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Inhibition of poly(ADP-ribosyl)ation does not prevent lymphocyte entry into the cell cycle

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The enzyme poly(ADP-ribosyl)transferase (ADPRT) becomes activated soon after a mitogenic stimulus is applied to lymphocyte cultures. It has also been reported that ADPRT inhibitors prevent cell proliferation when added to cultures at the same time as the mitogen. While this has been ascribed to the need to seal physiologically present DNA strand breaks before cells enter S phase, the presence of DNA strand breaks in quiescent human lymphocytes has been recently questioned. We demonstrate here that non-toxic concentrations of ADPRT inhibitors do not affect lymphocyte blastization and proliferation, as measured by thymidine incorporation and cytofluorimetry. We therefore suggest that ADPRT activation is required for late functions which are not needed for cell cycle progression.

NAD^+ ADP-ribosyltransferase; Enzyme inhibitor; Lymphocyte proliferation; Cell cycle

1. INTRODUCTION

The chromatin-bound enzyme poly(ADP-ribosyl)transferase (ADPRT; NAD^+ ADP-ribosyltransferase, EC 2.4.2.30) catalyses the transfer of ADP-ribose units from NAD^+ to nuclear proteins, thus forming long, sometimes branched, ADP-ribose homopolymers (review [1]). Although the presence of DNA strand breaks (SB) appears to be a prerequisite for ADPRT activation, the role of the enzyme in DNA repair is still poorly understood [2–4]. Moreover, ADPRT activity has been found to increase with cell cycle progression [5–10]; this suggests that the enzyme also plays a role in physiological processes other than repair.

In order to elucidate the function(s) of poly(ADP-ribosyl)ation in both DNA repair and other physiological mechanisms, so-called specific ADPRT inhibitors [11] have been widely employed. ADPRT inhibitors have been reported to prevent cell proliferation [5,12–14] when added at the onset of mitogen-stimulated lymphocyte cultures. So far, the presence of DNA SB has been generally accepted to be a prerequisite for ADPRT activation; therefore, research has been focused on searching for SB in quiescent lymphocytes. Recently, Greer and Kaplan [15] concluded that a large amount of SB is endogenously present in mouse splenocytes, although they were unable to support the previously held view of the continuous formation and sealing of such breaks [14,16]. At variance with this report, Boerrigter et al. [17] more recently failed to detect DNA SB in human peripheral lymphocytes. These discrepancies, together with the consideration that past studies employed ADPRT inhibitor concentrations higher than those required for ADPRT inhibition and potentially capable of exerting side effects [18,19], prompted us to take a fresh look at the reported effect of ADPRT inhibitors on lymphocyte proliferation. We demonstrate here that ADPRT inhibition does not affect PHA-induced human lymphocyte blastization and proliferation.

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2. MATERIALS AND METHODS

2.1. *Lymphocyte proliferation*

Mononuclear blood cells from healthy volunteers were prepared as described [20], and cultured in nicotinamide-free RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated human pooled AB serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (latter 3 from Gibco). 3-Aminobenzamide (3ABA) and cell culture-tested nicotinamide (NAM) were purchased from Sigma, and 3-methoxybenzamide from Aldrich; they were dissolved in RPMI 1640 medium on the day of the experiment. Cells were cultured in quadruplicate in 96-well tissue-culture microplates at a concentration of 5×10^5/ml in the presence of 1 μl/ml PHA-P (Wellcome). 0.1, 1, 5, or 10 mM 3ABA, 0.1, 1, 5, or 10 mM NAM, or 0.1, 1, or 2 mM 3MBA were added at the same time as PHA. Untreated and unstimulated untreated control cultures were also performed for every subject and each examined time of culture. Cultures were continued for 3, 4, 5, 6, or 7 days; 6 h before the end of the culture period, each well was pulsed with 0.5 μCi tritiated thymidine ([^{3}H]TdR, Amersham, spec. act. 5 mCi/mmol). [^{3}H]TdR incorporation was evaluated as in [20].

2.2. *Cytofluorimetry*

Propidium iodide binding was performed according to [21]. Cells were fixed in 70% ethanol for 30 min at 4°C, washed twice with cold PBS, incubated for 30 min at 37°C with RNase (40 μg/ml, Worthington), and then stained at room temperature for 1 h in a solution containing propidium iodide (18 μg/ml, Calbiochem). Before analysis, cells were passed several times through a 26 gauge needle to separate aggregates. Cytofluorimetry was performed by using a Facstar (Becton Dickinson), exploying the 488 nm line from an argon ion laser. Cell cycle analysis was performed by the DNA cell-cycle analysis software - Ver C 5/87 - sum of broadened rectangles model (Becton Dickinson).

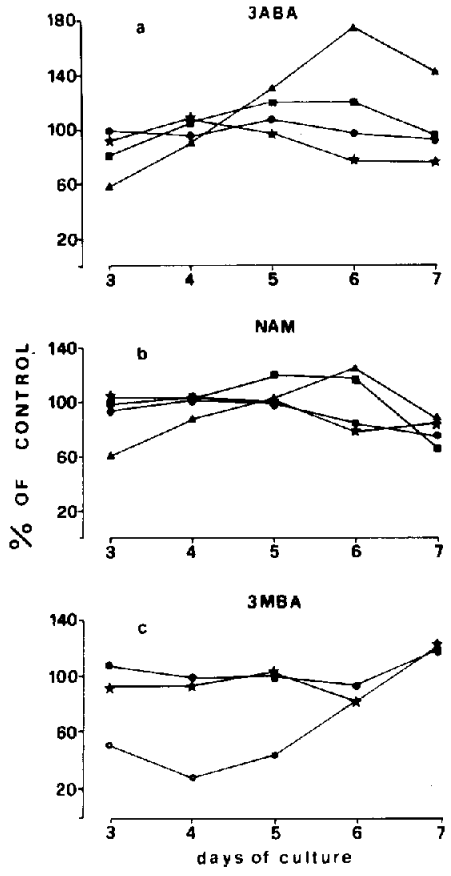


Fig.1. Effect of 3ABA or NAM on PHA-induced lymphocyte proliferation. Cells were cultured as described for 3–7 days in the presence of 0.1 (●), 1 (★), 2 (○), 5 (■), or 10 (▲) mM 3ABA, NAM, or 3MBA. Data are means of 10 experiments, and are expressed as % [^{3}H]TdR incorporation of untreated control cultures. SE did not exceed 10.

3. RESULTS

When 3ABA or NAM was added at the same time as mitogen (fig.1a,b), [^{3}H]TdR incorporation by PHA-stimulated lymphocytes was not significantly affected, except in 10 mM 3ABA- or NAM-treated cultures, where [^{3}H]TdR incorporation was initially reduced by 42–40%. At days 6 and 7, addition of 10 mM 3ABA caused an enhancement in [^{3}H]TdR incorporation, which often resulted in absolute values higher than those obtained in control cultures of the same subject on the day of peak [^{3}H]TdR incorporation (day 3 or 4).

Fig.1c shows the effects of 3MBA. It should be noted that the concentration of 2 mM, which appears to inhibit DNA synthesis, is probably cytotoxic. The two lower concentrations had no effect on [^{3}H]TdR uptake.

In one experiment, 3ABA was prepared from a stock solution made containing ethanol. We found that both 2.5% ethanol alone and 5 mM 3ABA dissolved in 2.5% ethanol inhibited [^{3}H]TdR incorporation (fig.2).

In order to obtain independent determination of the effect of ADPRT inhibitors on lymphocyte proliferation and to evaluate other parameters of PHA-induced lymphocyte blastization, including early events such as the increase in cell size [22,23], cytofluorimetric measures were sought. Fig.3 shows that the cell size, cell density, and DNA content of cells which received 5 mM 3ABA at the

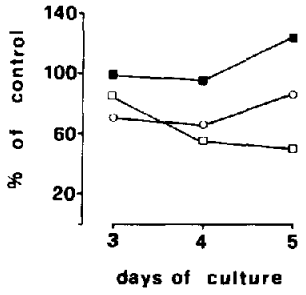
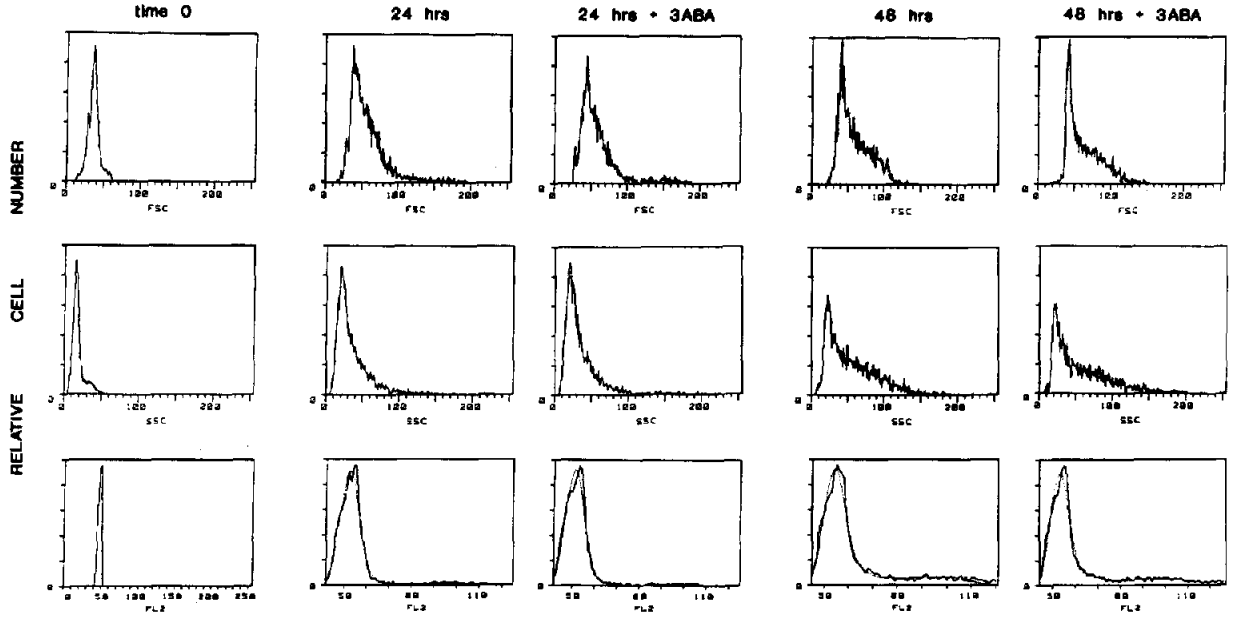


Fig.2. Effect of ethanol on PHA-induced lymphocyte proliferation. Cells were cultured as described for 3–5 days in the presence of 5 mM 3ABA (■) dissolved in culture medium, 2.5% ethanol (○), or 5 mM 3ABA dissolved in 2.5% ethanol (□). Data from one representative experiment are shown as % [^{3}H]TdR incorporation of untreated control cultures.

onset of the cultures were the same as those of untreated controls at all the examined times. Cell cycle analysis showed that the percentage of cycling cells was not affected by the presence of 3ABA. Three different subjects were analysed, with nearly identical results.

4. DISCUSSION

Our data partially contrast with those reported by others: (i) it has been reported that addition of 3MBA at the onset of lymphocyte cultures inhibits subsequent [^{3}H]TdR incorporation only at concentrations above 1 mM [15,24]; (ii) the effects of NAM were also described as being somewhat concentration-dependent: 0.1–0.5 mM NAM was found to enhance DNA synthesis [5], 2 mM being neutral [23], and 5–10 mM inhibitory [5,13,25]; (iii) at 5 mM, 3ABA was found to inhibit lymphocyte proliferation [12,13,15], but others observed that, within the range 0.5–2.0 mM, mouse thymus T-lymphocytes were stimulated [26]. In our hands, [^{3}H]TdR incorporation is not greatly affected by 0.1–1 mM 3MBA or 0.1–5 mM 3ABA or NAM. The higher concentrations appear to be initially in-



CELL CYCLE PHASE		PERCENT	OF	CELLS	
G0/G1	99	89	91	66	67
S	1	9	7	29	30
G2/M	0	3	2	5	3

Fig.3. Cell size (FSC, first row), cell density (SSC, second row), DNA content (FL2, third row), and percent of cells in G_0/G_1, S, or G_2/M (bottom), as analysed by a Facstar cytofluorimeter and DNA cell-cycle analysis software in lymphocytes cultured in the absence or presence of 5 mM 3ABA, as described in the text.

hibitory and in particular 10 mM 3ABA seems to alter the kinetics of lymphocyte proliferation. A delay in cell cycle progression due to the addition of 10 mM 3ABA has also been described as occurring in CHO cells [27]. Overall, however, our data are not indicative of inhibition of cell proliferation due to the initial addition of ADPRT inhibitors.

As pointed out by Cleaver et al. [18], little is known about the effective concentrations of ADPRT inhibitors in intact cells as opposed to permeable ones. Kun et al. [28] observed that, in intact fibroblasts, 1 mM 3ABA in the culture medium resulted in a concentration of 10 μM inside the cell, which apparently increased, rather than inhibited, ADPRT activity. Furthermore, very little is known about the rate at which benzamides and nicotinamide are metabolized in intact cells, i.e. concerning the concentration eventually reached at the time when DNA synthesis actually takes place. On the other hand, high benzamide concentrations are likely to have pleiotropic, and even toxic, side effects [19,29,30]. This uncertainty is even greater for NAM, since it is both a precursor of NAD^+, the ADPRT substrate, and an inhibitor of ADPRT activity. Therefore, both NAM starvation and addition have been used to inhibit the enzyme activity [31].

It may be added that in the past ethanol was widely used as a solvent for benzamide solutions [14]. It has been demonstrated that trace amounts of ethanol induce DNA SB in cultured lymphoid cells [29] and alter poly(ADP-ribose) metabolism [32]. We have obtained evidence suggesting that the presence of ethanol inhibits [^{3}H]TdR uptake in lymphocytes.

We approached these methodological problems via a carefully planned protocol: (i) three ADPRT inhibitors (3ABA, 3MBA, NAM) were used (NAM is considered to exert its inhibition in a different way vs benzamides); (ii) solutions were prepared in culture medium; (iii) a wide range of concentrations was tested, which is likely to comprise the inhibitory dose in intact cells, and higher concentrations were avoided in order to minimize both toxicity and side effects; (iv) cultures were continued for 7 days, in order to observe the entire course of the kinetics of the PHA-induced proliferative response; (v) in order to avoid possible variability among lymphocytes from different subjects, [^{3}H]TdR incorporation was studied in 10 different subjects; (vi) the effect of ADPRT inhibitors on lymphocyte proliferation was evaluated by two independent experimental systems.

Therefore, on the basis of this complete experimental protocol, we conclude that ADPRT inhibition does not prevent PHA-induced lymphocyte blastization and proliferation. This finding is in accordance with the observation that there are no DNA SB, requiring the intervention of ADPRT-related repair mechanisms, in quiescent lymphocytes [17]. ADPRT is induced at an early stage during mitogen-stimulated lymphocyte cultures [10], however its activity reaches maximum at around the 48–72 h [6,8]; this observation also supports the view that ADPRT is not required for early DNA repair. It has been suggested that ADPRT carries out other functions, probably related to specific changes in the chromatin structure [33–35]. Therefore, ADPRT expression may very well be cell cycle-regulated, rather than cell cycle-regulative, in the same way as other genes are [36].

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