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Can individual repair kinetics of UVC-induced DNA damage in human lymphocytes be assessed through the comet assay?

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Abstract

The suitability of the comet assay for quantifying DNA repair capacity at individual level was studied following the kinetics of nucleotide excision repair (NER) in human lymphocytes from four healthy donors, at various time steps after a single dose of UVC. A significant increase of DNA migration was seen as soon as 20 min after UV exposure, reaching the peak within 60–90 min. Afterwards, a rapid decline was observed, approaching the basal level at 180–240 min. The increase could be ascribed to excision activity, while the reduction to gap filling and rejoining, as demonstrated by the effects of phase-specific inhibitors, novobiocin and aphidicolin. Therefore, the comet assay should allow following the biphasic kinetics of NER. Wide inter-individual differences were observed, although repeated tests on the same donor cells revealed a large experimental variation. To quantitatively compare the individual patterns, a mathematical model was developed that adequately fitted the experimental results and estimated appropriate descriptors for each phase and for each donor. A second approach was also used to directly compare the distributions of damaged cells and to assess the differences between donors and between experiments visualizing them as reciprocal distances on a two-dimensional space computed with a principal component analysis (PCA). The results confirmed the inter-individual differences, but also the strong influence of experimental factors of the comet assay.

The two approaches provided the means of accurately comparing DNA repair kinetics at individual level, taking also into account the experimental variability which poses serious doubts on the suitability of the comet assay. Nevertheless, since this methodology allows a detailed analysis of repair kinetics and it is potentially very useful for identifying individual with reduced repair capacity, further efforts have to be addressed to improve the reproducibility of the comet assay.

Keywords: UVC irradiation; DNA repair capacity; Comet assay; DNA repair inhibitors; Experimental variability

1. Introduction

An important source of interindividual variability in cancer susceptibility is related to DNA repair capacity. In fact, individuals may widely differ in their capacity to repair DNA damage induced by both exogenous

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agents, such as tobacco smoke and sunlight exposure, and endogenous agents, such as oxidative stress by-products. For this reason, a number of epidemiological studies have been conducted to compare DNA repair capacity of cancer patients with controls [1–4]. There are interesting evidences that many polymorphisms of genes involved in different DNA repair pathways, such as base excision repair (BER), nucleotide excision repair (NER), recombination repair and mismatch repair (MMR) can modulate cancer susceptibility. This is not surprising because it is well known that genetic defects in DNA repair systems are responsible for an extremely high cancer proneness in several diseases, such as xeroderma pigmentosum (XP), hereditary non-polyposis colorectal cancer (HNPCC) or Li-Fraumeni syndrome.

NER is a very versatile and complex pathway, controlling the removal of UV-induced DNA damage and bulky adducts [5]. It is a highly conserved process among eukaryotes and is accomplished by at least 20–30 proteins [3]. NER pathway consists of the following four steps: recognition of the DNA lesion; excision of a 24–32 nucleotide stretch containing the lesion by dual incision of the damaged DNA strand on both sides; filling in of the resulting gap by DNA polymerase and ligation of the nick [6]. Therefore, during the process, DNA breaks are produced as intermediates, which can be visualized as DNA migration by the comet assay [7,8]. Polymerization and ligation steps subsequently rejoin the broken ends, determining a reduction of DNA breaks. Thus, the comet assay should allow following in details the kinetics of NER process, so that this test is potentially more informative than other cytogenetic assays (such as micronuclei, chromosome aberration and sister chromatid exchanges) which detect only indirectly the induction and repair of genetic damage [9–13].

Some chemicals are known to inhibit enzymes taking part in NER pathway. Novobiocin (NOV), interacting with the ATPase subunit of topoisomerase II, inhibits the incision step, thus markedly reducing repair-specific DNA cleavage [14–16]. On the other side, aphidicolin (APC), an inhibitor of DNA polymerase α, can block the rejoining of DNA ends, thus causing the persistence of DNA breaks [14,16,17]. Therefore, these two inhibitors can be used to separately suppress the two NER steps (incision step and polymerisation–ligation step) in UV-exposed human lymphocytes and their effects can be monitored by the comet assay [18,19].

The aim of this work is to assess whether the comet assay may be a reliable method for quantifying the two main phases of NER, i.e. DNA excision and DNA synthesis-ligation, controlled by several polymorphic genes. This would allow a careful assessment of the functional effect of specific alleles and allelic associations at individual level.

2. Materials and methods

2.1. Lymphocyte isolation

Heparinized venous blood samples were collected from two female and two male non-smoking healthy donors of similar age (25–27 years). Donors had never experienced photosensitivity or other unusual reactions consequent to sun exposure during their life. Three milliliters of whole blood and phosphate buffered saline (PBS, pH 7.4) (1:1) were carefully layered on 6 ml of Histopaque 1077 (Sigma, Milan, Italy) and centrifuged at 2100 rpm for 30 min. The upper layer was removed, the lymphocyte-containing buffy coat was carefully aspirated and the cells were washed in RPMI 1640 (Gibco, Invitrogen, s.r.l., San Giuliano Milanese, Italy), then resuspended in RPMI medium with antibiotics (100 IU penicillin/ml and 100 μg/ml streptomycin) and phytohemagglutinin (PHA, Gibco, Invitrogen, s.r.l., San Giuliano Milanese, Italy) for 20 h before UV exposure [20]. For lymphocyte cryopreservation and recovery, the procedures described by Visvardis et al. were followed [21].

2.2. UV irradiation and treatment with DNA repair inhibitors

Lymphocytes, suspended in PBS (pH 7.4), were irradiated for 15 s with a 254-nm UVC germicidal lamp (Philips, Milan, Italy) at a dose rate of 0.1 J/m^2/s. The intensity was measured with a short-wave ultraviolet intensity meter (UVP, USA). UVC irradiated cells were then suspended in RPMI 1640 medium supplemented with 10^{-4} M thymidine (Invitrogen) and incubated for eight time lengths (0, 20, 40, 60, 90, 120, 180 and 240 min) before proceeding to the comet assay. NOV (Sigma, Milan, Italy) was dissolved in dimethylsulfoxide (DMSO) and added to the cell suspension (900 μM) 1 h before UV irradiation and maintained in the medium until the end of the culture [16]. A stock solution of APC (Sigma, Milan, Italy), 2 mg/ml in ethanol, was stored at 4 °C [18,19]. APC was supplemented to the cell suspension (5 μM) immediately after UV irradiation and then kept in the culture for 1 or 4 h. After incubation, cells were washed in PBS. Lymphocytes to be processed for the assessment of DNA damage by means of the comet assay were divided into four vials as follows: (1) control, unirradiated cells; (2) unirradiated cells added with APC or NOV; (3) UV irradiated cells; (4) UV irradiated cells added with APC or NOV.

2.3. Single-cell gel electrophoresis (comet assay)

The alkaline (pH > 13) comet assay was performed according to Tice et al. [22]. Briefly, cells were suspended in pre-warmed low melting point agarose (LMA). Two solutions, 1%

normal melting point agarose (NMA) and 0.5% LMA were prepared in Ca^{2+}-Mg^{2+}-free PBS. Conventional microscope slides were dipped in 1% NMA and let dry to prepare the first layer of agarose. Then, 85 μl of 0.5% LMA containing 4×10^5 cells (10 μl cell suspension + 75 μl LMA) was poured onto the pre-coated slides. After solidification at 4 °C, further 85 μl of 0.5% LMA was layered onto the slides. Slides were immersed in ice-cold freshly prepared solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) to lyse cells and to allow DNA unfolding for at least 1 h at 4 °C in the dark. Then, slides were placed in a horizontal electrophoresis unit, covered with fresh buffer (1 mM Na_2EDTA, 300 mM NaOH, pH > 13) for 20 min. This allowed DNA unwinding and conversion of alkali-labile sites to single strand breaks (SSB). The electrophoresis run was performed at 25 V (1 V/cm) and 300 mA for 20 min. Both unwinding and electrophoresis were performed in an ice bath in order to maintain a constant the temperature (4 °C) throughout the whole procedure. The slides were then gently washed in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove alkali and detergent, placed in methanol for 3 min and stained with 60 μl ethidium bromide (50 μg/ml). All steps described above were conducted under yellow light or in the dark, to prevent additional DNA damage. For each experimental point, at least 50 cells were automatically analyzed using the Komet 5.5 Image Analysis System (Kinetic Imaging Ltd., Liverpool, UK) and DNA cleavage was expressed as the fraction of total DNA contained in the comet tail (TDNA).

2.4. Data analysis

Mixed model variance analysis for repeated measures (donors as random) with Bonferroni's correction was used to assess the significance of TDNA differences between sampling times. Normality of TDNA distributions was assessed by means of the Kolmogorov–Smirnov test, whereas variance homogeneity by means of Cochran's C test. All statistical evaluations, including fitting procedures, have been performed by means of Statgraphics Plus 5.1 software, Manugistics, Inc., Rockville, MD, USA and STATA 8, Stata Corporation, College Station, TX 77845, USA.

3. Results

3.1. The kinetics of formation and removal of DNA breaks following UVC irradiation

The kinetics of DNA damage repair after a single UVC dose was followed in lymphocytes incubated for different times before proceeding to the comet assay to evaluate separately the excision repair and synthesis-ligation activity in the same experiment. DNA cleavage (TDNA%) was measured in blood cells from four donors and in repeated experiments. In total four experiments were performed, but for technical reasons, each could include only two donors, thus donors #1 was paired with donor #2 and donor #3 with donor #4. As shown in Table 1, the greatest increase in DNA cleavage occurred between 60 and 90 min, for all subjects except donor #3, and then it decreased with time, approaching the basal level within 180–240 min after UVC irradiation, without reaching it. Prolonged incubation time (360 min), did not result in any further decrease of TDNA. On the contrary, a slight and constant increase of TDNA was observed for longer incubation times (data not shown).

As shown in Fig. 1, DNA breaks increased over the control level (TDNA = 4.34%, time = 0 min) already at the first harvesting (TDNA = 13.9%, time = 20 min; thus indicating that the excision phase takes place shortly after the UV-exposure. Then, DNA cleavage continued to increase with time, reaching the maximum extent (TDNA = 30.1%) at 60 min. Thereafter, a decrement of DNA breaks was observable at 90 min (TDNA = 26.2%) and further more at 120 min (TDNA = 18.8%), indicating that the process of gap filling may have started to prevail over the excision phase. The DNA repair process seemed to be almost completed between 180 and 240 min (TDNA = 12.0 and 10.4%, respectively). Therefore, the present experimental design seems to be adequate to follow the NER process kinetics.

Table 1
Mean ± S.E. (%) tail DNA (TDNA) of lymphocytes in four donors (two repetitions)

Time (min)	Donor 1		Donor 2		Donor 3		Donor 4	
	TDNA(1)	TDNA(2)	TDNA(1)	TDNA(2)	TDNA(1)	TDNA(2)	TDNA(1)	TDNA(2)
0	2.0 ± 7.0	10.1 ± 19.5	3.2 ± 11.2	3.7 ± 16.7	3.9 ± 12.1	4.8 ± 12.9	3.6 ± 14.1	3.3 ± 7.3
20	7.1 ± 13.6	22.2 ± 27.0	8.7 ± 19.4	12.2 ± 13.8	7.9 ± 15.7	30.5 ± 24.3	9.0 ± 17.2	13.9 ± 19.0
40	25.0 ± 22.0	34.6 ± 23.5	20.5 ± 21.1	20.9 ± 2.0	12.5 ± 18.1	43.2 ± 28.6	7.6 ± 17.5	26.6 ± 24.9
60	45.9 ± 21.1	30.1 ± 25.7	39.4 ± 19.1	23.8 ± 31.1	12.5 ± 21.5	33.9 ± 28.0	22.8 ± 25.0	38.5 ± 32.6
90	45.4 ± 18.9	41.8 ± 30.1	30.3 ± 22.5	12.9 ± 21.5	20.1 ± 21.6	15.8 ± 23.0	22.2 ± 26.0	21.0 ± 24.4
120	31.5 ± 21.6	19.4 ± 24.4	21.8 ± 22.7	12.1 ± 22.4	15.0 ± 21.5	11.2 ± 18.5	14.1 ± 23.3	25.9 ± 27.9
180	13.9 ± 27.9	27.8 ± 31.7	8.6 ± 17.7	13.4 ± 22.9	4.5 ± 9.2	10.3 ± 18.9	10.1 ± 19.2	7.7 ± 17.0
240	3.16 ± 3.1	17.2 ± 29.0	5.9 ± 14.0	28.2 ± 36.6	5.6 ± 15.1	7.0 ± 18.0	5.5 ± 14.1	10.5 ± 23.1

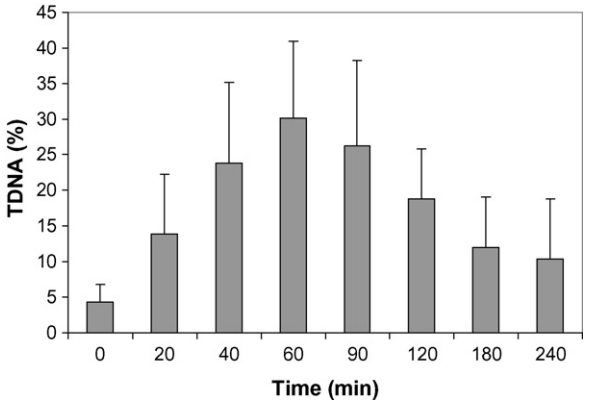

Fig. 1. Kinetics of DNA cleavage, as function of time elapsing from UVC challenge dose (1.5 J/m^2), measured by comet assay (TDNA means ± S.D.) in stimulated human lymphocytes from four donors, in twice repeated experiments. TDNA levels at sampling times between 40 and 120 min were significantly higher than those at control time (120 min = $p < 0.05$; 40 and 90 min = $p < 0.001$; 60 min = $p < 0.0001$).

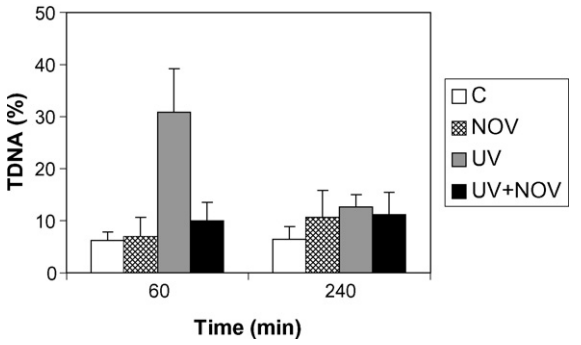

Fig. 2. Inhibitory effect of novobiocin (NOV) on the excision phase of NER process in lymphocytes 60 and 240 min after a challenge dose of UVC. White bars: TDNA means ± S.D. in untreated lymphocytes (control); Netlike bars: TDNA means ± S.D. in lymphocytes treated with novobiocin alone; Grey bars: TDNA means ± S.D. in lymphocytes treated with UV; Black bars: TDNA means ± S.D. in lymphocytes treated with UVC and novobiocin. TDNA levels of cells treated with NOV (±UV) were not significantly different both at 60 and 240 min.

In order to confirm that the observed kinetics was consequent to the activity of DNA excision and DNA polymerisation–ligation processes, two chemicals, novobiocin (NOV) and aphidicolin (APC), known to specifically inhibit these two steps, were also used. Because the peak of DNA cleavage was reached 60 min after the UVC exposure and the maximum reduction of DNA breaks appeared at 240 min (Fig. 1), we separately tested the effects of the two DNA inhibitors at these two times on the four donor lymphocytes.

In contrast with the increase in DNA migration, observed at 60 min in the lymphocytes treated with UVC alone, the presence of NOV completely abolished the effect of UV treatment, as shown in Fig. 2, demonstrating that the incision step is actively involved in the formation of DNA breaks following UVC exposure. As expected, at 240 min no effect of NOV was observed.

Nevertheless, the inhibitory effect of APC on DNA gap filling and end-rejoining was clearly evidenced, as shown in Fig. 3. In fact, at 60 min, APC treatment determined a dramatic increase of UV induced damage indicating that gap filling process is already fully operating. This effect is further increased at 240 min, demonstrating that the reduction of TDNA values observed in the second phase of the kinetics, shown in Fig. 1, was really due to the synthesis-ligation step of NER pathway.

These results confirm that the comet assay can be a suitable method to follow NER process in details. To further investigate on the early events, the UV-treated lymphocytes from the same donors were incubated for 20, 40 and 60 min with and without APC. APC determined similar increases of TDNA at all three sampling time, suggesting that the incision activity was already highly efficient at 20 min, although with a wide inter-experimental variation (data not shown).

3.2. Inter- and intra-individual variability assessment: a mathematical model

As shown in Table 1, a wide inter-individual and intra-individual variation was observed. In particular donor #1, unlike all other subjects, did not show any evident reduction of DNA damage at 90 min, compared with the effect seen at 60 min, in both experiments. On the other

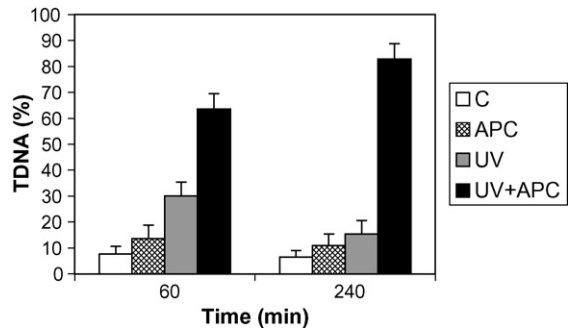

Fig. 3. Inhibition effect of aphydicolin (APC) on the polymerization–ligation phase of NER process in lymphocytes 60 and 240 min after a challenge dose of UVC. White bars: TDNA means ± S.D. in untreated lymphocytes (control); Netlike bars: TDNA means ± S.D. in lymphocytes treated with APC alone; Grey bars: TDNA means ± S.D. in lymphocytes treated with UV; Black bars: TDNA means ± S.D. in lymphocytes treated with UV and APC. TDNA levels of cells treated with APC + UV were significantly higher than these treated with UV alone ($p < 0.001$ and $p < 0.0001$ at 60 and 240 min, respectively).

side, intra-individual variation appeared to be particularly evident for donor #3: the DNA cleavage peak was very low (TDNA = 20%) and appeared at 90 min in the first experiment, while in the second experiment the peak was higher (TDNA = 43%) but occurred at 40 min.

To compare DNA repair kinetics at individual level by means of a quantitative estimation of the efficiency of the two NER steps, a first mathematical model was developed, based on few general assumptions. In our notation, the symbol $C(t)$ denotes a function representing the time-dependence of the quantity of DNA breaks measured by the comet assay. The function $E(t)$ denotes the total number of excisions and the function $F(t)$ denotes the total number of DNA fillings by synthesis-ligation activity. We cannot measure $E(t)$ and $F(t)$, but we have already assumed that $C(t) = E(t) - F(t)$. We also assume, on very general grounds that the system response is linear with respect to the entity of the phenomenon producing the response. In mathematical terms, linear response means that any effect is in general expected to be directly proportional to its cause, even if the phenomenon would be characterized by an S-shaped or exponential behavior.

Therefore, introducing the symbol D_0 to denote the total damage, we may write down the following response equations:

$$\frac{\mathrm{d}E(t)}{\mathrm{d}t} = \alpha(D_0 - E(t))$$

$$\frac{\mathrm{d}F(t)}{\mathrm{d}t} = \beta(E(t) - F(t) - C^*)$$

The first equation expresses the fact that the number of excisions per unit time must be proportional to the residual damage. The second equation expresses the fact that the number of fillings is proportional to the number of unfilled excisions that are present at a given time. The symbol C^* is introduced in order to allow for the possibility that the asymptotic value of $C(t)$ be different from zero even after a long time interval, as experimentally observed. We expect C^* to satisfy the condition $C^* \ll D_0$, but we do not need to assume $C^* = E_0$ (initial number of excisions), and we can leave it as a free parameter, whose value will be determined by fitting procedure.

In general, the coefficients α (excision efficiency) and β (filling efficiency) will be (unknown) functions of time, since the response is linear, but not instantaneous, and especially in the initial phase we may expect α and β to grow with time.

However, in order to get a quantitative picture of the asymptotic time-dependence, we may certainly approximate α and β with constants for sufficient large values of t, and under this assumption the general solution of the above equations can be explicitly found.

Including as an initial condition the fact that the initial number of excisions $\mathrm{E_o}$ is small ($\mathrm{E_o} \ll D_0$) but not completely absent, the solution is:

$$C(t) = \frac{\alpha D_0(\mathrm{e}^{-\alpha t} - \mathrm{e}^{-\beta t})}{\beta - \alpha} + \frac{E_0(\beta \mathrm{e}^{-\beta t} - \alpha \mathrm{e}^{-\alpha t})}{\beta - \alpha} + C^*(1 - \mathrm{e}^{-\beta t}) \quad (1)$$

The above result will hold after a sufficiently long time. However, this expression turns out to show a dependence from on the difference $\alpha - \beta$ which is too sharp for any practical use when the values of α and β are numerically similar, as we expect in practice. As a consequence in a best fitting procedure the determination of the numerical parameters would certainly be unstable. Therefore, it is convenient to give α and β a common value d and to take the appropriate limit. We then obtain the following simplified representation:

$$C(t) = (\mathrm{d}D_0\, t + E_0\,(1 - \mathrm{d}t))\,\mathrm{e}^{-\mathrm{d}t} + C^*(1 - \mathrm{e}^{-\mathrm{d}t})$$

This is still an asymptotic expression, but we can now describe phenomenologically the initial time-dependence of the coefficients by a power-law, and we can exploit the condition $\mathrm{E_0} \ll D_0$ to obtain further simplification. Our final proposal is the parametrization:

$$C(t) = C^* + (E_0 - C^* + b\,t^c)\,\mathrm{e}^{-\mathrm{d}\,t}$$

where E_0 is measured and the parameters C^*, b, c, d, can be determined by best-fitting the experimental results. By maximizing $C(t)$ we find that the time location t_M of the maximum value of this function should satisfy the relationship $\mathrm{d}t_\mathrm{M} = c$. Since t_M is an experimental parameter, the above condition might be used as an intrinsic test of accuracy for the parametrization.

The equation coefficients and their standard errors have been estimated by fitting the means of the percentage of migrated DNA (TDNA) in 50 cells for each experimental point (time = 0, 20, 40, 60, 90, 120, 240 min) by means of regression Marquardt estimation methods. Regression coefficients, a, b, c and d of DNA repair kinetics within and between individuals were compared by Student's t-test.

According to Eq. (1), each repair kinetics can be described by four parameters: $a = C^*$, which represents the basal TDNA level: b and c that characterize the excision activity, which is prevalent during the first phase of the repair process; and, finally, d that quantifies the ligation activity, which is predominant during the second phase.

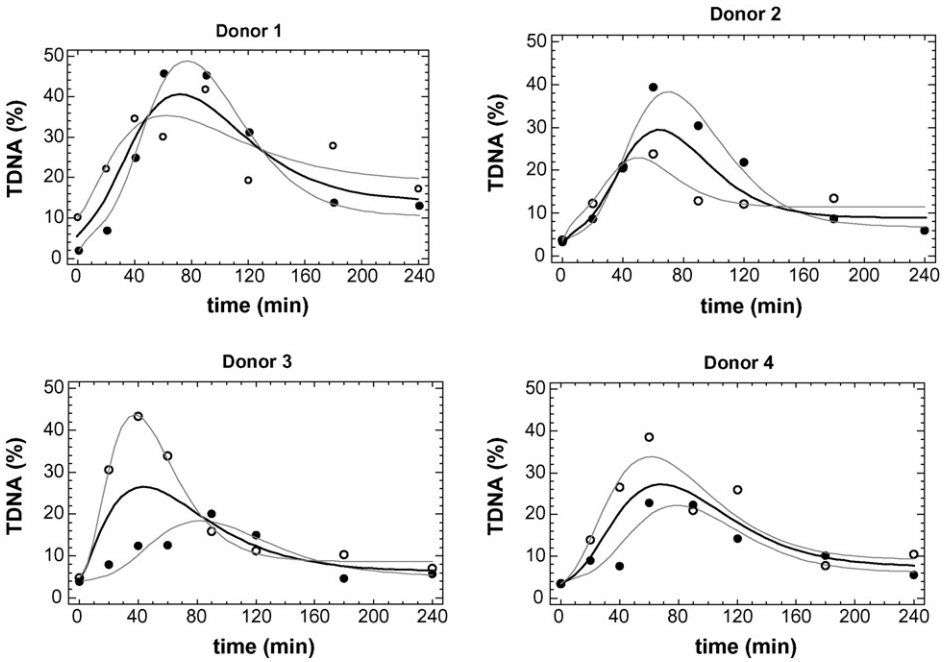

Fig. 4. Curve fitting of TDNA mean values in lymphocytes from four donors after a challenge dose of UVC (two experiments per donor) according with Eq. (1) (see text). Thick lines represent the fitting of two experiments taken together, while the thin lines the fitting of each experiment. Empty and filled circles allow distinguishing values obtained in each experiment.

The curves, fitting individual mean TDNA values obtained in repeated experiments, are reported in Fig. 4. When the two experiments are considered jointly (thick lines), the shapes are quite similar for all donors, although the peak of excised DNA was the highest for donor #1, as compared to the other three at the same time. Nevertheless, the remarkable intra-individual and inter-individual variability can be appreciated by comparing the curves referring to the single experiment on each donor sample (thin lines) and the estimated parameters, listed in Table 2. Hence, the values of a, the curve intercept, representing the estimated spontaneous levels of DNA damage, ranged from 5.2 to 19, similar to those experimentally observed, although with wide S.D. More remarkably, the b coefficients, which give an estimation of DNA cleavage efficiency, appeared to be very different, still with wide S.D. The highest b value (0.024) was obtained for donor #3 and the lowest (1.2×10^{-6}) for donor #2. But the differences between the two experiments on the same donor cells were also of several orders of magnitude, except for donor #2, indicating a relevant experimental variation.

The c coefficient, which also concerns the excision process, seemed less variable than b, although with wide S.D. The narrow range of c (1.8–5.4) indicates a relatively low inter- and intra-individual variability. However, since the coefficients b and c jointly describe the first phase of NER, the experimental variation seems

Table 2
Coefficients (a, b, c and d) of NER kinetic functions in four donors (two repetitions)

	$a \pm$ S.D.	$b \pm$ S.D.	$c \pm$ S.D.	$d \pm$ S.D.
Donor 1	10.5 ± 5.3	$1.9 \times 10^{-6} \pm 1.7 \times 10^{-5}$	5.1 ± 2.6	0.07 ± 0.04
	19.0 ± 26.8	$6.6 \times 10^{-2} \pm 1.6 \times 10^{-0}$	1.8 ± 7.1	0.03 ± 0.13
Donor 2	6.8 ± 5.8	$1.2 \times 10^{-6} \pm 2.4 \times 10^{-5}$	5.1 ± 3.4	0.07 ± 0.06
	11.5 ± 5.1	$1.7 \times 10^{-6} \pm 6.6 \times 10^{-5}$	5.4 ± 11.3	0.11 ± 0.27
Donor 3	5.2 ± 9.1	$3.1 \times 10^{-6} \pm 1.0 \times 10^{-4}$	4.5 ± 9.1	0.05 ± 0.14
	8.6 ± 2.5	$2.4 \times 10^{-2} \pm 6.0 \times 10^{-2}$	2.8 ± 0.8	0.07 ± 0.02
Donor 4	6.1 ± 9.6	$2.0 \times 10^{-6} \pm 7.2 \times 10^{-5}$	4.7 ± 9.3	0.06 ± 0.15
	9.1 ± 14.9	$2.8 \times 10^{-3} \pm 5.2 \times 10^{-2}$	2.9 ± 5.5	0.05 ± 0.10

definitely very wide, making any inter-individual comparison meaningless. Finally, the d coefficients, which estimate the efficiency of the DNA gap filling-ligation step, were very close (0.03–0.11), although with relatively wide S.D.

3.3. Inter- and intra-individual variability assessment: a 3-D representation and a principal component analysis (PCA)

The previous analysis based on the mean values of TDNA observed in 50 cells, for each experimental point, thus does not make use of the information at single cell level, as provided by the comet assay. Considering the high variability of the estimated parameters, especially of the *b* coefficient, we thought that it could be also interesting to examine and compare the distribution of TDNA values measured for each cell. With this aim, TDNA values were stratified into six classes: the first class included undamaged cells, and other five classes included cells with increasing damage ranges (1–20, 21–40, 41–60, 61–80, and 81–100% of TDNA). For each donor, experiment and time step, the distribution of cells in each class gives a 6-values vector. All the vectors referring to the same donor and experiment, grouped by the eight time steps, are presented visually in Fig. 5, as a 3-D graph, with the TDNA class on the *X*-axis, the time course on the *Y* and the relative frequency on the *Z*. At a first glance, the analytical results of the model (1) are confirmed. The two experiments for donor #1, for instance, gave quite different results. While the graph for the first experiment shows a distinct peak of damage around 90 min. (TDNA class: 41–60), in the second experiment the cell distribution is smoother and more dispersed, with the highest level of damage concentrating around 60 min. (TDNA class: 21–40). Analogous differences between the two tests were seen for the other donors, with exception of donor #4 for whom the kinetics had similar trend. Similarities can be observed by comparing, for instance, the patterns of donors #1 and #2, referring to the first experiment (left column), as well as those produced for donors #3 and #4 in the second one (right column). This representation of data provides a more detailed picture of the complexity of the evolution of the damage that was at least partially lost by simply considering the mean TDNA value. As with the mathematical model, data suggest that experimental conditions deeply influence results, making it difficult to fully appreciate the differences between individuals.

However, due to the complexity of the graphs, a formal definition of the differences between the distributions is definitely required to make reasonable any comparison. Thus, the distance *D* between experiments and between donors was taken as the square root of the sum of the squared differences of the corresponding points in the graphs according to equation:

$$D_{i,j} = \sqrt{\sum_{t=1}^{8}\sum_{c=1}^{6}(V_i(t,c) - V_j(t,c))^2} \quad (2)$$

where $V_i(t,c)$ is the distribution value for class c and experiment i at time t. This is actually equivalent to grouping the 6-valued vector for each time and each experiment, and taking the standard Euclidean distance on the resulting 48-valued vectors. Applying the above equation, we obtained the matrix of distances between experiments and between donors, reported in Table 3 that allows a quantitative estimation of the differences observed. The distances ranged from 0.064 for donors #3 and #4 in the same experiment (Exp = 2), to up to 0.18 for donors #1 and #2 in different tests (Exp 1 versus Exp 2). The distance between the two experiments on the same donor cells should in principle be the shortest. This was not the case, since the distances between two experiments were 0.14, 0.16, 0.14 and, as least, 0.10 for donor #1, #2, #3 and #4, respectively. Actually, the distance between donors #1 and #2 was short in the first experiment (0.08), but not in the second one (0.18). On the contrary, the distances between donors #3 and #4 were short in both experiments (0.064 and 0.093, respectively).

Table 3
Matrix of distances (values × 100) between donors (two repetitions)

Donor	#1	#2		#3		#4	
#1	*14.5*	**8.0**	18.3	16.1	16.2	15.2	12.1
		14.2	14.6	14.3	13.8	14.8	**11.4**
#2			*16.3*	12.8	14.1	11.0	**10.1**
				11.6	11.7	12.3	**11.0**
#3					*14.2*	**6.4**	9.3
						13.7	**9.3**
#4							***9.8***

N.B.: The closest distances per row are in bold, the distances between the two experiments on the same donor cells are in italic.

To preserve the information provided by the comet assay on the DNA damage at single cell level and, at the same time, to reduce the complexity of the 48-valued vector, in order to describe similarities and differences between the distributions, we also used the principal components analysis (PCA), a mathematical technique that translates a set of vectors into a new base. This base is chosen so that the first component of the projected vectors is along the first axis of maximum variability of

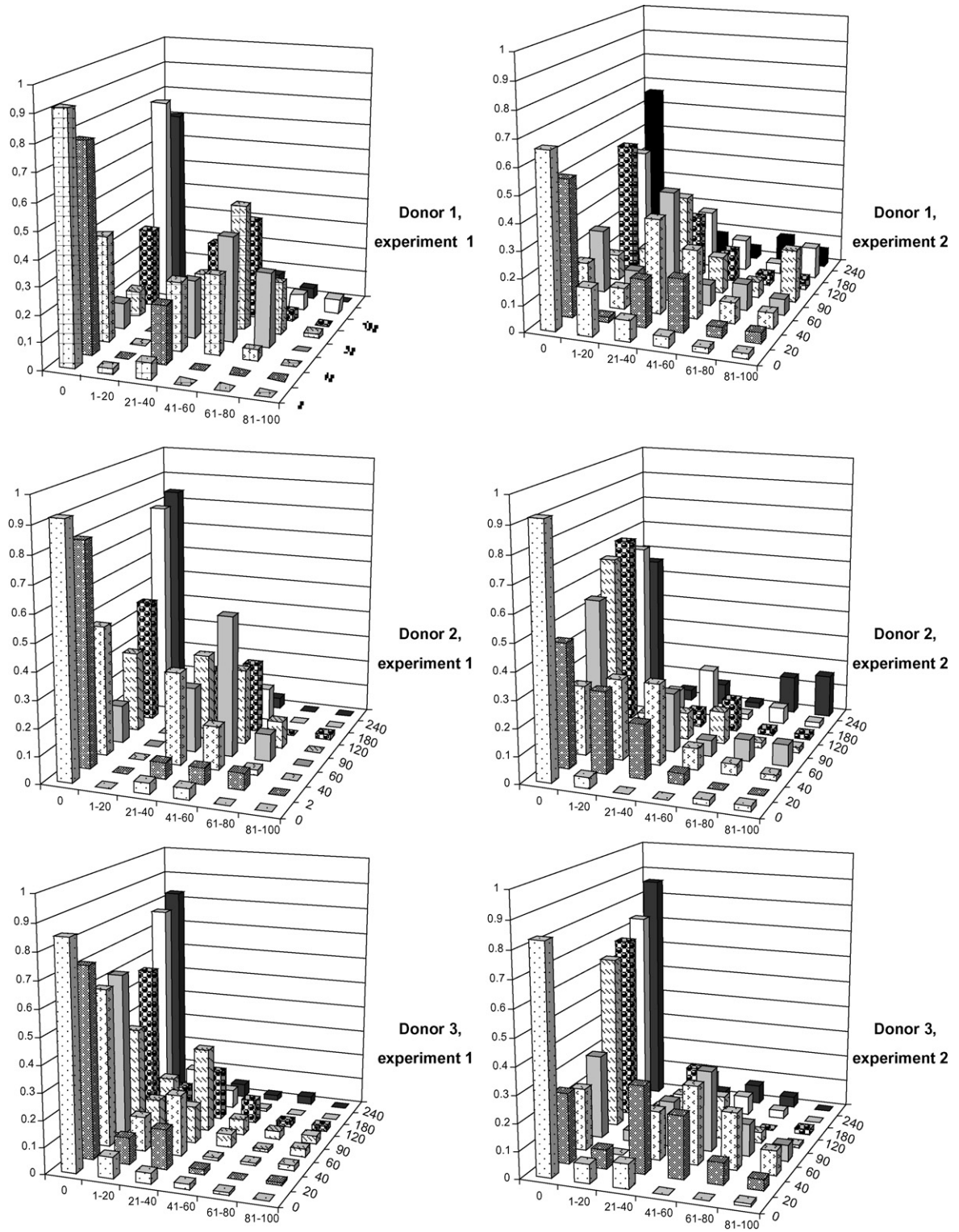

Fig. 5. Distributions of lymphocytes, stratified into six classes according to TDNA values measured after a challenge dose of UVC, from four donors in two experiments. TDNA class on width, time on depth, frequency on height.

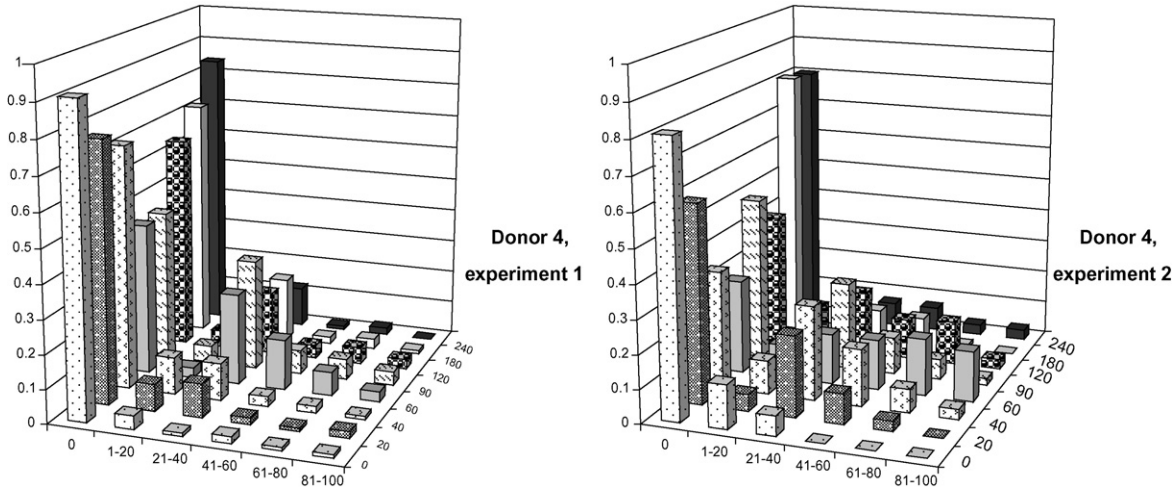

Fig. 5. (*Continued*).

the data, the second component along the second axis of maximum variability, and so on. PCA is typically used (as in this case) as a dimensional reduction tool.

Each 48-valued vector was thus transformed into a 2-valued vector, taking the first two components of the PCA-projected vectors, assuming that the loss of information is as small as possible, so that we could plot each experiment per each donor as a single point on a 2-D plane as shown in Fig. 6, although the transformation does not guarantee that the distances of the above matrix are preserved. This representation does not add much to our previous observations, apart from remarking, for instance, the extreme difference between the two tests of donor #2 and, although somewhat less, of donor #1.

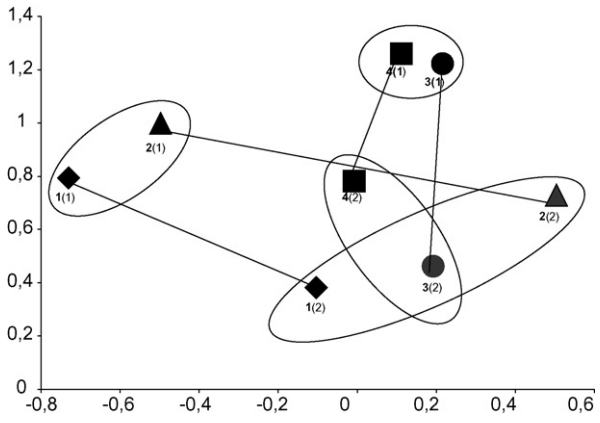

Fig. 6. Distances between donors and experiments plotted as result of PCA analysis on TDNA distributions. Individuals are identified by bold number, whereas experiments by number in brackets. The two experiments on the same donor cells are connected by straight lines, while the close lines include the two subjects tested within the same experiment.

The secluded position of the results on these two donors in the first experiment could, however, point to some methodological issues differentiating this experiment from the others. As previously shown in the matrix of distances, the cells from donor #3 and donor #4 that were tested at the same time gave results much closer to each other in both experiments. This method was also applied to the first part of the time course (0–60 min) and to the second part (90–240 min), separately. In this case, the variability between experiments was even greater than that between donors (data not reported), confirming the strong influence of experimental factors in the comet assay.

3.4. Intra-individual variability: further assessment on the test's reproducibility

Intra-individual variability may be due either to variations in the experimental conditions or to the donor physiological status that may be changed during the period elapsed between two blood samplings. In order to assess the relevance of this second factor, a single blood sample of donor #1 and donor #2 was divided in several vials, frozen at −80 °C. Eight repeated experiments for donor #1 and four for donor #2 were performed on thawed cells that were treated with UVC and processed for DNA repair assay at 0, 60 and 240 min within 4 months from the sampling.

As reported in Table 4, panel A, the coefficient of variation (CV) remained considerably high either for basal levels or for treated samples (65, 29 and 46%, respectively) for donor #1 (eight experiments) as well as for donor #2 (four experiments) (66, 17, and 49%). These high CV values suggest that some occasional, unidenti-

Table 4
Average TDNA damage (± standad deviations) observed in 50 cells, and coefficient of variation (CV) assessed at different times following UV exposure in different experiments

	Time (min)					
	0		60		240	
	TDNA (%)	CV (%)	TDNA (%)	CV (%)	TDNA (%)	CV (%)
A						
Donor 1 (eight experiments)	4.8 ± 3.1	65.2	40.9 ± 12.2	29.6	14.6 ± 6.8	46.1
Donor 2 (four experiments)	3.1 ± 2.1	66.4	40.4 ± 6.7	17.4	8.1 ± 4.1	49.2
B						
Donor 1 (four experiments)	3.4 ± 1.9	58.0	22.1 ± 10.5	47.2	4.9 ± 4.2	83.5

A: cryopreserved, PHA stimulated lymphocytes and B: unstimulated fresh lymphocytes.

fied modifying factors, with strong random effects, are occurring.

Because stimulation of lymphocytes with PHA may be another source of variation, fresh unstimulated cells from donor #1 were treated with UVC and processed for the comet assay at 0, 60 and 240 min. The observed coefficients of variation (CV = 58, 47 and 83%, respectively) were even greater than those observed in the previous sets of experiments. However, the peak of TDNA was much lower than that with fresh PHA-stimulated cells, suggesting that stimulation does not particularly influence the experimental variation, but can possibly affect the DNA cleavage efficiency in DNA repair process (Table 4, panel B). Indeed, because the TDNA means were about half those in stimulated lymphocytes, the inter-experiments variation in unstimulated lymphocytes is necessarily greater. This suggests that PHA-stimulated lymphocytes likely reach a more homogeneous metabolic level than unstimulated ones.

4. Discussion

Up to date, there are more than 120 genes known to control DNA repair and some of them are polymorphic. A number of these polymorphisms have been associated with an increased or decreased risk of cancer at one or multiple target sites. Therefore, the multiplicity of these alleles raises two major questions: (1) how can each detected variant modify the overall DNA repair capacity, i.e. are the discovered SNPs functionally effective? And (2) how can the effects of hundreds of allele combinations of these polymorphic genes be evaluated at individual level?

Due to the complexity of repair processes, simple in vitro allele-specific tests are subject to severe limitations, since each gene product interacts with many others, in different combinations, largely depending on damage-specific repair pathways. Thus, the answer to the above questions mainly relies on assays that evaluate in vivo, at least at the intact cell level, the individual repair capacity.

DNA repair challenge tests are suitable to this aim because they allow evaluating the amount of induced damage or its reduction following a treatment with an appropriate mutagenic agent [23]. By means of this approach several studies were carried out showing that some cancer patients presented a reduced DNA repair capacity after a challenge test [24].

Because most DNA repair processes consist of various steps, each controlled by several genes, we explored the suitability of a challenge test, based on the comet assay, to assess the individual repair capacity and to quantify the efficiency of the two main steps of NER pathway. Such a test could be of valuable relevance to establish sound phenotype–genotype relationships and to discriminate which genotypes are possibly associated with an impaired DNA repair capacity. The comet assay appears to be a good candidate, because it is very sensitive and offers the possibility of quantifying DNA cleavage at single cell level. Thus, this test can provide an unprecedented amount of information on DNA repair activity. Holmberg and co-workers firstly showed that the yield of DNA breaks increases as a function of incubation time, reaching the maximum within the first 30–60 min, and then declines with further incubation until 180 min [25]. Also Tuck and collaborators showed that the highest levels of strand breakage can be observed after 60 min of incubation [8]. It is likely that the damage observed at this time represents the balance between the two competing processes of excision and ligation of NER pathways. After 240 min the cells have already repaired most of the damage [8]. Peaks of DNA strand breaks were also observed in human lymphocytes 60 min after a UVC flash by Yamauchi et al. [26]. Our results were similar to those previously reported, further supporting the

hypothesis that the comet assay may allow the kinetics of DNA repair to be followed in human lymphocytes after exposure to a single UVC dose. The reliability of the observed dual-phase kinetics was strongly supported by the results of the experiments with two specific inhibitors of definite steps of NER pathway. Novobiocin, inhibitor of topoisomerase, completely suppressed the excision phase, abolishing the onset of the peak of DNA cleavage occurring in the first 60–90 min, whereas aphidicolin, inhibitor of DNA polymerases, completely prevented the gap filling-ligation phase, causing the persistence of the DNA breaks over 240 min.

To compare the individual pattern of DNA repair, a specific mathematical model was designed, based on the assumption that during NER process the two mechanisms, i.e. DNA excision and gap filling-ligation, have opposite effects on the formation of DNA breaks. It is remarkable how the model could adequately fit the experimental results, leading to a valuable estimation of the individual descriptors for both phases. This model can be potentially very useful for identifying individuals with reduced repair capacity and for quantifying the effects of genetic polymorphisms influencing the efficiency of the first as well the second phase of NER. The parameters estimated by the model indicated that there were wide differences in the DNA repair kinetics between four healthy donors, possibly attributable to genetic variation. However, the experiments repeated with the same donor cells some months later clearly showed that the intra-individual variability was, at least, as wide as inter-individual variability. This is possibly due to some technical aspect of the comet assay that can influence DNA migration and consequently TDNA value, for example the conditions of the electrophoresis run.

The difference between the two experiments on cells from the same donor became even more evident when the distribution of TDNA values (second approach) were considered, instead of the TDNA mean values, used in the mathematical model. Actually, in some instances, the results obtained within the same experiment on two donors were closer than those obtained for the same donor in two repeated experiments. However, the distances between experiments and between donors, calculated on the basis of PCA, provide a valuable means for comparing individuals for their DNA repair proficiency and, eventually for analyzing the effects of polymorphisms in genes involved in DNA repair processes.

A large influence of experimental factors on the reproducibility of the comet assay was reported also by Chazal et al. who observed a variation coefficient of about 37% in three independent experiments on the same primary keratinocyte cell line, irradiated with increasing doses of UVB [27]. On the other hand, Collins et al. reported that DNA repair capacity, measured by the comet assay on lymphocytes of 14 volunteers, was quite stable and reproducible after 5 weeks [10]. In this case, the intra-individual variation was lower than inter-individual variation, although the correlation coefficient (r^2) between the two experiments was 0.64 and the estimated mean excision activity was 40% greater in the second challenge test than in the first one, thus indicating a considerable experimental variation.

These experimental variations imply the need of performing several independent assays to accurately assess the DNA repair capacity at individual level. Another approach has been suggested to take into account experimental variability based on the use of an internal standard. De Boeck et al., observing a large inter-experimental variability in TDNA and TL in EMS-treated K562 cell line, suggested the use of calibration formulas to standardize electrophoresis runs [28]. However, the use of an internal standard does not improve reproducibility of the test. In fact, we performed up to eight repeated experiments with frozen lymphocytes from the same donors, at three sampling times (0, 60 and 240 min) following a UV-C challenging dose (Table 4). The experimental variation within the same electrophoresis run did not show a homogenous upward or downward fluctuation of TDNA. For this reason it does not seem possible to determine any suitable calibration formula to be used to standardize TDNA kinetics. So, we have evaluated some possible sources of experimental variation. We could exclude that lymphocyte stimulation with PHA plays any significant role: as reported in Table 4 unstimulated lymphocytes show even greater CV, as compared to PHA stimulated cells. In our experience, a very sensitive point in the comet assay is represented by the preparation of the three agarose layers that may cause visible changes in thickness of the lymphocyte layer from slide to slide. This may be a crucial point influencing the extent of both DNA denaturation and DNA migration. In addition, another source of experimental variation is the hydration level of the agarose layers. Up to now, attempts to reduce the influence of these experimental factors were unsuccessful and therefore we consider it mandatory to explore new approaches, modifying this step. Therefore, we conclude that the comet assay, carried out with the present procedures, is still subject to environmental and experimental variations, which render it not reliable enough to be used in individual DNA repair kinetic description. Once these technical problems would be solved, we are confident that comet assay can represent a reliable method for

assessing DNA repair kinetic at individual level by using, also, the suitable mathematical model we presented.

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