CORRELATION BETWEEN DNA SYNTHESIS AND INTRACELLULAR NAD IN CULTURED HUMAN LEUKEMIC LYMPHOCYTES

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SUMMARY

The NAD^+ level in lymphocytes obtained from an individual with acute monocytic leukemia increased five-fold and then remained constant when the cells were adapted to growth in suspension culture. When the NAD^+ level of established cells was lowered by means of a nicotinamide-poor medium or by the action of 1-methyl-1-nitrosourea, there was a concomitant decrease in the rate of DNA synthesis. These results indicate that there is a direct correlation between intracellular NAD^+ and the synthesis of DNA in cultured leukemic lymphocytes. However, the exact nature of the relationship remains speculative.

INTRODUCTION

Previous studies have provided evidence that links cell proliferation in tumors (1-3), regenerating tissues (4,5), and cultured mouse fibroblasts (6) with low intracellular levels of NAD^+. One interpretation of this relationship is that a low level of NAD^+ may limit the formation of poly(ADP-ribose), a nuclear polymer that is believed to inhibit DNA synthesis (7). In the present study, the relationship between DNA synthesis and intracellular NAD^+ was examined in a malignant line of human lymphocytoid cells that was adapted to tissue culture. Contrary to expectation, as the cells became established and began to proliferate, the level of NAD^+ per cell increased markedly. Furthermore, when an attempt was made to lower the level of NAD^+ by two different methods, the rate of DNA synthesis

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decreased. These results indicate that the previously established relationship (8) between cell proliferation and low intracellular NAD^+ is not necessarily universal but may vary with the cell type and possibly the conditions of growth.

METHODS AND MATERIALS

Leukemic Cells -- Blood (30 ml) was donated by a patient with acute monocytic leukemia (AML) before the administration of any therapeutic treatment. The blood was drawn into a heparinized, plastic syringe, and erythrocytes were allowed to settle by gravity. The leukocyte-rich plasma was expelled, diluted with an equal volume of 0.9% NaCl, layered in 5-ml aliquots on 5 ml of a 10:24 mixture of 33.1% sodium Metrizoate and 9.0% Ficoll (final density = 1.077 g/ml), and centrifuged at room temperature for 30 min at 400 x g (9,10). The leukocytes, which form a band above the interface, were removed, diluted with 0.9% NaCl, centrifuged as above for 10 min, washed with Hanks' balanced salt solution, resuspended in culture medium, and transferred directly to a spinner culture vessel. Sodium Metrizoate (Isopaque), 75%, was from Gallard-Schlesinger Corp., (Carle Place, NY), and Ficoll was from Pharmacia Fine Chemicals Inc. (Piscataway, NJ).

Culture Conditions -- The culture medium consisted of 80% RPMI-1640 and 20% heat-inactivated (56°C, 30 min) fetal calf serum and was supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). The nicotinamide concentration of this medium is 7.0 μM. The cells were stirred slowly at 37°C in closed vessels under a gas phase of 5% CO_2 and air, and fresh medium was added as necessary to maintain a concentration of 2-4 x 10^6 cells/ml. Cells were counted with a hemacytometer.

A nicotinamide-poor culture medium containing 10% heat-inactivated fetal calf serum was prepared by using RPMI-1640 formulated without nicotinamide. The nicotinamide content of this medium is about 0.2 μM and is derived from the serum (11). Growth media, fetal calf serum and antibiotics were obtained from Grand Island Biological Co. (Grand Island, NY).

NAD^+ -- Cells (1-2 ml) were centrifuged at room temperature for 10 min at 150 x g, washed with 2 ml of Hanks' balanced salt solution supplemented with 50 mM nicotinamide, resuspended in 1 ml of 0.9% NaCl supplemented with 50 mM nicotinamide, and acid-extracted as previously described (12) but without the N-ethylmaleimide step. NAD^+ was assayed by a cycling procedure (13).

DNA Synthesis -- Cells (1 ml) were incubated with [^{3}H]thymidine as described in the legend to Fig. 2, diluted with 1 ml of cold 0.9% NaCl, centrifuged at 4°C for 10 min at 150 x g, mixed in a vortex with 2 ml of cold, 5% trichloroacetic acid, and centrifuged as above at 800 x g. The precipitate was washed with 1 ml of cold methanol and dissolved with 0.1 ml of hot formic acid (boiling water bath, 1 min). Scintillation fluid (Aquasol, 10 ml) was added, and the radioactivity was determined by liquid scintillation counting. [^{3}H]Thymidine (19 Ci/mmole) was from Amersham/Searle Corp. (Arlington Heights, IL), and Aquasol was from New England Nuclear (Boston, MA). Other chemicals were of analytical grade quality, and water was deionized and glass-distilled.

RESULTS

Changes in NAD^+ During Establishment -- The NAD^+ level of freshly-isolated AML cells is 67 nmoles/10^9 cells (see Fig. 1), which is only slightly higher than that of freshly-isolated normal lymphocytes (56 nmoles/10^9 cells, mean value from 6 donors). Upon continued suspension culture, the NAD^+ content of the AML cells increases steadily and reaches a final level of about 350 nmoles/10^9 cells at about the same time that the cells are judged to be established (4 weeks). The NAD^+ content of normal lymphocytes also increases during tissue culture. However, culture conditions for normal cells are different, and the increase in NAD^+ is less pronounced. The increases in the level of NAD^+ may be caused, in part, by the extracellular concentration of nicotinamide, which in the complete culture medium is about 13-fold higher than its estimated concentration (0.5 μM) in human serum (11).

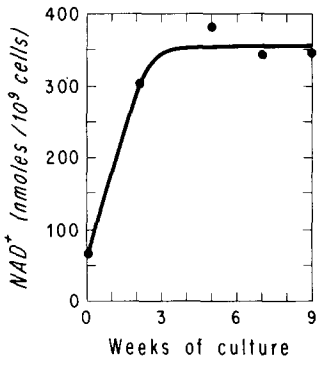

Figure 1 -- Changes in NAD^+ content of AML cells during adaptation to tissue culture.

Effect of 1-Methyl-1-Nitrosourea (MNU) -- MNU and related compounds have been previously shown to reduce NAD^+ levels in a variety of tissues (14-18). MNU was therefore used to test the effect of NAD^+ depression on DNA synthesis.

Upon incubation of AML cells with MNU, the NAD^+ level decreases rapidly, reaching 50% of its initial value in 24 min (Fig. 2A). Measurement of [^{3}H]thymidine incorporation into DNA (Fig. 2B) shows that the rate of DNA synthesis in MNU-treated cells immediately decreases to 7% of the rate in untreated cells. It is not known whether this inhibition of DNA synthesis is a consequence of the NAD^+ depletion or a direct effect of MNU upon DNA synthesis.

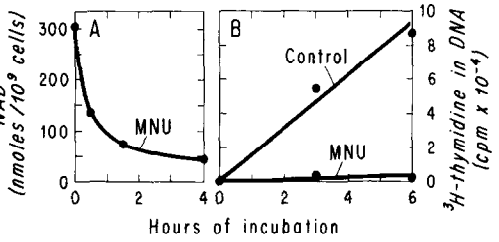

Figure 2 -- Effect of MNU on NAD^+ content and rate of DNA synthesis. (A) AML cells (4.8×10^6 cells in 2 ml of culture medium) were placed in 16 x 100-mm screw-cap tubes, supplemented with 4 mM MNU at zero time, and incubated at 37°C. At the times indicated, the cells were extracted for NAD^+ as described in Methods and Materials. (B) AML cells (2.4×10^6 cells in 1 ml of culture medium) were incubated as above with 2 μCi (1.25 nmoles) of [^{3}H]thymidine in the absence (control) and presence of 4 mM MNU. At the times indicated, the cells were extracted for DNA as described in Methods and Materials. Data are corrected for the small amount of [^{3}H]thymidine incorporated into the zero-time sample.

Effect of Nicotinamide Deprivation -- In order to test the effect of NAD^+ depletion on DNA synthesis in the absence of a drug that might have more than one mode of action, AML cells were transferred to a nicotinamide-poor medium. As shown in Fig. 3, the level of NAD^+ and the rate of DNA synthesis decrease in a parallel fashion and after 4 days are 61% and 47% of their initial values, respectively. In contrast, AML cells treated comparably but with complete medium (not shown) maintain a mean NAD^+ level of 307 nmoles/10^9 cells and incorporate [^{3}H]thymidine at a mean rate of 16.8×10^4 cpm under the conditions cited in the legend to Fig. 3.

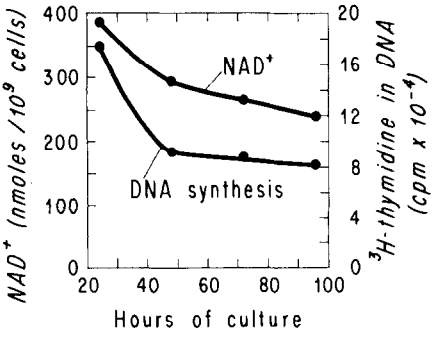

Figure 3 -- Effect of nicotinamide-poor medium on NAD^+ content and rate of DNA synthesis. AML cells in suspension culture (30 ml) were centrifuged, resuspended in 10 ml of nicotinamide-poor medium (see Methods and Materials), transferred to a 250-cc Falcon plastic bottle, and incubated at 37° C in a humidified atmosphere of 7% CO_2 and air. At the times indicated, 1-ml aliquots (17×10^6 cells) were extracted for NAD^+ or incubated for 3 hr with [^{3}H]thymidine (see legend to Fig. 2) and extracted for DNA. AML cells from 30 ml of suspension culture were also resuspended in 10 ml of complete medium containing 10% heat-inactivated fetal calf serum (control) and treated in a similar manner (see text for results).

DISCUSSION

The results presented here indicate that there is a direct relationship between the level of NAD^+ and the rate of DNA synthesis. However, this relationship appears to be complex. On one hand, an elevated NAD^+ level may stimulate metabolic activity and increase the amount of ATP available to support DNA synthesis. On the other hand, NAD^+ is the substrate for poly(ADP-ribose) (see 19 and 20 for reviews), and an elevated level of NAD^+ would have a greater potential for suppressing DNA synthesis through the formation of nuclear poly(ADP-ribose) (7).

If one assumes that a correlation exists between the synthesis of poly(ADP-ribose) and the intracellular level of NAD^+, then the results obtained with AML cells may be rationalized by further assuming that these cells contain an inactive poly(ADP-ribose) polymerase. MNU and its derivatives are believed to deplete intracellular NAD^+ by activating NAD glycohydrolase (14-18). Because poly(ADP-ribose) polymerase is a nuclear NAD glycohydrolase (21), it is possible that MNU triggers an ADP-

ribosylation reaction that has an immediate inhibitory effect on DNA synthesis. However, other interpretations are also possible, and further work is required to clarify the observed relationships.

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