EFFECTS OF FRACTIONATED IRRADIATION
ON SALIVARY GLANDS

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ABSTRACT

EFFECTS OF FRACTIONATED IRRADIATION ON SALIVARY GLANDS
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The thesis is a study of the effects of radiation on the salivary glands in an experimental and a clinical study. Irradiation is a cornerstone in the management of head and neck cancer and is as other modalities of cancer treatment, afflicted with adverse reactions. An optimal radiotherapy regime is limited by the sensitivity of the normal tissues with regard to early and late effects. In certain cases the early effects can be so troublesome that it will cause interruption in the irradiation and questioning of the curative intention. Although DNA is the lethal target, other parts of the cell have been proposed as sensitive targets to irradiation. Different in vitro secretory models and quantitative morphological characterization and immunohistochemical evaluation of neuropeptides were performed in rat salivary glands after irradiation. The irradiation was given unilaterally or bilaterally once a day for a five-day schedule with 6 MV photons (total dose 20, 30, 35, 40, 45 Gy) or a two fractions regime in five days with a total dose of 24 or 32 Gy. The contralateral gland served as a control for unilaterally treated animals and parallel analyses were done 10 days or 180 days following the last irradiation dose. An early, dose-dependent effect of fractionated irradiation on noradrenaline-stimulated potassium fluxes ($^{86}$Rb$^+$ fluxes) was demonstrated. In contrast, the exocytotic amylase release displayed no obvious alterations, and morphologically no changes were seen. Regarding late effects (180 days) the noradrenaline-stimulated electrolyte secretion was decreased at least for the higher doses of irradiation. Amylase content and loss of acini was also dose-dependently decreased. At 10 days after bilateral irradiation there was a marked increase in the expression of the neuropeptides substance P, leu-enkephalin and bombesin in the ganglionic cells associated with the submandibular glands and in nerve fibers of the glandular parenchyme.

In addition, a clinical prospective evaluation of 25 patients was performed before, during radiotherapy and 6, 12 and 18 months after the end of treatment. A great interindividual variation in the recovery was demonstrated with regard to salivary flow rate. Irradiation doses about 40-50 Gy caused generally reversible changes; sometimes salivary secretion was almost completely restored 6-18 months after the end of radiotherapy. Doses exceeding 65 Gy induced almost irreversible alterations.

Even if DNA is the target for the lethal effect of irradiation, other constituents, such as the cell membrane or neuropeptide expression can be significantly affected by irradiation and cause important physiological changes.

Key words: Salivary glands - irradiation - xerostomia - acinar cells - secretion - cell membrane - neuropeptides
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"People who make their own rules when they know they're right... people who get a special pleasure out of doing something well (even if only for themselves)..."

Richard Bach
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BACKGROUND

Irradiation is an important treatment modality in the management of head and neck cancer, but as other modalities of cancer treatment, it is afflicted with adverse reactions. Among normal tissues salivary glands display an inherent radiosensitivity and are severely affected by irradiation when treating cancer in the head and neck region with ensuing dryness and discomfort. The problems with oral health, eating, weight loss and sometimes total dryness and disappearance of salivary gland function reduce the quality of life. Although DNA is the lethal target, other structures in the cell have been proposed as sensitive targets. Nevertheless the mechanism is still obscure and further studies evaluating the mechanism of the glandular damage are of great importance in order to achieve methods to reduce the discomfort for the patients.

Irradiation -- normal tissue reaction

A radiotherapy regimen is limited by the sensitivity of the normal tissues with regard to early and late effects. The early changes begin with oral mucositis during the first weeks and include erythema, oedema and occasionally bleeding and pseudomembranes can occur as maximal side effects appearing about the fourth week or later. In certain cases the acute effects can be so troublesome that they cause interruption in the irradiation and questioning of the curative intention. The late effects in the oral mucosa, usually appearing at 6-24 months, are caused mainly by disturbances of the microvasculature and connective tissues (Rubin and Casarett, 1972). Atrophic mucosa, vulnerable for trauma and infections, can occur up to several years after the end of treatment. The atrophic mucosa can cause disturbing side effects even though the tumour is cured. These alterations are aggravated by the usually concomitantly depressed salivary gland function.

Salivary gland reaction

Radiotherapy of tumours in the head and neck region usually involves the salivary glands in the treatment volume and that decreases the salivary gland function. A sharp decrease in the flow rate occurs already in the first week with conventional fractions, i.e., 2 Gy/day (Dreizen et al., 1977; Mira et al., 1982; Shannon et al., 1978; Wescott et al., 1981), and continues to decline throughout the treatment period. When both parotid glands are affected by radiation to the maximal accepted dose to the normal tissue (MAD) (66 Gy) permanent xerostomia usually occurs (Cheng et al., 1981; Makkonen et al., 1987; Mira et al., 1982). The great sensitivity of salivary cells to irradiation is indeed
a unique radiobiological phenomenon (Junglee et al., 1986). Other well differentiated glandular cells (e.g., the pancreas) are known to be more or less radioresistant (Rubin and Casarett, 1972). The mechanism by which irradiation exerts its effect on salivary cells is not clearly outlined. It is generally accepted that in the case of radiosensitive, proliferating, immature cells, DNA damage causes mitotic delay and replication-linked cell death (Mitchell and Bedford, 1978; Munro, 1970). However, DNA damage can not solely cause glandular cell deaths (Farber Baserga, 1969; Lieberman, 1972), and irradiated salivary cells have been suggested to die in interphase (Stephens et al., 1986a; Stephens et al., 1986b). Cell membranes have also been proposed as an important target for injury leading to interphase cell death (Alper, 1977).

Salivary gland morphology and function

To better understand the effect of irradiation on salivary glands it is important to recall the normal structure and function (Junqueira and Carneiro, 1980).

Figure 1  The functional unit in a salivary gland consists of an acinus and a duct system

All the major and minor salivary glands have the same principal architecture; the salivary glands are divided into serous, mucous and seromucous types according to their secretory products. The glands are composed of distinct functional units (Fig. 1), and the secretory portion is composed of acinar cells, conducting intercalated ducts and striated ducts. The shape of a serous cell is pyramidal; it has ribosomal RNA in its basal region and secretory granules located at the apex (Fig. 2). The secretory granules are rich in proteins, glycoproteins, and amylase. The mucous acinar cell has principally the
same architecture as a serous cell but appears generally more "swollen" and the mucinous products push the nucleus basally.

![Serous acinar cell](image1)

Myoepithelial cells are found, surrounding the acini and the intercalated ducts. These cells are star-shaped and embrace the glands acini as an octopus would embrace a round boll. The presence of actin microfilaments similar to those of smooth muscle, as well as other muscle-like characteristics, suggests that these cells are contractile and may squeeze secretions out of the acinar cells. The presence of extensive lateral and basal membrane infoldings toward the vascular bed increase the ion-transport surface area 60 times, thus facilitating electrolyte and water transport during primary secretion (Fig. 3).

![Production of primary and secondary salivary fluid](image2)
The connective tissue which encapsulates the acini is of the reticular type and contains many plasma cells and lymphocytes. The plasma cells secrete an immunoglobulin, IgA, which may, in addition to other salivary components, constitute an immunologic defense mechanism against orally introduced pathogens. Surrounding the lumen of the intercalated ducts one finds small cuboidal cells, whereas the striated ducts are composed of more cylindrical cells with large nuclei. In the connective tissue the system ends with the excretory ducts, which are lined by pseudostratified epithelium that is gradually transformed into stratified squamous epithelium.

The function of the salivary glands is to produce saliva to moisten and protect oral mucosa and teeth, and to lubricate the food during mastication and swallowing. Saliva is composed of approximately 99% water, 0.5% inorganic ions and 0.1% protein. Human saliva consists of secretions from the parotid glands (25%), the submandibular glands (70%), and the sublingual glands (5%) in the resting condition (Junqueira and Carneiro, 1980). The primary saliva produced by the acinar cells has the same ionic composition as the isotonic blood but as the saliva progresses through striated and excretory ducts, the duct cells actively reabsorb sodium and excrete potassium. This explains why saliva is hypotonic with a higher concentration of potassium and a lower concentration of sodium than blood (Fig. 3).

One function of saliva is the hydrolysis of ingested carbon hydrates due to salivary amylase activity. The enzyme is secreted and released by the classical exocytotic route from exocrine cells. Proteins and glycoproteins are synthesized and stored in membrane-bound granules for storage in the cell. Stimulation causes discharge of the granule content into the acinar lumen after fusion of the membranes of the granules with the apical membrane (Butcher and Putney, 1980). The detailed effects of irradiation on the two main secretory processes, electrolyte/water secretion and exocytotic amylase release, have in fact never been outlined.

**Innervation**

Salivary gland function and integrity are mainly regulated by the two classical branches of the autonomic nervous system (Emmelin, 1981). The preganglionic sympathetic fibers arise in the spinal cord and end in the superior cervical ganglion. The submandibular ganglion, which lies close to the submandibular gland, supplies the parasympathetic fibers. Acetylcholine (ACh) is the classical neurotransmitter in the parasympathetic nervous system and has its major influence on the release of fluid and electrolytes. Sympathetic nerve stimulation or exogenously applied noradrenaline causes salivary
secretion with two separate responses. $\alpha_1$-adrenoceptor stimulation initiates release of fluid and electrolytes such as potassium (Butcher and Putney, 1980; Danielsson et al., 1988; Sundström et al., 1988), whereas $\beta$-adrenoceptor stimulation causes exocytotic enzyme release involving intracellular cyclic AMP as "second messenger" (Butcher and Putney, 1980; Carlsöö et al., 1981; Henriksson, 1982) (Fig. 4).

![Figure 4](image)

_Schematic illustration of the two main secretory processes in salivary glands._

NA, noradrenaline; $\beta_1$-adrenoceptor; $\alpha_1$-adrenoceptor

![Figure 5](image)

_Acetylcholine (Ach) and noradrenaline (NA) are "classical" neurotransmitters in the nervous system_

With the isolation and characterization of different neuronal peptides, a new group of transmitter molecules has emerged. These peptides often coexist with the "classical" transmitters (Hökfelt, 1986).

Substance P (SP) belongs to the tachykinin group of peptides. Tachykinins are involved in parasympathetic nerve-evoked salivation (Ekström et al., 1985; Ekström et al., 1988b) and act on the acinar cells of the submandibular gland (Iwabuchi et al., 1988). Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide (Amara et al., 1982) with a
wide distribution in the central and peripheral nervous system, particularly in the sensory neurons. CGRP is known to coexist with SP in a population of sensory neurons in salivary glands (Ekström et al., 1988a).

Vasoactive intestinal polypeptide (VIP) is a neuropeptide with a wide range of biological effects. Those include vasodilation (Said and Mutt, 1970), relaxation of smooth muscle and stimulation of secretion from several exocrine glands (Said and Mutt, 1972), including salivary glands (Lundberg et al., 1982). Postganglionic parasympathetic neurons innervating the submandibular gland have been shown to store and release vasoactive intestinal polypeptide (VIP), in addition to acetylcholine (Lundberg et al., 1980; Ekström et al., 1984) (Fig. 5). Neuropeptide Y (NPY) is widely distributed in the sympathetic nervous system, where it is colocalized with noradrenaline (Lundberg et al., 1982; Ekblad et al., 1984). Recently NPY has also been observed in portions of the parasympathetic nervous system (Forsgren, 1989). These observations raise the possibility that NPY acts as a neuromodulator in the parasympathetic as well as the sympathetic nervous system.

The tetradecapeptide bombesin has been isolated from amphibian skin. Bombesin-like peptides are present in the mammalian gastrointestinal tract (Erspamer and Melchiorri, 1975) and appear to have a direct action on pancreatic acinar cells (Christophe et al., 1977). Enkephalins (ENK), such as leu- and met-enkephalin, may function as neurotransmitters or as modulators of central and peripheral neuronal function. The endogenous enkephalins, produce analgesia similar to morphine (Belluzzi et al., 1976), exert crosstolerance and dependence to morphine (Waterfield et al., 1976). The distribution of met- and leu-enkephalin correlates very closely with the distribution of opiate receptors (Simantov and Snyder, 1976). ENK is present in the postganglionic parasympathetic neurons and nerve fibers of the submandibular gland (Soinila et al., 1991), but according to Kondo et al. (1988) ENK-like immunoreactivity is only transiently present in the parasympathetic nerve fibers and sympathetic fibers, during postnatal weeks 2 and 4 in the rat submandibular gland.

It is thus apparent that various neuropeptides are present in salivary gland parasympathetic innervation and that neuropeptides might induce or modulate secretory processes evoked by "classical" neurotransmitters and might cause changes in blood flow in salivary glands (Ekström et al., 1983, 1988; Gerstberger et al., 1988; Takeda and Krause, 1989). It would therefore be of great relevance to clarify whether irradiation influences the innervation pattern of salivary glands. Such studies have never previously been performed.
AIMS OF THE INVESTIGATION

The bilateral symmetry of salivary glands allows clinical and experimental studies on a gland from one side with the contralateral gland serving as a parallel control. Using this experimental approach the major aims of the present investigation were:

- To evaluate the early and late effects of fractionated irradiation on salivary gland morphology and function with special emphasis on the membrane-coupled fluid secretion and exocytotic enzyme release.

- To estimate the influence of irradiation on the expression of neuropeptides in the parasympathetic innervation of salivary glands.

- To characterize the salivary gland functional impairment and recovery in a group of consecutive, continuously followed patients irradiated for tumours in the head and neck region.
MATERIALS AND METHODS

Animals (I, II, III, IV, V)

White, albino, female rats of the Sprague-Dawley strain (8 week old and weighing approximately 200 g at the start of the study) (ALAB, Södertälje, Sweden) were used. They were fed water and chow ad libitum and kept on a diurnal light schedule. Ten days or 6 months following the last irradiation the rats were kept fasting for 18 h and were then sacrificed by an overdose of sodium pentobarbital.

Irradiation procedure (I, II, III, IV, V)

The rats were irradiated with x-rays from a medical linear accelerator (6 MV, 2.19 Gy/min; focus to skin distance 100 cm). The rats were anaesthetized by methohexital (Brietal®) and firmly fixed in a plastic mould during each irradiation period. The total radiation field was 8 x 20 cm when two rats were irradiated at the same time. When a unilateral technique was used, one side of the head -- the reference side -- was not irradiated, and was shielded with a thick lead block. The geometrical margins between the field boarders and the irradiated parotid were 10 mm in all cases and the distance between boarder and 95 % dose level was 6-7 mm. The minimum dose on the irradiated side was therefore at least 95 % of the prescribed dose. The dosimetry was checked with an ionization chamber in a rat-shaped plastic model and all scattering materials in the field were kept constant. The animals were irradiated with one of two different fractionation schedules. The first group of animals was irradiated on five consecutive days, Monday through Friday, with daily doses of 4, 5, 6, 7, 8 or 9 Gy for up to a total dose of 20, 25, 30, 35, 40 and 45 Gy. The second group was irradiated only twice, on Monday and the following Friday, with 12 or 16 Gy/dose up to total dose of 24 or 32 Gy. The endpoint time, 10 days, for early effects was chosen due to the clinical experience that hyposalivation is seen at that time in all cases. The erythema reaction seen on the animals was also most pronounced at 7-10 days following the last irradiation. However, at the present we have no data showing at which time after irradiation the maximal early effect on the rat parotid gland function is obtained. Some earlier studies have described degenerative processes at 60-180 days (Glücksmann and Cherry, 1962; Pratt et al., 1980) and in respect of this the analysis were performed 180 days after the last irradiation.
Rubidium Efflux (I, II, III)

The irradiated and the contralateral glands were rapidly excised and immersed in separate containers with basal medium consisting of a HEPES-buffered Krebs-Ringer solution supplemented with 5.0 mmol/l fumarate, 5.0 mmol/l glutamate, 5.0 mmol/l pyruvate and 1.0 mmol/l ascorbic acid. The pH was set to 7.40 and the gas phase was ambient air. The glands were dissected free of connective tissue under a stereo-microscope and cut into ten pieces weighing approximately 100 µg (wet weight) each. They were then pre-incubated in basal medium for 30 min at 37°C and preloaded in basal medium supplemented with 28 µm-86RbCl (10-15 TBq/mol) for 120 min. Specimens of each irradiated and control gland were processed separately and in parallel in each experiment.

Release of 86Rb+ in response to noradrenaline stimulation was studied by continuous perifusion. After preloading the pieces were rapidly washed in nonradioactive basal medium for 2 min and transferred to a perifusion chamber. The device consisted of polycarbonate membranes (8 µm pore size) in a Nucleopore 13 mm Pop-Top holder (Nucleopore Corp., Pleasanton, CA, U.S.A.). The peristaltic pump (Pharmacia Fine Chemicals, Uppsala, Sweden) and the media reservoirs were enclosed in a human infant incubator maintained at 37°C. The flow rate was 1.0 ml/min and fractions of the effluent were collected directly into scintillation vials. The medium was changed without interrupting the perifusion by using a three-way valve (Pharmacia Fine Chemicals, Uppsala, Sweden). The time for new medium to reach the chamber was 60 sec. Correction for this lag period has been made in the Figures. (For details see Danielsson et al., 1988; Sundström et al., 1985.)

Measurement of radioactivity (I, II, III)

The parotid tissue was freeze-dried overnight at -40°C, 0.1 Pa and dissolved in 100 µl Hyamine (Packard Instrument Corp., Downers Grove, IL, U.S.A.). The radioactivity of the effluent fractions and the tissue samples was measured by liquid scintillation spectrometry using Aquasol (NEN chemicals GmbH, Dreieich, Germany) as the scintillation medium. Triplicate samples from each labelling medium were used as external standards. Values are expressed as percentages of the mean efflux rate during minutes 11-15 (prestimulatory period). The experiments were performed in parallel with irradiated and control glands from the same animal (Danielsson et al., 1988; Sundström et al., 1988).
Exocytotic enzyme release (I, III)

Pieces of parotid tissue (approximately 5 mg each) were incubated in a Krebs-Henseleit bicarbonate buffer supplemented with pyruvate, glutamate and fumarate (5 mmol/l each), 1 g/l bovine serum albumin (BSA) and 0.6 g/l glucose. The medium was pretreated to 37°C and gassed with 95% O₂ and 5% CO₂. All pieces were preincubated for 15 min in 500 μl, rinsed and then incubated for 30 min in 500 μl of medium with different secretagogues in a shaking water bath. Control incubations without added drugs were included in each set of experiments. At the end of the incubation the wet weight of each tissue sample was recorded and the specimens were sonicated (Branson Inc., 50 W, 10 s) in a sodium-potassium-phosphate buffer (50 mmol/l, pH 6.9). Controls and irradiated glands were incubated in parallel (Carlsöö et al., 1981; Henriksson, 1982).

Amylase assay (I, III)

Incubation media and tissue homogenates were appropriately diluted with 50 mmol/l phosphate buffer (pH 6.9) and assayed for amylase activity using a micromodification of the 3,5-dinitrosalicylate method with 2% soluble starch as substrate (Henriksson, 1982; Sundström et al., 1988). One unit of amylase was defined as the activity liberating reducing groups corresponding to 1 μmol of maltose monohydrate per minute at 25°C. Amylase release was expressed as the percentage of amylase released into the medium in relation to the total amylase activity in medium plus homogenate.

Quantitative morphological examination (I, III)

Immediately after removal from the rat the parotid gland specimens were fixed in glutaraldehyde. After rinsing in buffer (0.2 N phosphate buffer) the salivary gland specimens were post-fixed in 1% osmium tetroxide in the same buffer. After a cold buffer rinse the specimens were dehydrated in graded ethanol solutions and embedded in epoxyresin. Semithin (1 μm) sections, cut on an LKB Ultrotome, and stained with toluidine blue, were used for light microscopical analysis and morphometry. The latter was performed by using an ocular with a 10 x 10 grid mesh and counting "hits" in the crossings. One-to-five hundred points in each section were counted (Weibel, 1979). Parotid gland specimens from irradiated glands and controls were fixed and embedded in parallel (Henriksson, 1982).
Immunohistochemical analysis of neuropeptides (IV, V)

The submandibular glands with adjacent tissue and the trigeminal ganglia were excised and fixed by immersion overnight at 4°C in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0. Thereafter the specimens were thoroughly washed in Tyrode’s solution, containing 10% sucrose, at 4°C overnight. The specimens were then mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, Ill) and frozen in propane chilled with liquid nitrogen. Specimens of submandibular glands of bilaterally irradiated and control animals were mounted together, as were specimens of the right and left submandibular glands of unilaterally-irradiated animals. Trigeminal ganglia of bilaterally irradiated and control animals were also mounted together.

Extensive series of 8-10 μm thick sections were cut using a cryostat. The sections were mounted on slides pre-coated with chrome-alum gelatin, dried and processed for immunofluorescence or stained for the demonstration of acetylcholinesterase activity (AChE) (Forsgren, 1986) or NADH-tetrazolium reductase (NADH-TR) (Dubowitz and Brooke, 1973). The sections processed for NADH-TR served as a reference for tissue morphology.

The immunohistochemical procedures were as described by Forsgren and Söderberg (1987). The sections were incubated for 30 min in a 1 % solution of detergent Triton X-100 (Kebo Lab, Stockholm, Sweden) in 0.01 M phosphate buffer saline (PBS), pH 7.2, containing 0.1 % sodium azide as preservative, rinsed in PBS, and incubated in 5 % normal swine serum in PBS supplemented with 0.1 % BSA for 15 min. The sections were then incubated in a humid environment with the primary antibody which was diluted in PBS with BSA,. Incubation was performed for 60 min at 37°C. After incubation with specific antiserum and after washing 10 min in PBS the sections were immersed in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark), diluted 1:40, for 30 min at 37°C in a moist chamber, washed in three changes of PBS, mounted in glycerol: PBS (1:1) and examined under a Leitz Orthoplane Photomicroscope equipped with epifluorescence optics. The specificity of the antisera was tested by incubating control sections with antisera preabsorbed with 10-20 μg of the respective peptide (Sigma) in 1 ml of antiserum.

Patients and irradiation

Twenty-five patients treated at the department of Oncology, University Hospital, Umeå, Sweden, during 1985-89 for malignancies in which radiotherapy involved at least one
parotid gland were included in the study. Informed consent was obtained from each patient. Sampling of saliva was made before the start of radiotherapy, every week during the irradiation period, and regularly after finishing the treatment period. All patients were checked by the same physician and dentist. In all patients the parotid glands were exposed to at least 95 % of the given dose as calculated by the isodose curve.

The details of the patients included and the treatment schedules used are shown in Table 1 in paper VI. As can be seen the irradiation delivered varied with the diagnosis according to generally accepted treatment strategies. Three main groups are outlined: one group given less than 46 Gy, another group of patients which were given 47-52 Gy, and a third group treated with full dose (MAD) (65 Gy or more). The first group included 14 parotid glands (8 patients), the second group contained 11 parotid glands (8 patients) and the MAD group 14 parotid glands (9 patients). Both parotid glands were irradiated in 14 patients and in 11 patients a unilateral technique was used in an attempt to protect the contralateral parotid gland from irradiation.

The irradiation treatment was performed with linear accelerators 4-6 MV, with opposed lateral, posterior-anterior or oblique fields with fixed-SSD or isocentric techniques. The target doses were between 1.45 and 2.37 Gy daily with a dose rate of 2.2 Gy/min, a focus to skin distance 80 cm (4 MV) or 100 cm (6 MV) and delivered with 5 fractions a week. To avoid doses exceeding 42 Gy to the spinal cord electrons were used with energies from 10-18 MeV (Microtron, Scanditronix).

Collection of saliva (VI)

Stimulated parotid saliva was collected prior to radiotherapy and weekly during treatment. Samples were also taken 2, 4, 6, 12 and 18 months after the end of radiotherapy. Parotid saliva was collected with Lashley cups which were placed over the orifice of Stenson's duct. Stimulation was carried out with a saliva stimulating tablet (Salix Pharma, Sweden), placed on the tongue. An aliquot of 1 ml saliva was collected between 9-12 a.m. and the collection time never exceeded 20 minutes. Whole saliva stimulated by chewing on paraffin was collected before radiotherapy.

Appearance of dryness (VI)

Subjective description of dry mouth conditions were recorded as a part of the patient interview. Distinction was made between lack of dryness (-), a slight dryness (+) which mainly occurred occasionally at night, and severe dryness (++).
RESULTS AND DISCUSSION

Early effects (I, II)

A dose-dependent effect of fractionated irradiation on potassium fluxes (\(^{86}\)Rb\(^+\) fluxes) was demonstrated in rat parotid gland as compared with contralateral controls when analyzed 10 days after the last irradiation. On the other hand the exocytotic amylase release displayed no obvious alterations, and morphologically no changes were seen at the light microscopical level.

Late effects (III)

The noradrenaline-stimulated \(^{86}\)Rb efflux 180 days after irradiation showed no obvious difference after the lower dose at five-day fractionation (total dose 35 Gy). In all other groups the \(^{86}\)Rb efflux was markedly reduced as compared with the contralateral side. It might have shown a more pronounced effect for total doses over 45 Gy given as 5 fractions/week or 32 Gy given as 2 fractions/week but these doses are close to the maximum dose the animals can survive with respect to early reactions.

Noradrenaline stimulation caused a significant secretion of amylase in both controls and in the irradiated groups. The amylase content and concentration in the irradiated gland specimens varied between individual animals but were, however, radiation dose-dependently decreased (Table 2 in III). The relative amylase content per acinar cell volume (amylase content/percentage of acinar cell density) was, however, higher in the irradiated side as compared with the control gland.

Morphologically a minor loss of acini was seen after 35 Gy and the changes were unevenly distributed. The acini were replaced by small duct-like structures and scattered amounts of fibrous stroma. Following 45 Gy a massive loss of acini was found (85 %) (Table 1 in III). In a few cases only one or two acini per mm\(^2\) were identified. The remaining acini were disorganized and were usually considerably larger than normal acini. The acinar cells otherwise had a normal configuration with huge amounts of secretory granules, often larger than in normal acinar cells. The ducts were generally better preserved. In certain areas only ducts were seen surrounded by fibrous stroma. Striated ducts appeared generally unaltered, whereas intercalated ducts often seemed to have wider than normal lumens.
Ultrastructurally the cells of the remaining acini sometimes appeared normal and were packed to a very high degree with secretory granules. However, the overall organisation of the acinus was altered with up to 30 acinar cells in a single acinus cross-section (Fig. 6). The acinar cells were in several layers and some cells seemed to be devoid of a secretory surface. Occasionally the lumen seemed bordered by intercalated ducts in the center of the acinus. In other areas acini were completely lost, and cells in various stages of degeneration and cell-death were seen. Swollen mitochondria, dilated endoplasmic reticulum, and all degrees of nuclear deterioration could be seen in cells neighbouring the well-preserved cells.

Figure 6  
Low-power electron micrograph showing acinar cells from irradiated gland (180 days, 45 Gy) packed with secretory granules, cells lying in a disordered manner, x 200. Franzén et al., unpublished data

In salivary glands the epithelial acinar cells belong to the renewing cell populations. Maximal life span is estimated as high as 2-4 months for tubular and acinar cells (Zajicek et al., 1985). Therefore, assuming that the lethal effect of irradiation is coupled to DNA replication and thus seen first after the expected mitosis, it would take a rather long time before the cellular architecture and content are affected. This can be valid for the present results since the observed changes in morphology were seen only in the late stage (180 days) and not in the early evaluation (10 days following irradiation). The morphological changes were concomitantly followed by alterations in amylase content (stored in secretory granules), i.e., a decrease was only seen 180 days after termination of irradiation. On the other hand, unlike most other cells, parotid acinar cell death
following irradiation has been denoted as an interphase death. The detailed mechanism is still unknown. Interphase death represents the sole mode of death of cells which are not capable of continuous cell division or are arrested in an extended G1 phase. Cellular targets other than DNA, for example plasma membranes, have been proposed as primary targets for the damage leading to interphase death (Alper, 1977; Altman et al., 1970; Desai et al., 1964; Wills and Wilkinson, 1966). Hence, it is plausible that parotid acinar cells are lethally damaged by disturbances in the cell membrane in addition to DNA injury.

**Cell membrane**

Ionizing radiation can produce a variety of changes in cellular structures and metabolism that may in turn cause abnormal cell function and/or cell death. The biochemical target effects are believed to involve nucleic acids, such as DNA and RNA. Double-stranded DNA breaks are the main theory of the lethal effects of radiation, but evidence for primary damage to the cell membrane can be obtained from our results which displayed changes in the noradrenaline-evoked $^{86}$Rb efflux. Note that the affected potassium efflux was the first processes changed, and preceeded all the other events analyzed, i.e., morphology and enzyme content. The sustained secretory process is ATP-dependent and requires activation by $\alpha_1$-adrenoceptors. All these initial events are produced in the cell membrane. Thus, as has been observed for several alkylating and redox-active chemicals that affect intracellular ion homeostasis through the inhibition of transport-ATPases in the plasma membrane, the initial disturbances of irradiation may be evoked in the plasma membrane. Indeed, earlier results suggested that impairment of membrane permeability is the primary cause of interphase death due to a rapid loss of sodium and potassium (Creasy, 1960; Myers, 1970). Effects on efflux of potassium and influx of sodium following irradiation have been seen within hours in different experimental systems (Dowben and Zuckerman, 1963; Merrick and Bruce, 1965; Shapiro et al., 1966; Sutherland et al., 1967). Moreover, in our studies the increased leakage of amylase, i.e., basal non-stimulated release (Fig. 2 in paper III), may further suggest an altered membrane structure with increased permeability following 5 x 9 Gy. Radiation-induced disturbances in ionic and water homeostasis in intact cells, somewhat in contrast to later appearing lysosomal, enzyme leakiness (Wills and Wilkinson, 1966), are early events and are elicited by relatively small doses of X-rays. Excessive leakiness of cells to K$^+$ and influx of Na$^+$ and water have been observed soon after irradiation of a variety of cells including yeast (Merrick and Bruce, 1965), bacteria (Shapiro et al., 1966), human red blood cells (Sutherland et al., 1967), and rat muscle cells (Dowben and Zuckermann, 1963). Moreover, destabilisation of ionic
homeostasis has also been observed following other types of injury and has been extensively documented in the case of toxic and anoxic cell death in the liver (Judah et al., 1974) and heart muscle. On the basis of these observations it has been postulated that lethal damage probably results from a sequence of events common to all or most non-dividing cells. Specifically, it has been suggested that membrane injury and a consequent uncontrolled accumulation of Ca\(^{2+}\) is the common pathway leading to cell death (El Mofty et al., 1975; Farber and El Mofty, 1975; Judah et al., 1974).

The mechanisms behind the differences in sensitivity to irradiation between the noradrenaline-stimulated \(\alpha_1\)-adrenoceptor-mediated potassium efflux and \(\beta_1\)-adrenoceptor exocytotic amylase release can only be matter of speculation. Although these two processes are initially activated by membrane receptor-coupled events, they are two clearly separate entities as outlined schematically in Fig. 4. One explanation could be that irradiation-induced free oxygen radicals cause oxidation of membrane lipids involved in the regulation of potassium channels. Moreover, potassium homeostasis requires intact transport mechanisms for ions; i.e., free passage of ions through cell membrane, whereas the production of enzymes such as amylase need at least an intact mechanism of protein synthesis, including transcription. Thus, the findings of unaffected amylase secretion and morphology may further point out the cell membrane as a plausible target for the early events of irradiation.

Nervous system -- peptidergic innervation

The submandibular gland, including the postganglionic parasympathetic cells, was subjected to immunohistochemical examination with respect to expression of the neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP), bombesin, leu-enkephalin (leu-ENK), neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP).

The pattern of SP-immunoreactivity in the ganglionic cells was greatly changed in animals subjected to bilateral irradiation, the pattern being almost the same 10 days and 180 days after treatment. A large number of nerve fibers showing SP-like immunoreactivity (SP-LI) was observed in the parenchyme in bilaterally-irradiated animals 10 days after treatment but at 6 months only a few such fibers were seen. Furthermore, only a few fibers showing SP-LI were observed in the parenchyme in animals in the control group and animals subjected to unilateral irradiation and examined 10 days or 6 months after irradiation (Table 1, Fig. 7a, b). The pattern of
CGRP-immunoreactivity in the ganglia and parenchyme of bilaterally-irradiated animals corresponded to that seen in control and unilaterally-irradiated animals.

Figure 7  The submandibular gland of a control animal (a) and an animal subjected to bilateral irradiation and examined after 10 days (b). The sections were processed for SP. Clearly there is a larger number of immunoreactive nerve fibers in (b) than in (a)

Figure 8  Submandibular ganglia of the irradiated side of a unilaterally-irradiated animal (a) and a bilaterally-irradiated animal that was examined after 10 days (b). The sections were processed for leu-ENK. The ganglionic cells show a marked leu-ENK-like immunoreactivity in (b)

A markedly enhanced expression of bombesin- and leu-ENK-LI in the ganglionic cells and an increase in the number of nerve fibers showing these immunoreactivities in the submandibular glandular tissue following bilateral irradiation in the sense of short term effects were seen (Table 1, Figs 8a, b). The changes were clearly less pronounced 180 days after irradiation. On the other hand, no changes in the pattern of VIP- and
NPY-immunoreactivities occurred. Thus, alterations in the expression of certain neuropeptides occurred in the submandibular gland and its associated ganglionic cells in response to bilateral irradiation of the head and neck region. Such changes have never previously been reported after irradiation, and may help to explain the inherent radiosensitivity of salivary glands.

Table 1  The relative frequencies of detectable nerve fibers in the parenchyme of the submandibular gland in control animals and 10 days after bilateral irradiation

<table>
<thead>
<tr>
<th>Substance</th>
<th>Control animals</th>
<th>Bilaterally-irradiated animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>Leu-Enk</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>Bombesin</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>CGRP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VIP</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>NPY</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = few or occasional fibers  
+ + = substantial number of fibers  
+ + + = large number of fibers

The presence of neuropeptides in the trigeminal ganglion which supplies the submandibular gland with the majority of its sensory nerve fibers was examined and no alteration was seen for the analyzed neuropeptides in respect to both early and late effects. The motor neurons of the cervical spinal cord also showed an unchanged pattern of neuropeptide expression after bilateral irradiation of the head and neck region (unpublished data). In the future a more detailed analysis of the sympathetic nervous system in response to irradiation should be performed even if the observations of an unaltered NPY-expression suggest that this system may not be affected to the same degree as the parasympathetic nervous system.
It is well known that parasympathetic nerve stimulation to salivary glands releases neuropeptides (Ekström et al., 1985). Therefore, the observations in the present study of changes in the expression of leu-ENK, SP and bombesin in the ganglionic cells and the nerve fibers in the glandular tissue may be of importance from a functional point of view in the situation examined, i.e., irradiation-induced effects on salivary gland function. Consequently, it is plausible to believe that there is a relationship between these changes and the altered salivary function which has been shown to occur in the same animals (I). As far as we know, no studies on the effects of SP, ENK, or bombesin in salivary glands have been performed following irradiation. Interestingly it has been shown that the bombesin family of peptides acts as growth factors in human tumors such as small cell lung carcinoma. Therefore our observations of an increase in bombesin expression after irradiation may be important from a clinical point of view in the aspect of radiotherapy for this group of patients (Thomas et al., 1991).

The difference in effects of irradiation with regard to unilateral (not affected) and bilateral treatment fields is not clearly understood. In animals subjected to unilateral treatment the contralateral side is not directly affected by irradiation and this may suggest that some compensatory mechanisms take place, which in turn "normalizes" or stabilizes the complex machinery of the autonomic nervous regulation of salivary gland function.

Radiobiological model evaluation (II, III)

The effects of irradiation with respect to early effects on the noradrenaline-stimulated efflux is dose dependent. In a more detailed radiobiological analysis of the results, the dose-incidence relationships could be given by the formula (Lange and Gilbert, 1968):

\[ P = \exp (-N \cdot \exp(-D/D_0)) \]  
(Eq. 1)

The inner exponent of this formula describes the surviving fraction of tissue-rescuing units, N, after dose, D. The dose D_0 reduces the assumed cell survival to 37%. This description is based on the common assumption that injury could be seen as a random inactivation of available targets in the tissue. If we further assume that the probability of tissue injury could be described by a similar model, then P in the equation above will give the probability that no tissue rescuing units will survive. This model gives a sigmoidal dose-response curve and is commonly used to describe the effect of irradiation on biological systems. The slope of this curve can be explained by heterogeneity of the responding tissues (Moore et al., 1983).
In order to make optimal use of our experimental results we made one further assumption about the dose-response curve. The model in use (Eq. 1) contains two parameters which make it possible to fit this model to experimental results. In our material we gave irradiation by different fractionation regimes. If we assume that the parameter \( N \) in Eq. 1 is proportional to the initial number of colony-forming cells (Moore et al., 1983) it should be a feasible approximation to keep this factor constant for the different treatment regimes. This would then limit the errors in the fitting procedure.

A commonly used model to interpret the iso-effect doses for different fractionation regimes is the so-called linear-quadratic (LQ) model (Douglas and Fowler, 1976):

\[
surviving\ \text{fraction} = \exp(-n(\alpha d + \beta d^2)) \quad (\text{Eq. 2})
\]

\( n \) is the number of fractions, \( d \) is the dose per fraction and \( \alpha \) and \( \beta \) are constants. This basic formula has been rewritten by pure algebraic operations and applied clinically to give a simple description of fractionation effects in radiation therapy (Barendsen, 1982; Thames and Hendry, 1987). A linearisation method to calculate the tissue and injury dependent \( \alpha / \beta \)-ratio has been described (Thames et al., 1982):

\[
1/D_n^{50} = (\alpha / E) + (\beta / E) \left(D_n^{50} / n\right) \quad (\text{Eq. 3})
\]

Where \( D_n^{50} \) is the dose that will give 50\% effect at \( n \) fractions and \( E \) is a constant factor which does not need to be solved. By the knowledge of the dose-response curve shape, as discussed above, \( D_n^{50} \) can be calculated from experimental data.

An \( N \)-value of 200 was estimated according to the curve shape and used in this data transformation procedure. Data have been plotted according to this formulation and linear approximations of the data can be made.\( \alpha / \beta \) is then given by the negative value of the intercept of the dose per fraction axis and gives an estimate value 22 Gy \pm 9 Gy (mean \pm S.D.). The data on late effects of potassium efflux do not show a significant dose-effect relationship in the investigated dose interval. It might have shown a more pronounced effect for total doses over 45 Gy with 5 fractions/week or 32 Gy with 2 fractions/week but these doses are close to the maximum dose that can be accepted with respect to early reactions. Subsequently, an \( \alpha / \beta \) ratio could not be calculated with reasonable accuracy.
The biological response to radiation (180 days) for the acinar cells has been calculated by comparing the irradiated and the non-irradiated glands of the same animal. Dose-response data were obtained with a high degree of significance and a rather simple mathematical model of cell survival described by the linear quadratic model (Douglas-Fowler, 1976) and the expression for probability of tissue injury (P) (Lange and Gilbert, 1968), a three parameter model, is obtained. The injury is then described by the equation:

\[ P = \exp(-R) \cdot \exp(-n(\xi d + \beta d^2)) \]  

(Eq. 4)

where R is a parameter proportional to the number of tissue rescuing-units (calculated to 52.6). In the analysis of different fractionation regimes we assume that the parameter (R) is characteristic for the tissue and not sensitive to fractionation regimes. From this model all parameters such as \( \xi, \beta, \xi/\beta \) and R were obtained by curve fitting according to the statistical model described by Nash (1987) and an \( \xi/\beta \) was estimated to 9.6 Gy \( \pm \) 2.0 Gy (mean \( \pm \) S.D.) for acinar cell for late effects (180 days). The error estimation for these parameters will be more straightforward using this model compared to a two stage model (Thames et al., 1986).

Clinical study (VI)

The inherited radiosensitivity, especially of parotid glands, is manifested by our results as well as others as a very early sign of decreased salivary flow seen already within days (Eneroth et al., 1972; Kashima et al., 1965; Mossman, 1983; Parsons, 1984; Mira et al., 1982; Marks et al., 1981; Wescott et al., 1978). The effects are clearly dose-related. At higher doses (>64 Gy) most glands in our study were without secretory capacity up to 12 months following the end of radiotherapy. In doses below 52 Gy almost all glands regained some function within 18 months. Nevertheless, it must be stressed that there is a great interindividual variation in the long term-effects of irradiation. Moreover, our results did not point out any obvious correlation between the used target dose (1.5-2.35 Gy) and the impaired parotid gland function at about the same total dose. Signs of recovery in the secretory rate were seen within 2 months in the lower doses (\( \leq 45 \) Gy). In a small number of patients a recovery was observed also in the higher doses at later stages. Earlier results indicate a salivary gland function recovery as late as five years after irradiation (Makkonen and Nordman, 1987). It must, however, be emphasized that no base-line pretreatment data were obtained in that study. It is tempting to speculate that the recovery seen in the clinical situation has explanation
from the suggestion in our animal studies where acinar cells have a potential regenerative capacity (Fig. 6).

Reports of discomfort with dryness during and following the irradiation were just as frequent among patients with high initial secretion rates as among the patients with low initial salivary flow when stimulated by chewing. Thus, no strict correlation seems to exist between the pretreatment baseline salivary secretion rate and the irradiation-associated dryness. All patients experienced dryness with variation in duration and severity during and following irradiation. The inconsistency between saliva flow and the dryness reported by the patients can be due to preserved function of the minor salivary glands. The secretion from the minor salivary glands in the mucosal membrane is of importance for the lubrication of the oral mucosa. From this point of view protection of the major salivary glands from irradiation should have a limited impact upon the problem with dry mouth. However, such protection is motivated by the importance of the secretion from the major salivary glands in maintaining oral health and food intake. More studies are of interest in evaluating the response of the minor salivary glands to therapeutic irradiation.
GENERAL CONCLUSIONS

Early effects

An early dose-dependent effect of fractionated irradiation on potassium fluxes ($^{86}$Rb$^+$ fluxes) was demonstrated in rat parotid glands as compared with contralateral controls as analysed 10 days after the last irradiation. On the other hand, the exocytotic amylase release displayed no obvious alterations, and morphologically no changes were seen at the light microscopical level.

Late effects

The noradrenaline-stimulated electrolyte secretion was decreased at least after the higher doses of irradiation.

Amylase content was radiation dose-dependently decreased. However, amylase secretion expressed as a percentage of glandular content was unaffected.

Loss of acini was also dose-dependently decreased.

Cell membrane

It can be speculated that the altered cell membrane-coupled potassium transport, as both early and late effects, can be a sensitive target in the radiation-induced damage. Thus, structures other than DNA may be radiation-sensitive targets.

Neuropeptides

Bilateral irradiation enhances the expression of the neuropeptides, substance P, leu-enkephalin and bombesin, and thus suggests that these aspects must be emphasized in the discussion of the mechanisms of action in radiation-induced damage with respect to salivary gland function.

It is obvious that, in addition to DNA, nongenetic targets such as the regulatory mechanisms for ion transport and neuropeptides may be targets in radiation-induced effects. The chronology and mode of injury to DNA and nongenetic macromolecules can be different. A lethal induced DNA damage is seen when the cell enters mitosis. The time lag from irradiation to the detection of chromosomal damage is to a major part dependent on the mitotic rate of the actual tissue. The effects on nongenetic constituents cause biophysical changes with immediate consequences but probably less ultimate lethality. Enhanced permeability of cell membranes can thus induce momentary influences on cell functions.
Clinical

Our prospective study of parotid gland function in patients subjected to irradiation of head and neck cancer demonstrated a great interindividual variation in the recovery with regard to salivary flow rate, and which was not correlated to the patients’ discomfort with dryness. Irradiation doses about 40-50 Gy caused generally reversible changes with sometimes almost a restored function of salivary secretion within 6-18 months following the end of radiotherapy. Doses exceeding 65 Gy generally induced irreversible alterations.
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