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Radiofrequency Field–Induced Radiosensitization Is Not Correlated with Induction of Reactive Oxygen Species

Angela Chinhengo, Antonio Serafin, & John Akudugu*

Division of Radiobiology, Department of Medical Imaging and Clinical Oncology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg 7505, South Africa

*Address all correspondence to: John Akudugu, Division of Radiobiology, Department of Medical Imaging and Clinical Oncology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg 7505, South Africa; Tel.: +27-21-938-9942, E-mail: jakudugu@sun.ac.za

ABSTRACT: The capacity of radiofrequency fields (RFFs) to modulate radiosensitivity in a variety of cells may render them useful as noninvasive agents for potentiating radiotherapy. Because ionizing radiation exerts its biological effectiveness via mediation by reactive oxygen species (ROS), any agent that increases ROS burden in a cellular system can be expected to enhance the cytotoxic effects of ionizing radiation. Therefore, it would be of significant interest to determine whether RFF exposure influences ionizing radiation–induced ROS production. In this study, we use four human cell lines, namely, MeWo and Be11 (melanomas), DU145 (prostate carcinoma), and L132 (normal lung fibroblasts), to assess the role of RFF modulation of cellular production of ROS in altering radiosensitivity. We measure radiosensitivity and ROS activity using standard assays. Although RFF exposure seems to consistently increase radiation-induced ROS activity, the extent of enhanced ROS activity does not correlate with the degree of radiosensitization. To confirm the role of ROS in radiofrequency-mediated radiosensitization, a similar study of an expanded cohort of cell lines with a wider span of radiosensitivity is warranted.

KEY WORDS: radiofrequency fields, reactive oxygen species, radiosensitization

I. INTRODUCTION

Having demonstrated in recent studies that radiofrequency fields (RFFs) can act as either radiosensitizers or radioprotectors,[1,2] it would be of significant interest to investigate potential mechanisms that mediate such effects of RFFs. One important nongenotoxic effect of RFFs is induction of reactive oxygen species (ROS).[3] RFFs may cause an increase in endogenous cellular factors that result in elevated levels of ROS and DNA damage.[4] This may be due to increased mitochondrial membrane permeability, pore formation, and nicotinamide adenine dinucleotide phosphate (NAD[P]H) content.[5] Increased NAD(P)H may lead to increased formation of ROS, which is mediated by NAD(P)H oxidase. NAD(P)H oxidase reduces oxygen to the ROS: oxygen radical and hydrogen peroxide. The primary and sole function of NAD(P)H oxidase is ROS production; thus, the absence of NAD(P)H prevents ROS formation.[6] NAD(P)H is a coenzyme for many enzymes in the cell. Other functions of NAD(P)H include xenobiotic detoxification, amino acid metabolism, and immune functions such as phagocytosis and synthesis of fatty acids. Therefore, depletion of NAD(P)H may prevent proper cell functioning.

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To maintain ROS homeostasis and avoid cell death, cancer cells increase their antioxidant capacity. This altered redox environment in cancer cells, compared to normal cells, may increase their susceptibility to ROS-manipulation therapies.[7] Cancer cells require a certain level of ROS, above or below which cell death is induced or promoted. This biochemical difference between cancer and normal cells can be exploited to develop and use therapeutic agents to preferentially target cancer cells.[6] Furthermore, ionizing radiation exerts its biological effectiveness via mediation by ROS.[8–11] Any agent that increases the ROS burden in a cellular system can be expected to enhance the cytotoxic effects of ionizing radiation. It would, therefore, be interesting to investigate the role of ROS in mediating radiomodulatory effects of RFFs.

In this study, the effect of RFF exposure on ionizing radiation–induced changes in the level of ROS was evaluated in human melanoma, prostate cancer cells, and normal lung fibroblasts. Our findings may decipher the potential link between RFF-induced changes in radiosensitivity and radiation-induced alterations in ROS burden.

II. MATERIALS AND METHODS

A. Cell Lines and Culture Maintenance

The four human cell lines used in the study were melanomas (MeWo and Be11), prostate carcinoma (DU145), and normal lung fibroblasts (L132). All were cultured, as described by Chinhengo et al.[12]

B. Clonogenic Survival

Cell survival data regarding the effect of 100- or 1000-Hz–amplitude modulated RFF exposure of the four cell lines investigated here are reported in Chinhengo et al.[12]

C. Cytosolic Superoxide Dismutase Activity Measurement

To evaluate the role of superoxide radicals generated in the RFF-mediated cellular response to X-ray treatment, cells were seeded in 25-cm^2 tissue culture flasks and incubated until they reached an ~ 80% confluence. The cells were subsequently exposed to a 1000-Hz modulated RFF for 30 min at 2 h before or following 6-Gy X-ray irradiation. This field was chosen for the assay because it produces the largest effect on radiosensitivity in all cell lines. The treated cells were incubated for 2 h at 37°C in a humidified atmosphere (95% air, 5% CO_2) and then harvested by gentle trypsinization. One million cells were harvested and washed three times by centrifugation in ice-cold phosphate-buffered saline (PBS) at 250*g* for 10 min and the supernatant was then discarded. The cells were then resuspended in 0.5 mL of ice-cold PBS and homogenized using a BeadBug 3 microtube homogenizer (Whitehead Scientific, Western Cape, South Africa; serial no. 1184070298). Homogenization was performed in plastic tubes containing 1.5 mm of high-impact zirconium beads at 4000 revolutions/min in three bursts. Each burst lasted for 30 s, with 30-s

rests between bursts. The cells were then centrifuged at 1500*g* for 10 min at 4°C, and supernatants were collected. The supernatants were spun again at 10,000*g* for 15 min at 4°C and collected to measure superoxide dismutase (SOD) 1, Cu/Zn.

Standards were prepared by serial dilution from the stock provided with the SOD kit (ThermoFisher Scientific, Waltham, MA, catalog no. EIASODC) to achieve concentrations of 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 U/mL, per supplier instructions. Samples were diluted at a ratio of 1:1 (sample:dilution buffer, v/v). Diluted samples (10 μL each) and standards were pipetted in triplicate into prelabeled wells of a 96-well plate, and 50 μL of 1× substrate were added to each well. The plate was blanked at 450 nm on a spectrophotometer (Labtech International, Sussex, UK; model LT-4000) to account for any nonspecific absorbance that might have been introduced by potential chemical contaminants (background), and 25 μL of 1× xanthine oxidase (the chromogenic SOD detection agent) was added to each well. The plate was incubated for 20 min at 25°C and read again at 450 nm. A standard curve that was plotted from the absorbance obtained from the standard samples was used to determine sample concentrations of SOD after subtracting background absorbance.

Data were further expressed as relative SOD activities as ratios of mean SOD concentrations in triplicate samples that were treated with X rays to those obtained for negative controls (SOD_{6Gy}/SOD_{0Gy}) or ratios of mean concentrations in samples treated with combinations of X rays and RFF to those obtained for positive controls ($SOD_{6Gy+1000Hz}/SOD_{1000Hz}$ or $SOD_{1000Hz+6Gy}/SOD_{1000Hz}$). Ratios of the former to the latter (or modifying factors) were then taken to represent the mode by which RFFs modified SOD activity in irradiated cells. Subscripts 6 Gy + 1000 Hz and 1000 Hz + 6 Gy denote RFF exposure after and before X-ray irradiation, respectively. A modifying factor of > 1, 1, or < 1 indicates a reduction, no effect, or an enhancement in SOD activity in irradiated cells by RFFs, respectively.

D. Statistical Analysis

Data analyses were performed with GraphPad Prism software (San Diego, CA). All data were presented as mean (± standard error of the mean) from three independent experiments. Where applicable, errors were determined by using appropriate error propagation formulae. For associations, we used linear regression analyses.

III. RESULTS

A. Effect of RFF Exposure on Radiosensitivity

The modulatory effect of RFF exposure on radiosensitivity was expressed as a modifying factor (*MF*), given as the ratio of surviving fractions at 6 Gy in the absence and presence of RFFs.[12] Modifying factors for the four cell lines are presented in Table 1. Modifying factors of < 1.0, 1.0, and > 1.0 indicate inhibition, no effect, and enhancement of radiosensitivity by RFFs, respectively.

TABLE 1: *MF*, relative to the surviving fraction at 6 Gy of X-ray treatment alone, for DU145, MeWo, Be11, and L132 cell lines when cells were treated with combinations of 100- and 1000-Hz modulated RFFs and 6 Gy of X rays, as described in Chinhengo et al.[12]

Treatment[a]	DU145	MeWo	Be11	L132
100 Hz + 6 Gy	1.15 ± 0.12	1.00 ± 0.56	4.10 ± 1.42	1.90 ± 0.40
6 Gy + 100 Hz	1.36 ± 0.15	1.16 ± 0.71	3.28 ± 1.55	3.22 ± 1.12
1000 Hz + 6 Gy	1.50 ± 0.32	2.42 ± 0.32	6.31 ± 2.22	9.67 ± 3.14
6 Gy + 1000 Hz	1.67 ± 0.39	3.22 ± 0.49	6.83 ± 2.07	12.89 ± 3.14

[a]100 Hz + 6 Gy and 6 Gy + 100 Hz denote radiofrequency exposures before and after X-ray irradiation, respectively. Errors were calculated by using error propagation formulae for ratios.

B. Effect of RFF Exposure on Radiation-Induced Changes in SOD Activity

SOD activity was measured in cell cultures 2 h after treatment with 6 Gy of X-rays alone or in combination with a 1000-Hz–modulated RFF. Figure 1 is the generated standard curve, as described under section IIIC below, from which SOD concentrations (an indicators of SOD activity) were determined.

The data in Fig. 2 show that X-ray exposure alone resulted in an ~ 40% increase in SOD activity in DU145 cells. Exposure of cells to 1000-Hz modulated RFFs led to an ~ 2.7-fold increase in SOD activity relative to the medium control. Relative to RFF exposure alone, X-ray irradiation before RFF treatment led to an ~ 40% reduction in SOD activity, whereas a reversed treatment sequence increased SOD activity to a similar extent.

Irradiation of the MeWo cells to 6 Gy yielded a 25% reduction in SOD activity relative to the medium control (Fig. 3). Relative to the medium control, RFF treatment alone

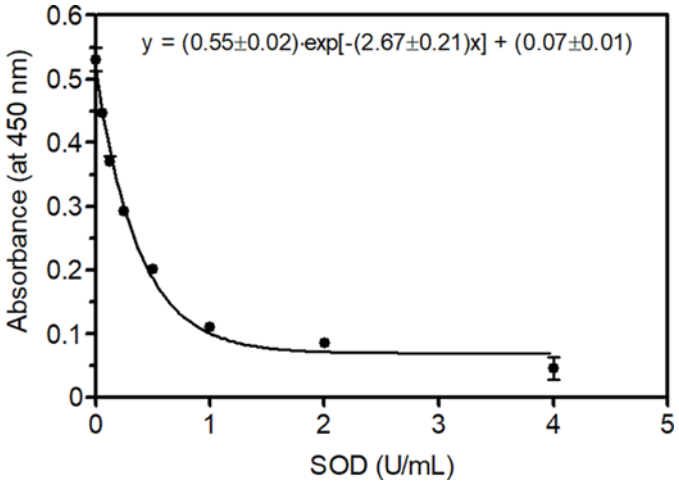

FIG. 1: Standard curve used to determine cytosolic SOD concentration from absorbance measurements (see section IIIC of the text). SOD, superoxide dismutase.

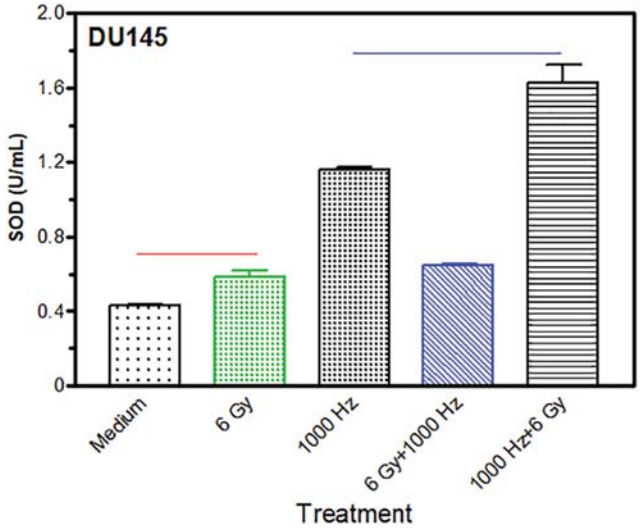

FIG. 2: SOD concentrations in DU145 cells following RFF exposure and X-ray irradiation, singly or in combination. X-ray treatment was compared with negative control (medium; lower horizontal line) and X-ray + RFF treatment was compared with positive control (1000 Hz; upper horizontal line) to generate relative SOD activities that were used to derive modifying factors (see section IIIC of the text). X-ray + RFF denotes X-ray irradiation before RFF exposure. SOD, superoxide dismutase.

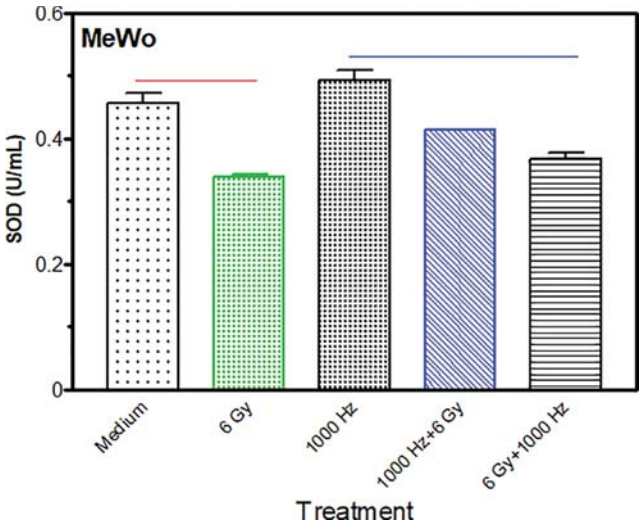

FIG. 3: SOD concentration in MeWo cells following RFF exposure and X-ray irradiation, singly or in combination. X-ray treatment was compared with negative control (medium; lower horizontal line) and X-ray + RFF treatment was compared with positive control (1000 Hz; upper horizontal line) to generate relative SOD activities that were used to derive modifying factors (see section IIIC in the text). X-ray + RFF denotes X-ray irradiation before RFF exposure. SOD, superoxide dismutase.

resulted in only an ~ 8% increase in SOD activity. The 6 Gy + 1000-Hz modulated RFF and 1000-Hz modulated RFF + 6-Gy treatments also reduced SOD activity by ~ 25% and 16% relative to the 1000-Hz control, respectively.

Data shown in Fig. 4 are SOD activities in Be11 cells for the various treatments. Treatment of cells with 6 Gy or 1000-Hz modulated RFFs led to an ~ 47% reduction or ~ 36% increase in SOD activity relative to medium control, respectively. When compared to the 1000-Hz control, treatment with 6 Gy + 1000-Hz modulated RFFs and 1000-Hz modulated RFFs + 6 Gy reduced SOD activity by ~ 85% and 50%, respectively.

When normal lung fibroblasts were exposed to 6 Gy or 1000-Hz modulated RFFs, a slight increase of ~ 6% SOD activity was observed (Fig. 5). Relative to the 1000-Hz control, 6 Gy + 1000-Hz modulated RFFs and 1000-Hz modulated RFFs + 6 Gy treatments resulted in ~ 8% and 11% reductions in SOD activity, respectively.

The dose-modifying factors, on the basis of SOD activity (as described in section IIIC below), are presented in Table 2. For the DU145 cell line, treatment with a combination of 1000-Hz modulated RFFs and 6 Gy did not change SOD activity (*MF* ≈ 1.0) compared to 6-Gy irradiation alone. However, when these cells were irradiated before RFF exposure, a larger than twofold reduction in SOD activity was observed. A similar treatment response was noted in the Be11 line.

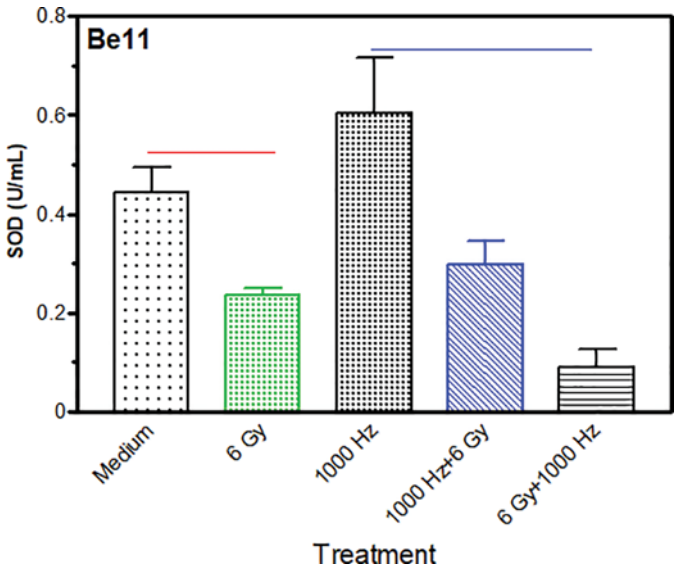

FIG. 4: SOD concentration in Be11 cells following RFF exposure and X-ray irradiation, singly or in combination. X-ray treatment was compared with negative control (medium; lower horizontal line) and X-ray + RFF treatment was compared with positive control (1000 Hz; upper horizontal line) to generate relative SOD activities that were used to derive modifying factors (see section IIIC of the text). X-ray + RFF denotes X-ray irradiation before RFF exposure. SOD, superoxide dismutase.

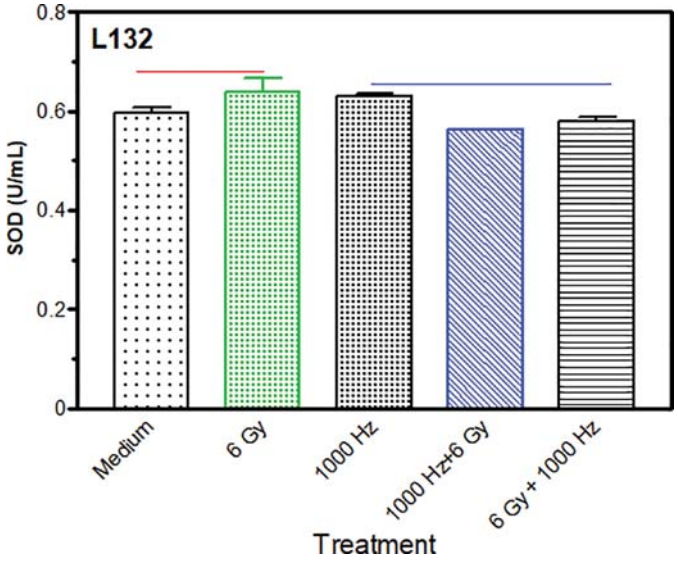

FIG. 5: SOD concentration in L132 cells following RFF exposure and X-ray irradiation, singly or in combination. X-ray treatment was compared with negative control (medium; left horizontal line) and X-ray + RFF treatment was compared with positive control (1000 Hz; right horizontal line) to generate relative SOD activities that were used to derive modifying factors (see section IIIC of the text). X-ray + RFF denotes X-ray irradiation before RFF exposure. SOD, superoxide dismutase.

TABLE 2: *MF*, relative to X-ray treatment alone, derived from SOD activities presented in Figs. 2–5 for DU145, MeWo, Be11, and L132 cell lines, as described in section IIIC of the text

Treatment[a]	DU145	MeWo	Be11	L132
1000 Hz + X rays	0.97 ± 0.09	0.88 ± 0.05	1.08 ± 0.30	1.20 ± 0.06
X rays + 1000 Hz	2.43 ± 0.18	0.99 ± 0.06	3.53 ± 1.71	1.16 ± 0.06

[a]1000 Hz + X rays and X rays + 1000 Hz denote radiofrequency exposure before and after X-ray irradiation, respectively. Errors were calculated by using error propagation formulae for ratios.

For the MeWo cell line, pre-exposure to RFFs resulted in an ~ 12% increase (*MF* < 1.0) in SOD activity. A treatment sequence reversal did not affect radiation-induced SOD activity. For lung fibroblasts L132, both combination treatments yielded an ~ 20% reduction (*MF* > 1.0) in SOD activity.

C. Relationship between RFF-Modulated Radiosensitivity and SOD Activity

To test whether treatment-induced changes in SOD activity were linked to modulatory effects seen in cellular radiosensitivity when the 1000-Hz modulated RFF was applied, modifying factors derived from SOD activity were plotted against those obtained from clonogenic cell survival. Figure 6 shows no relationship (Y = [0.0018 ± 0.0935] X +

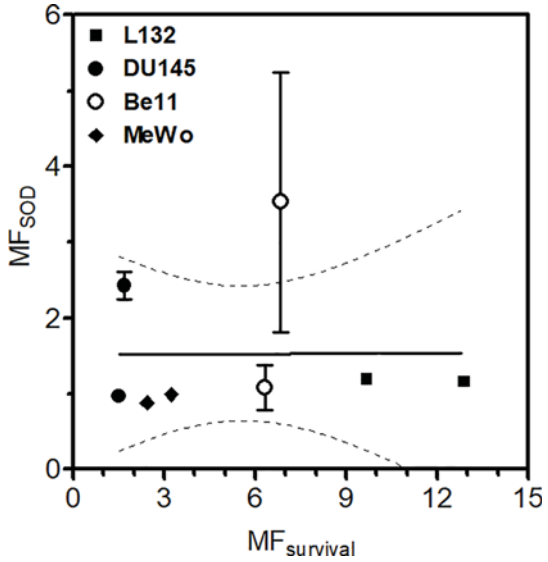

FIG. 6: Plot of modifying factors from SOD activity (measured 2 h after treatment) as a function of modifying factors from clonogenic cell survival for the four cell lines, following combined treatment with a 1000-Hz modulated RFF. $MF_{survival}$ represents the ratio of surviving fractions at 6 Gy of X rays in the absence of those in the presence of RFF exposure. Criteria for inhibition, no effect, and enhancement of radiosensitivity by RFF are $MF_{survival} < 1.0$, $MF_{survival} = 1.0$, and $MF_{survival} > 1.0$, respectively. Dashed lines represent a 95% confidence interval. MF, modifying factor; SOD, superoxide dismutase.

[1.52 ± 0.63]; $R^2 < 0.0001$; $p = 0.9856$) between RFF-induced radiosensitization and changes in SOD activity.

IV. DISCUSSION

A. Effect of RFF Exposure on Radiation-Induced Changes in SOD Activity and Its Relationship with Cell Survival

ROS has an important role in cellular signaling and a dual role in cancer. ROS can facilitate cancer cell proliferation, survival, and adaptation to hypoxia, but it can also cause oxidative stress, leading to cell death.[13–15] ROS is produced after increased metabolic activity in cells and mitochondrial dysfunction. Electromagnetic fields have been reported to influence the production of ROS in cells,[16–18] and its concentration may either enhance cell survival or cause macromolecule damages that lead to cell death. SOD is one of the enzymes responsible for removing or converting ROS to less harmful compounds. SOD concentration is relative to ROS levels in the cell. In this work, the effect of 1000-Hz modulated RFFs and 6 Gy of X rays on SOD concentration was assessed and deemed to signify ROS levels in response to treatment.

Unlike changes in metabolic activity that seem to be correlated with radiomodulatory effects of RFF exposure across cell lines, no link was apparent between alterations in SOD activity and RFF-mediated radiosensitization (Fig. 6). This finding is not surprising, given the multifunctional features of ROS.[13–15] SOD activity was only markedly reduced ($MF_{SOD} > 2.0$) in p53 mutant DU145 and p53 wild-type Be11 cells, when cells were pre-exposed to RFFs. No effect was apparent in the other treatments and cell lines, indicating that RFF-mediated changes in radiation-induced SOD activity do not appear to depend on p53 status or treatment sequence.

Although SOD has been known to have a key role in regulating cellular metabolism,[19] the current study did not identify a link between SOD and metabolic activity. The modes by which changes in SOD and metabolic activity may impact radiomodulatory effects of RFFs appear to be unrelated.

V. CONCLUSIONS

Data presented here show that levels of radiation-induced ROS generally increased when cells were concomitantly exposed to RFFs. However, elevated ROS levels did not correlate with observed RFF-mediated radiosensitization. Studies involving larger panels of cell lines might be required to confirm the role of ROS in radiofrequency-mediated radiosensitization.

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