of action may suggest new strategies for control of African trypanosomes in mammals.

ACKNOWLEDGMENTS

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mechanisms involved in the control of parasitemia in *Trypanosoma brucei* infected wildebeest (*Connochaetes taurinus*). *Immunology*, 2:231-238.


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Innate Resistance to Trypanosomiasis in Cape Buffalo

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Abstract: Cape buffalo are reservoir hosts of African trypanosomes. They bear infection with trypanosomes without signs of disease and have an innate capacity to curb parasitemia. We have shown that the animals also have a trypanocidal serum protein. The protein was isolated and identified as xanthine oxidase. In the presence of oxypurine, purine nucleobase and/or purine ribonucleoside in medium, cape buffalo serum and immunoaffinity-purified cape buffalo serum xanthine oxidase inhibited trypanosome growth or killed the parasites. Activity resulted from catabolism of purine, which trypanosomes have to obtain from their environment and from the concomitant generation of trypanocidal hydrogen peroxide. We hypothesize that the sustained presence of xanthine oxidase in cape buffalo serum is responsible for their superior capacity to limit parasitemia and thus their innate resistance to African trypanosomiasis.

Text: African trypanosomes are tsetse-transmitted protozoa that cause fatal sleeping sickness in humans and Nagana in cattle. Nagana excludes domestic livestock from about 70% of the land constituting the tsetse habitat [1], an area of 10 million square kilometers stretching from the southern aspect of the Saharan desert to a few degrees north of the Tropic of Capricorn. The wide distribution of African trypanosomes, the absence of conserved trypanosome antigens for use as vaccines and the continuous requirement by susceptible hosts for chemotherapy while under trypanosome challenge combine to make trypanosomiasis the major constraint to cattle-based agriculture in the humid and semi-humid zones of Africa. Although domestic cattle are excluded from most areas where trypanosomiasis is endemic, the niche supports many wild bovids including cape buffalo. Cape buffalo that are experimentally infected with African trypanosomes
rapidly control parasitemia [2] and subsequently maintain low level infections without signs of disease [3]. Effective control of trypanosome parasitemia by cape buffalo is not associated with superior acquired immune responses against the parasites and correlates with the presence of a constitutive serum protein that kills all species of African trypanosomes \textit{in vitro} [3]. Our preliminary characterization of the cape buffalo serum trypanocidal material [3] indicates that it is protein of about 150 kDa that is distinct in density and biologic activity from previously described trypanocidal material, namely very high density lipoprotein from human serum which is lytic for \textit{Trypanosoma brucei brucei} [4] but not for other species and sub-species of trypanosomes. Identification of the broad acting trypanocidal component of cape buffalo serum and elucidation of its mechanism of action might suggest novel and sustainable methods to control African trypanosomes in domestic animals.

Serum was prepared from uninfected cape buffalo that were bred in captivity from trypanosome-free parents and maintained outside the tsetse habitat. Exponentially growing \textit{T. brucei} from axenic cultures were incubated for 4 hours at 37°C with dilutions of cape buffalo serum or serum fractions in a phosphate saline glucose solution and the end titer that caused total loss of motility, determined by microscopic examination of the parasites, was taken as an indicator of trypanocidal activity [3; legend Fig 1]. Using this bioassay the trypanocidal component of cape buffalo serum was isolated by sequential chromatography on hydroxylapaptite (Fig 1A), Protein A/G (Fig 1B), Mono Q anion exchange resin (Fig 1C) and Superose 12 size exclusion matrix (Fig 1D) which jointly achieved a 1000-fold purification. The protein had an Mr of 150 kDa (Fig 1D) and trypanocidal activity correlated with the presence of a 146 kDa polypeptide detected by SDS-
PAGE under reducing conditions (Fig 2A). Trypanocidal activity was completely removed from cape buffalo serum by immunoaffinity chromatography using immune rabbit IgG raised against the 146 kDa polypeptide ("anti-146") [discussed in 5] indicating that the 146 kDa polypeptide was required for the trypanocidal activity. N-terminal amino acid sequences obtained from 3 peptide fragments of the 146 kDa polypeptide showed high homology with human, mouse and rat xanthine:oxygen oxidoreductase (1.2.3.2) (Fig. 3), a dual function enzyme that converts purines such as hypoxanthine and xanthine to uric acid generating NADH when exhibiting xanthine dehydrogenase activity, and hydrogen peroxide ($H_2O_2$) and superoxide anion when exhibiting xanthine oxidase activity [6, review].

Several lines of evidence show that xanthine oxidase was responsible for the trypanocidal activity of cape buffalo serum and that trypanosome death was caused by $H_2O_2$ generated during catabolism of purine in the assay buffer. First, antibodies specific for cow's milk xanthine oxidase (anti-CM.XO) [7] removed all trypanocidal activity from cape buffalo serum. Reducing SDS-PAGE of the anti-CM.XO immunopurified material indicated that this treatment removed the 146 kDa polypeptide (Fig 2B) together with polypeptides of 121 kDa, 84 kDa and 53 kDa that have not yet been characterized. Second, allopurinol which inhibits xanthine oxidase [8] abrogated trypanocidal activity of cape buffalo serum, the anti-146 immunopurified cape buffalo xanthine oxidase and cow's milk xanthine oxidase. The xanthine oxidase activity was determined in undiluted stock solutions by a standard technique [9]. A concentration of 0.1 mM allopurinol was required to inhibit 0.00013 units of cow's milk xanthine oxidase/ml buffer and 0.00006 units of immunopurified cape buffalo xanthine oxidase/ml buffer which were the minimal trypanocidal
concentrations of these preparations. Third, killing of trypanosomes by addition of cape buffalo serum, anti-146 immunopurified cape buffalo xanthine oxidase or cow's milk xanthine oxidase to the assay buffer was dependent on extracellular purine provided in the fetal bovine serum (FBS) component of the buffer. Dialysis (25 kDa cutoff membrane) of the FBS prevented expression of trypanocidal activity and activity was restored upon addition of 1μM hypoxanthine to the buffer. Fourth, addition of 1 μg (3.2 units) of bovine catalase (Sigma) to the assay buffer inhibited the trypanocidal activity of cape buffalo serum, anti-146 immunopurified cape buffalo serum xanthine oxidase and cow's milk xanthine oxidase irrespective of their concentrations. Catalase accelerates conversion of H2O2 to water and is not made by African trypanosomes [10]. Finally, while catabolism of purine by xanthine oxidase generates superoxide anion and uric acid in addition to H2O2, neither of these products was responsible for trypanocidal activity evidenced by a lack of effect of the latter up to a concentration of 1 mM and a lack of a sparing effect of superoxide dismutase up to a concentration of 1mg/ml buffer which was the highest non-toxic concentration.

Although cape buffalo serum and immunopurified xanthine oxidase killed trypanosomes in phosphate saline glucose assay buffer, they inhibited replication of the parasites when added to growth medium (Fig 4A). Cape buffalo serum depleted of xanthine oxidase by immunoaffinity chromatography on the anti-146 column had no effect on trypanosome growth (Fig 4A). T. brucei growth medium [11] contains pyruvate which non-enzymatically reduces H2O2 [12] and could accommodate an addition of up to 1.5 mM H2O2 before any inhibition of trypanosome growth was detected. Xanthine oxidase mediated growth inhibition was unlikely to be due to H2O2 as catabolism of the purine content of the medium ie., 10 μM hypoxanthine and 10 μM
adenosine, would not yield the required inhibitory concentration of \( \text{H}_2\text{O}_2 \). In contrast, trypanosomes died when the purine content was increased (above 0.1% saturation) in growth medium that contained intact cape buffalo serum or anti-146 immunopurified cape buffalo xanthine oxidase, but not in medium that contained xanthine oxidase-depleted cape buffalo serum (Fig 4B, 4C), suggesting that catabolism of the additional purine by xanthine oxidase resulted in the accumulation of a trypanocidal concentration of \( \text{H}_2\text{O}_2 \) in medium. Addition of catalase together with superoxide dismutase prevented trypanosome death in medium that contained cape buffalo serum and had a high (> 0.1% saturation) purine content (Fig 4C) but did not restore normal trypanosome replication in medium containing cape buffalo serum and low (<0.1% saturation) purine content (Fig 4C). Thus death of the trypanosomes in growth medium containing cape buffalo serum and high purine content was due to reactive oxygen intermediates produced during purine catabolism by xanthine oxidase, whilst reduced trypanosome population growth in medium containing cape buffalo serum and low purine content most likely resulted from purine depletion by xanthine oxidase. The organisms cannot synthesize purine \textit{de novo} [13] and have to obtain this essential nutrient from their environment.

Trypanosomes utilized any of a broad range of purines as a growth substrate (Table 1) consistent with their ability to synthesize all cellular nucleotides from each purine [14]. Cape buffalo serum and immunopurified xanthine oxidase inhibited trypanosome replication in medium containing single purines (Table 1). However, cape buffalo serum inhibited trypanosome replication to a greater extent than did immunopurified cape buffalo xanthine oxidase when evaluated in medium containing guanosine as the sole purine (Table 1) and the capacity of either preparation to limit
trypanosome replication in medium containing adenine as the sole purine was lower than that observed with the other purines (Table 1). The trypanosome growth-inhibitory activity of cape buffalo serum and immunopurified cape buffalo xanthine oxidase was consistent with their purine catabolic activities. HPLC analyses showed that cape buffalo serum and immunopurified cape buffalo xanthine oxidase had only low catabolic activity against adenine and catabolism of guanosine by cape buffalo serum required other enzymes in addition to xanthine oxidase (Table 2). Adenine is not present in cape buffalo serum, and the otherwise broad purine catabolic activity of the serum assures that changes in diet or in purine transport into blood are unlikely to improve its properties as a trypanosome growth medium.

Xanthine oxidase was only occasionally detected in serum collected from Holstein heifers and even then its activity was low (less than 0.5 units/liter of serum), and its presence in an individual animal sporadic. Activity was not detected in serum from other cattle breeds or from horses, goats, rabbits, rats, and mice. This may be fortuitous as cellular production of the enzyme can be induced by inflammatory cytokines and activated neutrophils [15] and its presence in serum of the domestic livestock and laboratory animals may vary with respect to the health status of the donor. The general absence of xanthine oxidase in the serum of domestic animals was not due to the presence of an inhibitor. Cape buffalo and cow's milk xanthine oxidase retained full activity when added to cow or mouse serum. Xanthine oxidase was always detected in cape buffalo serum where it was stably maintained at a concentration of 2 units xanthine oxidase/liter of serum. The donor cape buffalo were bred in captivity, had no signs of disease and were maintained under similar conditions to donor cattle and horses that had no serum xanthine oxidase activity. While
the sustained systemic presence of xanthine oxidase is a general feature of cape buffalo it is not known whether it reflects genotypic or environmental influences or both. Whatever the reason for the sustained systemic presence of xanthine oxidase in serum we hypothesize that it results in the superior capacity of cape buffalo to limit trypanosome parasitemia and is responsible for their innate resistance to African trypanosomiasis.

References and Notes


5. A slice of polyacrylamide gel containing the 146 kDa reduced polypeptide was excised after non-denaturing Commassie stain, emulsified in complete Freund's adjuvant and
injected into several subcutaneous sites on the back and flanks of a rabbit. The rabbit was
boosted after 4 weeks with the same antigen emulsified in incomplete Freund's adjuvant.
Blood was collected for serum after 10 days and immune IgG was isolated from the
immune serum on protein A/G (BioRad). 33 mg IgG coupled to 5 ml Affi-prep Hz
hydrazide support gel (BioRad) gel depleted the trypanocidal activity from 50 ml of
buffalo serum. 60% of the activity was recovered from the immunoaffinity matrix by
elution with 0.1 M triethylamine pH 11.5 and neutralization with 1M NaPO₄ pH 6.4 and
was 1600 fold purified. Trypanocidal material did not bind to pre-immune rabbit IgG, or
to immune IgG raised against either cape buffalo serum IgM and IgG, or total serum
lipoproteins (density < 1.23 gm/ml) conjugated to Affi-prep Hz.


7. Each rabbit was immunized with 200 µg of the 150 kDa fraction of cow's milk xanthine
oxidase (Grade III chromatographically purified suspension in 2.3 M ammonium sulphate;
Sigma) re-isolated by FPLC on Superose 12 and emulsified in Freund's complete adjuvant.
Each rabbit was boosted 4 and 8 weeks later with the same protein in Freund's incomplete
adjuvant and serum was prepared 10 days after the last boost. 10 mg of xanthine oxidase-
specific IgG was prepared from immune serum by binding to (in 200 mM PO₄ buffer pH
7.2) and elution from (with 100 mM triethylamine pH 11.5) 99 mg of cow's milk xanthine
oxidase immobilized on 10 ml Sepharose 4B, and in turn conjugated to 3 ml of CNBr-
activated sepharose 4B (Pharmacia).


17. The Superose 12 column was calibrated with blue dextran (2000 kDa), serum amyloid protein polymer (230 kDa, a gift from Dr. R. Mortensen), catalase (222 kDa), Mouse IgG (150 kDa), bovine serum albumin (67 kDa) and cytochrome C (12.4 kDa).
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Figure 1. Isolation of trypanocidal material from cape buffalo serum. Assay - *T. brucei* S 427 cl 1 organisms from axenic cultures [16] were washed twice by centrifugation at 1000g in 137 mM NaCl, 20 mM PO₄ pH 7.2 (PBS) at 20°C and suspended in PBS containing 1% wt glucose and 10% v/v heat inactivated (56°C, 30 min) fetal bovine serum (assay buffer). Serial dilutions (2 fold) of buffalo serum or serum fractions were made in assay buffer in wells of a 96 well tissue culture tray (Costar) with a final vol. of 100 µl/well. 10µl assay buffer containing 2x10⁴ trypanosomes was added to each well, the plate was incubated at 37°C for 4 h and trypanosomes scored for motility using an inverted tissue culture microscope. Results were recorded as the reciprocal end titer resulting in complete loss of parasite motility, which corresponds to complete loss of infectivity [3]. (A) African buffalo serum (50 ml) in 200 mM KPO₄ pH 6.8 was loaded at a flow rate of 0.2 ml/min on 80 ml bed vol. hydroxylapatite (Bio-Rad) pre-equilibrated with the same buffer, and eluted at 0.2 ml/min with increasing molarity of KPO₄ (-----). The protein elution profile at OD₂₈₀ is indicated by (-----). Trypanocidal activity (-----) eluted at 340 mM KPO₄. 5120 units of trypanocidal material (reciprocal end titer x vol.) was loaded and 3520 units recovered with a 10 fold purification based on activity relative to protein content. (B) Trypanocidal hydroxylapatite fractions were pooled, adjusted to 400 mM KPO₄ pH 6.8 by N₂ pressure dialysis over a YM 100 membrane (Amicon) and loaded at 0.1 ml/min on 5 ml bed vol. Protein A/G conjugated to agarose (Schleicher and Schull). The column was washed at 1 ml 0.1 M NaPO₄ pH 7.2/min and eluted at 0.1 ml 0.1 M glycine HCL pH 2.5/min. The protein elution profile at OD₂₈₀ is indicated by (-----) and trypanocidal activity (-----) was present in the column run-through only. 3520 units of trypanocidal material was both loaded and recovered with a 1.6 fold purification. (C) The protein A/G runthrough fraction was concentrated over a YM 100
membrane, dialyzed (25 kDa cutoff) into 20 mM Tris buffer pH 8.25 containing 52.5 mM NaCl and loaded at 0.1 ml/min onto 1 ml bed vol. Mono Q anion exchange resin (Pharmacia) pre-equilibrated with the same buffer. The column was eluted at 0.12 ml/min with increasing molarity of NaCl in Tris buffer (----). The protein elution profile at OD_{280} is indicated by (——). The bulk of trypanocidal material (——) eluted between 87 mM and 102 mM NaCl. 3520 units of trypanocidal material was loaded and 513 recovered with a 22 fold purification. (D) Trypanocidal fractions that eluted from Mono Q between 12.5 to 18.5 ml were pooled, concentrated to 100 µl by centrifugation on a Centricon 30 membrane (Amicon), and loaded at 0.1 ml/min on 25 ml bed vol. Superose 12 size exclusion matrix (Pharmacia) [17] pre-equilibrated with 150 mM NaCl, 20 mM PO_4, pH 7.2. Trypanocidal material (——) eluted as a single peak spanning vol. 9.5 - 11.0 ml indicating an Mr of 154 kDa consistent with previous values obtained by chromatography of whole buffalo serum [3]. 513 units of trypanocidal activity was loaded and 80 recovered with 3 fold purification.
Figure 2A. Reducing SDS-PAGE of Superose 12 fractions. Cape buffalo serum was fractionated by the scheme shown in Fig 1. Superose 12 column fractions were subjected to reducing SDS-PAGE on a homogeneous 7.5% gel and silver stained. Fraction elution vol. in ml is shown at the top of each track, reciprocal trypanocidal end titer at the bottom, MW marker (Pharmacia) positions in kDa on the left and the MW of isolated polypeptides on the right.

Figure 2B. Reducing SDS-PAGE of cape buffalo serum xanthine oxidase isolated by immunoaffinity chromatography on immobilized rabbit IgG prepared against cow's milk xanthine oxidase. The anti-cow's milk xanthine oxidase immunoaffinity column was prepared as described [7]. Buffalo serum (4 ml) was passed over this column using FPLC. Bound material was eluted with 0.1 M triethylamine pH 11.5, neutralized with 1M phosphate buffer pH 6.4, subjected to reducing SDS-PAGE on a homogeneous 10% gel and stained with Coomassie blue. MW markers and their molecular mass in kDa are on the left, eluted polypeptides and their molecular mass in kDa on the right.
Figure 3. The NH\textsubscript{2}-terminal sequences of 3 peptides, A, B and C from the CNBr digestion of the 146 kDa polypeptide of the trypanocidal protein, aligned with peptides from rat, human and mouse xanthine oxidase. Amino acid substitutions are underlined. Amino acid residues are represented by the abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Immunoadfinity chromatography on anti-146 kDa polypeptide IgG was used to obtain trypanocidal material from buffalo serum. The trypanocidal material was subjected to reducing SDS-PAGE on a homogeneous 7.5% gel, and transferred to Immobilon-P membrane. The PVDF-bound 146 kDa polypeptide was cleaved by CNBr and the N-terminal sequences of HPLC-purified peptides obtained by Edman degradation at the Wistar Protein Sequencing Facility, Philadelphia, PA. The homology search was conducted on the Swissport protein sequencing database.
Figure 4A. Effect of cape buffalo serum, xanthine oxidase-depleted cape buffalo serum, and cape buffalo xanthine oxidase on trypanosome replication. Exponentially growing *T. brucei* S 427 cl 1 from axenic cultures [16] were incubated in 150 µl volumes of Baltz-modified minimal essential medium [11] supplemented with 10% vol. fetal bovine serum (growth medium) ; or growth medium containing 50% vol. cape buffalo serum ; or growth medium containing 50% vol. xanthine oxidase-depleted cape buffalo serum ; or growth medium containing the xanthine oxidase content of 50% vol. cape buffalo serum isolated by immunoaffinity chromatography on the anti-146 column . Triplicate cultures, in wells of a 96 well culture plate (Costar), were seeded with $10^4$ organisms/ml medium ($1.5 \times 10^3$ organisms/well), incubated in a humid atmosphere of 5% CO₂ in air for 48 hours, resuspended and trypanosomes were counted in a hemocytometer under phase contrast illumination. Figure 4B. Effect of purine concentration on trypanosome growth inhibition by cape buffalo serum and immunopurified xanthine oxidase. Cultures were set up in growth medium ; or growth medium containing 50% vol. cape buffalo serum ; or 50% vol. xanthine oxidase-depleted cape buffalo serum ; or the xanthine oxidase content of 50% vol. cape buffalo serum ; as described in Fig 4A except that medium contained varying dilutions of a stock mixture of xanthine, hypoxanthine, adenine, guanine, adenosine and guanosine as indicated. The stock solution of purines contained 10 mg of each purine/ml growth medium, and was centrifuged (500 g) and filtered (0.22 µm pore) to remove undissolved material before use. Figure 4C. Effect of catalase and superoxide dismutase on trypanocidal cape buffalo serum activity. Cultures were set up as described in Fig 4B in growth medium ; or growth medium containing 20 µg bovine catalase (CAT; Sigma)/ml and 200 µg bovine superoxide dismutase (SOD; Sigma)/ml ; or growth medium containing 50% vol. cape buffalo serum ; or growth medium containing 50% vol. cape buffalo serum. CAT and SOD .
Table 1  Influence of cape buffalo serum xanthine oxidase on replication of *T. brucei* in medium with defined purine content

<table>
<thead>
<tr>
<th>Purine added</th>
<th>% vol. of purine preparation in incubation medium</th>
<th><em>T. brucei</em> x 10⁴(+/− 1SD)/ml medium containing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50% vol. caper buffalo serum</td>
<td>50% vol. xanthine oxidase-depleted cape buffalo serum</td>
</tr>
<tr>
<td>Xanthine</td>
<td>5</td>
<td>117 (2.6)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>5.6 (1.5)</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>7.0 (0.3)</td>
</tr>
<tr>
<td>Adenine</td>
<td>5</td>
<td>63.3 (1.5)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>84.3 (4.5)</td>
</tr>
<tr>
<td>Guanine</td>
<td>5</td>
<td>3.0 (0)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3.33 (0.6)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.6 (2.0)</td>
</tr>
<tr>
<td>Guanosine</td>
<td>5</td>
<td>24.0 (4.0)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>10.0 (7.0)</td>
</tr>
</tbody>
</table>

Saturated purine stock solutions were prepared by addition of 10 mg of each purine/ml medium containing 10% vol dialyzed (25 kDa cutoff) FBS. The stock preparations were centrifuged (500 g), filtered (0.22 µm pore size) and diluted as indicated. Xanthine oxidase-depleted cape buffalo serum was prepared by FPLC at 4°C on the anti-146 immunoaffinity column and added to a final vol of 50%. Cape buffalo xanthine oxidase was eluted from the anti-146 immunoaffinity column [5] dialyzed into PBS, adjusted to normal serum concentration, and added to a final vol. of 50%. Triplicate cultures of 150 µl vol. were seeded with 10⁴ *T. brucei* S 427 cl 1/ml, incubated for 48 hours at 37°C in a humid atmosphere of 5% CO₂ in air, suspended and trypanosomes counted in a hemocytometer.
Table 2  Influence of xanthine oxidase on purine catabolism in cape buffalo serum

<table>
<thead>
<tr>
<th>Cape buffalo serum component</th>
<th>Purine added (HPLC elution time, mins)</th>
<th>% purine remaining after incubation for:</th>
<th>New OD_{254} peaks at 120 min (HPLC elution time in min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 minute</td>
<td>120 minutes</td>
</tr>
<tr>
<td>intact serum</td>
<td>xanthine (4.0)</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>hypoxanthine (3.5)</td>
<td>53</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>guanine (3.5)</td>
<td>83</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>adenine (9.0)</td>
<td>79</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>guanosine (14.0)</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>adenosine (25.0)</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>inosine (12.5)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>xanthine oxidase-depleted cape buffalo serum</td>
<td>xanthine</td>
<td>ND</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>hypoxanthine</td>
<td>ND</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>guanine</td>
<td>ND</td>
<td>46</td>
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<td></td>
<td>adenine</td>
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<td>43</td>
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<td></td>
<td>guanosine</td>
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<tr>
<td></td>
<td>adenosine</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>inosine</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>immunopurified (anti-146) cape buffalo serum xanthine oxidase</td>
<td>xanthine</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>hypoxanthine</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>guanine</td>
<td>ND</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>adenine</td>
<td>50</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>guanosine</td>
<td>ND</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>adenosine</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>inosine</td>
<td>68</td>
<td>77</td>
</tr>
</tbody>
</table>

35 µl serum or serum component plus 35 µl of purine (100 µM in all cases except guanine which was saturated) in 10 mM KH2PO4 pH 7.2 were incubated for 1 or 120 min at 20°C, centrifuged for 6 min at 10,000g on a Centricon 3 (Amicon), and 15 µl filtrate was loaded onto a 3.9 x 150 mm NovaPac C18 column (Waters) using a Waters 600E HPLC with manual inject. The column was eluted with 20 mM KH2PO4 pH 5.6 for 5 min at 1 ml/min, then from 5 to 30 min with a linear gradient from 0% to 40% of CH3OH:H2O (6:4). Eluted material was scanned at OD_{254} using a Waters Lambda Max 481 LC spectrophotometer, and peak areas of OD plots estimated by the formula 1/2 base x peak height in all cases except adenine which eluted as an asymmetric peak and was estimated from 1/2 (product of the length of 2 sides and the sin of the included angle). ND denotes not done.
peptide A
1 TVPMDHTFFPSYR 13
rat XDH 381 TVRMDHTFFPGYR 393
human XDH 382 TVQMDHTFFPGYR 394
mouse XDH 384 TVWMDHTFFPGYR 396

peptide B
1 IPAFGSIPMEFR 12
rat XDH 1216 IPAFGSIPIEFR 1227
human XDH 1230 IPAFGSIPIEFR 1241
mouse XDH 1232 IPAFGSIPIEFR 1243

peptide C
1 VASIGGNIITASPISDLNPVEMASG 25
rat XDH 344 VASIGGNIITASPISDLNPVEMASG 368
human XDH 345 VASVGGNIITASPISDLNPVEMASG 369
mouse XDH 347 VASIGGNIITASPISDLNPVLMAS 370
The requirements for G₁ checkpoint progression of *Trypanosoma brucei* S 427 clone 1

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Abstract

*Trypanosoma brucei* S 427 clone 1 accumulated in G₁ when incubated under growth-limiting conditions. Further incubation of the G₁-restricted organisms in medium containing 10% fetal bovine serum (FBS) and 2 mM hydroxyurea resulted in their reversible arrest after a G₁ checkpoint beyond which serum was not required for progress into and through S. Progress of the G₁-restricted *T. brucei* through the G₁ checkpoint was linear and required continuous incubation with exogenous serum growth factors. These were principally low and high density lipoproteins; both lipoproteins triggered G₁ progression in a dose- and time-dependent manner whilst their removal by immunoaffinity chromatography severely reduced the capacity of FBS to stimulate G₁ progression. Serum-induced progress of *T. brucei* through G₁ was Ca²⁺-independent, but required gene transcription, protein synthesis, and continuous kinase activity that was inhibited by tyrphostin 51 and DAPH 1 which typically inhibit epidermal growth factor receptor protein tyrosine kinase activity. The tyrphostin 51-sensitive catalytic activity was not required for *T. brucei* protein synthesis, glycolysis, or S phase progression but was required for tyrosine phosphorylation of several polypeptides, none of which was specifically associated with serum-induced G₁ progression.

Keywords: *T. brucei*; G₁ checkpoint; LDL; HDL; Protein tyrosine kinase activity

1. Introduction

Pleomorphic *Trypanosoma brucei* are morphologically heterogeneous in mammals. Replicating slender forms predominate during rising parasitemia and are replaced via intermediates by non-dividing stumpy forms towards peak parasitemia, leaving a few slender antigenic variants to initiate the subsequent parasitemic wave [1,2]. Understanding the molecular basis for the slender to stumpy transition is central to understanding pop-
ulation dynamics of the hemoprotozoa. Analyses in vitro suggest that slender organisms transform to stumpy-like forms [3] in response to the accumulation in medium of differentiation factors [4] and growth inhibitors [5]. The growth inhibitors that arise in axenic cultures of *T. brucei* S427 cl I are neutralized by defatted albumin [5] which, together with serum low- or high-density lipoproteins (LDL, HDL), supports self-renewal of exponentially growing slenders in serum-free medium [5]. Omission of either lipoproteins or defatted albumin from the minimal growth medium causes irreversible (at 37°C) arrest of slender *T. brucei* in the first gap phase of the cell cycle (G1) prior to DNA synthesis (S) [5] indicating that they commit to divide or differentiate in G1.

Generation of growth competent slender *T. brucei* that are synchronized in G1 would allow analysis of the biochemical pathways that control G1 progression or differentiation. However, these have proven difficult to generate. Whereas incubation of procyclic *T. brucei* at high cell density in vitro causes G1 arrest and significant synchrony on subsequent re-entry into the cell cycle [6], this procedure causes slender *T. brucei* to lose the capacity to replicate at 37°C [5]. In addition, pharmacological agents that are commonly used to synchronize mammalian cells [7-11] do not synchronize bloodstream *T. brucei* [12], e.g. aphidicolin and nocodazole have little or no effect on the organisms and thymidine causes their irreversible arrest in S [12]. Here we report that growth-competent G1-restricted *T. brucei* arise during short-term incubation of exponentially growing organisms under growth-limiting culture conditions and describe the requirements for their re-entry into the cell cycle.

2. Materials and methods

2.1. Serum lipoproteins and lipoprotein-depleted serum

Fetal bovine serum (FBS) lipoproteins, lipoprotein-depleted-FBS, and LDL and HDL were prepared by flotation ultracentrifugation after adjusting serum density with KBr [13,14]. These were dialedyzed into 20 mM PO4 in 150 mM NaCl, pH 7.2 (phosphate-buffered saline, PBS) and stored at 4°C under argon for no longer than 1 week after preparation.

2.2. Lipoprotein-specific antibodies and immunoaffinity chromatography

Rabbit anti-FBS lipoprotein serum was prepared as previously described [13] and precipitated LDL and HDL but not lipoprotein-depleted FBS during radial immune diffusion in 1% agarose (Fig. 4A). IgG was isolated from the immune serum by binding to and elution from Protein A/G (Pharmacia), and conjugated to AffiPrep HZ (BioRad). The derivatized matrix was packed in a C-10/20 column (Pharmacia), equilibrated with PBS and used to deplete FBS of lipoproteins by FPLC (Pharmacia) at a flow rate of 0.1 ml min⁻¹. Run-through fractions were collected using a Frac 100 (Pharmacia) and screened for total cholesterol content using a Sigma Kit. FBS contained 1.22 mmol cholesterol l⁻¹. Initial column run-through fractions contained 0.05 mmol cholesterol l⁻¹. Later run-through fractions contained 1.22 mmol cholesterol l⁻¹ indicating complete column saturation. Lipoprotein-depleted and control fractions were diluted to 9.5 mg protein ml⁻¹ and assayed for their ability to support *T. brucei* G1 checkpoint progression as described in the text.

2.3. Trypanosomes, culture conditions and inhibitors

*T. brucei* S427 cl I of stock S427 was propagated during logarithmic growth in Baltz-modified minimal essential medium (BMEM) [15] containing 10% heat-inactivated (56°C, 30 min) FBS (Bio Whitaker Lot 4H0054) as previously described [5,13]. Exponentially growing *T. brucei* were incubated in complete or Ca²⁺-free BMEM supplemented with intact or Ca²⁺-depleted FBS, or LDL or HDL and fatty acid-free bovine serum albumin (FAF-BSA; Sigma) with or without inhibitors, as described in the text. FBS was depleted of Ca²⁺ by dialysis in 25-kDa cutoff
membranes against 3 changes of a 1000x volume of PBS containing 6 mM EGTA. The inhibitors tested were: staurosporine (Boehringer Mannheim Biochemica), genestein, lavendustin A (Gibco BRL), BAPTA-AM (Molecular Probes Inc.), 4,5-dianilinophthalimide (DAPI-I; a gift from Ciba Geigy), hydroxyurea, sphingosine, H7, H8, HA1004, tyrphostin 1, 23, 25, 46, 47, 51, 63, EGTA, cycloheximide and actinomycin D (Sigma). Cultures of 1-ml volume were made in triplicate, in wells of 24-well tissue culture trays (Costar) and were for varying periods of time at 37°C in a humid atmosphere of 5% CO₂ in air. In many instances T. brucei were incubated in medium containing one set of supplements, washed and incubations continued with other supplements. For washing, the organisms were centrifuged (10 min, 1200 x g, 4°C), medium removed, pellet dispersed by tapping, and organisms suspended in 1 ml PBS and the process repeated 3 more times. After incubations trypanosomes were counted in a hemocytometer, or analyzed for DNA content.

2.4. Cell cycle analysis

T. brucei (usually 5 x 10⁶ organisms) were fixed in ethanol/glycerol, treated with RNAase, stained with propidium iodide and screened for DNA content by flow cytometry as previously described [5]. Trypanosomes were ascribed to a cell cycle stage using Multicycle (Phoenix Flow Systems) or CELLFIT (Becton Dickenson) software, based on propidium iodide fluorescence which correlates directly with DNA content in RNAase treated cells.

2.5. Pyruvate secretion

T. brucei (2 x 10⁶ ml⁻¹) were incubated for 0–2 h at 37°C in BMEM containing 1% (wt) glucose, 10% FBS and 2 mM hydroxyurea (HU) with or without tyrphostins as indicated in the text. At intervals, 0.5-ml volumes of T. brucei-free culture supernatant was added to 1 ml ice-cold 5% perchloric acid in H₂O, vortexed, and incubated on ice for 5 min, centrifuged at 1000 x g for 10 min at 4°C, and supernatant assayed for pyruvate content by NADH consumption in the presence of lactate dehydrogenase (Sigma Kit # 726).

2.6. Protein synthesis

Triplicate 0.5 ml cultures of T. brucei (2 x 10⁶ ml⁻¹) were incubated in 48-well Costar plates in BMEM containing 10% FBS, 2 mM HU, 1 mCi [³⁵S]methionine (> 1000 Ci mmol⁻¹; Amersham) with or without tyrphostins as described in the text. After 2 h incubation at 37°C, trypanosomes were resuspended and 100-µl aliquots were mixed with 100 µl 20% (wt) trichloroacetic acid in PBS (TCA). The precipitate was collected on 0.45 µm pore size filter discs (Whatman) prewet with 10% TCA and washed with 50 ml 10% TCA followed by 20 ml 95% EtOH, air dried and immersed in scintillation fluid (Scintiverse) prior to counting [³⁵S]DPM.

2.7. T. brucei lysates, SDS-PAGE and phosphotyrosine blots

T. brucei (10⁷) prepared as described in the text, were centrifuged (1200 x g, 10 min, 4°C) and the pellet lysed by addition of 25 µl lysis buffer containing 150 mM NaCl, 50 mM Tris base, 3% (vol) NP-40, 10 mM EDTA, and freshly added 10 mM NaF, 10 mM sodium orthovanadate, 10 µg aprotinin ml⁻¹ and 10 µg leupeptin ml⁻¹ in deionized H₂O. Lysates were incubated on ice for 5 min, centrifuged at 10000 x g and the supernatant added to 12 µl SDS sample buffer comprised of 5% vol 2 mercaptoethanol in 0.4 M Tris, pH 6.8, 12% SDS, 50% glycerol, and 0.3% (wt) bromophenol blue. The mixture was illuminated at 95°C for 1.5 min. Aliquots (20 µl) of samples and broad range MW standards (BioRad) where electrophoresed in 10% homogeneous polyacrylamide gels [16]. The gel was blotted onto nitrocellulose (Amersham) using the BioRad wet blot system at a constant 100 V for 1 h at 4°C and transfer efficiency estimated using Ponceau Red [17]. Tyrosine phosphorylated proteins were detected on the blot using mouse monoclonal anti-phosphotyrosine 4G10 (UBI) at 1 µg ml⁻¹. Bound 4G10 was detected with horse radish peroxidase conjugated rabbit anti-mouse Ig (Southern Biotech)
Fig. 1. DNA content analysis of exponentially growing *T. brucei* incubated: (A) for 9 h at 37°C at a density of 5 × 10⁵ organisms ml⁻¹ BMEM + 10% FBS, or (B) for 9 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹ + 0.5% FBS, or (C) incubated as in (B), washed 2 times with PBS and incubated for 4 h at 37°C in BMEM + 10% FBS, or (D) incubated as in (B), washed 2 times in PBS and incubated for 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹. The graphs are DNA content histograms of sample populations (usually 10,000 events) under these four culture conditions. Doublet organisms have been excluded by a pulse peak vs. pulse area cytogram logic gate. The linear X axis (abscissa) represents relative propidium iodide fluorescence which is directly proportional to DNA content. The linear Y axis (ordinate) shows the frequency of events per channel. The dots are actual experimental data. The dashes are the summation of 3 curves representing G₁ (first peak), S (hatched peak), and G₂/M third peak as derived from the actual data by Multicycle software. The software uses a least squares curve fitting algorithm so that the departure of the fitted (dashed) line from the raw data (the Chi Square) is minimized.

using enhanced chemiluminescence and ECL-hyperfilm (Amersham).

3. Results

3.1. G₁-restricted *T. brucei*

Exponentially growing *T. brucei* from mainten-

ance cultures had a doubling time of 7.5 h and contained 41.6 ± 12.5% (mean ± 1 S.D.) members in G₁ determined by analysis of 10 populations. The population shown in Fig. 1A had 36% of members in G₁, 45% of members in S and 19% of members in G₂/M. During 9 h incubation at 37°C, at a density of 5 × 10⁵ organisms ml⁻¹
Fig. 2. DNA content analysis of G1-restricted T. brucei that were incubated at a density of 5 × 10^5 organisms ml⁻¹: (A) for 4 h at 37°C in BMEM + 10% FBS + 2 mM HU, or (B) as in (A), followed by washing 3 times with PBS and incubating for an additional 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹, or (C) for 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹ + 2 mM HU, or (D) as in (C), followed by washing 3 times in PBS and incubating for an additional 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹.

3.2. Effect of HU on G1 to S phase transition of G1-restricted T. brucei

G1-restricted T. brucei (5 × 10^5 ml⁻¹) that were incubated for 4 h at 37°C in BMEM + 10% FBS + 2 mM HU (Fig. 2A) had the same DNA content distribution as G1-restricted organisms that were incubated in BMEM + 5 mg FAF-
BSA ml⁻¹ + 2 mM HU (Fig. 2C). However, after washing and incubation for another 4 h at 37°C in serum-free BMEM + 5 mg FAF-BSA ml⁻¹, T. brucei that had been pre-incubated in the presence of serum flowed into S and G₂/M (Fig. 2B), whereas organisms that had been pre-incubated in FAF-BSA remained in G₁ (Fig. 2D). Thus HU caused a reversible block in serum-induced cell cycle progression after a G₁ checkpoint beyond which serum was no longer required for further progress into S and G₂/M.

3.3. Kinetics of G₁ checkpoint progression and role of lipoproteins

Most G₁-restricted T. brucei crossed the G₁ checkpoint during 4 h incubation at 37°C in BMEM + 10% FBS (Fig. 2). To examine whether serum was required throughout the 4-h period, G₁-restricted organisms were incubated in BMEM + 10% FBS + 2 mM HU for 0–240 min, washed and re-incubated for respectively 240–0 min in BMEM + 5 mg FAF-BSA ml⁻¹ + 2 mM HU. Thereafter the T. brucei were washed and incubated for 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹ prior to cell cycle analysis. Results presented in Fig. 3 show an almost linear decline in the percentage of G₁ organisms and a corresponding linear increase in organisms in G₂/M with increased time of exposure to serum. This indicates that the G₁-restricted T. brucei were spread throughout the G₁ interval and their progress through G₁ required continuous exposure to serum.

Two lines of investigation show that lipoproteins were mainly responsible for the capacity of FBS to stimulate T. brucei G₁ progression. Removal of lipoproteins severely compromised the capacity of FBS to support G₁ checkpoint progression (Fig. 4B), whereas LDL (Table 1) and HDL (not shown) in the presence of FAF-BSA but no other serum component stimulated G₁-restricted T. brucei to pass the G₁ checkpoint in a time- and dose-dependent manner (Table 1).

3.4. Protein kinase inhibitors prevent replication of T. brucei

T. brucei (5 × 10⁵ ml⁻¹) were incubated for 48 h at 37°C in BMEM + 10% FBS + varying concentrations of protein kinase inhibitors listed in the table (or carrier diluent), and viable cells were counted in a hemocytometer. Results are presented in Table 2. Staurosporine, the broadest acting protein kinase inhibitor [18] was the most potent drug. A variety of inhibitors of protein tyrosine kinases (PTKs) also prevented trypanosome replication with tyrphostin 1 and tyrphostin 51 being the most potent. Inhibitors of protein kinase C (PKC), cAMP-dependent protein kinases (PKA) and cGMP-dependent protein kinases (PKG) inhibited T. brucei replication only when used at high concentrations.

3.5. Effect of inhibitors on T. brucei G₁ checkpoint progression

G₁-restricted T. brucei (5 × 10⁴ ml⁻¹) were incubated for 4 h at 37°C in BMEM + 10% FBS + 2 mM HU with or without inhibitors listed in Table 3, washed and re-incubated for 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹ and screened for DNA content. Cycloheximide, an inhibitor of protein synthesis, or actinomycin D, which pre-

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Fig. 3. DNA content analysis of G₁-restricted T. brucei that were incubated at 37°C for 0–240 min at 5 × 10⁵ organisms ml⁻¹ BMEM + 10% FBS + 2 mM HU, washed and re-incubated for 240–0 min at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹ + 2 mM HU to give a total incubation time in each case of 240 min. The organisms were washed again in PBS and incubated for 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹ prior to cell cycle analysis.
vents DNA transcription, or tyrphostin 1, tyrphostin 51, or DAPH-1, which typically inhibit specific PTKs, prevented \textit{T. brucei} G, checkpoint progression (Table 3). Most (> 70%) \textit{T. brucei} that failed to pass the checkpoint in the presence of the drugs, were able to pass the checkpoint in response to FBS after removal of the drugs (data not shown).

A combination of EGTA, which chelates trace Ca\(^{2+}\) in medium, and BAPTA-AM, which chelates intracellular Ca\(^{2+}\), did not inhibit \textit{T. brucei} G, progression (Table 3). These studies were carried out using Ca\(^{2+}\)-free BMEM and Ca\(^{2+}\)-depleted FBS. The final concentration of EGTA was 6 mM and that of BAPTA-AM was 12.5 µM. This was the highest non-toxic concentration of BAPTA-AM and is slightly lower than that shown by Ruben et al. [19] to completely suppress Ca\(^{2+}\) spikes in \textit{T. brucei} organisms incubated with a Ca\(^{2+}\) ionophore in the presence of extracellular Ca\(^{2+}\).

\textit{G,}-restricted \textit{T. brucei} (5 × 10^5 ml\(^{-1}\)) that were incubated for 4 h at 37°C in BMEM + 10% FBS + 10^{-4}M tyrphostin 51, washed and re-incubated for varying times in BMEM + 10% FBS, crossed the \textit{G,} checkpoint with similar kinetics to \textit{G,}-restricted \textit{T. brucei} incubated only with FBS (as shown in Fig. 4). We conclude that tyrphostin 51-sensitive catalytic activity was required for \textit{G,} progression of \textit{T. brucei} irrespective of their position in \textit{G,}.

Although a concentration of 3.3 × 10^{-6} M tyrphostin 51 inhibited replication of 5 × 10^3 \textit{T. brucei} in 1 ml growth medium (Table 2), it was necessary to use 10^{-4} M tyrphostin 51 to prevent \textit{G,} progression of 5 × 10^5 \textit{T. brucei} ml\(^{-1}\) medium (Fig. 4) indicating that a critical amount of the tyrphostin is required for each trypanosome.

Incubation of exponentially growing \textit{T. brucei} (5 × 10^5 ml\(^{-1}\)) for 9 h at 37°C in BMEM + 10% FBS + 10^{-4} M tyrphostin 51 resulted in accumulation of 73% of cells in \textit{G,} indicating that the drug did not prevent transition through S and \textit{G,}/M at the concentration used.

**Fig. 4.** (A) Reaction of a rabbit anti-FBS-lipoprotein with FBS-LDL, FBS-HDL, total FBS lipoproteins (LP), lipoprotein-depleted FBS (LPD), and PBS following radial immunodiffusion. All protein contents were adjusted to 1 mg ml\(^{-1}\), and 5 µg of each preparation was added to peripheral wells. Rabbit anti-LP serum (25 µl) was added to the center well and the immunodiffusion slide was incubated at 4°C for 40 h prior to photography. (B) Effect of depletion of lipoproteins by immunoaffinity chromatography on the capacity of FBS to support \textit{G,} checkpoint progression of \textit{G,}-restricted \textit{T. brucei}. Both sera had 9.5 mg protein ml\(^{-1}\). The lipoprotein-depleted serum (■—■) contained < 0.5% of the cholesterol content of the control (○—○) serum which was collected after column saturation. \textit{G,}-restricted \textit{T. brucei} were incubated for 4 h at 37°C at 5 × 10^5 organisms ml\(^{-1}\) BMEM + 5 mg FAF-BSA ml\(^{-1}\), with or without dilutions of the two serum preparations at matched protein content (recorded in the figure as % column eluent), + 2 mM HU. The organisms were washed 3 times in PBS and incubated for 4 h at 37°C in BMEM + 5 mg FAF-BSA ml\(^{-1}\) and screened for DNA content by flow cytometry. Results are recorded as the % of \textit{T. brucei} that pass the \textit{G,} checkpoint calculated as (% \textit{T. brucei} that are in \textit{G,} after incubation in the absence of serum - % \textit{T. brucei} that are in \textit{G,} after incubation in medium containing a serum fraction).
3.6. Influence of tyrphostin 1, and tyrphostin 51 on pyruvate production and protein synthesis by G₁-restricted T. brucei

Inclusion of $10^{-4}$ M tyrphostin 51 in incubation medium containing 10% FBS and 2 mM HU had no effect on the capacity of G₁-restricted organisms to synthesize protein assayed by incorporation of $[^{35}]$methionine into the 10% (wt) TCA-precipitable fraction, but inclusion of $10^{-4}$ M tyrphostin 1 in the medium virtually eliminated protein synthesis (Table 4). Neither tyrphostins affected the capacity of the T. brucei organisms to catabolize glucose to pyruvate (Table 4), which is the sole energy generating pathway of replicating bloodstream stage T. brucei organisms [20].

Lysates of exponentially growing T. brucei, G₁-restricted T. brucei and G₁-restricted T. brucei that had been incubated for up to 20 min at 37°C in BMEM + 10% FBS contained the same abundant tyrosine phosphorylated polypeptides, detected after SDS-PAGE and Western blotting with phosphotyrosine-specific mab 4G10 (not shown). Furthermore, inclusion of $10^{-4}$ M tyrphostin 51 in the incubation medium resulted in reduced tyrosine phosphorylation of all of the polypeptides (not shown). Thus we have not identified any tyrosine phosphorylated polypeptide that is uniquely associated with serum-induced G₁ progression.

Table 1

| Incubation condition | BMEM + 2% FBS | % T. brucei in G₁ at | | |
|----------------------|---------------|----------------------|----------------------|
| 4 h                  | 8 h           |                      |                      |
| 5 mg FAF-BSA*        | 70.9          | 72.6                 |                      |
| 5 mg FAF-BSA* + 10 µg LDL* | 58.9 | 39.8 |                      |
| 5 mg FAF-BSA* + 30 µg LDL* | 27.0 | 10.6 |                      |
| 5 mg FAF-BSA* + 100 µg LDL* | 19.8 | 8.8 |                      |
| 10% FBS              | 3.3           | 3.0                  |                      |

Table 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target protein kinase</th>
<th>T. brucei growth-inhibitory concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staurosporin</td>
<td>PKC, PKA, PKG, PTK</td>
<td>$2.5 \times 10^{-7}$</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>PKC, PKA</td>
<td>$1.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>H7</td>
<td>PKC, PKA, PKG</td>
<td>$1.0 \times 10^{-4}$</td>
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<td>H8</td>
<td>PKC, PKA</td>
<td>$1.0 \times 10^{-4}$</td>
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<tr>
<td>HA1004</td>
<td>PKC, PKA, PKG</td>
<td>$1.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>Genistein</td>
<td>PTK</td>
<td>$4.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>Lavendustin A</td>
<td>PTK</td>
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<tr>
<td>Tyrphostin 1</td>
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<td>$4.8 \times 10^{-6}$</td>
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<td>Tyrphostin 23</td>
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<td>$2.4 \times 10^{-5}$</td>
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<td>Tyrphostin 25</td>
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</tr>
<tr>
<td>Tyrphostin 46</td>
<td>PTK</td>
<td>$2.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>Tyrphostin 47</td>
<td>PTK</td>
<td>$2.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Tyrphostin 51</td>
<td>PTK</td>
<td>$3.3 \times 10^{-6}$</td>
</tr>
<tr>
<td>Tyrphostin 63</td>
<td>PTK</td>
<td>$2.6 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

*The final drug concentration that inhibited parasite replication by 90% or greater of control cultures.

T. brucei S 427 clone 1 organisms were incubated for 48 h at 37°C in triplicate at a density of $5 \times 10^5$ organisms ml⁻¹ BMEM + 10% FBS + varying concentrations of protein kinase inhibitors.

4. Discussion

G₁-restricted T. brucei, prepared by incubation of exponentially growing organisms in a growth-limiting concentration of serum plus an adequate amount of FAF-BSA to neutralize endogenous growth inhibitors [5], re-entered the cell cycle in response to FBS (Fig. 1). Removal of lipoproteins by immunoaffinity chromatography severely reduced the capacity of FBS to support G₁ progression, whilst purified LDL (Table 1) and HDL (data not shown) caused G₁ progression in a dose- and time-dependent manner consistent with their established role as the principal T. brucei growth-supporting molecules in axenic cultures [5,13]. HU caused serum-, or lipoprotein-stimulated G₁-restricted T. brucei to arrest at the G₁/S boundary (Fig. 2) consistent with its inhibitory effect on ribonucleotide reductase [21]. HU also inhibits exponentially growing yeast and vertebrate cells at
Table 3
Inhibition of G₁ checkpoint progression

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
<th>Exp 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>87</td>
<td>86</td>
<td>82</td>
<td>83</td>
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<tr>
<td>50 µg Cycloheximide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>1 µg Actinomycin D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
<td>26</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>6 mM EGTA + 12.5 µM BAPTA-AM</td>
<td>74</td>
<td>72</td>
<td>76</td>
<td>79</td>
</tr>
<tr>
<td>10⁻⁴ M Tyrophostin 1</td>
<td>11</td>
<td>10</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>10⁻⁴ M Tyrophostin 51</td>
<td>17</td>
<td>19</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>5 x 10⁻⁵ M DAPH</td>
<td>14</td>
<td>12</td>
<td>18</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentration ml⁻¹.

G₁-restricted T. brucei were incubated for 4 h at 37°C at a density of 5 x 10⁵ ml⁻¹ BMEM + 10% FBS + 2 mM HU + inhibitors listed, washed 4 times in PBS and reincubated for 4 h at 37°C in BMEM + 5 mg FAF-BSA ml⁻¹ prior to cell cycle analysis. Results are calculated as:

% G₁-restricted T. brucei that cross checkpoint = 100 - % T. brucei in G₁ before incubation x 100% T. brucei in G₁ after incubation

Table 4
Effect of tyrphostin 1 and tyrphostin 51 on protein synthesis and pyruvate production by T. brucei S427 clone 1 organisms in vitro

<table>
<thead>
<tr>
<th>BMEM supplements (2mM HU +)</th>
<th>DPM (x 10⁻⁵) [³⁵S]methionine incorporated by 4 x 10⁵ T. brucei organisms</th>
<th>Moles (x 10⁻¹⁵) pyruvate excreted trypanosome⁻¹ min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FBS</td>
<td>3.11</td>
<td>1.32</td>
</tr>
<tr>
<td>10% FBS + 10⁻⁴ M tyrphostin 51</td>
<td>3.17</td>
<td>1.26</td>
</tr>
<tr>
<td>10% FBS + 10⁻⁴ M tyrphostin 1</td>
<td>0.09</td>
<td>1.25</td>
</tr>
</tbody>
</table>

For analysis of protein synthesis, triplicate 1-ml cultures of G₁-restricted T. brucei organisms (4 x 10⁵ organisms ml⁻¹ BMEM + 10% FBS + 2 mM HU + 1 mCi [³⁵S]methionine with or without tyrphostins) were incubated at 37°C for 2 h, resuspended and 100-µl aliquots were mixed with an equal volume 20% TCA. Precipitates were collected on 0.45 µm pore size filters, washed with 10% TCA then 90% ETOH, dried, immersed in scintillant and counted in a scintillation spectrometer. Results are the mean disintegrations min⁻¹ (DPM).

For analysis of pyruvate excretion, triplicate 1 ml cultures of G₁-restricted T. brucei organisms (2 x 10⁵ organisms ml⁻¹ BMEM + 10% FBS + 2 mM HU + 1% wt glucose with or without tyrphostins) were incubated at 37°C for 0–2 h and cell-free supernatants were collected and assayed for pyruvate content. In all cases pyruvate accumulation in medium was linear.

Virtually all G₁-restricted T. brucei became committed to divide during incubation with serum or lipoproteins + FAF-BSA. Commitment occurred in a linear manner over a period of time (Fig. 3) that varied with the concentration of serum (data not shown), or of isolated LDL in medium (Table 1). T. brucei population doubling time in exponential growth was 7.5 h and 41 ± 12% of exponentially growing organisms were in G₁ indicating that the G₁ interval occupied about 3 h. It required 4 h of incubation with 10% FBS (optimal condition) to commit most of the G₁-restricted T. brucei to division (Table 1, Fig. 3). Therefore it is probable that the G₁-restricted T. brucei were spread throughout G₁, had a short lag before re-entering the cell cycle, and each organism committed to division at, or close to, the end of its G₁ interval.

Exponentially growing Acanthamoeba in asynchronous cultures re-enter S after mitosis [26] and...
consequently bypass $G_1$ regulation. Exponentially growing *T. vaginalis* synchronize in $G_2$ in response to drug-induced stasis, or serum deprivation [27], and hence may commit to divide during the $G_2$ interval. Other protists do, however, regulate commitment to division in $G_1$ but with some differences to *T. brucei*. Commitment of *Paramecium tetraurelia* to mitotic division occurs at the end of $G_1$ of the preceding division just prior to initiation of macronuclear DNA synthesis [28]. Similarly, commitment of *Chlamydomonas* to division is determined just prior to DNA synthesis [29], but continued growth after commitment allows the cells to make further separate commitments prior to cytokinesis. In contrast, slender *T. brucei* commit to only 1 division in $G_1$ and commitment must be re-confirmed in the subsequent division.

Yeast and fibroblasts also become committed to a division in $G_1$. Commitment in fission yeast occurs at START which is close to $S$ and is regulated by nutrient availability and mating pheromones [30]. Commitment in fibroblasts is regulated by serum growth factors and occurs at the restriction point R, which is a few hours before $S$ [31]. Commitment corresponds to generation of a threshold complex of $G_1$ cyclins and cyclin-dependent kinase [32], which requires gene transcription and protein synthesis. Lipoprotein dose- and time-dependent progress of $G_1$-restricted *T. brucei* through $G_1$ to the point of commitment to divide (Table 1) also suggests accumulation of a regulatory molecule or complex of molecules to threshold concentration. Furthermore, inhibition of G1 progression by inclusion of actinomycin D and cycloheximide in incubation medium (Table 3) shows a requirement for gene transcription and protein synthesis. Protein synthesis has also been shown to be a requirement for $G_1$ to S transition of procyclic *T. brucei* [6]. Whilst a mitotic-like cyclin gene has been cloned from *T. brucei* and its product shown to complex with putative cyclin-dependent kinase p34cctcz [25], $G_1$ cyclins have not yet been described.

Removal of lipoproteins from culture medium caused progression of uncommitted *T. brucei* towards the $G_1$ checkpoint to cease (Fig. 4). Constant provision of a single FBS lipoprotein-associated component might therefore drive $G_1$ progression. In this regard, several biological processes that require long-term ligand and receptor interactions have been reported for mammalian cells, e.g. transcription of the retinoic receptor beta 2 gene in embryonal carcinoma cells [33], continued inhibition of exocrine and endocrine secretion by somatostatin [34], and myeloid CSF-induced expression of the pim-1 kinase which directly correlates with cell proliferation [35]. The possibility that several different components of FBS lipoproteins stimulate several distinct biochemical pathways each of which acts at a distinct stage during $G_1$ seems unlikely to us because $G_1$ progression was Ca$^{2+}$-independent, did not appear to require PKA, PKC and PKG activity (see below) and involved continuous tyrphostin 51-sensitive PTK activity as discussed below.

A combination of extracellular and intracellular Ca$^{2+}$ chelators (respectively EGTA and BAPTA-AM) did not prevent $G_1$ checkpoint progression in Ca$^{2+}$-free medium (Table 3), excluding the involvement of Ca$^{2+}$-binding *T. brucei* proteins [36] and kinases [37] in the process. Sphingosine, H7, HS and HA1004 which inhibit PKA, PKG and PKC catalytic activity [18,38,39] affected replication of $5 \times 10^3$ slender *T. brucei* ml$^{-1}$ medium only when used at $10^{-4}$ M (Table 2) which is 10–100-fold higher than the required concentration to affect target kinases in mammalian cells [38,39]. At this concentration the drugs are likely to exert non-specific inhibitory effects. Thus PKA, PKG and PKC catalytic activity might not be required for cell cycle progression of the organisms or else *T. brucei* kinase catalytic sites, with the exception of the H7-sensitive PKC-like *T. brucei* kinase [37], might differ from their mammalian counterparts. H7 and H8 have also been shown to have no effect on $G_1$ to S transition of procyclic *T. brucei* [6].

Specific inhibitors of PTKs prevented replication of $5 \times 10^3$ *T. brucei* organisms ml$^{-1}$ growth medium when used at between $10^{-5}$ and $10^{-6}$ M (Table 2), i.e. at concentrations that inhibit function of PTKs isolated from higher eukaryotes [38,40–42]. Tyrphostin 1 was one of the most potent inhibitors of *T. brucei* replication. The drug inhibited $G_1$ to $S$ cell cycle transition, did
not affect *T. brucei* energy metabolism but, like genistein [6], was a potent inhibitor of *T. brucei* protein synthesis (Table 4). The kinase target of tyrphostin 1 is unknown; the agent does not affect the EGFR-PTK or insulin receptor PTK [41].

Tyrphostin 51 and DAPH 1 were also potent inhibitors of replication of *T. brucei*. Tyrphostin 51 is known to competitively inhibit the interaction of the EGFR-PTK with its substrate [41] and DAPH 1 competitively inhibits the interaction of the PTK with ATP [42]. Tyrphostin 51 did not affect the capacity of *T. brucei* to synthesize protein, or to catabolize glucose to pyruvate which is the sole energy generating metabolic pathway in replicating bloodstream stage organisms [20]. Like serum (Fig. 3) or lipoproteins (Table 1), the tyrphostin 51-sensitive kinase activity was apparently required throughout G₁ and hence might affect a pathway or pathways that required continuous induction. We conclude that a growth regulatory kinase with a catalytic site that is operated by serum LDL and HDL via a Ca²⁺-independent kinase or kinases of which one or more has a catalytic domain that is inhibited by tyrphostin 51 and DAPH 1. *T. brucei* S 427 cl 1 is selected to grow in vitro but can be passaged in vivo without additional adaptation [13], consequently it is probable that the growth regulatory pathway operates in unselected organisms. Identification of the FBS-associated ligand(s) and elucidation of the G₁-regulatory signal pathway that it drives will contribute to our understanding of host and parasite interactions that regulate parasite population dynamics, and virulence.

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