Aromatic DNA adduct levels in human peripheral blood lymphocytes and total white blood cells by ^{32}P-postlabelling: need for validation

Giuseppina Brescia, Vito Foâ, Cristina Viezzer, Lucia Celotti and Giorgio Assennato

Long-lived lymphocytes tend to have higher ^{32}P-postlabelling-measured levels of adducts than short-lived granulocytes in environmental and life-style associated (i.e. smoking) exposures. With the aim of investigating this issue for occupational exposure to PAH and contributing to further validation of some technical aspects of the ^{32}P-postlabelling assay, two Italian laboratories analysed PAH–DNA adducts from lymphocytes and total white blood cells (WBC). Seventy-seven blood samples from coke-oven workers employed at a steel plant located in Taranto, Southern Italy, and 14 samples from control subjects were collected. At the University of Padua, DNA was purified from peripheral blood lymphocytes (PBL). Two years later, at the University of Bari, white blood cells (WBC) were isolated from replicate blood samples stored at – 80 °C and DNA purified by the same method. In both cases, the nuclease P1-modified postlabelling assay was used to determine aromatic DNA adduct levels. The mean adduct levels were 5.13 ± 3.37 (Padua) and 2.48 ± 1.27 (Bari) per 10^8 nucleotides. Both laboratories observed large inter-individual variations of adduct levels ranging from 0.09 to 18.93 per 10^8 nucleotides. Both the correlation and the agreement of the two sets of data were assessed. Slight correlation ($r = 0.39$; $p < 0.01$) and a poor level of agreement were found, the intra-class correlation coefficient being equal to 0.05. Better correlation coefficient ($r = 0.54$, $p < 0.01$) and intra-class correlation coefficient ($r' = 0.50$) were observed comparing only the adduct levels determined on the diagonal zone (DRZ). Our findings seem to confirm the same divergence reported in the literature on DNA adduct levels between lymphocytes and granulocytes.

Keywords: *^{32}P-postlabelling, aromatic DNA adducts, monitoring PAH exposure, lymphocytes, white blood cells.*

Abbreviations: *(anti)-B[a]PDE, (±)-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9, 10-tetrahydrobenzo[a]pyrene; B[a]P, benzo[a]pyrene; DMSO, dimethyl sulphoxide; DRZ, diagonal radioactive zone; PAH, polycyclic aromatic hydrocarbons; PBL, peripheral blood lymphocytes; TLC, thin-layer chromatography; WBC, white blood cells.*

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Introduction

The extent of binding of PAHs to DNA in humans may serve as a biomarker of exposure to genotoxic PAHs and therefore be useful to evaluate carcinogen exposure. Moreover, as the adduct measures also mirror some aspects of metabolism and DNA repair, DNA adducts may also reflect the individual variation in response to genotoxic exposure.

Several sensitive DNA adduct detection systems are available, including enzyme immunoassays (Santella 1988, Den Engelese *et al.* 1990), fluorometric assays (Weston and Bowman 1991, Alexandrov *et al.* 1992) and ^{32}P-postlabelling assays (Beach and Gupta 1992). The ^{32}P-postlabelling assay, being the most sensitive method, is especially suited to analyse DNA adducts resulting from the exposure to complex mixtures of unknown composition (Watson 1987) and the nuclease P1 modification of the assay is capable of measuring multiple lipophilic adducts of DNA, such as those formed by PAH exposure down to a limit of one adduct per 10^{10} nucleotides (Reddy and Randerath 1986).

Several studies have shown that levels of PAH–DNA adducts are significantly increased in peripheral WBC or lymphocytes of occupationally exposed coke-oven workers, foundry workers, and roofers (Schult and Shiverick 1992), although blood is not the target tissue of PAH carcinogenesis in humans.

A critical issue for molecular epidemiologists is the validity of surrogate tissue as sources of DNA adducts. Previous attempts to validate peripheral blood white cells as a surrogate tissue source for lung PAH–DNA adduct determinations used DNA isolated from total white blood cells. Approximately 70% of total white blood cells are short-lived granulocytes, whereas the remaining 30% are longer-lived lymphocytes. Aromatic DNA adduct levels in total white blood cells were found to be associated neither with current cigarette smoking (Phillips *et al.* 1988, 1990, Garner *et al.* 1990, Phillips *et al.* 1990), nor with adduct levels measured in lung tissue from the same individuals (van Schooten *et al.* 1992). On the contrary, in other studies, aromatic DNA adduct levels from lymphocytes were found to be increased in current smokers (Savela and Hemminki 1991) and were consistently reproducible by the ^{32}P-postlabelling assay over time (Savela and Hemminki 1993). Furthermore, as indicated by a recent study (Wiencke *et al.* 1995), lymphocytes could be a more valid and reliable surrogate tissue for estimating the burden of DNA adducts in respiratory tissue, in as much as a large correlation between tobacco-induced aromatic DNA adduct levels detected in lung tissue from 31 lung cancer patients and those detected in lymphocytes from the same individuals by the ^{32}P-postlabelling assay was found.

Therefore, validation is required to avoid systematic errors, and methodological attributes such as accuracy, reproducibility (intra- and inter-laboratory) and feasibility of using different surrogate cell types and stored samples must be determined by appropriately designed collaborative studies before the method can be applied in risk assessment.

With the aim of comparing DNA adduct levels between lymphocytes and total WBC for the assessment of cumulative carcinogen exposure in the workplace, of evaluating the influence of storage and of detecting potential tools to differentiate past and cumulative exposure from recent and

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ongoing exposure, two Italian laboratories analysed PAH–DNA adducts from total WBC samples (in Bari laboratory) and PBL samples (in Padua laboratory) taken from 77 coke-oven workers and 14 control subjects, using the nuclease P1-enhanced postlabelling method.

METHODS

Laboratories and blood sampling

The two laboratories involved in this trial were that of L. Celotti at Cell Biology Department, University of Padua, Italy and that of G. Assennato at Molecular Epidemiology Unit, DI.M.I.L., University of Bari, Italy.

The study sample consisted of 77 coke-oven workers employed at a steel plant located in southern Italy (Assennato *et al.* 1993). As a control group, 14 workers who were not occupationally exposed to PAH were recruited from the same area. A complete description of the features of the plant, of the job categories, of the study population, as well as of the environmental measures and of all the analytical systems used for the evaluation of different biomarkers, is reported in the final text of the research supported by the European Commission, Steel and Coal (Research contract no. 7280-01-04; Coordinator: Prof. Vito Foâ).

A trained interviewer completed questionnaires about smoking habits, occupational history, alcohol consumption, medication and use of protective equipment in the steel plant.

Duplicate blood-samples (25–30 ml) were collected in heparinized plastic tubes at the medical department of the plant and coded. One series was shipped to Padua immediately where lymphocytes were isolated within 24 h. The other series was centrifuged immediately in order to separate serum from cellular components. Both these components from the second series were transported to Bari where they were stored at –80 °C.

In the workplace, airborne B[*a*]P levels (range: 0.45–4.84 μg/mc; median: 2.82 μg/mc), PAH (range: 3.6–62.6 μg/mc; median: 27.09 μg/mc) and nitro-PAH (range: < 0.01–0.195 μg/mc; median: 0.029 μg/mc) concentrations were monitored by occupational hygienists using personal sampling devices in two different surveys. The relationships between the aromatic DRZ (Diagonal Radioactive Zone) adduct levels determined in Padua on the whole coded population under study and the external exposure, the cigarette smoking status and the urinary 1-hydroxypyrene levels in the exposed individuals (range: 0.018–5.587 μmol mol^{-1} creatinine; median: 0.741 μmol mol^{-1} creatinine), as well as comparisons in adduct levels between controls and exposed are reported in the work of Celotti *et al.* (1996).

Lymphocytes isolation (in Padua laboratory)

Lymphocytes were isolated within 24 h of sampling by a slight modification of the technique described by Boyum (Boyum 1977). The blood, diluted 1: 1 with Hank's balanced salt solution (HBSS), was carefully layered over a Ficoll Hypaque gradient (Sigma Chemical Co.) and centrifuged at 400 *g* for 40 min (Celotti *et al.* 1993). The interface of mononuclear cells, consisting of 85% lymphocytes and 15% monocytes was collected, washed three times with HBSS, and frozen at –80 °C until DNA extraction. A small contamination of erythrocytes was present in all the fractions separated by centrifugation, probably due to the 24 h delay in the isolation of lymphocytes, but it did not interfere with DNA extraction from PBL.

WBC isolation (in Bari laboratory)

Cellular components received from the medical department at the coke plant were stored in Bari for 2 years at – 80 °C. WBC were then isolated by centrifugation after incubation with 0.12 M NH_4Cl to lyse the erythrocytes. DNA was extracted immediately from the resulting WBC.

Reference-adducted DNA (in Padua laboratory)

^{3}H-(*anti*)-B[*a*]PDE, specific activity 1941 mCi $mmol^{-1}$, obtained from the NCI Chemical Carcinogen Standard Repository (Bethesda, MD, USA), and dissolved in DMSO at the concentration of 0.33 μM, was added to unstimulated human PBL (4×10^6 cells ml^{-1}) for 40 min in medium without serum. Treated cells were washed and DNA was isolated. Purified DNA was hydrolysed enzymatically to deoxynucleosides (Celotti *et al.* 1993). The level of DNA modification, as determined by scintillation counting, was 395 adducts per 10^8 nucleotides. A small aliquot of this reference-adducted DNA was sent to Bari on dry ice.

DNA extraction

In both laboratories, DNA was isolated according to a slight modification of the salting out procedure described by Leadon and Cerutti (1982). The DNA concentration was determined by measuring the absorbance at 260 nm and the purity was checked by evaluating the absorbance ratio $A_{260} : A_{280}$. This ratio was always within the range 1.79–1.91.

DNA adduct determination by ^{32}P-postlabelling assay

DNA adducts were analysed by the nuclease P1-enhanced procedure with slight modifications, according to the method described by Reddy and Randerath (1986). Five μg DNA was digested for 3.5 h at 37 °C with 250 mU micrococcal nuclease and 8 mU spleen phosphodiesterase (Sigma Chemical Co.) in a total volume of 5 μl containing 10 mM sodium succinate and 10 mM $CaCl_2$, pH 6. Samples were then digested further with 2 μg nuclease P1 (Boehringer Mannheim, Germany) in a total volume of 7.5 μl, containing 0.1 mM $ZnCl_2$ and 0.06 M sodium acetate (pH 5) for 1 h at 37 °C. After the addition of 2.5 μl 0.5 M tris base, the DNA digest was labelled with 2 μl (20 μci) [γ-^{32}P]ATP (5000 Ci $mmol^{-1}$, Amersham) using 2.5 units of T4 polynucleotide kinase (Boehringer). The reaction was terminated with 40 mM potato apyrase (Sigma) after 30 min. Purification and resolution of ^{32}P-labelled adducts were carried out by TLC on polyethyleneimine–cellulose sheets (Macherey-Nagel, Duren, Germany).

Chromatography was done according to a published method (Reddy and Randerath 1986) using the following solvents: Direction (D)1, 1.0 M sodium phosphate (pH 6.8); D2, 3.5 M lithium formate–8.5 M urea (pH 3.5) (opposite to D1); D3, 1.2 LiCl–0.5 M Tris–HCl–8.5 M urea (pH 8.0) (perpendicular to D2); and D4, 1.7 M sodium phosphate (pH 6.0) (directed as D3). The chromatograms were visualized by autoradiography at –80 °C for 72 h, using intensifying screens. Radioactive ink (seen as small spots at the edges of the chromatograms) was used to align the developed autoradiographs with the TLC sheets.

Adduct levels were determined by excising areas from the chromatograms and measuring their radioactivity by Cerenkov counting. Relative Adduct Labelling (RAL) was calculated from the amount of radioactivity on the chromatograms, the DNA amount loaded on the TLC sheet, and the specific activity of the [γ-^{32}P]ATP used for labelling, according to the method described by Reddy and Randerath (1986). A positive control of [^{3}H]-(*anti*)-B[*a*]PDE–DNA standard, a negative control of calf thymus DNA (Boehringer) and a sample without DNA were routinely assayed along with experimental samples. The reproducibility of the method was tested in Padua and Bari by repeated (triplicate) assays of each sample. The technical error, calculated as the standard deviation of the three replicates pooled over all individuals (Kahn and Sempos 1989), was 0.48 and 1.08 for total adducts, and 0.25 and 0.295 for DRZ adducts in Bari and Padua, respectively. The corresponding overall coefficients of intra-assay variation were 19% and 21% for total adducts and 20% and 21.5% for DRZ adducts. These values agree with previous recent studies in which the reproducibility of the assay is reported (van Schooten *et al.* 1995).

Statistical methods

Univariate analysis, log-transformation of data and correlation coefficients were determined with the EPILOG computer software

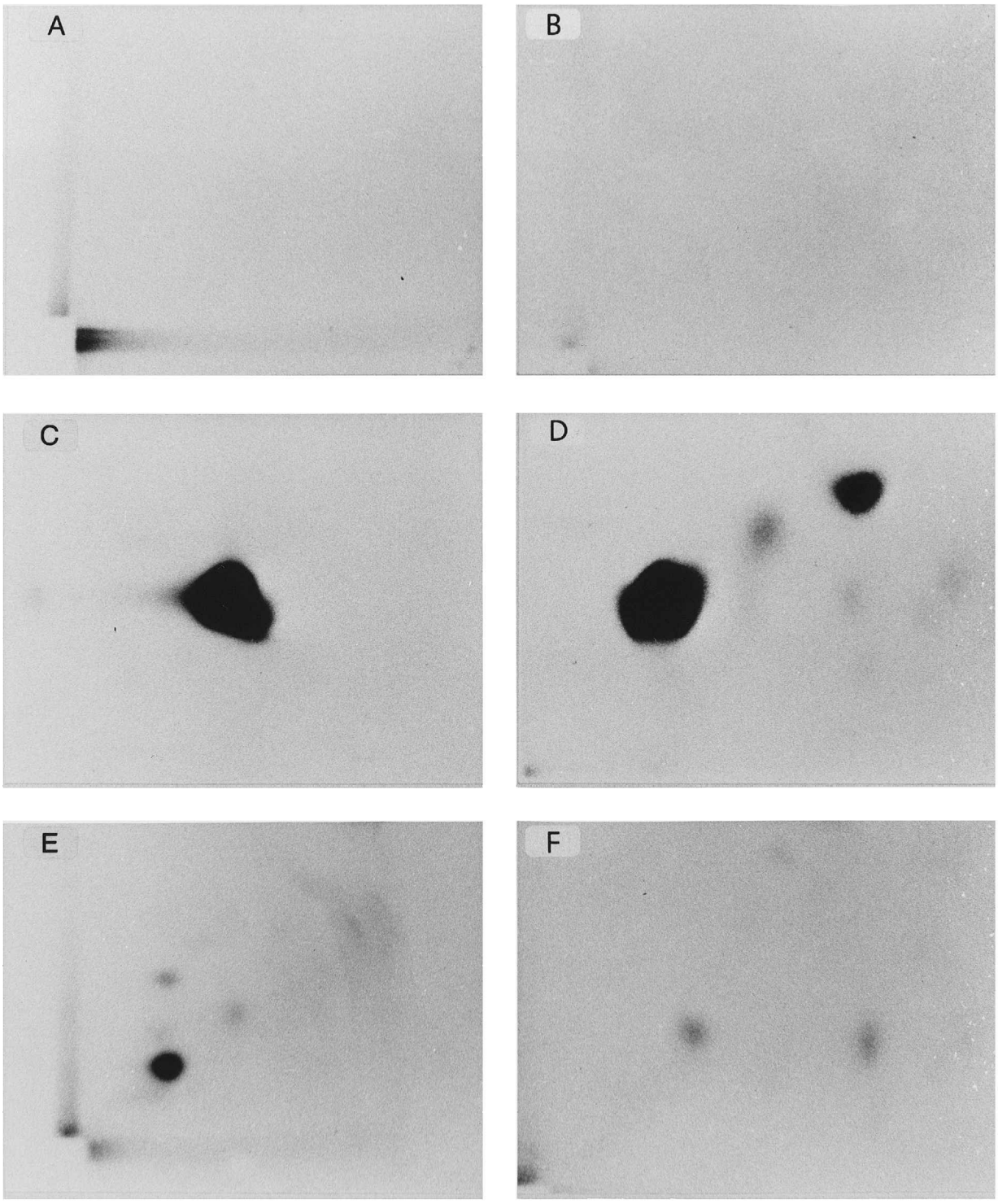

Figure 1. Representative autoradiograms of TLC maps of a control sample (A, B), of the reference (*anti*)-B[*a*]PDE-modified DNA (C, D) and of a coke worker (E, F) obtained in the two laboratories (Bari and Padua, respectively).

Results

Qualitative analysis

For control WBC and PBL DNA samples, chromatograms devoid of visible radioactive spots (Figure 1(A and B)) were obtained, although, in some cases (4/14), some faint spots arranged in a similar profile to that of the exposed group appeared. The presence of these spots is considered to be indicative of non-occupational external PAHs sources (e.g. smoking, charcoal-broiled food consumption, environmental pollution). Samples of calf thymus DNA were used as further controls and were free of spots. These observations suggest that the spots observed in controls represent PAH–DNA adducts and not artifacts.

With (*anti*)-B[*a*]PDE-modified DNA, both laboratories observed the same characteristic major spot (Figure 1(C and D)), although, a second minor spot was also evident in chromatograms from the Padua laboratory. We believe that the major spot represents the main adduct of (*anti*)-B[*a*]PDE to the exocyclic N^2 position of guanine, and the minor spot a less stable (*anti*)-B[*a*]PDE adduct, not always recovered after all the phases of the procedure.

DNA samples obtained from blood cells of the exposed workers showed evidence of aromatic DNA adducts and quite similar profile of adduct spots were detected for WBC and PBL. Autoradiograms from the two laboratories are shown in Figure 1(E and F). The familiar diagonal arrangement, DRZ, of the radiolabelled spots was apparent. There were variations, however, in the length and the width of radioactivity and in chromatographic mobility, sharpness and resolution of adduct spots. The pattern, indicative of adduct formation by a complex mixture of aromatic compounds, was qualitatively similar to those previously seen in other studies of PAH-exposed workers in iron foundries (Phillips *et al.* 1985), coke ovens (Hemminki *et al.* 1990), and aluminium plants (Schoket *et al.* 1991).

Some samples contained areas of diffuse labelling or smears suggesting the presence of multiple, poorly resolved adducts, resulting from the covalent binding to DNA of many different chemical compounds with similar characteristics (Phillips *et al.* 1985, 1988). Similar adduct profiles have been obtained following the application of carcinogenic mixtures of PAHs to experimental animals (Randerath *et al.* 1988, Schoket *et al.* 1988).

In a relevant fraction of the DNA samples (54% and 30% for WBC and PBL, respectively), an adduct spot, mapped in the DRZ and with similar chromatographic mobility to that obtained with (*anti*)-B[*a*]PDE-modified DNA, was detected. However, no further characterization was attempted.

Other adduct spots were detected outside the diagonal zone. In order to improve the qualitative comparison, the adduct spots of the TLC plates were mapped into specific areas, as follows. The plate was divided into three regions as shown in Figure 2: area 1 = DRZ; area 2 = left of DRZ; area 3 = right of DRZ. In most of the DNA samples (80% and 76% for WBC and PBL, respectively) adduct spots in area 2 were detected by both laboratories. Adduct spots in area 3 were observed only in 15% of the WBC DNA samples, and none in PBL DNA.

In conclusion, although a qualitative agreement is apparent, in order to perform a statistically correct qualitative evaluation, it is necessary to achieve a specific comparison for each sample and to define morphometric parameters.

Quantitative analysis

The same reference-adducted DNA with a modification level of 395 adducts per 10^8 nucleotides was used in both laboratories. The RAL values (number of adducts per 10^8 nucleotides) calculated after having performed the ^{32}P-postlabelling assays were compared with the expected values obtained by scintillation counting, and thus, labelling efficiencies were determined. Mean (± SD) labelling efficiencies of 45.6%

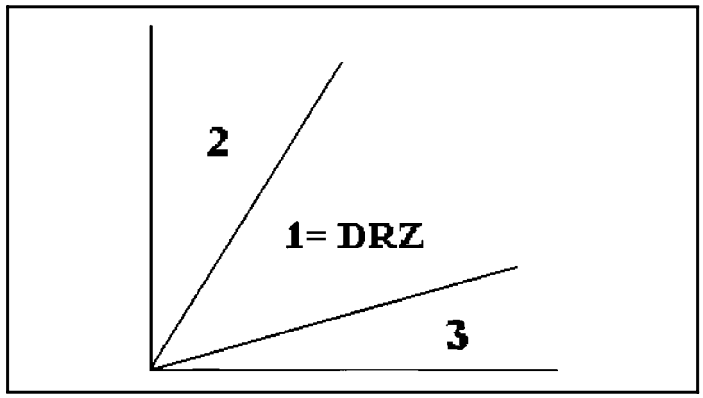

Figure 2. The autoradiograms were divided into three areas for mapping the adduct spots.

(± 1.93%) and 45.8% (± 1.37%) were obtained in Padua and Bari, respectively. The intra-class correlation coefficient (r') between the two sets of measurements obtained in the two laboratories was determined in order to assess the inter-laboratory reproducibility. A very good reproducibility ($r' = 0.924$) was observed.

The adduct levels determined in WBC and PBL for both control and exposed individuals are shown in Figure 3. The RAL method of quantitation was used, basing the determinations on the specific activity of the [^{32}P]ATP provided by the commercial supplier (Amersham). The lowest control value obtained was 0.09 adducts per 10^8 nucleotides and this compares closely with the arbitrary control value of 0.01 adducts in 10^8 nucleotides quoted by Phillips and Castegnaro (1993). The main statistics of the analysis are shown in Table 1.

Both laboratories observed large inter-individual variations of adduct levels amongst the 80 coke worker samples ranging from 0.69 to 6.06 (in WBC) and 0.86 to 18.93 (in PBL). These may be related to metabolic differences between individuals or to different exposure patterns. In the control group, a mean adduct level (range) of 0.3 (0.09–0.57) and 2.00 (1.15–3.53) per 10^8 nucleotides was obtained for WBC and PBL, respectively. The mean number of adducts and the standard deviations of

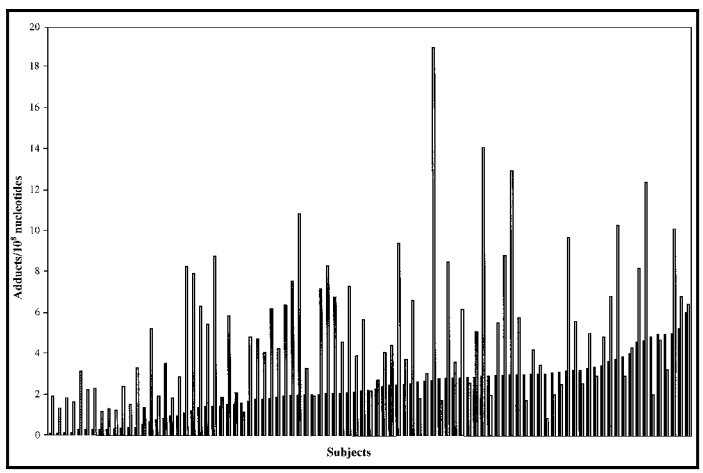

Figure 3. Total PAH–DNA adduct levels of individual coke-oven and non-oven workers obtained from total WBC (■) and PBL (□). The number of adducts in 10^8 nucleotides is arranged from the lowest to the h

Cell-type	Mean[a]	SD[a]	CV (%)	r (log)	r′
PBL (Padua laboratory)	5.13	3.37	65	0.39**	0.05
WBC (Bari laboratory)	2.48	1.27	51		

Table 1. Distribution of total aromatic adduct levels measured in total WBC and PBL for 77 coke-oven workers and 14 controls, and summary statistics.
[a] Adducts per 10^8 nucleotides.
** $p < 0.001$.

Cell-type	Mean[a]	SD[a]	CV (%)	r (log)	r′
PBL (Padua laboratory)	1.37	1.69	123	0.54**	0.50
WBC (Bari laboratory)	1.24	0.88	70		

Table 2. Distribution of DRZ aromatic adduct levels measured in total WBC and PBL for 30 coke-oven workers, and summary statistics.
[a] Adducts per 10^8 nucleotides.
** $p < 0.001$.

the two overall (exposed + controls) sets of data were 2.48 ± 1.27 and 5.13 ± 3.37 in 10^8 nucleotides for WBC and PBL, respectively.

Pearson's correlation was applied for the statistical analysis of the log-transformed data obtained for the two cell-types: WBC and PBL. Moderate correlation was found, the correlation coefficient being 0.39 ($p < 0.01$). An analysis of the agreement was carried out by determining the intra-class correlation coefficient (r'), used to assess the concordance on quantitative scales, which was equal to 0.05, so indicating a poor agreement.

A further inter-laboratory comparison was made for 30 coke workers' samples, for which DRZ RAL levels (adduct spots located on the diagonal zone of the chromatogram) were quantified in addition to the total RAL levels (total adduct spots on the chromatogram) (Figure 4). In spite of this relatively small number of samples analysed, a correlation of $r = 0.54$ ($p < 0.01$) and an agreement of $r' = 0.50$ were obtained between the laboratories. The mean levels (± SD) of DRZ RAL were 1.24 (± 0.88) and 1.37 (± 1.69) in 10^8 nucleotides in WBC and PBL, respectively (Table 2).

Repeat postlabelling assays were performed on a set of 10 WBC DNA samples randomly selected from those aliquots that had been stored in Bari at –80 °C for 2 years. An analysis of the correlation and the agreement between this and the first DNA adduct determinations was carried out. Both the correlation and the agreement were very high ($r = 0.93$ and 0.94, $p < 0.001$, $r' = 0.92$ and 0.93 for total and DRZ adduct levels, respectively), so indicating an excellent agreement between adduct levels measured 2 years apart in DNA samples stored at –80°C.

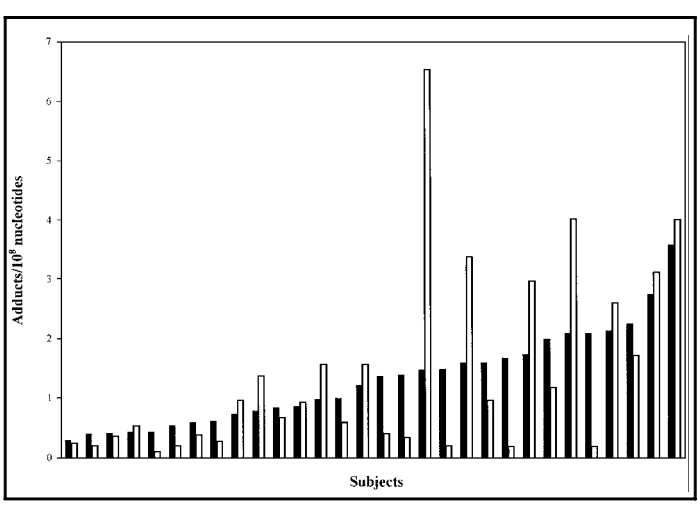

Figure 4. DRZ DNA adduct levels obtained from total WBC (■) and PBL (□) on 30 coke-oven workers. The number of adducts in 10^8 nucleotides is sequentially indicated on the basis of increasing levels measured in WBC.

Discussion

^{32}P-postlabelling assays have been used widely for the detection and estimation of aromatic–DNA adducts in biological samples. The types of PAH-exposed occupational groups studied include foundry, coke oven and aluminium workers (Hemminki *et al.* 1990, Óvrebo *et al.* 1992, Grzybowska *et al.* 1993, Santella *et al.* 1993, Perera *et al.* 1994, Óvrebo *et al.* 1994, Óvrebo *et al.* 1995), roofers, bus garage, terminal workers and car mechanics (Hemminki *et al.* 1994), and chimney sweeps (Ichiba *et al.* 1994). In many of these groups of workers, elevated levels of aromatic DNA adducts have been found in white blood cells or lymphocytes, at levels ranging from 1 to 10 adducts per 10^8 nucleotides. In three studies of coke-oven workers, the mean DNA adduct levels, measured by the ^{32}P-postlabelling assay, were 1.67, 6.1 and 11.6 per 10^8 nucleotides, respectively (Óvrebo *et al.* 1992, Grzybowska *et al.* 1993, Hemminki *et al.* 1990). However, none of these studies were able to define a statistically significant correlation between the adduct levels and external exposure, and relatively wide inter-individual variation in adduct levels was observed.

In our study, we applied ^{32}P-postlabelling assay to blood samples from 77 coke-oven workers occupationally-exposed to PAHs and from 14 control subjects. The results from two independent laboratories (Padua and Bari) were analysed in order to address the methodological issues of using different cell-types (WBC in Bari laboratory and PBL in Padua laboratory) and of storage time lags.

We found that blood samples taken from the same workers generally gave the same adduct profiles irrespective of the testing laboratory. Samples generally gave the characteristic diagonal zone of radioactivity (DRZ) observed by others (Phillips *et al.* 1985, Hemminki *et al.* 1990, Schoket *et al.* 1991) in PAH-exposed workers. These findings suggest that WBC or PBL, fresh or stored blood samples, are acceptable for simple qualitative analysis of aromatic–DNA adducts in coke-oven workers. However, our statistical methods have identified several issues that show that further standardization of test methods is needed for the ^{32}P-postlabelling assay to be a reliable quantitative biomonitoring t

The comparison of total aromatic DNA adduct levels indicated that the two sets of data, even if moderately correlating, were numerically different ($r = 0.39$ vs $r' = 0.05$). We hypothesize and provide evidence that this could be due to the use of different cell-types as DNA source (total WBC in Bari and PBL in Padua).

Lymphocytes and granulocytes are believed not to play a significant role in the metabolism of xenobiotics and there is no evidence that they differ in their capacity to metabolize PAHs. However, since they have the longer life-span in blood, lymphocytes might provide the more sensitive tool for assessing cumulative internal dose from long-term exposure. Furthermore, recent studies indicate that a large fraction of blood lymphocytes migrates to different non-lymphoid organs such as lung, intestine, liver, and only slowly redistributes to peripheral blood (Pabst and Binns 1989, Pabst *et al.* 1993). The prolonged residence of lymphocytes in the capillary network of these organs is predicted to result in a greater exposure to xenobiotics than will occur in granulocytes. In support of this, DNA adduct levels in the blood of smokers and individuals from a polluted industrial region of Poland have been found to be greater in the lymphocytic than the granulocytic fraction (Savela and Hemminki 1991, 1993, Mustonen and Hemminki 1992, Grzybowska *et al.* 1993, Moller *et al.* 1996). In the Polish study, a strong seasonal variation in exposure was revealed in adduct levels in lymphocytes but not granulocytes.

A correlation has been found between DNA adduct levels in blood mononuclear cells and tobacco-carcinogen induced genetic damage in human lung (Wiencke *et al.* 1995). However, in other studies, total WBC aromatic DNA adduct levels have been found to be associated neither with current cigarette smoking (Phillips *et al.* 1988, 1990, Garner *et al.* 1990), nor with adduct levels measured in lung tissue from the same individuals (van Schooten *et al.* 1992). Our results for total WBC and PBL DNA adducts believed to arise from workplace exposures complies with these earlier findings. In the comparison of the DNA adduct levels determined from PBL in Padua and from total WBC in Bari, the correlation level was only slight and the agreement level was poor. Moreover, the mean individual total DNA adduct level determined in Padua from lymphocytes was significantly higher than that determined in Bari from total WBC (5.13 vs 2.48 per 10^8 nucleotides, test $t = -6.9$, $p < 0.001$), and, in 82% of the individuals, the adduct levels obtained in PBL were higher than those in total WBC. We believe that our results provide some evidence that DNA adducts measured in lymphocytes might be considered as indicators of the cumulative dose resulting from long-time exposure, whereas DNA adducts measured in total white blood cells cannot.

In addition to the effect of the variation between cell types, both inter-laboratory differences and storage conditions could have influenced the divergence between the two sets of data. However, we believe that these two factors had a relatively low, if any, influence on the difference of DNA adduct levels between the two laboratories. Although it should be evident that samples may deteriorate on storage and that the stability of DNA adducts may decrease over a long period, there is no overall consensus reached on this matter. In our investigation, we detected a very good agreement ($r' = 0.92$ and 0.93 for total and DRZ adducts, respectively) between WBC DNA adduct levels measured after a lag time of 2 years storage at –80°C in a group of 10 randomly selected samples. This suggests that storage at –80 °C for 2 years had no effect on the stability of aromatic DNA adducts, and consequently, does not contribute to the discrepancy observed in DNA adduct levels between Padua and Bari.

We believe that systematic variations between the two laboratories could have accounted for only a small portion of the differences detected, as the comparison of the adduct levels measured in the two laboratories for the same (*anti*)-B[*a*]PDE-modified DNA gave a very good agreement ($r' = 0.924$), so indicating a high inter-laboratory reproducibility.

Finally, it is interesting to note that inter-laboratory comparisons of adduct levels improved considerably when they were limited to the DRZ instead of total adducts ($r = 0.54$ and $r' = 0.50$). It could be speculated that the adducts in the DRZ might represent the most reproducible, specific marker for occupational exposure to PAHs, regardless of the cell-type. This is in agreement with other evidence reported in literature on the characteristic pattern of adduct spots detected in PAH-exposed workers (Phillips *et al.* 1985, Hemminki *et al.* 1990, Schoket *et al.* 1991).

In conclusion, we have observed a variation in the levels of aromatic DNA adducts in lymphocytes and WBC from PAH-exposed workers. This inter-cell variation is consistent with findings reported by others on DNA adducts in smokers and individuals exposed to environmental pollutants. Thus, our findings provide further indication that there is a pressing need for standardization of the method and defined quality control of the ^{32}P-postlabelling assay. In particular, care should be taken with cell-type identity and the range of adducts being monitored (e.g. DRZ or total adduct levels). Only after this has been achieved, can multicentric collaborative trials be designed to investigate DNA adduction in human population.

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References

ALEXANDROV, K., ROJAS, M., GENESTE, O., CASTEGNARO, M., CAMUS, A.-M., PETRUZZELLI, S., GIUNTINI, C. AND BARTSCH, H. (1992) An improved fluorometric assay for dosimetry of benzo[*a*]pyrene diol epoxide–DNA adducts in smokers' lung: comparison with total bulky adducts and aryl hydrocarbon hydroxylase activity. *Cancer Research*, **52**, 6248–6253.

ASSENNATO, G., FERRI, G. M., TOCKMAN, M. S., POIRIER, M. C., SHOKET, B., PORRO, A., CORRADO, V. AND STRICKLAND, P. T. (1993) Biomarkers of carcinogen exposure and cancer risk in a coke plant. *Environmental Health Perspectives*, **99**, 237–239.

BEACH, A. C. AND GUPTA, R. C. (1992) Human biomonitoring and ^{32}P-postlabelling assay. *Carcinogenesis (London)*, **13**, 1053–1074.

BOYUM, A. (1977) Separation of lymphocytes, lymphocytes subgroups and monocytes. A review. *Lymphology*, **10**, 71–76.

CELOTTI, L., FERRARO, P., FURLAN, D., ZANESI, N. AND PAVANELLO, S. (1993) DNA repair in human lymphocytes treated in vitro with (±)-*anti*- and (±)-*syn*-benzo[*a*]pyrene diolepoxide. *Mutation Res*

CELOTTI, L., VIEZZER, C., PAVANELLO, S., FERRARO, P., LEVIS, A. G., FERRI, G. AND FOÀ, V. (1996) DNA adduct levels in peripheral blood lymphocytes of coke-oven workers. *Clean Technology, Environmental Toxicology & Occupational Medicine*, **5**, 167–177.

DEN ENGELSE, L., VAN BENTHEM, J. AND SCHERER, E. (1990) Immunocytochemical analysis of in vivo DNA modification. *Mutation Research*, **233**, 265–287.

GARNER, R. C., CUZICK, J., JENKINS, D., PHILLIPS, D. H., HEWER, A., KING, M. M. AND ROUTLEDGE, M. N. (1990) Linear relationship between DNA adducts in human lung and cigarette smoking. *IARC Scientific Publication*, **104**, 421–426.

GRZYBOWSKA., E., HEMMINKI, K., SZELINGA, J. AND CHORAZY, M. (1993) Seasonal variation of aromatic DNA adducts in human lymphocytes and granulocytes. *Carcinogenesis*, **14**, 2523–2526.

HEMMINKI, K., GRZYBOWSKA, E., CHORAZY, M., TWARDOWSKA-SAUCHA, K., SROCZYNSKI, S. W., PUTNAM, K. L., RANDERATH, K., PHILLIPS, D. H., HEWER, A., SANTELLA, R. M., YANG, T. L. AND PERERA, F. P. (1990) DNA adducts in humans environmentally exposed to aromatic compounds in an industrial area of Poland. *Carcinogenesis*, **11**, 1229–1231.

HEMMINKI, K., SODERLING, J., ERICSON, P., NORBECK, H. E. AND SEGERBÄCK, D. (1994) DNA adducts among personnel servicing and loading diesel vehicles. *Carcinogenesis*, **15**, 767–769.

ICHIBA, M., HEGMAR, L., RANNUG, A. O., HOGSTEDT, B., ALEXANDRIE, A. K. AND HEMMINKI, K. (1994) Aromatic DNA adducts, micronuclei and genetic polymorphism for CYP1A1 and GST1 in chimney sweeps. *Carcinogenesis*, **15**, 1347–1352.

KHAN, H. AND SEMPOS, C. T. (1989) The primacy of data collection. In *Statistical Methods in Epidemiology*, B. Macmahon, ed. (Oxford University Press, New York), pp. 239–244.

LEADON, S. A. AND CERUTTI, P. A. (1982) A rapid and mild procedure for isolation of DNA from mammalian cells. *Analytical Biochemistry*, **120**, 282–288.

MOLLER, L., GRZYBOWSKA, E., ZEISIG, M., CIMANDER, B., HEMMINKI, K. AND CHORAZY, M. (1996) Seasonal variation of DNA adduct pattern in human lymphocytes analyzed by ^{32}P-HPLC. *Carcinogenesis*, **17**, 61–66.

MUSTONEN, R. AND HEMMINKI, K. (1992) 7-Methylguanine levels of DNA in smokers' and non smokers' total white blood cells, granulocytes, and lymphocytes. *Carcinogenesis*, **13**, 1951–1955.

ÓVREBO, S., HAUGEN, A., PHILLIPS, D. H. AND HEWER, A (1992) Detection of polycyclic aromatic hydrocarbon–DNA adducts in white blood cells from coke oven workers: correlation with job categories. *Cancer Research*, **52**, 1510–1514.

ÓVREBO, S., HAUGEN, A., FJELDSTAD, P. E., HEMMINKI, K. AND SZYFTER, K. (1994) Biological monitoring of exposure to PAH in an electrode paste plant. *Journal of Occupational Medicine*, **36**, 303–310.

ÓVREBO, S., HAUGEN, A., HEMMINKI, K., SZYFTER, K., DRABLOS, P. A. AND SKOGLAND, M. (1995) Studies of biomarkers in aluminium workers occupationally exposed to polycyclic aromatic hydrocarbons. *Cancer Detection & Prevention*, **19**, 258–262.

PABST, R. AND BINNS, R. M. (1989) Heterogeneity of lymphocyte homing physiology: several mechanisms operate in the control of migration to lymphoid and non-lymphoid organs *in vivo*. *Immunology Reviews*, **108**, 83–109.

PABST, R., BINNS, R. M., ROTHKOTTER, H. J. AND WESTERMANN, J. (1993) Quantitative analysis of lymphocyte fluxes *in vivo*. *Current Topics in Microbiology and Immunology*, **184**, 151–159.

PERERA, F. P., DICKEY, C., SANTELLA, R., O'NEILL, J. P., ALBERTINI, R. J., OTTMAN, R., TSAI, W. Y., MOONEY, L. A., SAVELA, K. AND HEMMINKI, K. (1994) Carcinogen–DNA adducts and gene mutations in foundry workers with changing exposure to PAH. *Carcinogenesis*, **15**, 2905–2910.

PHILLIPS, D. H. AND CASTEGNARO, M. (1993) Results of an interlaboratory trial of ^{32}P-postlabelling. In *Postlabelling Methods for Detection of DNA Adducts*, D. H. Phillips, M. Castegnaro and H. Bartsch, eds, IARC Scientific Publication no. 124 (International Agency for Research on Cancer, Lyon), pp. 35–49.

PHILLIPS, D. H., HEMMINKI, K., ALHONEN, A., HEWER, A. AND GROVER, P. L. (1985) Monitoring occupational exposure to carcinogens: detection by ^{32}P-postlabelling of aromatic DNA adducts in white blood cells from iron foundry workers. *Mutation Research* , **45**, 66–68.

PHILLIPS, D. H., HEWER, A., MARTIN, C. N., GARNER, R. C. AND KING, M. M. (1988) Correlation of DNA adducts levels in human lung with cigarette smoking. *Nature*, **336**, 790–792.

PHILLIPS, D. H., SCHOCKET, B., HEWER, A., BAILEY, E., KOSTIC, S. AND VINCZE, I. (1990) Influence of cigarette smoking on the levels of DNA adducts in human bronchial epithelium and white blood cells. *International Journal of Cancer*, **46**, 569–575.

RANDERATH, E., MITTAL, D. AND RANDERATH, K. (1988) Tissue distribution of covalent DNA damage in mice treated dermally with cigarette 'tar': preference for lung and heart DNA. *Carcinogenesis* (*London*), **9**, 75–80.

REDDY, M. V. AND RANDERATH, K. (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabelling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543–1551.

SANTELLA, R. M. (1988) Application of new techniques of carcinogen adducts to human population monitoring. *Mutation Research*, **205**, 271–282.

SANTELLA, R. M., HEMMINKI, K., TANG, D. L., PAIK, M., OTTMAN, R., YOUNG, T. L., SAVELA, K., VODICKOVA, L., DICKEY, C., WHYATT, R. AND PERERA, F. P. (1993) Polycyclic aromatic hydrocarbon–DNA adducts in white blood cells and urinary 1-hydroxypyrene in foundry workers. *Cancer Epidemiology, Biomarkers & Prevention*, **2**, 59–62.

SAVELA, K. AND HEMMINKI, K. (1991) DNA adducts in lymphocytes and granulocytes of smokers and non-smokers detected by ^{32}P-postlabelling assay. *Carcinogenesis*, **12**, 503–508.

SAVELA, K. AND HEMMINKI, K. (1993) Analysis of cigarette-smoke-induced DNA adducts by butanol extraction and nuclease P1-enhanced ^{32}P-postlabelling in human lymphocytes and granulocytes. *Environmental Health Perspectives*, **101** (Suppl. 3), 145–150.

SAVELA, K., HEMMINKI, K., HEWER, A., PHILLIPS, D. H., PUTMAN, K. L. AND RANDERATH, K. (1989) Interlaboratory comparison of ^{32}P-postlabelling assay for aromatic DNA adducts in white blood cells of iron foundry workers. *Mutation Research*, **224**, 485–492.

SCHOKET, B., HEWER, A., GROVER, P. L. AND PHILLIPS, D. H. (1988) Covalent binding of components of coal-tar, creosote and bitumen to the DNA of the skin and lungs of mice following topical application. *Carcinogenesis* (*London*), **9**, 1253–1258.

SCHOKET, B., PHILLIPS, D. H., HEWER, A. AND VINCZE, I. (1991) ^{32}P-postlabelling detection of aromatic DNA adducts in peripheral blood lymphocytes from aluminium production plant workers. *Mutation Research*, **260**, 89–98.

SCHULT, H. A. J. AND SHIVERICK, K. (1992) DNA adducts in humans as dosimeters of exposure to environmental, occupational, or dietary genotoxins. *FASEB Journal*, **6**, 2942–2951.

VAN SCHOOTEN, F. J., HILLEBRAND, M. J., VAN LEEUWIN, F. E., VAN ZANDWIJK, N., JANSEN, H. M., DEN ENGELSE, L. AND KRIEK, E. (1992) Polycyclic aromatic hydrocarbons–DNA adducts in white blood cells from lung cancer patients: no correlation with adduct levels in lung. *Carcinogenesis*, **13**, 987–993.

VAN SCHOOTEN, F. J., JONGENEELEN, F. J., HILLEBRAND, M. J. X., VAN LEEUWEN, F. E., DE LOOF, A. J. A., DIJKMANS, A. P. G., VAN ROOIJ, J. G. M., DEN ENGELSE, L. AND KRIEK, E. (1995) Polycyclic aromatic hydrocarbons DNA adducts in white blood cell DNA and 1-hydroxypyrene in the urine from aluminium workers: relation with job category and synergistic effect of smoking. *Cancer Epidemiology, Biomarkers & Prevention*, **4**, 69–77.

WATSON, W. P. (1987) Post-radiolabelling for detecting DNA damage. *Mutagenesis*, **2**, 319–331.

WESTON, A. AND BOWMAN, E. D. (1991) Fluorescence detection of benzo[*a*]pyrene–DNA adducts in human lung. *Carcinogenesis*, **12**, 1445–1449.

WIENCKE, J. K., KELSEY, K. T., VARKONYI, A., SEMEY, K., WAIN, J. C., MARK, E. AND CHRISTIANI, D. C. (1995) Correlation of DNA adducts in blood mononuclear cells with tobacco carcinogen–induced damage in human lung. *Cancer Research*, **55**, 4910–4914.

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