Vol. 80, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1978 Pages 504-510

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD) STIMULATION OF DNA SYNTHESIS BY HUMAN BONE MARROW CELLS

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Received December 15,1977

SUMMARY Addition of 20 to 200 μg/ml of Nicotinamide Adenine Dinucleotide (NAD) to short term cultures of normal human bone marrow cells increases DNA synthesis only if human serum is present. Although NAD derivatives; reduced (NADH) and phosphorylated (NADP and NADPH) are equally effective, the components of NAD, Nicotinamide (NA) and Adenine (Ad) have no effect or reduce DNA synthesis at high concentrations. When malignant cells are tested; acute myeloblastic leukemia cell division is unaffected or reduced by NAD, NA or Ad.

Recently NAD (Nicotinamide Adenine Dinucleotide) has been shown to have functions beyond its well known role as a co-factor in oxidation-reduction reactions. It is a substrate for the adenosine diphosphate (ADP) ribosylation of elongation factor II catalysed by diphtheria toxin (1) and for the ADP ribosylation of mitochondrial and nuclear proteins by cellular enzymes (2,3,4). Further it is the substrate from which poly adenosine diphosphoribose (poly ADPR) is synthesized (5). The physiologic import of ADP ribosylation of cell proteins (excluding elongation factor II) is unknown. Nor has the role of poly ADPR been elucidated. We now present evidence that the simple addition of NAD but not of adenosine (Ad) or Nicotinamide (NA) to short term cultures of normal human bone marrow cells stimulates DNA synthesis by these cells.

We have previously described regulators of human bone marrow cell division in sera collected from patients undergoing chemotherapy (6,7).

0006-291X/78/0803-0504$01.00/0

While attempting to purify and characterize the factors in human serum which affect bone marrow cell division we found that dialysis of human sera against phosphate buffered saline (PBS) eliminates the ability of such sera to support DNA synthesis. Similarly, Sachs has reported that dialysed conditioned media cannot support granulocyte colony formation in agar gels (8). He also found that the addition of low concentrations of Ad or adenosine containing compounds to these dialysed media restored colony formation. We therefore tested the ability of various nucleosides and nucleotides to affect DNA synthesis by normal human bone marrow cells in the presence of untreated human serum, dialysed human serum and in the absence of serum. DNA synthesis was measured by the uptake of tritiated thymidine into acid insoluble material.

MATERIALS AND METHODS

Materials Normal human serum was collected from one of us and allowed to clot at room temperature for two hours. The clot was allowed to retract overnight at 4°C and the serum removed. Residual red cells were removed by centrifugation. Serum was sterilized by passage through a 0.45 μM filter (Millipore; Bedford, Mass.) and stored frozen at -20°C.

Dialysis tubing, obtained from Fisher Scientific, was prepared by being boiled for 15 minutes in 10% Na_2CO_3, washed in distilled water, boiled in 0.01 M EDTA pH 10 for an additional 15 minutes and then boiled in distilled water.

Adenosine, NAD, NADP, NADH, NADPH were obtained from Sigma (St. Louis, Mo.). Tissue culture media came from Grand Island Biologicals (Bethesda, Md.). Phosphate buffered saline (PBS) was prepared by the method of Dulbecco and Vogt (9).

Methods Cells were collected with informed consent from normal donors or patients with normal bone marrows prior to chemotherapy. Red cells were lysed by the method of Boyle (10). The remaining cells were washed and suspended at 1 million per milliliter in 70% RPMI 1640 medium with 30% normal human serum or 30% dialysed (against 50 volumes of PBS changed three times) serum or 30% PBS. Tritiated thymidine was added to a final concentration of 1 mcC per milliliter and the incubation mixture divided into one-half milliliter aliquots. One tenth milliliter of the appropriate nucleoside or nucleotide in PBS or PBS alone was added to individual tubes. Cultures were incubated at 37°C, 5% CO_2 concentration overnight and then stored frozen until the DNA could be extracted.

RESULTS

DNA synthesis in the absence of serum or in the presence of dialysed serum is only one third to one half the level observed in the presence of normal human serum. None of the nucleosides or nucleotides tested

could completely substitute for whole serum. They could, however, alter significantly the level of the amount of tritiated thymidine (^{3}HTdR) incorporated. Figure I shows the relative levels of DNA synthesis when adenosine and its derivatives and NAD and its derivatives were added to cultures of normal human bone marrow cells incubated in the presence of serum, dialysed serum or in the absence of serum. The addition of Ad stimulated DNA synthesis when dialysed serum was present or when serum was omitted entirely but not in the presence of normal human serum. The effects of adenosine monophosphate (AMP) were essentially the same as those of Ad, but 3', 5' cyclic adenosine monophosphate (CAMP) had no statistically significant effect on DNA synthesis.

In contrast, DNA synthesis was consistently increased by the addition of NAD and its derivatives (NADH, NADP and NADPH) to cultures incubated with normal serum or dialysed serum but not when these compounds were added to cultures in the absence of serum. The small changes shown in Figure I when NAD and its congeners were added to cultures in the absence of serum are not statistically significant.

Thymidine and cytidine, which can be converted to thymidine, lowered ^{3}HTdR incorporation, under all conditions, presumably by isotope dilution (data not shown). Guanosine and its derivatives, guanosine monophosphate (GMP) and 3', 5' cyclic guanosine monophosphate (CGMP) like adenosine, stimulated DNA synthesis by bone marrow cells in the absence of any serum or when dialysed serum was present. In normal human serum, however, guanosine compounds inhibited DNA synthesis by bone marrow cells.

We examined the effects of the parent compound, NAD and two of its subunits nicotinamide (NA) and adenosine (Ad) over a wide range of concentrations on the ability of normal bone marrow cells to incorporate ^{3}HTdR in the presence of normal serum. Using normal cells, only the entire NAD molecule stimulated DNA synthesis. Adenosine had no statistically significant effect; while high concentrations of NA inhibited ^{3}HTdR incorporation into acid insoluble material (Figure II).

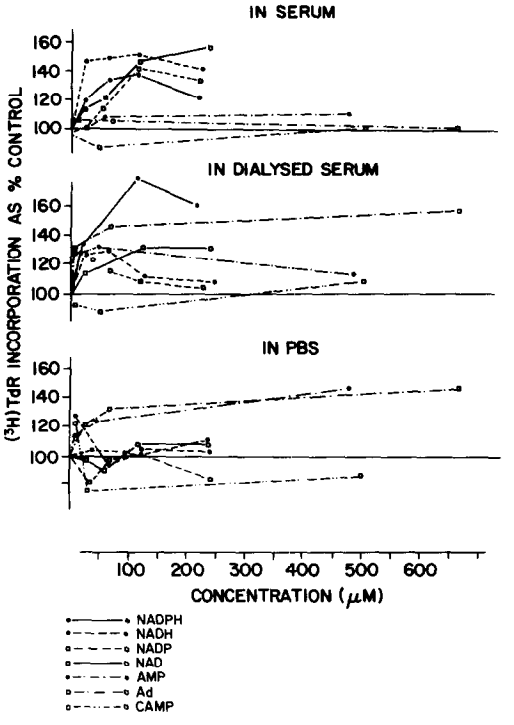

Figure I: The relative incorporation of tritiated thymidine into DNA by normal human bone marrow cells in the presence of various concentrations of different nucleosides and nucleotides. Cells were incubated in normal human serum, dialysed normal human serum or in the absence of serum. The 100% line in each graph is determined by the level of incorporation in control cultures to which no nucleosides or nucleotides had been added. Each point represents a means of from four to twelve individual determinations.

NAD has been shown to affect the kinetics of tumor development in vivo. Injected into mice, NAD delays the formation of methylcholanthrene induced tumors and reduces the rate of growth of Ehrlich Ascites and mastocytoma cells in the peritoneum (11,12,13). In our system, in the presence of serum, NAD had no statistically significant effect on the DNA synthesis of myeloblasts derived from patients with newly diagnosed and untreated acute myeloblastic leukemia (AML), while NA and Ad inhibited DNA synthesis by these cells (Figure III). In other experiments DNA synthesis by myeloblasts from two patients with chronic myeloblastic leukemia in blast crisis were unaffected by the addition of NAD. However, tritiated thymidine incorporation by lympho-

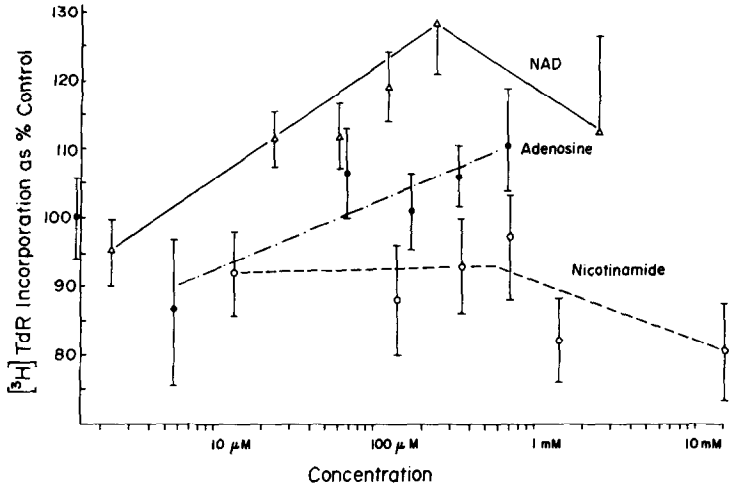

Figure II: The effect of Nicotinamide Adenine Dinucleotide (NAD), Adenosine and Nicotinamide on bone marrow cell division in short term cultures. Results represent average values plus or minus the standard error of the mean (error bars) in five separate experiments. Marrows from five different normal healthy donors were used. Normal human serum concentration, 30%.

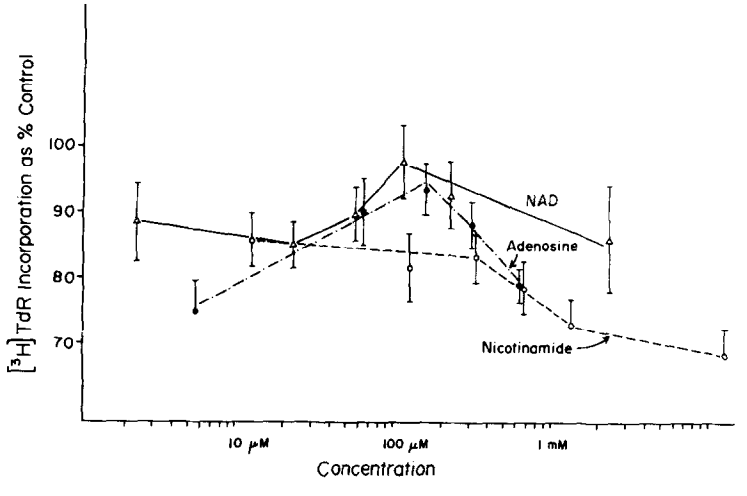

Figure III: The effect of Nicotinamide Adenine Dinucleotide (NAD), Adenosine and Nicotinamide on cell division by malignant myeloblasts. Bone marrow cells were collected from three patients with newly diagnosed and untreated Acute Myeloblastic Leukemia. The results represent the average values (plus or minus the standard error of the mean - error bars) of three experiments each with a different bone marrow. Normal human serum concentration, 30%.

blasts from two patients with acute lymphocytic leukemia was increased to 130% of control values in the presence of NAD while cell division by plasma cells from a patient with multiple myeloma was 135% of control when 60 mM NAD was present.

The concentrations of NAD, 2.4 μM to 2.4 mM used in these experiments, encompasses the physiologic concentrations reported. Normal tissues contain from two to three hundred mcg/g of NAD/NADH and five to ten mcg/g of NADP/NADPH. Whole blood is about 33 μM NAD and 4.6 μM NADH. The concentration of NADP in whole blood is some 11.6 μM and that of NADPH is 16.0 μM. NAD and its derivatives are largely intracellular. Only trace amounts of NAD are found in serum or plasma, about 0.7 mg/liter or 1.0 μM/liter by the fluorometric assay and none by microbiological methods. Nicotinamide is found both intra and extracellularly in the blood. Concentrations of this vitamin are 53.6 μM in whole blood and about 0.1 μM in plasma or serum (14,15,16).

In our system the lack of response to NA and Ad suggests that the entire NAD molecule is required for activity. Since stimulation of DNA synthesis by NAD requires the presence of serum or dialysed serum we conclude that some macromolecular (non-dialysable) component(s) is required. Either the oxidized (NAD, NADP) or reduced (NADH, NADPH) forms are equally active in stimulating DNA synthesis, therefore, they are probably not acting as terminal receptors in an electron transport pathway. Moreover since NAD is not known to cross the cell membrane and since the permeable parts of the molecule, NA and Ad, are inactive, NAD presumably acts extracellularly in our system.

We postulate two mechanisms by which addition of NAD to short term cultures of bone marrow cells could stimulate DNA synthesis by these cells. First, ADP ribosylation of cell surface proteins by serum enzymes could affect cell kinetics just as ADP ribosylation of elongation factor II by diphtheria toxin alters protein synthesis (1). Second, NAD could be acting as a co-factor of circulating dehydrogenases which form part of an extracellular enzymatic pathway. Numerous NAD requiring enzymes, such as lactate dehydrogenase, are

present in the circulation. Many of these enzymes are used clinically as markers of cellular breakdown. It is possible, however, that these "marker" proteins actually have a role in homeostasis and that some of them require NAD as a co-factor to perform this function. Our data suggests that an extracellular enzyme system exists which uses NAD as either a co-enzyme or a substrate, and that it affects DNA synthesis.

ACKNOWLEDGMENTS Supported in part by Grant CI-133 The American Cancer Society, Grants CA 05091 and CA 06973 The National Cancer Institute.

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