Oxygenated Carotenoid Lutein and Progression of Early Atherosclerosis

The Los Angeles Atherosclerosis Study

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Background—Carotenoids are hypothesized to explain some of the protective effects of fruit and vegetable intake on risk of cardiovascular disease. The present study assessed the protective effects of the oxygenated carotenoid lutein against early atherosclerosis.

Methods and Results—Epidemiology: Progression of intima-media thickness (IMT) of the common carotid arteries over 18 months was determined ultrasonographically and was related to plasma lutein among a randomly sampled cohort of utility employees age 40 to 60 years (n=480). Coculture: The impact of lutein on monocyte response to artery wall cell modification of LDL was assessed in vitro by quantification of monocyte migration in a coculture model of human intima. Mouse models: The impact of lutein supplementation on atherosclerotic lesion formation was assessed in vivo by assigning apoE-null mice to chow or chow plus lutein (0.2% by weight) and LDL receptor–null mice to Western diet or Western diet plus lutein. IMT progression declined with increasing quintile of plasma lutein (P for trend=0.007, age-adjusted; P=0.0007, multivariate). Covariate-adjusted IMT progression (mean±SEM) was 0.021±0.005 mm in the lowest quintile of plasma lutein, whereas progression was blocked in the highest quintile (0.004±0.005 mm; P=0.01). In the coculture, pretreatment of cells with lutein inhibited LDL-induced migration in a dose-dependent manner ($P<0.05$). Finally, in the mouse models, lutein supplementation reduced lesion size 44% in apoE-null mice (P=0.009) and 43% in LDL receptor–null mice (P=0.02).

Conclusions—These epidemiological, in vitro, and mouse model findings support the hypothesis that increased dietary intake of lutein is protective against the development of early atherosclerosis. **(*Circulation*. 2001;103:2922-2927.)**

Key Words: atherosclerosis ■ epidemiology ■ carotid arteries ■ ultrasonography ■ diet ■ lutein

Interest in the possible health effects of dietary carotenoids stems from the finding in epidemiological studies that persons with higher intake of fruits and vegetables are at reduced risk of coronary heart disease,[1] stroke,[2] and cancer at several sites.[3] Of the many phytochemicals found in these foods, the properties of carotenoids have suggested the hypothesis that they contribute to a reduced risk of cancer[4] and coronary heart disease.[5]

The present investigation focuses on the pathogenesis of early atherosclerosis and the impact of the oxygenated carotenoid lutein, a pigment found in dark green leafy vegetables, egg yolks, and other foods.[6] It is distinguished in chemical structure from the hydrocarbon carotenoid β-carotene by the presence of 2 hydroxyl groups. Data are presented from an epidemiological study of atherosclerosis progression[7] (as measured by change in carotid intima-media thickness, IMT[8]), an in vitro model of oxidation in the artery wall,[9] and in in vivo mouse models of atherosclerosis in the aortic arch.[10]

Methods

Epidemiology

Participants were from the Los Angeles Atherosclerosis Study, a cohort of 269 women (age 45 to 60 years) and 304 men (age 40 to 60 years), with no history of heart attack, angina, revascularization, or stroke, who were employees of a utility company. Participants were randomly sampled from all employees, with oversampling of Hispanics and smokers, with a participation rate of 85%. Ultrasound examination of the carotid arteries, venipuncture, and assessment of risk factors for atherosclerotic cardiovascular disease were performed at baseline and after 18 months.

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Carotid IMT

Common carotid artery IMT was measured at baseline and follow-up in the far wall of the left and right arteries by high-resolution B-mode ultrasound with an ATL scanner (7.5-MHz transducer). Procedures for image acquisition and processing have been reported.[8] Briefly, average thickness over a 1-cm segment of the common carotid artery 0.25 cm proximal to the bulb was measured with automated edge-tracking software. A reproducibility study found a mean absolute difference of 0.030 mm (coefficient of variation 4.0%) between repeated scans by 2 sonographers.[8] Both sonographers and readers were blinded to plasma lutein levels.

Risk Factors

Risk factor assessment occurred during the baseline and follow-up examinations, which took place in a mobile unit at work sites. Anthropometric measurements, blood pressures, and interview/questionnaire were conducted by the study nurse and sonographers.[7]

Plasma Carotenoids

Plasma lutein and β-carotene were determined by high-performance liquid chromatography from samples frozen at −70°C. Blood was drawn from fasting participants in the morning. Assays were performed by a participant (Heber Laboratory, UCLA) in the NIST/NCI micronutrients measurement quality assurance program[11] and by the analytic method described by Epler.[12] The coefficient of variation for plasma lutein was 5.3%. Serum cholesterol, HDL cholesterol, and triglycerides were determined by autoanalyzer. LDL cholesterol was estimated for fasting samples with triglycerides <3.95 mmol/L.

Statistical Procedures

Because of positive skew in the lutein variable, change in IMT was regressed on quintiles of plasma lutein in a linear model. Tests of linear trend across quintiles were computed by regression on a variable defined by the median lutein level within each sex-specific quintile. The age-adjusted model was also adjusted for sex, time interval between examinations, and an interaction between sex and lutein. The multivariate model included the additional covariates ethnicity, smoking status, systolic blood pressure, serum cholesterol, serum HDL cholesterol, plasma β-carotene, body mass index, alcohol intake, hours since last meal at blood draw, history of diabetes, current use of medication for high cholesterol or hypertension, and interactions of lutein with medication use.

LDL Oxidation by Artery Wall Cells

The impact of lutein on oxidative modification of LDL was evaluated in a coculture model of the artery wall formed from endothelial and smooth muscle cells from human aortas.[13]

The impact of lutein on the monocyte chemoattractant property of LDL incubated with the coculture was evaluated in 2 types of chemotaxis assays. In the first assay, the coculture was incubated with different concentrations of lutein overnight. Cells were then washed and coincubated with constant concentrations of LDL for 8 hours. In the second assay, LDL was first incubated with different concentrations of lutein for 4 hours and then added to the coculture for 8 hours. Lutein was reagent grade (79% lutein, 6% zeaxanthin) from plant sources, donated by Kemin Industries, Inc (Des Moines, Iowa). Tissue culture media, serum, and supplements were obtained from sources reported.[9] Blood monocytes were obtained from a large pool of healthy donors by modification of the Recalde procedure as described.[14]

Monocyte Chemotaxis Assay

The cocultures were treated with native LDL (250 μg/mL) in the absence or presence of HDL or lutein for 8 hours. The supernatants were collected and used for determination of lipid hydroperoxides. Cocultures were subsequently washed, and fresh culture medium 199 alone was added and incubated for an additional 8 hours. At the end of incubation, the supernatants were collected, diluted 40-fold, and assayed for monocyte chemotactic activity. The number of migrated monocytes was determined microscopically and expressed as the mean±SD of 12 fields counted in quadruple wells. Statistical analyses used ANOVA, followed by *t* tests.

Apolipoprotein E–Null Mouse

Mice deficient in apolipoprotein E (apoE) develop severe atherosclerotic lesions with lipid-laden macrophages, morphologically similar to human arterial lesions.[10] Two experiments of similar design were performed. In a pilot study, 10 female apoE-null mice (Jackson Laboratory, Bar Harbor, Me) were assigned to chow diet (TD95138, Harland Teklad) or chow plus lutein (0.2% by weight) starting at 8 weeks of age. In a replication, 20 female apoE-null mice were assigned to the same 2 conditions.

Lesion Size

Mice were euthanized after 8 weeks (age 16 weeks), and hearts were harvested. Measurement procedures were adapted from a standard protocol[15] described previously.[16] In brief, cryosections 10 μm thick were stained with oil red O and hematoxylin, counterstained with fast green, and examined by light microscopy for the identification of atheromatous lesions. Lesion size was measured with a (20×20) 1.0-cm optical grid (2500 μm^2/grid under ×10 magnification). Lesion size was the number of squares counted over the 20 consecutive stained sections superior to the appearance of the aortic valve. The technician was blinded in the second experiment.

Lipid Hydroperoxides and Red Cell Lysis

Lipid hydroperoxides in plasma,[17] lipoprotein levels in plasma, and lysis of erythrocytes[18] were measured from pooled samples in the second experiment. Hemoglobin release due to red cell fragility and lysis was estimated after 30, 60, and 120 minutes of incubation at 37°C. Hemoglobin release was estimated from absorbance at optical density 540 (OD 540).

Chemotaxis assays using LDL from the apoE-null mice in the second experiment were performed by methods similar to those described above for in vitro lutein supplementation. The statistical significance of group differences in lesion size was assessed with a nonparametric statistic.[19]

LDL Receptor–Null Mouse

Procedures for the LDL receptor–null mouse experiments were identical to those for the apoE-null mouse, except for the age of the mice and the diet. Ten female mice, age 10 months, were randomized to Western diet (No. 88137, high cholesterol and fat, Harlan Teklad) or Western diet plus lutein (0.2%).

Protocols were approved by the Institutional Review Boards of the Keck School of Medicine and the UCLA School of Medicine.

Results

Epidemiology

From the cohort of 573 participants with baseline examinations, carotid IMT was available at 18-month follow-up for 480 (84%). Characteristics of the group are given in the Table. The time interval between examinations averaged 18.1±2.4 months. Plasma lutein was missing or was from a nonfasting blood draw for 10 participants, resulting in a sample size of 470 for age-adjusted analysis. Covariate information was missing for an additional 8 persons, yielding a sample of 462 for multivariate analysis. The only difference in study variables between persons missing and followed up that approached significance was age (missing were 1.0 year older, $P=0.06$).

The only study variables significantly related to plasma lutein were HDL cholesterol (standardized $\beta=0.17$), LDL cholesterol ($\beta=0.21$), and body mass index ($\beta=-0.14$) ($P<0.01$ for all). Plasma lutein was correlated with plasma β-carotene in women (rank-order $r=0.28$, $P<0.001$) and men

Characteristics of Participants in the Cohort Study of Carotid Atherosclerosis and Plasma Lutein: The Los Angeles Atherosclerosis Study

Variable	n	Percent	
Female sex	480	47	
Smoking status	480		
Current		26	
Former		29	
Ethnicity	480		
Hispanic		30	
Non-Hispanic white		55	
Black		5	
Asian		8	
Other		3	
Diabetes history	480	3	
Antihypertensive medication	480	17	
Lipid-lowering medication	480	6	
		Mean	SD
Age, y	480	49.9	4.7
IMT, 18-month change, mm	480	0.015	0.042
Lutein, plasma, μmol/L	477	0.28	0.12
β-Carotene, plasma, μmol/L	477	0.74	0.80
Cholesterol, serum, mmol/L	471	5.58	0.95
HDL-C, serum, mmol/L	471	1.47	0.36
Triglycerides, serum, mmol/L	471	1.91	1.32
Fasting time interval, h	472	13.2	2.3
BMI, kg/m^2	480	28.0	5.5
Systolic BP, mm Hg	480	128.3	14.5

HDL-C indicates HDL cholesterol; BMI, body mass index; and BP, blood pressure. Fasting time interval is for subjects fasting ≥8 hours before blood draw.

(r=0.20, P<0.001). Statistical partialing for serum lipids reduced these associations slightly (r=0.23 for women, r=0.19 for men).

The regression of change in IMT on plasma lutein yielded an age-adjusted slope ($\beta\pm$SEM) of -0.052 ± 0.019 (mm IMT/18 months)/(μmol/L lutein) (P=0.007). The estimated slope was not attenuated by covariate adjustment in the multivariate model ($\beta=-0.071\pm0.021$, P=0.0007). The covariate-adjusted relation between change in IMT and quintile of plasma lutein is depicted by sex in Figure 1. Note that the inverse relations are similar for women and men (P for interaction=0.89; P for trend=0.03 for women and 0.007 for men). Combining the sexes, progression (mean±SEM) of IMT in the highest quintile of lutein was close to null (0.004 ± 0.005 mm/18 months), whereas progression of IMT in the lowest lutein quintile (0.021 ± 0.005 mm/18 months) was increased (P=0.01). Adjustment for plasma β-carotene level did not attenuate the inverse relation between lutein and change in IMT (linear trend $\beta=-0.072\pm0.021$; P=0.0006). Further adjustment for intake of supplemental vitamin C, vitamin E, and multiple vitamins also did not attenuate this inverse association.

No statistically significant interactions were detected between sex, ethnicity, smoking status, use of antihypertensive medication, or use of cardiovascular medications and the relation of plasma lutein to progression of IMT. The inverse relation was statistically significant among nonsmokers (P=0.03) and smokers (P=0.005).

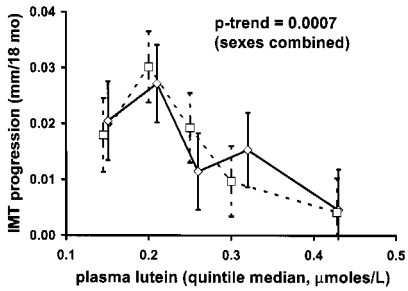

Figure 1. Inverse relation between change in carotid IMT and quintiles of plasma lutein. Graph depicts IMT means within lutein quintiles (median) for women (◇; n=214) and men (□; n=248) adjusted for cardiovascular risk factors (see Methods). Ranges of plasma lutein concentrations (μmol/L) for consecutive quintiles were 0.070 to 0.182, 0.184 to 0.240, 0.244 to 0.296, 0.297 to 0.360, and 0.367 to 0.805 for women and 0.019 to 0.180, 0.184 to 0.230, 0.231 to 0.279, 0.280 to 0.347, and 0.350 to 0.790 for men. Error bars indicate SEM. From the Los Angeles Atherosclerosis Study.

In contrast, the association between progression of IMT and plasma β-carotene was not significant (P for trend=0.15, multivariate model), and the point estimate was attenuated by inclusion of plasma lutein as a covariate (P for trend=0.22).

LDL Oxidation by Artery Wall Cells

Lutein was highly effective in a dose-dependent manner in reducing the attraction of monocytes in the coculture model of lipoprotein oxidation in the artery wall. Figure 2 depicts the results for the 2 types of chemotaxis assays. A dose-dependent reduction in chemotaxis for monocytes is apparent for increasing concentrations of lutein in each of the experiments. A dramatic inhibitory effect of lutein on chemotaxis occurs with pretreatment of the coculture cells. Note that lutein at 100 nmol/L inhibits monocyte migration at a level similar to that observed for human HDL. The measured

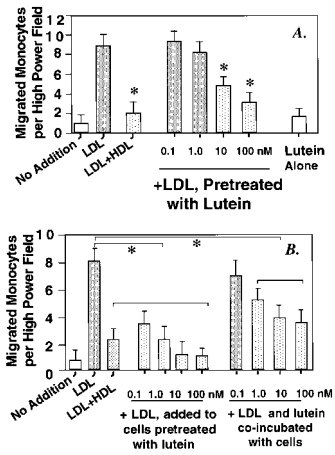

Figure 2. Effect of pretreatment with lutein on LDL oxidation and resulting monocyte chemotactic activity. A, LDL was incubated with lutein. Values are mean±SD for quadruple cocultures. B, Cocultures were preincubated with lutein. *P<0.05.

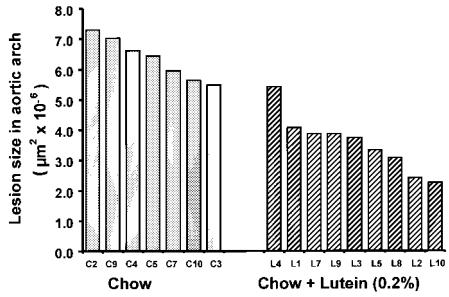

Figure 3. Aortic lesion size in ascending segment of aortic arch among apoE-null mice. Measurements were missing from 4 animals (3 chow, 1 lutein). Lesion size was reduced in lutein condition by 44% ($P=0.009$).

concentrations of lutein in plasma from the human cohort ranged from 20 to 930 nmol/L.

ApoE-Null Mouse

Mean body weight (±SD) was comparable at baseline between experimental groups of apoE-null mice in the pilot study ($P=0.33$) and in the second experiment (17.2±1.9 g for lutein and 17.4±2.0 g for controls, $P=0.81$). Increases in body weight from baseline to 8 weeks on trial were also similar across experimental conditions: 8.8±9.0% in the lutein-supplemented mice and 6.0±13.2% in control mice for the pilot ($P=0.86$); 4.2±11.3% versus 6.6±10.1%, respectively, for the second experiment ($P=0.64$). In the second experiment, plasma lutein (mean±SEM) was below our detection limit in the chow condition and 0.116±0.010 μmol/L in the lutein condition ($P<0.01$). Liver lutein concentrations were 0.0018±0.0011 and 0.0035±0.0022 μmol/g wet wt for the control and lutein-treated animals ($P=0.3$), respectively. These findings indicate that the lutein supplement was well tolerated and absorbed.

Measurements of atherosclerotic lesion size in the aortic arch were obtained from the 5 control animals and 4 of the 5 animals on the lutein-supplemented chow diet in the pilot study. Measurements at 8 weeks yielded an 86% reduction in average aortic lesion size in the lutein condition relative to controls (mean±SD, $0.6\pm0.7\times10^6$ versus $4.2\pm1.8\times10^6$ μm^2; $P=0.016$). In the second experiment, 2 mice were found dead, and slides from 2 other mice were not suitable for lesion quantification because of improper placement or movement of the heart in the cryomold before sectioning. In the replication, lesion size was reduced by 44% in the lutein condition ($5.5\pm1.5\times10^6$ versus $9.9\pm1.1\times10^6$ μm^2; $P=0.009$) (see Figure 3).

Lutein supplementation reduced the level of lipid hydroperoxides [13(s)HPODE] in plasma from apoE-null mice by 30% ($P<0.05$). Hemoglobin release (OD 540) due to in vitro red cell fragility was also significantly reduced ($P<0.05$) in the lutein condition. In addition, there was a significant 33% reduction in the levels of plasma VLDL+IDL in the group supplemented with lutein compared with the chow-fed mice ($P<0.001$). No significant changes were observed in plasma LDL or HDL levels.

Lutein supplementation resulted in a marked reduction in the lipid hydroperoxide formation when LDL from the 2 groups was incubated in cocultures (Figure 4A). In addition, monocyte chemotactic activity induced by LDL oxidation was markedly reduced when LDL from mice supplemented with lutein was incubated in the artery wall cocultures (Figure 4B).

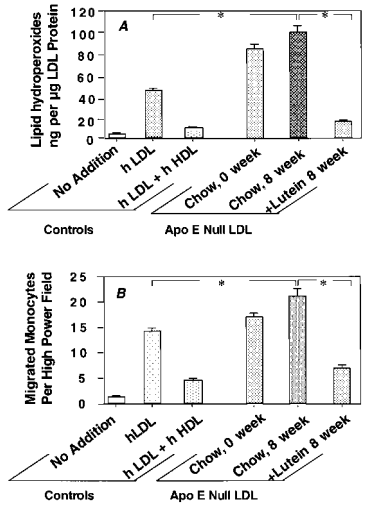

Figure 4. Effect of dietary supplementation with lutein on LDL oxidation by artery wall cocultures. Plasma was obtained from blood of apoE-null mice maintained on chow or chow supplemented with lutein (+Lutein). Samples consisted of those from 20 mice at week 0 (Chow, 0 week) or from 10 mice maintained on chow for 8 weeks (Chow, 8 week) or 10 mice fed lutein-supplemented chow for 8 weeks (+Lutein, 8 week). Monocyte chemotactic activity was determined (B) as described in Methods. Control values for background (No Addition), human LDL control (h LDL), and human LDL+human HDL control (h LDL+h HDL) are included. Values are mean±SD of quadruple samples assayed. *$P<0.05$.

LDL Receptor–Null Mouse Model

All 10 mice survived to completion of this experiment. Lesion size was reduced by 43% ($P=0.02$) in the lutein-supplemented condition ($22\pm10\times10^6$ μm^2) relative to controls ($38\pm5\times10^6$ μm^2).

Discussion

Results from 3 types of investigation were presented, and the findings from each were consistent with the hypothesis that increased intake of lutein was protective against the progress of early atherosclerosis. Progression of carotid IMT was increased substantially in the lower quintiles of plasma lutein, whereas progression was blocked in the highest lutein quintile (see Figure 1). Because the plasma level of lutein is affected by dietary intake of lutein,[20] the epidemiological finding of an inverse association between plasma lutein and progression of carotid IMT suggested that intake of lutein-rich foods has a protective effect on progression of early atherosclerosis. This finding motivated the coculture experiments in which lutein inhibited the inflammatory response of monocytes to LDL trapped in the artery wall. Pretreatment of the coculture cells with lutein at as low as 10 nmol/L reduced monocyte migration 8-fold.

These 2 findings were then followed by experiments in apoE-null mice and LDL receptor–null mice, in which addition of lutein to the diet resulted in marked reductions in

atherosclerotic lesion size in the aortic arch. The further finding in the apoE-null mice that indicators of oxidative stress (lipid hydroperoxides in plasma and red cell fragility) were reduced by the lutein supplement suggests that the protective effect of lutein was at least partially achieved via an antioxidant pathway. In addition, the LDL obtained from lutein-supplemented mice was markedly resistant to oxidation and induction of monocyte migration. The observation that lutein supplementation resulted in a 33% reduction in plasma VLDL+IDL points to additional pathways for potentially beneficial effects of lutein.

The importance of the observed epidemiological relation between progression of carotid IMT and plasma lutein levels follows from the strong association between IMT and risk of both coronary and cerebrovascular atherothrombotic events in men and women.[21] In addition, lipid-lowering therapy slows the progression of common carotid IMT.[22]

The importance of the coculture results stems from the findings in previous studies that this in vitro model shows a high correlation with lesion formation in animal models of atherosclerosis.[23] Also, the finding that lutein impacts monocyte recruitment at subnanomolar levels (Figure 2A) suggests that signal transduction with cellular receptors may mediate the antiatherogenic effects of lutein. Finally, the relevance of the in vivo findings follows from the similarity between lesions formed in these mice and human atherosclerosis and the substantial reduction achieved with lutein supplementation.

Epidemiological data concerning blood lutein levels and atherosclerotic disease are sparse. A nested case-control study with coronary heart disease incidence as end point[24] and a cross-sectional nested case-control study of IMT[25] found inverse relations with serum lutein (or lutein plus zeaxanthin[25]). Our present findings are from a prospective design, relating change in IMT to plasma lutein at baseline.

We know of no previous studies of lutein supplementation in an animal model of atherosclerosis. A recent study of β-carotene supplementation in the apoE-null mouse, however, did not find a significant effect on atherosclerosis in the aortic sinus.[26]

There are limitations to the interpretation of each of the findings reported here. Epidemiological associations are subject to possible confounding by unmeasured factors. For example, plasma lutein may be a marker for a healthy diet or lack of inflammation.[5] The coculture findings are limited by their in vitro nature, and animal models with high-dose, short-term exposure may not extrapolate to decades of low-dose exposure in humans. For example, the typical intake of lutein (+zeaxanthin) in the US diet has been estimated to range from 1000 to 14 000 μg/d (median 2800),[27] whereas the diet in the mouse experiments contained 5000 μg/d (3.5×10^1 versus 2.7×10^5 μg/kg body wt, respectively). This much higher dose in the mouse experiment achieved a plasma level of lutein (0.116 μmol/L) that falls into the bottom quintile of plasma levels in the human cohort (Figure 1). Nevertheless, the series of findings is more compelling, with the weaknesses of each model countered by results from other models. Replication of our findings and randomized trials involving dietary manipulation will be necessary to determine the impact of lutein-rich foods or supplements on human atherosclerosis and its sequelae.

Randomized trials of carotenoid supplementation have been reported only for the hydrocarbon carotenoid β-carotene, and results of these studies suggest null or adverse effects on both cancer and cardiovascular outcomes.[28–30] Given our results, an adverse effect of β-carotene supplementation is suggested by the finding of reduced lutein levels in plasma and tissues among supplementers.[31] Whole-food interventions could both raise plasma lutein levels[20] and avoid the adverse effects of single-compound supplementation. It is also plausible that supplementation with lutein would not yield the adverse effects observed in the β-carotene trials. Lutein is not a precursor of vitamin A; it has specific effects in enhancing immune function,[32] is more effective than β-carotene in preventing cell lipid oxidation[33] and oxidant-induced cell damage,[34] and is absorbed more efficiently.[35]

Our findings in epidemiological, in vitro, and in vivo investigations suggest that lutein may be a potent protective factor against the progression of atherosclerosis in humans and animals. Furthermore, the findings from the coculture and mouse models indicate that this antiatherogenic effect was achieved with lowering of VLDL and IDL, rather than LDL, and via pathways that involve reduced inflammation and oxidative stress in the artery wall.

Acknowledgments

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