Effect of Theophylline on Differentiation of Trypanosoma brucei

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Differentiation of Trypanosoma brucei in the mammal limits the degree of parasitemia and prepares the trypanosome for passage back into the tsetse fly. In an attempt to define the signals that control differentiation, we found that theophylline, in contrast to indomethacin, blocked differentiation, prolonged parasitemia, elevated prostaglandin and cyclic AMP concentrations of rat plasma, and depressed intratrypanosomal cyclic AMP. Relatively nontoxic drugs that alter differentiation are powerful tools for elucidating the events that control this important process.

African trypanosomes of the Trypanosoma brucei group were first noted to be “pleomorphic” in 1912, but this phenomenon attracted little attention until it was discovered that the two morphologic forms serve different functions in the trypanosomal life cycle (2, 19). Long slender trypanosomes predominate during the ascending phase of parasitemia. As the parasitemia peaks and remits, many of the rapidly dividing long slender forms differentiate into short stumpy trypanosomes, which do not divide in the mammal (2). Differentiation, which is accompanied by the development of a functional mitochondrion, prepares the parasite for passage back into the tsetse fly (19, 21). It also limits the degree of parasitemia because the life span of stumpy trypanosomes in the mammal is less than 24 h and the variable surface glycoprotein released from dying and disrupted stumpy trypanosomes induces the antibody response that eliminates undifferentiated slender trypanosomes (5, 17).

The biochemical events that trigger differentiation are unknown. We showed previously that the cyclooxygenase inhibitor indomethacin accelerates differentiation and lowers T. brucei parasitemia of C57BL/6 mice by as much as 99% (12), and Mancini and Patton have reported that intratrypanosomal cyclic AMP (cAMP) varies during parasitemia (15). The strategy of the present study was to compare the effects of indomethacin and theophylline, a cAMP phosphodiesterase inhibitor (7), on differentiation, degree of parasitemia, prostaglandin (PG) synthesis by the host, and cAMP concentrations of trypanosomes and host plasma. We reasoned that differences in the effects of these agents might help elucidate the events that mediate differentiation.

Accordingly, groups of 10 to 20 200-g female, Sprague-Dawley rats were infected intraperitoneally (i.p.) with \(5 \times 10^7\) to \(5 \times 10^8\) T. brucei GUTat3.1, a standard pleomorphic African trypanosome (12). Beginning the morning of infection, the groups were given either daily subcutaneous injections of theophylline (Aminophylline; Elkins-Sinn Inc., Cherry Hill, N.J.) at 175 mg/kg, i.p. indomethacin at 5 mg/kg, or held as untreated controls. After 1 h, the blood levels of Aminophylline were 7 to 10 mg/dl. Indomethacin (Sigma Chemical Co., St. Louis, Mo.), diluted in 0.3% Na2CO3 and adjusted to a pH of 7.0, produced more variable blood levels of 25 to 100 μg/ml. Parasitemias and differentiated forms were measured as described previously (12).

cAMP was extracted and measured by the method of Mancini and Patton (15) and expressed as picomoles per 107 trypanosomes or per milliliter of plasma.

Indomethacin dramatically accelerated differentiation and lowered the peak parasitemia of rats as shown previously (12) for mice. The parasitemia was 1 to 2 logs lower in indomethacin-treated rats and the trypanosome population contained 15 times more stumpy trypanosomes by days 3 and 4 (63 versus 4% in controls). Indomethacin did not change the plasma cAMP of infected rats (19.8 versus 20.3 pmol/ml in controls). The cAMP concentrations of trypanosomes harvested from treated rats on days 3 and 4 when more than half were stumpy trypanosomes, however, were one-third those of controls (3.75 versus 11 pmol/ml, \(P < 0.02\)). By day 5, immediately before remission of parasitemia in the controls, the difference was no longer significant.

In contrast, Aminophylline markedly increased (>1 log10) and prolonged parasitemia for at least an additional 5 days (Fig. 1). Although part of the Aminophylline-treated trypanosomes differentiated (50% on day 6; 90% on day 10), the remaining slender and intermediate trypanosomes continued to divide rapidly and maintain the parasitemia. The persistent parasitemia was not due to inhibition of antibody synthesis. Standard assays (3) of trypanocidal antibody showed that activity was equal among the treated day 5 in all groups (data not shown). Remission of parasitemia was delayed, however, to days 10 through 12, 5 to 7 days later than the control groups.

Unlike indomethacin, Aminophylline caused a dramatic and continuing difference in plasma and trypanosome cAMP levels (Fig. 2). Plasma concentrations were similar on day 3 postinfection, the first day of patency, but were markedly higher in treated rats on subsequent days (\(P < 0.05\)). Paradoxically, trypanosomes harvested from treated rats contained significantly less cAMP (\(P < 0.01\)). The different effects of Aminophylline on plasma and trypanosomal cAMP levels in vivo may be due to concentration-dependent, antagonistic mechanisms of action. At concentrations of \(10^{-3}\) to \(10^{-2}\) M, theophylline inhibits cAMP phosphodiesterase activity (6), but levels in the usual clinical range of \(10^{-4}\) to \(10^{-5}\) M competitively inhibit adenosine receptors (9). Plasma concentrations of 10 mg/dl (or about \(10^{-2}\) M) were high enough to inhibit rat phosphodiesterase and cause marked accumulation of cAMP, but trypanosome phosphodiesterase receptors may have been exposed to much less if they were buried deeply in the hydrophobic region of the
cytoplasmic membrane and were relatively inaccessible to the outer aqueous phase, as are the adenylcyclase receptors (20). Adenosine receptors, on the other hand, are known to be susceptible to exogenous stimulation by physiologic concentrations of the ligand (15). Alternatively, the phosphodiesterase of T. brucei may be unresponsive to theophylline, like that of T. cruzi (10), but this is unlikely because the cAMP concentrations of trypanosomes treated in vitro with 10^{-3} M Aminophylline are more than doubled (unpublished data).

To determine if the differences in trypanosomal cAMP were a function of their divisional stage, we compared the concentrations of untreated trypanosomes near the peak of parasitemia to those already on a plateau and those that were over the peak and declining in number. Trypanosomes near the peak of parasitemia contained significantly higher levels than did the other stages (P < 0.05) (Fig. 3).

These results suggested that stumpy trypanosomes might have lower cAMP levels than do slender trypanosomes that are near the peak of parasitemia and about to differentiate. We tested this possibility by treating C57BL/6 mice with 300 mg of cyclophosphamide per kg i.p. 2 days before infection with 10^7 T. brucei GUTat3.1. This manipulation selects for slender trypanosomes 3 to 5 days postinfection and stumpy trypanosomes 6 to 8 days after infection (unpublished data).

FIG. 1. Parasitemias of Aminophylline-treated and control rats. Mean parasitemias of control (□) and Aminophylline-treated rats (■) ± standard error from one representative experiment of four replicates. Parasitemias and differentiated forms were measured as described previously (12). During the first 8 days of parasitemia, Aminophylline-treated animals had persistently higher counts (P < 0.001). The parasitemia of treated rats did not remit during the entire 10-day observation period, while control rats experienced two waves of parasitemia.

FIG. 2. Comparison of cAMP levels in Aminophylline-treated and control rats. Mean plasma levels of cAMP (picomoles per milliliter) were compared in control (□) and Aminophylline-treated (■) rats. cAMP levels of Aminophylline-treated rats were significantly higher after day 4 (P < 0.05). The cAMP levels per 10^7 trypanosomes isolated from Aminophylline-treated rats (□) were always lower than those of control trypanosomes (■) and lower than the simultaneous plasma levels (P < 0.01). cAMP was extracted and measured by the method of Mancini and Patton (15). Briefly, trypanosomes from whole rat blood were collected over DEAE-cellulose for separation from blood cells as described by Lanham and Godfrey (14), pelleted by centrifugation, resuspended in 1 ml of buffer, and counted in a hemacytometer. Macromolecules were precipitated by cold 100% trichloroacetic acid; the supernatant was retained and extracted three times with anhydrous ether, adjusted to pH 5.0 to 6.0, and either assayed immediately or frozen at -70°C. Separated plasma was precipitated with 10% trichloroacetic acid, extracted four times with anhydrous ether, adjusted to pH 6.0, and frozen at -70°C. cAMP concentrations were assayed by ^{3}H radioimmunoassay (New England Nuclear Products, Billerica, Mass.) and corrected to either picomoles per 10^7 trypanosomes or per milliliter of plasma. ^{3}H]cAMP was added to all samples before trichloroacetic acid precipitation as a marker and confirmed 80 to 90% recovery.

FIG. 3. cAMP levels by divisional stage. cAMP levels were compared in plasma (□) and untreated trypanosomes (□) when trypanosomes were (A) approaching peak parasitemia (increase of >0.5 log_{10} in next 24 h), (B) on a plateau (increase of <0.5 log_{10} in next 24 h), or (C) declining (decreased during next 24 h). P, cAMP per milliliter of plasma; T, cAMP per 10^7 trypanosomes.
TABLE 1. Effect of aminophylline on plasma PG concentrations of normal and infected rats

<table>
<thead>
<tr>
<th>Group (no.)</th>
<th>PGF(_{1\alpha}) Uninfected control</th>
<th>PGF(_{1\alpha}) Infected control</th>
<th>P</th>
<th>PGF(_{1\alpha}) Uninfected control</th>
<th>PGF(_{1\alpha}) Infected control</th>
<th>P</th>
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<td>Uninfected</td>
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<td></td>
<td>----</td>
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<td></td>
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</tr>
<tr>
<td>Saline control (6)</td>
<td>100(^b)</td>
<td>100(^c)</td>
<td></td>
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<tr>
<td>Aminophylline (6)</td>
<td>400 &lt;0.01</td>
<td>177 &lt;0.01</td>
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<td></td>
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<tr>
<td>Indomethacin (4)</td>
<td>46 NS</td>
<td>46 NS</td>
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<tr>
<td>Trypanosomes (10(^7))</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Infected</td>
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<td>----</td>
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<tr>
<td>Saline control (6)</td>
<td>85 NS</td>
<td>74 NS</td>
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<tr>
<td>Aminophylline (6)</td>
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<td>623 &lt;0.01</td>
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<tr>
<td>Trypanosomes (10(^7))</td>
<td>0</td>
<td>0</td>
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\(^a\) Groups of four to six uninfected and infected rats were inoculated i.p. with 0.5 ml of normal saline, i.p. with 5 mg of indomethacin per kg, or subcutaneously with 175 mg of Aminophylline per kg 1 h before collection of plasma or trypanosomes. PG concentrations were determined by radioimmunoassay as noted in the text. PGF\(_{1\alpha}\) refers to the 6-keto PGF\(_{1\alpha}\) metabolite of PG\(_{1}\) (prostacyclin). NS, Not significant; ND, not done. \(P\) was calculated by Student's \(t\) test versus uninfected saline controls.

\(^b\) Actual value was 143 pg/ml.

\(^c\) 104 pg/ml.

\(^d\) \(P\) versus uninfected rats given Aminophylline was <0.02.

cAMP levels of trypanosomes harvested over DEAE-cellulose 4 days postinfection (>95% slender trypanosomes) were fivefold higher than those harvested 7 days postinfection (>95% stumpy trypanosomes). The mean value (± standard deviation) in picomoles per 10\(^7\) slender trypanosomes from five mice was 8.9 (± 6.5) compared with 1.5 (± 0.4) from five mice with >95% stumpy trypanosomes. Concentrations of PG\(_{1}\) (prostacyclin) measured as the more stable 6-keto PGF\(_{1\alpha}\) \(\alpha\) metabolite and PGF\(_{2\alpha}\) \(\alpha\) were assayed by a radioimmunoassay similar to the cAMP technique except that samples were collected into a citrate-indomethacin solution and the separated plasma or trypanosomes were column enriched before assay. Aminophylline profoundly elevated (\(P < 0.01\)) the concentrations of PGF\(_{1\alpha}\) \(\alpha\) and PGF\(_{2\alpha}\) \(\alpha\) in the plasma of both uninfected and infected rats (Table 1). The known effects of theophylline on different PGs, stimulation of PGF\(_{2}\) and PGF\(_{2}\) \(\alpha\) synthesis by renal tissue (1, 4), and inhibition of prostacyclin production by human umbilical vein endothelial cells (6, 11) did not prepare us for these major increases. PG levels of infected control rats did not change. Trypanosomes contained neither PGF\(_{1\alpha}\) \(\alpha\) nor PGF\(_{2\alpha}\) \(\alpha\).

Growth and differentiation of most cell lines are known to be mutually exclusive processes. Intracellular cAMP concentrations are negatively correlated with growth rate among a variety of cell lines (8), and either exogenous cAMP analogs or induction of endogenous cAMP slows or stops the growth of most cells (8, 13). Furthermore, transformed cells of many types contain lower cAMP levels than do their normal counterparts (13, 16, 18).

Combined with these observations on other eucaryotic cells, our results permitted us to construct the following model of intratrypanosomal events during differentiation. The cAMP levels of long slender trypanosomes rise parallel with parasitism until the high concentrations interfere with replication. At this point, differentiation occurs and the cAMP concentrations of the population fall drastically in association with the low levels of short stumpy trypanosomes. Although stumpy trypanosomes do not multiply in the mammal, they are prepared for rapid replication in the tsetse fly and would be expected to contain low levels of cAMP. The conclusions of Mancini and Patton (15) from observations on cAMP concentrations of T. brucei during cyclic parasitemia of untreated rats are also in accord with this model.

Thus, cAMP concentrations appear to play a pivotal role in the intracellular events that trigger differentiation. The role of PGs as extratrypanosomal mediators during natural infection is less clear. Although the PG levels of treated rats changed dramatically during treatment with indomethacin and Aminophylline, they did not vary in untreated animals, and the trypanosomes did not contain detectable PG (Table 1). The ability to manipulate and control differentiation with relatively nontoxic drugs should provide a powerful tool for further study of the complex and important differentiation process of African trypanosomes.

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LITERATURE CITED


