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Effects of vitamin A on doxorubicin-induced chromosomal aberrations in bone marrow cells of rats

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The present study was carried out to evaluate the role of vitamin A (VA) on the induction of chromosomal aberrations (CA) in rat bone marrow cells and to investigate its modulating effect on chromosomal damage induced by doxorubicin (DXR). Wistar rats were treated with VA (7.5, 15 and 30 μg/kg body wt) once a day for 2 days by gavage before injecting DXR (90 mg/kg body wt). Rats in the control group were treated with corresponding doses of water and olive oil. Animals treated with the medium dose of VA (15 μg/kg body wt) plus single dose of DXR presented a statistically significant reduction in total number of CA and in number of abnormal metaphases ($P < 0.05$). However, when compared with control and DXR groups, the low and high VA doses (7.5 and 30 μg/kg body wt) were found to be less efficient than the medium dose VA (15 μg/kg body wt) in terms of parameters analyzed. Furthermore, the high dose of VA group (30 μg/kg body wt) was found to be clastogenic ($P < 0.05$). This study concludes that the protective effect of VA against chromosome damage is dose dependent.

Introduction

Doxorubicin (DXR) (also known as adriamycin) is an anthracycline antibiotic that has been used for more than 30 years for the treatment of a wide variety of cancers. It can be obtained from *Streptomyces peucetius* or totally chemical synthesis is also possible. Breast and esophageal carcinomas, Hodgkin's and non-Hodgkin's lymphomas, osteosarcoma, Kaposi's sarcoma and soft tissue sarcomas have good responses to DXR. In spite of extensive and long-standing clinical utilization, the mechanisms responsible for the antiproliferative and cytotoxic effects of DXR are still uncertain and have been subject to considerable controversy (Buschini *et al*., 2003).

Doxorubicin is an antineoplastic which is cell cycle specific for the S phase of cell division (Kusyk and Hsu, 1976). Several mechanisms seem to account for the effects of this anthracycline, both in terms of anticancer action and cardiac and other organ toxicities. Antineoplastic activity of DXR may be due to binding to DNA by intercalating between base pairs resulting in inhibition of synthesis of DNA and RNA by template disordering and steric obstruction (Chachoua *et al*., 1988). In addition, reduction of DXR by membrane bound reductase enzymes leads to generation of various free radicals such as quinones (Powis, 1989; Ramos *et al*., 2001). Topoisomerase II is likely to be one of the primary targets for the activity of anthracycline antibiotics. The induction by DXR of strand breaks in the DNA of L1210 leukemic cells was described more than 20 years ago (Quiles *et al*., 2002). Tewey *et al*. (1984) have shown topoisomerase II to be a target enzyme for DXR.

The capacity of DXR to inhibit DNA synthesis has been assumed to be a mechanism of action of DXR. This mechanism may be related to DNA intercalation or inhibition of DNA polymerase activity. It is possible that this effect is due to growth arrest signaling events and p53 function. Another mode of action of DXR through alterations in DNA is induction of enzymatically or chemically activated DNA adducts and DNA cross-linking. Interference with DNA strand separation and helicase activity has also been postulated as mechanisms of action for DXR (Quiles *et al*., 2002).

Two different routes of free radical formation by DXR have been described. The first implicates the formation of a semiquinone free radical by the action of several NADPH-dependent reductases that produces a one-electron reduction of the DXR to the corresponding DXR semiquinone. In the second route, DXR free radicals come from a non-enzymatic mechanism that involves reactions with iron (Quiles *et al*., 2002).

DXR can undergo futile redox cycling that results in the production of oxygen free radicals; these reactive oxygen species may than oxidize proteins, lipids and nucleic acids and potentially cause DNA strand scission (Tewey *et al*., 1984; Barber and Harris, 1994; Ramos *et al*., 2001). The cytotoxic action of DXR may be exerted by various mechanisms, such as DNA binding, free radical formation, membrane composition differentiation and function alteration. Therefore, previous data indicated that DXR induces mutations and chromosome aberrations in normal and tumor cells (Au and Hsu, 1980; Larramendy *et al*., 1980; Chachoua *et al*., 1988; Ciaccio *et al*., 1993, 1994; Amara-Mokrane *et al*., 1996; Antunes and Takahashi, 1998; Tavares *et al*., 1998; Antunes *et al*., 1999; Quiles *et al*., 2002).

Antioxidant defenses against these damages include some vitamins, such as ascorbate, tocopherol and carotenoids. It is an important goal to reduce the cytotoxic effects of DXR to normal cells. Recently, retinoids have been found to be antioxidant and free radical scavengers in many *in vivo* and *in vitro* mammalian studies (Collins, 2001; Quiles *et al*., 2002).

Vitamin A (VA) was the first fat-soluble vitamin to be discovered and described. It has essential roles in vision, bone and muscle growth, reproduction and maintenance of healthy epithelial tissue. VA is one of the most important nutrients essential for normal growth and differentiation. It induces cellular differentiation, protects cells from injury by free radicals, decreases the expression of certain oncogenes and inhibits the growth of breast carcinoma cells *in vitro* (Ciaccio *et al*., 1993, 1994; Tesoriere *et al*., 1994; Badr *et al*., 1998).

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Previous reports have shown that retinol induces chromosomal aberrations in human lymphocyte cultures (Badr *et al*., 1998) and DNA damage in HL-60 cells via superoxide radical generation (Murata and Kawanishi, 2000). Oxidative cellular damage induced by retinol seems to be related to iron metabolism and highly reactive hydroxyl radical generation (Dal-Pizzol *et al*., 2000, 2001). Single- and double-strand breaks are a direct consequence of the attack of hydroxyl radicals on deoxyribose in the DNA molecule (Klamt *et al*., 2003).

In this study, we investigated the anticlastogenic and clastogenic effects of VA on bone marrow cells of rats *in vivo* and its modulating effect on chromosomal damage induced by DXR in the same test system.

Materials and methods

Drugs

Doxorubicin (Adriblastina® produced by Carlo Erba) and retinol palmitate (Sigma; CAS no. 79-81-2) were purchased from local pharmacies and chemical companies.

Organization of experimental groups and treatment with drugs

Albino Wistar rats, aged 6 weeks and weighing ~150 g, were used. The animals were supplied from the Experimental Medical Research Unit, Faculty of Medicine, Kocaeli University. Ethical approval was given by the Ethics Committee of Kocaeli University School of Medicine. Experimental groups were organized as 6 groups which included 6 animals (3 males and 3 females) each. The same experimental schedule was used by Tavares *et al*. (1998) and Antunes and Takahashi (1998).

The dose of DXR in this study was selected as 90 mg/kg body wt. This dose was chosen because it induced an increase in the frequency of chromosomal aberrations which was essential for an investigation of the anticlastogenic potential of VA (Antunes and Takahashi, 1998). The full protective efficiency of an agent is elicited when the frequency of aberrations induced is high enough and the concentration of the protector given is sufficient (Chatterjee and Raman, 1993; Antunes and Takahashi, 1998).

The animals were treated with DXR by the i.p. route since this mode of administration permits a marked exposure of bone marrow cells to the agent tested (Preston *et al*., 1987). Although chemotherapy affects virtually every organ system of the body, the cell populations that typically exhibit rapid cell turnover, such as those of the bone marrow and gastrointestinal mucosa, are the most sensitive (Morelli *et al*., 1996).

Three VA doses (7.5, 15 and 30 μg/kg body wt) were administrated on the basis of literature data (Ciaccio *et al*., 1993, 1994; Badger *et al*., 1996, 1998; Gradelet *et al*., 1996; Stoppie *et al*., 2000; Yamamoto *et al*., 2000; Gupta *et al*., 2001).

The appropriate amount of DXR was adjusted 0.5 ml/100 g body wt in sterile water and injected i.p. VA doses were prepared in olive oil.

DXR was injected 24 h before killing the rats in the DXR, $VA_{7.5}$ + DXR, VA_{15} + DXR and VA_{30} + DXR groups. Animals in the $VA_{7.5}$, VA_{15}, VA_{30}, $VA_{7.5}$ + DXR, VA_{15} + DXR and VA_{30} + DXR groups were treated with VA (7.5, 15 and 30 μg/kg body wt) once a day for 2 days by gavage before DXR injection (Ciaccio *et al*., 1993, 1994; Antunes and Takahashi, 1998). Rats in the control group were treated with water and olive oil.

Preparation of the rat bone marrow cell system

Bone marrow cell preparations for the analysis of chromosomal aberrations were produced by the colchicine–hypotonic citrate technique. Potassium chloride (0.075 M) was used in this technique.

Colchicine (0.1%, 1 ml/100 g body wt) was injected i.p. 90 min before killing the animals. Animals were killed and then bone marrow cells were flushed from the femora with 0.075 M potassium chloride. Slides were prepared by an air drying procedure and stained with 5% Giemsa stain.

The chromosomal abnormalities in cells with 42 ± 1 chromosomes were used for analysis. The frequencies of chromosomal abnormalities were estimated in 100 metaphase plates/animal. The mitotic index was obtained by counting the number of mitotic cells in 1000 cells/animal. The chromosomal aberrations were classified according to Savage's classification (Savage, 1976).

Statistical analysis

Statistical analyses for the difference in the mean number of chromosomal aberrations and mean mitotic index between groups were obtained by one-way ANOVA test. Because variances between groups were unequal, the mean values of each group were compared by the Tamhane multiple comparison test. A P value of 0.05 was considered significant.

Gaps were counted but not included in the statistical analysis, since their cytogenetic significance is not well established (Antunes and Takahashi, 1998).

Results

According to Savage's classification (Savage, 1976), seven structural chromosomal aberrations were determined in the control and the experimental groups. All groups treated with DXR showed a high frequency of total chromosomal aberrations when compared with the control group ($P < 0.05$).

In all DXR-treated groups we found a high frequency of chromosomal aberrations (i.e. gaps, chromatid breaks, isochromatid breaks, exchanges, triradial figures, acentric fragments and translocations) when compared with the control group and the VA groups ($P < 0.05$) (Table I). In the DXR-treated groups the most frequent chromosomal aberration was chromatid breaks.

When the VA groups ($VA_{7.5}$, VA_{15} and VA_{30}) were compared with the control group in terms of mean total number of chromosomal aberrations, the $VA_{7.5}$ and VA_{15} groups displayed no significant differences ($P < 0.05$), whereas in the VA_{30} group the mean total number of chromosomal aberrations was significantly higher ($P < 0.05$). However, in the combination groups ($VA_{7.5}$ + DXR, VA_{15} + DXR and VA_{30} + DXR) there was a significant difference ($P < 0.05$) in the total number of chromosomal aberrations compared with the control group (Table I).

A comparison of the combined groups ($VA_{7.5}$ + DXR, VA_{15} + DXR and VA_{30} + DXR) with the DXR group revealed that $VA_{7.5}$ + DXR and VA_{30} + DXR administration produced no significant difference in terms of mean total number of chromosomal aberrations, whereas in the VA_{15} + DXR group aberrations were significantly lower. Low and high doses VA (7.5 and 30 μg/kg body wt) displayed less efficacy than the medium dose (15 μg/kg body wt) concerning protection from the effects of DXR on cells (Table I).

The rats treated with VA or with combinations of VA and DXR ($VA_{7.5}$ + DXR, VA_{15} + DXR and VA_{30} + DXR) presented no significant differences in mean mitotic index compared with the control animals (Table I). However, in the rats treated with DXR there was a statistically significant difference in the mean mitotic index compared with the control group (Table I).

In the combination groups none of the VA doses restored the total number of chromosomal aberrations induced by DXR to the level of the controls.

Discussion

DXR is one of the most popular anticancer drugs because it is effective against a broad spectrum of human neoplasms (Blum and Carter, 1974; Au and Hsu, 1980). Like most anticancer drugs, DXR produces undesirable complications in chemotherapy. The main genetic effect of DXR and related compounds is binding to DNA. It is known that DXR and other anthracyclines induce peroxide production in various tissues. Cellular enzymes are capable of converting DXR into free radical metabolites. DXR cytotoxicity may be mediated by free radicals derived from this drug (Quiles *et al*., 2002).

Some vitamins may protect from harmful oxygen species, free radicals on electrophiles, which damage DNA and other cell targets (Antunes and Takahashi, 1998; Quiles *et al*., 2002).

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Table I. Mitotic index and frequencies of chromosomal aberrations and abnormal metaphases observed in Wistar rat bone marrow cells after treatment with vitamin A (VA) and/or doxorubicin (DXR)

Group	MI (%)[b]	Chromosomal aberrations[a]							Total (ΣCA/rat)	AM (AM/rat)
		Gap[c] (gaps/rat)	CB (CB/rat)	ICB (ICB/rat)	E (E/rat)	T (T/rat)	AF (AF/rat)	TC (TC/rat)		
Control	3.67 ± 0.82	0.67 ± 0.82	1.33 ± 0.52	0.00	0.00	0.00	0.33 ± 0.52	0.00	1.67 ± 1.03	1.67 ± 1.03
$VA_{7.5}$	3.17 ± 0.98	0.50 ± 0.55	1.67 ± 0.82	0.00	0.00	0.00	1.00 ± 0.63	0.00	2.67 ± 1.03[e]	1.67 ± 0.52[e]
VA_{15}	3.67 ± 0.82[e]	0.67 ± 0.52	2.67 ± 1.03	0.00	0.00	0.00	1.00 ± 0.63	0.00	3.67 ± 1.21[e]	2.17 ± 0.98[e]
VA_{30}	3.17 ± 1.17	0.67 ± 0.52	3.67 ± 1.03	0.00	0.00	0.00	1.50 ± 1.05	0.00	5.17 ± 1.60[d,e]	2.33 ± 1.03[e]
DXR	1.33 ± 1.03[d]	1.83 ± 1.17	23.33 ± 5.24	2.17 ± 1.17	5.00 ± 2.10	0.83 ± 1.17	3.33 ± 1.21	0.33 ± 0.52	35.00 ± 9.76[d]	33.67 ± 9.29[d]
$VA_{7.5}$ + DXR	1.67 ± 1.03	1.83 ± 1.17	13.50 ± 4.14	0.00	1.33 ± 0.52	0.00	1.50 ± 0.84	0.17 ± 0.41	17.33 ± 5.99[d]	13.50 ± 2.88[d]
VA_{15} + DXR	2.00 ± 0.63	1.17 ± 0.41	7.83 ± 0.75	0.50 ± 0.55	2.17 ± 0.75	0.67 ± 0.82	0.83 ± 0.75	0.00	11.17 ± 1.17[d,e]	9.50 ± 1.22[d,e]
VA_{30} + DXR	1.33 ± 1.21	1.33 ± 0.52	18.50 ± 4.59	1.50 ± 0.55	3.67 ± 1.63	0.67 ± 0.82	1.83 ± 1.94	0.50±0.55	26.67 ± 5.57[d]	23.17 ± 5.46[d]

MI, mitotic index; CB, chromatid break; ICB, isochromatid break; E, complex exchange; T, triradial figure; AF, acentric fragment; TC, translocation chromosome; AM, abnormal metaphase.
[a]Frequency per 100 cells. Each chromosomal aberration has been counted by analyzing 100 cells/animal (6 animals/group, for a total of 600 cells/treatment) and means ± SD were calculated per treatment group.
[b]Mitotic index has been calculated by analyzing 1000 cells/animal (for a total of 6000 cells/treatment) and percentage of the mitotic cells calculated for each treatment group.
[c]Gaps were not included in total chromosomal aberration.
[d]Significantly different from control ($P < 0.05$).
[e]Significantly different from DXR ($P < 0.05$).

Some of the protection studies have shown beneficial effects of vitamins against DXR toxicity (Myers *et al*., 1976; Pascoe and Reed, 1987; Amara-Mokrane *et al*., 1996; Antunes and Takahashi, 1998). Some related food substances, such as β-carotene, a precursor of VA and vitamin C, are known to cause significant reductions in the incidence of induced chromosomal changes (Winkler, 1987; el-Nahas *et al*., 1993; Badr *et al*., 1998; Cabrera, 2000).

Like vitamins C and E, VA also acts as an antioxidant by decreasing peroxidation products and it has an adjuvant effect with antineoplastic drugs (Ciaccio *et al*., 1993, 1994; Cabrera, 2000). Other studies have shown that the administration of VA resulted in a protective effect against the chromosomal damage induced by radiation in the somatic and germ cells of rodents (Badr *et al*., 1998; Quiles *et al*., 2002).

Regarding lipid peroxidation reduction after DXR administration, retinol has offered good results in several tissues of the rat, such as liver microsomes, heart, brain membranes and kidney, but failed to protect in some experiments in the retina and the liver (Quiles *et al*., 2002). Ciaccio *et al*. (1994) also described a protective effect exerted by VA (2.5–15 μM as all-*trans*-retinol) against lipid peroxidation toxicity in K562 human erythroleukemia cells treated with DXR and, additionally, retinol increased cell death. Finally, Nakagawa *et al*. (1985) reported that retinol did not enhance the antitumor effects of DXR in mice bearing ascites sarcoma 180, but increased the effectivity of the anthracycline in mice with P388 leukemia.

A great amount of available data supports the view that carotenoids and VA do not induce genotoxic effects *per se*. Even in the absence of any genotoxic agents, these nutrients appear, on the contrary, to display some mechanisms which play protective roles against tumor promotion and progression (De Flora *et al*., 1999).

VA and β-carotene could be acting as scavengers of reactive oxygen species, inhibitors of activation of promutagens or photoprotectors (Cabrera, 2000). In a review, De Flora *et al*. (1999) noted that most of the data were consistent with the conclusion that the major mechanism involved in the antigenotoxicity of carotenoids and VA is interference with the metabolism of xenobiotics, by inhibition of the cytochromes P450 system. Odin (1997) concluded that the modes of activity of VA and β-carotene were not fully understood.

Although it is not a general rule, carotenoids and VA appear to share similar mechanisms of protection against the genotoxicity and related effects induced by physical agents, biological agents, chemical oxidants, direct-acting genotoxicants, genotoxicants requiring metabolic activation in bacterial or mammalian cells and complex mixtures (De Flora *et al*., 1999). Some other reports indicate that retinol induces chromosomal aberrations in human lymphocyte cultures (Badr *et al*., 1998) and DNA damage in HL-60 cells via superoxide radical generation (Murata and Kawanishi, 2000). Oxidative cellular damage induced by retinol seems to be related to iron metabolism and highly reactive hydroxyl radical generation. Single- and double-strand breaks are direct consequences of hydroxyl radical attacks on deoxyribose in the DNA molecule (Klamt *et al*., 2003). The latter observation is reinforced by the demonstration that DNA fragmentation induced by retinol in V79 cells, also displayed in the Comet assay, was reduced by co-treatment with trolox, an analog of vitamin E. Retinol supplementation has a genotoxic effect in various animal models, including increased mitotic recombi-

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nation in the SMART assay with *Drosophila melanogaster*. Retinol also induced DNA fragmentation in Chinese hamster lung fibroblasts in the Comet assay and single- and double-strand breaks in primary Wistar rat Sertoli cell cultures (Klamt *et al*., 2003).

Retinol induced increased levels of sister chromatid exchanges (SCEs) in experiments with the cultured fetal female Syrian hamster pulmonary epithelial cell line M3E3rC3. Previous authors observed an SCE-inducing ability of retinoids in cultured human diploid fibroblasts, and this result was confirmed by other experiments. Retinoids alone, and in synergistic combinations with melphalan and caffeine, raised the numbers of SCEs in cultured human lymphocytes. Retinol in small doses, especially with high concentrations of proteins in S9 mix, potentiated the mutagenicity of 2-aminofluorene, an indirect-acting mutagen, in the Ames test and also the mutagenicity of 2-acetylaminofluorene in this test. In one study it was observed that SCE formation by polycyclic aromatic hydrocarbons in cultured V79 cells in the presence of retinol was accelerated, although retinol itself was not genotoxic to these cells. This increased SCE formation has been reviewed by Odin (1997).

A number of authors established that the genotoxicity of retinoids was mediated by S9 liver microsomes and, more precisely, was dependent on the protein concentration in this system. α-Naphthoflavone, an inhibitor of P448-dependent monooxygenase, prevented the retinol-induced increase in SCE formation. It is highly probable that retinol itself undergoes non-specific oxidation due to the presence of double bonds in its structure. It can be oxidized as easily as unsaturated fatty acids and products of this process, such as retinol epoxides, peroxides and others, are responsible for the observed genotoxic effects. In cases of retinol co-genotoxicity additional effects are probably due to its oxidation products, independent of other mutagens (Au and Hsu, 1980; Odin, 1997; Antunes and Takahashi, 1998).

In this study, the protective effect of a 15 μg/kg body wt dose of VA and the clastogenic effect of a 30 μg/kg body wt dose of VA on rat bone marrow cells is evident from the elevation in different types of chromosomal aberrations. These results are in agreement with data obtained in other studies (Juhl *et al*., 1978; Miller *et al*., 1981; Dozi-Vassiliades *et al*., 1985; Qin *et al*., 1985; Mohr and Emura, 1991; Badr *et al*., 1998). VA was suggested to exert its clastogenic effect through activation of mixed function oxidase (MFO) (Tetzner *et al*., 1980; Badr *et al*., 1998). Other vitamins, like vitamins C and E, have been shown to have genotoxic effects under certain conditions (Shamberger *et al*., 1979; Gebhart *et al*., 1985; el-Nahas *et al*., 1993).

The clastogenic effect of DXR in rodent bone marrow cells is well documented (Larramendy *et al*., 1980; Dulout *et al*., 1981; Antunes and Takahashi, 1998, 1999). In the present study, the medium dose of VA (15 μg/kg body wt) decreased the number of DXR-induced structural chromosomal aberrations significantly. Badr *et al*. (1998) used γ-irradiation as a clastogenic agent on human lymphocyte cultures *in vivo* and VA doses that were relatively low (2 and 8 μg/ml). These resulted in decreases of 60 and 40% in the frequency of induced chromosomal aberrations, respectively. On the other hand, when a high dose of VA (24 μg/ml) was given to cultures exposed to 3.0 Gy γ-rays it produced appreciable changes in the frequency of chromosomal aberrations, particularly dicentrics, whereas total chromosomal aberration frequency was not different from that caused by radiation alone (Badr *et al*., 1998).

Our results, along with previous data (Badr *et al*., 1998; Klamt *et al*., 2003), suggest that there is a threshold effect for VA where no further protective effect is evident. On the contrary, a rise in dosage of VA in combination with the antineoplastic drug causes additive harm.

A possible explanation for this biphasic action of VA on radiation- and DXR-induced chromosomal damage may be the toxicity of VA at these relatively high doses (Badr *et al*., 1998). Stress resulting from radiation and drugs renders the cells more vulnerable to drugs or substances with known toxicity or mutagenicity (Badr *et al*., 1998). High doses of VA of increased toxicity may slow down the protective role and, thus, facilitate the oxidative deterioration of cell structure and function (Ohki *et al*., 1984; Badr *et al*., 1998).

The inhibitory effect of retinol on carcinogenesis has been suggested to be tissue specific and associated with its embryonic derivation. VA regulation of cellular differentiation involves mainly epithelia of ectodermal and endodermal origin, whereas it enhances expression of threshold phenotype of mesenchymal cells (Badr *et al*., 1998).

Other related studies (Ohki *et al*., 1984; Badr *et al*., 1998; Klamt *et al*., 2003), together with our study, raise more questions concerning the understanding of underlying mechanisms of the mutagenicity of VA and its role as a DNA protector as well as an anticarcinogen. There are, however, indications that VA plays a biphasic role depending on its concentration at the cellular level and this role may be time dependent (Badr *et al*., 1998).

In conclusion, the protective role of retinol is strongly correlated with a threshold dose, confirming the intricate behavior of this compound. VA significantly inhibited DXR-induced chromosomal aberrations at 15 μg/kg body wt ($P < 0.05$). In contrast, VA did not exert protective effects at 30 μg/kg body wt. Even this dose significantly increased chromosomal aberrations in the absence of DXR ($P < 0.05$). The uncontrolled supplementation of VA could be more dangerous when compared with other vitamins (Bronzetti *et al*., 2001). VA can provide effective protection against chromosomal damage induced by DXR. Further investigations are needed to elucidate the complex relationship between threshold dose and treatment duration for VA. Specific knowledge about the target tissue, plasma and serum concentrations of VA and carotenoids in specific diseases is essential before therapeutic interventions can be planned.

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