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Original Paper

No Predictive Value of the Micronucleus Assay for Patients with Severe Acute Reaction of Normal Tissue After Radiotherapy

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In approximatively 5% of cancer patients undergoing radiotherapy, this treatment has to be interrupted because of an acute reaction of normal tissues. To test the possibility of predicting this type of reaction, the micronucleus assay was used to determine radiosensitivities of peripheral blood lymphocytes of 15 patients with severe acute reaction of normal tissue, 15 patients without this reaction and 15 healthy donors. Whole-blood cultures were irradiated with X-rays (4 Gy, 1.08 Gy/min) and treated with cytochalasin B. The micronuclei scores observed in irradiated cells were corrected for the scores in unirradiated cells. Intra-individual and interindividual variations in micronuclei scores were analysed in samples from healthy donors, and highly significant interindividual differences were found ($P < 0.001$). Scores of cells not irradiated *in vitro* were higher for cancer patients before radiotherapy than for healthy donors ($P < 0.001$), and those for cancer patients after radiotherapy were higher than for patients before radiotherapy ($P < 0.001$). Average micronuclei scores induced by *in vitro* irradiation were significantly higher in samples from cancer patients compared with those from healthy donors ($P < 0.01$). Moreover, all subgroups of cancer patients included individuals with very high levels of micronuclei after *in vitro* irradiation. There was, however, no relationship between the micronuclei scores and the occurrence of severe acute reactions in normal tissues.

Key words: radiosensitivity, lymphocytes, micronucleus, severe acute reaction
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INTRODUCTION

SEVERE ACUTE reactions of normal tissues represent a major problem in cancer radiotherapy. In approximatively 5% of patients, radiotherapy has to be interrupted because of high radiosensitivity of normal tissue. There is a generally held opinion that tumours, as well as normal tissues of individual patients, differ in their intrinsic radiosensitivity. The intrinsic radiosensitivity of the patient's normal cells appears, therefore, to be a major determinant of normal tissue reactions during radiotherapy [1]. Using colony-forming assays, a relationship between the radiosensitivity of fibroblast cells and the extent of normal tissue damage has been established [2–4]. In addition, significant differences in intrinsic radiosensitivity between normal individuals were observed in clonogenic assays with stimulated lymphocytes [5–7]. In a prospective study, using a clonogenic assay, peripheral blood lymphocytes from breast cancer patients exhibiting severe acute reactions to radiotherapy were, on average, more radiosensitive than those from normal donors [8].

In general, clonogenic assays are very time-consuming and, therefore, unsuitable for routine predictive tests. The micronucleus assay, being a relatively quick and simple method, is widely used to assess persistent DNA damage induced by radiation. Micronuclei are chromosome fragments which have been excluded from the nucleus during cell division. A good correlation between the formation of micronuclei and the loss of proliferative capacity has been demonstrated by different authors [9–11]. Introduction of the cytokinesis-block technique permits the number of cells containing micronuclei to be counted amongst the cells having undergone mitosis, providing results that are independent of the

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proliferation rate of the cell population. In the presence of cytochalasin B, binucleated cells are produced after the first cell cycle due to inhibition of cytokinesis [12, 13].

Blood cells are the most readily accessible type of cells from patients or from healthy donors. Results from a number of studies suggest that lymphocytes may be useful for predicting normal tissue radiosensitivity [1]. Significant inter-individual differences in radiation-induced increases in numbers of micronuclei of human lymphocytes have been reported by various authors [1, 14, 15]. In this study, we investigated the suitability of the micronucleus assay to predict excessive normal tissue reactions of cancer patients undergoing radiotherapy, by determining the *in vitro* radiosensitivity of their lymphocytes. 15 cancer patients without severe acute reactions were compared with 15 cancer patients suffering from severe acute reactions occurring during radiotherapy. In addition, samples from 15 healthy donors were assayed.

While the possibility of a relationship between *in vitro* radiosensitivity of lymphocytes and late reactions of normal tissue to radiotherapy should also be considered, the present report is restricted to acute reactions, since it may take 5 years or more for all late effects to develop in susceptible patients.

MATERIALS AND METHODS

Cultivation of lymphocytes

The cytokinesis-block micronucleus technique was performed as described by Fenech and Morley [12], with a few minor modifications, which appeared to provide better results. Blood was drawn by venepuncture from patients or from healthy donors, and heparinised aliquots of 0.12 ml were added to 1 ml of RPMI 1640 medium (Gibco, Life Technologies, Basel, Switzerland) supplemented with 20% fetal calf serum (Gibco), L-glutamine (459 μg/ml) (Merck, [Schweiz] AG, Dietikon), penicillin/streptomycin (39 μg/ml), fungizon (97 ng/ml) and phytohaemagglutinin (9.7 μg/ml) (Gibco).

Some of these blood microcultures were immediately irradiated at room temperature with X-rays (RT-250, Phillips, 250 kV, 0.5 mm Cu filter) using a dose of 4 Gy and a dose rate of 1.081 Gy/min, while control cultures were not irradiated. Subsequently, the cultures were incubated for 44 h at 37°C in a humidified atmosphere containing 5% CO_2. At this time cytochalasin B (Cyt-B) was added to the cultures (final concentration: 4 μg/ml) to block cytokinesis, as described [12], and the cultures were incubated for another 20–22 h. The cultures were then centrifuged, and the cells were exposed to a hypotonic shock by resuspending the pellet slowly in 1.5 ml of 0.125 M KCl with continuous vortexing. Six minutes later the cells were centrifuged again and the pellets were slowly resuspended by the addition of 1.5 ml fixation solution I (methanol, 0.9% sodium chloride and 96% acetic acid; 12:13:3) with vortexing. After 8 min, the cells were washed twice in fixation solution II (96% acetic acid and methanol; 1:4), resuspended in 170 μl fixation solution II and placed on to microscopic slides. After 15–20 s, the fixation solution was poured off and the preparations were dried in a current of air. Finally, the slides were stained for 20 min with Giemsa solution (Merck) diluted 20-fold with phosphate-buffered saline (PBS) pH 7.4.

Counting of micronuclei

1000 binucleated cells of each sample of irradiated and unirradiated blood cultures were scored microscopically for the number of micronuclei. The data are presented as the number of cells containing one or more micronuclei per 1000 binucleated cells (Mn cells/1000 BNC), or alternatively, as the number of micronuclei per 1000 binucleated cells (Mn/1000 BNC). As a measure of lymphocyte radiosensitivity, the values Mn cells/1000 BNC and Mn/1000 BNC obtained for non-irradiated lymphocytes were subtracted from the values obtained for lymphocytes irradiated *in vitro* with 4 Gy, and these corrected values were termed Mn cells/1000 BNC (4–0 Gy) and Mn/1000 BNC (4–0 Gy), respectively.

Patients

Verbal informed consent of 15 patients was obtained before radiotherapy was started (control patient group, consisting of patients without severe acute reactions). In addition, 15 patients for whom radiotherapy had to be interrupted or stopped because of severe acute reactions were asked for blood specimens. A severe acute reaction was defined as any event caused by radiotherapy that led to an interruption or cessation of treatment. All these 30 patients underwent radiotherapy without any antineoplastic chemotherapy. From the patients without severe acute reactions, blood samples were taken before and at the end of radiotherapy, whereas from the patients with severe acute reactions, blood samples were taken at the time of cessation of radiotherapy. The control patient group consisted of 7 males and 8 females with an average age of 52 years and an age range of 32–78 years, while the group of cancer patients with severe acute reactions included 9 males and 6 females with an average age of 64 years, ranging from 35 to 77 years. The characteristics of both groups were similar with regard to localisation of tumour (Table 1) and leucocyte count (data not shown).

Table 1. *Representation of the two groups of patients included in the study*

Tumour type	Patients without severe acute reaction ($n = 15$)	Patients with severe acute reaction ($n = 15$)	Type of acute reaction
Hodgkin's lymphoma	1	—	—
Meningoma	1	—	—
Head and neck cancer	8	4	4 mucositis
Cancer of the breast	3	1	1 epitheliolysis
Endometrial cancer	1	2	2 diarrhoea
Sarcoma	—	3	2 epitheliolysis 1 mucositis
Prostate cancer	—	2	1 diarrhoea 1 proctitis
Non-small cell lung cancer	1	2	2 epitheliolysis
Anal cancer	—	1	1 epitheliolysis

Data analysis

Intra- and interindividual variations were analysed by an ANOVA analysis, and differences between mean scores of individual healthy donors were also evaluated by Student's *t*-test. The different donor groups were compared by the Wilcoxon two-sample rank sum-test, and correlations between donor age and the micronuclei scores were analysed by linear regression.

RESULTS

Intra-individual and interindividual variation in radiosensitivity of lymphocytes from healthy donors

A total of 35 blood samples from 15 healthy donors was analysed. This donor group consisted of 3 females and 12 males with a mean age of 42 years and an age range of 24–67 years. In order to monitor intra-individual and interindividual variations in radiosensitivity, 5 samples were obtained from donor nos 1–5, at time intervals between two subsequent samples of 1 week to 18 months. The longest time interval between samples 1 and 5 was 19 months. The results obtained for these 5 healthy donors with respect to Mn cells/1000 BNC (4–0 Gy) are presented in Figure 1. Similar results have been obtained for Mn/1000 BNC (4–0 Gy; data not shown).

A relatively high intra-individual variation in radiosensitivity was observed, at least in some individuals, and in particular in donor 5, while results for donors 1 and 4 were highly reproducible. An ANOVA analysis of variance revealed highly significant interindividual differences (F value 8.53, $P < 0.001$). In addition, interindividual differences between donors 1 and 2, 1 and 3, and 2 and 4 were found to be highly significant, according to Student's t-test ($P < 0.001$). Qualitatively similar results were obtained for Mn/1000 BNC (4–0 Gy), with significant interindividual differences in the ANOVA analysis (F value 6.22, $P < 0.01$).

Micronuclei in lymphocytes not irradiated in vitro

In healthy donors, the mean micronuclei scores for lymphocytes not irradiated *in vitro* were highly significantly lower ($P < 0.001$) (means of 6.2 Mn cells/1000 BNC and 6.5 Mn/1000 BNC and standard deviations of 4.38 and 4.64, respectively) than those for patients with no severe acute reactions (means of 15.5 Mn cells/1000 BNC and 17.0 Mn/1000 BNC and standard deviations of 5.99 and 6.83, respectively), as determined before radiotherapy (Figure 2a versus b).

No significant correlation between donor age and micronuclei scores was obtained for healthy donors as well as for patients before radiotherapy. This may be explained by the small numbers of individuals in the two groups. For healthy donors linear regression analysis resulted in a slight but not significant ($r = 0.47$, $P = 0.08$) increase of the micronuclei score with donor age.

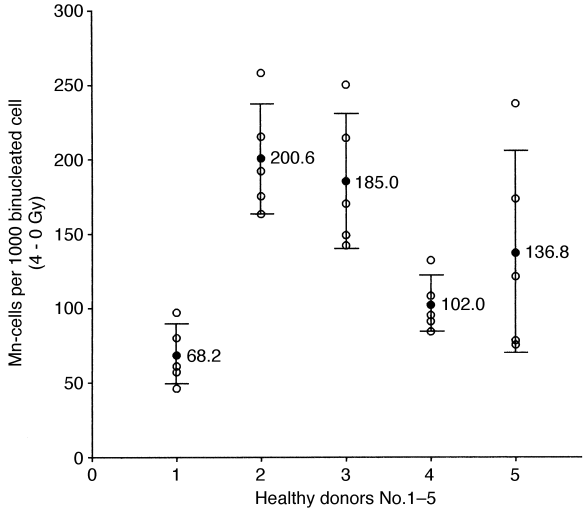

Figure 1. Data for radiation-induced micronuclei in lymphocytes from 5 healthy adult donors following *in vitro* irradiation with a dose of 4 Gy. The assays were carried out five times for each donor over time periods up to 19 months. The error bars give the standard deviations for each donor and the mean values are indicated.

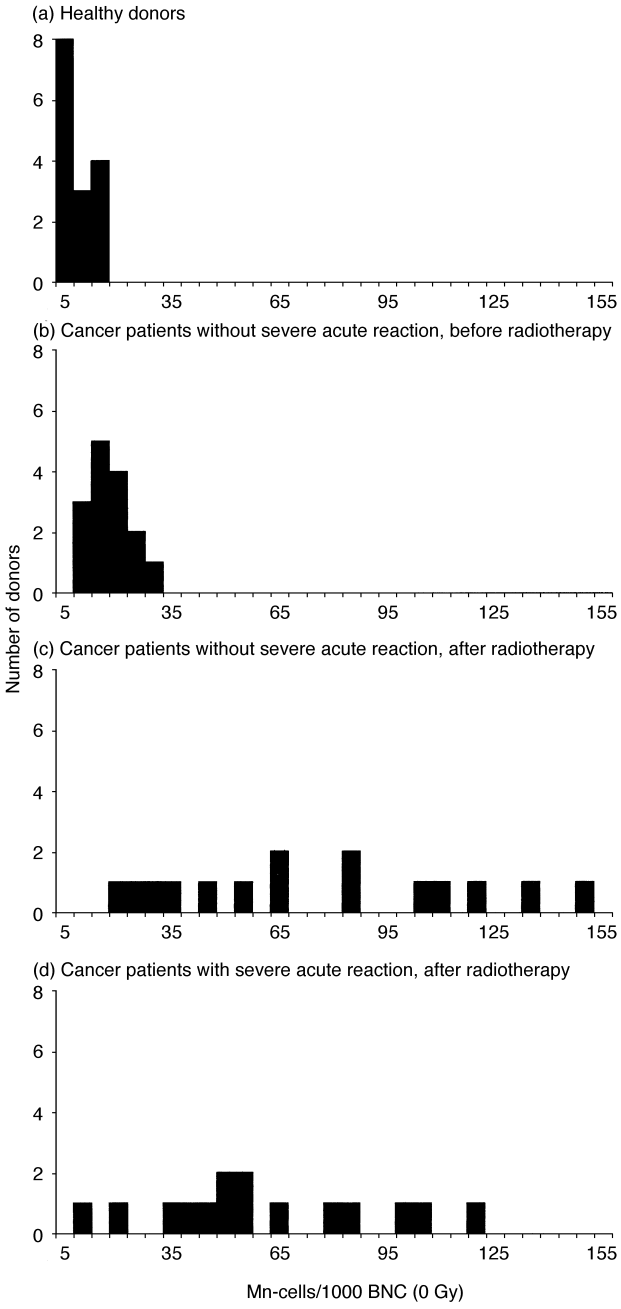

Figure 2. Micronuclei scores of lymphocytes which have not been irradiated *in vitro*. (a) Healthy donors, (b) and (c) cancer patients without severe acute reaction, before and after radiotherapy, (d) cancer patients with severe acute reactions after radiotherapy.

From the patients without severe acute reactions, blood samples were also taken before and after radiotherapy, and the number of micronuclei in lymphocytes that were not irradiated *in vitro* increased highly significantly (Figure 2b versus c, $P < 0.001$) after radiotherapy, obviously as a result of radiotherapy, and similar scores were also observed in patients with severe acute reactions (Figure 2b versus d, $P < 0.001$). There was no significant difference between patients with and without severe acute reactions after radiotherapy (Figure 2c versus d, $P = 0.44$). Similar results were obtained for Mn/1000 BNC (data not shown).

Radiosensitivity of lymphocytes from cancer patients

To present the results for all 15 healthy donors, the means for Mn cells and Mn/1000 BNC (4–0 Gy) were calculated for donors 1–5 and combined with the results obtained for the other 10 healthy donors. The results are presented in the form of histograms in Figures 3a and 4a.

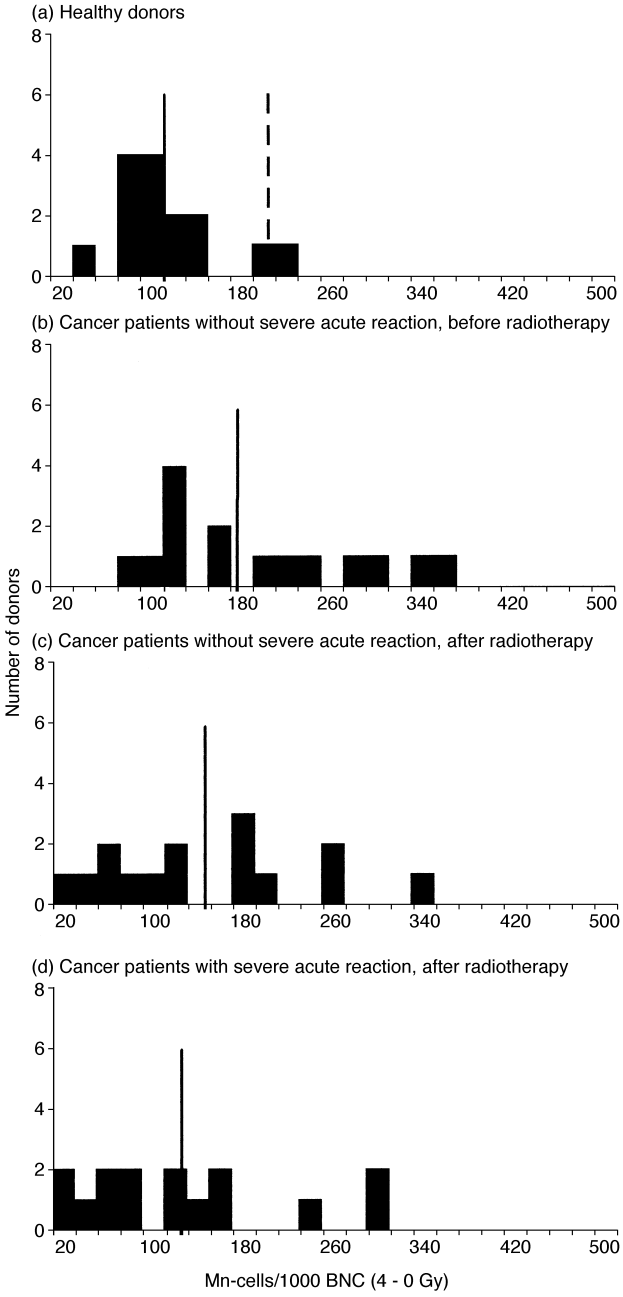

Figure 3. Induction of micronuclei (numbers of cells with micronuclei per 1000 binucleated cells, corrected for values in unirradiated cells) by *in vitro* irradiation of lymphocytes with a dose of 4 Gy. (a) Healthy donors, (b) cancer patients without severe acute reaction, before radiotherapy, (c) cancer patients without severe acute reaction after radiotherapy, (d) cancer patients with severe acute reactions after radiotherapy. The solid vertical lines indicate the mean values, the broken vertical line on (a) indicates the mean value + 2 S.D.

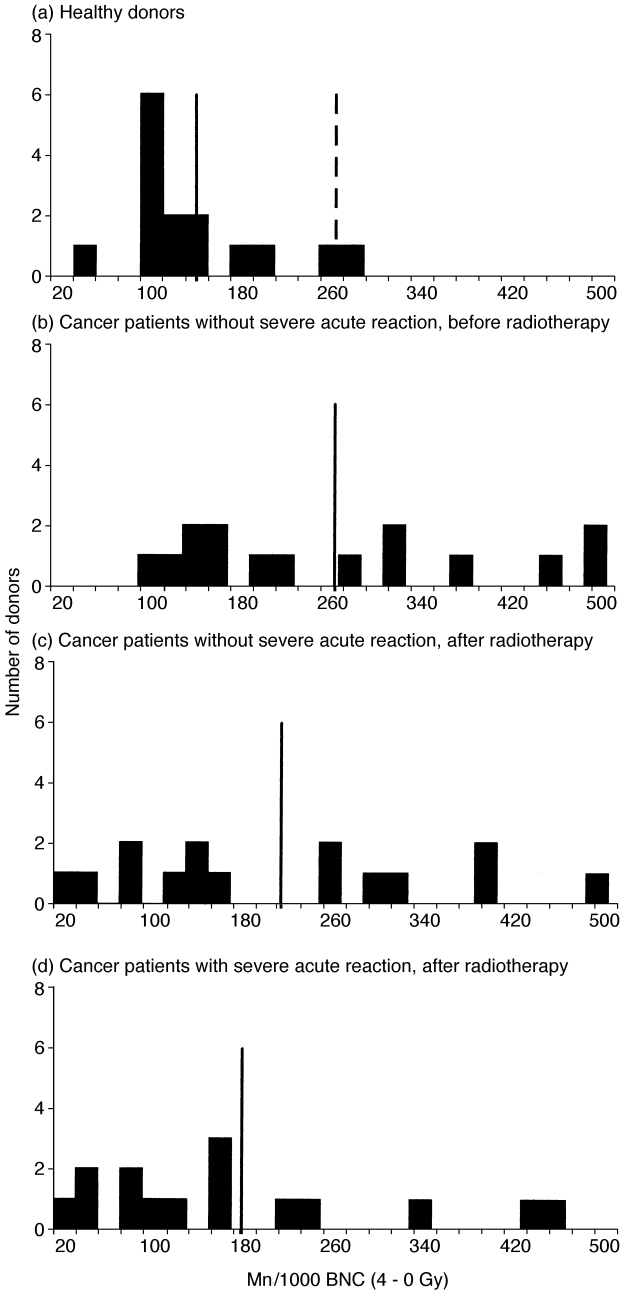

Figure 4. Induction of micronuclei (numbers of micronuclei per 1000 binucleated cells, corrected for values in unirradiated cells) by *in vitro* irradiation of lymphocytes with a dose of 4 Gy. (a) Healthy donors, (b) cancer patients without severe acute reaction, before radiotherapy, (c) cancer patients without severe acute reaction after radiotherapy, (d) cancer patients with severe acute reactions after radiotherapy. The solid vertical lines indicate the mean values, the broken vertical line on (a) indicates the mean value + 2 S.D.

In the group of patients without severe acute reactions, similar mean scores of micronuclei induced by *in vitro* irradiation (4–0 Gy) were observed before and after radiotherapy (Figures 3b versus c, 4b versus c), the values were also similarly spread in the histograms and no significant differences were found ($P = 0.12$ and 0.27, respectively). Furthermore, the mean scores and the resulting histograms for patients with severe acute reactions were comparable with those of cancer patients without acute reactions (Figures 3c versus d, 4c versus d), and no significant differences were found ($P = 0.53$ and 0.66, respectively). Thus, lymphocyte radiosensitivities, as determined by the micronucleus assay under the conditions used, did not permit any distinction between cancer patients with and without severe acute reactions.

It is also of interest that the mean scores of micronuclei induced by *in vitro* irradiation were significantly lower ($P < 0.01$) for cells from healthy donors (Figures 3a and 4a) than for cells from cancer patients without severe acute reactions, before or after radiotherapy (Figures 3b and c, 4b and c). Remarkably, with respect to the scores of Mn cells/1000 BNC (4–0 Gy), several cancer patients exhibited scores higher than the mean + 2 S.D. obtained for normal donors: 6 cancer patients without severe acute reactions before radiotherapy; 3 without severe acute reactions after radiotherapy and 3 with severe acute reactions after radiotherapy (Figure 3a versus b–d). Similarily, with respect to the scores of Mn/1000 BNC (4–0 Gy): 7 without severe acute reactions before radiotherapy, 5 without severe acute reactions after radiotherapy and 3 with severe acute reactions after radiotherapy (Figure 4a versus b–d) had scores higher than the mean + 2 S.D. for normal donors.

DISCUSSION

We scored both numbers of cells with micronuclei and numbers of micronuclei per 1000 binucleated cells. The number of cells with micronuclei is considered to be a more adequate measure of cells destined to die since a single micronucleus in a diploid cell correlates with a loss of proliferative capacity [15, 16]. The results obtained by these two methods of scoring micronuclei as expressed in histograms were qualitatively similar.

Intra-individual variations in numbers of micronuclei induced by *in vitro* irradiation of lymphocytes, when studied on samples from 5 healthy individuals, were relatively high in some cases (Figure 1). This may be attributed, at least in part, to the extended time intervals between collections of blood samples. However, highly significant ($P < 0.001$) inter-individual differences were also observed. Thus, differences in lymphocyte radiosensitivity between individual donors were detectable by the micronucleus procedure as applied in our studies. As reported previously [14, 15], statistically significant interindividual differences in lymphocyte radiosensitivity, as assessed by the micronucleus assay, may be observed using *in vitro* irradiation of lymphocytes at high dose rates (i.e. of the order of 1–2.5 Gy/min). In clonogenic assays, irradiation of lymphocytes at low dose rates has been found to be preferable to high dose rates for detecting interindividual differences in radiosensitivity [5], while other authors [6] have obtained similar interindividual differences using either high- or low-dose rate irradiation. We decided to determine lymphocyte radiosensitivity using irradiation at a relatively high-dose rate (1.08 Gy/min), because similar dose rates were applied during radiotherapy of the patients included in this study. In fact, results of our micronucleus assays on samples from healthy donors showed that highly significant inter-individual differences in radiosensitivity are obtained with high-dose rate irradiation of lymphocytes.

Average micronucleus frequencies in lymphocytes not irradiated *in vitro* were highly significantly higher ($P < 0.001$) in samples from cancer patients obtained before radiotherapy than in those from healthy donors (Figure 2a versus b). This may be attributed, at least in part, to the higher mean age of cancer patients without severe acute reactions (52 years) compared with that of healthy donors (42 years). However, it cannot be excluded that the cancer patients had, during their lives, been exposed to higher levels of genotoxic agents and/or had a lower capacity to repair damage caused by such agents.

After radiotherapy, micronuclei levels of lymphocytes were highly significantly higher ($P < 0.001$), both in patients with and without severe acute reactions, indicating that circulating lymphocytes had been damaged in the course of local radiotherapy.

The radiosensitivity of lymphocytes from healthy donors was compared with that of cancer patients before radiotherapy (Figures 3 and 4, a versus b). The average sensitivity of lymphocytes from cancer patients was significantly higher ($P < 0.01$) than that from healthy donors. This difference may be attributable to genetic factors such as heterozygous ataxia-teleangiectasia, resulting both in increased radiosensitivity and increased susceptibility to the development of neoplasia [1].

No significant differences in lymphocyte radiosensitivity of cancer patients without severe acute reactions were found, as determined before versus after radiotherapy (Figures 3 and 4, b versus c). The slightly lower values after radiotherapy may possibly be due to higher micronuclei scores of lymphocytes not irradiated *in vitro*, leaving a smaller difference from the maximal value.

The histograms of cancer patients with and without severe acute reactions after radiotherapy (Figures 3 and 4c versus d) were very similar and showed no relationship with severe acute reactions. The micronuclei test as applied in our study does, therefore, not permit any prediction of acute reactions in the course of radiotherapy. Similar conclusions have been reached on the basis of clonogenic assays with lymphocytes and fibroblasts, applying both high and low dose rates [4]. A relationship between radiosensitivity and acute reactions has been reported for clonogenic assays with cell lines from skin fibroblasts, irradiated at high-dose rates [2] or low-dose rates [3]. However, in another study, no correlation between human fibroblast radiosensitivity *in vitro* and early skin reactions in patients undergoing radiotherapy was detected [17]. In assays based on colony formation [8] and chromosome damage [18] of lymphocytes, a relationship of radiosensitivity with radiotherapy-related complications was obtained using low, but not high-dose rate *in vitro* irradiation.

Our failure to demonstrate a relationship between lymphocyte radiosensitivity and radiotherapy-induced severe acute reactions may also be attributable to differences in radiosensitivity profiles between lymphocytes and cell types involved in severe acute reactions, such as connective tissue and epithelial cells. In fact, no correlation between radiosensitivity of lymphocytes and that of fibroblasts was found in

clonogenic assays [6], whereas in another study, a reasonably good correlation of radiosensitivity between lymphoblastoid cell lines and corresponding fibroblast cultures has been observed [19].

The negative results with respect to a relationship between lymphocyte radiosensitivity and radiotherapy-induced severe acute reactions as documented in the present report do not exclude the possibility of a positive relationship with respect to late tissue reactions. This question may be addressed in a later report after appropriate follow-up of the patients included in this study.

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