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DIFFERENTIAL EFFECTS OF NAD, NICOTINAMIDE AND RELATED COMPOUNDS UPON GROWTH AND NUCLEOSIDE INCORPORATION IN HUMAN CELLS

PETER G. PARSONS* and IAN P. HAYWARD
Queensland Institute of Medical Research, Herston, Brisbane, Queensland, Australia, 4006

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Abstract—Two human melanoma cell lines, MM96 and MM127, were found to be highly sensitive to the toxicity of adenosine (D_{50} 100–150 μg/ml) compared with other melanoma lines, HeLa cells and a lymphoblastoid line (D_{50} > 500 μg/ml). The MM127 line was also sensitive to NAD (D_{50} 41 μg/ml) compared with the other lines (D_{50} > 400 μg/ml), and accumulated three-fold more NAD-derived isotopic label. Nicotinamide exhibited little toxicity in any cell type (D_{50} > 400 μg/ml); 25–100 μg/ml nicotinamide greatly increased the plating efficiency of melanoma cells and fibroblasts when low levels of foetal calf serum were used. The toxicity of DNA-damaging agents (alkylating agents and u.v.) in melanoma cells was not reduced in the presence of NAD, adenosine or nicotinamide. Studies of the effects of the latter compounds upon the incorporation of deoxynucleosides showed that: (a) melanoma cells have lower purine pools than fibroblasts; (b) [^{3}H]deoxyguanosine incorporation was inhibited more than [^{3}H]deoxyadenosine incorporation; (c) incorporation of [^{3}H]deoxyadenosine and [^{3}H]deoxyguanosine into RNA was inhibited by adenosine, thus providing a method for determination of guanine-specific DNA repair; and (d) NAD enhanced thymidine incorporation in intact melanoma cells but not in fibroblasts, in a pattern similar to the release from template restriction previously reported for permeabilised tumour cells.

The roles of NAD and its components in cell proliferation have been studied from several aspects. Mammalian cells require exogenous nicotinamide as a nutrient for synthesis of NAD [1]. In addition to being a cofactor, NAD may be involved in the regulation of cell proliferation via the NAD/NADH ratio [2] or as the precursor for the synthesis of poly(ADP-ribose) [3, 4]. Thirdly, NAD levels are depleted after treatment with DNA-damaging agents [5–9], presumably because of the association of poly(ADP)-ribosylation with DNA repair. This aspect has been extensively studied because of possible relevance to cancer chemotherapy [5–7]. Finally, cell growth can be blocked by exogenous adenosine at levels which vary widely (5–400 μM) depending upon the cell type [10]. This effect does not result from excessive incorporation of adenosine into nucleic acids [11] or, in mouse melanoma cells, from pyrimidine starvation [12].

The present study developed from work concerning the action of MTIC,† an antimelanoma DNA-methylating agent [13], and was prompted by three related considerations: the finding that increasing the nicotinamide level in culture medium, or adding the purine precursor AIC, enhanced the growth of human melanoma cells; the possibility of enhancing MTIC toxicity by external control of poly(ADP)-ribosylation; and the requirement for a method to divert [^{3}H]deoxyguanosine and [^{3}H]deoxyadenosine incorporation from RNA into DNA so that purine-specific DNA repair could be measured.

MATERIALS AND METHODS

The lymphoblastoid cell line AG, obtained by transformation with Epstein–Barr virus of lymphocytes from a normal donor, was provided by Dr D. Moss. The origins of the other human cell lines have been described [13–15].

Cells were cultured in Roswell Park Memorial Institute (RPMI) Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing penicillin (100 I.U./ml), streptomycin (100 μg/ml), 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (5 mM) and, unless otherwise stated, 10% (v/v) foetal calf serum. Periodic assays for *Mycoplasma* [13] were negative.

Plating efficiencies were determined by seeding 2×10^3 cells into 5 ml medium containing the appropriate additive, in duplicate 60-mm plates. After 7–14 days the colonies (>50 cells) were stained with Giemsa and counted. For assay of colony growth by isotopic labelling [14, 16], 2×10^3 cells were seeded in 16-mm dia. Linbro wells. After 7 days, the cells were labelled for 2–4 hr with [*methyl*-^{3}H]thymidine [2 μCi/ml (45 Ci/mmole)] (Radiochemical Centre, Amersham, U.K.), detached with trypsin–EDTA and washed with water onto glass fibre discs for liquid scintillation counting. Increase in cell number was determined by harvesting and counting cells (haemocytometer) at daily intervals after seeding of duplicates (2×10^4 cells/16-mm well).

* Please address all correspondence to: Dr P. G. Parsons, Queensland Institute of Medical Research, Herston, Brisbane, Queensland, Australia, 4006.

† Abbreviations: MTIC, 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide; AIC, 5-aminoimidazole-4-carboxamide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TNE, 0.1 M NaCl, 10 mM Tris and 2 mM EDTA, pH 6.8; TCA, trichloroacetic acid.

Nucleoside incorporations were determined using cells seeded 24 hr previously and treated during this time with the appropriate additive (5×10^5 cells/60-mm plate). After labelling for 90 min the cells were harvested (trypsin–EDTA), suspended in 200 μl TNE and 4×50 μl aliquots treated with 0.5 ml 1 N NaOH. Two aliquots were diluted with 2 ml cold 10% (w/v) TCA immediately and two after 2 hr at 37°, followed by filtration onto glass fibre discs and washing with ethanol for liquid scintillation counting. The temporal response of thymidine incorporation was determined using cells seeded 24 hr previously (2×10^4 or 2×10^5 cells/16-mm well). At various times after addition of NAD, cultures were labelled for 30 min with [^{3}H]thymidine (5 μCi/ml) and harvested onto glass fibre discs.

Thymidine pool sizes were determined using the isotopic dilution method of Clarkson [17]. Cultures (2×10^5/16-mm well) were prelabelled for 24 hr with [2-^{14}C]thymidine [0.005 μCi/ml (25 Ci/mole)], treated with NAD (100 μg/ml) for 24 hr and then labelled with [^{3}H]thymidine (10 μCi/ml) (0.2, 1, 3, 6 and 10 μM) for 60 min. The cells were harvested and the thymidine pool sizes determined from plots of the ^{14}C/^{3}H ratio vs thymidine concn as previously described [17].

For determination of the uptake and incorporation of NAD, cells seeded 24 hr previously (2×10^5/30-mm plate) were treated with 50 μg/ml [4-^{3}H]NAD (2.5 μCi/ml) (Radiochemical Centre) or [U-^{14}C-adenine]NAD (0.25 μCi/ml) (Radiochemical Centre) in 1 ml of complete medium at 37°. At various times, duplicate cultures were washed rapidly with phosphate-buffered saline (2×2 ml) and the cells detached with 0.5 ml TNE. Three 100-μl aliquots were diluted in 5% (w/v) TCA and washed onto glass fibre discs with ethanol for determination of NAD incorporation by liquid scintillation counting. The remainder was treated with Instagel scintillant (4 ml) (Packard Instruments, Zurich, Switzerland), for determination of NAD uptake. Blank values, obtained from dishes without cells, were typically 6000–14,000 ^{3}H dpm and 600–1300 ^{14}C dpm. No corrections were made for extracellularly-bound label. Cell size was determined using a Particle Data counter (Particle Data Corp., Elmhurst, IL).

RESULTS

Effects of medium additives on plating efficiency

Using a particular batch of foetal calf serum, human fibroblasts (PGP) and three melanoma lines seeded at very low cell densities had 50–100% greater plating efficiencies in the presence of non-toxic levels of nicotinamide or AIC (Table 1). NAD enhanced the plating efficiency of PGP fibroblasts. NAD and adenosine either had no effect on the melanoma cell lines or, using MM127 cells, were inhibitory. Growth enhancement was not additive when combinations of the former three compounds were used (results not shown). Colony size, determined by counting the number of cells in 50 colonies of MM200 and MM253c1, was not significantly increased by nicotinamide (25–100 μg/ml). Other batches of foetal calf serum, tested using MM253c1 cells, gave enhancements of 10–100% greater plating efficiencies in the presence of nicotinamide.

The enhancement of plating efficiency by nicotinamide was more marked at low serum levels in both fibroblasts and melanoma cells (Fig. 1A), In 2% serum, for example, the added nicotinamide raised the plating efficiency from barely detectable levels to >50% of the 10% serum value. Cell proliferation at high seeding densities, however, was not enhanced by these compounds at either low or normal serum levels when growth was compared over a 3-day period (Fig. 1B), or at daily intervals (results not shown).

To test the possibility that nicotinamide merely enhanced cell attachment, MM253c1 cells prelabelled for 24 hr with [^{3}H]thymidine (0.05 μCi/ml) were reseeded (2×10^4 cells/16-mm well) and harvested at various times up to 48 hr. Most cells attached within 4 hr, and at the same rate in the presence of nicotinamide (100 μg/ml) as in control medium (Fig. 1B).

The apparent sensitivity of MM127 to NAD and adenosine prompted a dose–response study in a larger number of cell lines (Table 2 and Fig. 2). Cell survival decreased in an approximately logarithmic manner with increasing doses of NAD or adenosine. MM127 was much more susceptible to killing by NAD than the other cell lines (Fig. 2A). This line

Table 1. Enhancement of plating efficiency by non-toxic concns of medium additives

Cell	Plating efficiency of controls (%)	Plating efficiency with medium additives (% of control)*			
		Nicotinamide (50 μg/ml)	AIC (100 μg/ml)	NAD (25 μg/ml)	Adenosine (50 μg/ml)
PGP	1.8 ± 0.3†	210 ± 19	185 ± 20	210 ± 10	105 ± 13
MM96	6.9 ± 2.0	232 ± 14	159 ± 28	96 ± 12	83 ± 6
MM127	10.0 ± 0.4	70 ± 6	94 ± 9	53 ± 4	32 ± 7
MM200	4.4 ± 0.8	177 ± 16	130 ± 26	68 ± 7	91 ± 14
MM253c1	12 ± 3.0	150 ± 11	175 ± 14	101 ± 5	120 ± 5

* Determined by visual counting of colonies (>50 cells) 7–14 days after treatment commenced.
† Mean and S.D. of duplicates.

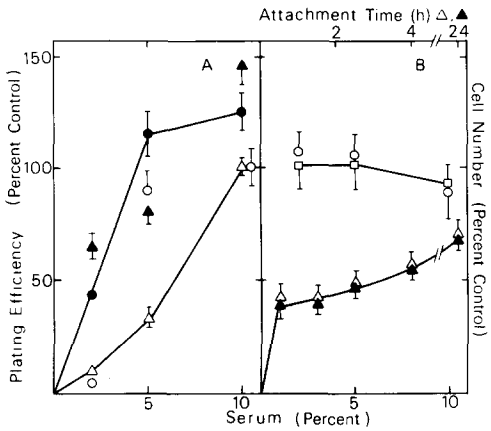

Fig. 1. Effect of nicotinamide on the attachment of cells, and on the plating efficiency and growth rate of cells in varying levels of foetal calf serum. Panel A, plating efficiences: △, PGP; ▲, PGP plus 100 μg/ml nicotinamide; ○, MM253c1; ●, MM253c1 plus 100 μg/ml nicotinamide. Panel B, attachment of MM253c1 cells in 10% foetal calf serum, with (△) or without (▲) 100 μg/ml nicotinamide. Increase in number of MM96 (□) or MM253c1 cells (○) after growth for 3 days in 100 μg/ml nicotinamide. Each point represents the mean and S.D. of duplicates.

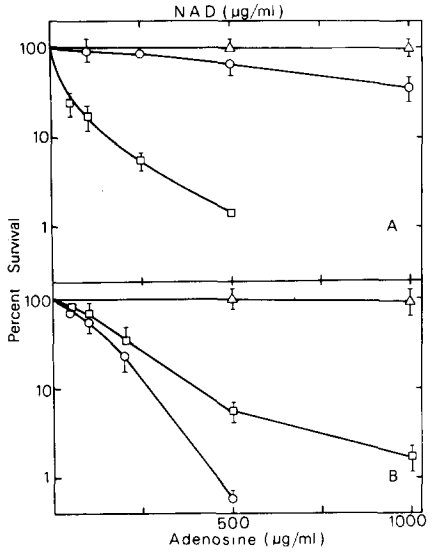

Fig. 2. Toxicity of NAD (panel A) and adenosine (panel B) in MM96 (○), MM127 (□) and MM253c1 cells (△). Each point represents the mean and S.D. of duplicates.

was also sensitive to adenosine, as was the MM96 line (Fig. 2B). Human fibroblasts (PGP), lymphoblastoid cells (AG) and four other tumour lines were relatively resistant to the toxicity of adenosine and NAD (Table 2). Nicotinamide exhibited little toxicity in any of these lines at levels up to 1 mg/ml.

The possibility of modifying the toxicity of MTIC using NAD, nicotinamide and adenosine was tested in MM253c1. This cell line is highly susceptible to killing by MTIC and MNNG, probably because of a DNA repair defect [13]. The results showed (Table 3) that, unlike previous reports using other agents [5, 7], neither NAD nor its components had any significant effect upon the toxicity of MNNG, MTIC or u.v.

Incorporation of nucleosides

A melanoma line (MM253c1) and a fibroblast strain (PGP) were used to ascertain the effects, if any, of medium additives on the incorporation of isotopically-labelled nucleotide precursors and to determine whether any such additions could divert the incorporation of purine deoxyribosides into DNA instead of into RNA as was found in preliminary experiments.

Table 2. Selective toxicity of medium additives used at high concns

Cell	D_{50} (μg/ml)*		
	Nicotinamide	Adenosine	NAD
PGP	1000	>1000	1000
MM96	950	110	750
MM127	400	150	41
MM170	>1000	>1000	810
MM200	>1000	>1000	850
MM253c1	>1000	>1000	>1000
HeLa	>1000	320	980
AG	>1000	580	450

* Dose required to reduce survival by 50%, determined by [^{3}H]thymidine labelling of colonies after treatment for 7 days.

Separate studies using DNAse, RNase and proteinase digestions of cell lysates showed that the alkaline treatment used to detected incorporation of label into RNA removed 4–7% of thymidine-labelled DNA, 75–85% of uridine-labelled RNA and 3–5% of leucine-labelled protein. Using this test, cells grown in normal medium were found to incorporate thymidine and uridine into DNA and RNA respectively (Table 4). The incorporation of deoxyadenosine and deoxyguanosine occurred almost completely into RNA in both cell types, DNase digestion showing that only 10% of the label was incorporated into DNA. Nicotinamide added 24 hr previously had little effect upon the labelling patterns, except for a slight inhibition of incorporation into the RNA of MM253c1 cells.

The effects on pyrimidine nucleoside incorporation of the purine precursor AIC and the naturally-occurring purine derivatives can, with important exceptions, be considered as a single group. Apart from AIC, these compounds inhibited thymidine incorporation in PGP but either enhanced or had no effect upon incorporation in MM253c1. The greatest difference was obtained using NAD, where thymidine incorporation was 67% of controls in PGP and 160% in MM253c1. NAD and, to a lesser extent, deoxyadenosine, enhanced uridine incorporation in both cell types whereas the other compounds had a somewhat negative effect.

The purine-related additives inhibited the incorporation of deoxyadenosine and deoxyguanosine, and the level of inhibition was 5–10-fold greater in MM253c1 than in PGP. Secondly, adenosine was the most inhibitory of the five additives tested, to the extent that deoxyadenosine incorporation was

Table 3. Toxicity of DNA-damaging agents to MM253c1 cells in the presence of NAD, nicotinamide or adenosine

Agent	Do*			
	Control	NAD (100 μg/ml)	Nicotinamide (100 μg/ml)	Adenosine (100 μg/ml)
MNNG	0.011	0.012	0.010	0.011
MTIC	1.2	1.3	1.1	1.3
254-mm u.v.	7.5	7.3	7.1	8.2
	(7.0)†	(7.1)	(6.5)	(6.7)

* Dose (μg/ml or $J.m^{-2}$) required to reduce survival (thymidine-labelling method) by 37% on the linear part of the survival curve. NAD, nicotinamide and adenosine were added 1 hr beforehand, and the culture medium was not changed during the course of the experiment.

† D_q, in parentheses, the size of the shoulder on the survival curve.

Table 4. Incorporation of nucleosides into nucleic acids

Medium additive (100 μg/ml)	Incorporation (% of control dpm)							
	PGP fibroblasts				MM253c1 melanoma cells			
	[^{14}C]Thymidine	[^{14}C]Uridine	[^{3}H]Deoxyadenosine	[^{3}H]Deoxyguanosine	[^{14}C]Thymidine	[^{14}C]Uridine	[^{3}H]Deoxyadenosine	[^{3}H]Deoxyguanosine
None	100 (6)	100 (81)	100 (80)	100 (76)	100 (4)	100 (82)	100 (76)	100 (79)
Nicotinamide	95 ± 9* (5)†	95 ± 8 (82)	80 ± 5 (79)	93 ± 2 (75)	97 ± 5 (5)	75 ± 3 (83)	73 ± 9 (75)	72 ± 3 (81)
AIC	102 ± 5 (7)	100 ± 14 (80)	46 ± 16 (80)	31 ± 5 (49)	135 ± 10 (6)	91 ± 3 (82)	8.8 ± 0.1 (80)	2.9 ± 0.1 (58)
Adenosine	52 ± 7 (6)	73 ± 3 (79)	23 ± 5 (72)	6.4 ± 0.3 (35)	89 ± 2 (7)	61 ± 4 (83)	6.5 ± 0.2 (71)	0.77 ± 0.01 (33)
Deoxyadenosine	79 ± 11 (5)	113 ± 2 (84)	53 ± 7 (78)	17 ± 3 (79)	101 ± 12 (2)	109 ± 11 (81)	8.4 ± 0.6 (65)	2.3 ± 0.2 (45)
Guanosine	81 ± 3 (8)	68 ± 5 (80)	41 ± 7 (70)	11 ± 1 (32)	119 ± 3 (7)	85 ± 3 (85)	8.6 ± 0.3 (78)	1.2 ± 0.1 (12)
Adenosine + guanosine	49 ± 12 (7)	71 ± 9 (78)	15 ± 3 (68)	3.6 ± 0.5 (29)	95 ± 6 (8)	55 ± 6 (84)	5.8 ± 0.2 (76)	0.37 ± 0.02 (11)
NAD	67 ± 5 (4)	121 ± 11 (93)	57 ± 4 (87)	27 ± 5 (86)	160 ± 7 (5)	149 ± 5 (80)	8.6 ± 0.7 (63)	2.3 ± 0.5 (38)

* Mean ± S.D. (N = 2).

† % Alkali-labile.

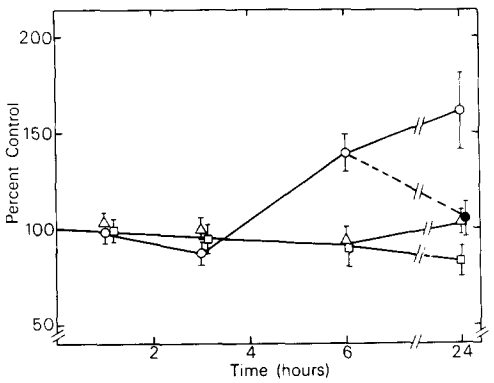

Fig. 3. Temporal effect of NAD (100 μg/ml) on thymidine incorporation. ○, 2×10^5 MM253c1 cells at 10^3 cells/mm^2; ●, 2×10^5 MM253c1 cells washed and refed control medium after 6 hr; △, 2×10^4 MM253c1 cells at 10^2 cells/mm^2; □, 2×10^5 PGP fibroblasts. Each point represents the mean and S.D. of duplicates.

inhibited more by adenosine than by isotopic dilution with unlabelled deoxyadenosine. Thirdly, in both cell types the purine nucleosides inhibited the incorporation of deoxyguanosine five-fold more than that of deoxyadenosine. Since most of the deoxyguanosine-derived isotope was alkali-resistant and hence in DNA after labelling in the presence of exogenous purines, the inhibition primarily affected incorporation into RNA. Simultaneous treatment with adenosine and guanosine did not relieve the inhibition produced when the compounds were used separately.

The temporal response of thymidine incorporation following NAD treatment was explored using PGP and MM253c1 (Fig. 3). At a high cell density (10^3 cells/mm^2), thymidine incorporation in MM253c1 exhibited a slight inhibition during the first 3 hr of treatment, followed by enhancement which was close to maximal after 6 hr. Removal of NAD at this time and culture for a further 18 hr in fresh medium reversed the effect. NAD did not effect the thymidine pool size (0.5 μM) in MM253c1. No enhancement of thymidine incorporation was detected using a lower density of MM253c1 or PGP cells (10^2 cells/mm^2). PGP cells at the higher density showed a gradual decline in thymidine incorporation during the 24-hr period studied, in keeping with the results given in Table 4.

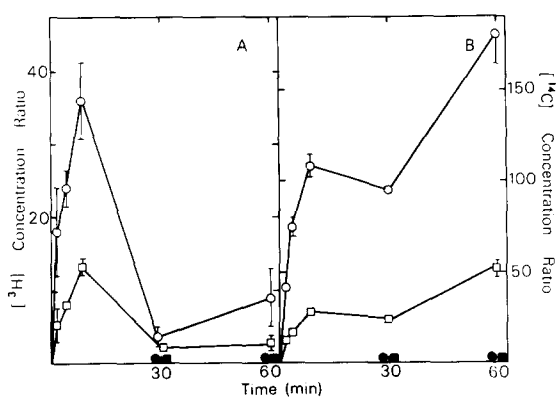

Fig. 4. Uptake and incorporation of dual-labelled NAD. Panel A, [^{3}H]nicotinamide label. Uptake by MM127 (○) and MM253c1 cells (□). Incorporation (TCA-insoluble label) in MM127 (●) and MM253c1 cells (■). Panel B, [^{14}C]adenosine label. Same symbols as in panel A. Each point represents the mean and S.D. of triplicates.

Uptake and incorporation of NAD

The uptake of dual-labelled NAD was compared in MM127 and MM253c1 cells (Fig. 4). In both cell lines, [^{3}H]nicotinamide-derived label accumulated within the first 10 min but then decreased to very low levels during the next 30 min. The initial uptake of [^{14}C]adenosine-derived label on the other hand was two- to three-fold greater than that of the ^{3}H label and continued to increase. After correction for differences in size, MM127 cells accumulated three- to four-fold more of each label compared with MM253c1. The incorporation of TCA-insoluble label was very low (approx 5% of uptake) in both cell lines (Fig. 4).

DISCUSSION

The enhanced plating efficiency of human cells obtained using 25 μg/ml nicotinamide (normally 1 μg/ml in RPMI 1640 culture medium) and the absence of significant effects upon isotopic labelling of DNA and RNA suggest that elevated nicotinamide could be used routinely for many experiments, particularly when low serum levels are required. Cell requirements for nicotinamide appear to be analogous to that for thymidylate precursors where growth enhancement was detected using low serum or low cell densitites [18]. The absence of any additive enhancement of colony formation using a mixture of AIC and nicotinamide and the lack of effect on colony size suggest a common nutrient mechanism effective in isolated, freshly-plated cells.

The levels of adenosine required for lethality in most of the lines were similar to those found for other human cells and 10–100 times those reported when adenosine deaminase inhibitors were used [10, 12, 19, 20]. Considering the brief half-life (approximately 50 min) (unpublished observations) of adenosine in culture medium due to the deaminase activity in foetal calf serum [10, 12], the effective treatment time of less than 24 hr was sufficient to cause the irreversible growth block in human melanoma cells. This contrasts with the reversible cytostasis found using mouse melanoma cells [12].

The most important finding concerning adenosine toxicity was the sensitivity of the melanoma lines MM96 and MM127. The two lines differ markedly in various properties such as size, morphology, chromosome and melanin content, doubling time, and resistance to anti-tumour drugs [15, 16], but no other information is presently available to suggest which, if any, of the various mechanisms proposed for adenosine toxicity could explain the sensitivity to adenosine. These lines could represent solid tumour

counterparts of the adenosine-sensitive chronic leukaemia lymphocyte [21], and may, in conjunction with the resistant lines, be useful for elucidating and testing adenosine-based control of at least some human tumours and for describing more completely the complexities of adenosine metabolism in human cells.

The toxicity of NAD in the MM127 line was unexpected in view of the lack of reports concerning NAD toxicity in mammalian cells. The results here suggest that exogenous NAD merely acts as a source of adenosine, possibly in the form of ADP-ribose, a known hydrolysis product of NAD [3]. Adenosine sensitivity of the NAD-resistant MM96 cells would therefore involve a different mechanism than adenosine sensitivity in MM127 cells. On the other hand, the NAD enhancement of thymidine incorporation in dense cultures of melanoma cells presents a close analogy to the effect of NAD on the replication of template-restricted DNA in permeabilised, resting HeLa cells [22], an effect which was much less marked in fibroblasts [4]. The present results therefore raise the question of whether some NAD is transported intact across the cell membrane. Some NAD appeared to accumulate as an intact molecule during the first 10 min of treatment, and nicotinamide, known to increase intracellular NAD levels in rat liver cells [23], was mildly toxic to MM127 cells. Thus apart from suffering degradation, sufficient NAD may cross the cell membrane to allow template release to be detected using intact cells.

The labelling experiments using exogenous guanosine, adenosine and AIC achieved the aim of reducing the incorporation of deoxyguanosine into RNA relative to DNA. Measurement of guanine-specific DNA repair synthesis as described for alkylated mouse cells [24] may now be feasible in MM253c1 cells and MTIC-resistant sublines [13] despite the very low levels of deoxyguanosine kinase reported for non-lymphoid human cells [21]. Preliminary experiments have shown that deoxyguanosine is rapidly decomposed to guanine by complete medium. The exogenous purines may competitively inhibit this pathway. It is apparent that deoxyadenosine is utilized differently from deoxyguanosine in these cells, and that the purine pools in the melanoma cells are lower than in fibroblasts.

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