

Metabolism of 2-Aminofluorene by Human Polymorphonuclear Leukocytes: More Evidence for the Association between Inflammation and Cancer

by Vicki J. Isola,¹ Tahnee C. Hartman,¹ Scott J. Trumble,¹ Melanie C. Ruzek,¹ and James M. Gentile¹

Recent investigations have demonstrated the ability of leukocytes to metabolize promutagens or procarcinogens into their genotoxic forms. As a possible explanation for the association between inflammation and cancer, we and others have hypothesized that local accumulations of leukocytes could take up nearby promutagens, metabolize them, and release genotoxic agents that may cause damage in the surrounding tissue. Using a modified, two-step preincubation protocol with *Salmonella*, we have tested this hypothesis. We have shown that total human peripheral blood leukocytes, cultured in the presence of 2-aminofluorene for 18 hr, can metabolize 2-aminofluorene into agents mutagenic to *Salmonella typhimurium* strain TA98. Furthermore, experiments in which polymorphonuclear leukocytes were separated from mononuclear leukocytes demonstrated that the PMNs metabolized 2-aminofluorene to a much greater extent than the MNs.

Introduction

We are interested in the association between parasite infestation and cancer. It is well established that a high correlation exists between the occurrence of some parasite diseases and enhanced incidence of neoplasia (1,2). One hypothesis to explain this relationship is an altered metabolism in the parasitized host, which renders that host more susceptible to chemically induced carcinogenesis. Previously, we studied the metabolic potential of S9 preparations from the livers of *Fasciola hepatica* infected and noninfected mice and found that S9 from the infected livers had a greater ability to activate the promutagen aflatoxin B₁ into a mutagen detectable by the Ames assay than S9 from noninfected livers (3).

In addition to the infected organ(s), parasitized hosts differ from their noninfected counterparts by the presence of a chronic inflammatory state. Thus, the inflammatory cells, or leukocytes, could also contribute to the altered metabolic capability of an infected host. We and others (4,5) have hypothesized that local accumulations of leukocytes during inflammation could take up nearby promutagens, metabolize them, and release genotoxic agents

that may cause damage in the surrounding tissue. To study this possibility further, we wished to develop a system whereby we could test the overall ability of human leukocytes to activate a wide range of promutagens and release agents that could cause damage in a nearby cell.

Many investigators have used the generation of nucleic acid binding species as an indicator of metabolism of a xenobiotic by leukocytes (4,6-12), but only a few have analyzed for the ability of metabolites to cause genetic damage in cells other than the leukocytes themselves (4,5,13). In addition, these studies have only investigated xenobiotic metabolism in leukocytes stimulated to undergo respiratory burst by a tumor promoter. We are interested in the inherent ability of "resting" or nonstimulated human leukocytes to metabolize the promutagen 2-aminofluorene (2AF). It has previously been shown that stimulated human and guinea pig leukocytes and HL-60 cells can metabolize 2AF into nucleic acid binding species (6,7,12). In addition, purified leukocyte peroxidase (myeloperoxidase) can metabolize 2AF into nucleic acid binding species (7), and other peroxidaselike enzymes, such as prostaglandin endoperoxide synthetase, can metabolize 2AF into a *Salmonella* mutagen (14). Using a modified, two-step preincubation protocol with whole and separated peripheral blood leukocytes, we demonstrate in this paper that resting human leukocytes can also metabolize 2AF into a *Salmonella* mutagen.

¹Biology Department, Hope College, Holland, MI 49423.

Address reprint requests to J. M. Gentile, Biology Department, Hope College, Holland, MI 49423.

Materials and Methods

Chemicals

RPMI 1640, fetal calf serum, glutamine, and Mono-poly Resolving Media were obtained from Flow Laboratories (McLean, VA). 2-Aminofluorene was purchased from Aldrich (Milwaukee, WI). All other chemicals were obtained from Sigma Chemical (St. Louis, MO), and the microbiological media were obtained from Difco (Detroit, MI).

Bacterial Tester Strains

Salmonella typhimurium strain TA98 was provided by B. N. Ames, Berkeley, CA, and maintained as a frozen stock at -80°C as recommended by Maron and Ames (15). Confirmation of strain identity was made using the methods of Zeiger et al. (16). The range of spontaneous mutant colonies per plate for this strain in our laboratory was 20–60.

Isolation and Culture of Human Leukocytes

Heparinized human venous blood obtained from healthy donors was overlaid onto 6% dextran (molecular weight 100,000–200,000) at a 3:1 ratio (blood:dextran) and allowed to sit for 1 hr at room temperature. The top layer, containing the leukocytes, was removed and centrifuged at 330g for 10 min. The pellet was treated once with lysing buffer (0.15M NH_4Cl , 0.1 M Tris base, pH 7.2) to lyse any remaining erythrocytes. The pellet was washed twice with RPMI 1640, resuspended in complete media (RPMI 1640, 10% FCS, 1% glutamine), and seeded at 3–4 million cells/mL in a total volume of 4 mL complete media in 25 cm^2 tissue culture flasks. In some experiments, leukocytes were separated directly from blood on Mono-poly Resolving Media, according to the manufacturer's instructions. The mononuclear fraction and polymorphonuclear fraction were individually treated with lysing buffer, washed, then cultured in complete media as described above, at varying cell densities. Cell densities were determined by mixing an aliquot of culture with trypan blue and counting under a hemacytometer.

Preincubation Assay with Salmonella

Step 1. Appropriate dilutions of 2AF dissolved in dimethyl sulfoxide were added to the culture flasks in 25- μL aliquots resulting in final concentrations of 0–375 μM 2AF. Control flasks contained 2AF but no leukocytes. The culture flasks were then incubated vertically with loose caps at 37°C in a candle jar for 18 hr. Preliminary experiments showed the leukocytes remained viable in the presence of up to 375 μM 2AF for the duration of the preincubation period. The 18-hr incubation period was chosen based on the results of Gupta et al. (8), who found a maximum amount of DNA adducts in lymphocytes incubated with 2AF for 18 hr. Our own preliminary data (not shown) also demonstrated the greatest amount of mutagenic metabolites after incubating 2AF with leukocytes for 18 hr.

Step 2. After 18 hr, culture supernatants were obtained by centrifugation and transferred to scintillation vials. We added 0.2 mL of an overnight broth culture of *Salmonella typhimurium* strain TA98 per milliliter of supernatant, and the vials were placed in a shaking water bath for 3 hr. A typical incubation volume was 2.4 mL. We then added 0.6 mL of this mixture to 2 mL of top agar containing 0.05 mM histidine-biotin. The top agar was overlaid onto plates of Vogel-Bonner medium E, and plates were incubated for 48–72 hr at 37°C . The visible colonies (revertants) that formed were counted. Background plates contained 0.5 mL of complete media and 0.1 mL of overnight broth culture to correct for the added amount of histidine.

Each group of experiments was repeated a minimum of three times, and three plates were averaged for each data point. Because of the day-to-day variation of the white blood cells and volunteer-to-volunteer variation, individual experimental data could not be pooled. However, all of the trends remained the same in each experiment, and the specific results presented are representative of all the results obtained. This daily variation in metabolic capacity of cells from the same person as well as variation from person to person has been noted by other authors (8,12).

Results

To determine if human leukocytes could activate 2AF into an agent mutagenic to *Salmonella typhimurium* TA98 and release that agent into the surrounding media, leukocytes were incubated with increasing concentrations of 2AF for 18 hr, and the supernatants were examined for mutagenic activity in a typical Ames preincubation assay (Fig. 1). Under these conditions, the resulting supernatant was mutagenic to the bacteria in a dose-dependent manner. Supernatant from cultures containing leukocytes and increasing concentrations of 2AF up to 375 μM caused increasing numbers of revertants in the *Salmonella*. Supernatants from cultures of human leukocytes alone, 2AF alone, or 2AF incubated in the presence of boiled human leukocytes (data not shown) failed to induce mutations in the bacteria.

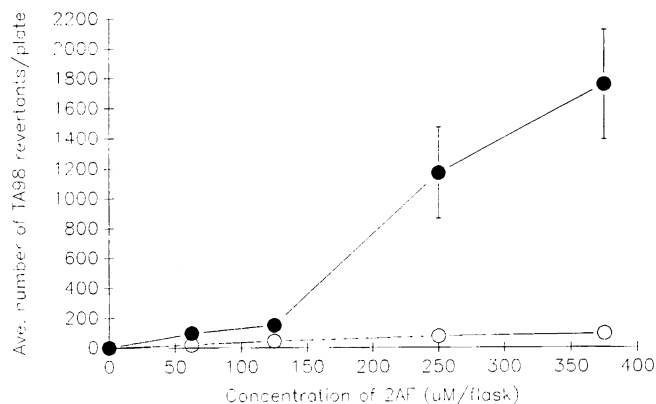


FIGURE 1. Activation of 2-aminofluorene incubated in the presence (●—●) and absence (○—○) of human leukocytes.

Because total peripheral blood leukocyte cultures contain two major groups of leukocytes, the polymorphonuclear leukocytes (PMNs) and the mononuclear leukocytes (MNs), we were interested in which group was responsible for the activation of 2AF. Therefore, we separated the leukocytes into mononuclear and polymorphonuclear fractions and tested their ability to activate 2AF individually compared to collectively (Fig. 2). PMNs did the most activation, similar to that performed by both PMNs and MNs together, whereas the MN fraction alone only slightly activated the 2AF.

We tested the effect of cell density on the activation of 2AF (Figs. 3, 4). Increasing concentrations of PMNs in the culture flasks from 0 to 1.2 million cells/mL produced an increasingly mutagenic supernatant. A second experi-

ment using PMN concentrations from 0 to 4.7 million cells/mL also showed increasing activation up to a density of between 1 and 2 million cells/mL, with the amount of activation leveling off beyond 2 million cells/mL. In both experiments, MNs showed only a slight activation of 2AF.

Discussion

Human leukocytes metabolize xenobiotics into agents that bind nucleic acid and cause genetic damage in various cell types. For example, leukocytes have been shown to activate *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BaP-7,8-diol) into agents that bind DNA (4,11), cause mutations in *Salmonella* (4), and induce sister chromatid exchanges in V79 cells (13). In addition, human leukocytes have been shown to activate 2AF into agents that bind to leukocyte DNA (7,12). In this paper we show that human leukocytes can also metabolize 2AF into agents mutagenic to *Salmonella*.

Polymorphonuclear leukocytes appear to do the majority of the metabolizing of 2AF in this system, although a small portion of activation is seen with mononuclear cells. This finding supports other data describing the ability of PMNs to bioactivate many different xenobiotics. Phorbol-ester-stimulated human and rodent PMNs have been shown to activate bleomycin (17), procainamide (18), diethylstilbestrol (19), eugenol (20), BaP-7,8-diol (4,11,13), and many arylamines (6,7,9,12). Phagocytic cells (neutrophils and monocytes) but not lymphocytes contain the enzyme myeloperoxidase. Previous workers have described the ability of peroxidase-like enzymes such as prostaglandin endoperoxide synthetase (PES), horseradish peroxidase (HRP), and myeloperoxidase (MPO) to activate 2AF. PES can co-metabolize 2AF into a *Salmonella* mutagen in the presence of arachidonic acid (14), PES and HRP can oxidize 2AF (21), and HRP and MPO can activate 2AF into a DNA binding species (6,7). If

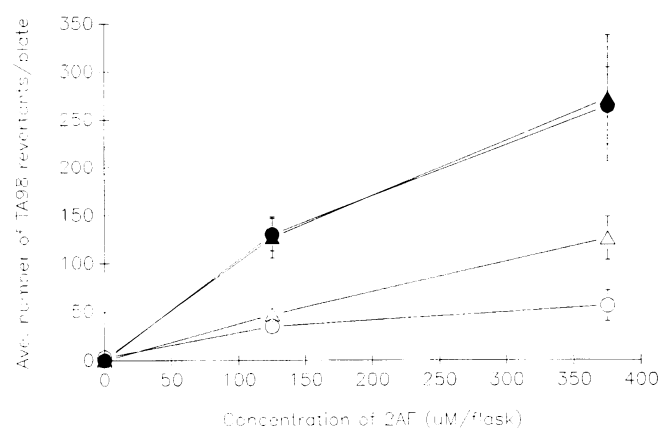


FIGURE 2. Activation of 2-aminofluorene in cultures containing no leukocytes (○—○), mononuclear leukocytes only (△—△), polymorphonuclear leukocytes only (▲—▲), and both polymorphonuclear leukocytes and mononuclear leukocytes (●—●).

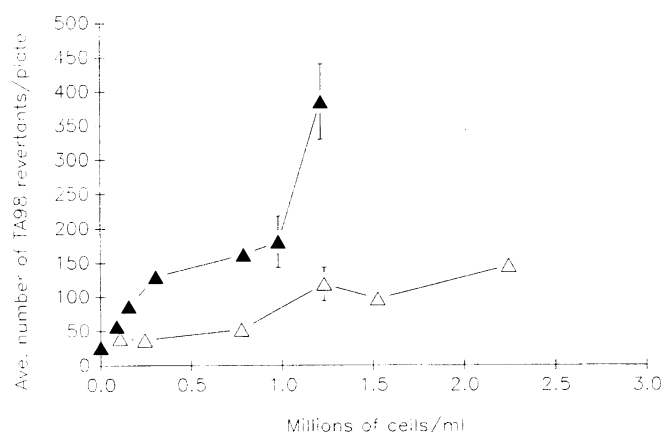


FIGURE 3. Activation of 2-aminofluorene by increasing concentrations of mononuclear leukocytes (MNs) (△—△) and polymorphonuclear leukocytes (PMNs) (▲—▲). MNs were incubated at 0.11, 0.24, 0.78, 1.23, 1.52, and 2.24 million cells/mL, and PMNs were incubated at 0.09, 0.16, 0.31, 0.79, 0.98, and 1.21 million cells/mL with 250 µM 2-aminofluorene.

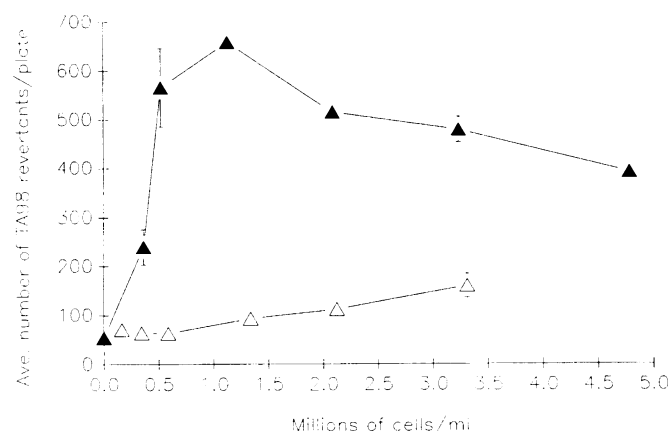


FIGURE 4. Activation of 2-aminofluorene by increasing concentrations of mononuclear leukocytes (MNs) (△—△) and polymorphonuclear leukocytes (PMNs) (▲—▲). To confirm and extend the results shown in Figure 3, the same procedure was done with higher cell densities. MNs were incubated at 0.16, 0.34, 0.58, 1.34, 2.12, 3.31 million cells/mL, and PMNs were incubated at 0.36, 0.52, 1.13, 2.09, 3.23, and 4.78 million cells/mL with 250 µM 2-aminofluorene.

myeloperoxidase is activating 2AF in this system, one would predict results similar to ours, i.e., that PMNs would show the greatest activation because most are neutrophils and the MNs would show a small amount of activation because most are lymphocytes and only some are monocytes. In a study of the oxidation of procainamide, MNs oxidized the chemical 10 times less than the PMNs (18), and in another study on the formation of DNA adducts by PMNs and MNs, the MNs showed 5 times less adduct formation than the PMNs (10). These results are consistent with the lower level of activation seen by the MNs in our system.

Initially, we supposed our system would reflect the uptake of 2AF by the leukocytes and the release of mutagenic metabolites into the surrounding media, which were detected with the Salmonella assay. Actually, it is unclear whether metabolism of 2AF is occurring inside the cells with the metabolites being released or whether the leukocytes are releasing agents that metabolize the 2AF outside of the cells because in either case one would detect the metabolites in the surrounding media. Preliminary experiments suggest the metabolism is occurring within cells (i.e., the cells need to be present during the incubation period because "conditioned media" [media incubated with cells for 18 hr] cannot activate 2AF [Isola, unpublished observations]). Other investigators have detected metabolites of 2AF bound to DNA found both outside (10) and inside (6-8,12) of the activating cell. Although, in these situations as well, metabolism could occur either outside, inside, or perhaps both outside and inside the cell (7).

It is important to note that the metabolism of 2AF seen in this system is being performed by "unstimulated" leukocytes, whereas the majority of evidence for 2AF metabolism in the literature comes from "stimulated" leukocytes only. Corbett and Corbett (7) found human PMNs activate 2AF into a nucleic acid binding species only in the presence of a phorbol ester (which stimulates respiratory burst), in up to a 1-hr incubation period. Similarly, Tsuruta et al. (6) found guinea pig PMNs activated 2AF into a DNA binding species only in the presence of phorbol ester during a 20-min incubation period. It is likely that the length of the incubation period (18 hr) in our system contributes to the detection of 2AF metabolites. This phenomenon was also seen by Gupta et al. (8) who saw maximal production of 2AF DNA adducts formed within mononuclear cell cultures incubated with 2AF for 18 hr. The mechanism of activation of 2AF by the leukocytes in this system is unknown. Both the reactive oxygen species produced by phagocytic cells during the respiratory burst and the enzyme myeloperoxidase have been implicated by others in the activation of 2AF (6,7,10,12) and many other xenobiotics (4,9,11,13,17-20). Further experimentation is needed to determine the mechanism of activation of 2AF in this system. Interestingly, Twerdok and Trush (22) compared the response of resting and phorbol-ester-stimulated bone marrow neutrophils to myeloperoxidase-dependent and superoxide-anion-dependent chemiluminescence and found both responses in stimulated neutrophils but only a superoxide anion response in resting cells. Superoxide anion may be involved in the activation of 2AF in this resting system.

In addition, the nature of the 2AF metabolites formed in this system is currently unknown. It is well documented that cytochrome P450 enzymes metabolize 2AF into N-hydroxy-2-aminofluorene (N-OH-2AF), which is considered the ultimate mutagenic metabolite. There are some reports of human leukocytes activating 2AF to produce the DNA adduct C8-(N2-aminofluorenyl)-deoxyguanosine-3'-5'-diphosphate (C8-dG-2AF), which is the only metabolite formed from the ultimate mutagen N-OH-2AF (8,10). In contrast, other investigations have revealed that some enzyme systems metabolize 2AF to DNA binding species other than that seen by N-OH-2AF (7,12,21). In addition, both C8-dG-2AF and other 2AF DNA adducts have been found *in vivo* (23). Perhaps the metabolites produced in this system are different from the expected N-OH-2AF because mutagenesis of the bacteria in our system is enhanced by an extended preincubation period (3 hr) with the unknown metabolites and incubation of 2AF with rat liver S9, which produces N-OH-2AF only requires a 30-min preincubation to be detected as mutations in Salmonella. Alternatively, this system could be producing a very small amount of N-OH-2AF whose detection is enhanced by the longer preincubation period.

We and others have hypothesized that the accumulation of PMNs at sites of infection could generate genotoxic metabolites if the individual is exposed to a promutagen, and these metabolites may affect the surrounding tissue in a way to initiate or promote tumor formation. The results presented in this paper further support this hypothesis. However, more research must be conducted if the association between inflammation and cancer is to be fully understood.

This work was supported by grants from the National Science Foundation (NSF-REU grants BB5-8712566 and DIR-9000984) and a 1990 Pew Midstates Science and Mathematics Consortium Faculty Development Award.

REFERENCES

1. Gentile, J. M. Schistosome-related cancers: a possible role for genotoxins. *Environ. Mutagen.* 7: 775-785 (1985).
2. Kramers, P. G. N., Gentile, J. M., Gryseels, B. J. A. M., Jordan, P., Katz, N., Mott, K. E., Mulvihill, J. J., Seed, J. L., and Froberg, H. Review of the genotoxicity and carcinogenicity of antischistosomal drugs; is there a case for a study of mutation epidemiology? Report of a task group on mutagenic antischistosomes. *Mutat. Res.* 257: 49-89 (1991).
3. Gentile, J. M., and DeRuiter, E. Promutagen activation in parasite-infected organisms: preliminary observations with *Fasciola hepatica*-infected mice and aflatoxin B₁. *Toxicol. Lett.* 8: 273-282 (1981).
4. Trush, M. A., Seed, J. L., and Kensler, T. W. Oxidant-dependent metabolic activation of polycyclic aromatic hydrocarbons by phorbol ester-stimulated human polymorphonuclear leukocytes: possible link between inflammation and cancer. *Proc. Natl. Acad. Sci. U.S.A.* 82: 5194-5198 (1985).
5. Isola, V. J., Trumble, S. J., Ruzek, M., and Gentile, J. M. Activation of 2-aminofluorene into a Salmonella mutagen by human leukocytes. *Environ. Mol. Mutagen.* 15(suppl. 17): 27-28 (1990).
6. Tsuruta, Y., Subrahmanyam, V. V., Marshall, W., and O'Brien, P. J. Peroxidase-mediated irreversible binding of arylamine carcinogens to DNA in intact polymorphonuclear leukocytes activated by a tumor promoter. *Chem.-Biol. Interact.* 53: 25-35 (1985).

7. Corbett, M. D., and Corbett, B. R. Nucleic acid binding of arylamines during the respiratory burst of human granulocytes. *Chem. Res. Toxicol.* 1: 356-363 (1988).
8. Gupta, R. C., Earley, K., and Sharma, S. Use of human peripheral blood lymphocytes to measure DNA binding capacity of chemical carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* 85: 3513-3517 (1988).
9. Corbett, M. D., Corbett, B. R., Hanothiaux, M.-H., and Quintana, S. J. Metabolic activation and nucleic acid binding of acetaminophen and related arylamine substrates by the respiratory burst of human granulocytes. *Chem. Res. Toxicol.* 2: 260-266 (1989).
10. Shen, J.-H., Wegneke, M., and Wolff, T. Capability of human blood cells to form the DNA adduct, C8-(N2-aminofluorenyl)-deoxyguanosine-3'-5'-diphosphate from 2-aminofluorene. *Carcinogenesis* 11: 1441-1444 (1990).
11. Trush, M. A., Twerdok, L. E., and Esterline, R. L. Comparison of oxidant activities and the activation of benzo(a)pyrene-7,8-dihydrodiol by polymorphonuclear leucocytes from human, rat and mouse. *Xenobiotica* 9: 925-932 (1990).
12. Corbett, M. D., Hanothiaux, M.-H., Corbett, B. R. and Quintana, S. J. A comparison of the HL-60 cell line and human granulocytes to effect the bioactivation of arylamines and related xenobiotics. The binding of 2-aminofluorene to nucleic acids as the result of the respiratory burst. *Chem.-Biol. Interact.* 78: 33-54 (1991).
13. Seed, J. L., Kensler, T. W., Elia, M., and Trush, M. A. Induction of sister-chromatid exchanges by polycyclic aromatic hydrocarbons following metabolic activation with phorbol ester-stimulated human polymorphonuclear leukocytes. *Res. Commun. Chem. Pathol. Pharmacol.* 67: 349-360 (1990).
14. Robertson, I. G. C., Sivarajah, K., Eling, T. E., and Zeiger, E. Activation of some aromatic amines to mutagenic products by prostaglandin endoperoxide synthetase. *Cancer Res.* 43: 476-480 (1983).
15. Maron, D. M., and Ames, B. N. Revised methods for the Salmonella mutagenicity test. *Mutat. Res.* 113: 173-215 (1983).
16. Zeiger, E., Pagano, D. A., and Robertson, I. G. C. A rapid and simple scheme for confirmation of Salmonella tester strain phenotype. *Environ. Mutagen.* 3: 205-209 (1981).
17. Trush, M. A. Activation of bleomycin A₂ to a DNA-damaging intermediate by phorbol ester-stimulated human polymorphonuclear leukocytes. *Toxicol. Lett.* 20: 297-302 (1984).
18. Uetrecht, J., Zahid, N., and Rubin, R. Metabolism of procainamide to a hydroxylamine by human neutrophils and mononuclear leukocytes. *Chem. Res. Toxicol.* 1: 74-78 (1988).
19. Eastmond, D. A., French, R. C., Ross, D., and Smith, M. T. Metabolic activation of diethylstilbestrol by stimulated human leukocytes. *Cancer Lett.* 35: 79-86 (1987).
20. Thompson, D., Constantin-Teodosiu, D., Norbeck, K., Svensson, B., and Moldeus, P. Metabolic activation of eugenol by myeloperoxidase and polymorphonuclear leukocytes. *Chem. Res. Toxicol.* 2: 186-192 (1989).
21. Boyd, J. A., and Eling, T. E. Evidence for a one-electron mechanism of 2-aminofluorene oxidation by prostaglandin H synthase and horseradish peroxidase. *J. Biol. Chem.* 259: 13885-13896 (1984).
22. Twerdok, L. E., and Trush, M. A. Neutrophil-derived oxidants as mediators of chemical activation in bone marrow. *Chem.-Biol. Interact.* 65: 261-273 (1988).
23. Krauss, R. S., Angerman-Stewart, J., and Eling, T. E. The formation of 2-aminofluorene-DNA adducts in vivo: evidence for peroxidase-mediated activation. *J. Biochem. Toxicol.* 4: 111-117 (1989).