Biomedical Reviews 1998;9: 121-129. ©The Bulgarian-American Center, Varna, Bulgaria
ISSN1310-392X

IONIZING IRRADIATION AND THE SALIVARY GLAND SEQUELAE

R.M.Nagler
Department of Oral and Maxillofacial Surgery and Oral Biochemistry Laboratory, Rambam Medical Center and Faculty of Medicine, Technion - Israel Institute of Technology, Haifa, Israel

SUMMARY

• *Salivary gland damage due to radiotherapy, leading to xerostomia and causing a great of suffering to patients, is a phenomenon known since the beginning of this century. The mechanism responsible for it has not been elucidated and no adequate treatment for patients is available. According to the mechanism suggested for the parotid irradiation-induced specific damage, the injurious agents resulting in delayed serous cell death, leading to specific parotid radiosensitivity, are transition, highly redox-active metal ions, such as Fe and Cu, associated with secretion granules. These ions enhance the lethal effect that irradiation has on DNA, resulting in a reproductive delayed cell death. The immediate effects of metal-mediated enhancement of irradiation damage in cells may occur but does not seem to play a major role in the underlying mechanism. Indeed, in a series of recent experi ments, it was succeeded in positively correlating an extended time point (two months) protection of parotid function with preirradiation degranulation and redox active metal ion mobilization out of the gland into the secreted saliva prior to irradiation. In contrast, a negative correlation in the submandihular gland, with no protection, no degranulation, no metal ion mobilization and no redox activity was demonstrated. The ability to protect the parotid function at two months with Zn-DFO, a specific transition metal ionmobilher, from sensitive intracellular targets lends further credence to these studies.* ***(Biomed Rev** 1998; 9: 121-129)*

Reccivcd lor publication 15 May 1998 and accepted 15 November 1998.
Corespondence and reprint requests to Dr R.M. Nagler. Department of Oral and Maxillofacial Surgery. Rambam Medical Center. FOB 9602, Haifa 31096. Israel. Tel: 972 (4) 8520 989. Fax: 972 (4) 8515 710.

INTRODUCTION

• In 1911, Bergonie *el al* (1) were the first to describe salivary gland swelling shortly following irradiation, a mode of therapy for cancer used since the beginning of this century. Salivary gland damage due to radiotherapy which leads to xerostomia, while not life-threatening, causes a great deal of suffering. Xerostomia due to irradiation will engage annually 30 000-50 000 individuals treated for head and neck cancer in the United States alone (2-4). Due to their size, location and bilateral symmetry, it is inevitable that at least a portion of the major salivary glands will be included in most radiation fields, deliv ered to control and abate the primary malignant neoplasm and/ or its common cervical lymph node metastasis (5). Even radio therapy modalities such as total body irradiation delivered pri or to bone marrow transplantation, mouth field irradiation ad ministered while treating Hodgkin's lymphoma or radioactive iodine therapy given for thyroid carcinoma, all expose salivary glands to the severe effect of ionizing irradiation (6-8). The se vere negative impact that xerostomia has on the patient's life results from various secondary effects, such as impairment of taste, mastication, swallowing, speech and sleep patterns. Fur thermore, xerostomia often causes a reduction in the oral cavity protection for both hard and soft tissues, alters microbial flora to a more pathogenic one, initiates dry ulcerated painful mucosa, limits the wearing of oral prostheses and often causes consti pation (4,9-11). No adequate treatment for xerostomia is cur rently available. Thus, one may speculate that a better under-

standing of the xerostomia phenomena may help in developing a proper treatment or even to prevent the problem. The lack of understanding the phenomena, i.e. the "enigma" as it is often defined (12), is generally based on the fact that salivary glands are highly differentiated and metabolically active tissues with a low mitotic rate which are considered as "reverting post-mitotic" tissue (13,14), and presumably belong to the "flexible tissues" (15). These kinds of tissues are not expected to be radiosensitive according to the rules for high cellular radiosensitivity, as suggested in 1906 by Bergonie and Tribon-dean, as they do not have a high mitotic rate, have no expected future mitoses and are largely differentiated. The commonly accepted characteristics of irradiation-iduced xerostomia are that *(i)* it occurs rapidly following relatively low doses of irradiation, *(ii)* the parotid is the main if not the only salivary gland involved, and *(in)* often there is no objective recovery. Recovery, however, may occur if the irradiation dose and the portion of the exposed gland are limited enough and if it correlates well with the preirradiation secretion capacity (16). These characteristics are widely accepted and the biologic-mechanistic point of view is probably the basis for the large amount of literature dealing with the different sources of ionizing irradiation, modalities of delivery, doses, volumes, and irradiated fields in different species, and with studies of numerous parameters, mainly clinical and morphological. The most studied species are human and rodent, although studies have been done on monkeys, dogs, cats, swine and rabbits. However, we remain far from understanding the development of xerostomia.

The purpose of this review is to provide an updated description of early and late irradiation effects on salivary glands in humans, other primates and in rodents. Based on this description, the mechanism underlying xerostomia will be discussed.

IONIZING IRRADIATION EFFECTS ON SALIVARY GLANDS OF HUMAN AMD OTHER PRIMATES

• The usual total irradiation dose given for controlling head and neck tumors lies in the range of 40-70 Gy, although in rare cases the dose can be as low as 20 Gy or as high as 80 Gy. Deeg *el a!* (6) and Rubin and Cassaret (13) suggested that xerostomia, as the most severe end-point complication, has a TD 5/50 (probability of 5% within 5 years) when 5 0 Gy are delivered, and aTD 50/50 when 60 Gy are delivered. This xerostomia is ra pid in appearance following low doses. Doses of up to 10 Gy, usually given within the first week of therapy, may reduce the salivary flow by as much as 50-60% (5-12,17-20). The parotid gland is affected early following irradiation, demonstrating a rapid reduction of its secretion capacity and especially "at rest" rather than "at stimulated" conditions (17,20). After the initial sharp reduction in secretion rate, there is a less rapid rate of reduction until it eventually reaches barely measurable values (18,21-23). Recovery of the secretion capacity occurs in few cases, depending on the radiation dose. It is a dose-dependent phenomenon which seems to be completed when the administered dose is up to 25-30 Gy, whereas only a partial recovery is achieved at doses up to 50-60 Gy; the recovery does not occur following higher doses. The volume of parotid gland exposed to irradiation seems to be an even more important factor for the prognosis of both damage and recovery. Other factors that may also play a role are the primary functional capacity of the glands, the age of the patient, the personal sensitivity, and the sex (14,16-18,20,23-26).

In contrast to the parotid, the other major salivary glands have lesser been studied in respect to their response to irradiation. In the only available human direct submandibular/sublingual study which dealt with the long-term secretion capacity under stimulated and unstimulated conditions, the irradiation-related flow reduction was found to be comparable yet smal ler than that of the parotid gland (12).

The most sensitive indicator of salivary irradiation is an immediate induced hyperamylasemia. Within a few hours after low-dose irradiation (1 -4 Gy), a profound, 10 to 80 fold increase ofthe parotid amylase isoenzyme is found. This elevation reaches its peak within 12-36 hours and may be the result of immediate serous cell death ofthe parotid gland, accompanied by disruption ofthe cellular membrane and leakage ofthe secretory enzyme into the extracellular space and the blood circulation (6,7,27-33). Another immediate clinical finding is enlargement ofthe major salivary glands, occasionally painful. This infrequently occurring phenomenon may be the result of induced edema and inflammation, is noticed within a few hours after irradiation and subsides within a few days (1,5,16,23,27,34).

Contrary to the numerous chronic phase postirradiation studies, only one large human study and a few primate studies have been published on the acute phase for both parotid glands and sumandibular glands (SMG) (27,29). Dead serous cells were consistently observed as early as one hour after irradiation and even after as low a dose as 2.5 Gy. However, the amount of serous cell destruction was dose-dependent and reached saturation at 10-15 Gy. Extensive destruction reached its maximal extent at 24 hours when the acute inflammatory cells were replaced by chronic ones. At 16-22 and 40 weeks post-15 Gy irradiation, primate salivary glands revealed a comparable extent of atrophy with approximately 100% loss of serous acini and a relative radioresistant state of mucous cells. Although loss of serous acini occurs very quickly, early gross atrophy of the salivary glands may be concealed by the swelling induced by the inflammatory, hyperemic and edematous reaction. Only after this swelling subsides can the salivary atrophy be evaluated, as was also demonstrated in sialograms (35,36) and ^{67}Ga-citrate accumulation studies (37-39). ^{97}Tc-sialograms examining the functional impairment of both parotid glands and SMG of 20-

70 Gy demonstrated that the effect was similar at later times in both glands, although the parotid was more affected up to 3 months (40). Clinically, the SMG may become firm and en larged, whereas histologically, the characteristic principle features of the salivary chronic changes are atrophy and loss of parenchy ma (mainly serous acini), fibrosis, chronic inflammation and occasional adipose tissue replacement. The duct system increa ses its prominence relative to a loss of acinar tissue and the duct epithelium commonly demonstrates squamous metaplasia. Vas cular changes of hyaline thickening of arterioles, teleangiecta-sia. arterial internal proliferation and endothelial cell enlarge ment are inconsistent changes of variable severity (41-43). Salivary compositional changes leading to a reduction in the protective capacity of the saliva were also widely reported. These changes included reduction in pH and buffer capacity (bicarbonate levels), increase in viscosity, increase in specific immunoglobulins, lysozymes and lactoferrin levels, but an overall reduction due to the secretion decrease (5,12).

IONIZING IRRADIATION EFFECTS ON SALIVARY GLANDSOF RODENTS

• The rodent is the most studied species regarding ionizing effects and salivary glands. The factors which rendered the rodent into the animal of choice include the relative conve nience of harvesting glands for morphological and histoche-mical studies, the ease in comparing various factors between different animal groups or in comparing some factors at different time points in the same animal, and the relatively low costs in volved. There are, however, somenotable differences in bioche mical, physiologic and morphologic characteristics between salivary glands of humans and rodents. The submandibular/ sublingual size compared with the parotid is relatively larger in the rat. The rodent salivary glands are under endocrine control and the effects of irradiation differ between the sexes. Some morphological studies have indicated that the rat and mouse parotid glands are more radioresistant than the human glands. Contrary to humans, acute inflammatory cell infiltration does not occur in rat salivary glands and hyperamylasemia does not consistently develop after irradiation (44-56). While the chronic irradiation damage to the human salivary glands is fully devel oped and stabilized by 1 -2 years (13,14), it is suggested that this period is much shorter, 60-90 days, in the rat (57). However, one of the major differences between human and rat studies seems to be the severe and systemic effects that head and neck irra diation has on rats, mediated by the oropharyngeal mucositis and leading to substantial reduction in food and water intake, total body weight and to reduction in the survival of rats dur ing the second week post-irradiation. This reduction in food and water intake could be at least partially responsible for various parameter alterations (gland weight, flow rate, amylase activity) which are considered to be related directly to the irradiation effects (58,59). Even morphological and histochemical enzy-matic activity changes have been demonstrated to result from total body irradiation with neck shielding or starvation (60).

When analyzing the post-irradiation period studies, it seems that the acute phase is the "weakness" in human studies, while the chronic phase is poorly dealt with in rodents, with a few exceptions such as the studies of Cherry and Glucksmann (61, 62), who followed the morphological alterations of all three rat major salivary glands up to ayear after irradiation. There are very few flow rate functional studies with the few available con-centratingmainly on the parotid. *Viss'mkelal* (44,45,63) are the only authors who studied submandibular/sublingual functional parameters, comparing them up to 30 days to those of the parotid and demonstrating extensive functional similarity. Mor-phologically, the first cellular alterations, including cell death, are demonstrated by electron microscopy, during the first few hours after irradiation. This cellular destruction reached its nadir after 3-4 days, which is estimated to be relatively low when compared to the functional loss (44,45,53,63-70). However, following this nadir, there is a recovery, invilving not only morphological alterations but other parameters as well, such as glandular weight, cellular "Tc and leucine uptake, amylase activity, proliferation, functional parameters such as flow rate, flow volume and lag phase, and salivary composition parameters such as sodium, potassium and amylase (44,45,48,52,55,58, 63,64,68-74). After this intermediate phase of recovery, it seems that another phase of decline occurs for some of the parameters studied, starting at the third week after irradiation and progressing gradually until at least 6 weeks postirradiation (44,45,63, 71,72). In 1970, Phillips(71)dividedthepost-irradiationperiod into three: the first phase characterized by a decline, reaching nadir in the middle of the first week; the second phase being recovery up to the 16th day; the third period another degenerative phase. It seems, therefore, reasonable to conclude that the traditional classification of the postirradiation period as acute *versus* chronic phases may be too simplistic and to suggest a new, four-phase classification: *(i)* the immediate phase; the first few hours postirradiation, in which most of the sublethal damage is repaired and the first signs of immediate cell death become apparent, *(ii)* the short phase; the two weeks following irradiation, in which the oropharyngeal syndrome predominates while a major part of the potential tissue repopulation, ede-matous changes and recovery are expressed to their most advanced extent. *(Hi)* the late phase; further progress at the cellular and tissue levels, until a state of stabilization is achieved, a period which is yet to be defined but that presumably takes months; this progress may have a pattern of further decline until stabilization is achieved, and *(iv)* the extended phase; stabilized state, at a level which is presumably dependent on the irradiation dose given, as well as on other general and specific parameters which may play a role, such as the protraction and frac-tionation modalities and the irradiation linear energy transfer, presence of pharmacological modifiers, and level of tissue

oxygenation. Salivary gland recovery will be complete following doses of up to 2.5 Gy but compromised at higher doses. However, there is no consistency in the literature regarding threshold doses (55). Since most studies were based on a single rather than a fractionated dose, drawing conclusions from the results would not be warranted when compared to those in humans which are based on a fractionated modality. It seems that maximal damage for the rodent is achieved with doses of 7.5-15 Gy, as concluded from a series of rat studies examining various salivary and general parameters following irradiation doses in the range of 2.5-15 Gy(44,45,48,52,63,68,69,73-80).

IRRADIATION EFFECT ON RAT SALIVARY GLANDS AT THE FUNCTIONAL LEVEL

• Duringthe first two weeks following 15 Gy irradiation, there was a distinct dissociation between the parotid glands and SMG. While there were no significant alterations in the submandibular flow rate during this period, the parotid function was reduced drastically. Nevertheless, it almost completely recov ered towards the end of the second week. These reduction values were significant at 1,4,8 and 11 days were by 42%, 74%, 75% and 90%, respectively. On the 14th day post 15 Gy irradia tion, there was no significant reduction in the parotid flow rate compared with control animals (81,82). During the first two weeks postirradiation, with doses at 15 Gy, food and water intake is profoundly reduced in the rodent due to the induction of severe oropharyngeal mucositis (83). As a result, dehydration, dysphagia and reduction in mastication are inflicted, all known to cause salivary gland atrophy and reduction of secretion ca pacity. This phenomenon mainly involves the parotid glands and not the SMG. To examine the assumption that the so-called ' irradiation[1] effects on the parotid gland of the rat during the first two weeks are actually mucositic effects and, thus, are transient, both function and partitution-coefficient parameters of the sa livary glands were examined in both irradiated and pair-fed but not irradiated rats (81,84). It was clearly shown that during the first two weeks postirradiation in the rat it is the mucositis rather than the irradiation which predominates in the parotid func tional response. However, at later time points and after a short recovery phase of a few weeks, there was a functional deterio rating phase for both parotid glands and SMG. At two months post-15 Gy irradiation, the flow rate reduction of both glands was 84% and 68%, respectively. The functional reduction of both glands becomes similar and the deleterious effect of even very low irradiation doses on both salivary glands was reveal ed only at delayed time points. It was shown that even the low est dose of only 2.5 Gy caused over 60% of the maximal damage resulting from lOGyat 12 months (82,85,86). Also demonstrated was that during the year following irradiation there was a dose-dependent relation in the rat salivary functional damage for various doses in the range of 2.5-15 Gy.

IRRADIATION EFFECT ON RAT SALIVARY GLAND AT THE BIOCHEMICAL AND MOLECULAR LEVELS

• In 1996, we evaluated the expression of early response proto-oncogenes *(c-fos* and *jun-B),* tissue specific genes (proline-rich protein and kallikrein), and proteolysis linked ubiquitin gene following exposure to 15 Gy irradiation alone or in com bination with P-adrenergic stimulation of the rat SMG (87). Head and neck irradiation resulted notonly in dysfunction andtissue loss of the salivary glands but also in a systemic effect express ed as profound body weight loss. Irradiation alone was found to induce expression of the *jun-B* but not the *c-fos* proto-oncogenes. The combination of irradiation and p-adrenergic stimulation by isoproterenol induced earlier expression *ofjun-B* and profound expression of the *c-fos* proto-oncogene in comparison to irradiation alone. In contrast, the kallikrein and ubiquitin genes were expressed constitutively and were not af fected by irradiation alone or in combination with p-adrenergic stimulation. In addition, irradiation had no effect on SMG mRN A translation. We observed that the expression of these genes was enhanced by irradiation alone or in combination with isoproterenol administration. In contrast, the expression of genes associated with the functional integrity of the cell, i.e. kallikrein, ubiquitin, and proline-rich protein, was unaffected. These findings, in addition to delayed gland dysfunction, led us to believe that the irradiation-induced injury to the SMG is to be attributed to reproductive stem cell death. Further, we exa mined various sialochemical parameters in parotid gland and SMG secreted saliva of irradiated rats (88). Various doses of radiation from 2.5 to 15 Gy were administered to the head and neck region and the saliva was evaluated for its amylase activi ty and the concentration of sodium, potassium and total protein. Saliva samples containing equal amounts of proteins were also electrophoresed on separately sodium dodecyl sulphate gels, silver-stained and examined for possible qualitative altera tions. The total protein concentrations of parotid saliva showed a radiation dose-dependent reduction at 3 days and 3 and 9 months following 15 Gy of 93%, 82% and 73%, respectively. Forty days after the 15 Gy irradiation, the reduction was not as severe (55%). Three and 40 days post 15 Gy, amylase activity demonstrated a similar pattern of reduction, 98% and 89%, respectively. In contrast to the parotid, no quantitative changes in the protein concentrations of the SMG saliva were detected. As for the qualitative profiles of separated proteins, no radia tion-induced changes were found for either parotid glands or SMG at 3 and 40 days or 3 and 9 months, as compared with con trols. The electrolyte concentrations were found to be flow-rate dependent. The Na concentrations of parotid saliva at 3 and 40 days following 15 Gy were reduced by 65% and 83%, respec tively. For SMG saliva, the Na concentration was reduced at 40 days by 58%. The K concentration of parotid saliva increased at 40 davs by 79%. We believed that the data suggested that the various observed sialochemical changes could result from

a number of surviving parenchymal cells. Thus we presumed that the observed salivary compositional alterations were not directly induced by radiation but, rather, were secondary effects. Further, we examined the hypothesis that intracellular and redox-acti ve ions of iron and copper, which are associated with the secretion of granules, play a catalytic role in the irradiation-induced damage (89). Rats were subjected to head and neck ilTadiation (15 Gy) and allowed to recover for two months. The function of the parotid glands and SMG was then determined by pilocarpine-stimulated salivary secretion. A 45% decrease in the function of both glands was obtained when compared to non-irradiated controls. Treatment prior to irradiation (90 min) with cyclocytidine (200 mg/kg) led to massive degranulation of the parotid gland and yielded nearly complete protection from irradiation-induced damage. In contrast, pilocarpine stimulation prior to ilTadiation led to marginal degranulation of the parotid gland and yielded only 13% protection. Neither agent caused degranulation of the SMG mucous cells or yielded functional protection of this gland. Treatment with both agents yielded a marked increase in iron, copper and manganese levels in the parotid gland saliva. An analogous marked increase in the redox activity of iron and copper ions was recorded for the parotid saliva stimulated by pilocarpine and cyclocytidine. Pilocarpine-stimulated SMG saliva contained metal levels similar to those of the parotid gland saliva. However, no redox activity and no increase in metal mobilization could be demonstrated in the SMG saliva stimulated by both agents. We suggested that the correlation between the patterns of the gland degranulation, mobilization of redox-active metals and the protection of gland function for both parotid and SMG focuses attention on the catalytic roles played by transition metal ions in promoting free radical reactions which likely participate in the process of injury to the tissue.

CONCLUSION

• Based on the literature available and on our own studies, we believe that one can suggest an overall mechanism for the damage induced by irradiation to the salivary glands. Our results have shown a mutual delayed expression of irradiation-induced damage in both parotid glands and SMG, more evident in the parotid gland. We have demonstrated that the short-term effect of irradiation on the parotid gland during the first two weeks was transient and secondary to the oropharynge syn drome. In the rodent, this syndrome is predominated by severe and transient mucositis resulting in dehydration, malnutrition and reduced mastication. All these are known to induce pro found hypofunction of the parotid, unrelated to the direct salivary effect (83,90-92). We have supported this hypothesis by a study in which we mimicked the "two week irradiation" effects (including recovery) by pair feeding the animals (81,84). The direct effect was expressed later, and the morphological analysis demonstrating short- *versus* long-term sparing of the serous cells adds credence to this observation. Furthermore, while immediate cell death cannot be excluded, it does not play a major role in the long-term accumulating damage, due to the nearly total recovery from the short-term effects (81).

What is responsible for the delayed effect on the one hand and the specific radiosensitivity of the parotid gland on the other? The mechanism of radiobiological delayed damage is usually considered to reveal DNA latent damage being expressed during mitosis in cells with a low mitotic rate. This damage results in reproductive cell death. The mitotic rate of salivary parenchymal cells is reported to be one-three months, with a parotid rate twice as high as that of the SMG (61,62). The accumulative nature of the delayed hypofunction of both glands in conj unction with the maj or component being expressed at three months postirradiation and the lagging behind of the SMG seems to be in accordance with this data. DNA is considered to be a very radiosensitive cellular target and was shown to be so in salivary glands as well (75,76,93). The profound effect induced by 2.5 Gy that we observed undoubtedly reflects a very radiosensitive target, a peculiar enhancement of the irradiation effect, or both. The "sudden" disappearance of normally functioning serous cells as demonstrated by morphology is suggested by the accumulative reduction in volume of secreted saliva whose normal composition is preserved. This seems to be well in accordance with reproductive cell death and is also the case in the unaltered expression pattern of salivary functional tissue-specific genes, such as amylase, proline-rich protein and kallikrein. Concomitantly, the irradiation-induced injury to DNA leading to reproductive cell death is further supported by the profound high expression following irradiation of DNA damage-induced genes, such as *c-fos andjun-B* (94,95).

Two more questions have yet to be addressed: (/) why is the parotid gland specifically affected, and *(if)* are there any enhancing agents which increase the effect of ilTadiation even if DNA is the target? According to the hypothesis suggested by Abok *et al* (47), heavy metal ions such as Zn, Mn and Fe contained within the secretion granules are the damage-enhancing agents and are responsible for irradiation-induced immediate death of serous cells. This hypothesis could explain the rapid response of the parotid gland, as serous cells contain high levels of these secretion granules and are in a much higher prevalence in the parotid gland. However, when examining this hypothesis, we faced two major problems: (/) the predominant salivary effect of irradiation is the induced delayed cell death rather than an immediate one, and (//) according to basic principles of radiobiology, heavy metal ions as such cannot participate in the enhancement of irradiation-induced biological damage, which is mediated by a greater production of hydro-xyl free radicals. Metal ions that may be involved in such a process should fulfil three conditions. They must be transition metal ions, redox active in physiologic conditions, and in a

"free" state to participate in the process. Fe and Cu may fulfil these conditions, but Zn and Mn do not. .

The following mechanism for the irradiation-induced parotid specific damage is suggested: the injurious agents resulting in delayed serous cell death leading to the specific parotid radiosensitivity are transition, highly redox-active metal ions, such as Fe and Cu, associated with secretion granules. These ions enhance the lethal effect that irradiation has on DNA, resulting in a reproductive delayed cell death. The immediate effects of metal-mediated enhancement of irradiation damage in cells may occur, but does not seem to play a major role in the underlying mechanism. Indeed, in a series of experiments, we succeeded in positively correlating an extended time point (two months) protection of parotid function with preirradiation degranulation and redox-active metal ion mobilization out of the gland into the secreted salivapriorto irradiation. In contrast, we demonstrated a negative correlation in the SMG with no protection, no degranulation, no metal ion mobilization and no redox activity (89,96,97). Our ability to protect the parotid function at two months with Zn-DFO, which is a specific transition metal ion mobilizer, from sensitive intracellular targets gives further credence to our suggestion (97). We believe that the mechanism we suggested is fairly comprehensive for the parotid gland, although we are not aware of the specific "trigger parameter" which induces the profound, even if delayed, injury to the SMG at this time.

ACKNOWLEDGMENT

• The author wishes to thank Mrs M. Perlmutter for her assistance in the preparation of the manuscript.

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