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Rapid screening assay for mutagen sensitivity and DNA repair capacity in human peripheral blood lymphocytes

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Individual susceptibility to carcinogens, an important determinant of disease risk, is influenced by host factors such as the ability to repair DNA lesions. In order to identify subjects who are at high risk, we have developed a microgel electrophoresis assay for use in molecular epidemiological studies. The assay was validated in a pilot case–control study: Peripheral blood lymphocytes were collected from 100 patients with lung cancer and 110 control patients without cancer and from the same hospital, and stored at –80°C. After thawing, phytohaemagglutinin-stimulated cells were treated with bleomycin at 20 μg/ml for 30 min and the extent of DNA damage and DNA repair capacity were determined by microgel electrophoresis. Peripheral blood lymphocytes from patients with lung cancer were significantly more sensitive to mutagens than those from controls and showed reduced DNA repair capacity (both $P < 0.001$). Both endpoints were independent risk factors for smoking-related lung cancer. Repeated analysis of peripheral blood lymphocytes from the same individual demonstrated good reproducibility of the assay. Cryopreservation of the lymphocytes for ≤12 months did not significantly affect their sensitivity. Our standardized microgel electrophoresis assay is suitable for determining individual sensitivity to mutagens and DNA repair capacity: it is sensitive and faster than cytogenetic assays, and can be applied to native and cryopreserved peripheral blood lymphocytes.

Introduction

Exposure to carcinogens such as tobacco smoke and individual cancer susceptibility play key roles in the development of disease (Markham *et al.*, 1994). Several biomarkers for genetic susceptibility which are also associated with increased cancer risk have been identified (McGlynn and Buetow, 1994). These markers allow estimation of inter-individual differences in response to carcinogenic agents and facilitate the identification of susceptible subjects. Since it is unlikely, however, that a single marker will be sufficient for accurate prediction of the cancer risk of an exposed individual, the probability of identifying such risk will be increased by using a panel of biomarkers.

The ability to repair DNA lesions is strongly associated with the risk of cancer and other diseases (Friedberg, 1985; Bohr *et al.*, 1989), as it is a ubiquitous defence mechanism that is essential for cell survival and the maintenance of cell cycle control (Hartwell and Weinert, 1989). An epidemiological review of markers of DNA repair and suceptibility to cancer in humans revealed positive and consistent associations between DNA repair capacity and cancer occurrence (Berwick and Vineis, 2000). DNA repair capacity affords protection against genotoxic carcinogens, whereas a decrease in that capacity results in greater susceptibility to mutations and enhanced genetic instability. Consequently, individual rates of repair of DNA damage are a crucial determinant of cancer susceptibility. Cellular DNA repair capacity can be measured in several ways: as the rate of damage removal, uptake of substrates for unscheduled DNA synthesis or the expression of damaged reporter genes in host cells. Hsu (1987) (see also Hsu *et al.*, 1989) developed an assay in which the frequency of chromatid breaks induced by bleomycin in cultured lymphocytes *in vitro* was quantified as a combined measure of mutagen sensitivity and DNA repair capacity; the number of bleomycin-induced breaks per cell was used to identify sensitive subjects. This cytogenetic assay has been used in a number of studies that have shown that patients with cancers of the head and neck, lung, or colon express the mutagen-sensitive phenotype significantly more often than cancer-free control subjects (Hsu *et al.*, 1989; Schantz and Hsu, 1989; Hsu *et al.*, 1991; Spitz *et al.*, 1993, 1994; Spitz and Hsu, 1994; Wu *et al.*, 1995, 1996). This assay suffers from some limitations, however. Since chromatid breaks can be detected only in cells in metaphase, other DNA modifications such as point mutations and cross-links cannot be detected, and sensitivity to mutagens and DNA repair capacity cannot be determined independently. Athas *et al.* (1991) developed a host cell reactivation assay to measure DNA repair using a transient expression vector harbouring the chloramphenicol acetyl transferase (*cat*) reporter gene. In this assay, pCMVcat plasmid DNA damaged by the test agent is transfected into unexposed host cells and reactivation of the damaged plasmid is used as a measure of the DNA repair activity of the host cells. This assay allows indirect measurement of the extent and efficiency of overall cellular repair of DNA but is less suitable for evaluating the repair of single- or double-strand breaks, as they reduce plasmid transfer frequencies (Nickoloff and Reynolds, 1992).

Smoking-induced lung cancer causes more deaths than any other cancer worldwide (Kabat, 1993; Davila and Williams, 1993). The fact that only 10–15% of smokers develop lung cancer (Mattson *et al.*, 1987) suggests that genetic and acquired host factors modulate susceptibility to this disease. Spitz *et al.* (1995) used chromatid breaks as a marker in bleomycin-treated lymphocytes in a case–control study to demonstrate that sensitivity to mutagens is a risk factor for lung cancer. In a *cat*-based host cell reactivation assay, reduced DNA repair activity was observed more frequently in lung cancer patients than in controls (Wei *et al.*, 1996). These assays have been used routinely, but sample preparation and evaluation of

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results are lengthy and the cytogenetic method is restricted to proliferating cells.

We have developed a single-cell microgel electrophoresis assay which allows simultaneous measurement of sensitivity to mutagens and DNA repair capacity in readily accessible biological specimens such as peripheral blood lymphocytes. We treated the cells with the radiomimetic antibiotic bleomycin, which is a known inducer of single- and double-strand breaks and AP sites in DNA and was shown previously in a microgel electrophoresis assay (Anderson *et al.*, 1994) to elicit a positive response in peripheral blood lymphocytes. In order to validate our standardized assay, we applied it in a pilot case–control study of lung cancer. We also investigated the effect of cryopreservation for 1 year on the sensitivity of the cells to mutagens and DNA repair capacity, to ensure that cell samples can be accumulated for molecular epidemiological studies. Additionally, we investigated the effect of seasonal sampling on basal DNA damage in these cells. In the present paper, we demonstrate the suitability of our assay for epidemiological studies with focus on technical aspects. A detailed analysis of our ongoing lung cancer case–control study, including larger numbers of subjects and detailed information on epidemiological variables, will be published elsewhere.

Materials and methods

Study subjects and data collection

In the context of an ongoing study of lung cancer, 100 patients with newly diagnosed, previously untreated lung cancer were identified by the Thoraxklinik Heidelberg–Rohrbach after ethical clearance; informed consent was obtained from all. The group was not restricted by age or tumour stage, but the cases were histologically confirmed as adenocarcinoma (n = 40), squamous cell carcinoma (n = 43), large-cell carcinoma (n = 5), small-cell lung cancer (n = 3) and unspecified (n = 9). The controls consisted of 110 tumour-free subjects recruited from the same hospital. Information on age, gender, family history of cancer, occupational exposure and exposure to radiation, smoking habits, alcohol consumption and nutritional factors was collected by means of a questionnaire. At this stage of the ongoing case–control study, samples were analysed as collected and the two groups were not formally matched by age, gender, race or socio-economic status, but they were stratified by smoking habit as current smoker (including people who had stopped smoking <1 year previously), former smoker (those who had stopped smoking ⩾1 year ago) and those who had never smoked. Microgel electrophoresis analyses were performed with no knowledge of case or control status.

Lymphocyte isolation and treatment

Approximately 10 ml of blood from each subject was drawn into heparinized tubes, carefully layered over 10 ml of Lymphoprep at room temperature and centrifuged at 2000 r.p.m. for 30 min. The buffy coats were removed and washed with phosphate-buffered saline (Gibco-BRL). For cryopreservation, the cells were suspended in 1 ml of freezing medium containing 10% dimethylsulfoxide in heat-inactivated fetal calf serum (Gibco-BRL), transferred to a cryotube, cooled from 4°C to –80°C at a rate of –1°C/min in a Nalgene container and stored at –80°C until the day of use. The frozen samples were then rapidly thawed and washed with RPMI 1640 medium (Gibco-BRL) and adjusted to a concentration of 1×10^6 cells/ml. The cells were then resuspended in RPMI 1640 supplemented with 2 mmol/L of L-glutamine, 10% fetal calf serum, 2% phytohaemagglutinin (Gibco-BRL) and 100 U/ml of penicillin and of streptomycin (Gibco-BRL) and cultured for 20 h at 37°C in 5% CO_2.

In order to establish the optimal conditions for bleomycin treatment and for testing DNA repair capacity, lymphocytes were isolated from seven healthy non-smoking volunteers (three female and four male, aged 30–52 years). Treatment with bleomycin at concentrations of ⩽20 μg/ml induced a dose-dependent increase in DNA damage (Figure 1A), but higher concentrations led to no further increase. At a concentration of 20 μg/ml, DNA damage was induced in 100% of the lymphocytes from all seven donors, with a concomitant high cell viability of 90–100%. DNA repair capacity was tested after removal of bleomycin and incubation of the lymphocytes for various times (Figure 1B). A fast, statistically significant decrease in tail moment, i.e. the product of the percentage of DNA in the tail region and the mean migration distance in the tail, was observed in cells from all seven individuals within the first

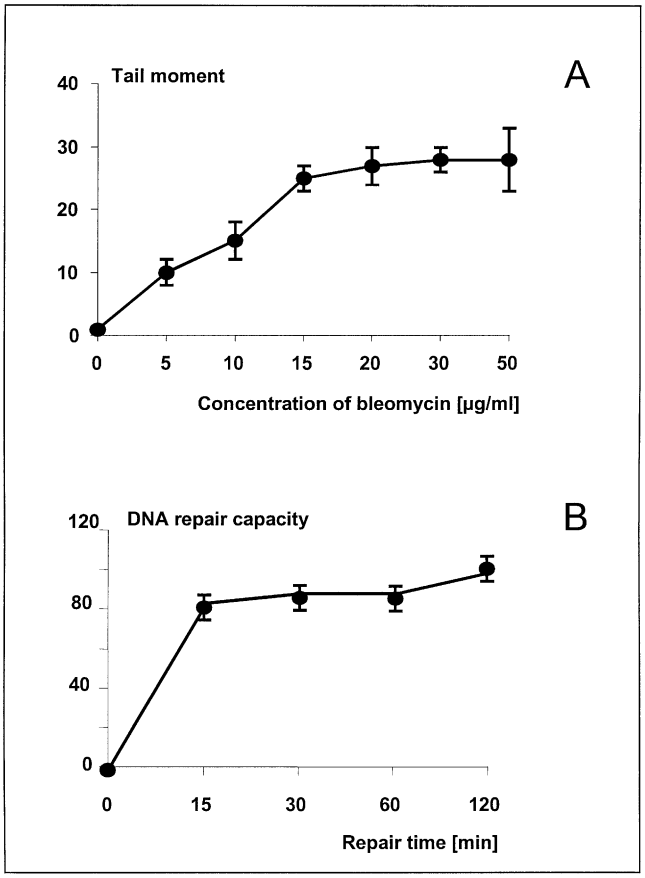

Fig. 1. (**A**) Dose–response curve for bleomycin-induced DNA damage in human peripheral blood lymphocytes. (**B**) Kinetics of repair of bleomycin-induced damage. Each data point represents mean ± SEM for seven individuals.

15 min ($P < 0.001$), by which time ~80% of the induced DNA damage had been removed. Much slower repair rates were observed thereafter; repair was still not complete after 2 h. On the basis of these initial results, a bleomycin dose of 20 μg/ml was used to induce DNA damage and a repair time of 15 min was used to determine DNA repair capacity in all assays with lymphocytes from hospital patients. As an inhibitory effect of serum on bleomycin-induced DNA damage has been described (Proctor *et al.*, 1989) and was also observed in preliminary experiments (data not shown), bleomycin was added to the lymphocytes in the absence of serum.

The cells were cultured for 20 h in 5% CO_2 at 37°C and subsequently treated with bleomycin (Sigma) at 20 μg/ml for 30 min. After centrifugation, the cell pellet was processed for microgel electrophoresis to determine sensitivity to mutagens. Cells that were treated identically except for bleomycin treatment were used as controls. To measure DNA repair capacity, the cells were additionally incubated in RPMI 1640 at 37°C for 15 or 60 min before being subjected to the microgel assay. Cell viability was checked by Trypan Blue exclusion and was always >90%.

Alkaline microgel electrophoresis assay

The assay was performed essentially as described by Singh *et al.* (1988), with some modifications. Fully frosted microscope slides were covered with a layer of normal melting point agarose followed by $6–7 \times 10^5$ cells mixed with 65 μl of 0.7% low melting point agarose at 37°C. The slides were then placed on ice for 10 min to allow solidification of the agarose. The layer of cells was covered with a layer of 75 μl of low melting point agarose. The slides were carefully immersed in lysis solution (100 mmol/L sodium EDTA, 10 mmol/L Tris, 2.5 mmol/L NaCl, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethylsulfoxide, pH 10) and kept for ⩾1 h at 4°C. After cell lysis, the slides were placed in a horizontal gel electrophoresis unit filled with alkaline electrophoresis buffer (1 mmol/L sodium EDTA, 300 mmol/L NaOH, pH 13). After 20 min of alkali treatment to allow DNA unwinding, electrophoresis was performed for 20 min at 25 V and 300 mA. The slides were then rinsed with neutralization buffer (0.4 mol/L Tris, pH 7.5), stained with 20 μg/ml ethidium bromide and kept in a humidified box. In order to

Table I. Characteristics of patients who participated in the study

Group	Age (years)	Gender (%)		Smoking status (%)		
		male	female	current	former	never
Controls	52 ± 14	65	35	34	29	37
Cases	60 ± 9.9	74	26	53	32	15

prevent additional DNA damage, all of the steps described were conducted under red light. The coded slides were evaluated under a fluorescence microscope coupled to an image analysis system (Kinetic Imaging Ltd, Liverpool, UK). DNA of individual cells was seen as a comet-like spot with a head and a tail region. The more DNA damage that has been introduced, the more DNA migrates during electrophoresis into the tail region. The results are expressed either as tail moment or as the percentage of undamaged cells (defined as those with a tail moment of <2). Tail moment and percentage of undamaged cells were determined for 51 randomly selected cells per slide, usually with three parallel slides per data point. DNA repair capacity was calculated after a fixed repair time of 15 or 60 min, as (percentage of undamaged cells after bleomycin treatment divided by the percentage of undamaged cells in control preparation) × 100.

Effect of long-term croypreservation

To determine the effects of freezing and various storage times on the sensitivity of human peripheral blood lymphocytes to bleomycin, mononuclear cells from four healthy adult volunteers were cryopreserved for 1, 20, 42, 166 or 371 days and the bleomycin-induced DNA damage and subsequent DNA repair capacity of the cells was then measured.

Statistical analysis

Samples were decoded and data were analysed by non-parametric Wilcoxon rank-sum methods. P values of <0.05 were regarded as statistically significant. The effects of possible confounding factors were analysed by calculating rank correlation coefficients by Kendall's rank test.

Results

The characteristics of the patients with lung cancer and the control subjects are summarized in Table I. As the study was performed without knowledge of the case or control status of the patients and as there was no restriction on factors such as age or gender, perfect matching could not be achieved; there was a statistically significant difference in the mean age of cases and controls (60 and 52 years, respectively; $P < 0.005$). The gender distribution was not significantly different, but men were overrepresented in both groups (cases: 74% male and 26% female; controls: 65% male and 35% female). As was to be expected, the smoking habits differed significantly between the patients with lung cancer and the tumour-free patients: only 15% of the case subjects but 37% in the control group had never smoked, and 53% of the lung cancer patients and 34% of the controls were smokers ($P < 0.002$). In an analysis of mean age, gender and smoking habit by three histological types (adenocarcinoma, 40%; squamous cell carcinoma, 43%; other subtypes, 17%), significant differences were observed only for gender (more males; $P < 0.015$) and smoking habit (fewer people who had never smoked; $P < 0.008$) in the group of squamous cell carcinomas when compared with the other groups (Table II).

The background level of DNA damage, determined in untreated aliquots of all samples of lymphocytes, in cells from the lung cancer patients (85% ± 9%) was not significantly different from that in cancer-free control subjects (82% ± 10%), and the tail moments were also similar (0.8±0.3). Gender, age, smoking habits and, in the case of cancer patients, histological tumour type had no effect on the basal level of DNA damage.

We used the tail moment measured immediately after bleomycin treatment as an indicator of sensitivity to mutagens. Large inter-individual differences were observed in both case and control subjects, with tail moments ranging from 5 to 60, but the patients with lung cancer were more sensitive to bleomycin than the control subjects ($P < 0.001$). The frequency distribution of sensitivity to bleomycin values is shown in Figure 2: >50% of the control subjects, but only 18% of the lung cancer patients, had low sensitivity to mutagens (tail moment ⩽ 20), while 8% of the controls but >40% of the cases were highly sensitive. Regression analyses showed that age and gender did not affect sensitivity to mutagens in lung cancer patients or control subjects; histological subtype of cancer also showed no correlation. Smoking status, however, did affect sensitivity to mutagens: among both controls and lung cancer cases, smokers were significantly more sensitive than people who had never smoked ($P < 0.05$), although cancer patients were more sensitive than controls in all three smoking categories.

DNA repair capacity within the first 15 min after removal of bleomycin was significantly lower in lung cancer patients (68%) than in control subjects (81%; $P < 001$). The frequency distribution of values for DNA repair capacity (Figure 3) showed that lymphocytes from >95% of the controls but from only 59% of the cancer patients were able to remove >60% of the bleomycin-induced DNA damage. Conversely, >40% of the cases but <5% of the control subjects had a DNA repair capacity of ⩽60%. Regression analyses revealed no effect on these values of gender, age or (in lung cancer patients) histological subtype. Interestingly, there was also no apparent correlation between sensitivity to bleomycin and DNA repair capacity. Smoking habits affected DNA repair capacity: in both controls and cases, smokers had a significantly lower DNA repair capacity than those who had never smoked ($P < 0.05$). Cancer patients in all three smoking categories were, however, less efficient in DNA repair than control patients.

Repeated experiments on isolated lymphocytes from the same donor resulted in similar mean values for sensitivity to mutagens and DNA repair capacity and no significant difference was observed between lymphocytes from frozen samples and freshly isolated cells (data not shown). Frozen lymphocytes from four healthy non-smoking volunteers aged 30–52 years were analysed repeatedly after different periods of storage. Cryopreservation of the cells for ⩽371 days did not significantly affect their sensitivity to mutagens or their DNA repair capacity (Figure 4). Samples of peripheral blood lymphocytes were also collected at different seasons over 1 year, in order to evaluate seasonal variation in background DNA damage. The tail moments showed no significant seasonal variation.

Discussion

Comparison of bleomycin-treated peripheral blood lymphocytes from patients with lung cancer and patients without tumours revealed significant differences: After adjustment for gender, age and smoking habit, an increased risk for cancer was associated with increased sensitivity to mutagens and with decreased DNA repair capacity. Both endpoints were independent risk factors for smoking-related lung cancer. Our results are in agreement with those of previous studies in which the sensitivity of human peripheral blood lymphocytes to mutagens was determined as bleomycin-induced chromatid breaks (Spitz *et al.*, 1995) and DNA repair capacity was measured indirectly as host cell reactivation (Wei *et al.*, 1996).

Table II. Distribution of selected variables by histological type of lung cancer

Tumour type	No. of patients	Age (years)	Gender (%)		Smoking status (%)			Sensitivity to bleomycin	DNA repair capacity
			male	female	current	former	never		
Adenocarcinoma	40	62	63	37	45	37	18	28	69
Squamous cell carcinoma	43	59	88[a]	12	63	30	7	29	68
Other	17	60	65	35	47	24	29[b]	29	64

[a]Significantly different from males with adenocarcinoma ($P = 0.015$).
[b]Significantly different from current smokers ($P = 0.008$).

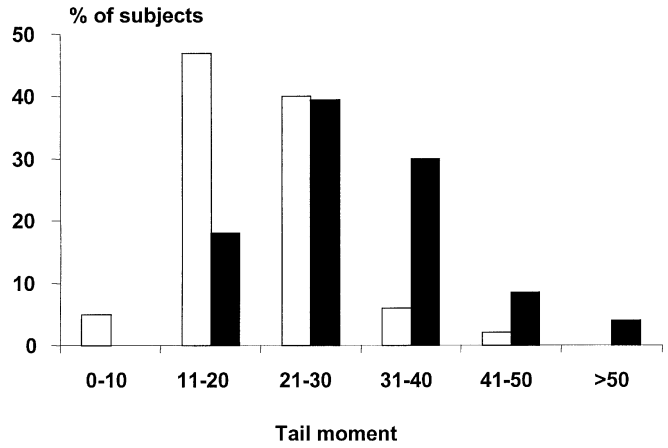

Fig. 2. Frequency distribution of classes of sensitivity to bleomycin of patients with lung cancer (black bars) and cancer-free control subjects (open bars).

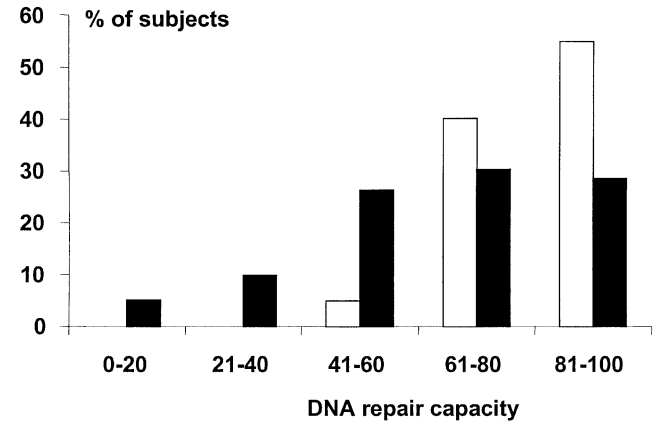

Fig. 3. Frequency distribution of DNA repair capacity for patients with lung cancer (black bars) and cancer-free control subjects (open bars).

An important advantage of our microgel electrophoresis assay over many other techniques is its applicability to virtually any cell type. Use of human peripheral blood lymphocytes in our case–control study allowed comparison of our results with those of earlier studies based on cytogenetic or host cell reactivation assays for measuring sensitivity to mutagens or DNA repair capacity (Hsu *et al.*, 1989, 1991; Schantz and Hsu, 1989; Athas *et al.*, 1991; Spitz *et al.*, 1993, 1994; Spitz and Hsu, 1994; Wu *et al.*, 1995, 1996). As bleomycin has been used in most of these studies as a mutagenic challenge, this drug was also selected for our assay. This drug is known to generate DNA single-strand breaks by forming a complex with ferrous ions and molecular oxygen, resulting in the release of oxygen radicals at the site of DNA intercalation

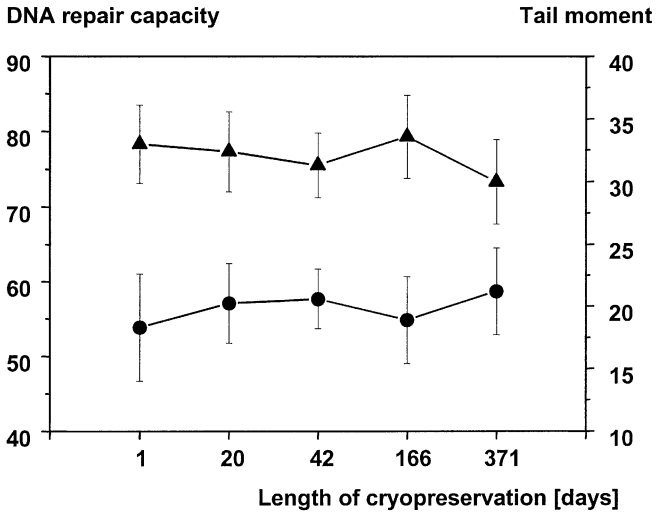

Fig. 4. Effect of length of cryopreservation on DNA repair capacity (▲) and sensitivity to bleomycin (●) in human peripheral blood lymphocytes. Each data point represents mean ± SEM for four individuals.

(Cunningham *et al.*, 1984; Sikic, 1986). Only a very small proportion of single-strand breaks can be rejoined directly by DNA ligase, while the rest are repaired mainly by base excision. The repair of single-strand breaks in normal cells is generally rapid, with a half-life of a few minutes, while the half-life of repair of double-strand breaks is generally >1 h (Frankenberg-Schwager, 1989; Lopez-Larraza *et al.*, 1990). In our microgel electrophoresis assay, the cells were allowed to repair DNA for 15 min, which was considered optimal since mainly single-strand breaks are formed under alkaline conditions.

The possibility of freezing and storing peripheral blood lymphocytes samples before analysis is an important technical advantage for conducting molecular epidemiological studies. Initial experiments confirmed that neither the amount of background and bleomycin-induced DNA lesions nor DNA repair capacity were significantly affected by freezing and storage. Furthermore, under our conditions, cryopreservation for ⩽371 days did not affect the endpoints tested. The report by Visvardis *et al.* (1997) that cryopreserved lymphocytes could not repair hydrogen peroxide-induced DNA lesions within 2 h after treatment might indicate that repair is blocked for some time after cells have been frozen. Those authors did not stimulate their cells with phytohaemagglutinin, however. The ability of mammalian cells to remove DNA damage is closely correlated with cell proliferation (Carson *et al.*, 1986; Sirover, 1990; Boerrigter and Vijg, 1992; Kaminskas and Li, 1992) and the relatively low DNA repair capacity of unstimulated lymphocytes has been linked to the low activity

of repair enzymes within these cells (Scudiero *et al.*, 1976). Furthermore, many DNA repair enzymes are cell cycle-dependent, so stimulated cells should have more activity than resting cells (Sirover, 1990). Our microgel electrophoresis assay was therefore based on mitogen-stimulated peripheral blood lymphocytes.

Microgel electrophoresis analysis of untreated peripheral blood lymphocytes in our study revealed no relationship between the extent of background damage and gender, smoking habits, age or seasonal variations in cases or controls. Other studies have shown that background DNA damage measured by microgel electrophoresis in unstimulated, untreated lymphocytes is related to smoking habits (Betti *et al*., 1994, 1995) and to the season in which blood samples were taken (Moller *et al.*, 1998). In all these studies, however, the analyses were carried out on resting lymphocytes, 99% of which are normally in G_0 phase. In our microgel electrophoresis assay, we cultured and stimulated the peripheral blood lymphocytes for 20 h in order to bring them into G_1, when they have a greater DNA repair capacity (Boerrigter and Vijg, 1992). In mitogen-stimulated cells, therefore, background DNA lesions undergo more efficient repair, possibly abolishing inter-individual differences of any pre-existing damage. In our study, a comparison of the initial damage in cancer patients and controls showed no significant difference.

Cytogenetic analyses of DNA lesions induced in peripheral blood lymphocytes by ionizing radiation (Cloos *et al.*, 1999) or bleomycin (Roberts *et al.*, 1999) in studies of families and twins indicate that the mutagen-sensitive phenotype has a genetic basis. Our new assay is less time consuming than the established cytogenetic tests; sensitivity to mutagens and DNA repair capacity can be assessed as independent endpoints. Elucidation of the underlying genetic basis of increased sensitivity to mutagens or reduced DNA repair capacity and prospective studies to identify high-risk populations are feasible with our new assay which can be conducted with stored, cryopreserved peripheral blood lymphocytes samples.

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