T-cell Receptor Assay and Reticulocyte-Micronuclei Assay as Biological Dosimeters for Ionizing Radiation in Humans

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In radiation accidents, biological methods are used for dosimetry if the radiation dose could not be measured by physical means. The knowledge of individual dose is a prerequisite for planning medical treatment and for health risk evaluations. In this paper we represent the summary of biodosimetrical methods used in our laboratory in the patients treated with radioiodine for thyroid cancer. The dose-response relationship was measured by the flow cytometry-based micronucleus assay in transferrin receptor positive reticulocytes (MN-Tf-Ret assay) and by the T-cell receptor (TCR) assay in CD4 lymphocytes. Compared to our previous works, the database for TCR assay was significantly improved, and two groups of thyroid cancer patients (from Belarus and Germany) were studied. In the MN-Tf-Ret test, mutant frequency increased proportionally with the radiation dose, whereas in the TCR-test the dose relationship could be described by an exponential equation which takes into account the limited lifespan of TCR mutants. Finally, these assays are compared with other biodosimetrical assays used in patients treated with radioiodine.

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Introduction

After the Chernobyl disaster, the frequency of thyroid cancer dramatically increased in children from Belarus. The therapy includes the removal of the tumor by thyroidectomy followed by several courses of radioiodine ablation. In about a quarter of Belarusian patients (225 persons), radioiodine therapy was performed at the Clinic of Nuclear Medicine of the University of Wurzburg as supported by the Association of the German Electricity Companies (VDEW) and the Association "Medizinische Hilfe fur Tschernobyl-Kinder."

The main known risks of radioiodine treatment are the induction of pulmonary fibrosis and secondary cancers arising as a consequence of radiation exposure.[1] The frequency of somatic mutations in several genes in erythrocytes and lymphocytes has been used as a surrogate indicator of genotoxic effect of radiation assessable by hypoxanthine-guanine phosribosyl transferase (HPRT), glycophorin A (GPA) and T-cell receptor (TCR) assays.[2-7] In this study, TCR assay was applied to two groups of patients to quantify genotoxic effect of the radioiodine therapy and to identify patients who might be at risk of developing secondary cancer because of higher rates of mutations

acquired during the treatment. The first group was represented by young patients from Belarus. The second one included older patients from Germany with thyroid cancer who were undergoing the radioiodine therapy in Clinic of Nuclear Medicine.

An additional method of biological dosimetry used in our study was the reticulocytes micronuclei assay. This method is long known and used in mice as a micronucleus (MN) assay of polychromatic erythrocytes. In humans, the application of this technique has been problematic since all the aberrant (i.e. micronucleated) cells are rapidly entrapped in and eliminated by the spleen and therefore for a long time this assay was limited to spleenectomized patients. Recently the flow-cytometric *in vivo* micronucleus assay has been adapted for use in man employing a preliminary step of immunomagnetic separation of immature transferrin-positive reticulocytes (Tf-Ret) from the peripheral blood.[8] The results of a previous study indicated that micronuclei in human nascent reticulocytes could be a sensitive biomarker of chromosomal damage. To evaluate the applicability and sensitivity of this method for the detection of radiation-induced DNA damage, we performed a study of MN in Tf-Ret in 46 patients undergoing radioiodine therapy. These patients received the defined activi-

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ties of ^{131}I, and precise dosimetry was performed during the course of treatment. Determined were the individual frequencies of MN-Tf-Ret (f(MN-Tf-Ret)).

Subjects and Methods

Patients

In all our assays we used peripheral blood from thyroid cancer patients after obtaining informed consent. The Belarusian group included 72 young thyroid cancer patients (30 males and 42 females) aged from 14 to 25 years with the mean of 19 years. Most of the patients were from the southern part of Belarus, which suffered heavily from the Chernobyl fallout. These patients had been diagnosed for thyroid cancer during their childhood and surgically treated at the Republican Thyroid Cancer Center in Minsk, Belarus, before radioiodine therapy (RIT) in Würzburg. Among the patients some were with nodal disease without (T2 N1 M0, n=23) or with distant metastases (T2 N1 M1, n=7). Several cases displayed capsular invasion on histology without (T4 N1 M0, n=2) or with distant metastases (T4 N1 M1, n=14). The group of German patients consisted of 66 adult thyroid cancer patients, 19 males and 47 females. All the patients received similar RIT in our clinic.

In TCR assay, both groups, Belarusian and German, were analyzed. For MN-Tf-Ret assay mostly Belarusian group was considered (23 women and 21 men) with an addition of 2 elderly patients from Germany.

Assays

TCR-assay

The assay was performed as described by Kyoizumi et al.[9] Briefly, lymphocytes were isolated using ficoll gradient centrifugation from a total of 6 mL heparinized blood. For flow cytometry about 5 million nucleated cells were stained with fluorescein-labeled anti-Leu3a

(CD4) and phycoerythrin-labeled anti-Leu4 (CD3) antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA) and propidium iodide (PI) (20 µg/mL), and the frequency of CD3+ CD4+, CD3- CD4+, CD3+ CD4- and CD3- CD4- cells was determined with Coulter EPICS XL-MCL flow cytometer (Coulter Co, Hialeah, Fl). Dead cells were excluded by the PI staining. TCR-Mf was the number of CD4+ CD3- cells divided by the total number of CD4+ cells. Every lymphocyte fraction was analyzed twice and the mean value was taken.

MN-Tf-Ret assay

Procedure of transferrin-positive reticulocytes separation and staining has been described by Abramsson-Zetterberg et al.[8] Cells were isolated using the mouse anti-human CD71 antibody and magnetic beads (CELLection™ Pan Mouse IgG Kit, Dynal, Oslo), and then fixed with SDS/2% formaldehyde. The stained samples were analysed on FACSVantage SE flow cytometer or LSR flow cytometer (BD Immunocytometry Systems, Sunnyvale, CA).

Statistical analysis

For non-linear regression analysis, SigmaStat 1.0 (Jandel Scientific, Erkrath, Germany) software was used, which utilizes the Marquardt-Levenberg algorithm to calculate the parameters of the variables that yield the best fit.

Results and Discussion

TCR-assay

Results shown in Figure 1 are representative examples of the flow cytometric analysis of TCR-mutants in a healthy person and in a patient treated by RIT several times. The mutant window used in the present study was set as described by Kyoizumi et al.[9]; it is the region with the CD3 level (FL2) < 4% of that of normal CD4+ cells.
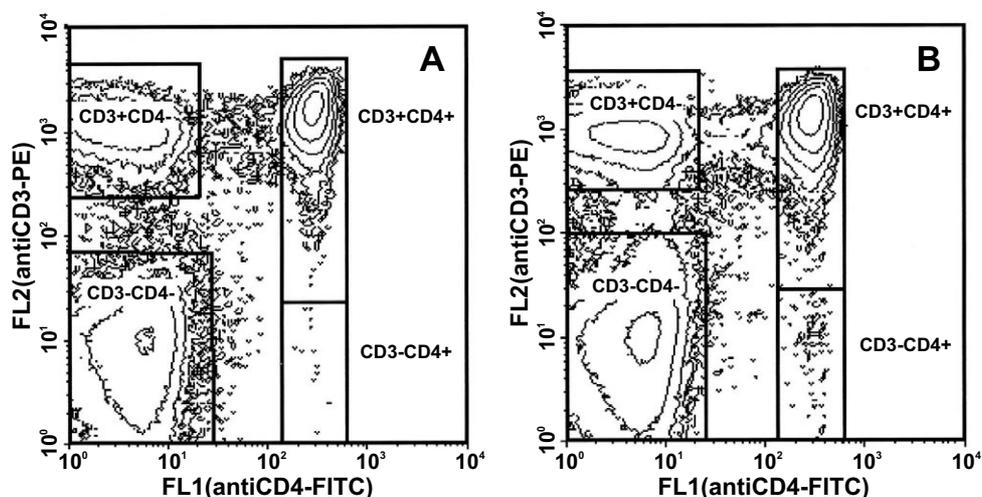

Figure 1. Flow cytometric analysis of TCR mutant lymphocytes. **A.** Healthy donor with normal level of TCR-Mf of 1.73×10^{-4}. **B.** Patient treated with radioiodine 8 times between 1994 and 1999; TCR-Mf=4.89×10^{-4}.

The left and right limits of FL1 were set at values half of and two times greater than, respectively, the mode intensity of FL1 for normal CD4 T-cells. The mean TCR-Mf in 25 patients before ^{131}I treatment, the so-called spontaneous mutant frequency, was 2.0±0.6 (×10^{-4}) (mean ± SD). After the first RIT, Mf reached its maximum about half a year later. The Mf decay in the following years can be described by the three-parameter single exponential decay function:

$$Mf = Mf_0 + k \times D \times e^{-b(T-0.5)}, \qquad (1)$$

where Mf_0 is the spontaneous mutant frequency, k coefficient showing the increase in Mf per 1 mGy radiation dose to red marrow, D total dose in mGy per treatment, and b elimination coefficient of mutants. The dimension of b, which corresponds to the reciprocal mean lifespan of TCR mutants, is year^{-1}. T is the time (in years) between Mf measurement and the treatment. The value of -0.5 considers the fact that the maximum of Mf is reached not earlier than about half a year after RIT. Since most of the patients were treated with radioiodine more than once, the cumulative Mf level is described by the sum of exponential decay functions as follows:

$$Mf = Mf_0 + \sum_{i=1}^{n} k \times D_i \times e^{-b(T_i-0.5)} \qquad (2)$$

where n is the number of treatments. In the present groups of patients the highest number of treatment courses was 10.

Our goal was to find the values of the coefficients Mf_0, k and b, which fit best the measured mutant frequencies in the case where the absorbed dose for every treatment (D_i) and the time between treatment and the measurement (T_i) were known. The values of these coefficients are given in Table 1 as found by nonlinear regression analysis based on the model of Equation (2). The correlation between calculated Mf values and measured values was satisfactory (Figure 2). The values of Mf_0 in Table 1 partly support the results of previous investigations,[11] where the age dependency of TCR-Mf was described by the following equation: $Mf_0 = (1.9+0.02 \times age) \times 10^{-4}$. The parameter k reflects the increase in TCR-Mf per 1 mGy radiation dose to red marrow. Their values are 6.5×10^{-7} mGy^{-1} and 13.2×10^{-7} mGy^{-1}. Akiyama et al.[11] have measured the increase in TCR-Mf in radioiodine treated patients as a function of the ^{131}I activity administered. They found that Mf increases by 6×10^{-5} per 1 GBq of administered activity. Assuming the body weight of ca. 70 kg, this activity would result in a red marrow dose of ca. 50 mGy. The corresponding value of k is 12×10^{-7} mGy^{-1}, which is very close to the value corresponding to the cohort of German patients, while it is approximately 2 times higher than that of our young Belarussian patients. If there was only one RIT or one X-ray exposure, TCR-Mf might be used as a biological dosimeter since the relationship between the radiation dose and the maximum of Mf can be described by Equation (3a) for Belarusian patients and Equation (3b) for German patients as demonstrated in Figure 3.

$$Mf_{MAX} = 2.03 \times 10^{-4} + 6.5 \times 10^{-7} \times D \qquad (3a)$$
$$Mf_{MAX} = 3.36 \times 10^{-4} + 13.2 \times 10^{-7} \times D \qquad (3b)$$

Table 1. Parameter values calculated for patients from Belarus and Germany

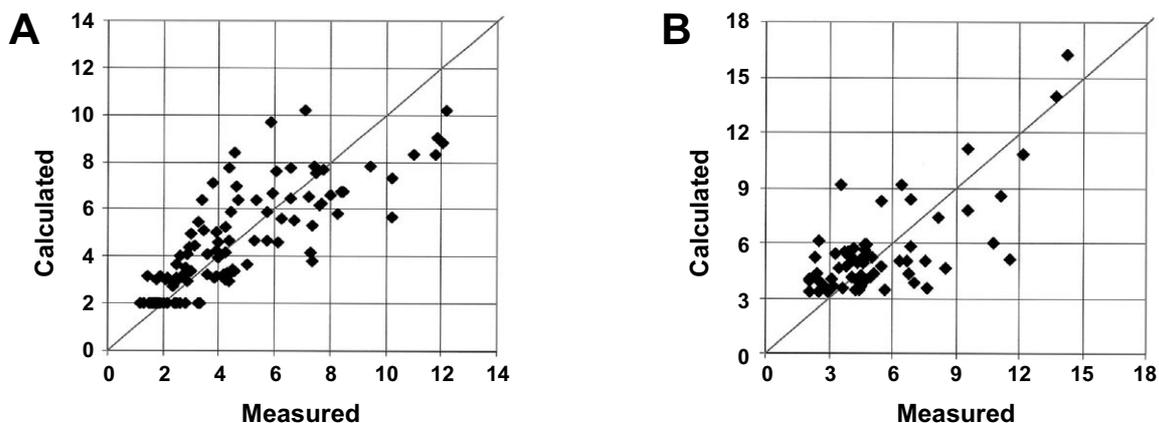| Parameter	Patients from Belarus	Patients from Germany
Mf_0	2.03×10^{-4}	3.36×10^{-4}
k	6.5×10^{-7}	13.2×10^{-7}
b	2.46×10^{-1}	2.23×10^{-1}
$T_{1/2}$	2.8 years	3.1 years
Doubling Dose	310 mGy	255 mGy

Figure 2. Correlation between measured and calculated values of TCR-Mf in patients who received RIT. In none of our patients from Belarus (A) and Germany (B), the TCR-Mf level deviated considerably from the regression curve. Among German patients, there was one patient with calculated value of 5.96×10^{-4} and measured value of 10.75×10^{-4}, suggesting a possibility of higher radiosensitivity in this patient (B). Such patients might be at an increased risk for secondary cancer and should be followed closer than usually.[10]
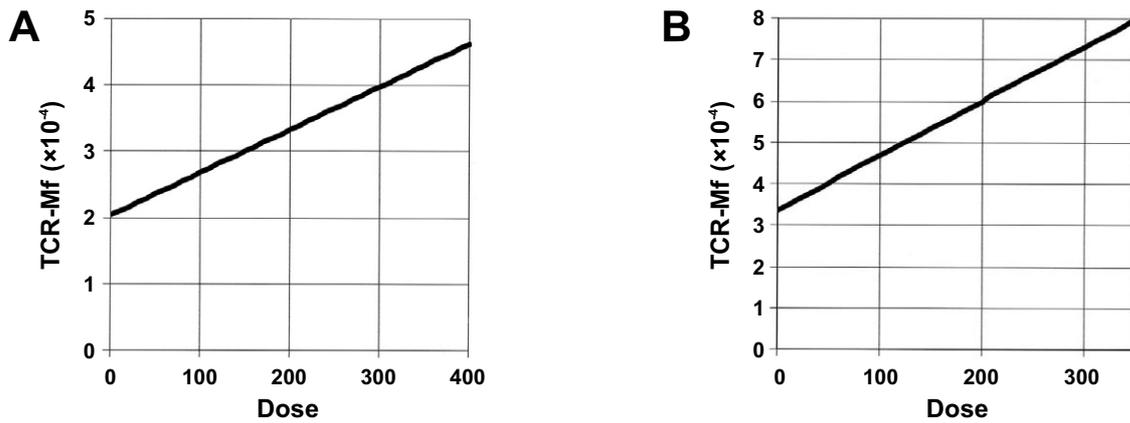
Figure 3. TCR-*Mf* as a function of radiation dose to red marrow. Equations (3a) and (3b) are derived from Equation (1) by setting the time between treatment and measurement as 0.5 years. The radiation dose which doubles TCR-*Mf* is 310 mGy for Belarusian (A) and 255 mGy for German (B). These calibration curves can only be used if the quality of the radiation, dose rate and dose range are all comparable with those in radioiodine therapy. A disadvantage of the TCR-assay as a biological dosimeter is that it cannot be used before about half a year after exposure.[10]
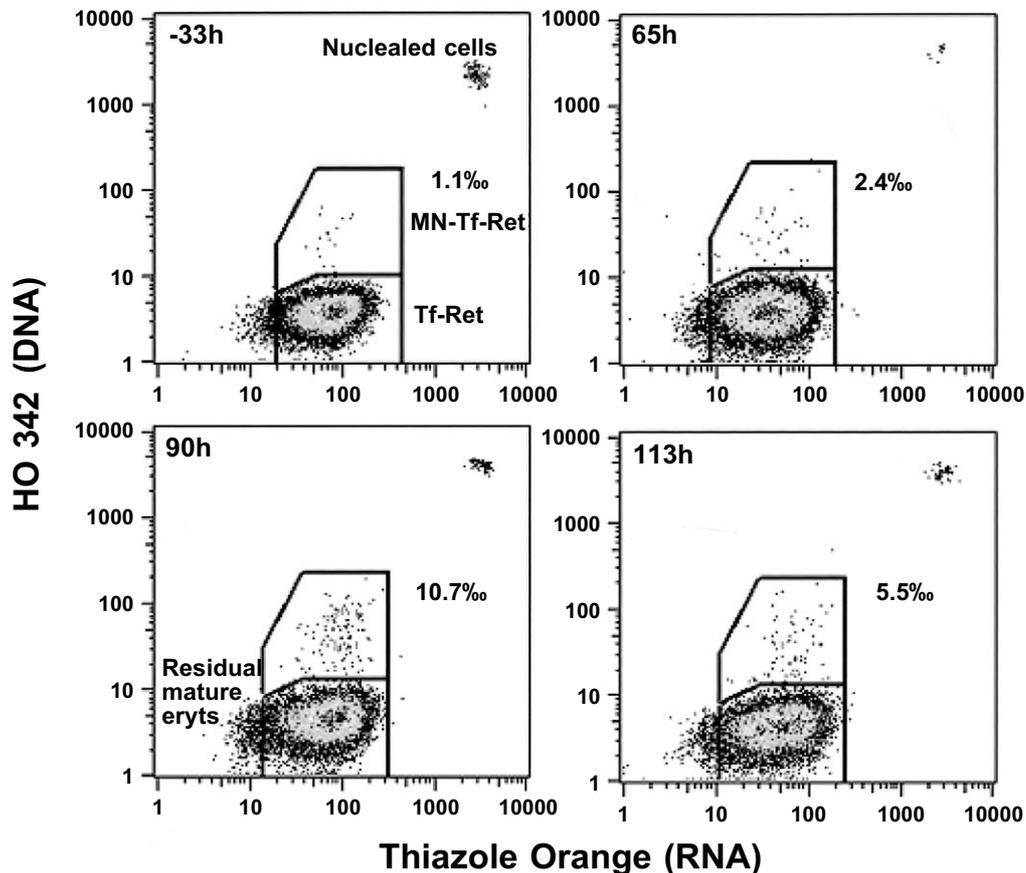

Figure 4. Flow cytometric dot plots of 4 Tf-Ret samples taken from a representative patient before and after RIT. Hour indications in each panel is relative to the time of ^{131}I administration. Each dot represents one cell. Values for the frequency of MN-Tf-Ret are given for each time point. FL1: Thiazole orange fluorescence (RNA); FL4: HO342 fluorescence (DNA).[12]

MN-assay

Since Tf-Ret are very rare, they were immunomagnetically isolated from total blood before f(MN-Tf-Ret) determination by flow cytometry. Figure 4 shows analyses of Tf-Ret fractions in patient before and after ^{131}I administration. Note a high purity of the Tf-Ret fraction: in all preparations mature erythrocyte contamination was below 10%. Typical time curves of f(MN-Tf-Ret) are presented in

Figure 5, where therapeutic ^{131}I activity of about 50 MBq per kg body-weight and about 100 MBq per kg body-weight in the first RIT and the following RITs, respectively. The MN-Tf-Ret frequency was determined once per day. After a latency period of about 2 to 3 days, f(MN-Tf-Ret) rose to the maximum within one day, and thereafter slowly decreased and attained the pre-treatment level within 3 to 6 days. The "short transit" curves are from two patients with an unusually short latency period. Figure 6 shows the dose-response of f(MN-Tf-Ret). Within the dose range of 100 to 600 mSv the dose dependence is weak. The bold line is the linear regression line for all data. Its parameters as well as those of the gender-specific regression lines are given in Table 2. On the average, the dose-dependent increase in MN frequency is higher in males than in females.

The knowledge of the erythropoiesis timetable is mandatory for understanding of the MN-Tf-Ret course in radioiodine-treated patients. An overview of the size of the different cell fraction of the erythron and the timetable of the erythropoiesis is represented in Figure 7. The term "erythron" refers to the combined populations of erythrocytes and their haemoglobin-containing precursors in blood and bone marrow. Tf-Ret in blood is a very small fraction of the erythron. They resemble marrow Tf-Ret of similar size, containing cytoplasmic organelles (mitochondria and ribosomes) and displaying transferrin receptors. In blood they belong to the so called immature reticulocyte fraction.[13] f(MN-Tf-Ret) in blood was measured as an indicator of the f(MN-Tf-Ret) in bone marrow, where radiation-induced MN form during erythroblast's mitosis. The red pulp of the spleen plays a key role in Tf-Ret maturation and the elimination of MN-Tf-Ret since it temporally sequesters reticulocytes[14] and removes the micronucleated ones. In erythrocytes of patients whose spleen has been removed, not only micronuclei (Howell-Jolly bodies) but also autophagosomes containing mitochondria and ribosomes were found.[15] Autosomes are normally absent from mature erythro-
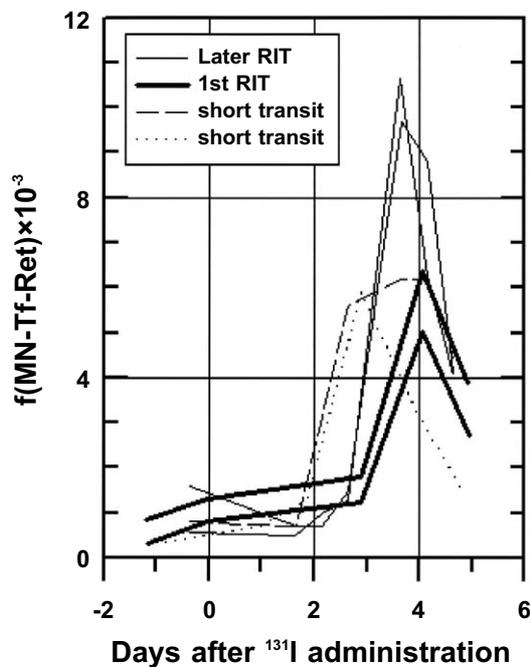

Figure 5. Course of f(MN-Tf-Ret) after therapeutic ^{131}I application. Dotted curve (1st RIT) and dashed curve (2nd RIT) are from patients, where an increase in f(MN-Tf-Ret) was detected earlier than usual.[12]
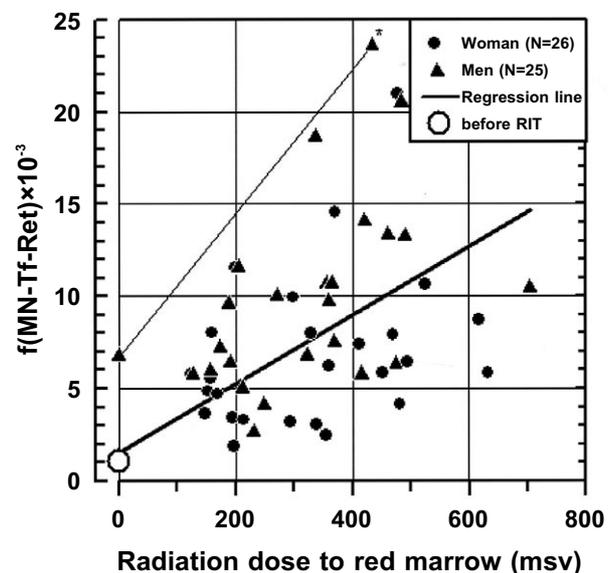

Figure 6. Dose and gender dependence of f(MN-Tf-Ret) in RIT. Maximal MN frequency was attained 3 to 4 days after ^{131}I administration in 51 RITs in 24 female and 22 male patients. Corresponding frequencies before treatment are given as a function of the total red marrow dose (Table 2). The bold line is the linear regression for the data of all RITs except one.[12]

Table 2. Dose dependence of *f(MN-Tf-Ret)* in patients after RIT. Parameters of the regression line: $f(MN\text{-}Tf\text{-}Ret) = Y_0 + a \times D$

Gender	Number of patients receiving RIT	$Y_0 \times 10^{-3}$	a (mSv^{-1}) $\times 10^{-5}$	R^2	DoublingDose (mSv)
Man	24	1.58 (0.53)[a]	2.27 (0.22)	0.69[b]	70
Women	26	1.39 (0.53)	1.52 (0.21)	0.52	91
Both	50	1.55 (0.53)	1.86 (0.16)	0.58	83

[a]Estimate of parameter (standard error of estimate in parentheses).
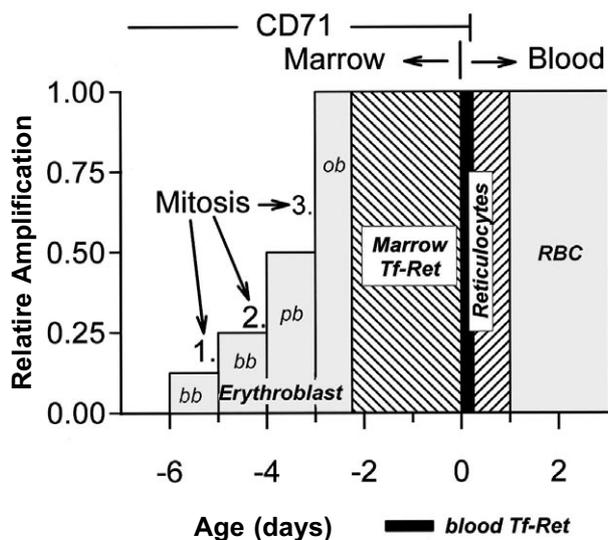[b]Coeeficient of determination.

Figure 7. Timetable of erythropoiesis in man and relative frequency of red blood cell precursors according to investigations with radioiron. The day when reticulocytes enter the bloodstream is assigned zero. Most micronuclei arise at the last mitosis and cells enter the circulation about 70 hours later. The lifespan of Tf-Ret in blood was found to be 3.5±4 (hours). bb=Basophilic erythroblast; pb=Polychromatic erythroblast; ob=Orthochromatic erythroblast.[12]

cytes. MN-Tf-Ret assay has some features that may make it one of the most useful biological dosimeter in humans. First is the high sensitivity of the assay, which allows the detection of radiation doses as low as 50 mSv. Another advantage, under some exposure circumstances, is the short memory of this endpoint. After exposure, f(MN-Tf-Ret) returns to normal range within a fortnight. This allows the retrospective comparison between the spontaneous and the radiation induced f(MN-Tf-Ret) on an individual base. The assay also has a well-documented animal model counterpart. Thus, the method may be of use for monitoring individuals after a suspected accidental radiation exposure provided blood samples can be obtained during a few consequential days after the presumed exposure.

Biodosimetry in RIT patients

Other methods of biological dosimetry applied to patient treated with radioiodine include the following:

1. MN in lymphocytes assay[16-22]

For this assay lymphocytes are cultured for 44 hours. Afterwards, cytochalsin B is added which blocks cytokinesis and makes it possible to detect lymphocytes dividing in the culture. Cells that have undergone the first mitosis are recognized as binucleated, and they are screened for presence of micronuclei.

2. Chromosome aberration assays
2.1. Fluorescence in situ hybridization (FISH)[23-28]

Painted chromosomes are analyzed under the fluorescence microscope by visual scoring of translocations, insertions, deletions and breaks.

2.2. Sister chromatid exchange (SCE) method

The test is based on the exchange of 5-bromodeoxyuridine with tymine nucleotide in the chromosomes of newly divided lymphocytes. The chromosomal uptake site of the 5-bromodeoxyuridene does not permit staining, while thymine does.

3. Glycophorin A assay[29-32]

The glycophorin A (GPA) assay concurrently detects and quantifies two types of erythrocytes with variant phenotypes arising due to the autosomal locus responsible for the polymorphic MN blood group. It uses a pair of allele-specific monoclonal antibodies and flow cytometry to analyze a standard population of 5 million cells efficiently. The two phenotypes detected correspond to single allele loss and allele loss followed by reduplication of the remaining allele; both are consistent with the mechanisms underlying "loss of heterozygosity" of tumor suppressor genes.

A comparison of MN and TCR assay to these methods is given in Table 3.

Conclusion

TCR assay and MN-Tf-Ret assay were calibrated in patients treated with radioiodine for thyroid cancer. During the course of therapy, bone marrow and lymphatic organs are exposed to ionizing radiation at a dose of 0.1 to 0.75 Sv within about 2 days. Since several

Table 3. Methods of biodosimetry applied to radioidine treated patients

Method	Sensitivity	Period of applicability	Dose characteristics	Time to consume	Biologically equivalent dose (Gy)
TCR assay	100 mGy	0.5-5 years	Cumulative	6 hours	0.4-0.6
TCR assay after cell culture	400 mGy	1 day-6 months	Cumulative	1 week	0.4-0.6
Chromosome damage	200 mGy	1 day-1 year	Cumulative	2 days	0.5
MN in lymphocytes	50 mGy	1 day-6 months	Cumulative	3 days	0.3-0.5
MN in reticulocytes	50 mGy	3-6 days	Recent	2 days	NA
GPA assay	200 mGy	0.5 years or over	Cumulative	8 hours	NA

NA=Not available.

RITs have to be given to patient with an interval of from 6 months to ca. 1 year, total accumulated dose can be up to 2 Sv within 2 to 3 years. The dose for thyroid tissue is approximately 1000 times higher. In the MN-Tf-Ret test, mutant frequency increases proportionally with the radiation dose whereas in the TCR-assay, time- and dose-relationship could be described by an exponential equation which takes into account the limited lifespan of TCR mutants. The calibration curves measured in the described patients can be used for dose estimates in humans if the radiation conditions correspond to those in RIT. This limits their applicability to low dose-rate b- and g-irradiation and to doses not exceeding about 0.5 Sv per session. If higher doses or dose-rates as well as the other types of ionizing radiation are involved, calibration curves in animals are indispensable. In the case of MN-Tf-Ret test, mouse models are established and may be used.

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