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CHANGES IN NAD LEVELS IN HUMAN LYMPHOCYTES AND FIBROBLASTS DURING AGING AND IN PREMATURE AGING SYNDROMES

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SUMMARY

NAD levels markedly increase upon mitogen stimulation of lymphocytes from young subjects. In contrast, lymphocytes from old subjects do not increase NAD levels upon stimulation. A survey of 35 individuals aged 18–79 years revealed a significant age-dependent decrease in the NAD response to mitogen stimulation. No significant differences were noted in lymphocytes from age-matched individuals with Down's syndrome or diabetes mellitus. On the other hand, cultured skin fibroblasts showed elevated NAD levels with age. However, this effect appears to be due to increased size of the cells since the NAD/protein ratio is unchanged. Skin fibroblasts from patients with progeria exhibit much higher levels of NAD and protein per cell than age-matched controls.

Key words: Diabetes mellitus; Down's syndrome; Progeria; Werner's syndrome

INTRODUCTION

The ability of peripheral lymphocytes to undergo blast transformation upon mitogenic stimulation becomes impaired with aging [1–4]. This defect has been attributed to an intrinsic defect in the lymphocyte [5–7]. The numbers of circulating T-lymphocytes, distribution of subpopulations, and the number and affinity of mitogen receptor sites per cell do not appear to be altered with age [8,9].

When lymphocytes from a young population are stimulated with mitogen, the levels of all glycolytic enzymes increase markedly [10]. This ability to increase glycolytic flux appears to be essential for blast transformation since the specific inhibition of glycolytic enzymes prevents blastogenesis [11]. We have observed that lymphocytes from old subjects exhibit an impaired ability to increase the levels of the glycolytic enzymes [12]

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upon mitogenic stimulation. Similarly, lymphocytes from old subjects display an impaired response in the production of nicotinamide adenine dinucleotide (NAD*) levels upon stimulation, whereas lymphocytes from young subjects rapidly increase NAD levels [13].

The resemblance between certain premature aging syndromes and pellagra has led to the idea that a dysfunction of NAD(H)- or NADP(H)-dependent enzymes may play a major role in aging. Horita *et al.* [14], using an antimetabolite of NAD, postulated that a dysfunction in some NAD(H)- or NADP(H)-dependent enzyme could account for the central nervous system disorders associated with the Creutzfeld-Jacob premature aging syndrome.

The present study was undertaken with three objectives: first, to examine the mitogen-stimulated NAD responsiveness of lymphocytes from a spectrum of age groups. Secondly, the study was designed to examine the lymphocyte NAD response in two groups considered to be premature aging syndromes: diabetes mellitus and Down's syndrome. Thirdly, NAD levels with respect to age in other cell types (*e.g.* fibroblasts) were examined in normal and other premature aging syndromes (progeria and Werner's syndrome).

MATERIALS AND METHODS

Lymphocytes

Fasting blood samples were collected from three groups of subjects. Group I consisted of five donors over the age of 60 years (mean = 72) and five donors less than 25 years' old (mean = 22). Group II consisted of nine subjects diagnosed as having diabetes mellitus, and nine age- and sex-matched non-diabetic controls. Four of the nine subjects were diagnosed as juvenile-onset type; the remaining were adult-onset type diabetics. These subjects ranged in age from 18 to 54 years (mean = 36). Group III was composed of 11 institutionalized subjects with Down's syndrome. Simultaneous lymphocyte isolations were performed on ten age- and sex-matched control subjects from the same institutional setting. This group consisted of ages 18 through 37 years (mean = 27).

In the above studies peripheral blood lymphocytes were isolated as described previously [13]. For Group III, 10–20 ml of heparinized blood were collected, mixed with equal volumes of phosphate-buffered saline and immediately layered over Ficoll-Hypaque for lymphocyte isolation [13]. The freshly isolated lymphocytes were cultured as previously described [13] with the addition of 5% CO_2 to the incubation atmosphere. Aliquots were removed at specified intervals from culture flasks in experiments involving Groups I and II. In Group III studies, 2.1-cm^2 flat-bottomed culture dishes were employed, with individual dishes being extracted at indicated intervals (initial cell density for Group III cultures was 0.71×10^6 cells/cm^2).

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*NAD represents the total [both oxidized (NAD^+) and reduced (NADH)] concentrations of nicotinamide adenine dinucleotide.

Fibroblasts

Ten skin fibroblast cell lines from male donors aged 3–96 years were obtained from the Human Genetic Mutant Cell Repository. The eight normal cell lines were GM 2938A (3 years), GM 2037 (13 years), AG 4054 (29 years), AG 2602 (35 years), AG 4147 (49 years), AG 2261 (61 years), AG 4057 (81 years), and AG 4059 (96 years). Cell line AG 3513 was from a 13-year-old male with progeria and AG 780 was from a 60-year-old male with Werner's syndrome.

All cells were grown at 37 °C in a humidified CO_2 atmosphere in Eagle's minimum essential medium (Earle's Balanced Salt Solution) supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin sulfate (Schering Diagnostics) and fetal bovine serum (10% for normal cells, and 20% for AG 4059 and the premature aging cell lines). Maintenance of cells consisted of three feedings per week and subculturing monolayers 1–2 days post-confluence at a split ratio of 1:2 for cultures AG 4057, AG 4059 and those with premature aging, and a ratio of 1:3 for all other normal cell lines. Cultures were discontinued if confluency was not reached within two weeks. Cultures were harvested at confluency (G_1) with 0.05% (w/v) trypsin, 0.02% (w/v) EDTA, washed twice with phosphate-buffered saline, counted with a hemacytometer and assessed for viability by the exclusion of 0.4% trypan blue.

Pyridine nucleotides

Cells were extracted with ice-cold 0.1 N NaOH with 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 for determination of the total NAD concentration. An equal portion was heated at 60 °C for 10 min (this extract contained only the reduced pyridine nucleotides). Phenazine ethosulfate (20 mM) was added to cycle all NAD to the oxidized form before analysis. NAD^+ content was determined in extracts by the enzymatic cycling assay [15]. For fibroblasts, 250 μl of a 0.1×10^6 cells/ml extract was used in the assays.

Proteins

The protein concentration of cell extracts was determined by a modification of the method of Lowry *et al.* [16]. Cells were extracted with ice-cold 0.1 N NaOH containing 1 mM nicotinamide and were incubated at room temperature with 670 μl of a solution of 1% $CuSO_4$, 2% sodium tartrate, and 2% Na_2CO_3 in 0.1 N NaOH (1:1:100). For lymphocytes, 330 μl of a 1.0×10^6 cells/ml extract were assayed, and for fibroblasts 25 μl of a 0.5×10^6 cells/ml extract were used. 2 N phenol (33 μl) was added and duplicate measurements were made at 720 nm.

Thymidine uptake

DNA synthesis was monitored using 0.2 μCi of [^{3}H] thymidine per 2×10^5 cells in flat-bottomed culture dishes [13]. Initial cell densities were 0.71×10^6 cells/cm^2. The blastogenic index (BI) was calculated as BI = (stimulated cell cpm − background cpm)/(control cell cpm − background cpm).

Statistical analyses

Statistical analyses were as reported previously [13]. Three-way analysis of variance was performed on the data. When interaction was found, a series of one-way analysis of variance (ANOVA) was conducted, controlling for the other variables. Duncan's multiple-range test was used to indicate significant differences ($p \leq 0.05$). Correlations with donor age were determined by use of linear regression and Pearson product-moment correlations and significant probabilities.

RESULTS

Lymphocyte NAD and age of the subject

Lymphocytes from five subjects less than 25 years' old, when stimulated with phytohemagglutinin (PHA, 5 μg/ml) responded with significant increases in NAD levels. However, the response to the PHA in cells from five old subjects (62–79 years) did not show corresponding significant differences in NAD when compared to the non-stimulated control cells. Figure 1 shows that there were significant differences in the NAD concentrations of stimulated lymphocytes from subjects less than 25 years' old when compared with subjects over the age of 60 ($p = 0.0530$ at 48 h, $p = 0.0074$ at 72 h). In studying an age spectrum, 25 other subjects ranging in age from 18 to 59 years were examined. Linear regression analysis of 72-h results revealed a significant ($p = 0.0128$) negative correlation (correlation coefficient = −0.4166) between the age of the donor and the NAD levels in mitogen-stimulated cells (Fig. 2). NAD^+ levels at 72 h of culture were significantly ($p = 0.0096$) decreased as the subject age increased (correlation coefficient = −0.5282) while NADH levels with a p value of 0.0944 were lowered to a lesser degree as a function of age.

Lymphocyte NAD and premature aging syndromes

The NAD levels in stimulated lymphocytes from subjects with Down's syndrome increased significantly ($p = 0.0012$ at 72 h) when compared to non-stimulated cells from the same individuals (Fig. 1). Information on reduced and oxidized forms of NAD was not possible due to the limited sample volumes. Similar responses were seen in the age- and sex-matched control subjects without Down's syndrome ($p = 0.0019$ at 72 h). When total NAD from the group with Down's syndrome was compared to the control group, no significant differences were seen in this response to PHA (Fig. 1, Table I).

Subjects previously diagnosed as having diabetes mellitus were also studied and compared with an age- and sex-matched control group (Fig. 1, Table I). Total NAD in the diabetic group was significantly increased in cells with PHA when compared to control cultures without PHA ($p = 0.0010$ at 72 h). Significant increases in both NADH ($p = 0.0102$ at 48 h, and $p = 0.0042$ at 72 h) and NAD^+ ($p = 0.0135$ at 72 h) were observed. The non-diabetic control group also responded to PHA by significantly ($p = 0.0116$ at 48 h, $p = 0.0003$ at 72 h) increasing NAD levels. The NADH concentrations increased significantly at 48 h ($p = 0.0357$) and at 72 h ($p = 0.0264$), and the NAD^+ was found to be significantly ($p = 0.0144$) elevated at 72 h. The stimulated lymphocytes from the

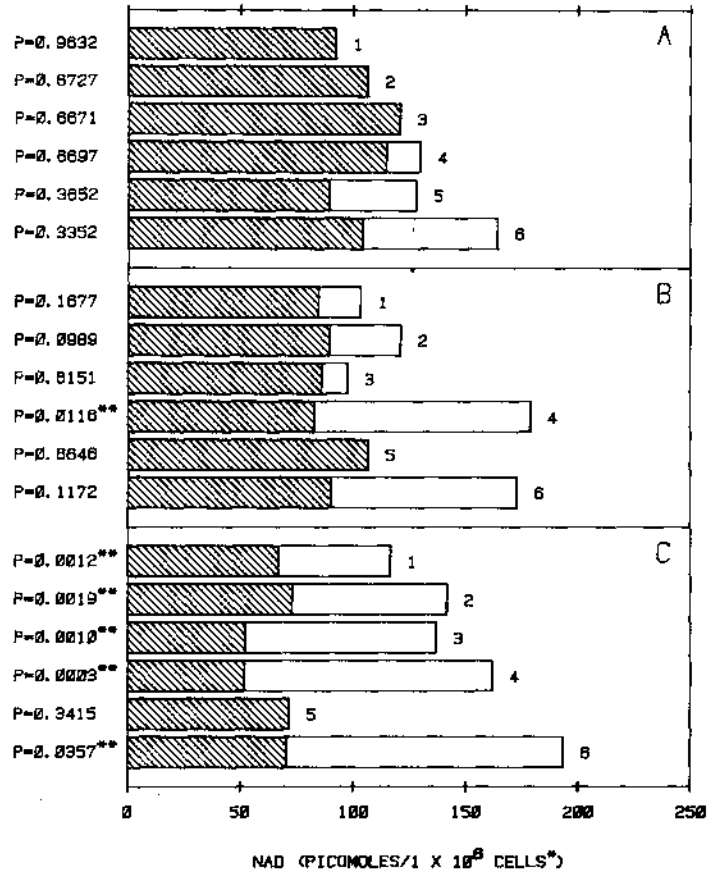

Fig. 1. Total NAD in non-stimulated control (hatched area) and PHA stimulated (open area) lymphocyte cultures (A, after 24 h of incubation; B, 48 h; C, 72 h) from subjects with Down's syndrome (1), age-matched controls (2), diabetics (3), age-matched controls (4), donors > 65 years' old (5), and young donors (6). Significance levels are shown for all data. **Significant ($p \leq 0.05$) differences between control and stimulated cultures. *NAD results are based on an initial lymphocyte concentration of 1×10^6 cells/ml.

diabetic population exhibited a significantly lower ($p = 0.0167$) increase in NAD at 48 h when compared with their age- and sex-matched control group of non-diabetics.

Of the 35 subjects studied there were never significant differences in the NAD levels in the non-stimulated cells from 0 to 72 h of incubation.

Lymphocyte blastogenic index, cell viability, and protein concentration

Cell proliferation was monitored by the uptake of tritiated thymidine in the control and stimulated cultures at 24 h, 48 h and 72 h of incubation. There were no age-related differences in the blastogenic index. Moreover, there was no significant difference in the blastogenic index of cells from subjects with Down's syndrome or diabetes mellitus when compared with their control groups. Protein determinations were also carried out on

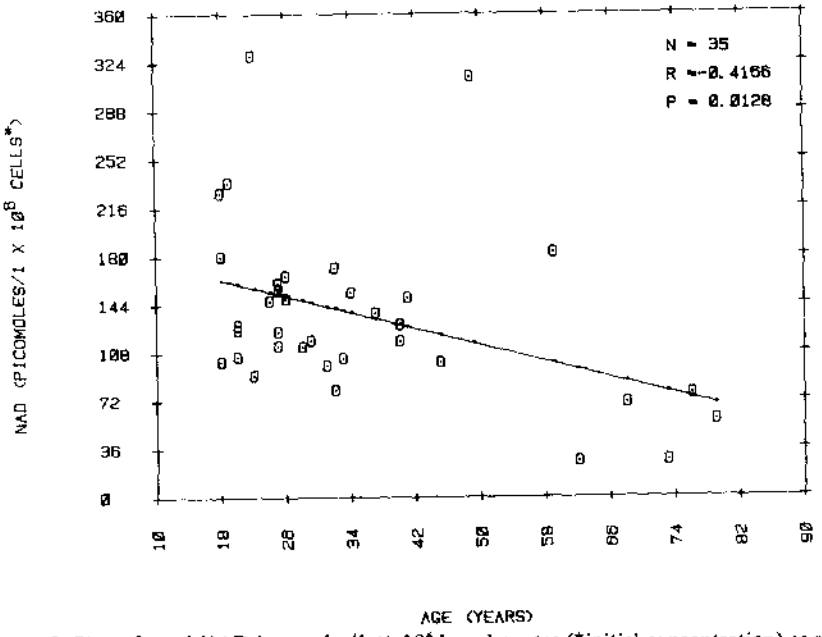

Fig. 2. Plot of total NAD in pmoles/l X 10^6 lymphocytes (*initial concentration) as related to donor age after 72 h of PHA stimulation. Data points are the mean of duplicate assays for each donor. Regression line was calculated by Pearson product–moment correlations and significant probabilities. The number of donors participating is represented by the letter N. The regression coefficient is given as the letter R and is shown as a straight line through the data points. P represents the significance level of the regression line.

TABLE I

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD) IN STIMULATED LYMPHOCYTES FROM OLD SUBJECTS OR THOSE WITH EARLY AGING SYNDROMES AND NORMAL CONTROLS

	24 h		*48 h*		*72 h*	
	NAD ± 1 S.D.	*p*	*NAD ± 1 S.D.*	*p*	*NAD ± 1 S.D.*	*p*
Old	128.0 ± 70.8	0.5605	87.8 ± 55.7	0.0530[a]	51.5 ± 23.6	0.0074[a]
Young	164.6 ± 114.7		173.2 ± 63.1		193.7 ± 86.2	
Diabetic	106.2 ± 69.1	0.4786	97.7 ± 37.7	0.0167[a]	137.1 ± 47.4	0.3700
Control	129.9 ± 72.6		179.2 ± 84.7		162.0 ± 67.5	
Down's	91.7 ± 28.4	0.8094	103.4 ± 26.1	0.2078	116.8 ± 27.7	0.1423
Control	95.8 ± 35.4		121.2 ± 18.7		141.9 ± 30.5	

NAD values [pmoles/l X 10^6 lymphocytes (initial concentration)] ± 1 standard deviation (S.D.) for all measurements within each group.

[a]Significant ($p \leq 0.05$) differences between groups.

TABLE II

PERCENTAGE VIABILITY, NAD AND PROTEIN IN FIBROBLASTS FROM NORMALS AND PREMATURE AGING SYNDROMES

Subject	*Age (years)*	*Mean population doubling (± 1 S.D.)*	*n*	*Viability (%)*	*NAD (pmoles/l × 10^6 cells)*	*Protein (μg/1 × 10^6 cells)*
Normal						
GM 2938A	3	14 ± 7	3	89.3	1867.0	628.2
GM 2037	13	19.5 ± 1.5	3	87.1	2847.6	621.4
AG 4054	29	14 ± 1	2	94.1	1891.6	464.0
AG 2602	35	11 ± 1	1	98.4	3266.6	608.0
AG 4147	49	10.5 ± 2	5	91.3	2353.4	454.4
AG 2261	61	10 ± 2	3	93.2	2917.0	669.4
AG 4057	81	11 ± 1	4	83.3	3674.4	706.0
AG 4059	96	17 ± 1	3	91.1	3614.2	901.4
Progeria						
AG 3513	13	10 ± 1	4	93.8	3123.2	746.0
Werner's Syndrome						
AG 780	60	7 ± 4	4	88.4	3910.0	858.6

the subjects with diabetes and Down's syndrome and their respective control groups. No significant differences were observed with respect to the controls. Cell viability also did not appear to differ with respect to age or in the two pathological syndromes.

Fibroblast NAD and protein levels: normal and premature aging syndromes

Table II shows the mean NAD values for normal fibroblast cell lines from subjects at ages varying from 3 to 96 years, and from fibroblasts from subjects with progeria and Werner's syndrome. While there was no change in the cell viability with age, there was a gradual increase in the NAD content with the age of the fibroblast donor (correlation coefficient = 0.6326, $p = 0.0009$). However, unlike the case of the lymphocytes, there was a significant difference in protein content of the cells with age. A positive correlation coefficient of 0.4338 with a significance of $p = 0.0342$ was found. Thus, the content of NAD per mg of protein showed no correlation with age. In the case of the two premature aging syndromes, the NAD and protein content of the progeria and Werner's syndrome fibroblasts were much higher than their age-matched controls (Fig. 3). The predicted age (based on NAD content) of the progeria cells was 69 years (actual age 13 years) and for the Werner's syndrome 128 years (actual age 60 years). Similarly, based on protein content per cell, the progeria cells were 97 years and the Werner's cells were 148 years.

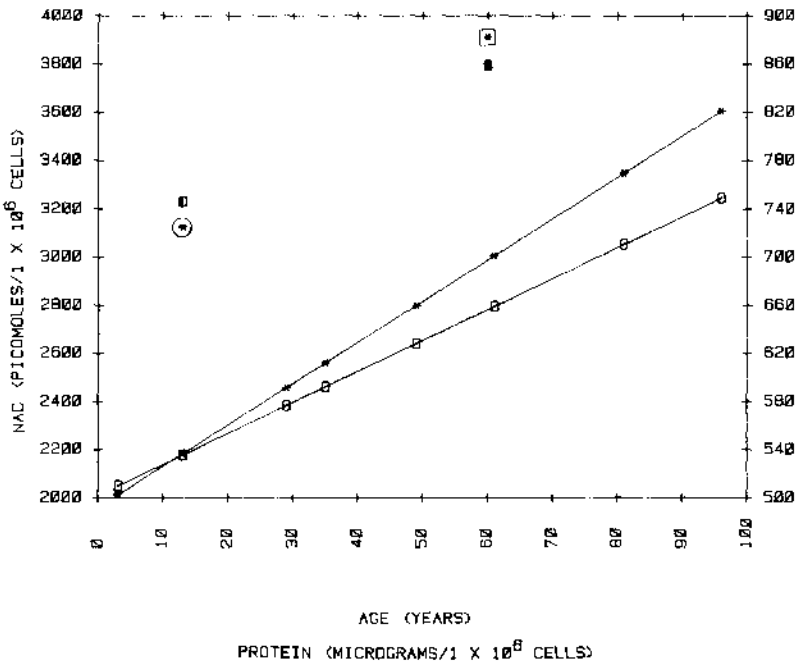

Fig. 3. Relationship of NAD and protein of normal skin fibroblast cultures with the age of subject. Skin fibroblast cell lines from normal subjects ($n = 24$) ranging in age from 3 to 96 years were assayed (1–5 cultures/cell line) for NAD and protein. The mean values for each cell line were used for linear regression analysis of NAD *vs.* age of subject (*) (correlation coefficient = 0.6326, $p = 0.0009$), and protein *vs.* age of subject (⊙) (correlation coefficient = 0.4338, $p = 0.0342$). The mean values of NAD for progeria ($n = 4$) (⊛) and Werner's syndrome ($n = 4$) (▣) are plotted as well as the mean values of protein for progeria (◧) and Werner's syndrome (■).

DISCUSSION

Under the conditions of this study, lymphocyte viability, protein concentration and blastogenic index were not significantly affected by the age of the subject. Thus, the changes observed in NAD levels occur prior to observed declines in cell viability or blastogenic index which are seen only after 72 h [13]. The present study verifies our previous observation that peripheral lymphocytes from an old population exhibit an impaired ability to increase NAD levels in response to mitogen stimulation. Moreover, the present study showed a significant age-dependent correlation of this impairment when a spectrum of ages was examined. It is also interesting that in all of the studies there was no significant difference in the NAD levels in freshly isolated cells or in controls which were not subjected to mitogens. Thus, it appears that only when the cells are exposed to this environmental stress do the latent biochemical age-related defects become apparent. A similar situation appears with respect to the ability of lymphocytes to increase the levels of the glycolytic enzymes upon mitogen stimulation [12].

The basis for the increase of NAD during blastogenesis is not clear. One obvious possibility is that with increased glycolytic flux and increased levels of the glycolytic

enzymes, there is an additional need for NAD as a coenzyme for glycolysis. However, there are other possibilities. For example, NAD is a substrate for the synthesis of poly-(ADP-ribose), and Sims *et al.* [17] have shown changes in NAD levels and poly(ADP-ribose) in lymphocytes forced to carry out unscheduled DNA synthesis.

It is also unclear whether the age-related inability to increase glycolytic enzymes as well as NAD are related. We have recently shown that at least one glycolytic enzyme, triosephosphate isomerase, accumulates in old cells as an unstable form due to specific deamidation of two asparagines [18]. Similarly, glucose 6-phosphate dehydrogenase [19–24], 6-phosphogluconate dehydrogenase [19,20,22,23,25] and hypoxanthine–guanine phosphoribosyltransferase [20,23,25] also appear to accumulate labile forms in old cells. It is possible that the accumulation of these "abnormal" enzymes interferes with the synthesis of new enzymes and elevation of NAD levels under conditions of "metabolic stress" (*e.g.* mitogen stimulation).

The study of premature aging syndromes can provide important information relating to basic normal biochemical age-dependent changes. In thepresent study, age-matched subjects with Down's syndrome showed no significant impairment in the ability to elevate NAD in response to mitogens. However, it should be recognized that the subjects with Down's syndrome (and age- and sex-matched controls) ranged in age from 18 to 37 years, perhaps too narrow an age range.

In the case of the fibroblast studies, a different situation exists. The cells were growing without exposure to the mitogen PHA. Under these conditions, the age-related changes in NAD levels appear to be simply due to the fact that cell size increases with age and thus NAD per cell increases, but the NAD/protein ratio remains essentially constant. It is also interesting in this respect that the fibroblasts from individuals with progeria and Werner's syndrome exhibited elevated NAD and protein levels well beyond the age-matched controls. This is also probably a function of cell size since the cells from these premature aging syndromes are much larger.

In conclusion, these studies support and extend our original findings [13] that lymphocytes from old subjects are impaired in their ability to increase NAD levels in response to mitogenic stimulation. The effect correlates with age over a wide age spectrum. Further studies on the metabolism of NAD should be of interest in eludicating the basic reason for the NAD increase and the site(s) of the impairment in lymphocytes from old persons.

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