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EFFECTS OF AGE ON ENERGY STATUS AND REDOX STATE OF LYMPOCYTES DURING BLASTOGENESIS

M. Lynne Chapman, M. Rebecca Zaun and Robert W. Gracy

Departments of Chemistry and Biochemistry
North Texas State University/Texas College
of Osteopathic Medicine
Denton, Texas 76203

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SUMMARY: The concentrations of the adenine and pyridine nucleotides were determined during mitogen-induced blastogenesis of human peripheral lymphocytes from young and old subjects. Significant differences were found in the energy status (ATP/ADP) and levels of the pyridine nucleotides. While lymphocytes from young subjects maintained a relatively constant energy status over 72 hours, the energy status decreased in both stimulated and control cells from old subjects. In addition, lymphocytes from young subjects show a marked increase in both oxidized and reduced NAD during blastogenesis. In contrast, cells from old subjects are not capable of increasing NAD levels.

Numerous studies have demonstrated that cell mediated immunity decreases with age. Human and animal lymphocytes from old subjects exhibit impaired responses to plant lectins (1), mixed lymphocyte reaction (2), graft-vs-host response (3), cutaneous-delayed hypersensitivity (4) and cell-mediated cytolysis (5). These observations have led to the hypothesis that impaired T-cell function may account for the increased incidence of cancer and autoimmunity with age (6-8).

Relatively little is known of the fundamental biochemical changes which occur in the T-lymphocyte as it undergoes the dramatic transition from the relatively dormant lymphocyte to the metabolically active lymphoblast. It has been demonstrated that there is no decrease in the number of blood T-lymphocytes (9,10) or the affinity and number of lectin binding sites (11) with aging.

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* To whom correspondence should be addressed

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Two of the most important factors in increased metabolic activity associated with cell growth are the adenine and pyridine nucleotide coenzyme levels (12-14). The present study was designed to determine the levels of ATP, ADP, AMP, NAD^+, NADH, $NADP^+$, and NADPH during mitogen-induced lymphocyte transformation. The study was conducted on cells isolated from young and old subjects.

MATERIALS AND METHODS

Cell Isolation: Fasting blood samples (100 ml) were collected by sterile venipuncture from subjects 60 years or older (median age 72) and from subjects less than 25 years of age (median age 22). Subjects were free from diseases or medication known to affect the reactivity of lymphocytes. Lymphocytes were isolated free of platelet contamination by a modification of the method of Mendelsohn et al (15). Blood was defibrinated by continuous rotation for 15 min in 250 ml erlenmeyer flasks containing 6% dextran (20% of blood volume) and 10-15 glass beads (5mm diameter). The blood was then allowed to sediment by gravity in a 100 ml graduated cylinder at 37° for 45 min with frequent removal of the leukocyte rich supernatant solution. Isopycnic centrifugation at 250 x g for 20 min in 50 ml conical styrene tubes was performed on 40 ml portions of the supernatant solution layered over 8 ml of Ficoll (Sigma)-Hypaque (Winthrop). The lymphocyte layer was collected and washed twice with phosphate buffered saline by centrifugation at 1000 x g for 2.5 min and resuspended in Medium 199.

Cell Culturing: Freshly isolated lymphocytes were adjusted to a concentration of 1×10^6 cells/ml in Medium 199 (KC Biological) containing 25 mM HEPES buffer, 0.2 µM nicotinamide, 10% fetal calf serum (heat inactivated at 56°, 30 min), 75 units/ml penicillin and 75 µg/ml streptomycin (Gibco). The mitogen, 5 µg/ml phytohemagglutinin or 20 µg/ml Conconavalin A (Sigma), was added to cell aliquots to induce blastogenesis. Cells were incubated in 90% humidity at 37°. Controls were incubated in the absence of mitogen under identical conditions. At various times over a 72 hour period, cell aliquots were harvested by centrifugation at 1700 x g for 10 min at 0°. Cell counting was performed with a hemacytometer. Viability was determined by 0.4% trypan blue uptake.

Pyridine Nucleotides: The NAD and NADP pools were determined using a modification of the procedure of Jacobson et al (16). Cells were extracted with cold 0.1 N NaOH, containing 1 mM nicotinamide in a volume equal to the original cell suspension. A portion of each sample was immediately neutralized with 0.37 M H_3PO_4 and later used for the total NAD and NADP assays. An equal portion was heated at 60° for 10 min with agitation to destroy the NAD^+ and $NADP^+$ and immediately neutralized with 0.37 M H_3PO_4. This extract contained only the reduced pyridine nucleotides. Phenazine ethosulfate (20 mM) was added to cycle all NAD and NADP to the oxidized form before analysis. NAD^+ and $NADP^+$ were determined on 0.5 ml of extract (5×10^5 cells) by the enzymatic cycling assay (14). All tests were performed in duplicate. Oxidized pyridine nucleotides were determined as the difference between total and reduced levels.

Adenine Nucleotides: Energy assays were performed on aliquots of neutralized lymphocyte extracts following a modification of the luciferase method of Chapman et al (17). For concentrations of ATP, 50 µl of cell extracts (5×10^4 cells) were added to 25 µl buffer (75 mM potassium phosphate, pH 7.4, 15 mM $MgCl_2$) and 40 mM phosphoenolpyruvate. The concentrations of ATP + ADP

were analyzed as above but with 45 I.U. of pyruvate kinase. Total adenylates (ATP + ADP + AMP) were assayed as above but included 125 I.U. of adenylate kinase. Each reaction was incubated at 30° for 30 minutes and stored at 0° until analyzed. Luminescence was measured on 20 µl of the incubation mixture. 140 µl of reaction buffer and 40 µl of 10 mg/ml luciferin-luciferase. All assays were carried out in triplicate. The energy status is defined as ATP/ADP (18).

Thymidine Uptake: ^{3}[H] Thymidine (0.2 µCi) was added to 0.2 ml of the lymphocyte suspension (2 x 10^{5} cells) in flat-bottom microtiter plates. Analyses of controls and mitogen-stimulated samples were conducted in triplicate. The cells were labeled for 24h at 37°. Using a cell harvesting system (Brandel 24-V), the cells were aspirated and deposited onto filter paper discs (Reeve Angel 934 AH), air dried and counted in 5 ml Aquascint (ICN). The blastogenic index is defined as: $\frac{\text{stimulated cpm - blank cpm}}{\text{control cpm - blank cpm}}$

Statistical Analysis: Three-way analysis of variance was performed on the data. When interaction was found, a series of one way ANOVA were performed controlling for the other variables, i.e. time, culture conditions or age. Duncan's multiple range test (19) was used to indicate significant differences ($p \leq 0.05$).

RESULTS

Thymidine Uptake: Table I shows the blastogenic index for stimulated lymphocyte cultures from young and old individuals. No significant differences were ascertained in the rate of incorporation of tritiated thymidine during 72 hours of culture between young and old subjects.

Adenine Nucleotides: Table I also displays the adenine nucleotide levels in lymphocytes and lymphoblasts from young and old individuals. ATP in mitogen-stimulated cells from young subjects was significantly greater at 48h ($p=0.0136$) and 72h ($p=0.0008$) than in the cells from old subjects. This is reflected also in the relatively constant energy status (ATP/ADP) in the lymphocytes from young subjects in contrast to the decreasing energy status of cells from old subjects in the presence or absence of mitogen.

Pyridine Nucleotides: Fig. 1 shows the total NAD levels in mitogen-stimulated lymphocytes from young and old subjects. Total NAD in stimulated cultures from young people was significantly higher at 48h ($p=0.0267$) and 72h ($p=0.0008$) than NAD in cells from old subjects. Statistical difference due to age was also reflected in NAD^{+} ($p=0.0565$) and NADH ($p=0.0429$) at 72h in mitogen-stimulated lymphocytes. In comparing stimulated cells with non-

Table I. BLASTOGENIC INDEX, PYRIDINE AND ADENINE NUCLEOTIDES IN LYMPHOCYTES FROM YOUNG AND OLD SUBJECTS

Subjects	Conditions	Hours of Culture	Blastogenic Index	NAD^+	NADH	$NADP^+$	NADPH	ATP	ADP	AMP	$\frac{ATP}{ADP}$
				pmoles/10^6cells							
Young N=5 Age: 18-25	Control	0		46.2	7.4	0.2	24.0	1332	2490	488	0.53
		24		78.2	13.8	2.8	44.0	1108	2108	1058	0.53
		48		71.6	9.4	1.4	11.2	990	2310	534	0.41
		72		54.6	12.0	0.6	8.6	882	2140	1264	0.44
	+Mitogen	0	0	49.6	5.6	0	14.0	1254	2406	736	0.52
		24	1.6	121.4	28.8	0	18.8	1166	2262	620	0.56
		48	27.2	125.8	26.4	0.4	13.2	1304	2168	886	0.54
		72	178.5	123.2	41.4	2.2	10.0	1592	2404	964	0.71
Old N=5 Age: 62-79	Control	0		51.0	14.6	0.2	20.8	1104	2090	1768	0.52
		24		86.6	16.8	1.6	17.0	726	2084	1204	0.37
		48		91.6	18.0	2.6	10.2	674	2282	798	0.30
		72		53.6	10.8	0.4	10.8	574	2098	792	0.29
	+Mitogen	0	0	44.8	15.0	2.8	17.4	884	1924	1004	0.47
		24	1.1	101.8	25.4	1.0	24.8	792	2086	598	0.39
		48	87.3	74.8	19.0	1.2	8.8	618	2164	672	0.32
		72	150.8	45.6	12.8	0.8	5.2	530	1786	612	0.31

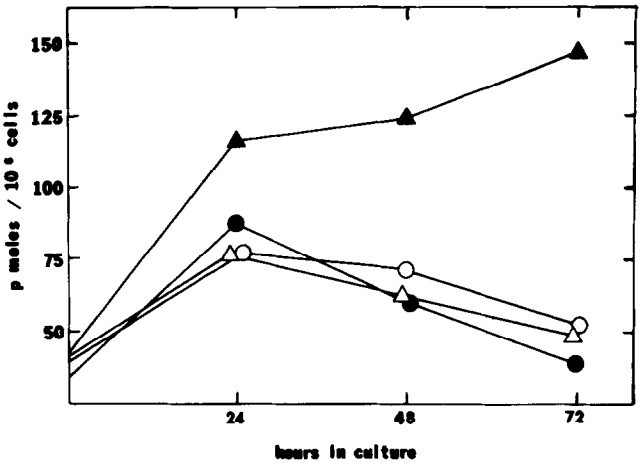

Figure 1: Total NAD in control and mitogen stimulated lymphocytes from young and old subjects. Lymphocytes were isolated and cultured in the presence (closed symbols) and absence (open symbols) of mitogens as described in "Methods". △ = young subjects non-stimulated controls; ▲ = young subjects mitogen-stimulated; ○ = old subjects non-stimulated controls; ● = old subjects mitogen-stimulated.

stimulated cells from old individuals, no significant increase of total NAD, NAD^+ or NADH was found. While NAD^+ and NADH levels in lymphocytes from young subjects increased during blastogenesis, NAD^+ and NADH levels decreased in mitogen-stimulated cells from old people (Table I). The redox state (NAD^+/NADH) of cells from young subjects decreased during blastogenesis but no change was observed in the stimulated lymphocytes of old subjects.

Levels of NADP in lymphocytes from young people did not increase during blastogenesis, but the levels decreased in cells from old subjects. Thus, the total NADP in stimulated cells from young people was significantly higher at 48h ($p=0.0175$) and 72h ($p=0.0128$) than NADP in cells from old subjects. Essentially 100% of the NADP pools was NADPH in all cases.

No difference due to age was seen in NAD or NADP levels of control cultures from young or old persons.

DISCUSSION

Under the conditions of this study, stimulated-lymphocytes from young and old subjects showed equal abilities to incorporate tritiated thymidine over a

72-hour period. Viability of the cells and visual determination of blast transformation confirmed these findings. No significant differences were observed using concanavalin A or phytonemagglutinin as the stimulating mitogen, and no difference was found with respect to sex. These observations are similar to those of Czlonkowska and Korlak (20) who found no difference in tritiated thymidine uptake in concanavalin A stimulated cells from young and old populations but observed depressed abilities of the lymphocytes from old subjects with respect to other measures of cell mediated immunity. As Pakin et al (21) have pointed out, tritiated thymidine incorporation is highly dependent on incubation conditions, mitogen concentration, cell density, tissue medium, temperature and other conditions. From the present study it is clear that major biochemical differences in energy status and NAD levels occur under conditions when tritiated thymidine uptake is unchanged.

The energy status of lymphocytes from young persons cultured in the presence or absence of mitogens remained fairly constant over the 72 hour period. In contrast, cells from old individuals showed a decrease in the energy status in control and mitogen-stimulated cultures.

A more profound difference between the young and old cells was the relative inability of lymphocytes from old persons to increase their pools of NAD. During blastogenesis of lymphocytes from young persons NAD levels increased markedly. These data are consistent with the observations of Blomquist et al (22) who reported increased levels of NAD^+ and NADH in phytohemagglutinin stimulated lymphocytes. On the other hand, cells from old subjects were not capable of increasing NAD levels.

The question arises as to the basis for the NAD increases during blastogenesis. Roos and Loos (23) and Hedeskov (24) showed that an increase in glycolytic flux is a prerequisite for blastogenesis. Kester et al (25) and Rogers et al (26) have demonstrated marked increases in glycolytic enzymes during blast transformation. It is possible that the increased NAD is required for redox purposes associated with the increased metabolism of the lym-

phoblast. On the other hand, there are other functions of NAD in the cell such as serving as the substrate for poly(ADP-ribose) (27,28). Several studies (14,29-35) have shown that NAD levels are significantly altered during cell proliferation and have suggested that this nucleotide may be more directly involved in the control of cell division (13).

Whatever the basis for the increased need for NAD in blastogenesis, it appears that lymphocytes from old persons are defective in making this adaptive response when challenged with mitogens.

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