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GOLD SUPPRESSION OF HUMAN NEUTROPHIL FUNCTION *IN VITRO*

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Abstract—The effect of Myochrysine and Auranofin on leukocyte function were measured using quantitative leukocyte iodination. Both suppressed iodination at concentrations achieved in patients. Under conditions of leukocyte submaximum stimulation, enhanced gold suppression was observed. The active portion of Myochrysine appeared to be protein bound while the active portion of Auranofin appeared to be free. Preincubation experiments indicated suppression of the myeloperoxidase–halide system. Inhibition of this probable mediator of inflammation may be one of the modes of action of gold.

Recently, in an introduction at the International Workshop and Symposium on Gold in the Rheumatic Disease [1], Davis and Harth stated: "Despite extensive clinical and laboratory research there is still no clear, unified concept to account for the mode of action by which gold salts exert their clinical effects." This symposium reviewed research previously done on the proposed mechanisms of action of gold salts and suggested that gold salts may have both immunologic [2–10] and anti-inflammatory [11, 12] activities. Jessop *et al.* [12], using skin window techniques, have shown that the elevated phagocytic index of macrophages and neutrophils in patients with active rheumatoid arthritis was decreased to normal during treatment with gold salts. Whether the effect was a nonspecific result of the suppression of inflammation or a direct effect of gold on the phagocytic cells is not clear. Additional *in vivo* experiments in rats following a single 5 mg dose of gold sodium thiomalate resulted in suppression of carbon uptake by the rat neutrophils [13]. Other investigators have demonstrated gold salt inhibition of: lysosomal enzymes from peritoneal macrophages [14], cathepsin isolated from rheumatoid synovium [15], liver and rheumatoid synovial hydrolase [16, 17], phagocytosis and killing of bacteria [18], and inactivation of Cl [19, 20]. A new oral gold compound, Auranofin, has been shown to inhibit release of lysosomal enzymes from adjuvent arthritic rat peripheral blood leukocytes whereas gold thiomalate did not [21]. A recent study has shown suppression of some monocyte functions with a concentration of 25 μg/gold thiomalate in a 10% serum test mixture [22].

Most of the animal experiments used higher doses of gold that are used to treat patients. Many of the *in vitro* experiments contain very little serum protein in relation to the concentrations of unbound gold that are achieved during gold treatment of patients. The following experiments measured the effects of gold thiomalate and Auranofin on human neutrophil function utilizing quantitative leukocyte iodination and concentrations of protein bound gold that can be achieved clinically. This test system measures myeloperoxidase activity during phagocytosis, an important mechanism in microorganism and cell killing.

MATERIALS AND METHODS

The procedures described by Klebanoff *et al.* [23, 24] were modified as follows: venous blood from healthy laboratory personnel was collected into 20 ml tubes (Vacutainer No. 4787) previously injected with 2 ml of ACD solution (Grand Island Biological Co., Grand Island, NY). Ten milliliters of citrated blood was gently mixed with an equal volume of 2% dextran (average mol. wt 234,000, Sigma Chemical Co., St. Louis, MO) in 0.85% NaCl, and the red blood cells were sedimented at room temperature for 30 min in siliconized 16 × 150 mm glass test tubes. The leukocyte rich supernatant fracture was transferred to 50 ml siliconized glass centrifuge tubes and 35 ml of 0.86% NH_4Cl was added to lyse the remaining blood cells. The suspension was allowed to stand for 10 min at room temperature and the leukocytes were sedimented at 150 *g* for 10 min. The cell pellet was resuspended and washed in calcium-free Krebs–Ringer solution (KR), pH 7.4, collected by centrifugation, and adjusted to 5×10^7 neutrophils (PMN) per ml of KR.

Pooled normal serum (PNS) was collected from healthy laboratory volunteers, aliquoted, and stored at −20° until used. Zymosan particles were washed in distilled water, centrifuged, suspended in PNS, and incubated for 60 min at 37°. The opsonized zymosan (Z) was centrifuged, washed three times with KR, and resuspended in KR.

The test reaction mixture consisted of 5×10^6 PMN, 0.05 μCi $^{125}I^-$ (Cambridge Nuclear Radiopharmaceutical Corp., Cambridge, MA), with or without 5 nmoles carrier NaI, 36 μg glucose, 0.5 or 0.05 mg Z, 10% or 20% PNS, drug if used, and KR to 0.5 ml. Triplicate samples were incubated in 12 × 75 mm plastic tubes (Falcon 2054) on a "Roto Rack" (Fisher

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Scientific Co., Silver Spring, MD) at 20 rpm for 1 hr at 37°. The samples were centrifuged at 200 *g* at 4° for 35 min; 0.1 ml of 0.1 M sodium thiosulfate and 1 ml of 10% trichloroacetic acid (TCA) were added to each tube. The precipitate was collected by centrifugation at 1200 *g* for 5 min and washed three times with 1 ml of cold TCA.

The ^{125}I-labelled precipitates were counted in a Searle Analytic model 4227 Gamma well scintillation counter. Appropriate corrections were made by subtraction of cell-free control and the results were expressed as percent of radioiodide converted to a TCA precipitable by 5×10^6 PMN/hr.

Preincubation of 5×10^6 PMN, Z, glucose and PNS for 15 min was followed by the addition of iodide and drug used to differentiate between inhibitors of myeloperoxidase (MPO) and endocytosis [24].

Means were compared using unpaired, one-tailed Student's *t*-test as described by Steel and Torrie [25] and are expressed as means ± S.E.

Compounds used in these experiments were gold sodium thiomalate (Myochrysine, Merck, Sharp & Dohme, Rahway, NJ) and Auranofin (donated by Smith Kline & French Laboratories, Philadelphia, PA).

RESULTS

Effects of gold on leukocyte iodination. Leukocyte iodination in the absence of drug was 50.6 ± 0.6% of added ^{125}I. The two gold compounds suppressed leukocyte iodination in a dose-dependent fashion (Table 1). Gold thiomalate at concentrations of 1, 2, and 5 μg/ml of test mixture suppressed iodinations 10, 19.4, and 30.7% in tests containing 20% serum and 0.5 mg Z. This suppression was significant at $P < 0.01$. Concentrations of 0.2 and 0.4 μg/ml suggested suppression; however, it was not significant at $P < 0.05$. Auranofin at concentrations of 1, 2, and 5 μg/ml suppressed leukocyte iodination 3.9, 13.3 and 33.3% ($P < 0.01$).

Effects of carrier iodine addition. The addition of 5 nmoles carrier iodine to the control test resulted in 57.6 ± 1.6% of the added ^{125}I becoming protein incorporated. This is equivalent to 6.76 nmoles·10^7 leukocytes^{-1}·hr^{-1}. The addition of carrier iodine increased the suppression induced by both compounds. Gold thiomalate suppression increased from 10 to 13.7%, from 19.4 to 24.2%, and from 30.7 to 33.8% at gold concentrations of 1, 2, and 5 μg/ml (Table 1). However, the change was not significant at $P > 0.05$. Auranofin at the same concentrations of gold increased suppressions from 3.9 to 9.9%, from 13.3 to 27.6% and from 33.3 to 50.9%. This increased suppression was significant at $P < 0.01$.

Effects of increasing the ratio of free drug/protein bound drug. The effect of increasing the percent of drug that was non-protein bound while the total concentration was unchanged was studied by decreasing the amount of serum present to bind the drug. The concentration of serum was decreased from 20 to 10%. This resulted in slightly less leukocyte iodination, 48.6 ± 0.7% in the absence of carrier iodine. Gold thiomalate suppression did not increase (Table 1). This finding is consistent with previous studies that have shown that almost all gold thiomalate is protein bound at concentrations up to

Table 1. Gold suppression of leukocyte iodination*

	Suppression† (%)			
	Carrier-free		Carrier I^-	
Au (μg/ml)	20% PNS	10% PNS	20% PNS	10% PNS
Myochrysine				
0.2	0.2 ± 1.5 (8)	0.3 ± 1.6 (4)	0 (4)	4.8 ± 2.3 (3)
0.4	2.7 ± 1.9 (8)	1.4 ± 2.3 (4)	5.7 ± 1.4 (4)	7.7 ± 2.5‡ (3)
1.0	10.0 ± 2.6§ (7)	10.6 ± 2.2§ (4)	13.7 ± 3.1‡ (4)	16.5 ± 2.9§ (3)
2.0	19.4 ± 2.7§ (7)	15.2 ± 3.0§ (4)	24.2 ± 3.3§ (4)	20.8 ± 4.8§ (3)
5.0	30.7 ± 2.0§ (7)	21.6 ± 2.9§,‖ (4)	33.8 ± 1.3§ (4)	30.1 ± 2.5§ (3)
Auranofin				
0.2	0 (5)	0 (4)	0 (3)	1.3 ± 1.2 (2)
0.4	0 (5)	0 (4)	1.6 ± 1.8 (3)	3.8 ± 0.7‡ (2)
1.0	3.9 ± 1.7 (6)	7.4 ± 3.3‡ (5)	9.9 ± 2.4 (3)	27.2 ± 1.4‡,¶ (2)
2.0	13.3 ± 2.3§ (5)	20.5 ± 2.9§,¶ (4)	27.6 ± 3.1§ (3)	53.8 ± 0.8§,¶ (2)
5.0	33.3 ± 2.3§ (4)	55.1 ± 4.9§,¶ (3)	50.9 ± 5.1§ (3)	76.0 ± 2.5§,‖ (2)

* Column one is the amount of gold added per ml of test. Column two is % suppression from control in 20% serum and column three is % suppression from control in 10% serum solution in the absence of carrier iodine. Column four is % suppression from control in 20% serum and column five is % suppression in 10% serum with 5 nmoles carrier iodine added. The numbers in parentheses are the number of tests.

† Suppression $= 1 - \frac{\text{test with drug}}{\text{test without drug}} \times 100 \pm$ S.E.

‡ Significant suppression, $P < 0.05$.

§ Significant suppression, $P < 0.01$.

‖ Significant difference between column three and column two or column five and column four, $P < 0.05$.

¶ Significant difference between column three and column two or column five and column four, $P < 0.01$.

50 μg/ml serum, which is equivalent to 5 μg/ml in a 10% serum mixture [26–28]. In contrast, there was a marked increase in Auranofin suppression when the concentration of serum was reduced to 10%. Auranofin suppression increased significantly ($P < 0.05$) from 3.9 to 7.4%, from 13.3 to 20.5% and from 33.3 to 55.1% at concentrations of 1, 2, and 5 μg/ml. These results indicate that the active portion of Auranofin is non-protein bound.

The addition of 5 nmoles carrier iodine to the 10% serum test mixture increased control iodination to 62.5 ± 6% [3]. The presence of carrier iodine further increased Auranofin suppression from 7.4 to 27.7%, from 20.5 to 53.8%, and from 55.1 to 76.0% at gold concentrations of 1, 2, and 5 μg/ml ($P < 0.01$). The increased suppression noted with the addition of carrier iodine suggests either a pharmacological effect of the carrier iodine or more ideal experimental conditions to demonstrate suppression.

Effects of changing experimental conditions from maximum to submaximum stimulation. The data above were obtained under conditions of maximum leukocyte stimulation. However, *in vivo*, the leukocytes may not always be functioning under maximum stimulation, so conditions of submaximum stimulation were studied by decreasing the number of Z particles from 0.5 mg to 0.05 mg per test. This decreased leukocyte iodination to 16.1 ± 1.1% of added ^{125}I and 10.0 ± 0.1% in the presence of carrier iodine. Gold thiomalate under conditions of submaximum stimulation showed more suppression than under conditions of maximum stimulation (Table 2). Suppression was increased from 0.2 to 6.7% and 2.7 to 16.7% at gold concentrations of 0.2 to 0.4 μg/ml. This increase was significant ($P < 0.05$) compared to maximum stimulation. However, because of the day-to-day variability of control, these were not statistically different from control ($P > 0.05$). In each instance, the leukocytes with drug incorporated less ^{125}I than leukocytes without drug. Submaximum stimulation with gold thiomalate at concentrations of 1, 2, and 5 μg/ml increased suppression from 10 to 27.7%, from 19.4 to 39.1%, and from 30.7 to 50.9% ($P < 0.01$). The addition of carrier iodine masked this change so that there was no difference in gold thiomalate suppression between maximum and submaximum stimulation.

Auranofin caused similar effects. Under conditions of submaximum stimulation, suppression increased from 0 to 8% and from 0 to 16.6% at gold concentrations of 0.2 to 0.4 μg/ml. This increased suppression was significant ($P < 0.01$). Again, because of day-to-day variations of control, it was not different from control ($P > 0.05$). However, in every experiment, iodination was less when drug was present at these low concentrations. At higher Auranofin concentrations of 1, 2, and 5 μg/ml, submaximum stimulation revealed increased suppression from 3.9 to 31.7%, 13.3 to 46.7%, and 33.3 to 67.1% when compared to maximum stimulation ($P < 0.01$). The change from control was significant ($P < 0.01$). Addition of carrier iodine did not increase suppression; in fact less suppression was noted. However, the difference from control was significant ($P < 0.01$).

Preincubation. To separate the effect of gold com-

Table 2. Gold suppression of leukocyte iodination—submaximum stimulation*

	Suppression† (%)			
	Carrier-free		5 nmoles carrier I^-	
Au (μg/ml)	0.5 mg Z	0.05 mg Z	0.5 mg Z	0.05 mg Z
Myochrysine				
0.2	0.2 ± 1.5 (8)	6.7 ± 3.9‡ (3)	0 (4)	2.2 ± 6.7 (2)
0.4	2.7 ± 1.9 (8)	16.7 ± 3.5§ (4)	5.7 ± 1.4 (4)	7.6 ± 3.1 (2)
1.0	10.0 ± 2.6‖ (7)	27.7 ± 3.6§,‖ (4)	13.7 ± 3.1¶ (4)	15.4 ± 5.2¶ (2)
2.0	19.4 ± 2.7‖ (7)	39.1 ± 2.8§,‖ (4)	24.2 ± 3.3‖ (4)	24.6 ± 3.0‖ (2)
5.0	30.7 ± 2.0‖ (7)	50.9 ± 2.0§,‖ (3)	33.8 ± 1.3‖ (4)	32.9 ± 4.6‖ (2)
Auranofin				
0.2	0 (5)	8.0 ± 0.8§ (4)	0 (3)	0 (2)
0.4	0 (5)	16.6 ± 0.7§ (5)	1.6 ± 1.8 (3)	2.8 ± 1.7 (2)
1.0	3.9 ± 1.7 (6)	31.7 ± 0.6§,‖ (6)	9.9 ± 2.4 (3)	13.1 ± 2.9 (2)
2.0	13.3 ± 2.3‖ (5)	46.7 ± 1.6§,‖ (5)	27.6 ± 3.1‖ (3)	28.2 ± 2.1‖ (2)
5.0	33.3 ± 2.3‖ (4)	67.1 ± 2.1§,‖ (4)	50.9 ± 5.1‖ (3)	52.5 ± 3.7‖ (2)

* Results when the amount of Z was reduced from 0.5 mg to 0.05 mg per test. Column one is the amount of gold added per ml of test. Column two is % suppression with 0.5 mg Z and column three is % suppression with 0.05 mg Z in the absence of carrier iodine in 20% serum. Column four is % suppression with 0.5 mg Z and column five is % suppression with 0.5 mg Z in the presence of 5 nmoles carrier iodine in 20% serum. The numbers in parentheses are the number of tests.

† Suppression $= 1 - \frac{\text{test with drug}}{\text{test without drug}} \times 100 \pm \text{S.E.}$

‡ Significant difference between column three and column two or column five and column four, $P < 0.05$.

§ Significant difference between column three and column two or column five and column four, $P < 0.01$.

‖ Significant suppression, $P < 0.01$.

¶ Significant suppression, $P < 0.05$.

Table 3. Gold suppression of leukocyte iodination—preincubation*

	Suppression† (%)			
	Carrier-free		5 nmoles carrier I^-	
Au (μg/ml)	Control	Preincubation	Control	Preincubation
Myochrysine				
0.2	0.2 ± 1.5 (8)	0 (4)	0 (4)	0 (2)
0.4	2.7 ± 1.9 (8)	3.2 ± 1.6 (4)	5.7 ± 1.4 (4)	0‡ (2)
1.0	10.0 ± 2.6§ (7)	10.5 ± 1.2§ (4)	13.7 ± 3.1‖ (4)	1.6 ± 4.2‡ (2)
2.0	19.4 ± 2.7§ (7)	20.1 ± 4.9§ (4)	24.2 ± 3.3§ (4)	7.8 ± 7.7‡ (2)
5.0	30.7 ± 2.0§ (7)	27.5 ± 5.1§ (4)	33.8 ± 1.3§ (4)	14.3 ± 5.7¶ (2)
Auranofin				
0.2	0 (5)	0 (4)	0 (3)	0 (2)
0.4	0 (5)	0 (4)	1.6 ± 1.8 (3)	0 (2)
1.0	3.9 ± 1.7 (6)	1.1 ± 2.6 (4)	9.9 ± 2.4 (3)	0‡ (2)
2.0	13.3 ± 2.3§ (5)	3.1 ± 2.9‡ (3)	27.6 ± 3.1§ (3)	0‡ (2)
5.0	33.3 ± 2.3§ (4)	35.0 ± 4.6§ (3)	50.9 ± 5.1§ (3)	32.0 ± 2.2‡,‖ (2)

* Results when drug and ^{125}I were added after 15 min of preincubation of PMNs and Z. Column one is the amount of gold added per ml of test. Column two is % suppression when drug and ^{125}I were added at the start of incubation and column three is % suppression when drug and ^{125}I were added after 15 minutes of preincubation in the absence of carrier iodine. Column four is % suppression when drug and ^{125}I were added at the start of incubation and column five is % suppression when drug and ^{125}I were added after 15 min of preincubation in the presence of 5 nmoles carrier iodine. The numbers in parentheses are the number of tests.

† $\text{Suppression} = 1 - \frac{\text{test with drug}}{\text{test without drug}} \times 100 \pm \text{S.E.}$

‡ Significant difference between column three and column two or column five and column four, $P < 0.05$.

§ Significant difference between column three and column two or column five and column four, $P < 0.01$.

‖ Significant suppression, $P < 0.01$.

¶ Significant suppression, $P < 0.05$.

pounds on activation of the leukocyte from the effect of the MPO–halide enzyme system, the cell mixture was preincubated for 15 min before the drug and iodine were added. If the effect is on leukocyte activation, then no suppression would be expected after preincubation. If the effect is on the MPO–halide enzyme system, then the suppression should be the same with or without preincubation. The results obtained were different depending on the absence or presence of carrier iodine.

Preincubation tests without drug incorporated 52.0 ± 1.2% in the absence of carrier iodine and 52.2 ± 3.4% with carrier iodine. In the absence of carrier with gold thiomalate at concentrations of 1, 2, and 5 μg/ml, preincubation did not change suppression. Suppression was 10.0, 19.4 and 30.7% before preincubation and 10.5, 20.1 and 27.5 after preincubation (Table 3). There was no significant change. In the presence of iodine carrier, suppression at these same gold thiomalate concentrations changed from 13.7 to 1.6%, from 24.2 to 7.8% and from 33.8 to 14.3%. This change was significant. Preincubation in the presence of carrier iodine abolished all significant gold thiomalate induced suppression. Similar results were obtained with Auranofin. In the absence of carrier iodine and with gold concentrations of 1, 2, and 5 μg/ml, suppression went from 3.9 to 1.1%, from 13.3 to 3.1% and from 33.3 to 35.0% with preincubation. This change was significant only at the 2 μg concentration ($P < 0.05$). In the presence of carrier iodine, suppression with preincubation changed from 9.9 to 0%, from 27.6 to 0%, and from 50.0 to 32.0%. These changes were significant at $P < 0.05$. It is quite clear that the presence or absence of carrier iodine greatly influenced the results. In the absence of carrier iodine, no difference was demonstrable between preincubation and normal incubation, indicating a drug effect on the MPO–halide enzyme system. In the presence or carrier iodine, preincubation abolished most of the suppressive effect consistent with drug effect on leukocyte activation by Z.

DISCUSSION

All the information obtained in these experiments shows suppression of leukocyte iodination by gold salts. However, changes in the *in vitro* experimental conditions greatly influence the results observed. The variables studied included changes in concentration of drug, changes in the amount of serum present to bind the gold compounds, the presence

or absence of carrier iodine, preincubation of leukocytes with zymosan before the addition of radioactive iodine and drug, and, finally, the effect of changing the degree of leukocyte stimulation.

Under conditions of maximum stimulation in 20% serum, both gold compounds suppressed iodination at 2 μg/ml concentrations. Higher concentrations of gold resulted in increased suppression as expected. Since it is unknown whether leukocytes *in vivo* are always under maximum stimulation, studies were also done with less stimulation of leukocytes by decreasing the amount of Z in the test. This resulted in enhanced suppression of leukocyte iodination when compared to maximum stimulation. Even the lowest concentration of gold studied (0.2 μg/ml) induced suppression ($P < 0.05$). This concentration of gold is easily obtainable during treatment of patients and is equivalent to using 0.2 ml of a patient's serum containing 1 μg/ml gold in each test mixture.

Many previous *in vitro* experiments on the effect of gold salts on various biological reactions have ignored the fact that, *in vivo*, gold thiomalate is virtually 100% protein bound. At concentrations above 50 μg/ml serum, a measurable amount of gold is non-protein bound. This means that, if *in vitro* experiments contain less protein that serum, the concentration of gold needs to be proportionally decreased. If it is not, then free gold thiomalate will be present which most likely has a different effect than protein bound gold thiomalate.

The present studies using two different concentrations of serum in the test mixture did not show any different in effect. This is because even at the lower serum concentrations of 10% it is likely that all the gold thiomalate was protein bound. Five μg/ml as a 10% serum mixture is the same as using 0.1 ml of serum containing 50 μg/ml gold thiomalate in a 1-ml test. The results indicate that the active portion of gold thiomalate at these concentrations is protein bound. If it were not, then decreasing the serum should increase its effects.

In contrast, the binding of Auranofin to serum protein is unknown. Decreasing the amount of serum to bind Auranofin resulted in increased suppression. This suggests that the active drug is most likely the non-protein bound portion. Any *in vitro* experiments need to take this into account and either decrease the amount of gold proportional to the amount of serum protein present in the test system, or increase the amount of serum protein present. It is for this reason that the effects of gold on isolated myeloperoxidase are not reported in this paper. The test system is protein free and, although concentrations of gold as low as 0.1 μg/ml induced significant inhibition, the results cannot be interpreted.

The need of adding carrier iodine to the test systems is unclear. The ^{125}I is a marker of the MPO–H_2O_2 system rather than a reactant. The addition of carrier iodine resulted in an increased percentage of ^{125}I becoming protein bound. Under most of the experimental conditions studied, there was an increased apparent gold-induced suppression of leukocyte iodination with carrier iodine present. This suggests that the carrier iodine either by itself has a pharmacological effect, or created experimental conditions more ideal for demonstrating suppression.

The addition of carrier iodine changed results when added to tests involving preincubation of cells and Z before the addition of drug and ^{125}I. These tests were utilized to differentiate the effect of gold on Z activation of the leukocyte from the effect on the MPO–halide enzyme system. If the effect was only on the MPO–halide enzyme system, then the amount of suppression with and without preincubation should have been the same, because most of the leukocyte activation occurred during the first 15 min of incubation [24]. In the absence of carrier iodine, there was no significant difference between preincubation and no preincubation. This indicates the effect of gold on the MPO–halide enzyme system. The addition of carrier iodine resulted in quite different results. Preincubation markedly decreased or abolished the gold suppression of leukocyte iodination. This suggests that the combination of gold and carrier iodine effects Z stimulation of the leukocyte rather than acting directly on the MPO–halide enzyme system. Since these large amounts of iodine would not be found *in vivo*, it is likely that most of the *in vivo* effect of gold is on the MPO–halide enzyme system.

The clinical significance of gold-induced suppression of leukocyte iodination is unknown. The MPO–halide enzyme system is known to be toxic to bacteria, most likely involving the deamination and decarboxylation of amino acids in the presence of chloride ions to produce ammonia, carbon dioxide and aldehydes corresponding to the oxidised amino acids [29–31]. In addition to being toxic to bacteria, this system has been shown to be injurious to fungi, viruses, mycoplasma, sperm and tumor cells [32]. It is likely, therefore, to be a mediator of tissue injury in the inflammatory reactions. Inhibition of this H_2O_2 MPO–halide enzyme system may be one of the mechanisms of action of gold salts used for treatment of rheumatoid arthritis.

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