Specific Inhibition of the Differentiation of *Trypanosoma cruzi*

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ABSTRACT The morphological transformation of *Trypanosoma cruzi* amastigotes was studied in both mammalian cells and an extracellular differentiation system. Inhibitors of ADP-ribosyl transferase were found to block differentiation in both cases, without affecting proliferation. The inhibitory effect was reversible and was not observed with chemical analogues that do not inhibit ADP-ribosyl transferase. As inhibitors of ADP-ribosyl transferase have recently been shown to block the differentiation of several cell types from vertebrates (Farzaneh, F., R. Zalin, D. Brill, and S. Shall, 1982, *Nature (Lond.)*, 300:362–366; Johnstone, A. P., and G. T. Williams, 1982, *Nature (Lond.)*, 300:368–370), our results suggest that the enzyme is of general importance in eucaryotic differentiation both in multicellular and unicellular organisms. In addition, since the compounds can block *T. cruzi* differentiation inside mammalian cells, these results suggest that it may be possible to exploit such inhibition in a new and potentially powerful approach to the chemotherapy of several important parasitic diseases.

Protozoan differentiation is a cyclic process that appears different in many ways from the differentiation of the cells of higher eucaryotes. The full molecular basis of differentiation has yet to be firmly established for any eucaryotic cell. However, several reports have suggested that nuclear ADP-ribosyl transferase (ADPRT),[1] an enzyme found in the nuclei of both higher eucaryotic cells (1–3) and protozoa (4, 5), may be involved (6–9). Most recently, a requirement for ADPRT activity has been demonstrated in the differentiation of three cell types (10–12). However, before the present investigation a requirement for ADPRT activity had been shown only in cells from higher animals. The data presented here show that inhibitors of ADPRT also block the transition between different morphological forms of the protozoan *Trypanosoma cruzi*, indicating that the enzyme is widely employed in cell differentiation in eucaryotes.

T. cruzi differentiates between three morphological stages during its life cycle (13, 14). Two stages, the epimastigote and the trypomastigote, possess flagella. The amastigote stage, which is nonmotile, normally proliferates inside mammalian cells (for example, see references 15, 16), but can also be grown extracellularly in a number of systems (references 17–19). In the experiments described here, amastigotes derived from extracellular culture were induced to differentiate extracellularly. If amastigotes were grown with murine S2 cells at 37°C, purified, and transferred to Warren's medium (20) at 27°C, 30–50% differentiated and produced a rudimentary flagellum after 3 to 4 d. Subsequently both epimastigotes and trypomastigotes were formed. Amastigote differentiation in this system was also blocked by compounds which inhibit ADPRT.

MATERIALS AND METHODS

Chemicals: 3-Aminobenzamide was obtained from the Sigma Chemical Co. (London, UK); 3-methoxybenzamide and 3-aminobenzoic acid were obtained from Aldrich Chemical Co. (Gillingham, UK). 5-Methylnicotinamide was a gift from Professor S. Shall (University of Sussex, Brighton, UK).

T. CRUZI: This parasite was handled as is appropriate for this type of human pathogen. *T. cruzi* Y strain was maintained in continuous-flow culture at 4×10^7 parasites/ml. Wel Tryp 2AII, a Y strain clone, was co-cultivated with the adherent mouse muscle tumour line S2, in Dulbecco's modification of Eagle's minimal essential medium (DME) containing 160 μg/ml gentamycin (Roussel, Ltd., London, England) and 10% fetal calf serum (Gibco Europe Ltd., Paisley, Scotland) to produce amastigotes (19).

Extracellular Differentiation of T. cruzi Amastigotes

The nonmotile amastigotes were harvested from the supernatants of cultures containing S2 cell monolayers. These supernatants usually contained a small

[1] *Abbreviation used in this paper:* ADPRT, ADP-ribosyl transferase.

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number of the mammalian cells that were removed by centrifugation for 1 min at 600 *g*. The amastigotes in the culture medium were then pelleted by centrifuging for 15 min at 600 *g*. The supernatant medium was removed and the amastigotes were resuspended at 5×10^5 cells/ml in Warren's medium (20) consisting of brain-heart infusion (Oxoid Ltd., Basingstoke, England) containing 10% fetal calf serum and 20 mg/liter haemin (BDH, Poole, England), pH 7.4. The cultures were then transferred to 27°C and samples were removed at intervals under sterile conditions for examination using a phase-contrast light microscope. Differentiating parasites were not fixed before examination because the development of the rudimentary but active flagella that occurred after a few days incubation was much more easily detected using live parasites. The concentration of parasites possessing these whip-like flagella, together with the concentrations of mature epimastigotes and trypomastigotes, were determined using a haemocytometer.

Quadruplicate 2-ml cultures in closed 10 ml screw-top glass bottles were normally used for the incubations, but the rate of parasite differentiation was not substantially affected by varying the culture volume between 500 μl and 10 ml. Similarly, the rate of differentiation (percent of total parasites possessing flagella at day 5) was largely independent of initial parasite concentration between 5×10^5 cells/ml and 5×10^6 cells/ml.

The effects of chemical inhibitors on parasite differentiation were determined by incubating the amastigote cultures with the inhibitor for 18 h at 37°C before the transfer to Warren's medium containing inhibitor at 27°C. Pre-incubation, to allow complete penetration of the inhibitors into the cells, was performed at 5×10^5 amastigotes/ml in Dulbecco's modified Eagle's medium (DME) with 10% fetal calf serum and 160 μg/ml gentamycin.

INTRACELLULAR DIFFERENTIATION OF T. CRUZI: S2 cells to be infected with *T. cruzi* were grown in DME with 10% fetal calf serum and 160 μg/ml gentamycin in plastic 24 well tissue culture plates (Costar 3524, L. H. Engineering, Stoke Poges, UK). S2 cells were dispensed into wells at a density of 1×10^5 cells/ml. After 24 h, each S2 cell culture was infected with 1×10^5 Y strain trypomastigotes derived from previously infected S2 cell cultures. After 24 h incubation at 37°C all cultures were washed 5× with phosphate-buffered saline to remove extracellular trypomastigotes and incubated in 1 ml DME with fetal calf serum and gentamycin. Cultures were re-fed after a further period of 24 h. In experimental cultures, potential inhibitors were present in all medium added after the removal of trypomastigotes. The emergence of trypomastigotes was monitored using an inverted microscope.

RESULTS AND DISCUSSION

After 3 d of incubation at 27°C, an increasing number of amastigotes began to develop active whip-like flagella (Fig. 1). About 1 d later, mature *T. cruzi* trypomastigotes and epimastigotes began to appear. At progressively later times, the numbers of mature epimastigotes and trypomastigotes increased and the numbers of parasites with only rudimentary flagella reached a plateau and later declined (Fig. 1). After prolonged incubation and subculturing, the epimastigote stage predominated, presumably because of the proliferation of this form of the parasite at 27°C. The flagellated forms of the parasite together made up 30–50% of the total number after 5 d of incubation.

The time course of appearance of the flagellated stages of the parasite suggested that both the epimastigotes and the trypomastigotes were derived from parasites with whip-like flagella that were the first to appear. However, since trypomastigotes and epimastigotes began to appear at approximately the same time, there was no indication that trypomastigotes were derived from mature epimastigotes (Fig. 1).

The differentiation of amastigotes grown in extracellular culture reported here has proved, over a period of 2 yr, to be a convenient and reliable experimental system with which to investigate the molecular processes involved in protozoan differentiation.

3-Methoxybenzamide and 5-methylnicotinamide are powerful inhibitors of ADPRT (21, 22) and strongly inhibit the differentiation of chick myoblasts (9, 10) and human peripheral blood lymphocytes (12). Both these compounds blocked the differentiation of amastigotes to the flagellated stages (Fig. 2, *A* and *B*). It is important to note that these inhibitors did not affect the differentiation-independent proliferation of *T.*

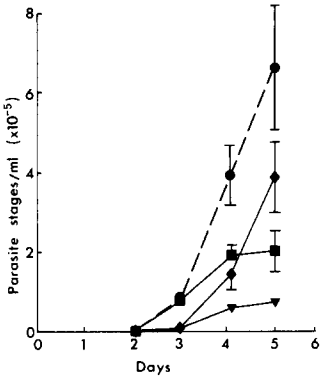

FIGURE 1 Differentiation of *T. cruzi* amastigotes at 27°C. Y strain clone Wel Tryp 2AII amastigotes were obtained from cultures of *T. cruzi* and S2 mouse muscle tumor cells (19). Contaminating S2 cells were removed by centrifugation for 1 min at 600 *g* and the amastigotes incubated for 18 h at 37°C in DME with 10% fetal calf serum and 160 μg/ml gentamycin at 5×10^5 cells/ml. The amastigotes were then centrifuged for 15 min at 600 *g* and resuspended at 5×10^5 cells/ml in Warren's medium (20) containing 10% fetal calf serum and incubated at 27°C. Samples were removed under sterile conditions at intervals and the total concentration of parasites which had differentiated was determined by phase-contrast light microscopy using a haemocytometer (●). Parasites with rudimentary but active flagella (■) first appeared, followed by epimastigotes (▼) and trypomastigotes (◆). The means and standard errors (except where encompassed by the symbols) of four replicate cultures are shown in each case. Zero time is the time of transfer of amastigotes to Warren's medium (20) at 27°C.

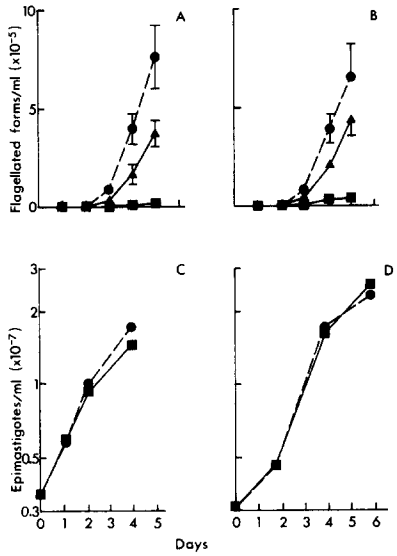

FIGURE 2 Effect of ADPRT inhibitors on differentiation and proliferation of *T. cruzi*. (*A* and *B*) Effect on differentiation. Amastigotes were obtained as described in the legend to Fig. 1, and were incubated for 18 h at 37°C in DME with 10% fetal calf serum and inhibitor (except in controls). The amastigotes were centrifuged and resuspended at the same cell density, 5×10^5 cells/ml, in Warren's medium (20) with 10% fetal calf serum at 27°C with inhibitor (except in controls) (0 hours). Total numbers of flagellated forms were counted daily using a haemocytometer. (*C* and *D*) Effect on proliferation. Y strain epimastigote forms were grown at 27°C in Warren's medium (20) with 10% newborn calf serum. Parasite numbers were measured after fixation using a model ZB Coulter Counter. (■) 2 mM 3-Methoxybenzamide (*A* and *C*) or 5 mM 5-methylnicotinamide (*B* and *D*) continuously present. (▲) 2 mM 3-Methoxybenzamide (*A*) or 5 mM 5-methylnicotinamide (*B*) present up to 24 h. (●) No inhibitor. The mean and standard error (except where contained within the symbol) of four cultures is shown for each time point.

cruzi epimastigotes (Fig. 2, *C* and *D*), and that inhibition of differentiation was abolished by removal of the inhibitors after 24 h (Fig. 2, *A* and *B*) indicating that the parasites remained viable. The effect of ADPRT inhibitors on myoblast differentiation was also reversible (9, 10). Other ADPRT inhibitors, 3-aminobenzamide (21), benzamide (23), nicotinamide (22), and theophylline (24) also inhibited *T. cruzi* differentiation (25), which indicates that specific inhibition of differentiation is a general property of these compounds. Nicotinic acid, which does not inhibit ADPRT, had no effect. The ADPRT inhibitors inhibited *T. cruzi* differentiation at concentrations that were the same as, or slightly lower than, those used for myoblasts (9, 10), lymphocytes (12) and hepatocytes (11).

The presence of 3-methoxybenzamide or 5-methylnicotinamide for only the first 24 h of culture did significantly delay the appearance of flagellated parasites (Fig. 2, *A* and *B*), indicating that an early stage of the differentiation process was blocked. To establish more precisely the stage at which the protozoa were susceptible to inhibition, 5-methylnicotinamide was added to differentiating amastigotes at different times before or after transfer to Warren's medium at 27°C (Fig. 3). It is clear that differentiation was strongly inhibited when 5-methylnicotinamide was added before or within a few hours of transfer, but was affected only marginally if the inhibitor was added after 20 h. Since the flagellated forms did not appear in significant numbers until ~90 h after transfer, 5-methylnicotinamide appears to act very early in the transition between stages. This is markedly similar to its effect on the mitogen-stimulated activation of human peripheral blood lymphocytes (12).

The process of cell differentiation is of crucial importance for *T. cruzi*. In its mammalian hosts, the protozoan parasite exists in two inter-dependent forms. The trypomastigote stage infects many cell types but does not proliferate and it differentiates to the amastigote soon after penetration. The amastigotes multiply intracellularly and differentiate to trypomastigotes before rupture of the host-cell to continue the infection. This intracellular transition from amastigote to trypomastigote form is again blocked by ADPRT inhibitors. 3-Aminobenzamide substantially delayed the appearance of trypomastigotes, but the non-inhibitory analogue, 3-aminobenzoic acid had no effect (Fig. 4*A*). The more powerful inhibitors, 5-methylnicotinamide and 3-methoxybenzamide, inhibited the appearance of trypomastigotes more strongly (Fig. 4, *B* and *C*). However, this inhibition could again be reversed simply by removal of the inhibitor (Fig. 4*C*). The intracellular proliferation of amastigotes was unaffected and in infected cells treated with 3-methoxybenzamide, >95% of the parasites were present as amastigotes, illustrating that differentiation rather than release was blocked.

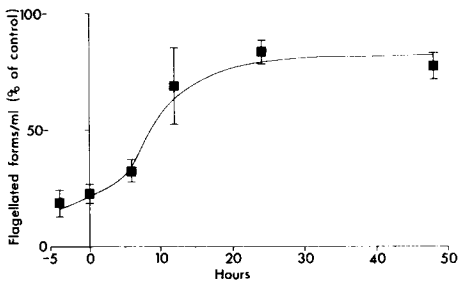

FIGURE 3 Effect of time of addition of 5-methylnicotinamide on *T. cruzi* differentiation. Amastigotes were prepared as described for Fig. 1 and transferred to Warren's medium at 27°C at time zero. 5-Methylnicotinamide was added, to a final concentration of 5 mM, to separate cultures at the times indicated and the numbers of flagellated cells counted with a haemocytometer at 96 h. This was expressed as a percentage of the flagellated cells in control cultures without 5-methylnicotinamide. The mean and standard error (except where contained within the symbols) of four cultures is shown for each time point.

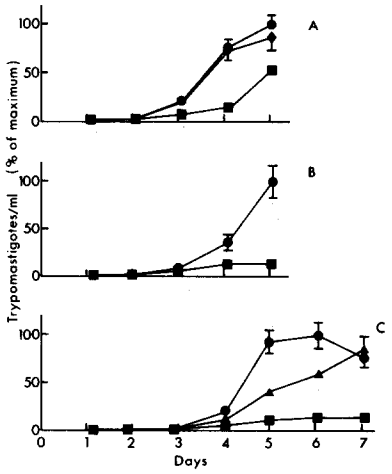

FIGURE 4 Inhibition of the intracellular infection cycle of *T. cruzi*. S2 cells were plated into 24 well tissue culture plates (Costar, U.K.) in DME with 10% fetal calf serum at a density of 1×10^5 cells/ml (1 ml/well). After 48 h, 2×10^5 Y strain trypomastigotes (from *T. cruzi*/S2 cell cultures) in complete medium with or without inhibitor were added to each well. After a further day, all cultures were washed five times with phosphate-buffered saline and fed with 1 ml of DME with 10% fetal calf serum with or without inhibitor. In C, 500 μl of each culture was replaced with appropriate fresh medium at 4 d and at daily intervals afterwards to prolong the culture period. The appearance of trypomastigotes in the supernatant was monitored by using an inverted microscope to examine each culture without removing any of the medium. The average number of trypomastigotes per microscope field was directly proportional to the number per milliliter. The concentration of trypomastigotes was expressed as a percentage of the maximum observed in control cultures (at day 5 for experiments A and B, and at day 6 for experiment C) to allow experiments A, B, and C to be compared. The mean and standard error (except where contained within the symbol) of four cultures is shown at each time point. (♦) 5 mM 3-aminobenzoic acid (Aldrich) present continuously. (■) *A*: 5 mM 3-aminobenzamide present continuously. (■) *B*: 5 mM 5-methylnicotinamide present continuously. (■) C: 2 mM 3-methoxybenzamide present from day 1 onwards. (▲) 2 mM 3-methoxybenzamide present day 1–3. (●) No inhibitor.

Several different inhibitors of ADPRT therefore block the morphological differentiation of *T. cruzi* without blocking proliferation in both of the experimental systems studied. The absence of an effect on proliferation illustrates the specificity of action of the inhibitors at the concentrations used. Chemical analogues that do not affect ADPRT had no effect. This suggests that ADPRT activity is required for the cell differentiation of this protozoan cell, although it is not needed for growth or division. This interpretation is considerably strengthened by reports that ADPRT activity is required for the differentiation of chick myoblasts (9, 10) and human lymphocytes (12), and also for the induction of fetal enzymes in cultured hepatocytes (11). These observations together suggest that ADPRT may be involved in the activation of cell-type-specific genes by a general mechanism employed by

higher animals, by protozoa and also quite possibly, by many other eucaryotes. This mechanism may well involve important structural changes in the DNA of the differentiating cell (10, 12, 27).

These observations may have significant practical implications. Several of the protozoa that cause important diseases in man and animals, notably *T. cruzi* and the malaria parasite, *Plasmodium*, are heavily dependent on morphological differentiation during their infection cycles. It should therefore be possible to use inhibitors of parasite differentiation in the chemotherapy of such infections. These could be more powerful inhibitors of ADPRT developed from those already in use, or drugs that interfere with any other process specifically involved. The mechanism of action of such compounds is fundamentally different from the conventional cytotoxic and cytostatic drugs in current use. Since inhibitors of ADPRT also block the differentiation of mammalian cells, it is likely that such chemotherapy would be most effective when compounds have been developed that are specific for parasite ADPRTs, and when ADPRT inhibitors are used in conjunction with conventional anti-parasitic drugs. It has recently been reported that even currently available ADPRT inhibitors, given without other drugs, do provide significant protection to mice experimentally infected with *Plasmodium yoelii* (26).

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