Abstract. Aim: Salivary glands (SG) can become atrophic following radiation exposure. Malignant transformation of SG in a radiation field is another known sequela of patients who have been treated by radiotherapy for a malignant tumor in the head and neck region. The aim of this study was to investigate cytogenetic alterations and to determine the proliferation index (PI) of SG of rats subjected to various total dosages of fractionated X-rays. Materials and Methods: We investigated rat SG, subjected to 20, 40, or 60 Gy exposure by X-rays to the left neck and skull base. Non-irradiated rats served as a control group. Tumors originating from the SG were histologically-diagnosed following the descriptions for human SG tumors. The MIB-5 antibody was used to determine the PI. The ploidy was determined by flow and image cytometry (FCM, ICM). Results: We consistently recorded diploid histograms in the FCM in irradiated glands. ICM revealed aneuploid histograms in 6/22 tumors, 3 of them were Auer Type III or IV. The PI showed a dose- and time-dependent course, indicative of variable regeneration properties of the parenchyma. Statistically significant differences were found for the PI within the irradiation groups and comparing irradiated SG and tumors. Conclusion: Irradiation of rat SG can cause almost complete loss of function. On the other hand, the PI remained in animals subjected to 40 Gy and investigated 1 year after completion of radiation at a level up to 10-fold higher than in untreated controls. The PI in carcinoma is higher in this species than in irradiated SG. Constantly elevated PI could support the development of cancer in SG.

Salivary glands can become inflamed and later on atrophic following radiation exposure (18). On the other hand, malignant transformation of salivary glands in a radiation field is a known sequela of patients who have been treated by radiotherapy from a malignant tumor in the head and neck region (20, 45), who were treated with radiotherapy in the head and neck region for benign conditions (49), or who survived atomic bomb explosions (58). Functional and morphologic lesions following radiotherapy were repeatedly recorded (37, 40, 45, 49, 51, 56). Further on, exposure to X-ray sources in dental practice has come under scrutiny for causing some salivary gland tumors (47, 48). Data from several studies suggest that in malignant tumors the proliferation activity and the DNA content are positively correlated (6, 7, 42, 52). The aim of this experimental study was to investigate cytogenetic alterations and to determine the proliferation of salivary glands of rats subjected to various total dosages of fractionated X-rays (2, 3, 8, 17, 19, 26, 46, 62, 63). Rat salivary gland tumors were included in the study in order to compare cytogenetic alterations in irradiated salivary glands and salivary gland tumors (9). This model of fractionated irradiation in rats has been proven to be suitable for the study of irradiation effects in vivo (10-15).

Materials and Methods

We investigated rat salivary glands [submandibular and sublingual glands (28)] of 56 animals, subjected to 20, 40 or 60 Gy exposure of roentgen rays to the left neck and skull base. The female Wistar rats (Charles River, WIGA) had body weights ranging from of 250g...
to 350g at the beginning of the experiments. The irradiation experiments were performed according to the principles of laboratory animal care and were repeatedly approved by the local ethics committee of Hamburg University, Germany (No. 3/94-3/96).

Irradiation. The equipment for irradiation of small animals and the running of the experiments were described elsewhere in detail (24). The fractionation protocol was adopted from human therapeutic regimes for the treatment of head and neck cancer (59). The irradiation field allowed the restriction of radiation damage to the region of interest, avoiding inflammation of the whole upper aerodigestive tract and impairment of nutrition. The low impact of these experiments on the body weight of Wistar rats as a parameter of constant nutrition intake has been recently published (25).

Further on, this small irradiation field allowed the intraindividual comparison of shielded glands and those directly exposed to the central beam of the roentgen ray source (10-15).

Salivary glands. Salivary glands (both sides) of non-irradiated rats served as a control group (Table I). The glands of the shielded side of irradiated rats were investigated for scattering irradiation, doubling the number of rat salivary glands from irradiated animals. We investigated both glands that are situated below the mandibular corpus and are closely attached to each other, the submandibular and the sublingual gland. The radiation effects on the rat salivary glands of this study have been described in detail (10-15). Tumors originating from the salivary glands were collected according to the macroscopic appearance and were histologically diagnosed following the descriptions for human salivary gland tumors (Table II). These tumors were recently described in detail (9, 14).

Histology. Seifert’s classification of radiation damage in human salivary glands was used for histological classification of the radiation injury in rat salivary glands (18, 53, 54).

Immunohistochemistry. A Ki-67 antibody reactive on formalin-fixed, paraffin-embedded rat tissue (MIB-5, Molecular Research Institute, Borstel, Germany, provider: Dianova, Hamburg, Cat.-No. DIA 5055, Lot No. 8/98) was used to determine the proliferation index (PI) in salivary glands. Four-micron-thin sections were mounted with Histo-Bond® on Super Frost/plus™ adhesion slides (Fa. Menzel-Gläser). The sections were deparaffinized and rinsed in distilled H2O. Then the slides were incubated with citrate buffer (pH 6.0; 1:21 g/l) in a pressure cooker for 4 min. The endogenous peroxidase was blocked with H2O2 (3%) for 20 min. Following the incubation with the MIB-5 antibody (1:30), goat anti-mouse IgG (Dianova, Hamburg, Germany No. 115-035-062, 1:20) and then rabbit anti-goat IgG (Dianova, No. 305-035-045, 1:50) were applied. The antibodies were diluted with TBS/FCS 10% at room temperature. The sections were rinsed with TBS buffer after every incubation with any agent (TRIS 1.21 g/l, pH 7.4-7.6, Merek, Darmstadt, Germany No. 8382; NaCl 8.78 g/l, Merek No. 6404).

The urea hydrogen-peroxidase reaction was used for the detection of the antigen-antibody reactions (Sigma Fast/DAB peroxidase substrate tablet set, No. D 4168, Sigma, Deisenhofen, Germany). Hemalaun according to Mayer (Merek, No. 940341684) was used for colouring the sections. The principles of this immunohistochemical technique and the reliability of the staining results were recently confirmed by Ito et al. (34).

MIB-5 positivity of cells was determined as the brownish coloration of nuclei but not of the cytoplasm. One thousand cells per salivary gland were evaluated in a meander-like pattern. The numbers of positive cells were recorded in percent.

Determination of the DNA content. For quantifying the DNA content we used both flow-cytometry (FCM) and image-cytometry (ICM). In order to attain comparability of the results determined by two imaging methods, sections of the same paraffin blocks were cut one after the other.

FCM. Thirty-μm-thin paraffin slices were dewaxed with xylol and degrading alcohols, then dissolved in a pepsin solution (5.8 ml 1N HCl and 100 mg pepsin in 100 ml Aqua dest.) at 37°C for 1 hour. A cell suspension was then produced with a Pasteur pipette, put through a gauze into a centrifuge test tube. The suspension was centrifuged at 900 rotations per minute for 5 min. The supernatant was discarded, the pellet was dissolved in 800 μl PBS (pH 7.4) and put into an Eppendorf receptacle. One hundred μl RNase (1 mg RNase/ ml PBS) and 100 μl propidium iodide solution (0.4 mg/ml H2O) were added to the receptacle. The suspension was mixed and incubated at 37°C for 30 min. The samples were investigated with the Ortho Cytoron Absolut (Fa. Ortho-Diagnostics). The principles of FCM are described elsewhere in detail (7, 16, 23, 27, 29-31).

The DNA-index (DI) was determined as the mean value of the quotient of the aneuploid peaks and the diploid values. The internal standards for the diploid peaks were determined on diploid cells in each sample (leukocytes, stroma cells). No

| Table I. Number of animals, explantation of the glands after different total dosages of fractionated roentgen rays exposure and different explanation times (50 animals). |
|-----------------|--------|--------|--------|--------|-----------------|
| Time            | 20 Gy  | 40 Gy  | 60 Gy  | Non-irradiated |
| 6 Months        | 7      | 7      | 8      | 5      |
| 12 Months       | 9      | 10     | 10     | 5      |

| Table II. Tumors following irradiation of the left neck and skull base region in Wistar rats. |
|-----------------------------------------------|-----------------|
| Diagnosis (in analogy with human tumors)      | Number          |
| Adenocarcinoma                               | 7               |
| Fibrosis/Kuettner’s tumor                    | 4               |
| Sclerosis/Atrophy                            | 7               |
| Squamous cell carcinoma                      | 5               |
standardized method currently exists for the evaluation of the histograms. Auer’s classification (6, 7) was used for this study, i.e. the graphical distribution of the values was assigned to one of the 4 types of histograms.

ICM. Seven-µm-thin slices were fixed on silane-coated slides, dewaxed, rehydrated and rinsed. Then the hydrolysis was performed with 1N HCl (15 min, 60°C). The slides were rinsed 3x in Aqua dest. and then incubated with Schiff’s reagens at 20°C for 90 min. Then the slides were washed for 2 min in a natrium sulfite solution (1%), rinsed in Aqua dest. for 30 min and then were dehydrated in graded ethanols and xylol. Finally the sections were covered with Eukitt. The Feulgen-stained sections (60) were investigated with a Reichert-Jung Polivar microscope (green filter, spectral area about 546 nm) with a 40x magnification. The section area depicted in the visual field was transformed with a video camera on a monitor. The DNA-content was calculated from the grey scales, using the Ahrens Cytometry Analyse System. The calibration of the DNA scale with the colour intensity of the individual section was performed on a reference section prior to any measurement. The internal standard was determined after photometry of 20 fibrocytes. Every salivary gland was independently investigated. The section was scanned in a meander-like route. In every sublingual gland 200 cells and in every gland 100 cells were analysed. In tumors, representative areas were chosen and 200 tumor cells were photometrically evaluated. The same classification for the histograms was used as for FCM.

The data were computed with the Statistical Package for the Social Sciences (SPSS™) computer program.

Results

Morphology. The analysis focussed on the special serous cells of the parenchyma of the submandibular glands. According to Seifert’s classification, the irradiated glands (18 rats) were predominantly in stage 2 (11/18), in one case only in stage 3 (1/18). Transitional stages were also noted (stage 1-2: 1/18; stage 2-3: 2/18). A late stage 1 was seen in 3 out of 18 animals. In the contra-lateral, shielded glands (14 rats) a late stage 1 was determined in 5/14, a transitional stage 1-2 in 2, no animal had a terminal stage, and normal findings were obtained in 7/14. Normal findings were found in non-irradiated rats, excluding a focal fibrosis and a diffuse, moderate increase of connective tissue in one animal each.

A detailed description of the tumors is provided in a current publication (9).

<table>
<thead>
<tr>
<th>Submandibular Gland</th>
<th>Euploid</th>
<th>Aneuploid Proliferation</th>
<th>No Measurement possible</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>-</td>
<td>-</td>
<td>110</td>
<td>2</td>
</tr>
<tr>
<td>6 Months, 20 Gy</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>12 Months, 20 Gy</td>
<td>-</td>
<td>-</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>6 Months, 40 Gy</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>12 Months, 40 Gy</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>6 Months, 40 Gy</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Sublingual Gland (All Glands)</td>
<td>98</td>
<td>-</td>
<td>14</td>
<td>112</td>
</tr>
<tr>
<td>Tumors (Total)</td>
<td>8</td>
<td>3</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>- Adenoca.</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>- Fibrosis/ Kuettner’s tumor</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>- Sclerosis/ Atrophy</td>
<td>5</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>- Squamous cell carcinoma</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
We recorded consistently diploid histograms in the flow cytometry in irradiated glands (100%). Both glands (sublingual and submandibular) were calculated jointly. It is likely that the sublingual gland does not provide a remarkable impact on the values due to the small size of this gland (1/4 to 1/3 of the submandibular) and its mucous secretion type with poor radiosensitivity. A representative histogram of an irradiated gland is shown in Figure 1.

The results of ICM were uniform for irradiated glands (Table III). Salivary glands from both irradiated and non-irradiated control animals showed variable degrees of proliferation and were not further specified. On the other hand, in 6 tumors aneuploid histograms were recorded. Three tumors were classified according to Auer (6, 7). One squamous cell carcinoma and one adenocarcinoma were type III and one squamous cell carcinoma type IV (Figures 2-4).

MIB-5. MIB-positive nuclei were predominantly found in indifferent tubules but scarcely in acinus cells (Figure 5). The staining results of the carcinomas varied considerably, between 4.0% to 28% and 0.1% and 15%. On the other hand, the results from fibroses and scleroses were uniform, even when sections of different layers were investigated. The results are summarized in Table IV.

The statistical analysis of the MIB-5 proliferation rate (PI) in irradiated glands was determined separately for each side and each gland (sublingual or submandibular). In non-treated control animals the PI of the left-sided salivary glands was between 0% and 1%, mean 0.32% (submandibular), and 0% and 0.3%, mean 0.0075% (sublingual), and of right-sided glands 0% to 0.6%, mean 0.16% (submandibular) and 0% (sublingual).

In irradiated animals different findings were calculated. The submandibular gland of the left side (in-the-radiation-field) showed a tendency for a higher PI than the right (shielded side) \((p=0.091;\) all irradiated glands). The latency period influenced significantly the proliferation (6 and 12 months latency vs. non-irradiated controls: \(p=0.002\)). Interestingly, following an increase of the PI in the submandibular gland after 6 months latency, the reduction of the PI compared to the 12 months groups was significant for both sides (6 months vs. 12 months: \(p=0.024\); right side: 6 months vs. 12 months: \(p=0.005\)). The sublingual glands showed no side different PI (left vs. right side: \(p=0.178\)). In addition, the latency period had no impact on the PI (\(p=0.193\)). A trend was noted for the reduction of the PI comparing 6 to 12 months sublingual glands.

The comparison of the PI of all salivary glands as a function of side and latency revealed the latency period as the most effective parameter [latency 6 and 12 months vs.
controls (12 months): \( p = 0.001 \). In particular, this correlation was evident for the submandibular gland, showing the above mentioned increase after 6 months and a decrease in the 12-month groups (decrease of the PI of left-sided submandibular glands 6 vs. 12 months: \( p = 0.015 \); right-sided: \( p = 0.001 \)). Further on, the PI increased significantly in right-sided submandibular glands after 6 months (6 months vs non-irradiated ipsilateral control glands: \( p = 0.045 \)). However, no difference was proven in the comparison of submandibular glands 12 months following irradiation and non-irradiated animals. Bonferroni-calculation could not prove any significant difference for the above mentioned parameters for sublingual glands, although a tendency for an increased PI was noted in the 6-month groups compared to controls.

No significant difference was evident when both glands of each side were grouped together, irrespective of total dosage and latency (\( p = 0.312 \)). No differences were also calculated for the separate evaluation of all submandibular glands (\( p = 0.139 \)) and sublingual glands (\( p = 0.178 \)) in the comparison of both sides.

The comparison of all glands as a function of radiation exposure revealed a significant global effect of this parameter (irradiation with 20, 40 or 60 Gy vs. non-
irradiated animals: $p=0.001$), but no side specificity (right-sided vs. left-sided salivary glands, including all Gy-stages: $p=0.215$).

Differences became evident after analysis of glands with a different secretion type. The submandibular gland showed a tendency for an increased PI, starting with exposures to 20 Gy (left vs. right: $p=0.065$). However, the effect of radiation was not significantly different (radiation, all stages, vs. controls: $p=0.361$). The same findings were recorded for the sublingual gland (left vs. right as a function of irradiation: $p=0.133$; radiation, all stages, vs. controls: $p=0.548$).

A significant difference of the PI was determined in left submandibular glands for the parameter latency, in particular for the 6-month groups (high PI compared to 12-month groups, 20 and 60 Gy exposures). The irradiation of 20 Gy vs. 40 Gy vs. 60 Gy as a function of latency period showed no difference ($p=0.773$). However, the course of the PI in the latency groups showed same differences as a function of the variable total dosage: a 6-month latency period resulted in 20-Gy-irradiated glands (in-the-field) an increase of the PI, in 40-Gy-irradiated glands a decrease, and in 60-Gy-irradiated glands an increase. After 12 months the PI increase was remarkably high for the 20-Gy-exposed animals (scattering effect). After 12 months the differences between the 3 groups were minimal.

The evaluation of the left sublingual glands revealed no effect for the differences of the latency periods ($p=0.153$) nor the differences of the total dosages ($p=0.252$). There was a tendency for an increased PI for 40- and 60-Gy-exposed animals after 6 months. In addition, the course of the PIs showed no differences in the comparison of 6- and 12-month groups as a function of irradiation.

A significant difference of the PI was determined in left submandibular glands for the parameter latency, in particular for the 6-month groups (high PI compared to 12-month groups, 20 and 60 Gy exposures). The irradiation of 20 Gy vs. 40 Gy vs. 60 Gy as a function of latency period showed no difference ($p=0.773$). However, the course of the PI in the latency groups showed same differences as a function of the variable total dosage: a 6-month latency period resulted in 20-Gy-irradiated glands (in-the-field) an increase of the PI, in 40-Gy-irradiated glands a decrease, and in 60-Gy-irradiated glands an increase. After 12 months the PI increase was remarkably high for the 20-Gy-exposed animals (scattering effect). After 12 months the differences between the 3 groups were minimal.

The evaluation of the right sublingual gland revealed no statistically significant differences for the tested parameters.

The application of interaction variables allows a combination of every latency period with every irradiation group, leading to 6 groups. In submandibular glands there was a tendency for differences between the groups ($p=0.052$), but the Bonferroni calculation excluded any significant difference. However, for the right-sided glands (outside-the-radiation-field) the group comparisons revealed differences ($p=0.0001$) that were specified by Bonferroni calculations. In particular, the PI increase of 20-Gy-irradiated animals, explanted after 6 months, was intriguing. The values of this group were significantly higher than those of all other groups (6 months, 20 Gy vs. 6 months, 40 Gy: $p=0.001$; 6 months, 20 Gy vs 6 months, 60 Gy: $p=0.006$; 6 months, 20 Gy vs. 12 months, 20 Gy: $p=0.0001$; 6 months, 20 Gy vs. 12 months, 40 Gy: $p=0.0001$; 6 months, 20 Gy vs. 12 months, 60 Gy: $p=0.0001$). Differences between the other groups could not be demonstrated.

In sublingual glands differences between the groups could not be revealed (left side; $p=0.238$; right: $p=0.216$) and were not detected using sub-specifications and Bonferroni calculations.

### Table IV. MIB-5 Measurement of salivary glands, depending on total irradiation dosage and the latency period.

<table>
<thead>
<tr>
<th>Glands Irradiation Dosage and Latency Period</th>
<th>Irradiated Submandibular Gland (Mean Value)</th>
<th>Irradiated Sublingual Gland (Mean Value)</th>
<th>Non-Irradiated Submandibular Gland (Mean Value)</th>
<th>Non-Irradiated Sublingual Gland (Mean Value)</th>
<th>Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Months, 20 Gy</td>
<td>5.029</td>
<td>0.520</td>
<td>3.333</td>
<td>0.350</td>
<td>14</td>
</tr>
<tr>
<td>12 Months, 20 Gy</td>
<td>0.477</td>
<td>0.025</td>
<td>0.533</td>
<td>0.000</td>
<td>18</td>
</tr>
<tr>
<td>6 Months, 40 Gy</td>
<td>1.550</td>
<td>0.100</td>
<td>0.786</td>
<td>0.200</td>
<td>13</td>
</tr>
<tr>
<td>12 Months, 40 Gy</td>
<td>2.220</td>
<td>0.250</td>
<td>0.760</td>
<td>0.089</td>
<td>20</td>
</tr>
<tr>
<td>6 Months, 60 Gy</td>
<td>3.950</td>
<td>1.114</td>
<td>1.188</td>
<td>0.725</td>
<td>16</td>
</tr>
<tr>
<td>12 months, 60 Gy</td>
<td>0.600</td>
<td>0.260</td>
<td>0.170</td>
<td>0.060</td>
<td>20</td>
</tr>
<tr>
<td>Controls</td>
<td>-</td>
<td>-</td>
<td>0.240</td>
<td>0.038</td>
<td>10</td>
</tr>
</tbody>
</table>
As the mean result of these investigations, a PI increase was evident for submandibular glands subjected to 20 or 60 Gy, followed by a decrease in the 12-month group to values similar to non-irradiated animals. In sublingual glands the radiation effect on the PI was low, excluding an increase of 60-Gy-exposed animals after 6 months. The calculations revealed scattering effects in shielded glands that resulted in significant PI increase.

PI in tumors. The statistical analysis was restricted to a few evaluations due to the small sample size. The PI in tumors of non-irradiated animals was 1.12% (SD±3.32%). Irradiation (irr) had an effect on the PI (irr. vs. non irr: \( p=0.004 \)). However, this comparison was mainly restricted to tumors following 60 Gy exposure (only 1 tumor following 40 Gy) and those arising in non-irradiated animals. Animals irradiated with 60 Gy developed tumors with a significantly higher PI than those from non-irradiated animals [non-irr. 1.12%±SD 3.32% vs. 60 Gy irr 15.86%±SD 10.7%: \( p=0.01 \)]. The latency period had some effect on the PI, i.e. tumors that developed prior to 6 months after completion of the irradiation had significantly higher PI than those that developed later [latency < 6 months (mean 20.5%±SD 7.32) vs. latency > 6 months (mean 7.0%±SD 7.54%): \( p=0.041 \)].

The highest PI was found in malignant tumors. The PI of cystadenocarcinoma (n=2, 23% and 28%) and one microcystic carcinoma (27%) were much higher than the PI in adenoid cystic carcinoma (12%), squamous cell carcinoma (15%) and one further microcystic carcinoma (10%). The PI of the remaining carcinoma were lower than 10%. Benign proliferations had low PIs between 0 and 0.1% (Table V).

**Discussion**

This study reveals differences in the ploidy and proliferation rate between irradiated salivary gland cells and salivary gland tumors with respect to total dosage, the latency period, the position of the salivary gland inside or outside the radiation field, the different effects of irradiation damage on salivary glands depending on the secretion type and ageing.

Fajardo (20) reported that the fractionation schemes and radiation doses used in animal studies were not comparable with radiotherapy protocols in humans. During the last 20 years only a few experimental studies on radiation injury to the salivary glands were based on a fractionation scheme equivalent to therapeutic irradiation of humans (for review: 15). A single fraction of 2 Gy/d, 5 days a week, coming to a maximum dosage of 60 Gy, is a standard dosage in radiation oncology (59). In analogy to irradiation of tumors in humans we used a Roentgen ray equipment. In recent studies we evaluated that extreme salivary gland damage does not occur following this protocol (10-15).

FCM analysis of tissues is an established method for the investigation of tumor tissue (1, 16, 23, 27, 43, 50, 55, 57). However, cases with non-classifiable ploidy should be analysed with ICM (33). The analysis of fresh tissue is preferred for FCM (4). However, Hedley et al. (29-31) showed that the validity of DNA content determination is high in paraffin-embedded tissues. This is in contrast to findings published by Zalupski et al. (64) who evaluated an error rate of 14% in paraffin-embedded tissues compared to fresh samples. Autolysis can cause erroneously aneuploid peaks (4, 5). In our study, the animals were perfusion fixed with buffered formalin, followed by immersion fixation and immediate embedding in paraffin. Autolysis was not recorded on representative slices that were routinely stained with Hematoxylin-eosin prior to our investigation. Repeated investigation of the same tissue leads to different results. These differences are caused by the method, where the quality of the measurements strongly depends on the heterogeneity of the tissue. Therefore, different layers of the same tissue sample can cause different results. Kallioniemi et al. (36) concluded on their experience with FCM of 1394 tumors that repeated measurements and the calculations of mean values are mandatory in order to provide reliable data of the tumor.
usable for prognostic calculations. In this series the ploidy of irradiated glands was repeatedly measured and the differences between the single measurements were very low.

The ICM enables a morphologic analysis of the sections prior to the cytometric procedures. Thereby the evaluation of autolytic areas can be avoided (6, 42). Earlier ICM studies with paraffin-embedded and fresh tissues showed that the differences of the cytometric results between both groups are very low (Caselitz et al., unpublished results). This result is in accordance with findings from other institutions (29, 30).

**MIB-5.** To the knowledge of the authors this is the first study that uses the rat-specific MIB-5 antibody on irradiated salivary glands and tumors of salivary gland origin. MIB-1 is a well described antibody that identifies the Ki-67 antigen in paraffin-embedded tissue (32, 33, 39, 41). MIB-5 identifies the same epitope in rats as MIB-1 in human tissues. The MIB-1 staining pattern proved to be of prognostic impact in salivary gland carcinoma (35) and other malignant tumors (32, 39). MIB-5 immunostaining is a useful method for the assessment of proliferative activity in rodent lung tumors (34). In this study MIB-5 positivity was restricted to the nuclei of cells. Differences in staining reaction were rarely seen, predominantly due to differences of section thickness. Leukocytes are stained with MIB-5 and have to be distinguished from the cells under study. Our statistical evaluation identified differences in the proliferation of salivary gland tissue that were reproducible as indicated by the immunohistochemical staining pattern. Thus the antibody provided reliable data for the assessment of radiation damage and repair in the salivary glands of the rat.

**Comparison of methods.** The combined assessment of tissues with FCM and ICM was recommended (21, 22, 35). Discrepancies between the results obtained with FCM and Ki-67 proliferation activity have already been noted by Isola et al. (33), who analysed breast carcinomas. These authors found that the Ki-67 immunoreactivity correlates positively with aneuploid tumors only. The S-phase fraction of diploid tumors, determined with FCM, does not correlate with Ki-67, because in diploid tumors connective tissue cells, inflammatory cells and the tumor cells are lying in the same peak. Therefore, these authors argued in favour of the immunohistochemical analysis of tumor tissue in breast cancer patients, providing more reliable prognostic data. A lack of correlation between FCM and immunohistochemical proliferation measurements was also reported by Linden et al. (38) and others (22, 61).

We also found no correlation of the results obtained by the 3 methods in rat tumor tissue. A high PI (> 15%) did not inevitably result in a proliferation or aneuploidy indicating histogram. On the other hand, the MIB-5 PI showed some correlation with the ICM (about 15%), but the number of proliferating cells in FCM was not sufficient to influence the histograms.

For the analysis of rat salivary gland the ICM seems to be superior to FCM. A detailed study of the proliferation can be provided by the MIB-5 antibody, calculated as a proliferation index. For example, the PI remained in animals subjected to 40 Gy and investigated 1 year after completion of radiation on a level up to 10-fold higher than in untreated controls. This high proliferation could be, at least in part, attributable to the development of cancer in salivary glands.

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**References**


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