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Validation of an immunochemical assay for the detection of DNA damage as a tool for biological dosimetry of human exposure to ionizing radiation

A method for biological dosimetry based on the immunochemical detection of DNA damage in human white blood cells has been validated. To this end the method developed at TNO (Rijswijk, the Netherlands) was also set up at IChTJ (Warszawa, Poland). Blood samples of 11 individuals were irradiated with 0 or 5 Gy of 170 kV X-rays at IChTJ and analysed both at IChTJ and TNO. It appeared that in both laboratories damage could be detected to the same extent. The average background level of DNA damage amounted to 1.0 Gy-eq with an inter-individual standard deviation of 0.25 Gy. The contribution of the sample variance to the total variance is only 14%. The radiosensitivity showed only a variation of about 10% and can, therefore, be neglected in estimating the radiation dose from the amount of DNA damage detected.

Ocena testu immunochemicznego wykrywania uszkodzeń DNA jako metody dozymetrii biologicznej ekspozycji ludzi na promieniowanie jonizujące

Oceniono metodę immunochemiczną wykrywania uszkodzeń DNA w limfocytach ludzkich. W tym celu, metodę opracowaną w TNO (Rijswijk, Holandia) wdrożono także w IChTJ. Próbki krwi 11 osobników napromieniono w IChTJ (0 lub 5 Gy) promieniowaniem X (170 kV) a następnie wykonano oznaczenia równolegle w TNO i IChTJ. W obu laboratoriach wykrywano podobny poziom uszkodzeń DNA. Średni poziom tła wynosił 1 Gy-eq (równoważnik Gy) z międzyosobniczym odchyleniem standardowym 0,25 Gy. Udział wariancji próbki w stosunku do całkowitej wynosił tylko 14%. Wariancja promieniowrażliwości wynosiła ok. 10% a zatem może być zaniedbywana przy ocenie dawki pochłoniętej na podstawie poziomu uszkodzeń DNA.

CONTENTS

1. INTRODUCTION

For an accurate assessment of the extent of damage, induced by exposure to ionizing radiation, a complementary approach that combines physical dosimetry and biological indicators of damage is necessary. To this purpose TNO (Rijswijk, the Netherlands) is developing a method for biological dosimetry based on the immunochemical detection of DNA damage in human white blood cells. A rapid immunochemical assay, the sandwich ELISA, has been developed [1]. With this technique the extent of radiation-induced single-strandedness in DNA is assessed, which results from the partial unwinding of DNA containing single-strand breaks [2,3]. Because directly induced single-strand breaks are rather rapidly repaired, this dosimetry method should be applied within a short period after exposure.

In the present report part of the results of a co-operative research project between the Netherlands and Poland are presented. The data described in this report are a contribution to the validation of the immunochemical assay. It contains the experiments carried out in Warsaw with the set-up of the method, developed in the Netherlands, and the subsequent application of the method in a study on the inter-individual variation of background levels and of radiosensitivity of blood of 11 donors. The same alkali-treated blood samples were also analysed in the immunochemical assay at TNO in Rijswijk.

2. MATERIALS AND METHODS

2.1. Blood sampling

Venous blood of 11 human volunteers (10 ml, with consent of the donor) or pigs was collected in evacuated glass tubes, containing 15 mg Na_2EDTA. The donors were 8 females ranging in age from 35-50 years and 3 males ranging from 35-40 years, all non-smoking and in good health.

2.2. Alkaline treatment

The procedure described by Timmerman et al. 1995 [4] was used for the detection of radiation-induced strand breaks in DNA. Briefly, 30 µl of 10-fold diluted blood was brought in a cluster tube in quadruplicate. Then, 100 µl 20 or 25 mM NaOH in 1.3 M NaCl (calculated pH of 12.3 or 12.4 respectively) was added at 20°C. After 6 min at 20°C the solutions were neutralized with 25 µl 0.25 M NaH_2PO_4, followed by about 1-second sonication.

2.3. Sandwich ELISA

The sandwich ELISA has been described in detail previously [4]. The 100%ssDNA values were determined as a mean of 16 determinations for each donor. The %ssDNA was determined in quadruplicate both for samples treated at pH 12.3 and pH 12.4. Each plate contains one sample of each donor. To eliminate plate-to-plate variations the mean %ssDNA over the 11 donors of each plate was normalized to the mean %ssDNA on the first plate. This normalization procedure is required since in these experiments we had chosen not to apply on each plate an internal control for calibration.

2.4. Irradiation with 180 kV X-rays

Human blood, collected as described above, was irradiated in vitro, after pre-cooling to 5°C. X-irradiation was performed using a Stabilipan-250 machine Siemens, Erlangen, German.

Conditions of irradiation were: 180 kV, 18 mA, 1 mm copper filter and dose rate of 1.28 Gy/min. Irradiations were performed after cooling the blood samples on ice.

2.5. Microtiter plate reader

The microtiter plate reader used at IChTJ was a Fluoroskan (Eflab, Finland). At TNO the Cytofluor II (Perceptive Biosystems, USA) was applied.

3. RESULTS

For studying the inter-individual variation of all blood samples the background level of breaks was determined, expressed in both %ssDNA and in Gray-equivalents (Gy-eq). One Gy-eq corresponds to the amount of breaks induced by 1 Gy of X-rays. The radiosensitivity was determined by comparing the %ssDNA after a dose of 5 Gy with that of the unirradiated sample. Alkali-treated blood samples were analysed in the sandwich ELISA both at IChTJ and TNO.

As shown in Fig. 1, there is a large variation in the 100%ssDNA values indicating that the blood of the donors contained large variations in the total amount of WBC.

The results in Fig. 2 show that for the pH 12.4 samples there is a good correlation between the %ssDNA data obtained in Poland and those in the Netherlands. At pH 12.3 there is somewhat less correlation which may be due to the low fluorescence values obtained after treatment at pH 12.3.

In Fig. 3 the %ssDNA data are presented after irradiation of the blood samples with a dose of 5Gy.

It is surprising that the variation in the data for the at pH 12.3 and 12.4 treated blood samples and analysed at TNO is more or less the same, whereas that variation is not found in the corresponding data analysed at IChTJ. This suggests that during freezing-thawing some alteration occurs which is donor-dependent. It should be mentioned that the observed difference in level between the data obtained at IChTJ and TNO may be due to the application of the plate-to-plate normalization procedure (by the absence of an internal control).

In Fig. 4 the radiosensitivity, expressed as %ssDNA/Gy, is presented for the 11 donors.

Also in this Fig. the close correlation between the data of the pH 12.3 and 12.4 bloodsamples is surprising (determined at TNO), whereas the pattern for the sample analysed at IChTJ is somewhat different. In the latter case the sample variance is the main contribution to the total variance.

The patterns of the data presented in Fig. 2-4 show some differences when comparing those produced in IChTJ and TNO. The main reason appeared to be the fact that the 100%ssDNA values for the data of IChTJ are based on only 2 determinations per person. When we apply the 100%ssDNA values determined at TNO (based on 16 determinations per person) to the analysis carried out at IChTJ, together with the same plate-to-plate normalization as applied on the samples analysed at TNO, then the pattern resemble much better (Fig. 5-7).

In Fig. 8 the background level of DNA damage is expressed in Gy-eq. For these calculations, in principle, the absolute value of the total amount of DNA, i.e. the values for 100%ssDNA are not required. The average level of damage calculated from the pH 12.3 samples corresponds very well with those of the pH 12.4 samples (determined at TNO).There appeared to be a good correlation between the average level of background damage, expressed in Gy-eq, of the pH 12.3 and 12.4 blood samples assayed at TNO and the pH 12.3 samples assayed at IChTJ. The pH 12.4 data obtained at IChTJ are somewhat higher (possibly inherent to the plate-to-plate normalization procedure), but follow very well the pattern observed at

TNO. This indicates that again a clear inter-individual variation with respect to the background level of DNA damage exists.

When the plate-to-plate normalization of TNO was applied to the data obtained at IChTJ, both curves became more close to one another (Fig. 9).

In Table the results of the variance analysis are summarized.

Table. Variance analysis of data obtained with the sandwich ELISA carried out at IChTJ and TNO. Sandwich ELISA's were carried out on blood of 11 individuals irradiated with 180 kV X-rays with doses of 0 and 5 Gy. To eliminate plate-to-plate variations the mean %ssDNA over the 11 donors of each plate was normalized to the mean %ssDNA on the first plate. This normalization procedure is required since in these experiments we had chosen not to apply on each plate an internal control for calibration. Due to this operation the mean values (with its STD between brackets) observed at IChTJ may differ from those obtained at TNO.

	site of analysis	mean± (STD)*	mean ± (STD)	total variance	sample variance
%ssDNA (0 Gy, 12.3)	TNO	0.69 (0.19)	1.00 (0.27)	0.074	0.010
	IChTJ	1.06 (0.18)		0.032	-
%ssDNA (0 Gy, 12.4)	TNO	3.22 (0.70)	4.13 (0.94)	0.886	0.140
	IChTJ	3.77 (0.64)	3.72 (0.69)	0.470	0.040
%ssDNA (5 Gy, 12.3)	TNO	6.81 (0.65)	6.65 (0.64)	0.406	0.123
	IChTJ	8.42 (1.57)		2.48	-
%ssDNA (5 Gy, 12.4)	TNO	19.64 (2.31)	26.22 (2.88)	8.28	3.11
	IChTJ	20.72 (2.19)	14.67 (1.04)	1.08	0.57
%ssDNA/Gy (12.3)	TNO	1.22 (0.13)	1.13 (0.13)	0.016	0.005
	IChTJ	1.47 (0.30)		0.089	-
%ssDNA/Gy (12.4)	TNO	3.28 (0.42)	4.42 (0.52)	0.269	0.130
	IChTJ	3.39 (0.41)	2.19 (0.18)	0.031	0.020
Gy-eq (0 Gy, 12.3)	TNO	0.57 (0.16)	0.90 (0.28)	0.076	0.012
	IChTJ	0.74 (0.15)		0.023	-
Gy-eq (0 Gy, 12.4)	TNO	0.99 (0.25)	0.95 (0.25)	0.063	0.013
	IChTJ	1.23 (0.26)	1.71 (0.36)	0.127	0.020

In the column marked with * data are presented without application of the normalization procedure.
The data of the pH 12.3 samples of IChTJ are the means of single determinations.
The sample variance was calculated as the average of the variances of the quadruplicate samples of each individual.

The variance analysis of %ssDNA (0 Gy) revealed that the sample variance contributes for only 9-14% to the total variance, indicating that variance is mainly due to inter-individual variation of the background DNA damage. The same can be concluded when the background damage is expressed in Gy-eq. For %ssDNA/Gy, again the variance analysis suggest that above

the sample variance also an inter-individual variance exists with respect to radiosensitivity, albeit less pronounced than with respect to the background level.

In addition to the series of experiments described above, at IChTJ another experiment was carried out in order to check, whether the technique was adequately mastered. The results are presented in Figs 10-11.

Fig. 10 shows the variation in the 100%ssDNA values. The variation corresponds reasonably to that observed earlier.

In Fig. 11 (upper panel) the data of the background level and radiosensitivity are presented.

The variation in background level is about the same as observed earlier. Moreover, the variation is larger than that of the level after 5 Gy. Nevertheless, the radiosensitivity also shows much inter-individual variation. Also this is in agreement with the earlier data.

Finally, the background level expressed in Gy-eq (Fig. 11, lower panel), resembles reasonably that observed earlier, taking into account that this time only two determinations have been carried out.

4. DISCUSSION AND CONCLUSIONS

The data presented indicate that both at TNO an IChTJ radiation-induced damage in DNA of WBC can be detected with the use of the sandwich ELISA. The average amount of background damage in the blood of the 11 individuals is about 1 Gy-eq with a STD of 0.25 Gy-eq. Damage can be detected, in comparison to the unirradiated sample, at a lower detection limit of 0.2 Gy in a single determination. When determination is carried out in quadruplicate, the lower detection limit is 0.1 Gy. However, when the background level of DNA damage is unknown, one has to take into account the inter-individual variation in the background level.

Both the analysis at pH 12.3 and 12.4 lead to reasonable results, albeit, with respect to the reproducibility of the background levels, there is a slight preference for the pH 12.4 treatment. On the other hand, one has to take into account that blood contains different populations of cells (lymphocytes, granulocytes and possibly apoptotic cells) which may vary for the different individuals and which may behave differently at different pH's.

The radiosensivity showed only a 12% variation between the 11 individuals. Therefore, the pattern of the background level does not change very much when expressed either in %ssDNA or Gy-eq. This indicates that for a quick estimate of the amount of (radiation-induced) damage the inter-individual variation in radiosensitivity can be neglected.

In the absolute value of the amount of background damage there is still a larger uncertainty than necessary, due to plate-to-plate variations. When repeating the analysis it should be worthwhile to apply an internal control DNA sample to be able to correct for these plate-to-plate variations.

For a better comparison of the analysis carried out at IChTJ and TNO, it should be necessary to carry out the analysis at IChTJ to the same extent as was done at TNO (all samples in quadruplicate and the 100% values in 16-fold).

Acknowledgements

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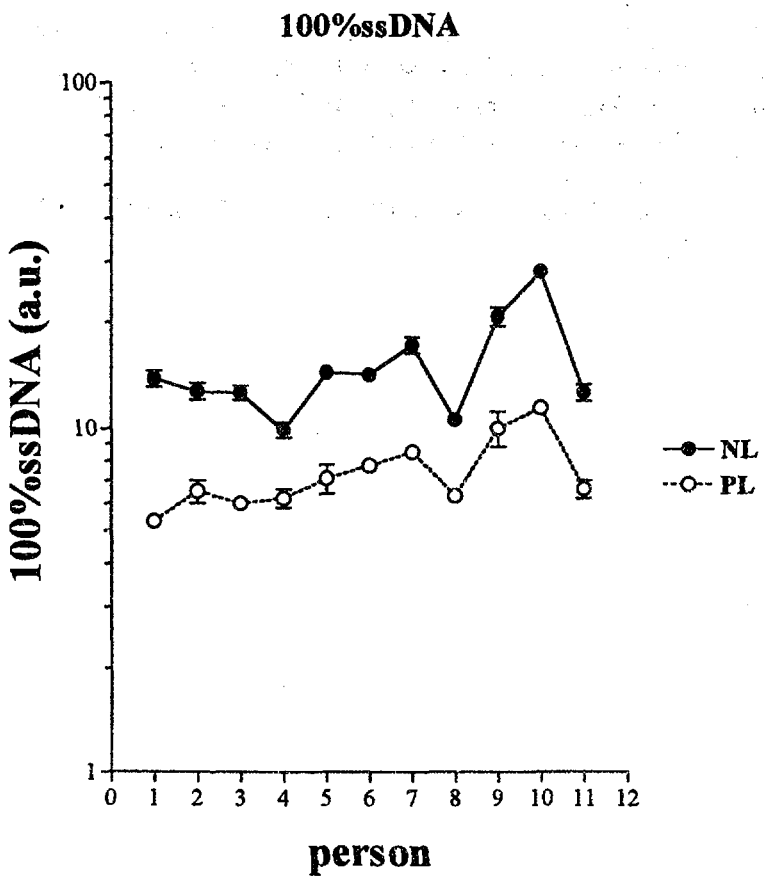

Fig. 1. Inter-individual variation in the total amount of DNA in each blood sample, expressed as 100%ssDNA value in arbitrary units (which are different at TNO and IChTJ, depending on the type of microtiter plate reader used). The data obtained at TNO are the mean of 16 determinations. The error bars represent SEM. The data obtained at IChTJ are the mean of 2 determinations. The error bars represent the range between the two data.

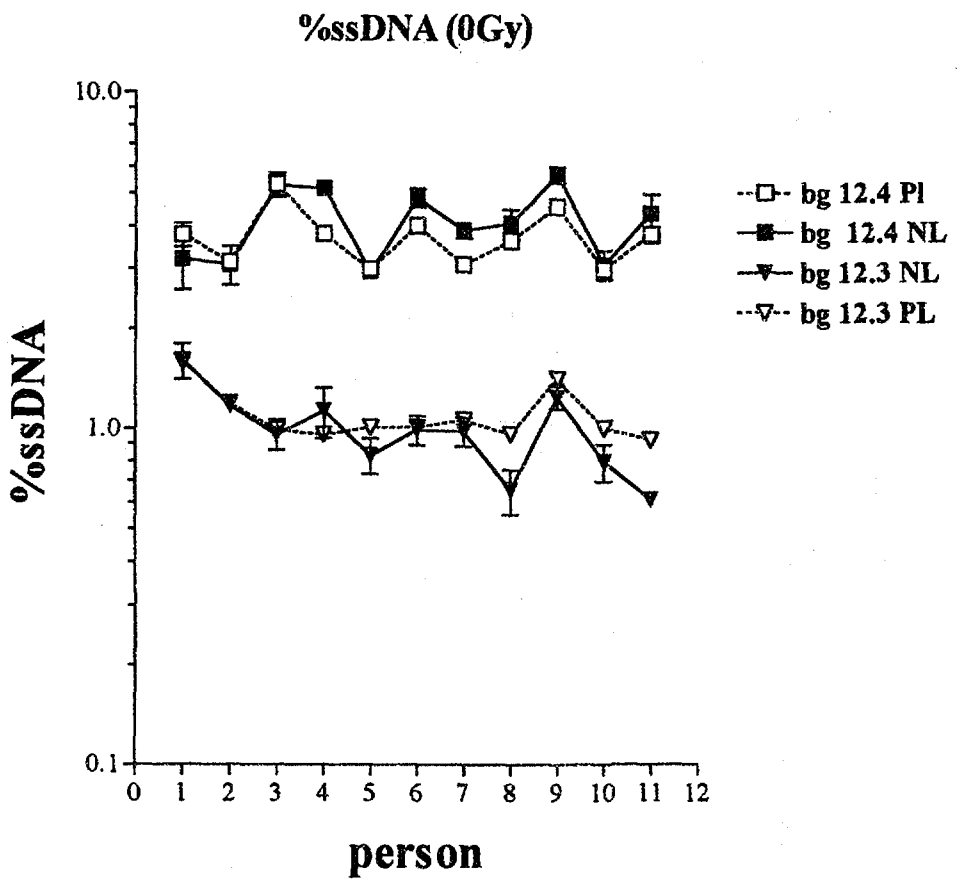

Fig. 2. Inter-individual variation in background level, expressed as %ssDNA, in blood of 11 individuals. The data represent the mean of 4 blood samples, split from the same donor and divided over 4 microtiter plates. The error bars represent the SEM. The pH 12.3 samples were only carried out as a single determination at IChTJ. The 100%ssDNA fluorescence values, used for the calculations, were averaged over 16 determinations per donor (at TNO) or 2 determinations per donor (at IChTJ).

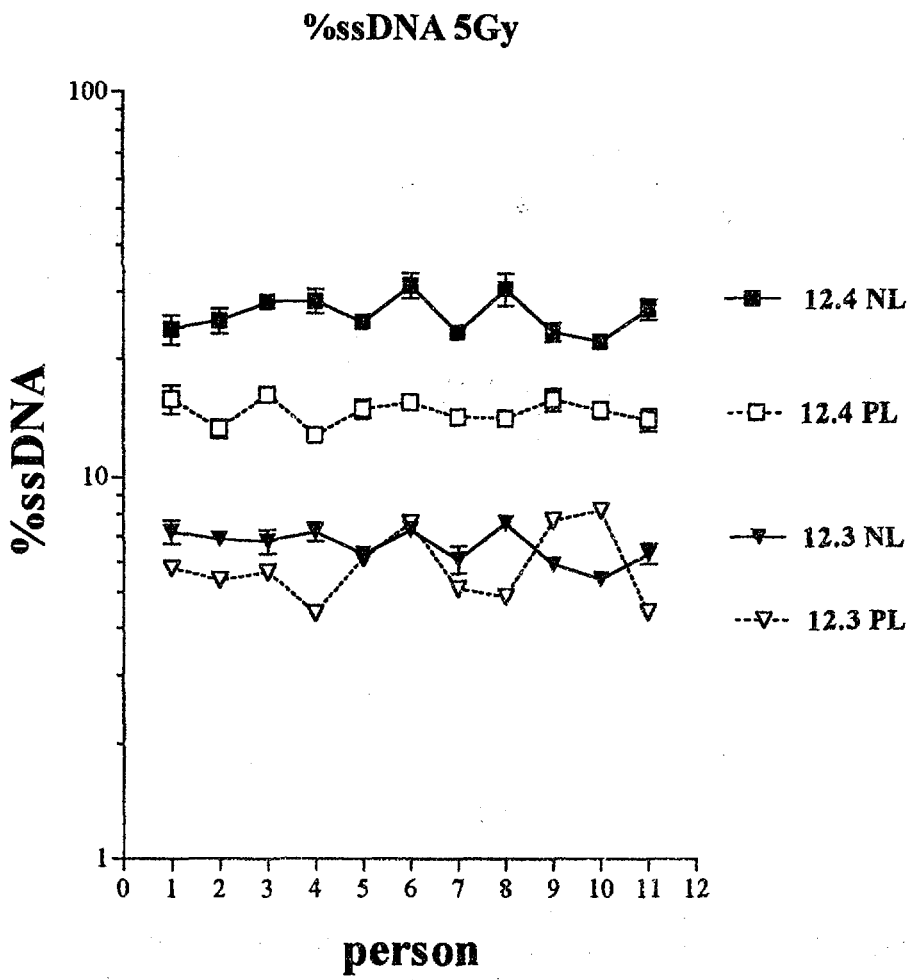

Fig. 3. Inter-individual variation in the %ssDNA after irradiation with a dose of 5 Gy, in blood of 11 individuals. The data represent the mean of 4 blood samples, split from the same donor and divided over 4 microtiter plates. The error bars represent the SEM. The pH 12.3 samples were only carried out as a single determination at IChTJ. The 100%ssDNA fluorescence values, used for the calculations, were averaged over 16 determinations per donor (at TNO) or 2 determinations per donor (at IChTJ).

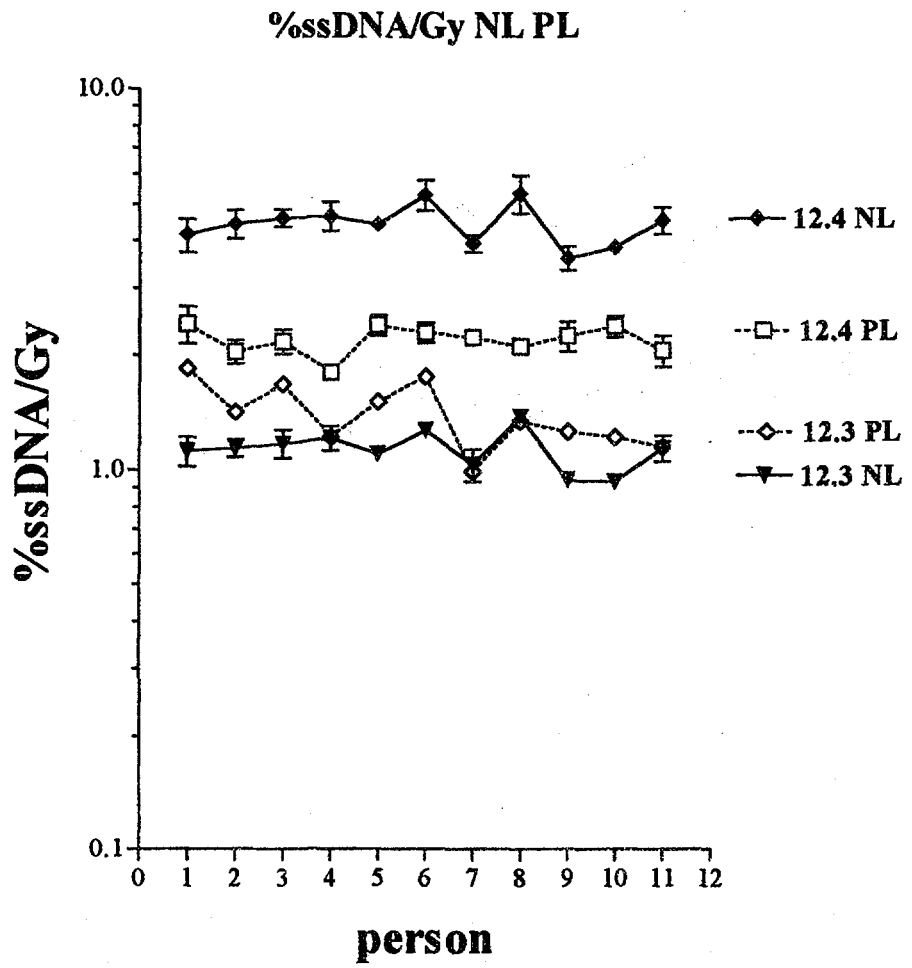

Fig. 4. Inter-individual variation in radiosensitivity, expressed as %ssDNA/Gy, of blood of 11 individuals. The data represent the mean of 4 blood samples, split from the same donor and divided over 4 microtiter plates. The error bars represent the SEM. The pH 12.3 samples were only carried out as a single determination at IChTJ. The 100%ssDNA fluorescence values were averaged over 16 determinations per donor (at TNO) or 2 determinations per donor (at IChTJ). The data are calculated from the data presented in Figures 2 and 3.

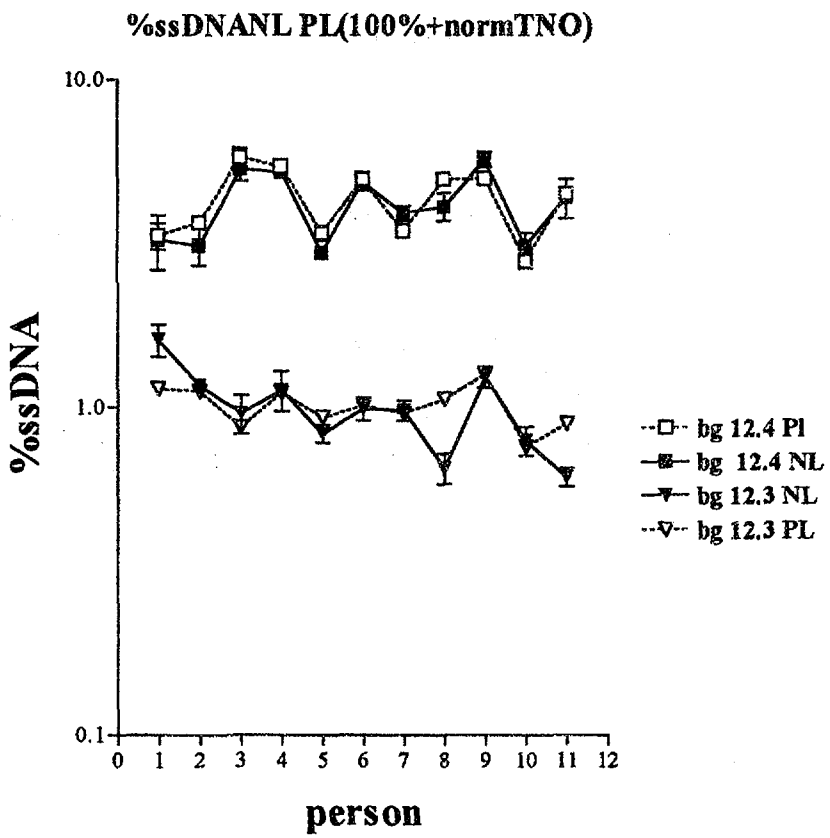

Fig. 5. Inter-individual variation in background level, expressed as %ssDNA, in blood of 11 individuals. The same data are presented as in Fig. 2 but calculated with the 100%ssDNA fluorescence values, determined at TNO, and with the TNO plate-to-plate normalization.

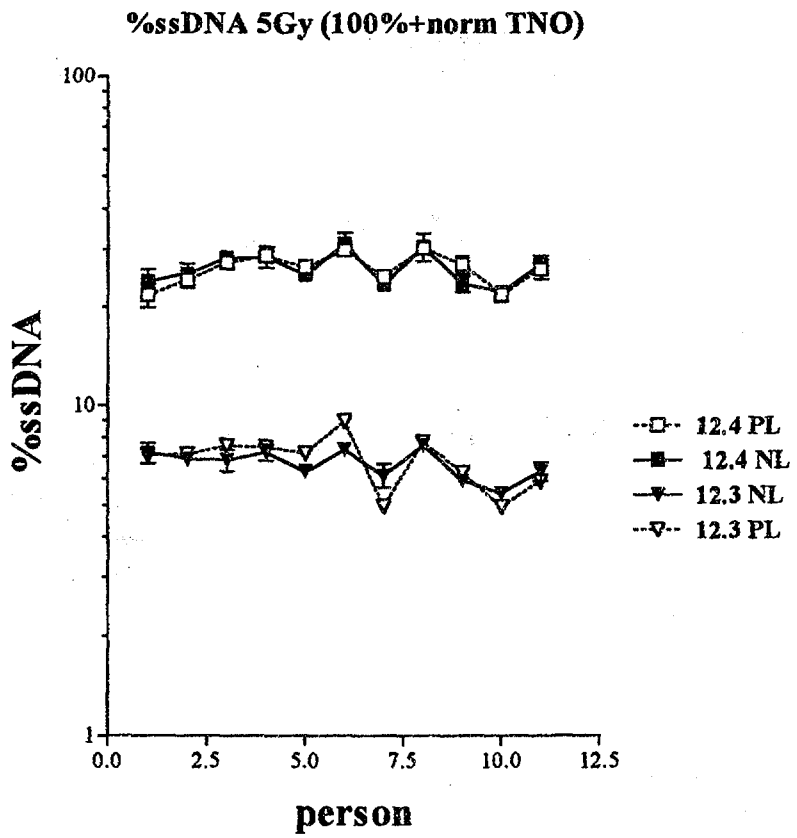

Fig. 6. Inter-individual variation in the %ssDNA after irradiation with a dose of 5 Gy, in blood of 11 individuals. The same data are presented as in Fig. 2 but calculated with the 100%ssDNA fluorescence values, determined at TNO, and the TNO plate-to-plate normalization.

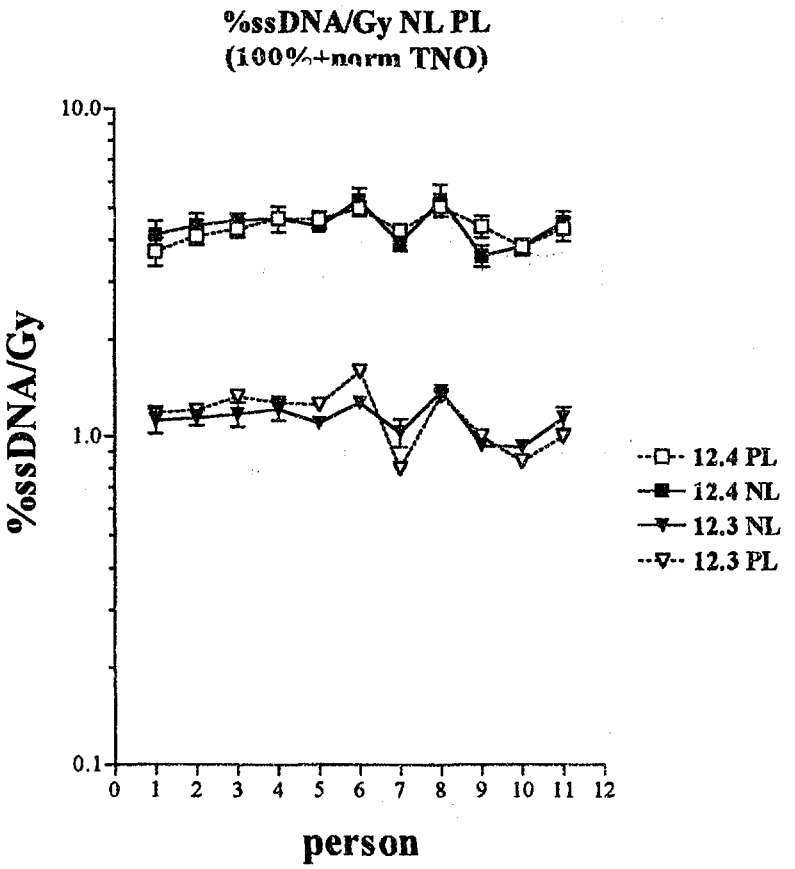

Fig. 7. Inter-individual variation in radiosensitivity, expressed as %ssDNA/Gy, of blood of 11 individuals. The same data are presented as in Fig. 2 but calculated with the 100%ssDNA fluorescence values, determined at TNO, and the TNO plate-to-plate normalization.

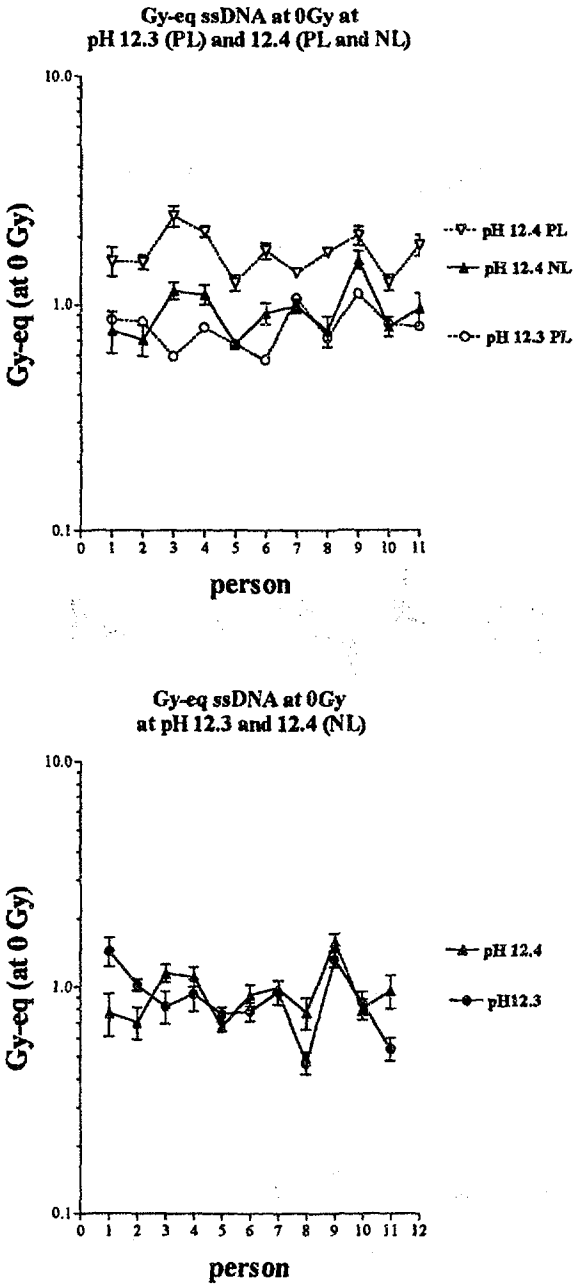

Fig. 8. Inter-individual variation in background level, expressed as Gy-eq, in blood of 11 individuals. The data represent the mean of 4 blood samples, split from the same donor and divided over 4 microtiter plates. The error bars represent the SEM. The pH 12.3 samples were only carried out as a single determination at IChTJ. The 100%ssDNA fluorescence values, used for the calculations, were averaged over 16 determinations per donor (at TNO) or 2 determinations per donor (at IChTJ).). The data are calculated from the data presented in Figs 2 and 3.

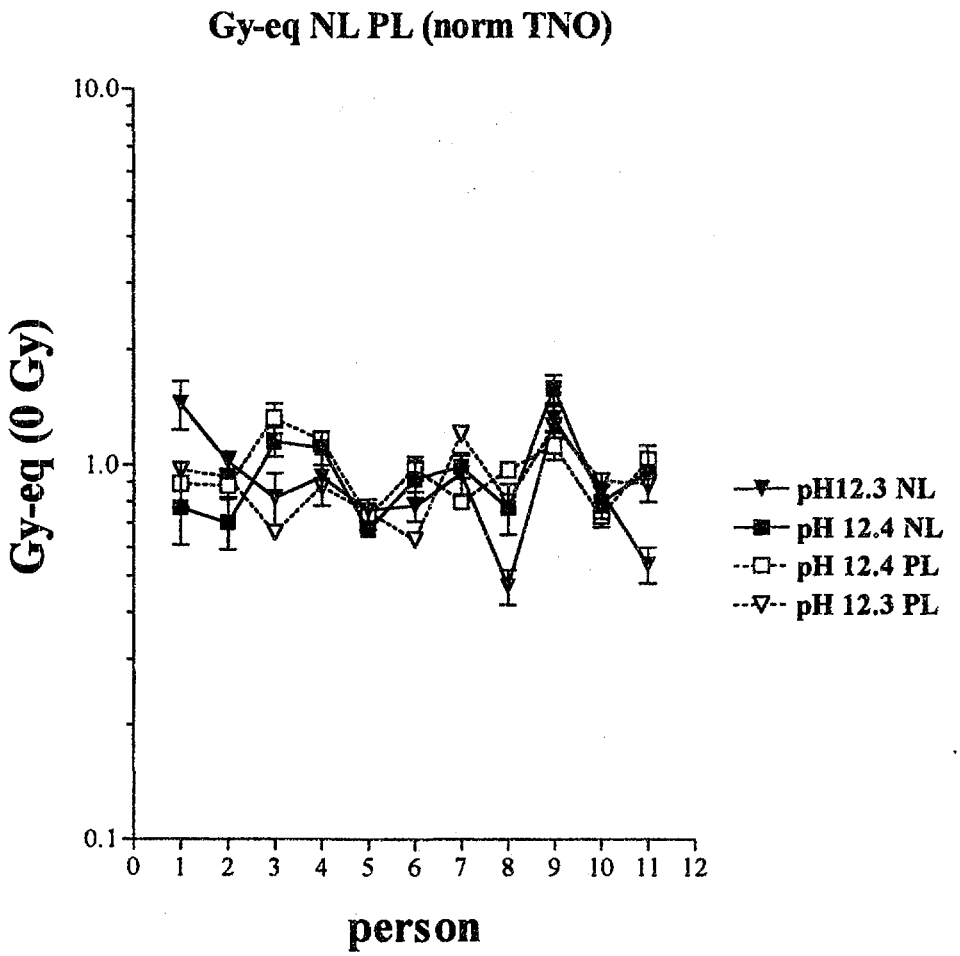

Fig. 9. Inter-individual variation in background level, expressed as Gy-eq, in blood of 11 individuals. The same data as presented in Fig. 8 but the IChTJ-data are normalized according to the plate-to-plate normalization applied at TNO.

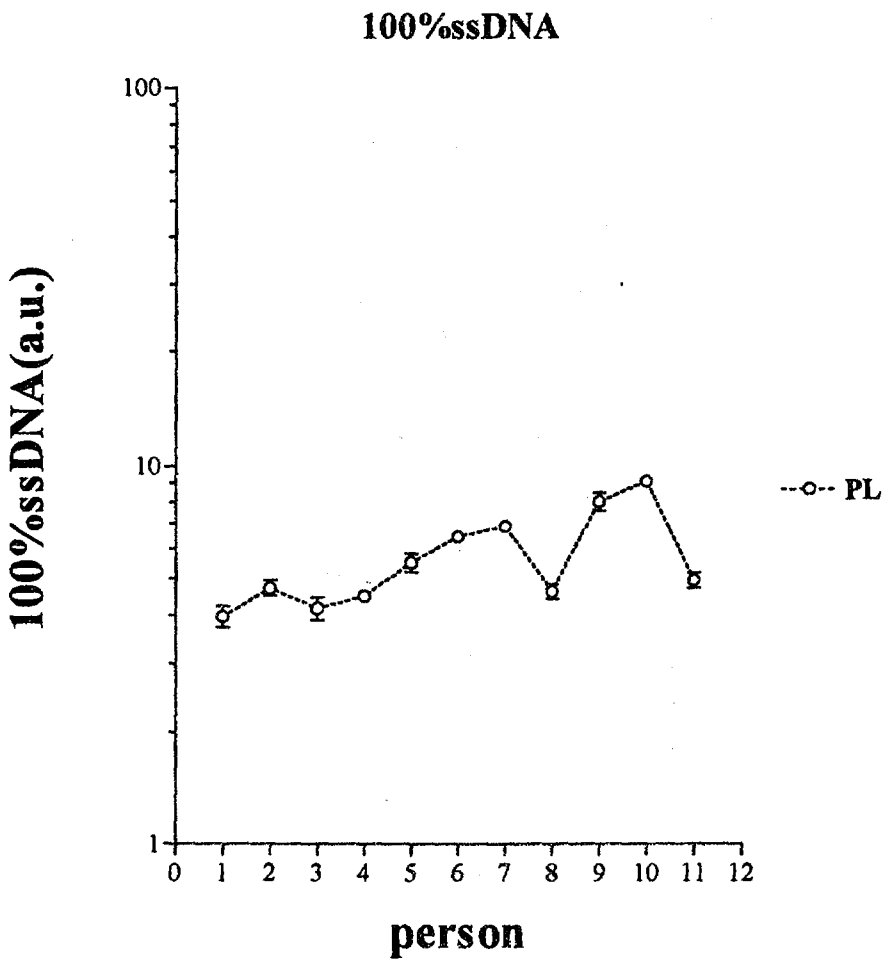

Fig. 10. Inter-individual variation in the total amount of DNA in each blood sample, expressed as 100%ssDNA value in arbitrary units. The data obtained at IChTJ are the mean of 4 determinations normalized to the average 110%ssDNA values of the first plate. The error bars represent the SEM.

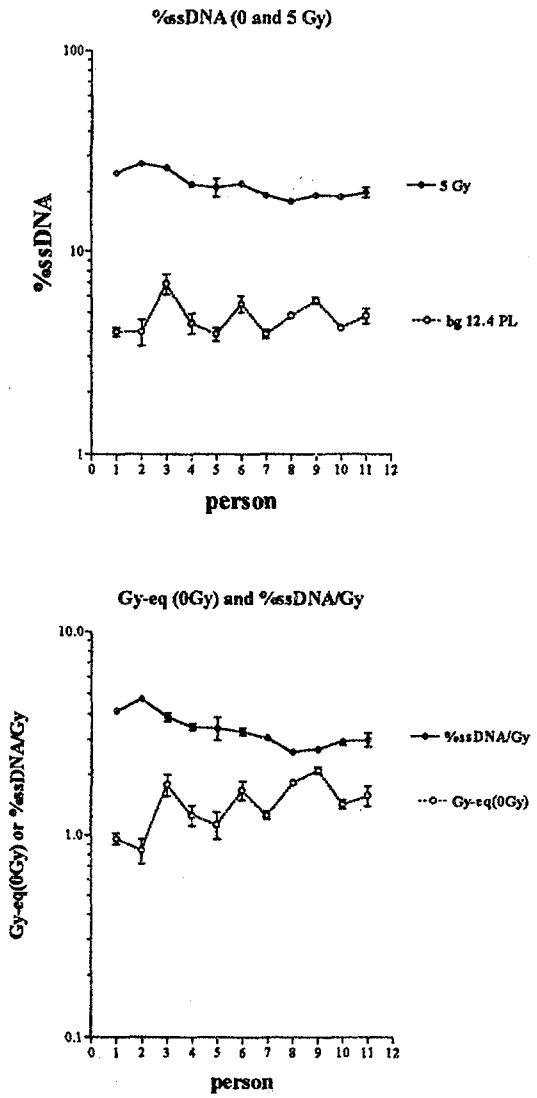

Fig. 11. Upper panel: Inter-individual variation in background level and the level after irradiation with a dose of 5 Gy, expressed as %ssDNA, in blood of 11 individuals. The data represent the mean of 2 blood samples, split from the same donor and divided over 2 microtiter plates. The error bars represent the range between the two data. The 100%ssDNA fluorescence values, used for the calculations, were averaged over 4 determinations per donor (at IChTJ) and normalized to the average of the first plate.
Lower panel: Inter-individual variation in background level (expressed in Gy-eq) and radiosensitivity, expressed as %ssDNA/Gy, of blood of 11 individuals. The data represent the mean of 2 blood samples, split from the same donor and divided over 2 microtiter plates. The error bars represent the range between the two data.