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SHORT COMMUNICATION

Capability of human blood cells to form the DNA adduct, C8-(*N*2-aminofluorenyl)–deoxyguanosine-3′-5′-diphosphate from 2-aminofluorene

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Human blood cells, separated by Ficoll–Hypaque centrifugation, were tested for their ability to catalyze the formation of DNA adducts of 2-aminofluorene (AF), using the ^{32}P-postlabeling procedure for adduct analysis. Incubation of neutrophils with AF, hydrogen peroxide and exogenous DNA yielded a single DNA adduct identified as C8-(*N*2-aminofluorenyl)–deoxyguanosine-3′-5′-diphosphate (AFdG) by cochromatography with a standard sample. AFdG levels in intact cells, lysed cells and in the granule fraction prepared from cell lysates were 102, 894 and 240 AFdG adducts/10^9 nucleotides/30 min respectively. AFdG levels corresponded to the activity of neutrophil peroxidase in these preparations. The monocyte/lymphocyte fraction yielded a low amount of 30 and 40 AFdG/10^9 nucleotides/30 min in the presence of hydrogen peroxide and of NADPH respectively. Erythrocytes did not generate a detectable level of AFdG, neither as intact cells nor as cell lysates. Whole blood samples likewise did not generate AFdG. Our findings reveal that, among blood cells, only neutrophils are capable of forming a biologically significant DNA adduct of aminofluorene in reasonable amounts and suggest that myeloperoxidase was the catalyzing enzyme.

Blood has long been regarded as a vehicle transporting foreign compounds to the sites of metabolism and excretion. Evidence has now accumulated that blood cells by themselves are capable of metabolizing certain drugs and chemicals. A classic example is the finding that hemoglobin in erythrocytes converts hepatic metabolites of aniline to yield methemoglobin and nitrosobenzene (1). Hemoglobin also metabolizes aniline and derivatives *in vitro* to the corresponding *N*- and *C*-oxides (2). Conversion of styrene to phenyl glycol by erythrocytes and hemoglobin was also reported (3,4). The meaning of these findings for the *in vivo* situation is still a matter of debate.

Other blood cells exhibit oxidizing enzyme activities and are also capable of metabolizing xenobiotics. For example, peripheral blood lymphocytes express low activities of benzo[*a*]pyrene (B[*a*]P*) monooxygenase (5,6). Lymphocytes generate DNA adducts from carcinogenic polycyclic aromatic hydrocarbons and aromatic amines (7) and convert styrene to styrene glycol (3). Myeloperoxidase of neutrophils is another well known oxidase activity (8). In neutrophils and in mononuclear cells this enzyme was found to oxidize the anti-inflammatory aniline derivative, dapsone, to yield the monohydroxylamine (9). Recently, a peroxidase-mediated DNA binding of arylamine carcinogens (10,11) and of a carcinogen metabolite of B[*a*]P (12) was detected in neutrophils stimulated with phorbol myristate acetate. DNA binding was assayed by determining radioactivity incorporated into DNA from the radiolabeled compounds, but the bound material was not identified.

The purpose of this study was to examine the capability of human erythrocytes, lymphocytes and neutrophils to form DNA adducts from the carcinogen, 2-aminofluorene (AF). AF is a substrate of cytochrome P450 monooxygenases (13) and of peroxidases (14) and is therefore a suitable compound to register the activity of those enzymes in blood cells. To examine DNA adduct formation the ^{32}P-postlabeling method (15,16) was used.

Blood samples were collected from healthy male and female volunteers by EDTA coated syringes. Samples of 7 ml were layered on 4 ml Ficoll–Hypaque separation medium (Flow Lab, Meckenheim, FRG) and centrifuged at 400 *g* for 30 min at room temperature. A layer containing lymphocytes and monocytes, a neutrophil fraction and a pellet of erythrocytes were separable by this procedure. The layer comprising neutrophils was withdrawn and washed with ice-cold physiological saline. Neutrophil lysates and the granular fraction, i.e. the supernatant of lysate centrifugations, were prepared according to Pember *et al.* (17).

To test for the involvement of peroxidase or cytochrome P450 in the formation of AF–DNA adducts, incubations were carried out either in the presence of hydrogen peroxide or of an NADPH regenerating system. After 30 min incubation at 37°C, the incubation mixtures were extracted with phenol–chloroform (18) and the DNA purified by the hydroxyapatite method (19). The DNA-containing fractions were precipitated with cetyl pyridinium bromide (20).

Incubation of freshly prepared neutrophils with AF, calf thymus DNA and hydrogen peroxide give rise to the formation of several spots in the radio TLC of the ^{32}P-labeled DNA hydrolysates (Figure 1a). One major spot 'A' and two minor spots termed 'B' and 'C' were detectable. Spot A was identified to be C8-(*N*2-aminofluorenyl)–deoxyguanosine-3′-5′-diphosphate (AFdG) by cochromatography with a reference sample prepared from *N*-2-fluorenyl-hydroxylamine and deoxyguanosine-3′-monophosphate (21). Spots B and C were also observable in control incubations performed in the absence of hydrogen peroxide and in the presence of catalase, with densities similar to experimental samples (Figure 1b). These spots were not detectable in control incubations performed in the absence of AF (Figure 1c). They probably indicate AF–DNA adducts generated by a non-enzymic oxidation reaction.

Formation of AFdG was determined in various neutrophil preparations known to express different levels of peroxidase activity. As shown in Table I, lysed cells exhibited a 4-fold increased peroxidase activity and a 9-fold increased level of AFdG, compared to intact cells. Peroxidase activity of the 'granular fraction' prepared from cell lysates was 50% higher than the activity determined for intact cells and showed a 2-fold higher level of AFdG. Control incubations performed in the

*Abbreviations: B[*a*]P, benzo[*a*]pyrene; AF, 2-aminofluorene; AFdG, C8-(*N*2-aminofluorenyl)–deoxyguanosine-3′-5′-diphosphate; N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; AAF, 2-acetylaminofluorene.

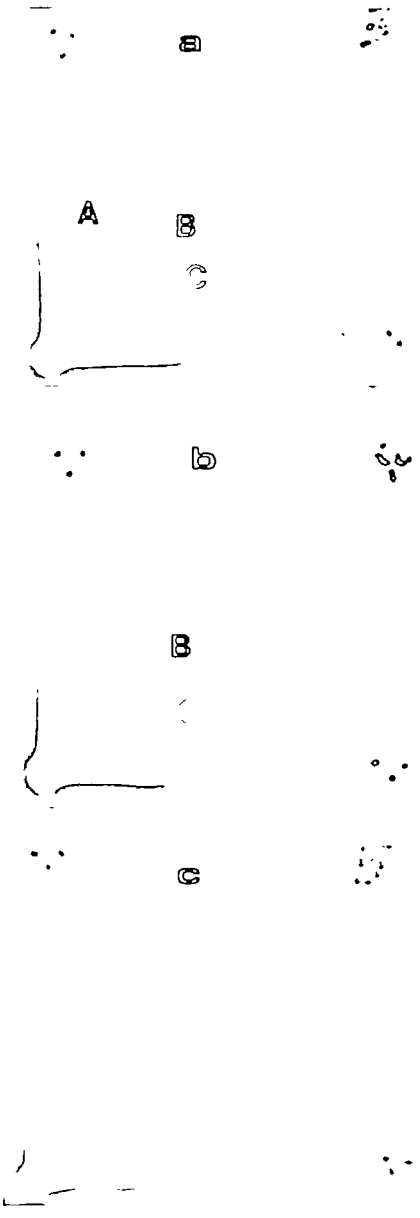

Fig. 1. Autoradiograms of TLC maps of ^{32}P-postlabeled digests of DNA isolated from incubations of intact neutrophils with AF and processed according to Gupta (2). After enzymatic hydrolysis of DNA (2 μg) the adducts were enriched by extraction with *n*-butanol. The dried extracts were 5′-end-labeled in the presence of carrier free [γ-^{32}P]ATP (150 μCi) and chromatographed by four directional PEI–cellulose chromatography. The solvents were: direction 1 (D1), 1.1 M lithium chloride; D2, 2.5 M ammonium formate, pH 3.5; D3, 3.5 M lithium formate, 8.5 M urea, pH 3.5; D4, 0.5 M Tris, 0.8 M lithium chloride, 8.5 M urea, pH 8.0. Small amounts of residual [^{32}P]phosphate were removed by reverse D3 chromatography with 1 M sodium phosphate, pH 6.8. Complete incubation mixture (for composition see Table I) (**a**), complete mixture minus hydrogen peroxide (**b**), complete mixture minus hydrogen peroxide minus AF (**c**).

absence of hydrogen peroxide and in the presence of catalase exhibited a low level of AFdG in intact cells and in cell lysates. These results suggest a catalytic function of myeloperoxidase in the formation of AFdG.

The monocyte/lymphocyte fraction was isolated from 10 ml blood, equivalent to ~25 × 10^6 cells, and was incubated in 2 ml 10 mM sodium phosphate buffer, pH 7.0, with 0.1 mM AF and 1 mg calf thymus DNA. Cofactors were either 0.2 mM

Table I. Formation of AFdG and peroxidase activity in neutrophil preparations

Preparation	AFdG/10^9 nucleotides/30 min		Peroxidase activity $\left[\frac{\text{guajacol units}^a}{10^6 \text{ cells}}\right]$
	Complete system (minus H_2O_2)	Complete system	
Intact cells	33 ± 17 (4)	102 ± 74 (6)	53 ± 11 (6)
Cell lysates	49 ± 47 (4)	894 ± 308 (6)	207 ± 63 (6)
'Granular fraction'	ND	240 ± 140 (3)	76 ± 16 (3)

Freshly prepared neutrophils, ~2 × 10^6 cells, were incubated for 30 min with calf thymus DNA and AF and the DNA processed as described in the legend to Figure 1. Cell lysates were prepared by addition of 100 μl of distilled water to the pellet of ~2 × 10^6 cells. The supernatant of a lysate centrifuged for 5 min at 500 *g* was taken as the 'granular fraction'.

About 2 × 10^6 neutrophils, equivalent to the cell number in 0.5 ml human blood, or cell lysate or the granular fraction prepared both from 2 × 10^6 neutrophils were incubated in 2 ml 30 mM sodium phosphate buffer, pH 7.0 containing 0.1 mM AF (Aldrich, FRG), 0.2 mM hydrogen peroxide, and 1 mg calf thymus DNA (Serva, FRG). Control incubations in the absence of hydrogen peroxide contained 1600 U catalase.

Data are means ± SD of individual blood samples the numbers of which are given in parentheses. Adduct frequencies are corrected for background radioactivity of control incubations carried out in the absence of cofactors and AF. Peroxidase activity of neutrophils was determined according to Hummelhoch *et al.*, using guajacol as substrate (22).

[a]mE/min at 470 nm.

ND, not determined.

hydrogen peroxide as a cosubstrate for the peroxidase of monocytes or NADPH to fortify the monooxygenase activity of lymphocytes. NADPH was generated by a system consisting of 5 mM NADP, 1 U/ml glucose-6-phosphate and 1 mM glucose-6-phosphate dehydrogenase respectively. Under both conditions, AFdG was detectable in the radiochromatograms. Quantitation exhibited 24 ± 12 and 35 (mean of 31 and 38) adducts/10^9 nucleotides/30 min formed in the presence of hydrogen peroxide and NADPH respectively.

Erythrocytes and whole blood samples were incubated in 50% Hank's medium. AF, DNA and the cofactors were used at the same concentrations as described for neutrophil incubations (see Table I). In incubation systems containing erythrocytes, AFdG was neither detectable in the absence nor the presence of hydrogen peroxide. This observation was made at cell numbers of 10^{10}–10^{11} cells/ml, a number by far exceeding the erythrocyte count in blood. Erythrocyte lysates similarly did not convert AF to AFdG. The same result was obtained when whole blood samples, 0.5 ml, were incubated either with hydrogen peroxide or with NADPH.

The present findings reveal that among various types of blood cells, neutrophils expressed the highest capacity to form the DNA adduct, AFdG, and suggest that myeloperoxidase was the activating enzyme. The highest activity was observed with neutrophil lysates in the presence of hydrogen peroxide. With respect to peroxidase dependent reactions, this experimental system corresponds to phorbol ester stimulated neutrophils undergoing an oxidative burst. Therefore, the radioactive material incorporated into DNA upon incubation of radiolabeled AF with phorbol ester stimulated neutrophils (10,11) probably contains AFdG as a major component. We cannot rule out the possibility that further DNA binding products are formed by peroxidase dependent reactions that are not recognized by the ^{32}P-post-labeling assay. However, the detection of AFdG implies that neutrophils form one of the biologically significant adducts of AF. Evidence for the significance of AFdG in the initiation step

of carcinogenesis is provided by the finding that the level of AFdG in mammalian cell lines correlated with mutagenic, cytotoxic and chromosome damaging properties of the AF metabolite, *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) (23,24). *In vivo* AFdG is the predominant DNA adduct of AF and its acetylated derivative, 2-acetylaminofluorene (AAF), persists in various tissues (25).

The question arises whether neutrophils or peroxidase containing eosinophils contribute to AFdG formation *in vivo*, in addition to the known activation pathway for AF via hepatic cytochrome P450 enzymes (21,25). Although there is no conclusive answer, the possibility should be considered that granulocytes, upon stimulation by endogenous compounds, may form AFdG and may release reactive AF metabolites forming AFdG in the surrounding tissue. It is also conceivable that AF is activated by intact cells, when hydrogen peroxide is present in the adjacent tissue. In this context, it is interesting to note that AFdG was detected in uterus DNA of AF-treated rats and that formation of this adduct appeared to be dependent on the activity of uterus peroxidase (26). Uterus peroxidase has been shown to originate, at least in part, from invaded eosinophils (27,28).

Compared to neutrophils, the lymphocyte/monocyte fraction did not form remarkable levels of AFdG, indicating a weak metabolic activity of these cells, compared to neutrophils. Similar results were obtained in long term incubations of primary cultures of peripheral lymphocytes with AF. Low levels of AFdG indicating a low metabolic activity of these cells were observed (7).

The finding that neither intact nor lysed erythrocytes generated any detectable amount of AFdG is somewhat surprising, in view of previous observations suggesting that oxyhemoglobin is capable of bioactivating chemicals. For example, styrene was reported to be metabolized by erythrocytes and oxyhemoglobin to styrene oxide, a mutagenic, carcinogenic and DNA binding metabolite (3,29). However, the metabolic rate for styrene oxide formation was low, even at substrate concentrations much higher than generally used for microsomal incubations (4). The same is true for hemoglobin-dependent *p*-hydroxylation and *N*-oxygenation of aniline derivatives (2,13). Therefore, we assume that the concentration of AF in our incubation system might have been too low to generate significant amounts of reactive AF metabolites. In the case that reactive AF metabolites were indeed formed by oxyhemoglobin, they may have been bound to hemoglobin or to other cellular proteins, before reaching the DNA. The fact that erythrocytes constitute >99% of whole blood cells, explains our finding that whole blood samples did not generate detectable levels of AFdG.

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