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Serum Xanthine Oxidase: Origin, Regulation, and Contribution to Control of Trypanosome Parasitemia

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ABSTRACT

African trypanosomiasis is caused by Salivarian trypanosomes, tsetse fly-transmitted protozoa that inhabit the blood plasma, lymph and interstitial fluids, and, in the case of *Trypanosoma brucei* species, also the cerebrospinal fluid of mammal hosts. Trypanosomiasis in people and domestic animals manifests as recurring waves of parasites in the blood and is typically fatal. In contrast, trypanosomiasis in Cape buffaloes, which are naturally selected to resist the disease, is characterized by the development of only one or a few waves of parasitemia, after which the infection becomes cryptic, being maintained by the presence of 1–20 mammal-infective organisms/ml of blood. The control of the acute phase of parasitemia in Cape buffaloes correlates with a decline in blood catalase activity and the generation of trypanocidal H_2O_2 in serum during the catabolism of endogenous purine by xanthine oxidase. Here we review features of this response, and of trypanosome metabolism, that facilitate H_2O_2-mediated killing of the parasites with minimal damage to the host. We also discuss the origin and regulation of serum xanthine oxidase and catalase, and show how recovery of serum catalase in infected Cape buffaloes precludes a role for H_2O_2 in the long-term, stable suppression of trypanosome parasitemia. Antioxid. Redox Signal. 4, 161–178.

TRYPANOSOMES AND TRYPANOSOMIASIS

THE AFRICAN TRYPANOSOMIASES represent a group of diseases caused by African trypanosomes, flagellated protozoan parasites belonging to the order *Kinetoplastida* of the class *Zoomastigophora*. These diseases affect man and livestock. Clinically they are characterized by intermittent fever, lymphoid organ enlargement, massive elevation in serum IgM, immune complex disease, and progressive emaciation, accompanied, in the case of human trypanosomiasis, by nervous system pathology and, in the case of bovine trypanosomiasis, by severe anemia. The distribution of African trypanosomes coincides with that of their tsetse fly vector. Tsetse flies infest the humid and semihumid zones of Africa affecting a land mass of 10 million km^2 and embracing 36 countries lying between latitudes 15°N and 20°S. Trypanosomiasis precludes cattle-based agriculture from much of this area, presently results in a yearly loss of about U.S. $2.7 billion in production revenue in areas where cattle ranching is possible, and threatens up to 60 million people, of whom about a half million are presently infected.

African trypanosomes are spindle-shaped protozoans of about 20 μm long and 2 μm

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maximum diameter and bearing a single flagellum. They inhabit the blood plasma and interstitial fluids of their mammal hosts. Although exposed to attack by plasma components, the parasites have been selected through evolution to evade elimination. Trypanosome survival in the bloodstream relies on a sheath of glycosylphosphatidylinositol-anchored glycoprotein, the so-called variable surface glycoprotein (VSG). The VSG coat masks complement-activating membrane components and hence protects the parasites from complement-mediated opsonization and lysis. Furthermore, it provides trypanosomes with a means of evading immune elimination. The expression of the coat genes is regulated so that only one of the 1,000 or so genomic VSG genes (hundreds of VSG genes and pseudogenes) present in each trypanosome, and recombinants of these, is expressed at a time. The extensive diversity of VSG genes, together with their clonal expression and stochastic transcriptional switching, results in a practically unlimited parade of antigenically distinct trypanosomes. This ensures chronic infection by out-pacing immune response mechanisms. The infection manifests as recurring waves of parasitemia that are resolved by succeeding waves of trypanosome VSG-specific antibody production. The magnitude of parasitemic waves varies among hosts, being highest in the most trypanosomiasis-susceptible species.

Although trypanosomiasis is fatal to people and domestic animals, this is certainly not the case in all mammals. Some sub-Saharan mammals have been selected through evolution to coexist with tsetse flies and trypanosomes. Blood meal analyses of captured tsetse flies show that they feed on a variety of wildlife species, including Cape buffaloes (6). Serological analyses of samples collected from Cape buffaloes in the field show that they contain antibodies against African trypanosomes, the titer of which increases with age of the buffaloes, consistent with continuous challenge and infection (46). These naturally infected Cape buffaloes display few or no signs of disease.

TRYPANOSOME CONTROL IN CAPE BUFFALOES: THE ROLE OF SERUM XANTHINE OXIDASE

Cape buffaloes that are bred in captivity from trypanosome-free parents and maintained in a tsetse fly-free environment are resistant to trypanosomiasis (17, 23, 48, 55, 66, 80), showing that the trait has a genetic basis. Trypanosome growth is initially similar in experimentally infected cattle and captive Cape buffaloes (66, 80). However, the parasites attain higher peak levels in the blood of infected cattle as compared with the Cape buffaloes. Furthermore, waves of parasitemia recur in cattle until their death, whereas experimentally infected Cape buffaloes display only a single, or occasionally a few, waves of patent parasitemia.

Experimentally infected Cape buffaloes do not mount faster, or higher-titer, VSG-specific antibody responses than similarly infected cattle (66). However, they do develop nonspecific trypanocidal activity in blood serum (80), which may account for their rapid control of parasitemia. A protein responsible for this activity has been purified from Cape buffalo serum using column chromatography, and was identified as xanthine oxidase (XO) by a combination of amino acid sequence analysis, immunoaffinity chromatography, substrate dependence, and sensitivity to external effectors (48). Trypanosomes do not remove XO from culture medium during incubations at 4°C and 37°C (Wang and Black, unpublished observations). Consequently, the parasites are unlikely to bind, endocytose, or catabolize the enzyme. This indicates that XO-dependent killing of the parasites relies on an interaction between the enzyme and its substrate in medium.

XO is one conformational form of xanthine:oxygen oxidoreductase (XOR), a flavoprotein enzyme that catalyzes the oxidation of hypoxanthine and xanthine to uric acid. The enzyme is composed of two identical, noncovalently associated subunits each of 146 kDa. The reaction mechanisms of XOR are partially resolved (10, 25, 41, 64). In brief, XOR reacts with purines at a sulfated molyb-

dopterin center and captures electrons from them via the sulfido group. The electrons are channeled to a flavin adenine dinucleotide (FAD) center via two, nonidentical, iron:sulfur centers. Electrons are then passed from the FAD center to an oxidizing substrate, either NAD^+ or O_2. XOR can exist as xanthine dehydrogenase (XDH), XO, or a dual-purpose enzyme (XDH/XO) depending on its conformation. XDH solely uses NAD^+ as an electron acceptor, yielding NADH as a by-product of purine catabolism. In contrast, XDH/XO preferentially uses NAD^+ as an electron acceptor, but also uses O_2 and does so when NAD^+ is limiting, or NADH is in excess. XO exclusively uses O_2 as the electron acceptor, yielding O_2^- and hydrogen peroxide (H_2O_2) as reaction by-products. XO is one of the most widely distributed oxidases and has been inculpated in a legion of biological phenomena ranging from H_2O_2-mediated signal transduction, through the amplification of inflammatory responses, to organ failure (12, 22, 53, 85).

In the presence of Fe^{2+}, H_2O_2 and O_2^- can yield OH^- via the Haber–Weiss reaction and Fenton chemistry. Although each of these reactive oxygen intermediates has microbicidal activity, our investigations show that the trypanocidal activity of Cape buffalo serum and purified Cape buffalo XO is mainly, if not entirely, due to H_2O_2 (48). The trypanocidal activity is inhibited by addition of catalase, which catalyzes conversion of $2H_2O_2$ to $2H_2O + O_2$, but is unaffected by addition of superoxide dismutase (SOD), mannitol, or taurine. The SOD catalyzes conversion of $2O_2^-$ to $O_2 + H_2O_2$, whereas mannitol and taurine scavenge OH^-.

TRYPANOSOME GLUCOSE CATABOLISM AND SENSITIVITY TO H_2O_2

Trypanosomes are highly motile. Exposure to trypanocidal Cape buffalo serum results in a progressive loss of motility, until the parasites became moribund. A combination of purified XO and xanthine is similarly lethal to trypanosomes, as is addition of H_2O_2. As little as 1 μM H_2O_2 can kill some bloodstream stage trypanosomes, whereas others require up to 10 μM concentration of the reactive oxygen intermediate, suggesting differences in their redox pathways or in the target of the H_2O_2, or both. The cytosolic energy coinage of trypanosomes [adenosine triphosphate (ATP)] plunges to 10% or less of its normal concentration within seconds after exposure of the parasites to trypanocidal Cape buffalo serum, or a mixture of xanthine and XO (48), suggesting extreme sensitivity of trypanosome energy metabolism to H_2O_2. Furthermore, trypanocidal activity is abrogated by addition of ATP to the reaction mixture (47), lending credence to the hypothesis that energy metabolism is the primary target of the reactive oxygen intermediate.

Consideration of trypanosome energy metabolism, and their pentose phosphate and redox pathways, suggests an explanation for the high sensitivity of the replicating bloodstream form parasites to H_2O_2. Firstly, bloodstream stage trypanosomes generate all of their ATP by the glycolytic pathway (16, 39). Secondly, they lack ribulose-5-phosphate 3′-epimerase (EC 5.1.3.1) and transketolase (EC 2.2.1.1) (14), and thus terminate their pentose phosphate pathway at ribulose-5-phosphate instead of recycling this product back into the glycolytic pathway. Thirdly, the parasites lack catalase, and instead couple redox reactions that convert H_2O_2 to H_2O using the reducing power of NADPH (20, 54, 60). NADPH negatively regulates glucose-6-phosphate dehydrogenase (G6PD), the enzyme that operates the gateway into the pentose phosphate pathway. Consequently, upon the consumption of NADPH, catabolism of glucose-6-phosphate may be deflected from the glycolytic into the truncated pentose phosphate pathway and, by favoring production of NADPH over ATP in the presence of H_2O_2, may account for the rapid loss of ATP from trypanosomes in the presence of XO and xanthine (48). The advantage conveyed to the parasites by selection for metabolic susceptibility to H_2O_2 is not known.

REGULATION OF TRYPANOCIDAL H_2O_2 IN CAPE BUFFALO SERUM BY SUBSTRATE AVAILABILITY

Undiluted preinfection Cape buffalo serum is not trypanocidal (66), whereas undiluted serum that was collected during clearance of the first parasitemic wave in infected Cape buffaloes had XO-dependent trypanocidal activity (80). The level of XO in Cape buffalo serum did not change after infection with trypanosomes (80). Thus, the acquisition of XO-dependent trypanocidal activity in postinfection serum must reflect a change in substrate availability or an increase in the longevity of H_2O_2 in serum, or a combination of these. In fact, there is both an infection-associated elevation in purine substrate and a decline in blood (erythrocyte and serum) catalase activity, the latter prolonging the survival of H_2O_2 in serum and allowing its accumulation during purine catabolism (80).

Several purines have been shown to elicit production of trypanocidal H_2O_2 when added to Cape buffalo serum, some of which are not directly catabolized by XO (81). This is because serum has additional purine catabolic enzymes. The serum contains guanine deaminase (GA) (Kimball and Black, unpublished observations), adenosine deaminase (ADA), and purine nucleoside phosphorylase (PNP) in addition to XO (81). Upon addition to the serum, adenosine, inosine, guanosine, guanine, hypoxanthine, and xanthine are catabolized to uric acid in a series of steps, yielding H_2O_2 as diagramed in Fig. 1. A similar range of enzymatic activity is found in sera from cattle (80), although amounts of the purine catabolic enzymes differ from those found in Cape buffalo serum, particularly with respect to XO. Mouse serum also contains these enzymes and, in addition, has a limited capacity to catabolize adenine to hypoxanthine (24). However, mouse serum has a lower capacity than Cape buffalo serum for XO-dependent production of H_2O_2 (Fig. 2).

In addition to the presence of extracellular purine-catabolic enzymes, the utilization of purines by host cells and filtration by the kidney prevent the buildup of purine nucleosides, bases, and oxypurines in blood plasma. However, these processes do not fully deplete purines required for the growth of African trypanosomes, which are purine auxotrophs (19, 26), or prevent the development of XO-dependent and catalase-sensitive killing of trypanosomes in undiluted postinfection Cape buffalo serum (80). The trypanocidal activity of postinfection Cape buffalo serum was not affected by dialysis (12 kDa), suggesting that purine substrate was obtained from a pool sequestered with macromolecules and made accessible to XO in the presence of trypanosomes (80). In support of this hypothesis, we have observed that a portion (variable, dependent on the purine species) of added purines becomes associated with serum macromolecules (24) and is not removed by dialysis even against buffer containing 2% (by weight) activated charcoal (Black, unpublished observations). Evidence for a sequestered purine pool is also suggested by the observation that trypanosomes were able to make several divisions in purine-free medium supplemented with fetal bovine serum that had been dialyzed against 2% activated charcoal (Li and Black, unpublished observations). Perhaps the mining of sequestered purines by trypanosomes results

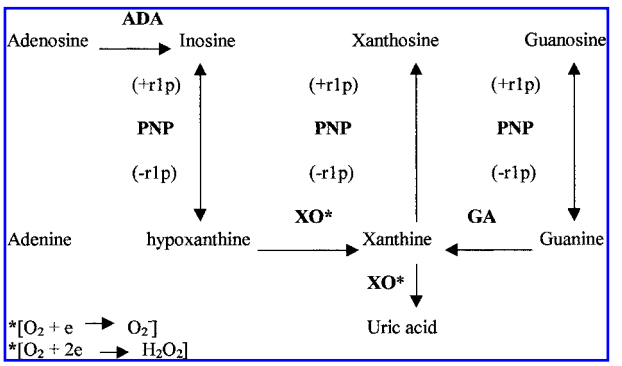

FIG. 1. Purine catabolism and generation of reactive oxygen species in Cape buffalo serum. The diagram is based on data from Wang *et al.* (81) and from Kimball and Black (unpublished observations). The symbol ± r1p denotes the presence or absence of ribose-1-phosphate in the reaction. **ADA** denotes adenosine deaminase, **PNP** denotes purine nucleoside phosphorylase, **GA** denotes guanine deaminase, and **XO** denotes xanthine oxidase. The direction of each reaction is indicated by an arrow, and the absence of an arrow, indicates that the reaction does not occur. *Whether single or two-electron reduction of oxygen occurs depends on the degree of electron loading of XO.

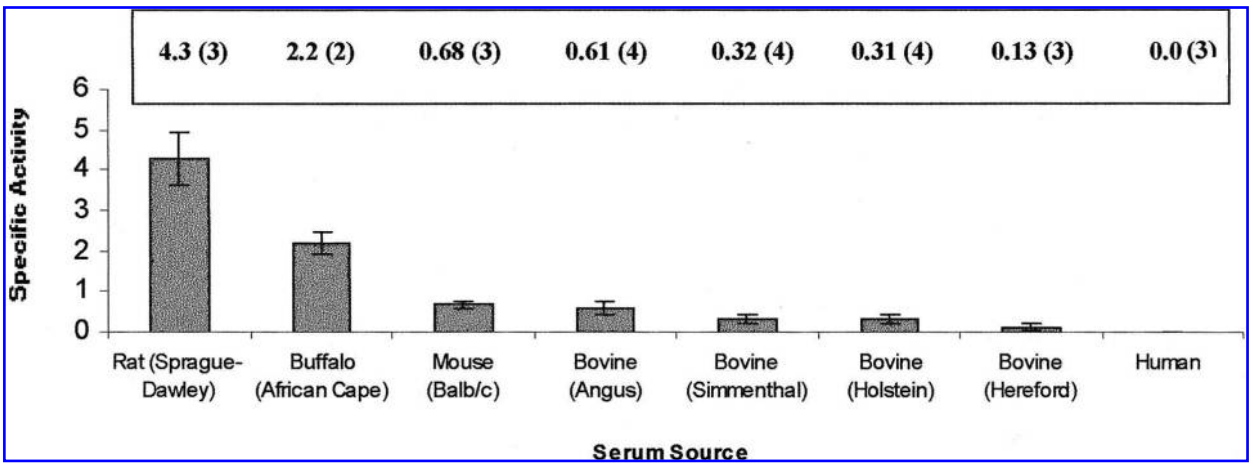

FIG. 2. A comparison of serum XO specific activities. XO specific activities were determined by a kinetic peroxidase assay [derived from Le Tissier *et al.* (36)], under steady-state conditions. In brief, triplicate 20-μl samples were incubated in 180 μl of reagent solution [0.1 m*M* xanthine, 8 U of horseradish peroxidase (HRP)/ml, 0.1 m*M* 4-aminoantipyrine (4AAP), and 1.0 m*M* 2,4,6-tribromo-3-hydroxybenzoic acid (TBHB) in 1× phosphate-buffered saline, pH 7.0] and were continuously monitored by an MRX spectrophotometer over time at 510 nm. (In the presence of HRP, XO-derived H_2O_2 couples with 4AAP and TBHB, forming a chromagen with a peak absorbance at 510 nm.) Serum XO specific activities are presented in terms of H_2O_2 nmol produced/min/ml of serum. Error bars are one standard deviation. Numerical mean values for serum XO specific activities are given above the respective columns in the graph, as are *n* values. Activity means were statistically compared by a one-sided, two-sample *t* test for independent samples. All comparisons showed statistically significant differences ($p < 0.05$) except for Mouse vs. Angus and Simmenthal vs. Holstein.

in local catabolism and H_2O_2 production by XO in the microenvironment of the parasites. If so, this process would minimize oxidative damage to the host.

REGULATION OF TRYPANOCIDAL H_2O_2 IN SERUM BY CATALASE ACTIVITY

Catalase in undiluted serum from uninfected Cape buffaloes prevents expression of XO-dependent trypanocidal activity (80). Indeed, massive addition of XO (orders of magnitude greater than in Cape buffalo serum) or purine substrate, or both, to undiluted serum from naive Cape buffaloes does not result in trypanocidal activity, whereas removal of catalase by immunoaffinity chromatography, or its inhibition by 3-amino-1,2,4-triazole, does. These studies strongly suggest that catabolism of H_2O_2 by catalase is the limiting factor in expression of trypanocidal activity in Cape buffalo serum, consistent with the observation that an infection-associated decline in blood catalase parallels the acquisition of peak H_2O_2 production and trypanocidal activity by postinfection Cape buffalo serum (80; Table 1). Cape buffaloes are not unique in regulating antioxidant defenses to control pathogens more efficiently. Some *Plasmodium falciparum*-malaria resistant people have a G6PD deficiency that limits NADPH production in infected erythrocytes, leading to their oxidative damage and selective clearance (13), whereas the black rhinoceros has "marked deficiencies of intracellular ATP, catalase, ADA, and other enzymes involved in glycolysis, glutathione cycling and nucleotide metabolism," which "may be an evolutionary adaptation conferring selective advantage against common hemic parasites" (56).

The mechanism that inhibits catalase activity in trypanosome-infected Cape buffaloes is not resolved. Both erythrocyte-associated and serum catalase are similarly affected, implicating a diffusible effector. Incubation of Cape buffalo serum or red blood cells with large numbers of trypanosomes in the presence or absence of VSG-specific antibody had no impact on its catalase activity, suggesting that the putative inhibitor was neither a

TABLE 1. TRYPANOSOME CONTROL IN CAPE BUFFALO

*Day after infection**	*Mean number of mammal-infective trypanosomes/ml of blood*	*Nonspecific trypanocidal activity of undiluted serum*	*nmol of H_2O_2 ± ISD catabolized/ml of serum/min*
0	None	None	2.5 ± 0.5
9	10,000	None	1.5 ± 0.5
11	<1† (possibly none)	Yes‡	0.5 ± 0.1
16	<1† (possibly none)	Yes‡	0.7 ± 0.3
31	10†	None	2.4 ± 0.4
38	10†	None	2.5 ± 0.6
41	10†	None	2.6 ± 1.1

*Cape buffalos 7810, 7813, and 7752 were injected with 5×10^4 *T. brucei* ILTat 1.1 + 5×10^4 *T. congolense* IL1180, which were in exponential growth and have the capacity to transit between *in vitro* and *in vivo* without a lag.

†Jugular blood was collected with 10 IU of heparin/ml and centrifuged (1,000 g for 10 min); plasma was removed; blood cells were washed in trypanosome culture medium (7), serially diluted in triplicate in the medium, and incubated at 37°C, and the cultures were observed daily for the presence of trypanosomes. The proportion of blood to medium did not exceed 20% vol, and preliminary studies using blood spiked with *T. brucei* ILA4 showed that all cultures containing 1 ml of blood with 1 trypanosome gave rise to a trypanosome cell line. A value of <1/ml denotes that no parasites grew in cultures containing 1 ml of blood. A value of 10 trypanosomes/ml of blood of denotes that cultures containing 100 µl of blood grew trypanosomes, but cultures containing 50 µl of blood did not.

‡Assayed as described (80) in the absence of any added purine.

metabolic product of the parasites nor generated during immune destruction of the parasites (79). Analyses of catalase activity in model systems have shown that it can be inactivated by exposure to H_2O_2, alone or in combination with O_2^-, if stabilizing NADPH becomes depleted (5, 27, 54). Catalase can also be inactivated by nitric oxide (NO) (11), which is elevated after trypanosome infection (21, 73). Finally, catalase can be inactivated by monochloramines that are formed when ammonia, released upon decay of an unstable *N*-chloramine, reacts with OCl^-, the latter generated from H_2O_2 and Cl^- by myeloperoxidase released from activated neutrophils (40). Our *in vitro* analyses show that catalase in Cape buffalo serum was not affected by addition of XO and saturating xanthine (~100 µ*M*) (Zou and Black, unpublished observations), but could be inactivated by incubation with >5 m*M* H_2O_2, a concentration far in excess of any that could be reached in blood. Thus, we consider it unlikely that direct exposure to H_2O_2, alone or in combination with O_2^-, accounts for the loss of blood catalase activity in infected Cape buffaloes. We have as yet no information on the role of NO and monochloramines, and do not know whether Cape buffaloes have a G6PD deficiency.

Depletion of catalase from cattle sera does not result in the acquisition of trypanocidal activity, consistent with their low serum XO activity (Fig. 2), whereas this activity can be elicited by depletion of catalase accompanied by supplementation with XO to mimic the concentration found in Cape buffalo serum (80). Understanding the regulation of XO activity in serum and the mechanism of infection-associated loss of catalase activity in blood is required for the complete characterization of this novel defense system and evaluation of the feasibility of implementing this defense in other mammals. In the following sections, we summarize our current understanding of the former of these processes.

ORIGIN OF SERUM XO

XOR is synthesized without a signal peptide in all species examined to date (2, 8, 30, 74, 86), and in mammals it is found in the cytosol of hepatocytes, endothelium, epithelium, kidney, muscle, and a variety of other

tissues (45, 57, 70), but is not found in the endoplasmic reticulum or Golgi apparatus (31, 70), or in association with other organelles with the possible exception of peroxisomes (4). Cellular XOR typically exists as XDH, but is reversibly converted to XO by thiol oxidation and irreversibly converted to XO by proteolytic removal of a 20-kDa peptide fragment from the C-terminus of one subunit essential for maintaining the conformation required to bind NAD^+ (41). Proteolytic conversion of XDH to XO is mediated by an enzyme found in mitochondrial extract (69). The identity of the enzyme and its role in irreversible XDH to XO conversion *in vivo* have not been established.

Conditions that elevate cellular XDH and its conversion to XO include the exposure of expressing cells to a period of anoxia followed by oxygen sufficiency (58, 65, 87) or to inflammatory cytokines (62, 74), elastase released from activated neutrophils (63), adhesins expressed by neutrophils (77), or bacterial lipopolysaccharide (33), or, in the case of mammary epithelium, exposure to glucocorticosteroids and prolactin (34). Although XO is a cytosolic enzyme, it is also detected in plasma (48), on the surface of vascular endothelium *in vivo* (1), and on the surface of vascular endothelium in primary cultures (76) where it is attached through electrostatic reaction with heparan sulfate proteoglycans (1). Many conditions that elevate cytosolic XO also result in an increase in the concentration of XO bound to heparan sulfate proteoglycans on vascular endothelium (29, 82), suggesting that elevated cellular expression of XO can be accompanied by elevated release. The presence of XO on the surface of primary cell lines of vascular endothelium *in vitro* is suggested to reflect specific export from the cell (76), as do related studies showing constitutive release of XO by pulmonary microvascular endothelial cells (59) and the presence of XO on the surface membrane of human endothelial and epithelial cell lines (69). However, we have found no transport of XO or XDH onto the cell surface or into the culture medium from a primary cell line of bovine vascular endothelium that constitutively expresses XOR, from mouse mammary HC-11 epithelium cells that were induced to express XOR by treatment with prolactin, or from XOR overexpressing 3T3 cells that had been stably transfected with bovine XOR under control of an inducible promoter (78). Thus, the pathway that leads to the presence of XO in serum remains controversial. It may be that XOR is exported from unique cell types, or under conditions that we did not replicate in our *in vitro* studies, or that the cytosolic enzyme may simply leak from ailing cells as has been shown for other macromolecules in model systems (32).

Extracellular XOR is mainly in the XO conformation (48), consistent with an origin from cells with an oxidizing environment. Analysis of immunoaffinity-purified serum XO by polyacrylamide gel electrophoresis after sample treatment with sodium dodecyl sulfate (SDS-PAGE) showed that both the 146-kDa uncleaved XO subunit and the 126-kDa cleaved subunit are detected, suggesting that a portion of the enzyme may be derived from cells in which the putative XO-processing enzyme leaked from mitochondria into the cytosol (Van Praagh and Black, unpublished observations).

H_2O_2 that is produced upon addition of xanthine to serum can be readily measured by a coupled reaction with horseradish peroxidase (HRP) (36). The HRP reduces the H_2O_2 to H_2O in the presence of 4-aminoantipyrine (4AAP) (an electron donor) and 2,4,6-tribromo-3-hydroxybenzoic acid (TBHB), yielding a chromagen with a peak absorbance at 510 nm. The assay is not affected by serum catalase (36) and is considered indicative of the level of serum XO (36). The capacity to produce H_2O_2 in serum upon addition of xanthine differs among species and breeds, but is relatively constant among healthy individuals of a species or breed (Fig. 2), suggesting species/breed-specific homeostatic control of serum XO activity levels. Of the sera tested, rat serum had the highest level of xanthine-dependent H_2O_2 production, and human and cattle sera the lowest, although there was significant variation in xanthine-dependent production of H_2O_2 among breeds of cattle.

TABLE 2. H_2O_2 CATABOLISM IN SERUM

Serum donor	*n*	*nmol of H_2O_2 ±ISD catabolized/μl of serum/min*
Rat (Sprague–Dawley)	5	5.9338 ± 1.9133
Cape buffalo	4	0.0179 ± 0.0040
Mouse	5	3.8284 ± 1.3896
Bovine (Holstein)	5	0.0356 ± 0.0080
Human	5	1.6012 ± 0.0849

Serum (100 μl), diluted with phosphate-buffered saline, was added to an equal volume of H_2O_2 (0.625 m*M*) and incubated at 37°C for 20 min. Remaining H_2O_2 was detected by addition of 800 μl of assay buffer (1 m*M* TBHB, 0.1 m*M* 4AAP, and 8 U of HRP/ml of 0.05 *M* potassium phosphate buffer, pH 7.4) by reading at OD512 and comparing the value obtained with those obtained using a series of dilutions of H_2O_2 in assay buffer. *n* denotes number of donor animals, and SD denotes standard deviation.

Interestingly, rats are readily infected by trypanosomes and develop high levels of parasitemia, confirming that a high capacity to produce H_2O_2 during catabolism of xanthine in serum, as determined by the HRP-dependent assay, does not automatically lead to trypanocidal activity and resistance to infection (80). Rat serum also has a very high capacity to catabolize H_2O_2 (Table 2) indicating high catalase activity. Serum catalase activity does not decline in trypanosome-infected rats (Black, unpublished observations), accounting for the absence of XO-mediated serum trypanocidal activity in this species.

REGULATION OF SERUM XO LEVELS: IMPACT OF CODING SEQUENCE

Rat sera have much higher levels of XO activity than sera from cattle (Fig. 2). However, rat liver, which is by far the major XOR-containing organ of the body, contains an equivalent amount of XOR as cow liver on a per gram tissue basis (Van Praagh and Black, unpublished observations). Thus, the level of XO activity that accumulates in serum is not directly associated with total cellular XOR content. To evaluate the possibility that the XOR coding sequence includes information that predisposes toward high serum XO activity levels, we cloned and sequenced the expressed XOR gene from a Cape buffalo (GenBank accession no. AF104501), an eland (GenBank accession no. AF104502), and members of two breeds of cattle, Boran and N'Dama, which are, respectively, *Bos indicus* and *Bos taurus* from Africa. We compared sequence data of the expressed XOR from these species with each other and with the published sequences of human (30, 86), bovine (8), mouse (74), and rat (2) expressed XOR. Serum XO activity is high in Cape buffalo, eland (data not shown), and rat serum, intermediate in mouse, and low in cattle and human serum (Fig. 2). Cape buffalo and eland have similar trypanocidal serum activity (66).

A comparison of inferred amino acid sequences (Fig. 3) derived from the coding sequence of Cape buffalo, eland (GenBank AF104501, AF104502), and *Bos taurus* (8) XORs shows that sites vital to enzyme activity are identical:

(I) The two Fe/S-cluster-binding subdomains of bovine milk XOR are "co-ordinated respectively to Cys 43, Cys 48, Cys 51 and Cys 73, and Cys 113, Cys 116, Cys 148 and Cys 150" (18). These amino acids and intervening sequences are fully conserved in Cape buffalo and eland XOR, and correspond to equivalent sequences in rat, human, and mouse XOR (2, 30, 74, 86).

(II) The amino acids around the NAD^+ binding site have been identified by site-directed peptide chemical modification (52) and sequence motif analysis (50). The sequence TFFPSYRKTLLGPE (amino acids 388–401) corresponds to this NAD^+ binding site and is completely conserved in mouse, rat, human, cattle, buffalo, and eland XOR.

(III) Sequences essential for cofactor FAD binding have been predicted using amino acid sequence "fingerprint" (83). Common to the binding site of the nucleotide cofactor of many enzymes, Cape buffalo and eland XOR sequences are compatible with the proposed sequence

of this binding sites, a βαβ-fold revolved around a highly conserved consensus sequence GXGXXG. In the buffalo and eland sequence, GCGEGGCG (amino acids 42–49) matches the first β-turn, and the α-helix, GGGFGG (amino acids 795–800), could be the second β-turn (74). A similar motif is present in rat, fruit fly, bovine, and human XOR, suggesting that a common conformation is required for flavin binding.

(IV) The molybdopterin factor contacting segments have been identified using the crystal structures of aldehyde oxidase (67) and bovine milk XOR (18). In XOR from Cape buffalo, eland, and cattle, GGGFGG (amino acids 795–800), AFRGFGGPQA (amino acids 909–918), GQG (amino acids 1,039–1,041), PTAAS (amino acids 1,076–1,080), and VGEPPL (amino acids 1,258–1,263) correspond to the contacting segments. Cys^{825}, which has been proposed as the actual cofactor binding residue (42), is fully conserved in Cape buffalo and cattle XOR.

(V) Conversion of XDH to XO occurs irreversibly through limited proteolysis. This causes structural rearrangement of a loop close to the flavin ring, partially blocking access of NAD^+ and altering the electrostatic environment of the active site reflecting the change in substrate specificity (18). The putative tryptic digestion sites, Lys^{185} and Lys^{551}, are well conserved in Cape buffalo, eland, and other mammalian XORs.

(VI) The amino acid residues involved in the reversible conversion of XDH to XO have been identified by thiolmodification and sequence analysis of rat XOR (2, 51). In Cape buffalo XOR, Cys^{534}, Cys^{992}, and $Cys^{1,325}$ correspond to the proposed residues. All the corresponding residues are completely conserved among human, rat, mouse, and, bovine XOR.

These data show that differences in serum XO activity among mammal species and breeds are not due to differences in the amino acid sequences of the enzyme functional sites.

Conserved XOR coding sequence outside that encoding vital domains could account for the higher XO activity levels in Cape buffalo and eland sera as compared with cattle sera. A comparison of deduced amino acid sequences from Cape buffalo, eland, and cattle XORs (excluding residue 552, which might differ among cattle, see below) showed that the former two species had conserved amino acids at 12 positions that differed from those in matched positions in cattle. These are listed in Fig. 4 together with the matched amino acids of domestic bovine, rat (Sprague–Dawley), mouse (BALB/c), and human XOR. A comparison of these residues shows that replacements at residues 348, 472, and 752 conserve the side-chain characteristic, whereas replacements at residues 679, 684, 855, and 1,323 are moderately conservative, *e.g.*, polar charged residues are replaced with polar uncharged residues or with glycine, which can fit into hydrophobic or hydrophilic environments, and replacements that occur at residues 304, 447, 943, and 1,321 are varied. There is no obvious association between replacement fidelity with respect to these 12 residues and serum XO activity.

A comparison of inferred amino acid sequences of XORs from Boran, N'Dama (78), and an improved *Bos taurus* (8) showed that they were practically identical, with the exception that the amino acid at position 552 in Boran and N'Dama was aspartic acid (GAC) in contrast to the published sequence of the improved *Bos taurus,* CAC, which encodes histidine. In addition, XOR from the improved *Bos taurus* (8) and the N'Dama had glutamine (CAG) at position 976, whereas that of the Boran had glutamic acid (GAG) (78). Thus, in contrast to the significant differences in the baseline levels of H_2O_2 production upon addition of xanthine to serum of different breeds of cattle (Fig. 2), there was very little coding difference in the expressed XOR gene.

Although not conclusive, the comparative analyses of expressed XOR coding sequences suggest that differences in xanthine-dependent serum H_2O_2 production among species are not due to differences in XOR coding sequence.

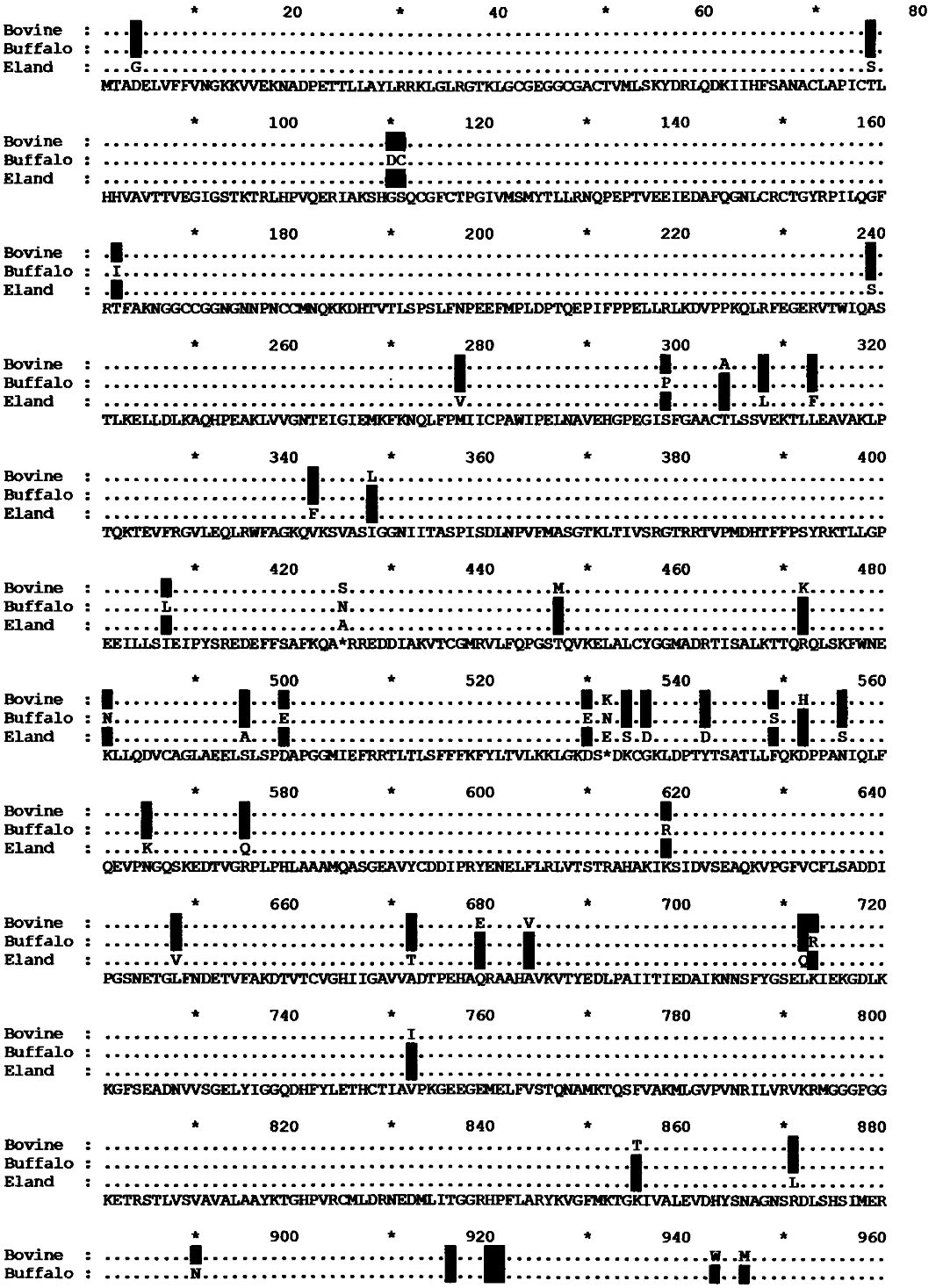

FIG. 3. The deduced amino acid sequences of bovine, Cape buffalo, and eland XOR. Total RNA was isolated from peripheral blood leukocytes. cDNA was synthesized and amplified by a standard RT-PCR method. Both strands were sequenced using the Sequenase version 2.0 system (Amersham). Consensus sequence corresponding to dots is shown on the fourth line. Single residue differences are noted at the appropriate position on lines 1–3. Where three residues differ at the same position, an asterisk is marked at that position in the consensus sequence.

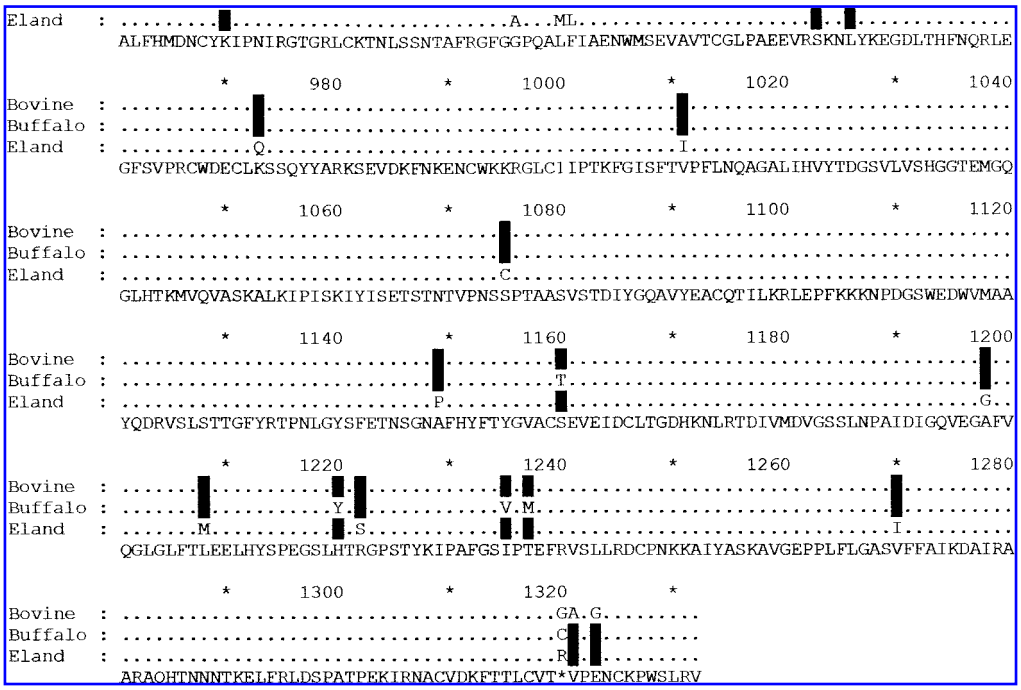

FIG. 3. *Continued*

Bovine Residue #	Matched amino acid residue, and its group characteristic (*), from the inferred amino acid sequences of xanthine oxidases of:					
	C. buffalo	Eland	Rat**	Mouse**	Human**	Bovid
304	Thr (2)	Thr (2)	Pro (4)	Pro (4)	Pro (4)	Ala (3)
348	Ile (3)	Ile (3)	Ile (3)	Ile (3)	Val (3)	Leu (3)
447	Thr (2)	Thr (2)	Ile (3)	Thr (2)	Thr (2)	Met (3)
472	Arg (1)	Arg (1)	Lys (1)	Lys (1)	Arg (1)	Lys (1)
679	Gln (2)	Gln (2)	Gln (2)	His (1)	Gln (2)	Glu (1)
684	Ala (3)	Ala (3)	Gly (4)	Gly (4)	Gly (4)	Val (3)
752	Val (3)	Val (3)	Val (3)	Val (3)	Val (3)	Ile (3)
855	Lys (1)	Lys (1)	Thr (2)	Thr (2)	Thr (2)	Thr (2)
943	Ser (2)	Ser (2)	Arg (1)	Arg (1)	Arg (1)	Trp (3)
946	Leu (3)	Leu (3)	Met (3)	Met (3)	Leu (3)	Met (3)
1321	Val (3)	Val (3)	Val (3)	Thr (2)	Val (3)	Ala (3)
1323	Glu (1)	Glu (1)	Glu (1)	Glu (1)	Glu (1)	Gly (4)

FIG. 4. Comparison of amino acid residues that are conserved in Cape buffalo and eland, but not cattle, with matched amino acids from other XOs. *(1) denotes polar charged; (2) denotes polar uncharged; (3) denotes nonpolar; (4) denotes the presence of a side chain that has a unique property. **Numbered relative to data in Fig. 3 and adjusted for maximal alignment.

POSSIBLE IMMUNE CONTROL OF SERUM XO CONCENTRATION

It is noteworthy that polyclonal (48) and monoclonal (Van Praagh and Black, unpublished observations) antibodies that are raised against the 146-kDa subunit of bovine XO capture a variety of polypeptides from plasma or serum in addition to XO as determined by SDS-PAGE under reducing conditions. Ongoing analyses show that the additional polypeptides captured from serum are immunoglobulins of the IgM and IgG classes, some of which are in complex with XO and others of which bind to idiotypic determinants on XO-specific antibody (Van Praagh and Black, unpublished observations). Immune complexes containing XO have been reported in normal human serum (49), and it is possible that XO-specific antibody as well as antiidiotype antibodies contribute to XO homeostasis in plasma. In this regard, a portion of serum XO from cattle could be isolated by immunoaffinity chromatography on protein A, indicating an association with IgG, whereas that was not the case with XO from Cape buffalo serum (Van Praagh and Black, unpublished observations). Perhaps serum XO homeostasis is set by the specificity and immunoglobulin class of the autoimmune antibody response to the enzyme, and by the efficiency with which XO-binding antibody provokes an antiidiotype response and binds to Fc receptors on phagocytes facilitating clearance of immune complexes. If so, it remains unclear how this process is regulated in outbred animals to achieve species- and breed-associated differences in serum XO levels.

POSTTRANSLATIONAL PROCESSING AND SERUM XO ACTIVITY

Functional XO is a homodimer of 146-kDa subunits, each containing one sulfated molybdopterin center, two nonidentical iron-sulfur centers, and a single FAD. The electron-transfer pathway is xanthine to sulfidomolydopterin to 2Fe/2S to FAD to O_2. Thus, differences in XO activity among sera may result from differences at any of these centers, from differences in the conformation of the molecule or its integrity, from the presence in serum of an inhibitor, or from a combination of these. We have no information on these parameters, and the copurification of some serum immunoglobulins with XO during immunoaffinity chromatography using monoclonal anti-XO, as well as an isolation-associated degree of XO activity loss that varies among donor mammal species and breeds, has limited our capacity to characterize accurately the isolated XO in terms of quantity and specific activity.

It is also possible that differences in XO levels among sera are not due to differences in specific activity or to differences in immune clearance. They may reflect differential binding of XO to vascular endothelium and extracellular matrix among mammal species and breeds. We have no information on this interesting possibility.

SYNERGY BETWEEN XO AND SOD ENHANCES H_2O_2 PRODUCTION DURING CATABOLISM OF XANTHINE AT NEUTRAL pH UNDER AEROBIC CONDITIONS

During reoxidation of reduced XO, electrons are passed to molecular O_2, yielding H_2O_2 and O_2^-. Our ongoing studies show that the yield of H_2O_2 from a combination of commercially purified cows' milk XO (Sigma) and xanthine, at pH 7.4 in air, doubles when excess SOD is added to the reaction mixture (unpublished observations), showing that the ratio of H_2O_2 to O_2^- that is produced under these conditions in the absence of SOD is 1:2. These experiments show that detection of H_2O_2 upon addition of xanthine to serum is not a quantitative measure of XO activity unless SOD is either absent from all samples or present in excess in all samples. Variation in SOD concentrations among sera from different mammal species or breeds may therefore contribute to species-specific differences in xanthine-dependent H_2O_2 generation in serum, up to a maximum value of twofold.

For example, differences in serum SOD concentration, if such occur, might explain differences in cattle breed-associated and xanthine-dependent serum H_2O_2 production (Fig. 2), but could not alone account for the more than twofold difference in xanthine-dependent H_2O_2 production between Cape buffalo and cattle sera. We are in the process of reanalyzing the samples tested in Fig. 2, this time in the presence of excess SOD. In the samples tested so far (rat, Cape buffalo, mouse, Holstein), there is close to a twofold increase in xanthine-induced H_2O_2 production in the presence of excess SOD, indicating that these serum samples contain little or no endogenous extracellular-SOD (EC-SOD) activity. H_2O_2 that is produced upon addition of xanthine to serum in the absence of added SOD is therefore likely to result from the spontaneous dismutation of O_2^-.

CONTRIBUTION OF OXIDATIVE RESPONSES TO CONTROL OF TRYPANOSOMES IN SPECIES OTHER THAN CAPE BUFFALO

The discovery of an infection-associated reduction in blood catalase activity that unleashed the latent trypanocidal activity of Cape buffalo serum prompted an attempt to modify the capacity of mice to control African trypanosomes. It proved possible to enhance significantly trypanosome control in C3H/He mice by long-term provision of the catalase inhibitor, 3-amino-1,2,4-triazole, in their diet (79). However, parasitemias were lowered by only threefold ($p < 0.01$) in the treated mice compared with normal C3H/He mice throughout the course of infection (79), and this effect was not improved by the presence of the cas-1 mutation, which also depletes catalase activity (71). It is likely that the level of serum XO (or XO and SOD) (see Fig. 2) and purine substrate in these hosts was inadequate to support efficient killing of trypanosomes.

With the exception of XO-dependent killing of trypanosomes in Cape buffalo serum, and suppressed parasitemia in catalase-depleted C3H/He mice, there is little evidence of a role for host oxidative defenses in control of African trypanosomes. For example, although trypanotolerant breeds of cattle have elevated levels of serum polyamine oxidase relative to more susceptible breeds, the level of substrate is not adequate to promote the generation of trypanocidal H_2O_2 (75), and the retention of blood catalase levels in these animals after infection (80) further argues against a role for H_2O_2 in their control of blood trypanosomes. Killing of *Trypanosoma brucei brucei* by human serum is hypothesized to involve peroxidase activity of a very-high-density lipoprotein particle that uses endogenous parasite H_2O_2 to oxidize and destabilize the lipid membrane of an acid endosome (72). However, questions have been raised with respect to both the peroxidase activity of the lytic factor and the involvement of endogenous parasite H_2O_2 in the lytic process (44). Furthermore, whereas a number of *in vitro* investigations show that macrophages from trypanosome-infected mice release NO that kills trypanosomes, the killing does not occur in the presence of red blood cells (38), because NO is rapidly inactivated by hemoglobin. This suggests that NO does not contribute to the control of trypanosome parasitemia *in vivo.* Indeed, disruption of the gene that encodes inducible NO synthase in mice has no impact on their capacity to control trypanosome parasitemia (43). As further evidence against a general role for host oxidative defenses in control of African trypanosomes, we have found that administration of ethanol, in a regime (61) that enhances the host oxidative response, or *N*-acetylcysteine, in a regime (3) that sustains glutathione and reduces oxygen radicals, did not affect parasitemia in normal BALB/c mice even when the modified diets were provided for several days before, and for weeks after, the infection was initiated (24). In addition, we have found that levels of parasitemia and survival times are identical in wild-type and interferon-γ knock-out female Balb/c mice infected with *Trypanosoma brucei brucei* GUTat 3.1 (24), suggesting that the interferon-γ-dependent respiratory burst plays little or no role in trypanosome control in this host.

CONCLUDING COMMENTS

The body of evidence discussed above indicates that XO-dependent killing of trypanosomes in serum will be restricted to animals that resemble Cape buffaloes in terms of their serum concentrations of XO, SOD, and purines, and in which catalase drops during infection to a level that permits the accumulation of H_2O_2. The little information we have obtained on regulation of serum XO concentrations indicates that this is a complex process, and control of soluble EC-SOD, purines, and catalase adds additional complications. Clearly, it will be difficult, if not impossible, to modify other mammals to resemble Cape buffaloes with respect to these serum parameters. Perhaps an effort to achieve this would still be justified if the XO-dependent trypanocidal response that arises in infected Cape buffaloes actually terminated the infection. However, even in Cape buffaloes the nonspecific trypanocidal serum response is short-lived and does not result in complete clearance of the infection. Results presented in Table 1 show that the concentration of catalase in blood returns to preinfection levels by a month after infection of the Cape buffaloes, and consequently H_2O_2 cannot accumulate in serum at that time. The restoration of blood catalase to its preinfection level is accompanied by repopulation of the blood with trypanosomes (Table 1), presumably from another niche, such as the lymph or interstitial fluids, and the onset of the cryptic phase of infection, which is characterized by the sustained presence of 1–20 mouse-infective (66) and tissue culture growth-competent (Table 1) parasites/ml of blood. Our ongoing studies suggest that an acquired immune response involving antibodies reactive with trypanosome growth factor receptors may control the organisms at this stage of infection (9).

SUMMARY

Our investigation of trypanosome control in Cape buffaloes revealed a novel extracellular XO-dependent oxidative defense that contributes to control of the acute phase of parasitemia, but is not responsible for the long-term, stable suppression of parasitemia in these hosts (9, 80). An interesting aspect of the defense system is the infection-induced transitory decline in erythrocyte-associated and serum catalase. This feature of infection in Cape buffaloes may also convey protection against parasites that replicate within erythrocytes by enhancing the red-cell sensitivity to H_2O_2. It remains to be determined whether the decline in catalase function is due to a metabolic deficiency in the Cape buffaloes, *e.g.*, a G6PD deficiency, or the generation of a diffusible inhibitor. It also remains to be established how XO reaches the serum and is regulated therein. Our comparative analyses of expressed XOR genes suggest that differences in serum XO activity among mammals is not due to differences in XOR coding sequence. However, our finding that some immunoglobulins copurify with XO during immunoaffinity chromatography on XO-specific polyclonal and monoclonal antibodies raises the tantalizing possibility that autoantibodies may play a role in XO regulation and, by inference, in the regulation of other serum enzymes that derive from the cell cytosol and are transported, or leak, into plasma after acquisition of immune competence. The level of XO in serum may also be affected by how extracellular XO partitions between cell surfaces and plasma. Although it is known that XO is bound to sulfated glycosaminoglycans on the surface of vascular endothelium *in vivo* (1, 29, 82), species comparisons of bound XO have not been done, and, to our knowledge, information is not available on the tissue distribution of cell-bound XO in any mammal. It may prove useful to obtain this information for economically important mammal species, because the distribution of cell-bound XO could conceivably have an impact on tissue damage during inflammation and other conditions that elevate purine substrate, *e.g.*, ischemia/reperfusion. Finally, our studies indicate a role for SOD in determining the amount of H_2O_2 that is generated during catabolism of xanthine by XO under aerobic conditions. This observation may have broad biological relevance. Like XO, EC-SOD binds to heparan sulfate proteoglycans on cellular

surfaces (37) and may, through colocalization on cells, protect them from oxidative damage wrought by O_2^- that is generated by membrane-bound extracellular XO.

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ABBREVIATIONS

4AAP, 4-aminoantipyrene; ADA, adenosine deaminase; ATP, adenosine triphosphate; EC-SOD, extracellular superoxide dismutase; FAD, flavin adenine dinucleotide; GA, guanine deaminase; G6PD, glucose-6-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; IgM, immunoglobulin with μ heavy chains; NO, nitric oxide; PNP, purine nucleoside phosphorylase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TBHB, 2,4,6-tribromo-3-hydroxybenzoic acid; VSG, variable surface glycoprotein; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine:oxygen oxidoreductase.

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