Protective Effect of Curcumin and Chlorophyllin against DNA Mutation Induced by Cyclophosphamide or Benzo[a]pyrene

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The current study was carried out to evaluate the potency of curcumin and chlorophyllin as natural antioxidants to reduce the oxidative stress markers induced by cyclophosphamide (CP) and benzo[a]pyrene [B(a)P] which were used as free radical inducers.

For this purpose, 126 male albino rats were used. The animals were assigned into 4 main groups: negative control group; oxidant-treated group (subdivided into two subgroups: cyclophosphamide-treated group and benzo[a]pyrene-treated group); curcumin-treated group; and chlorophyllin-treated group. Liver samples were collected after two days post the oxidant inoculation and at the end of the experimental period (10 weeks).

These samples were examined for determination of liver microsomal malondialdehyde (MDA), DNA fragmentation, restriction fragment length polymorphism (RFLP) and 8-hydroxy deoxyguanosine (8-OHdG) concentration.

Both CP and B(a)P caused increments in DNA fragmentation percentages, liver microsomal MDA, concentration of 8-OHdG and induced point mutation. Treatment of rats with either curcumin or chlorophyllin revealed lower DNA fragmentation percentages, liver microsomal MDA concentration, concentration of 8-OHdG and prevented induction of mutations, *i.e.*, reversed the oxidative stress induced by CP and B(a)P and proved that they were capable of protecting rats against the oxidative damage evoked by these oxidants.

Key words: Antioxidants, Mutation, DNA Fragmentation

Introduction

Reactive oxygen species (ROS) is a collective term that encompasses all highly reactive oxygen-containing molecules including free radicals. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function (Percival, 1998). The oxidative stress caused by free radicals is capable of causing damage to various cellular constituents such as DNA, proteins and lipids leading to carcinogenesis and aging and have been implicated in the pathogenesis of many diseases (Kasai, 1997).

Raising the intracellular free calcium due to oxidative stress might fragment the DNA by activation of Ca-dependent endonucleases in a mechanism resembling apoptosis (Farber, 1990). Single intraperitoneal (i.p.) administration of cyclophosphamide (CP) was previously found to cause destructive changes particularly within mitochondria and the smooth endoplasmic reticulum of hepatocytes. The changes were accompanied by an increase in the malondialdehyde (MDA) level in liver tissue homogenate (Das *et al.*, 2002). 8-Hydroxy deoxyguanosine (8-OHdG) was produced due to benzo[a]pyrene [B(a)P] exposure (Zhang *et al.*, 2004).

Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants that are capable of stabilizing or deactivating free radicals before they attack cells. The highly sophisticated and complex antioxidant protection system involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to eliminate free radicals. From exogenous antioxidants, numerous phytonutrients are present in a wide variety of plant food (Percival, 1998). Two good examples are curcumin found in *Curcuma longa* and chlorophyllin derived from chlorophyll. The former has been identified to reduce radiation-induced DNA damage in rat lymphocytes by modulating the SOS repair system (Thresiamma *et al.*, 1998). Chlorophyllin appears to be one of the most promising antimutagens because of its high response against a number of substances (Dashwood *et al.*, 1991).

Although intensive studies were made on anti-inflammatory, antioxidant, anticancer, antibacte-

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rial and antiprotozoal activities of curcumin and chlorophyllin, little is known about their roles in establishing the balance between DNA damage and its repair.

The aim of the present work is to get more approach towards the effects of the naturally occurring endogenous free radicals on the levels of the DNA damage parameters in normal control rats, and, in addition, to study the alterations in the levels of the DNA damage markers after injection of CP or B(a)P and to indicate how and to what extent the treatment with either curcumin or chlorophyllin can minimize the oxidative stress markers in the oxidant-treated rats.

Materials and Methods

Experimental design

A total number of 126, apparently healthy, male albino rats, aged 4 weeks, weighing 80–90 g, was obtained from the farm of experimental animals in Helwan belonging to Vaccine and Sera Authority (VACSERA). The animals were acclimatized for 2 weeks from starting the experiment. After the adaptation period the rats were randomly assigned into 4 main groups as follows:

I) Negative control group: This group comprised 14 rats that were kept on a basal diet only without any treatment.

II) Oxidant-treated groups: These groups were composed of 28 rats which were injected intraperitoneally (i.p.) at the sixth week from the beginning of the experimental period with a single dose of either CP or B(a)P. Thus, this group was subdivided into two equal subgroups namely IIa and IIb:

IIa) Cyclophosphamide-treated group: A dose of 40 mg of CP/kg body weight dissolved in distilled water was injected i.p. at the sixth week of the experimental period (0.2 ml/rat) (Shukla *et al.*, 2002).

IIb) Benzo[a]pyrene-treated group: At the sixth week, rats were given 50 mg of B(a)P/kg body weight dissolved in paraffin oil (0.2 ml/rat) (Madrigal-Bujaidar *et al.*, 1997).

III) Curcumin-treated groups: They were comprised of 42 rats and at the third week of the experimental period, rats were orally administered 100 mg of curcumin/kg body weight (0.2 ml/rat) suspended in phosphate buffer saline (Shukla *et al.*, 2002) three times weekly until the end of the experiment. The animals of this category were allocated to three equal subgroups, namely IIIa, IIIb, IIIc, of 14 rats each:

IIIa) Curcumin positive control group: It was kept only on the curcumin treatment.

IIIb) Curcumin- and cyclophosphamide-treated group: The animals were injected by a single dose of 40 mg of CP/kg body weight i.p. dissolved in distilled water at the sixth week (3 weeks post curcumin treatment) (Shukla *et al.*, 2002).

IIIc) Curcumin- and benzo[a]pyrene-treated group: At the sixth week (3 weeks post curcumin treatment), the curcumin-treated rats were given a single i.p. injection of 50 mg of B(a)P/kg body weight in paraffin oil (Madrigal-Bujaidar *et al.*, 1997).

IV) Chlorophyllin-treated group: At the third week of the experimental period, 42 rats were given 3 mg of chlorophyllin/kg body weight (0.2 ml/rat) dissolved in distilled water, three times weekly through an oral intubation until the tenth week (Madrigal-Bujaidar *et al.*, 1997). This group was categorized into 3 equal subgroups of 14 rats each, namely IVa, IVb and IVc:

IVa) Chlorophyllin positive control group: It was given chlorophyllin only throughout the whole experimental period.

IVb) Chlorophyllin- and cyclophosphamide-treated group: The rats were administered a single i.p. injection of 40 mg of CP/kg body weight dissolved in distilled water at the sixth week (3 weeks post chlorophyllin treatment) of the experimental period (Shukla *et al.*, 2002).

IVc) Chlorophyllin- and benzo[a]pyrene-treated group: At the sixth week (3 weeks post chlorophyllin treatment) of the experimental period, the animals were given a single i.p. injection of 50 mg of B(a)P/kg body weight dissolved in paraffin oil (Madrigal-Bujaidar *et al.*, 1997).

Sample collection

The animals were starved for 12 h, anaesthetized with diethyl ether, and then they were killed by cervical dislocation. The livers were rapidly removed and perfused with 50 to 100 ml of ice-cold 0.9% NaCl solution and then stored at −40 °C.

Samples (7 rats in each time) were collected twice at the sixth week (2 d after the oxidant treatment) and at the tenth week (the end of the study).

Analysis of liver tissue samples

DNA fragmentation assay

Diphenylamine incubated with the DNA fragments, present in the cell lysate, develops a blue colour that was quantified spectrophotometrically at 578 nm (Perandones *et al.*, 1993).

Determination of liver microsomal MDA

The liver microsomes were separated by the method described by Rizzo *et al.* (1994) and MDA was determined according to Albro *et al.* (1986). MDA is expressed as μmol/ml of microsomal preparation.

Isolation of hepatocytes genomic DNA

The isolation of the genomic DNA was done according to Maniatis *et al.* (1982).

Restriction fragments length polymorphism (RFLP)

The technique of RFLP was conducted according to Ausubel *et al.* (1987) using two types of endonucleases; Bam H1 and Eco R1.

Agarose gel electrophoresis

Agarose gel electrophoresis was done according to Maniatis *et al.* (1982).

HPLC quantitation of 8-OHdG

The DNA solution was applied to a HPLC column after enzymatic hydrolysis. 8-OHdG was eluted isocratically with a buffer and detected by a UV detector at 254 nm using 8-OHdG standard (Ide *et al.*, 1997). Concentration is expressed in ng/ml of prepared DNA hydrolysate. Statistical analysis of the obtained data was done according to Snedecor and Cochran (1980).

Results and Discussion

DNA fragmentation assay

In the present study, the cytotoxic effects of CP and B(a)P are shown by elevation of the DNA fragmentation percentage (Fig. 1). This effect of CP may be explained through induction of apoptosis after being metabolized in liver by P450 into phosphoramide mustard and acrolein (Lopez and Luderer, 2004) or by activating caspase-9 (Schwartz and Waxman, 2001). In a similar manner, B(a)P has been shown to induce apoptosis (Solhaug *et al.*, 2004).

Curcumin is a unique compound having both phenolic and β-diketone groups; therefore, it has free radical scavenging activities (Wargovich,

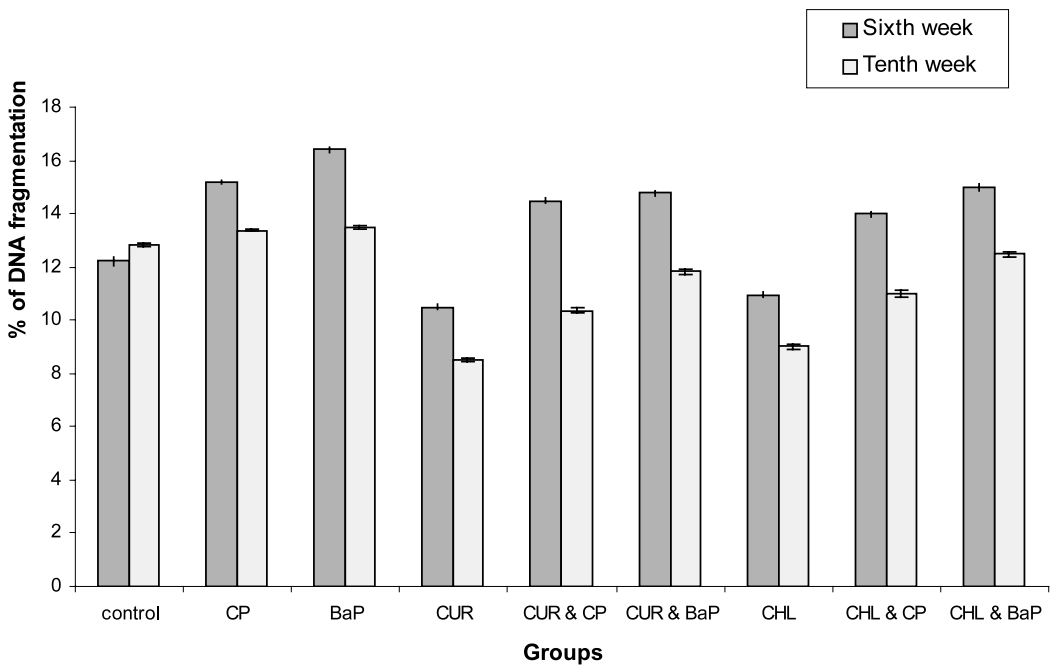

Fig. 1. DNA fragmentation percentage at the sixth and tenth weeks.

1997). This might explain the decrease in the fragmentation percentage in the curcumin positive control group. This scavenging activity of curcumin lead to attenuated DNA fragmentation due to the elevation of glutathione (Piwocka *et al.*, 2001).

Inoculation of CP following curcumin supplementation produced low DNA fragmentation percentage at the end of the experiment when compared to the CP-treated group. Such differences between the two effects can emerge the benefit of curcumin supplementation to minimize the risk of DNA fragmentation caused by CP inoculation. In this respect, Shukla *et al.* (2002) reported that curcumin was found to inhibit CP-induced mutagenic damage in a dose-response manner. Similarly, curcumin reduced DNA fragmentation caused by B(a)P at the 10th week of treatment. These data confirmed those obtained by Azuine and Bhide (1992) who demonstrated that curcumin had a chemopreventive effect on B(a)P-induced DNA adduct formation.

DNA fragmentation was decreased by chlorophyllin administration in both CP- and B(a)P-treated groups. These results coincide with that of Harttig and Bailey (1998) who stated that skin tumorgenesis in female rats induced by B(a)P was inhibited by chlorophyllin given by gavage. This action of chlorophyllin was explained by Botelho *et al.* (2004) who stated that chlorophyllin binds irreversibly to mutagens through hydrophobic interactions and thus inhibits nuclear fragmentation.

Liver microsomal MDA

CP and B(a)P produced a significant increase in the MDA concentration compared to the other treated groups (Fig. 2). These findings were found to be in accordance with those of Lahouel *et al.* (2004) who suggested that exposure to CP leads to 120% increase in MDA concentration. In addition, Garcon *et al.* (2001) stated that B(a)P caused oxidative stress which was seen by an approximately 2-fold increases in the MDA production.

The significant decreased level of MDA in the curcumin positive control group is in close agreement with those reported by Garcea *et al.* (2004) where curcumin was supposed to enhance the activities of antioxidative enzymes such as SOD and CAT.

Curcumin is shown to lower the MDA level in both CP- and B(a)P-treated groups at the end of the treatment. In a similar study, Huang *et al.* (1992) reported that curcumin lowered the MDA production thus inhibited tumorgenesis in skin induced by B(a)P.

Chlorophyllin is found to decrease the MDA level in both CP- and B(a)P-treated groups. These results were found to be closely related to the previous findings obtained by Kamat *et al.* (2000) who recorded that chlorophyllin was a highly effective antioxidant capable of protecting mitochondria against oxidative damage induced by various ROS.

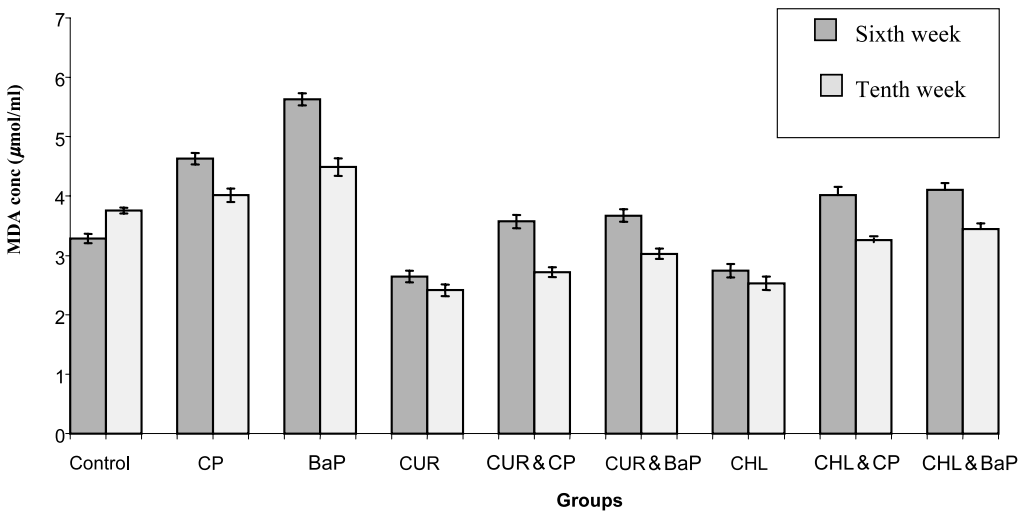

Fig. 2. MDA concentration in liver microsomes at the sixth and tenth weeks.

Restriction fragments length polymorphism (RFLP)

Restriction analysis of DNA using Bam H1 enzyme revealed that CP and B(a)P induced certain mutations at the recognition site of Bam H1 resulting in different patterns of DNA fragments at both 6th and 10th week (Fig. 3). At the same time, no mutation was found in Eco R1 site where digestion of rats DNA of all groups revealed the same pattern of DNA fragments (data not shown).

In the CP-treated group, only four DNA fragments resulted from Bam H1 digestion, whereas in B(a)P-treated rats, 6 DNA fragments were produced which are completely different from those of all other groups (Tables I, II). The effect of B(a)P on DNA polymorphism has been previously discussed (Wang *et al.*, 2003). This DNA polymorphism occurred in different genes as P53, K-ras and glutathione *S*-transferase (Mao *et al.*, 2004).

On the other hand, curcumin and chlorophyllin could prevent the effect of these oxidants resulting in a normal pattern of digestion as in the negative control group that gave five fragments. This role of the antioxidant activities of curcumin and chlorophyllin were shown to inhibit the mutagenic effect of CP or B(a)P (Azuine and Bhide, 1992; Botelho *et al.*, 2004).

HPLC estimation of 8-OHdG

8-OHdG is a major product of oxidized bases of damaged DNA with a clear mutagenic potential (Kasai, 1997). During bioactivation of CP, ROS are also formed, which can modify the components of both healthy and neoplastic cells leading to decreased antioxidative capacity (Stankiewicz *et al.*, 2002). In the light of this context CP caused an elevated level of 8-OHdG reaching to 260% and 137.2% of the negative control at the 6th and 10th weeks, respectively (Fig. 4). Several carcinogenic polycyclic aromatic hydrocarbons such as B(a)P are involved in the production of H_2O_2. This is closely associated with a marked increase in the 8-OHdG production. The mechanism is postulated to be due to the production of ROS such that there is a linear relationship between ROS production and 8-OHdG formation in cells exposed to B(a)P (Zhang *et al.*, 2004).

Curcumin can be considered as one of the potent antioxidants as it showed a decreased level of 8-OHdG (88% of control at the 6th week and 40% of control at the 10th week). This result is supported by the finding of Inano and Onoda (2002) that adding curcumin to diet before and/or after irradiation reduced the elevated 8-OHdG level by 50–70% of the control level.

Both curcumin- and CP-treated group and curcumin- and B(a)P-treated group showed a higher concentration of 8-OHdG relative to the negative control group at the 6th week. This value decreased at the end of the experimental period.

In consistence with this finding, it has been demonstrated that curcumin can inhibit B(a)P-induced DNA adduct formation, that can be caused by the higher content of 8-OHdG (Huang *et al.*, 1997), and also can inhibit CP-induced mutagenic damage in a dose-response manner (Shukla *et al.*, 2002).

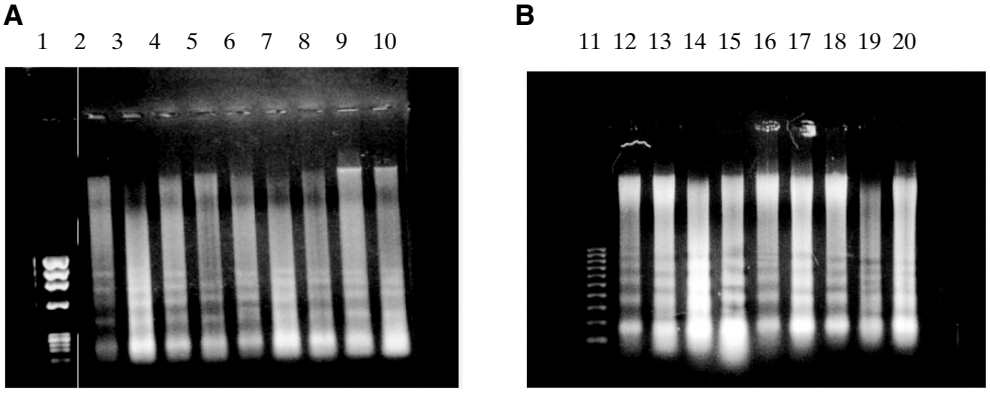

Fig. 3. The pattern of DNA digested by Bam H1 at (A) the 6th week and (B) the 10th week. Lanes 1, 11, DNA molecular weight markers; 2, 12, negative control groups; 3, 13, curcumin-treated groups; 4, 14, chlorophyllin-treated groups; 5, 15, CP-treated groups; 6, 16, B(a)P-treated groups; 7, 17, CUR- and CP-treated groups; 8, 18, CUR- and B(a)P-treated groups; 9, 19, CHL- and CP-treated groups; 10, 20, CHL- and B(a)P-treated groups.

Table I. The molecular weights of DNA fragments obtained by Bam H1 digestion at the sixth week.

DNA molecular weight marker*	Negative control group	CUR positive control group	CHL positive control group	CP-treated group	B(a)P-treated group	CUR- and CP-treated group	CUR- and B(a)P-treated group	CHL- and CP-treated group	CHL- and B(a)P-treated group
1353	1151	1151	1151	789	1095	1151	1151	1151	1151
1078	789	789	789	556	985	789	789	789	789
872	556	556	556	376	745	556	556	556	556
603	376	376	376	178	544	376	376	376	376
310	178	178	178		357	178	178	178	178
276					156				
234									
194									
118									
72									

* Molecular weight expressed in base pair (BP).

Table II. The molecular weights of DNA fragments obtained by Bam H1 digestion at the tenth week.

DNA molecular weight marker*	Negative control group	CUR positive control group	CHL positive control group	CP-treated group	B(a)P-treated group	CUR- and CP-treated group	CUR- and B(a)P-treated group	CHL- and CP-treated group	CHL- and B(a)P-treated group
1000	1151	1151	1151	789	1095	1151	1151	1151	1151
900	789	789	789	556	985	789	789	789	789
800	556	556	556	376	745	556	556	556	556
700	376	376	376	178	544	376	376	376	376
600	178	178	178		357	178	178	178	178
500					156				
400									
300									
200									
100									

* Molecular weight expressed in base pair (BP).

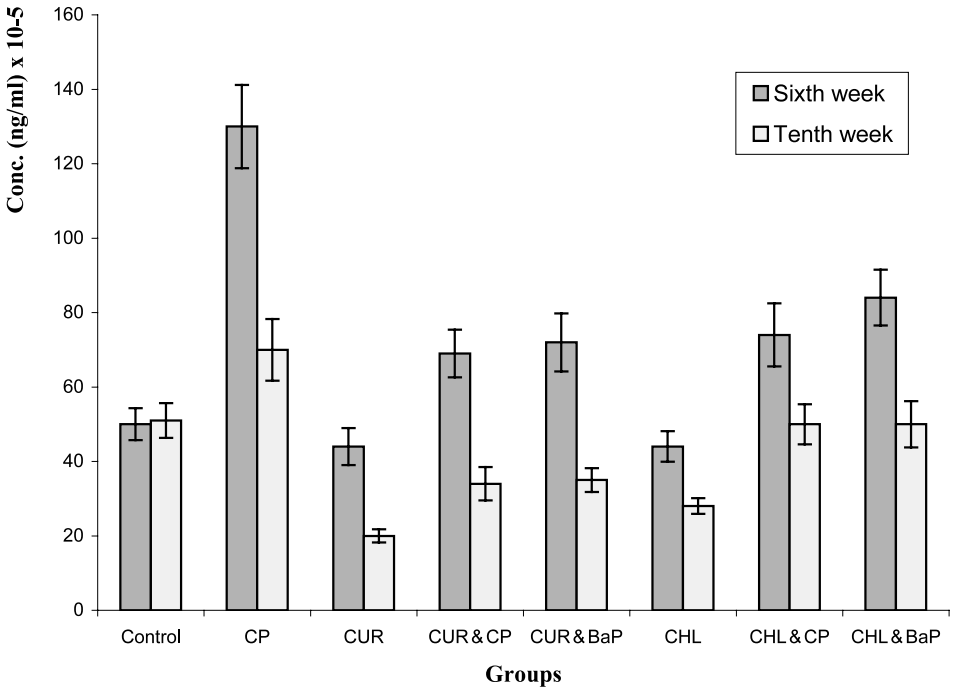

Fig. 4. Concentration of 8-OHdG in hepatitic DNA at the sixth and tenth weeks.

Chlorophyllin administration in both CP- and B(a)P-treated groups showed a higher concentration of 8-OHdG relative to the negative control group at the 6th week but a decreased concentration of 8-OHdG at the end of this experiment. It was also reported by Park *et al.* (2003) that DNA strand breaks by ROS and the formation of 8-OHdG in calf thymus DNA were inhibited markedly in a chlorophyllin concentration-dependent manner. Several mechanisms have been proposed to explain the antimutagenic activity of chlorophyllin including its antioxidant properties and its ability to form complexes with mutagens. These properties might include acceleration of the degradation of the ultimate carcinogen B(a)P to inactive tetrols, along with inhibition of carcinogen activation (Ardelt *et al.*, 2001).

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