Brief Definitive Report

INHIBITION OF GLUTATHIONE SYNTHESIS AS A CHEMOTHERAPEUTIC STRATEGY FOR TRYPANOSOMIASIS*

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Despite the advances in chemotherapy made during the first part of this century, trypanosomes and their insect vector, the tsetse fly, continue to pose a serious threat to the health and economic well-being of millions of Africans (1). Recent advances in the elucidation of trypanosomal metabolism have encouraged the development of new chemotherapeutic agents specifically designed to exploit known biochemical differences between trypanosomes and their mammalian hosts. Reports that trypanosomes lack catalase (2), a deficiency thought to be secondary to defective heme biosynthesis (3), focused attention on hydrogen peroxide (H_2O_2) and the regulation of its intracellular concentration. Measurements in bloodstream forms of *Trypanosoma brucei brucei* demonstrated an intracellular H_2O_2 concentration of about 70 μM (4), a value at least 100-fold higher than that reported in mammalian cells (5, 6). The present and previous (4, 7, 8) studies suggest that this unusually high concentration of H_2O_2 and the absence of at least one important enzyme of H_2O_2 catabolism (i.e., catalase) constitute a therapeutically exploitable difference in trypanosome biochemistry. We have used buthionine sulfoximine (BSO), a potent and specific inhibitor of glutathione biosynthesis (9), to deplete trypanosomes of glutathione, an important component of the cellular defense mechanism against both H_2O_2 and free radicals (10). Although the administration of buthionine sulfoximine to mammalian cells or whole animals is without apparent toxic effect, it was anticipated that bloodstream forms of *T. b. brucei* would suffer substantial oxidative and free radical damage when depleted of intracellular glutathione.

Materials and Methods

Animals. Female Swiss Webster mice, 18–24 g, were obtained from Taconic Farms, Germantown, N. Y.

Organisms. *T. b. brucei* (EATRO 110) were obtained from Dr. W. Trager (The Rockefeller University) and stored as stabilates at −80°C in 10% glycerol. Thawed organisms were routinely passaged in rodents before use. Intraperitoneal injection of 1×10^5 motile organisms into mice resulted in high parasitemia within 3–4 d with organisms of uniformly slender morphology.

Isolation of Trypanosomes. Trypanosomes were isolated from blood according to the procedure of Lanham and Godfrey (11) 3–4 d after infection. Infected mice were bled by axillary incision with heparinized pipettes under ether anesthesia. Whole blood (1–2 ml) was applied to a column of DE-52 cellulose (Whatman Inc., Clifton, N. J.) and eluted with phosphate-buffered

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saline (3:7) and glucose (PSG). Isolated organisms were washed twice with PSG by centrifugation.

DL-Buthionine-SR-Sulfoximine (BSO). BSO was prepared by a modification of the published procedure (9). Plasma levels of BSO in mice after intraperitoneal administration were determined by amino acid analysis as previously described (12).

Measurement of Total Intracellular Glutathione (GSH). GSH was measured by a modification of the method of Tietze (13). Isolated trypanosomes ($5–10 \times 10^8$) were suspended in 1 ml PSG containing 5 mM HCl. Each cell suspension was divided in two and a known amount of GSH (Sigma Chemical Co., St. Louis, Mo.) was added to one half to act as an internal standard. The preparation of acid-soluble extracts of trypanosome lysates using sulfosalicylic acid (Sigma Chemical Co.) at 2.8%, as well as the measurement of GSH content, was performed on both the sample and the sample plus standard in parallel. Extracts were stored at −80°C and assayed within 1 wk. Protein concentration was determined by the method of Bradford (14), using bovine serum albumin as standard.

Assessment of Trypanocidal Activity In Vivo. Groups of six mice were infected with *T. b. brucei* by intraperitoneal injection of 1×10^5 organisms in 0.5 ml minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.). 10 h after infection, injections of BSO (4 mmol/kg) were begun. BSO was administered intraperitoneally at a concentration of 200 mM every 1.5 h. At the time treatment was begun, parasitemia was confirmed in each animal by microscopic examination of blood smears obtained by tail snip and stained with Diff-Quik Solution II (Harleco, Gibbstown, N. J.). Mice surviving 40 d were considered cured.

Results

Effect of BSO Administration on the Trypanosomal GSH Levels of Parasitemic Mice. Because BSO is a reversible inhibitor of GSH biosynthesis, successful long-term inhibition in bloodstream forms of *T. b. brucei* requires the continual presence of a substantial plasma BSO concentration. Preliminary experiments indicated that intraperitoneal injection of mice with 4 mmol/kg of BSO produced plasma levels of inhibitor that reached 5–6 mM in 15–20 min and then declined to about 1 mM at 1.5 h after injection. Based on these findings, a protocol was adopted in which 4 mmol/kg of BSO was administered intraperitoneally every 1.5 h. Control experiments indicated that this procedure produced no overt toxicity in uninfected mice, even when continued for 18–27 h (a total administration of 48–72 mmol/kg of BSO).

To assess the effect of BSO on trypanosomal GSH levels, parasitemic mice were given BSO, and bloodstream forms of *T.b. brucei* were subsequently isolated at various times and assayed for GSH. The results are given in Fig. 1. It is evident that the GSH level of trypanosomes falls to ~50% of its normal value after 5 h of exposure to BSO and that the rate of decline decreases progressively thereafter. Trypanosomes isolated from infected animals after 10 or more h of BSO treatment lysed spontaneously at 25°C and could not be accurately assayed for GSH.

Effect of BSO Administration on Trypanosome Survival in Parasitemic Mice. The marked instability of isolated trypanosomes containing $<\sim 1$ nmol GSH/mg protein suggested that continued inhibition of GSH biosynthesis might be lethal to the parasites in vivo. When mice with very high parasitemias were given BSO for 18 h (4 mmol/kg every 1.5 h) and then examined, blood samples were found to be free of parasites. Despite the absence of parasites, the treated mice were hypothermic, exhibited ruffled fur, and generally appeared more ill than untreated parasitemic controls. The treated mice died within 3 d while still apparently free of parasites; untreated parasitemic controls died 1–2 d later.

The results given above suggested a toxic reaction to lysed trypanosomes. A similar

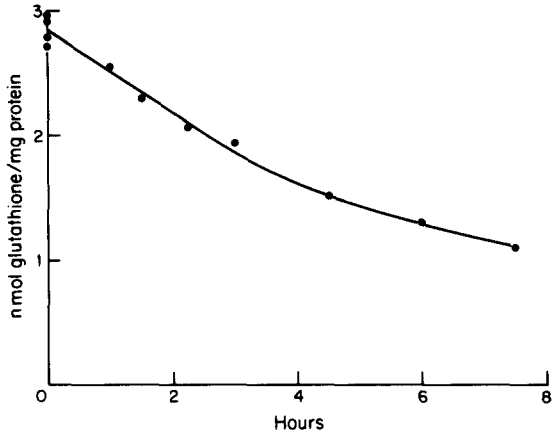

FIG. 1. Level of GSH in *T. b. brucei* after administration of BSO to parasitemic mice. Parasitemic mice (3–4 d after infection with 1–2 × 10^5 organisms) were injected intraperitoneally with 4 mmol/kg BSO at time zero and at 1.5-h intervals thereafter. At the times indicated, trypanosomes were isolated from the blood and analyzed for GSH as described in Materials and Methods. The values reported represent GSH equivalents (i.e., GSH + 2 × GSSG); previous studies indicated that GSSG represents <5% of GSH (8).

TABLE I

Effect of BSO on the Survival of T. b. brucei-infected Mice

Number of injections*	Survival time‡
0	4, 4, 5, 5, 5, 5
6	4, 4, 4, 5, 5, 5
12	5, 5, 5, 5, 8, 9
18	11, 11, 12, 16, C, C§

* 4 mmol/kg every 1.5 h. Mice were parasitemic at the onset of treatment with 1–5 × 10^3 parasites/ml blood.

‡ Days to death after infection. Deaths were associated with high parasitemia (0.5–5 × 10^9 parasites/ml blood within 24 h of death).

§ C, cured (survived 40 d without detectable parasitemia).

experiment was therefore carried out using mice with lower initial parasitemias. These mice were divided into groups that received 4 mmol/kg BSO intraperitoneally every 1.5 h for a total of 0, 6, 12, or 18 injections. The survival times of the individual animals are given in Table I. Of the mice receiving 18 injections, two were cured. Four others had significantly prolonged survival, but eventually died with marked parasitemias. In a similar experiment in which animals received 0, 4, or 12 injections (data not shown), two of the four animals in the most extensively treated group showed a clearing of parasitemia and significantly prolonged survival (15 and 16 d); however, these animals eventually succumbed to trypanosomiasis.

Discussion

The formation of H_2O_2 by cells appears to be an unavoidable consequence of aerobic metabolism. The steady-state intracellular concentration of H_2O_2 represents a balance between rates of production, catabolism, and diffusion out of the cell (6). Thus, because bloodstream forms of *T. b. brucei* lack catalase (2) and are without detectable GSH peroxidase activity (8), H_2O_2 accumulates to a concentration of 70 μM, a value much higher than that observed in mammalian cells (4).

Earlier work in this laboratory concerned with the development of therapeutic agents exploitive of increased trypanosomal H_2O_2 has identified a class of active compounds that promote homolytic cleavage of H_2O_2 into the highly reactive hydroxyl and hydroperoxy radicals (4, 7). Consistent with the role of GSH in cellular defense against free radical damage, it has been proposed that decreased GSH levels sensitize trypanosomes to these agents (8). However, the interpretation of experiments in which GSH is depleted with chemically reactive compounds, such as diamide or arsenicals, is complicated by questions of reagent specificity (15, 16).

An alternative approach to GSH depletion is possible with BSO, a potent and specific inhibitor of γ-glutamylcysteine synthetase, the first enzyme of GSH biosynthesis (9). Studies with mice and rats have shown that inhibition of GSH biosynthesis is followed by a depletion of intracellular GSH by the continuing reactions of GSH utilization, including translocation of intact GSH out of cells. In tissues that have a naturally high rate of GSH turnover, the depletion of GSH after inhibition of new synthesis can be rapid and dramatic; kidney and liver GSH levels can be reduced by >80% in less than 2 h, for example. In other tissues (e.g., erythrocytes, heart, lung, and spleen), GSH turnover is intrinsically slow, and temporary inhibition of synthesis does not produce a marked depletion (12).

In the present studies, BSO was administered to parasitemic mice, and the GSH content of the trypanosomes isolated from the blood was shown to decline 50% in about 5 h. Because the trypanosomes divide with a generation time of 7–9 h (data not shown), much of the observed depletion of GSH is probably attributable to dilution of preexisting GSH into an increasingly large cytoplasmic volume. It should be noted, however, that the unknown effect of GSH depletion on the generation time and cell volume of *T. b. brucei* precludes a firm conclusion as to the mechanism by which the GSH concentration drops after inhibition of biosynthesis.

Trypanosomes depleted of >75% of their initial GSH content were found to be extremely fragile when isolated and examined in vitro. A similar parasite fragility apparently also develops in the parasitemic mouse given BSO. In all cases examined, *T. b. brucei* could not be identified in blood samples collected 16–18 h after BSO administration was begun. As shown in Table I, it was possible in some cases to cure the infection if plasma BSO concentrations were maintained for about 27 h. In other cases, mice apparently rendered aparasitemic by BSO were found to relapse after several days. It is probable in these cases that a small number of parasites survived the initial treatment and then multiplied after BSO had been cleared from the plasma. It is notable in this regard that, in contrast to diamide or the arsenicals, BSO does not react directly with GSH. For BSO to cause GSH depletion, the parasites must either lose GSH through some natural metabolic process or they must divide and thereby dilute the GSH pool. Metabolically quiescent trypanosomes are therefore expected to be resistant to BSO-induced lysis. It is also possible that, in some cases, surviving trypanosomes were initially sequestered at sites within the central nervous system and were not exposed to a sufficiently high concentration of BSO. Amino acid analysis of the tissues of mice given BSO shows that the concentrations of inhibitor in the brain are substantially lower than those found in other tissues (12). Recrudescence of trypanosomiasis from the central nervous system after chemotherapy has recently been demonstrated (17).

The large doses of BSO required to cure parasitemic mice precludes the chemo-

therapeutic use of the compound in larger animals. The present results nevertheless indicate a means by which a biochemical difference between mammals and *T. b. brucei* may be therapeutically exploited. Thus, BSO is a drug with no known mammalian toxicity and with little intrinsic chemical reactivity. It apparently acts solely to inhibit GSH biosynthesis and thus does not directly affect other cellular thiols. At present, *T. b. brucei* is the only organism known to be damaged by BSO-induced GSH depletion, a susceptibility attributable to the protozoan's limited ability to deal with self-generated H_2O_2. It is apparent that practical therapies should be possible with the development of more tightly bound inhibitors of GSH biosynthesis and with the concurrent administration of compounds such as hematoporphyrin that intensify even further the increased endogenous oxidative stress to which *T. b. brucei* is exposed. It is further noted that other pathogenic organisms are also catalase deficient (e.g., *T. cruzi*) (18, 19) and may be susceptible to similar therapies.

Summary

With the expectation that trypanosomal glutathione (GSH) plays a major protective role against the endogenous oxidant stress that results from high intracellular levels of H_2O_2, we sought to deplete *Trypanosoma brucei brucei* of their GSH through inhibition of its biosynthesis. Administration of buthionine sulfoximine (BSO), a reversible inhibitor of γ-glutamylcysteine synthetase, to parasitemic mice resulted in a progressive decrease in trypanosome GSH content, such that parasites isolated after 5 h of BSO treatment contained 50% of normal values. When BSO administration was continued for 18 h (intraperitoneal injection of 4 mmol/kg every 1.5 h), parasitemias temporarily cleared. When inhibitory plasma levels of BSO were maintained for about 27 h, two out of six infected mice were cured and the rest had significantly prolonged survival. These findings demonstrate the potential value of GSH depletion for the treatment of trypanosomiasis.

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