POLY(ADP-RIBOSE) SYNTHESIS AND CELL DIVISION

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Summary:

A clone of HeLa cells has been selected in the presence of 5-methylnicotinamide. In the presence of 10 mM 5-methylnicotinamide the resistant cells grow at 70% of the rate of the same cell culture without 5-methylnicotinamide. 10 mM 5-methylnicotinamide completely inhibits the growth of normal HeLa cells. Both cell types in the absence of 5-methylnicotinamide have the same generation time. Poly(adenosine diphosphate-ribose) synthesis is less sensitive to 5-methylnicotinamide in nuclei isolated from the resistant cells than in nuclei from sensitive cells.

Introduction:

Since the detection of poly(adenosine diphosphate-ribose) synthesis from NAD *in vitro*, (1, 2, 3) a search has been under way for the role of the polymer in eucaryotic cell metabolism. Ample evidence has been presented for the synthesis of the polymer *in vivo* (4, 5, 6) but its function has not been elucidated nor has its synthesis been demonstrated to be a requirement for cell growth and division. In the present paper we describe the selection of a variant HeLa cell clone whose growth compared to normal HeLa cells is resistant to 5-methylnicotinamide, a potent inhibitor of poly(adenosine diphosphate-ribose) synthesis *in vitro* (7, 8). Poly(adenosine diphosphate-ribose) synthesis *in vitro* is also shown to be less sensitive to 5-methylnicotinamide in nuclei from resistant cells compared to synthesis of the polymer in nuclei from sensitive cells. These results offer the first direct proof that poly(adenosine diphosphate-ribose) synthesis in intact cells is obligatory for cell growth and division.

Materials and Methods:

HeLa cells were propagated in Eagles' Medium, modified as previously

described (9). Cells were synchronized with 2 mM hydroxyurea and S-phase was induced 16 hours later by pelleting the cells and resuspending in growth medium minus hydroxyurea (9). Cells resistant to 5-methylnicotinamide were selected by adding the compound (Eli Lilly & Co., Indianapolis) to a final concentration of 10 mM. After 48 hours incubation in shake culture, the cells were plated on plastic tissue culture dishes and any surviving cells allowed to attach for 16 hours. At this point almost all cells had lysed. The selective medium was replaced with fresh growth medium and after a week, one colony was detected (from an original innoculation of 20 X 10^6 cells. The selective medium was added again for 4 hours then removed and growth medium added again. One clone of cells survived and was propagated and the characteristics of this cell were compared to those of the starting cell population denoted sensitive cells.

DNA synthesis was assessed in synchronous cultures by uptake of [^{3}H]thymidine as described previously (10). Nuclei were prepared from the two cell types by the method reported (9).

Poly(adenosine diphosphate-ribose) Synthesis:

Nuclei from 10^6 cells were incubated in 0.25 ml of buffer which contained 1.5 mM [2-^{3}H]adenosine labeled NAD, 3 mM dithiothreitol, 2 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8. [^{3}H]NAD synthesized and purified according to Ueda, et al (11) had a specific activity of 3 X 10^6 cpm/μmole. Incubations were performed at 25°C and the reaction terminated by snap-freezing in dry ice-ethanol. Acid precipitable radioactivity was collected on Whatman GF/C filters and counted in a cocktail consisting of 20 parts of Spectrafluor (Amersham) to 473 parts toluene.

Results and Discussion:

The effect of 10 mM 5-methylnicotinamide on the growth of the resistant and sensitive cell lines is given in Fig. 1. As seen, the two cell types in the absence of 5-methylnicotinamide have the same doubling time (approx. 24 hours). In the presence of 5-methylnicotinamide resistant cell doubling time is increased

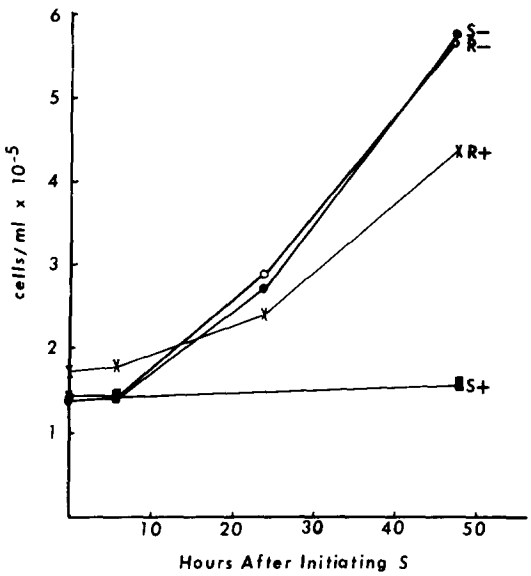

Fig. 1: Effect of 5-methylnicotinamide on cell growth. Cells which had been blocked for 16 hours with 2 mM hydroxyurea were spun down at low speed and resuspended in fresh growth medium with (+) or without (-) 10 mM 5-methylnicotinamide. S. Cells whose growth is sensitive to 5-methylnicotinamide. R. Cells whose growth is resistant to 5-methylnicotinamide.

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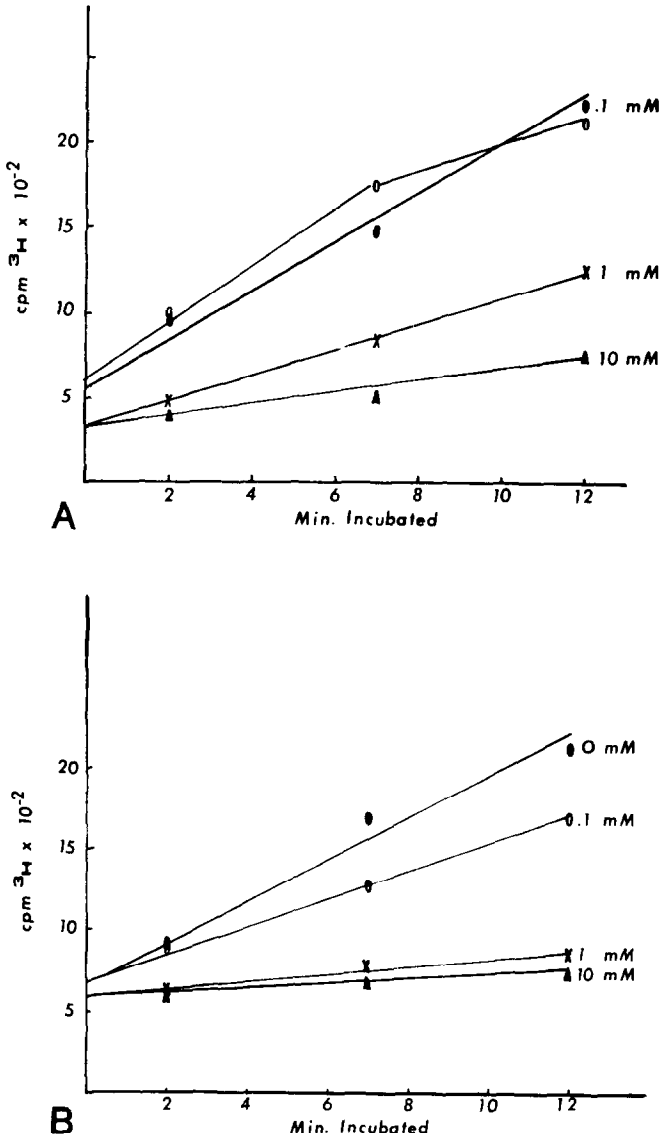
to about 30 hours. The inhibitor produces almost total blockage of growth in the sensitive cells.

Since 5-methylnicotinamide has been shown to be a potent inhibitor of poly(adenosine diphosphate-ribose) synthesis in vitro (7, 8), we examined the effect of this compound on [^{3}H]NAD incorporation into the polymer in nuclei isolated from both cell types. The kinetics of NAD incorporation at various concentrations of 5-methylnicotinamide is depicted in Figs. 2a and b. The inhibition based on changes in initial velocity calculated from the figures (Table 1) show that polymer synthesis in resistant cell nuclei is less sensitive to all concentrations of 5-methylnicotinamide tested than comparable synthesis in the nuclei of sensitive cells. For example, at 1.0 mM 5-methylnicotinamide, NAD incorporation is reduced by 63% in resistant cell nuclei and 92% in sensitive cell nuclei. Also evident from the data is the fact that polymer synthesis in vitro in the absence of 5-methylnicotinamide is



Figs. 2a and b: Effect of 5-methylnicotinamide on [^{3}H]NAD incorporation into poly(adenosine diphosphate-ribose) in isolated nuclei. S-phase was initiated by release of the hydroxyurea block. Eight hours later nuclei were prepared and assayed for poly(adenosine diphosphate-ribose) synthesis in the presence of various concentrations of 5-methylnicotinamide. Fig. 2a: Resistant cell nuclei. Fig. 2b: Sensitive cell nuclei.

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equivalent for equal numbers of nuclei from both cell types.

At what stage of the growth cycle does 5-methylnicotinamide act? On the basis of [^{3}H]thymidine incorporation into cells released from hydroxurea block

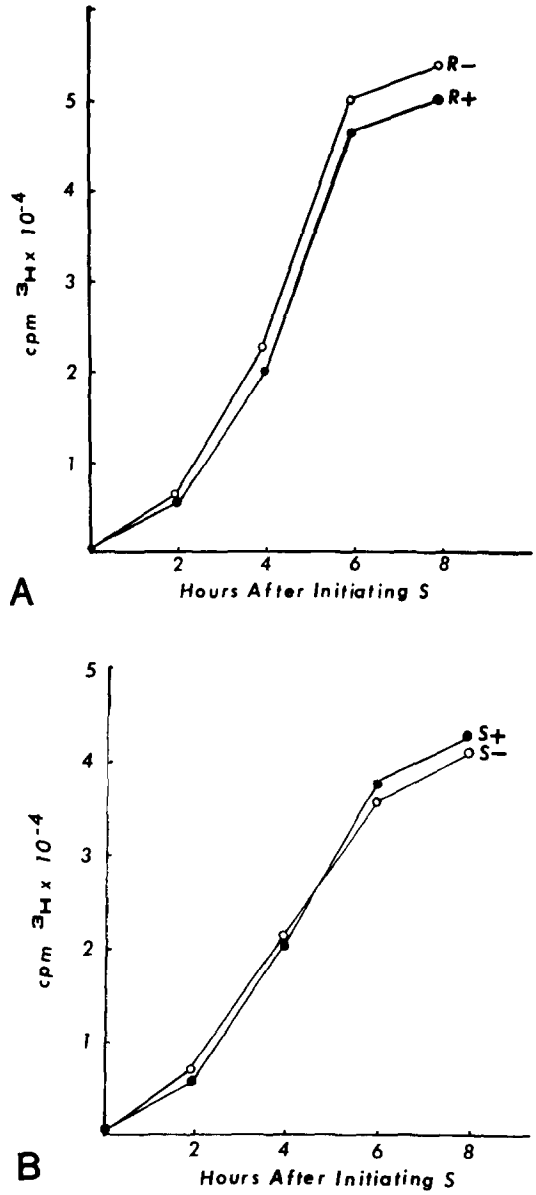
Table I

Effect of 5-methylnicotinamide on [^{3}H]NAD Incorporation in vitro[a]

Inhibitor Concentration (mM)	Resistant Cell Nuclei[b]		Sensitive Cell Nuclei[b]	
	cpm Incorp./10 min.	% Inhibition	cpm Incorp./10 min.	% Inhibition
0	1486	--	1520	--
0.1	1081	27	804	47
1.0	553	63	124	92
10.0	280	81	101	93

[a]Data calculated from initial velocities of Figs. 2a and b.

[b]See legend of Fig. 1.



Figs. 3a and b: Effect of 5-methylnicotinamide on DNA synthesis. Cells were treated as described in Fig. 1 except that [methyl-^{3}H]thymidine (0.5 Ci/μmole) was added to all cultures at a final concentration of 2 x 10^{-6} M. Fig. 3a: Resistant cells. Fig. 3b: Sensitive Cells.

it is apparent that DNA synthesis is not significantly affected either in resistant or sensitive cells (Fig. 3). Furthermore, cell division in sensitive cells was completely blocked when 5-methylnicotinamide was added to synchronous cultures that had traversed through S (7 hours after medium change). Examinatio

of this culture for several hours thereafter did not reveal an accumulation of cells in mitosis. Thus one apparent locus of 5-methylnicotinamide action is during G_2. This is consistent with the fact one peak of poly(adenosine diphosphate-ribose) synthesis *in vitro* (12, 13) and *in vivo* (13) occurs during G_2 in continuously dividing cells. Previously we reported that DNA synthesis was inhibited *in vivo* by nicotinamide with a concommitant reduction of poly-(adenosine diphosphate-ribose) synthesis (9). Since DNA synthesis was not inhibited by 5-methylnicotinamide in sensitive cells, poly(adenosine diphosphate-ribose) synthesis in S-phase is independent of DNA synthesis or there are two or more poly(adenosine diphosphate-ribose) polymerase activities. The latter hypothesis is favored by the finding that the K_m for polymer synthesis is 0.42 mM in S-phase nuclei and 0.75 mM in G_2 nuclei.

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