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Original Article

Serum Markers Variation Consistent with Autoschizis Induced by Ascorbic Acid–Menadione in Patients with Prostate Cancer

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Abstract

In vitro exposure of malignant prostate cell lines to ascorbic acid–menadione showed that tumor cells were killed through a mechanism named *autoschizis.* Experimental animal studies showed that autoschizis is also evident when ascorbic acid–menadione is administered to nude mice with implanted human prostate tumors. Prostate-specific antigen (PSA) is a known serum marker of prostate tumor cells specific activity. Recently, total serum homocysteine has been identified as a marker of tumor cell numbers with sensitivity for early detection of tumor cell death induced by treatments. A clinical trial with prostate cancer patients submitted to the association of ascorbic acid–menadione was performed and PSA/homocysteine was assessed in the follow-up. The early response in serum PSA and homocysteine levels was reported. The results showed that ascorbic acid–menadione produced an immediate drop in tumor cell numbers as assessed by homocysteine levels. Serum PSA levels showed an early rise and later dropped. These results suggest that autoschizis can also be induced by this pharmacological association at the clinical level in prostate cancer patients. Further studies are being performed in order to research if these results can be found with other primary tumors as it was shown in in vitro and experimental models. Ascorbic acid–menadione could be emerging as a new antitumoral chemotherapy.

Key Words: Menadione; ascorbic acid; chemotherapy; tumor cell death; prostate cancer.

Introduction

A high dose of ascorbic acid has an antitumoral effect in some individual cases of human solid tumors *(1),* but the statistical significance of these results has been controversial *(2).* Menadione has also inhibited the growth of human solid tumors in preclinical studies *(3–5)* and it was also reported that it has a clinical antitumoral effect acting as a radiosensitizer *(6).* When technological advances in plasma determination of menadione were available *(7),* the difficulty of achieving in patients the plasma concentration/time of in vitro cell exposure to menadione in order to reproduce the effect of this drug as a single antitumoral agent at the clinical level was evidenced *(8).*

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Tests in models of human tumor cell lines *(9–11),* animal transplantable tumors *(12,13),* and human tumors implants in nude mice *(14,15)* have shown that the association of ascorbic acid and menadione in a ratio of 100/1 induces antitumoral effects in a concentration 10 to 50 times lower than each agent acting alone. In the same conditions, an antitumoral synergist effect with radiotherapy and chemotherapy was also demonstrated *(12,13).* Scanning and transmission electron microscopies were employed to characterize this cytotoxic effects of ascorbic acid–menadione association. Following 1-h treatment of a human bladder carcinoma cell line (T24 line), the cells display membrane and mitochondrial defects as well as excision of cytoplasm fragments that contain no organelles. These continuous self-excisions reduce the cell size. Concomitantly, nuclear changes, chromatin disassembly, nucleolar condensation and fragmentation, and decreased nuclear volume lead to cell death via a process similar to karyorrhexis and karyolysis. Because this cell death is achieved through a progressive loss of cytoplasm because of self-morsellation, the authors reporting this study named this mode of cell death *autoschizis,* from the greek *autos,* self, and *schizein,* to split *(16,17).* Flow-cytometric studies have also identified autoschizis as an ultrastructural perturbation with the break of organoids *(18).*

Among the different primary human solid tumors submitted to ascorbic acid–menadione in the referred preclinical studies, prostate cancer cell lines and human prostate tumor implants have been well documented *(11,14,15).* It has been reported that in nude mice implanted with human prostate tumor, a week of oral ascorbic acid–menadione treatment induced a more significant antitumoral effect than the same drugs administered by injection *(15).* Therefore, clinical trials to assess the oral association ascorbic acid–menadione as antitumoral treatment in patients with prostate cancer have started.

As autoschizis is a mechanism of tumor cell death, the study of the ascorbic acid–menadione effect upon tumor cells in vivo in patients with prostate cancer required a marker of tumor cell death induced by antitumoral treatments. Serum level of the prostate-specific antigen (PSA) has been considered as a serum marker of most prostate cancers. However, the correlation with alive malignant cell mass can be difficult because the serum level can be modified, among other factors, by the synthetic activity of tumor cells and the content release from cells to serum during and after the tumor cell death. In this framework, it has been important the report that serum total homocysteine (HCY) is a good marker of the number of alive tumor cells, including human prostate tumor cell lines, and that it falls immediately after the death of tumor cell induced by antitumoral treatments. Therefore, in prostate cancer, serum measure of both markers, PSA and HCY, has been proposed as a very efficient procedure to obtain early information about the tumor cell death induced by treatments *(19).*

Patients and Methods

Treatment

Two 7-d courses of menadione at 50 mg/m^2/d and ascorbic acid at 5 g/m^2/d were administered orally. The 7-d courses of treatment began d 1 and d 22 of the study. Only two treatment courses were tested in this study because the purpose was to identify the immediate effect of this drug's association in a clinical model reproducing the previously proven immediate *autoschizis* elicited by them in preclinical models.

Assay for Homocysteine

The measure of total HCY in serum was performed weekly, with a previously reported high-performance liquid chromatographic (HPLC) procedure *(17).* The first measure was taken prior to starting the first course of treatment (d 1) and the last one on the d 42 of the study.

Assay for PSA

Total PSA was measured in the same blood specimens where homocysteine was measured, weekly, d 1 through d 42.

Patients

The study was conducted in 20 prostate cancer patients admitted in medical centers that referred medical data to the Cooperative Trials Center (CTC) of PharmaBlood, R&D Department (Florida, USA). A prospective and randomized trial was performed. Written informed consent was obtained from all

Table 1
Characteristics of the 20 Patients with Prostate Cancer Included in This Study

	Group 1	Group 2	Group 3	Group 4
Age! (yr), mean (range)	64 (56–72)	62 (55–73)	67 (58–70)	65 (56–70)
PSA mean pretreatment (μg/mL) (range)	16.6 (11.2–26.4)	14.3 (10.8–29.2)	17.2 (13.7–26.1)	16.2 (12.4–27.1)
Performance status (range)	1–2	1–2	2–2	2–2
Race (%), white	100	80	100	100
% Bone metastases	100	100	100	100
% Lymph node metastases	40	20	20	20
% Other sites metastases	0	0	0	0
Median (range) Hb (g/dL)	13.1 (11.4–14.2)	13.0 (12.2–14.0)	13.2 (11–14.1)	12.8 (11.4–13.6)
Median (range) creatinine (mg/dL) (0.70–1.52)	1.32 (0.74–1.67)	1.28 (0.82–1.44)	1.36 (0.78–1.62)	1.28

Note: The patients were randomized in four groups in order to be submitted to one of the tested treatments: group 1, ascorbic acid–menadione; group 2, menadione; and group 3, ascorbic acid. Group 4 is the control group receiving an appropriate placebo.

patients. The Institutional Ethical Committee approved the trial.

All patients fulfilled the following eligibility criteria: pathologically proven prostate cancer, advanced stages (M1), osseous metastasis; resistant to hormonotherapy, performance status (PS) according to the Eastern Cooperative Oncology Group (ECOG) ≤ 2; age ≤ 74 yr old; adequate hematological function (WBC count ≥ 4000/μL, neutrophils count ≥ 2000/μL, hemoglobin level ≥ 9.0 g/dL, platelet count ≥ 10×10^4/μL); renal function (serum creatinine ≤ 1.5 mg/dL, 24-h creatinine clearance ≤ 60 mL/min), hepatic function (total bilirubin ≤ 2.0 mg/dL, serum transaminases ≤ 2.0 × upper limit of normal range) and pulmonary function ($PaO_2 \geq 60$ torr. The patients were randomly distributed in four groups of five patients each. Group 1 was submitted to the described two courses of ascorbic acid–menadione treatment. In group 2, ascorbic acid was omitted. In group 3, menadione was omitted. A placebo was administered to patients of group 4. The homocysteine and PSA assays were performed in the four groups as was described.

Statistics

Three-way analysis of variance (ANOVA) was performed using Statsdirect statistical software. The two-way interactions were tested at the 0.005 level of significance.

Results

Table 1 shows that the four groups of patients were comparable in most relevant parameters. Figure 1 shows the results registered in the 42-d follow-up of this clinical trial. The two courses of treatment were administered the first and fourth week of the study. Serum homocysteine and PSA were measured the first day of each week (d 1, 8, 15, 22, 29, 36, and 42 of the study). In group 1, treated with ascorbic acid–menadione, the pretreatment serum level of HCY (d 1) fell in all posttreatment samples (d 8, 15, 22, 29, 36, and 42). Also in the group 1, the PSA serum levels rose in the two initial weeks (d 8 and 15) and, afterward, it fell at d 36 and 42. Variations that were not relevant were observed in homocysteine and PSA serum levels in groups 2 and 3, which were treated with a single agent—menadione and ascorbic acid, respectively.

Table 2 shows the statistical analysis of weekly measures in the three drug-treated groups comparative with the placebo (control) group (group 4). Group 1 showed a fall of HCY associated to ascorbic acid–menadione treatment, with high statistical significance ($p < 0.01$), in all weekly measures after starting the first series (d 8–42). Nonsignificant variation of HCY was evidenced for group 2 (menadione, single agent) and group 3 (acid ascorbic, single agent) comparative to group 4 (control placebo).

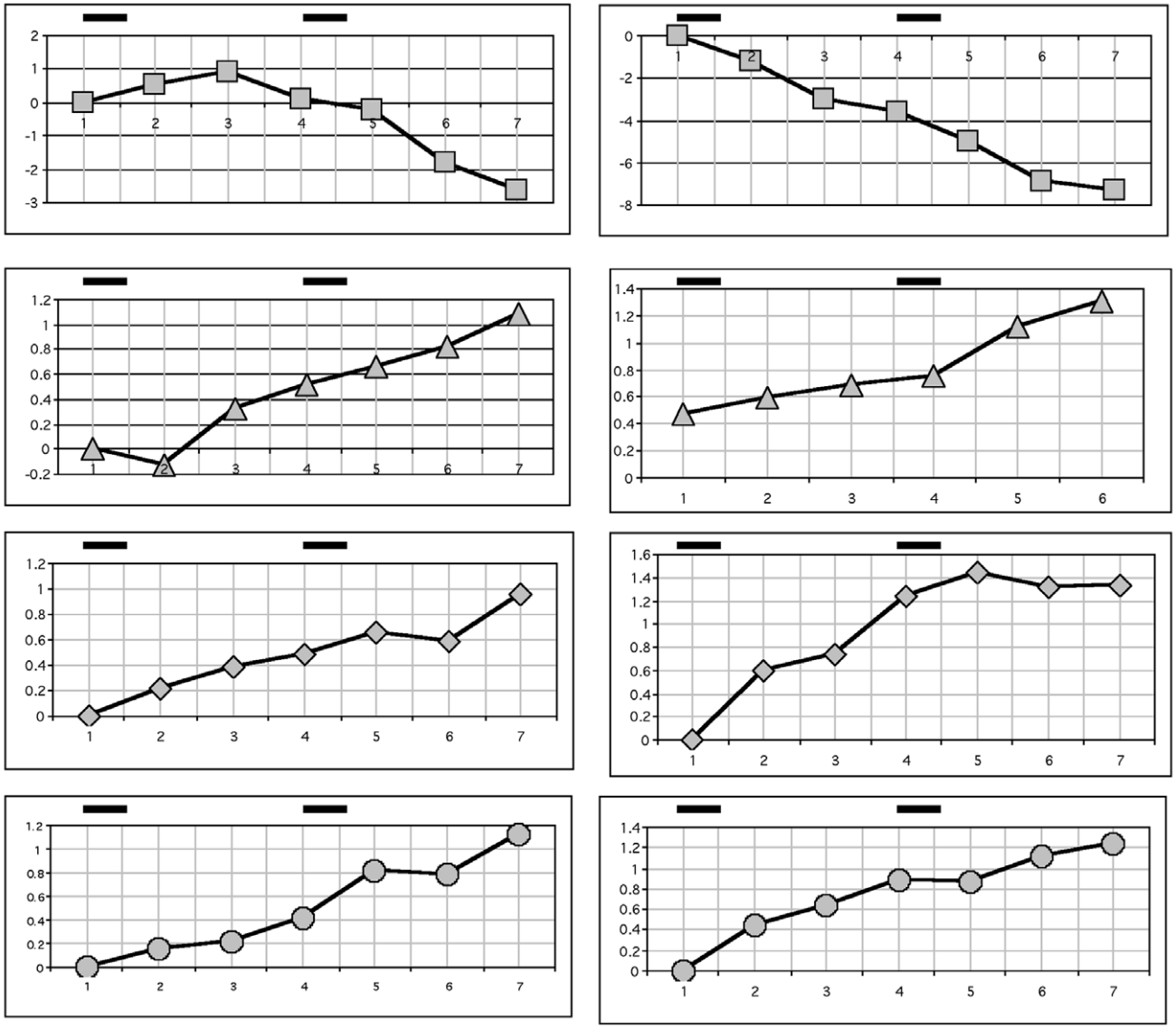

Fig. 1. **Left:** Variation of PSA serum level (μg/mL) (*y*-axis); **right:** Variation of HCY serum level (μ*M*/mL) (*y* axis). *(x)*-Axis: weeks of the study, marking the days of PSA and HCY measures. Assuming that the initial value (pretreatment measure) is 0, the PSA and HCY were measured each week during the 42 trial days. At d 1 and d 22, each one of the two series of 7-d treatment (—) included in this study was started (two-course treatment). In each one of these courses, group 1 was treated with ascorbic acid–menadione association; group 2 was treated only with menadione; group 3 was treated only with ascorbic acid; and group 4 received an appropriate placebo. The figure shows the mean PSA values and HCY values obtained from the five patients of each group, weekly. Groups: 1,Square- 2, Diamond-3,Triangle- 4, Circle

Table 2
Variation of Serum HCY after two treatment courses (d 1–8 and 22–28)

Assay day	Group 1 (mean ± SD) (95% CI)	Group 2 (mean ± SD) (95% CI)	Group 3 (mean ± SD) (95% CI)	Group 4 (mean ± SD) (95% CI)	Group 1 vs group 4	Group 2 vs group 4	Group 3 vs group 4
1	0	0	0	0	—	—	—
8	–1.2 ± 0.568 (–1.904/–0.496)	0.468 ± 0.362 (0.018/–0.918)	0.59 ± 0.508 (–0.040/1.220)	0.45 ± 0.335 (0.034/0.866)	$p = 0.0029$	$p = 0.9462$	$p = 0.5608$
15	–3 ± 1.712 (–5.126/–0.874)	0.59 ± 0.227 (0.310/0.874)	0.74 ± 0.349 (0.304/1.172)	0.64 ± 0.221 (0.365/0.915)	$p = 0.0057$	$p = 0.7936$	$p = 0.6608$
22	–3.6 ± 1.437 (–5.385/–1.815)	0.68 ± 0.210 (0.419/0.941)	1.24 ± 0.448 (0.684/1.796)	0.88 ± 0.316 (0.487/1.273)	$p = 0.0012$	$p = 0.2133$	$p = 0.2703$
29	–5.0 ± 1.595 (–6.980/–3.020)	0.76 ± 0.341 (0.337/1.183)	1.45 ± 0.498 (0.832/2.068)	0.86 ± 0.298 (0.490/1.230)	$p = 0.0016$	$p = 0.4260$	$p = 0.0519$
36	–6.9 ± 1.799 (–9.134/–4.666)	1.12 ± 0.400 (0.624/1.616)	1.32 ± 0.448 (0.763/1.877)	1.12 ± 0.273 (0.781/1.459)	$p = 0.0009$	$p > 0.9999$	$p = 0.1634$
42	–7.3 ± 1.394 (–9.030/–5.570)	1.3 ± 0.432 (0.763/1.837)	1.34 ± 0.486 (0.736/1.944)	1.24 ± 0.365 (0.786/1.694)	$p = 0.0001$	$p = 0.8405$	$p = 0.6909$

Note: Twenty prostate cancer patients, all of them resistant to hormonotherapy, with bone metastases were randomized in four groups of five patients each. In group 1, the treatment course was ascorbic acid–menadione; in group 2, it was menadione; and in group 3, it was ascorbic acid. Group 4 received a placebo. Assuming the initial value (pretreatment measure) is 0, HCY variation was measured each week during the 42-d trial period (assay days). The mean ± standard deviation and 95% confidence interval (95% CI) of each group is shown. The statistical significance of mean variation in treated groups comparative with the control group (p-value) for each assay day, calculated by ANOVA, of is also shown.

Table 3
Variation of serum PSA after two treatment courses (d 1–8 and 22–28)

Assay day	Group 1 (mean ± SD) (95% CI)	Group 2 (mean ± SD) (95% CI)	Group 3 (mean ± SD) (95% CI)	Group 4 (mean ± SD) (95% CI)	Group 1 vs group 4	Group 2 vs group 4	Group 3 vs group 4
1	0	0	0	0	—	—	—
8	0.54 ± 0.286 (0.185/0.895)	–0.12 ± 0.275 (–0.462/0.223)	0.21 ± 0.244 (–0.093/0.513)	0.16 ± 0.157 (–0.035/0.355)	$p = 0.0799$	$p = 0.0200$	$p = 0.7629$
15	0.94 ± 0.266 (0.610/1.270)	0.32 ± 0.131 (0.157/0.483)	0.38 ± 0.191 (0.143/0.617)	0.21 ± 0.223 (–0.067/0.487)	$p = 0.0024$	$p = 0.3893$	$p = 0.1923$
22	0.07 ± 0.193 (–0.170/0.310)	0.52 ± 0.290 (0.160/0.880)	0.48 ± 0.186 (0.249/0.711)	0.42 ± 0.199 (0.173/0.667)	$p = 0.0022$	$p = 0.6075$	$p = 0.5466$
29	–0.24 ± 0.237 (–0.534/0.054)	0.66 ± 0.369 (0.201/1.119)	0.66 ± 0.241 (0.361/0.959)	0.81 ± 0.260 (0.488/1.132)	$p<0.0001$	$p = 0.4438$	$p = 0.4931$
36	–1.8 ± 0.380 (–2.272/–1.328)	0.82 ± 0.301 (0.446/1.194)	0.58 ± 0.258 (0.260/0.890)	0.78 ± 0.311 (0.394/1.166)	$p < 0.0001$	$p = 0.8063$	$p = 0.3268$
42	–2.6 ± 0.570 (–3.307/–1.893)	1.08 ± 0.235 (0.789/1.371)	0.96 ± 0.223 (0.683/1.237)	1.12 ± 0.360 (0.674/1.566)	$p = 0.0002$	$p = 0.8309$	$p = 0.4050$

Note: Twenty prostate cancer patients, all of them resistant to hormonotherapy, with bone metastases were randomized in four groups of five patients each. In group 1, the treatment courses was ascorbic acid–menadione; in Group 2, it was menadione; and in group 3, it was ascorbic acid. Group 4 received an appropriate placebo. Assuming that the initial value (pretreatment measure) is 0, the PSA was measured each week during a 42-d trial period (assay days). The mean ± standard deviation and 95% confidence interval (95% CI) of each group is shown. The statistical significance of mean variation in treated groups comparative with control group (p-value) for each assay day, calculated by ANOVA, is also shown.

Table 3 shows the statistical analysis of PSA serum levels. For group 1, the rise of PSA at d 15 and the fall of PSA at d 22, 29, 36, and 42 were significantly different ($p < 0.01$) compared to the control group (group 4). A nonsignificant difference was observed between group 2 or group 3 and group 4.

Discussion

This article reported a study about the variation of two serum tumor markers in prostate cancer patients who were treated with two 7-d courses of oral ascorbic acid–menadione. The total homocysteine was one of the assessed serum markers. It was previously identified as a marker of tumor proliferative mass and its rise was linked to tumor cell folate depletion. This depletion of folate inactivates the remethylation reaction catalyzed by the methionine synthetase, and as consequence, HCY cannot be converted to methionine and increase. High levels of HCY have been shown in cancer patients treated with antifolate and nonantifolate drugs; therefore, it was concluded that folate depletion is not dependent on antifolate treatments. It is caused by the high proliferation rate and, obviously, by the high number of cells. The fall of this high serum HCY level in cancer patients, induced by treatments, could be a marker of the deacceleration of the tumor cells proliferation rate and/or a marker of the kill of tumor cells with the decrease in proliferating tumor cells number. Briefly, the serum HCY level has been previously identified as an indicator of the number of alive and proliferative tumor cells with early response (fall) to death and/or slowing the growth of tumor cells induced by treatments. Serum is not recommended for HCY determination, but when it is carefully collected, a nonsignificant difference was found between the serum and the plasma measures *(19)*. The results reported in this article show that the HCY serum level drops in the first day after the treatment series in group 1. This decrease in tumor cell number induced by ascorbic acid–menadione association persists the following 15 d in the interseries of this short treatment. No similar HCY variation was evident in groups 2 and 3, which were treated with each drug separately. It was reported that the association of ascorbic acid–menadione produces a potentiation of the antitumoral activity of each isolated agent by a factor of 50–100. Therefore, it is possible that any antitumoral effect of each isolated tested agent in groups 2 and 3 did not reach the level of minimum sensitivity of our marker. PSA was the other marker assessed. It is known that serum PSA is a specific indicator of tumor cell activity in most of prostate tumors. It is a glycoprotein with protease activity synthesized by epithelial prostate cells, including prostate tumor cells. The permeation of PSA from inside the tumor cells to extracellular media is responsible of the high serum level of PSA in most prostate cancer patients. The main mechanism through which the treatments kill prostate tumor cells is apoptosis. The serum PSA falls as consequence of the decrease of tumor cells mass; the total source of PSA synthesis decreases and the total PSA permeated from tumor cells to serum also decreases. However, this fall in serum PSA is frequently evident with a delay of days or weeks after tumor treatment. Apoptosis time, changes in the level of remnant tumor cell activity, new conditions of PSA permeation, and the efficiency in the clearance of serum PSA are parameters used to explain the delay in the response of PSA serum level to prostate cancer treatments. In vitro studies have shown that dead prostate tumor cells can release their PSA within hours or days *(19)*. An early increase of PSA after the induction of tumor cells death by treatments can be observed as a consequence of the release of cell contents into the blood. As a consequence, PSA is not recommendable as a measure of early tumor cell death induced by treatments. In this trial, PSA increases in the initial serum specimens taken immediately after treatment with ascorbic acid–menadione and it drops slowly afterward. In the frame of this short treatment, PSA reaches a significant drop at the end of the follow-up. The variations of serum homocysteine and PSA suggest that malignant cell death is induced in prostate cancer patients by the administration of ascorbic acid–menadione. Preclinical studies have shown that this drug association induces prostate tumor cell death by autoschizis in vitro and in experimental animal models *(11,14,15)*. Our results suggest that ascorbic acid–menadione also produces autoschizis in vivo in human prostate cancer patients. The membrane and cell injury produced by oxidative radicals generated by H_2O_2 has been proposed as the mechanism of action involved in the autoschizis induced by ascorbic acid–mena-

dione. It is well known that the ascorbic acid–menadione association generates H_2O_2. It is also known that most malignant cells are often catalase deficient. This mechanism of action seems to be confirmed because autoschizis is inhibited by exogenous catalase *(9,11)*. Cytoskeletal structures and ATP pool have been considered as the target of this peroxidative mechanism of action *(5,11)*. Activation of DNAses with decrease of cell DNA is also a major fact in autoschizis *(14)*. These oxidative mechanisms of action and these targets can be involved in other primary tumors submitted to the same treatment as suggested by the pioneer preclinical studies *(9)*. Further studies are necessary to investigate the optimal antitumoral conditions and clinical relevance of ascorbic acid–menadione, which could make this association a new antitumoral chemotherapy.

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