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Distribution of –844 G/A and *Hind III* C/G *PAI-1* Polymorphisms and Plasma PAI-1 Levels in Mexican Subjects: Comparison of Frequencies Between Populations

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Several polymorphisms have been described in the *PAI-1* gene including the –844 G/A and *Hind III* C/G polymorphisms. These polymorphisms have been associated with different diseases such as preeclampsia and cardiovascular diseases. The allele and genotype frequencies of both *PAI-1* polymorphism where investigated in Mexican subjects and compared with other healthy worldwide populations. The hematological and biochemical parameters where classified according each genotype in our studied group. One hundred Mexican subjects were recruited. Demographic data and hematological and biochemical parameters were collected, and genomic DNA isolation was performed in all the participants. Screening of both polymorphisms studied was made by polymerase chain reaction and restriction analysis. Levels of plasminogen activator inhibitor-1 in plasma were measured by ELISARA plasminogen activator inhibitor antigen kit. The –844 and *Hind III* genotypes frequencies were as follows: 49% (G/G), 40% (G/A), 11% (A/A) and 50% (C/C), 44% (C/G), 6% (G/G), respectively. The wild-type genotypes (G/G and C/C) were significantly higher with respect to the compared populations. In addition, a significant increase of apolipoprotein A1 in the carriers of G/A –844 and C/G *Hind III* genotypes was observed. However, when the plasma plasminogen activator inhibitor levels were analyzed with respect to each genotype and haplotype, no significant differences were found.

Keywords: Plasminogen activator inhibitor 1; polymorphism; Mexican subjects

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Plasminogen activator inhibitor 1 (PAI-1) belongs to the family of serin protease inhibitors. PAI-1 is a linear glycoprotein that is composed of 379 amino acids and has a molecular weight of 48 kDa.[1,2] PAI-1 is a key inhibitor of the plasminogen activation system (PAS), which comprises an inactive proenzyme, plasminogen, that can be converted to the active enzyme, plasmin. The plasmin degrades fibrin into soluble products. Two physiologic plasminogen activators (PAs) have been identified: the tissue type PA (tPA) and the urokinase type PA (uPA). The tPA regulates plasminogen activation in the circulation, whereas uPA binds to a specific cellular receptor (uPAR), resulting in enhanced activation of

cell-bound plasminogen. The inhibition of the PAS may occur either at the level of the PA, by specific PAIs such as PAI-1, or at the level of plasmin, mainly by α2-antiplasmin.[3]

The *PAI-1* gene is located on chromosome 7 bands q21.3-q22, is approximately 12.2 kb in length, and consists of 9 exons and 8 introns.[4] Several polymorphisms have been described within the gene, including the –844 G/A and *Hind III* C/G polymorphisms. Both polymorphisms have been associated with various diseases including deep vein thrombosis, coronary artery disease (CAD), and preeclampsia.[5-8] Also, –844 G/A has been associated with lower triglycerides and higher high-density lipoprotein cholesterol in lean subjects. Variations of apolipoprotein A1 and apolipoprotein B according to genotypes were observed in CAD.[9,10] *Hind III* C/G polymorphism has been related to high levels of very low density lipoprotein cholesterol and insulin according to genotype in myocardial infarction patients.[11]

We designed a population-based study to find out the allele and genotype frequency of both *PAI-1* polymorphisms in healthy Mexican subjects compared with previously reported populations. In addition, in our population we established the association of –844 G/A and *Hind III* C/G *PAI-1* polymorphisms with hematological and biochemical parameters.

Materials and Methods

Healthy Subjects

We recruited 100 subjects without kinship who were residents from Guadalajara, Jalisco, Mexico, consecutively from February 2005 to August 2006. All subjects were of the Mexican Mestizo population between 18 and 67 years old, 78 females and 22 males. According to the National Institute of Anthropology, the definition of a Mexican Mestizo states that the person must have been born in the country, have a Spanish-derived last name, and have a family history of Mexican ancestors, at least back to the third generation.[12] Included were subjects who were older than 18 years and were clinically healthy. Individuals with evidence of infectious and chronic disease or with any treatment that could influence the biochemical and hematological test were excluded from the study. Informed written consent was obtained from all subjects before enrollment in the study. The investigation was performed according to the ethical guidelines of the 2004 Declaration of Helsinki and was approved by the ethical committee of the Department of Molecular Biology and Genomics from the University of Guadalajara.

Laboratory Assessment

Early morning venous blood samples were obtained after a 12-hour overnight fast from all study subjects. Serum total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c), very low density lipoprotein cholesterol (VLDL-c), triglycerides, and serum glucose were determined according to Synchron Clinical System LX20 (Beckman Coulter System, Fullerton, Calif). Apolipoprotein A (apo A-1), apolipoprotein B (apo B), and fibrinogen levels were measured according to a manufacturer's assay (Immage™ Immunochemistry (Beckman Coulter System). White blood cell, red blood cell, hemoglobin, and platelet counts (Cell-Dyn 3500R, Abbott Diagnostics, North Chicago, Ill) were assayed in all participants.

PCR-RFLP Screening of –844 G/A and *Hind III* C/G *PAI-1* Single Nucleotide Polymorphisms

The genomic DNA (gDNA) was isolated from peripheral blood leukocyte according to the salting out method.[13] The –844 G/A and *Hind III* C/G *PAI-1* single nucleotide polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Amplification of –844 *PAI-1* promoter region was done in a thermal cycler (Techne, TC-312, Cambridge, UK) using the following oligonucleotides: 5′CAGGCTCCCACTGATTCTAC3′ (Forward) and 5′GAGGGCTCTCTTGTGTCAAC3′ (Reverse).[14] PCR was carried out in a final volume of 25 μL containing 1 μg of gDNA, 20 μM of each oligonucleotide, 1.25 U/μL *Taq* DNA polymerase, supplied buffer enzyme 1X, $MgCl_2$ 2.5 mM, and 2.5 mM of each deoxynucleotide triphosphate (dNTP) (Invitrogen Life Technologies, Carlsbad, Calif). PCR reaction was performed by initial denaturation at 94°C for 3 minutes, 30 cycles of amplification at 94°C for 30 seconds for denaturation, 60°C for 30 seconds for annealing, and 72°C for 30 seconds for extension. Finally, 72°C for 1 minute was used for ending extension, resulting in a 510 base pair (bp) amplified fragment analyzed on a 2% agarose gel (Invitrogen Life Technologies) stained with ethidium bromide. Amplified fragments of –844 *PAI-1* polymorphism were digested for 1 hour at 37°C with

3 U of *Xho I* (New England Biolabs, Beverly, Mass) restriction enzyme. Afterward, restriction fragments were analyzed by electrophoresis on a 2% agarose gel (Invitrogen Life Technologies); it was stained with ethidium bromide. The G/G wild-type genotype was digested and appeared as 364 and 146 bp fragments, whereas the A/A polymorphic genotype (absence of the *Xho I* site) migrated as a 510 bp fragment.

The *Hind III* polymorphism was detected using the following oligonucleotides: 5′GCCTCCAGCTACCGTTATTGTACA3′ (Forward) and 5′CAGCCTAAACAACAGAGACCCC3′ (Reverse).[15] PCR was carried out in a final volume of 25 μL containing 1 μg of gDNA, 3 μM of each oligonucleotide, 1.25 U/μL *Taq* DNA polymerase, supplied buffer enzyme 1X, $MgCl_2$ 1.5 mM, and 2.5 mM of each dNTP (Invitrogen Life Technologies). PCR reaction was performed by initial denaturation at 94°C for 3 minutes, 30 cycles of amplification at 94°C for 30 seconds for denaturation, 60°C for 30 seconds for annealing, and 72°C for 30 seconds for extension. Finally, 72°C for 1 minute was used for ending extension, resulting in a 755 bp amplified fragment analyzed on a 2% agarose gel (Invitrogen Life Technologies) stained with ethidium bromide. Amplified fragments of *Hind III PAI-1* polymorphism were digested for 1 hour at 37°C with 5 U of *Hind III* (New England Biolabs) restriction enzyme. Afterward, restriction fragments were analyzed by electrophoresis on 2% agarose gel (Invitrogen Life Technologies); it was stained with ethidium bromide. The C/C wild-type genotype was digested and appeared as 567 and 188 bp fragments, whereas the G/G polymorphic genotype (absence of *Hind III* site) migrated as a 755 bp fragment.

Antigen PAI-1 Assay

PAI-1 levels were measured using plasma samples from healthy subjects (HS) by ELISARA (Hyphen Biomed, Neuville-sur-Oise, France). The detection threshold was 0.5 ng/mL or less. PAI-1 levels were calculated from a standard curve using the corresponding recombinant human PAI-1.

Study Design and Statistics

This was a comparative study. Allele and genotype frequencies were estimated by the gene counting method. Genotype distribution was tested for Hardy-Weinberg expectations, and it was pairwise compared with different healthy worldwide populations by chi-square test (MedCalc Statistical Software, StatPoint Inc, Herndon, Va). Lipid profile, hematological parameters, and plasma PAI-1 levels were compared between subjects with different *PAI-1* genotype by 1-way analysis of variance, according to the sample size. Analysis was performed using Statgraphics Centurion XV (StatPoint Inc, Herndon, Va) and SPSS version 10.0 software (SPSS Inc, Chicago, Ill). In each test, $P < .05$ was considered statistically significant.

Results

A total of 100 Mexican subjects (200 chromosomes) were studied. They were unrelated and apparently healthy. None of them received any medication. The age range was 18 to 67 years old and the median age was 38. The demographic and clinical characteristics are shown in Tables 1 and 2.

Counts and percentage of –844 G/A and *Hind III* C/G *PAI-1* polymorphisms are presented in Table 3. The genotype distribution (–844 and *Hind III*) in Mexican subjects was in Hardy-Weinberg expectation: $P > .05$. The wild-type genotypes frequencies of –844 and *Hind III* polymorphisms were GG (49%) and CC (50%). Both polymorphisms were significantly higher in Mexican subjects with respect to the 10 previous reports used for comparison (≥10 and ≤25.3), whereas the polymorphic homozygous genotype frequencies were A/A (11%) and GG (6%), the lowest frequencies in comparison with other worldwide healthy subjects studied (Table 3). A notable excess of heterozygosity was found. With respect to allele frequencies, the same relationship was observed.

Hematological and biochemical parameters were compared among Mexican subjects classified according the *PAI-1* polymorphism. In addition, we compared the parameters in each decade, but no significant differences were found. Significant increases of apo A1 in the carriers of G/A –844 and C/G *Hind III* genotypes (148 ± 27.5 mg/dL and 147 ± 27.5 mg/dL, respectively) were observed. However, no significant difference was found in the other parameters studied (Tables 1 and 2).

Plasma PAI-1 antigen was measured in all HS (Fig. 1). Higher levels for the wild-type genotype (GG 26.19 ng/mL; CC 26.33 ng/mL) and heterozygous genotype (G/A 24.69 ng/mL; C/G 23.12 ng/mL) than for A/A and G/G polymorphic genotypes were found. However, no significant differences were obtained. When we analyzed the combination of –844 and *Hind III PAI-1* polymorphisms with the

Table 1. Demographic Characteristics and Hematological and Biochemical Parameters in Healthy Mexican Subjects Classified According to –844 *PAI-1* Polymorphism

		–844 Genotypes		
Variable	Total (N = 100)	G/G (n = 49)	G/A (n = 40)	A/A (n = 11)
Sex, F/M	75/25	37/12	30/10	8/3
Age, y	38 ± 12	38 ± 11	38 ± 12	38 ±11
WBC, κ/μL	6.20 ± 1.5	6.38 ± 1.7	6.17 ± 1.7	6.09 ± 1.2
RBC, M/μL	4.95 ± 0.5	4.93 ± 0.5	4.97 ± 0.5	5.04 ± 0.5
HGB, g/dL	14.7 ± 1.5	14.6 ± 1.5	14.8 ± 1.6	14.9 ± 1.4
PLT, κ/μL	259 ± 65.7	258 ± 61.4	267 ± 67.1	241 ± 78.7
TG, mg/dL	134 ± 86.3	137 ± 85.4	141 ± 95.8	114 ± 59.8
TC, mg/dL	196 ± 41.1	188 ± 32.0	210 ± 47.9	199 ± 52.2
HDL-c, mg/dL	45 ± 13.1	43 ± 12.2	47 ± 11.9	48 ± 18.6
LDL-c, mg/dL	126 ± 34.3	118 ± 28.6	139 ± 40.9	132 ± 38.7
VLDL-c, mg/dL	27 ± 17.2	27 ± 17.1	28 ± 19.2	23 ± 11.9
apo A1, mg/dL[b]	139 ± 29.9	132 ± 30.7	148 ± 27.5[a]	143 ± 28.4
apo B, mg/dL	100 ± 30.5	93 ± 27.3	107 ± 33.8	99 ± 27.7
Glucose, mg/dL	84 ± 11.9	83 ± 11.3	86 ± 13.8	80 ± 4.7
Fibrinogen, mg/dL	404 ± 96.7	399 ± 100.9	414 ± 93.0	340 ± 69.0

NOTE: WBC = white blood cells; RBC = red blood cells; HGB = hemoglobin; PLT = platelet count; TG = triglycerides; TC = total cholesterol; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; VLDL-c = very low density lipoprotein cholesterol; apo = apolipoprotein.
[a]G/A *PAI-1* genotype vs G/G and A/A *PAI-1* genotypes.
[b]$P = .008$; no other variables significant.

Table 2. Demographic Characteristics and Hematological and Biochemical Parameters in Healthy Mexican Subjects Classified According to *Hind III PAI-1* Polymorphism

		Hind III Genotypes		
Variable	Total (N = 100)	C/C (n = 50)	C/G (n = 44)	G/G (n = 6)
Sex, F/M	75/25	38/12	34/10	3/3
Age, y	38 ± 12	37 ± 11	39 ± 12	39 ± 10
WBC, κ/μL	6.20 ± 1.5	6.22 ± 1.7	6.02 ± 1.3	7.37 ± 0.9
RBC, M/μL	4.95 ± 0.5	4.92 ± 0.4	4.93 ± 0.5	5.34 ± 0.5
HGB, g/dL	14.7 ± 1.5	14.5 ± 1.5	14.7 ± 1.5	15.9 ± 1.1
PLT, κ/μL	259 ± 65.7	258 ± 67.3	261 ± 67.4	259 ± 43.9
TG, mg/dL	134 ± 86.3	136 ± 87.2	132 ± 86.1	155 ± 110.5
TC, mg/dL	196 ± 41.1	188 ± 37.4	205 ± 43.8	210 ± 50.2
HDL-c, mg/dL	45 ± 13.1	43 ± 12.3	47 ± 13.9	43 ± 10.1
LDL-c, mg/dL	126 ± 34.3	119 ± 31.0	135 ± 36.4	135 ± 41.1
VLDL-c, mg/dL	27 ± 17.2	27 ± 17.4	26 ± 17.2	31 ± 22.1
apo A1, mg/dL[b]	139 ± 29.9	134 ± 31.8	147 ± 27.5[a]	129 ± 19.4
apo B, mg/dL	100 ± 30.5	95 ± 30.4	105 ± 30.9	101 ± 28.5
Glucose, mg/dL	84 ± 11.9	83 ± 12.3	84 ± 11.8	88 ± 10.4
Fibrinogen, mg/dL	404 ± 96.7	401 ± 98.7	407 ± 95.4	330 ± 65.9

NOTE: WBC = white blood cells; RBC = red blood cells; HGB = hemoglobin; PLT = platelet count; TG = triglycerides; TC = total cholesterol; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; VLDL-c = very low density lipoprotein cholesterol; apo = apolipoprotein.
[a]C/G *PAI-1* genotype vs C/C and G/G *PAI-1* genotypes.
[b]$P = .025$; no other variables significant.

plasma PAI-1 levels, no significant differences were found. Nevertheless, the most frequent haplotypes were GG/CC (21.66 ng/mL) and GA/CG (18.41 ng/mL).

Discussion

This is a first study in Mexican subjects to investigate the genetic variability of the –844 and *Hind III*

Table 3. –844 and *Hind III PAI-1* Polymorphisms Frequencies in Healthy Mexican Subjects and Comparison With Healthy Populations Previously Reported

		–844 Genotypes				Alleles		
Country (Reference No.)	N	G/G, % (n)	G/A, % (n)	A/A, % (n)	*P* Value	G, % (n)	A, % (n)	*P* Value
Mexico	100	49 (49)	40 (40)	11 (11)	[a]	69 (138)	31 (62)	[a]
Europe[5]	50	10 (5)	62 (31)	28 (14)	0.0001	41 (41)	59 (59)	.0001
France[14]	180	22 (40)	43 (77)	35 (63)	0.0001	43.6 (157)	56.4 (203)	.0001
Northern Ireland[14]	65	21 (14)	51 (33)	28 (18)	0.0005	47 (61)	53 (69)	.0001
France[17]	859	17 (146)	49 (424)	34 (289)	0.0001	41.7 (190)	58.3 (266)	.0001
France[18]	74	23 (17)	53 (39)	24 (18)	0.001	49 (73)	51 (75)	.0003
China[19]	91	25.3 (23)	53.8 (49)	20.9 (19)	0.002	52 (95)	48 (87)	.001
Germany[10]	534	15 (80)	46 (245)	39 (209)	0.0001	38 (405)	62 (663)	.0001
Italy[8]	80	20 (16)	51 (41)	29 (23)	0.0001	46 (73)	54 (87)	.0001
		Hind III Genotypes				Alleles		
		C/C, % (n)	C/G, % (n)	G/G, % (n)		C, % (n)	G, % (n)	
Mexico	100	50 (50)	44 (44)	6 (6)	[a]	72 (144)	28 (56)	[a]
United States[6]	59	25 (15)	57 (34)	17 (10)	0.003	54.2 (64)	45.8 (54)	.002
United States[7]	47	51 (24)	32 (15)	17 (8)	NS	67 (63)	33 (31)	NS

NOTE: NS = not significant.
[a]Mexican population vs other worldwide populations.

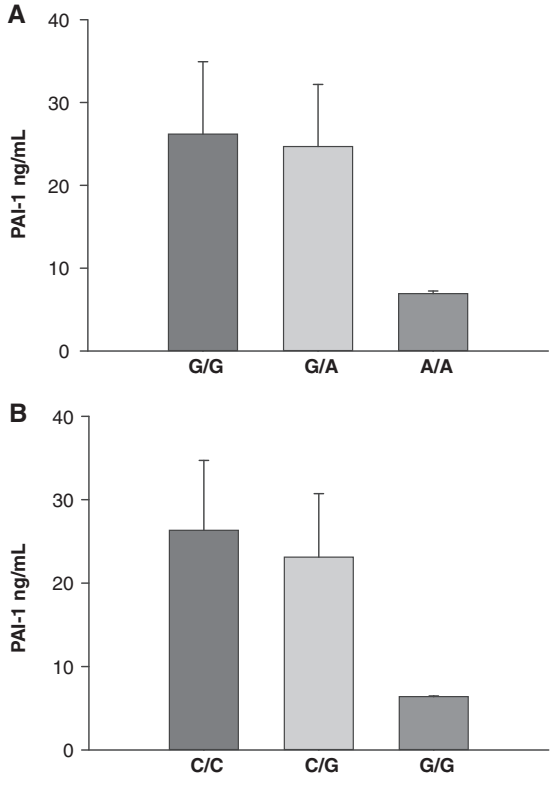

Figure 1. Plasma plasminogen activator inhibitor-1 (PAI-1) levels in Mexican healthy subjects. A, PAI-1 levels classified according to –844 *PAI-1* polymorphism. B, PAI-1 levels classified according to *Hind III PAI-1* polymorphism. No significant differences were found.

polymorphisms in the *PAI-1* gene. The comparison of hematological and biochemical parameters with *PAI-1* genotypes showed significant differences, with high apo A1 levels according to G/A and C/G *PAI-1* heterozygous genotypes, respectively, that have not been documented previously. The –844 *PAI-1* polymorphism has been associated with low triglycerides and increased fasting glucose concentration, insulin, and HDL-c in the Caucasian population.[9]

The –844 *PAI-1* polymorphism has been determined in some populations, with the highest frequencies for the G/G genotype in the Chinese population and for the A/A genotype in the German population. However, high frequency in G/G genotype and low frequency in A/A genotype carriers were found in Mexican subjects. In the other hand, just 1 previous report in healthy subjects looking for *Hind III PAI-1* polymorphism indicated a similar C/C genotype frequency in comparison with our results (51% vs 50%, respectively); however, the G/G genotype frequency was lower (17% vs 6%) (Table 3). According to our results, the *PAI-1* distribution that we found in Mexican subjects is a consequence of the admixture process between Amerindians and Spaniards after the Conquest when the Mestizo population had risen.[16] In addition, the City of Guadalajara, capital of Jalisco State, Mexico, is considered a Mexican Mestizos population resulting from an admixture of Spaniard, Indian, and black genes

Table 4. –844 and *Hind III PAI-1* Polymorphisms Frequencies in Several Diseases Previously Reported

		–844 Genotypes			Alleles	
Disease (Reference No.)	N	G/G, % (n)	G/A, % (n)	A/A, % (n)	G, % (n)	A, % (n)
DVT[5]	83	2.4 (2)	60.2 (50)	37.4 (31)	32.5 (54)	67.5 (112)
VT[18]	168	17 (28)	45 (76)	38 (64)	39 (132)	61 (204)[a]
MI[19]	87	27.6 (24)	56.3 (49)	16.1 (14)	56 (97)	44 (77)
CAD[10]	2710	15.6 (424)	48.7 (1319)	35.7 (967)	40 (2167)	60 (3253)
Preeclampsia[8]	52	10 (5)	48 (25)	42 (22)[a]	34 (35)	66 (69)
		Hind III Genotypes			Alleles	
		C/C, % (n)	C/G, % (n)	G/G, % (n)	C, % (n)	G, % (n)
CAD[6]	49	27 (13)[a]	43 (21)	31 (15)[a]	48 (47)	52 (51)
CAD[7]	22	77 (17)	9 (2)	14 (3)[a]	82 (36)	18 (8)

NOTE: DVT = deep vein thrombosis; VT = venous thrombosis; MI = myocardial infarction; CAD = coronary artery disease.
[a]Association with the disease.

that is similar to any other group from Mexico.[12] The contribution of Amerindian genes to the genetic profile of Mexican Mestizos is very strong, a reason that may explain the genetic and allele differences in our population in comparison with other populations worldwide. To confirm this genotype and allele *PAI-1* frequencies, it is necessary to study a larger population sample and/or more Mexican population samples including ethnic groups in the Jalisco State of Mexico.

Grubic et al[5] reported the lack of association between plasma PAI-1 level according to the –844 *PAI-1* genotype in HS; likewise, Dawson et al[11] demonstrated no association between *Hind III* genotypes and plasma PAI-1 levels. We found no significant differences in this study; nevertheless, in both *PAI-1* polymorphisms, the A/A (–844) and G/G (*Hind III*) polymorphic genotypes, we observed low plasma PAI-1 levels. This trend suggests that the plasma PAI-1 levels in the HS Mexican population can be dependent on each genotype; however, in the future it is necessary to design studies with samples big enough to permit investigators to identify more polymorphic genotypes.

The main importance of the *PAI-1* gene is its implication for the pathogenesis of several diseases. The A allele –844 polymorphism has been associated with vein thrombosis with factor V Leiden carriers[18] and with mild preeclampsia independent of thrombophilic mutations.[8] Moreover, the homozygote forms of the *Hind III PAI-1* polymorphism are associated with CAD.[6,7] Other studies of thrombotic events and cardiovascular diseases have been reported; however, no significant associations were found (Table 4). These associations are very important because these diseases have a high prevalence in Mexico. Pre-Hispanic populations supported the low incidence or absence of cardiovascular diseases, but interestingly at the beginning of the admixture process between Spaniards and Indian natives, heart diseases appeared.[20]

Conclusions

The frequencies of wild-type –844 and *Hind III* polymorphisms are higher in our subjects than in other populations worldwide, whereas the homozygous polymorphic genotypes are the lowest reported so far. Further studies are required to elucidate the relationship between apo A1 according to heterozygous genotype in these polymorphisms.

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