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Indomethacin Promotes Differentiation of *Trypanosoma brucei*

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The treatment of mice with indomethacin lowered *Trypanosoma brucei* parasitemia 1 to 2 $\log_{10}$ because it quickly promoted the differentiation of rapidly dividing long, slender trypanosomes into short, stumpy forms that do not divide in the mammal but do develop a functional mitochondrion and the ability to infect the tsetse. Since natural resistance correlates with the rate of differentiation, this observation may provide important information about factors that control the severity of trypanosomiasis.

Trypanosoma brucei undergoes morphological changes during its life cycle in the mammalian host and the tsetse fly vector. During the rising phase of parasitemia in the mammal, long, slender trypanosomes predominate. As the parasitemia peaks, many of the rapidly dividing slender forms differentiate into shorter, broader, stumpy trypanosomes, which divide slowly or not at all (1). Differentiation, which is accompanied by development of a functional mitochondrion, increase in cellular volume, and loss of a free flagellum (3), prepares the parasite for passage back into the insect (22). Laboratory strains that have lost the capacity to differentiate (monomorphic strains) are more virulent for experimental rodents than pleomorphic strains but are noninfective for the tsetse (2). The signals that initiate trypanosome differentiation are unknown. Because prostaglandins (PGs) regulate differentiation of some mammalian cells (9), we investigated the effect of a PG inhibitor on *T. brucei*. Inhibition of PG synthesis was associated with a reduced level of parasitemia and accelerated differentiation of all three clones of *T. brucei* that were studied.

Male C57BL/6 mice aged 3 months, from the International Laboratory for Research on Animal Diseases colony, were infected intraperitoneally (i.p.) with 10^3 pleomorphic *T. brucei* GUTat 3.1 trypanosomes, cloned from TREU 667 stock (20). One group was inoculated i.p. daily with 5 mg (100 μg) of indomethacin (Sigma Chemical Co., St. Louis, Mo.) per kg in 1% bicarbonate-buffered normal saline (13), and the control group received buffer alone. This dose of indomethacin has been shown to inhibit PG synthesis in rodents without causing demonstrable toxicity (19). The group treated with indomethacin developed a peak parasitemia that was 2 logs lower than the peak parasitemia of controls, and the treated group cleared their blood of parasites 1 day faster (Fig. 1A). Indomethacin also lowered the peak of the second wave of parasitemia from 7.02 to 6.42 $\log_{10}$ trypanosomes per ml of blood (Fig. 1A).

Two other experiments showed that the effect of indomethacin was not specific for GUTat 3.1. Indomethacin lowered the first wave of parasitemia with pleomorphic *T. brucei* ILTat 3.3 (isolated by S. Shapiro at the International Laboratory for Research on Animal Diseases from ILTat 1.4 [4]) from 9.04 to 7.36 $\log_{10}$ per ml of blood (Fig. 1B) and the peak of the second wave from 7.39 to 6.39 $\log_{10}$ (not shown). Even monomorphic (or weakly pleomorphic) ILTat 1.4 (4), which kills mice during the first wave, responded to indomethacin treatment. Although mortality was equal, the peak parasitemia was lowered from 9.2 $\log_{10}$ in controls to 8.2 $\log_{10}$ in treated mice (Fig. 1C).

Indomethacin did not select for slow-growing trypanosome subpopulations that were resistant to the drug. Trypanosomes harvested from indomethacin-treated or untreated mice during the ascending phase of the first wave of parasitemia were equally susceptible to indomethacin. Normal parasites in untreated mice peaked at 7.95 $\log_{10}$ per ml of blood compared with 6.47 $\log_{10}$ in indomethacin-treated mice (Fig. 2A). Parasites that had been exposed to indomethacin before passage to naive, untreated mice caused a peak parasitemia 2 $\log_{10}$ per ml higher than the same parasites inoculated into indomethacin-treated mice (Fig. 2B). These results not only demonstrate that the effect of indomethacin is not due to selection, but they also show that the drug does not limit the size of the infecting dose.

The lower parasitemia and earlier clearance of *T. brucei* in mice treated with indomethacin were associated with accelerated differentiation of slender trypanosomes into stumpy forms. Daily smears from tail blood of each mouse infected with *T. brucei* GUTat 3.1 were first stained with Giemsa for differentiation of slender, intermediate, and stumpy forms. The ratio of slender to stumpy forms was 15.25 times higher at day 5 postinfection in untreated mice than in mice treated with indomethacin (Table 1). This difference diminished as the trypanosomes in untreated mice reached the peak parasitemia and began to differentiate. Similar results were obtained with ILTat 3.3 and even with ILTat 1.4, the weakly pleomorphic strain (Table 1). We compared the accuracy of scoring the different morphological stages by the Giemsa stain with a stain for NAD diaphorase, a mitochondrial enzyme that is absent in slender forms but appears early in their differentiation to intermediate and stumpy forms (21). Optimal staining of the enzyme was achieved with Burstone's modified stain, using ethanol and malate as substrates (6) and 0.1 M cacodylate-buffered 5% glutaraldehyde for poststaining fixation (21). In three separate experiments the differences in ratios of slender to stumpy forms in controls versus indomethacin-treated animals were statistically significant at days 4 and 5 postinfection whether scored by Giemsa or the NAD diaphorase stain. Figure 3 illustrates the appearance of slender and stumpy forms stained for NAD diaphorase.

To minimize the participation of the immune system, we repeated the indomethacin experiment in lethally irradiated

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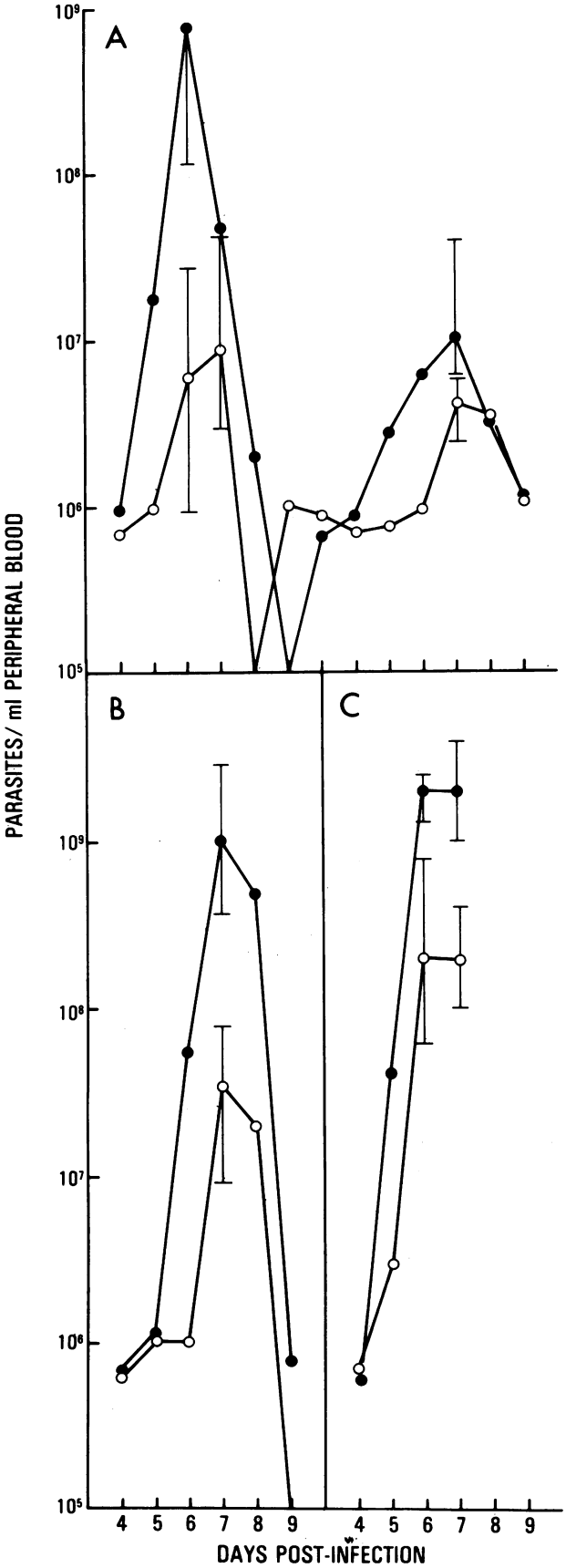

FIG. 1. In each experiment 3-month-old male C57BL/6 mice were infected i.p. with 10^3 *T. brucei* GUTat 3.1 (A), 10^3 *T. brucei* ILTat 3.3 (pleomorphic [B], or 10^2 *T. brucei* ILTat 1.4 (virtually monomorphic [C]) trypanosomes. Groups consisted of at least 10 mice. Treated mice received 100 μg of indomethacin in 1% bicarbonate-buffered normal saline, i.p., daily. Untreated mice received buffer alone. Parasitemia was monitored by daily wet mounts of tail blood and quantified by counting trypanosomes in a hemacytometer. Each point represents the mean number of parasites per milliliter of peripheral blood for each group. Symbols: ●, untreated mice; ○, treated mice. Bars = 1 standard deviation from the mean peak of parasitemia. One representative experiment from at least three replicates is shown.

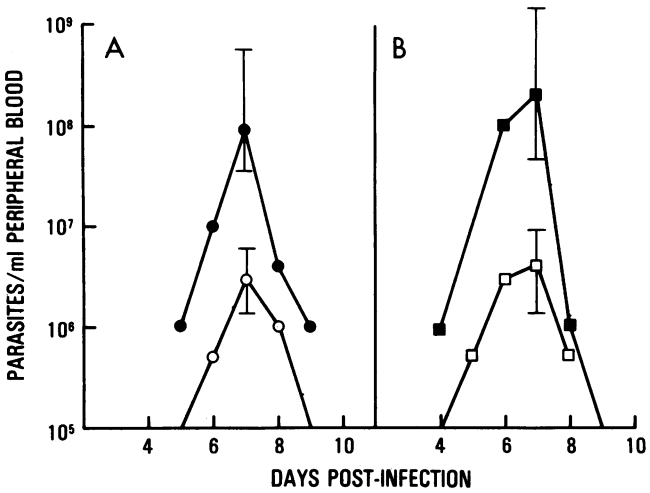

FIG. 2. Five untreated and five treated (see legend to Fig. 1) C57BL/6 mice were infected with 10^3 GUTat 3.1 trypanosomes. On day 5 during the ascending phase of the first wave of parasitemia, blood was collected from each group, parasites were enriched over a DE-52 column (12), and 10^2 parasites were injected into 10 treated and 10 untreated mice. Parasitemias in the four groups were followed as detailed in the legend to Fig. 1. Symbols: ●, untreated mice infected with untreated trypanosomes; ○, indomethacin-treated mice infected with untreated trypanosomes; ■, untreated mice infected with treated trypanosomes; □, treated mice infected with treated trypanosomes.

mice (800 R). The drive to differentiation by indomethacin was as great in irradiated mice as in intact ones, but the parasitemia was affected less than in intact animals (Fig. 4). These results suggest that radiosensitive components of the immune system are not essential to the effect of indomethacin on differentiation but may be important in the clearance of parasites from the peripheral blood.

These studies show that indomethacin controlled expansion of the population of *T. brucei* in the bloodstream of mice by promoting differentiation of long, slender trypanosomes into slowly dividing or nondividing, short, stumpy forms. Premature loss of rapidly dividing, long, slender trypanosomes in mice treated with indomethacin resulted in a lower peak parasitemia. The percentage of trypanosomes that finally differentiated in control animals was almost as great as it was in treated animals. Thus, the rate of differentiation, not the extent, was affected by indomethacin treatment.

The mechanism by which indomethacin promotes differentiation of *T. brucei* is unknown, but PGs and cyclic nucleotides have been shown to control differentiation of

TABLE 1. Effect of indomethacin on differentiation of *T. brucei*[a]

T. brucei	Treatment	Day 5		Day 6		Day 7		Day 8	
		Slender-to-stumpy ratio	Fold increase in stumpy forms with treatment	Slender-to-stumpy ratio	Fold increase in stumpy forms with treatment	Slender-to-stumpy ratio	Fold increase in stumpy forms with treatment	Slender-to-stumpy ratio	Fold increase in stumpy forms with treatment
GUTat 3.1	None	11.75	15.25	1.27	4.70	0.14	1.40	0.05	5.00
	Indomethacin	0.77		0.27		0.10		0.01	
ILTat 3.3	None	4.66	11.65	0.85	10.62	0.20	10.00	0.10	3.33
	Indomethacin	0.40		0.08		0.02		0.03	
ILTat 1.4	None	83.00		32.00		1.50		ND	
	Indomethacin	10.33	8.03	2.40	13.33	0.25	6.00	ND	

[a] Air-dried, methanol-fixed, Glemsa-stained films of parasites were made in duplicate from mouse tail blood. At least 100 trypanosomes per slide were counted for differentiated forms (slender, intermediate, or stumpy). Slender parasites were needle shaped with long flagella, an anterior nucleus, and a tightly apposed, undulating membrane. Intermediates had more cellular volume, a shorter flagellum, a more obvious undulating membrane, and a more centrally located nucleus. Stumpies were characterized by the virtual absence of a free flagellum, the presence of a highly developed undulating membrane and posterior nucleus, and a large increase in the cellular volume (3). Results are expressed as the ratio of slender to stumpy forms of the untreated and indomethacin-treated mice infected with each of the three strains of *T. brucei*. The increase in the stumpy forms after treatment was calculated by dividing the ratio of slender to stumpy forms of the untreated mice by the ratio of slender to stumpy forms of the treated mice. Intermediates were not included in the calculations. By the chi-squared test statistic, the accelerated differentiation of *T. brucei* in mice treated with indomethacin differed from that in control mice at confidence levels of >99% (ILTat 1.4) to >99.9% (GUTat 3 and ILTat 3.3). ND, Not determined.

other eucaryotic cells. Hopkins and Gorman (9) reported that differentiation of 3T3-4 fibroblasts to adipocytes was enhanced by indomethacin and blocked by the addition of prostaglandin E_1 or prostaglandin I_2. Reproduction of *Trypanosoma lewisi* (18), differentiation of some strains of *T. brucei* (14), and gametocytogenesis of *Plasmodium falciparum* (11) are all accompanied by changes in the levels of cyclic AMP. Although the powerful effect of indomethacin on *T. brucei* may also be mediated by inhibition of PG synthesis, indomethacin has many other biological effects. It has been reported to inhibit phospholipase A_2 (10), cyclic AMP-dependent protein kinase (7), calcium flux into cells (15), cyclic nucleotide phosphodiesterase (8), and cyclic GMP production (16). The effect of indomethacin on cultivated *T. brucei* and in vivo and in vitro investigations of the activity of other PG inhibitors should help to uncover the mechanism of the action of indomethacin.

The rate and extent of trypanosome differentiation appear to be important factors in the interaction between host and parasite. Sendashonga and Black (17) have shown that the kinetics of the immune response are determined by the rate of parasite differentiation, since stumpy and irradiated, slender trypanosomes are better immunogens than untreated, long, slender forms. Furthermore, the ability of mice and cattle to control *T. brucei* parasitemia correlates with the rate of parasite differentiation (5). Accordingly, agents that promote trypanosome differentiation may provide important information about systemic or microenvironmental factors that determine the severity of African trypanosomiasis.

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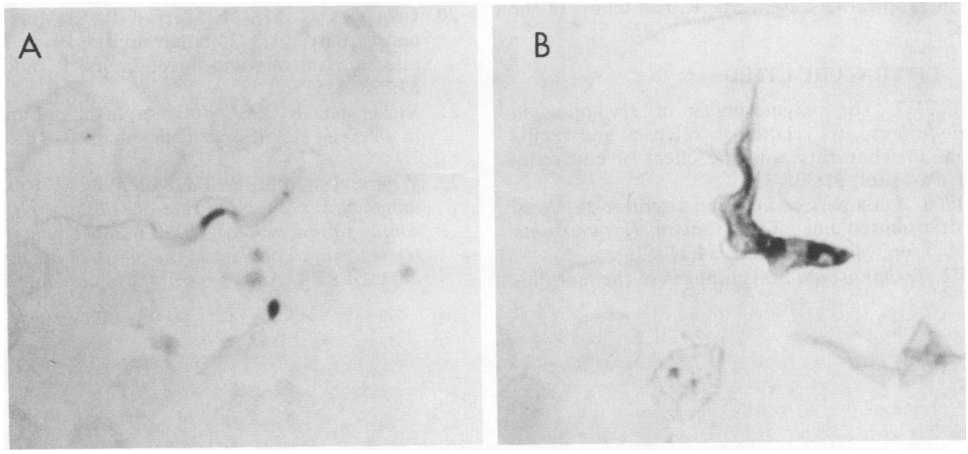

FIG. 3. Appearance of slender (A) and stumpy (B) forms of *T. brucei* GUTat 3.1 stained for NAD diaphorase as described in Table 1, footnote *a* (×1,800). Dark-staining areas in the stumpy forms indicate NAD diaphorase activity.

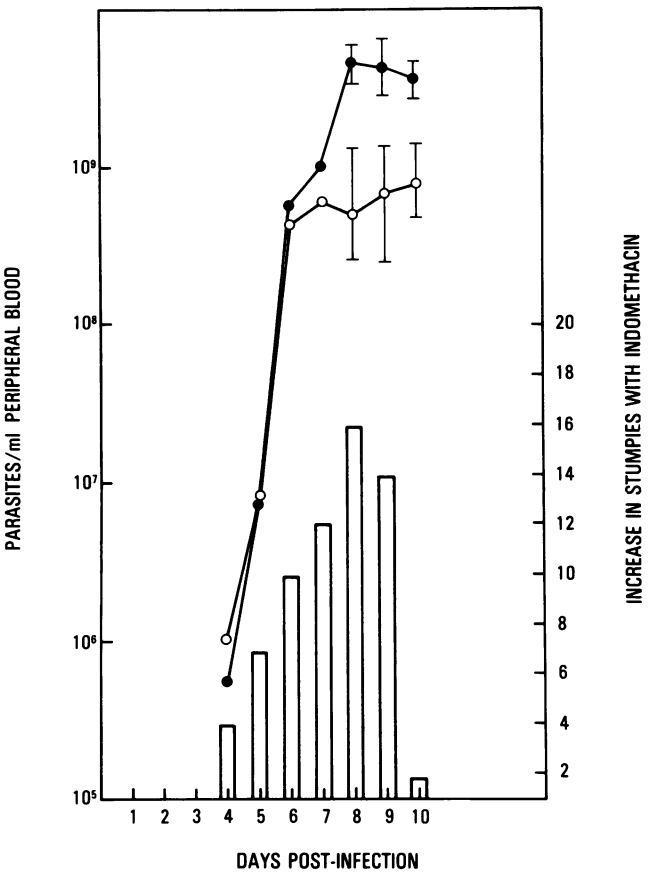

FIG. 4. Twenty C57BL/6 mice were lethally irradiated (800 R, ^{137}Cs source at 3.7 rads/s), infected i.p. 24 h later with 10^3 GUTat 3.1 trypanosomes, and divided into two groups of 10. One group was treated with indomethacin, and the other was treated with buffer alone. Parasitemias were monitored as detailed in the legend to Fig. 1, and smears for differentiated forms were stained and examined as indicated in Table 1, footnote *a*. Symbols: ●, irradiated with buffer; ○, irradiated with indomethacin. The increase in stumpy forms with indomethacin was calculated by dividing the ratio of slender to stumpy forms of the untreated mice by the ratio of slender to stumpy forms of the treated mice (bar graph).

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