DNA-Damaging Effects of Genotoxins in Mixture: Nonadditive Effects of Aflatoxin B₁ and N-Acetylaminofluorene on Their Mutagenicity in Salmonella typhimurium


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Received 16 April 1999; accepted 1 July 1999

Most animal genotoxicity studies have used exposures to single chemicals; humans, however, are potentially exposed to mixtures of genotoxins. Cancer and developmental toxicity risks associated with genotoxins in mixture are generally estimated by assuming additivity of the components. Two or more genotoxins acting sequentially or simultaneously may present a greater or lesser hazard than that predicted by simple addition of their potencies. Previously, we studied the effect of one genotoxin on the binding of a second genotoxin to DNA in an in vitro system and demonstrated that consecutive binding of the two toxins was not additive. In the present study, the effect of one genotoxin on the mutagenicity of another was evaluated for two well-known genotoxins using the Salmonella assay. Pretreatment of frameshift strains TA98 and TA1538 with AFB₁-8,9-epoxide (17.3 ng/plate) enhanced the mutagenicity induced by subsequent exposure to N-acetoxy-acetylaminofluorene (N-AcO-AAF) ~2–3 times above theoretical values for additivity. Pretreatment of base-substitution strain TA100 with N-AcO-AAF (0.1 μg/plate) inhibited the mutagenicity following subsequent exposure to AFB₁-8,9-epoxide by 3 times below the theoretical additive value. Concentration-response relationships for these enhancing or inhibitory effects were demonstrated using increasing concentrations of the first genotoxin during pretreatment. These results demonstrate effects, other than additive, of sequential exposures to two genotoxins on the induction of mutations in a bacterial system.

Key Words: aflatoxin B₁; aflatoxin B₁-8,9-epoxide (AFB₁-8,9-epoxide); genotoxins; mixtures; mutagenicity; N-acetylaminofluorene; N-acetoxy-acetylaminofluorene (N-AcO-AAF); Salmonella assay.

In order to reflect the real world exposure scenario, where humans are in contact with an array of chemically distinct genotoxins, it is important to study interactive, in addition to individual, effects. Previous studies have shown the inadequacy of making estimates of risk based on assumed additivity of individual chemical effects (Feron et al., 1995; Sexton et al., 1995; Smythe et al., 1969). Modulation of the DNA adduct-forming potential of one type of genotoxin by another is an example of toxicodynamic interactions by genotoxic mixtures. Sites of DNA damage by a genotoxin can be modulated by both the chemical nature of the genotoxin and by the sequence of nucleotides neighboring the target guanine (Said and Shank, 1991). Furthermore, formation of DNA adducts derived from one genotoxin class may alter the susceptibility of a second site to adduction by a second class of genotoxin. Our working hypothesis has been that preexisting DNA adducts in the genome can modulate the rate and site of formation of an additional adduct upon exposure to a second genotoxin. Recent work supports this expectation by demonstrating that exposure of DNA to multiple genotoxins leads to DNA damage that cannot always be predicted by assuming additive effects (Said et al., 1995; Said and Shank, 1991).

The biological relevance of these in vitro results and their potential impact on human risk assessment require further investigation. Although studies using naked DNA (Said et al., 1995; Said and Shank, 1991) or oligonucleotides (Ross et al., 1999) can address mechanisms by which genotoxic mixtures form DNA adducts, they were without the influence of normal DNA structure and repair. Results from the mutagenicity assay in Salmonella clearly demonstrate the biological relevance of these studies on genotoxic mixtures.

Several co-carcinogens have been found to enhance the mutagenicity of chemical carcinogens in the Salmonella assay. 2,4-Diaminotoluene, which is not a mutagen in the standard Ames assay, enhanced the mutagenicity of the potent mutagen, 2-aminofluorene, when both compounds were administered in combination (Pan and Reed, 1997). Cadmium chloride increased the mutagenicity of both N-methyl-N-nitrosourea and N-methyl-N'-nitro-N-nitrosoguanidine in a synergistic fashion up to 30-fold greater than expected from simple additivity (Mandel and Ryser, 1984). 3,3-Dichlorobenzidine enhanced the mutagenicity of di-tert-butylated hydroxytoluene, while the latter was found to inhibit the mutagenicity of N-AcO-AAF (Weng et al., 1997). Wang and co-workers (1991) showed that 1,3-indandione inhibited the mutagenicity of N-methyl-N-nitrosourea, benzo(a)pyrene and AFB₁. Inhibitory effects of

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the phenolic compound, ellagic acid, on AFB1, and benzo-
(a)pyrene mutagenicity has also been reported (Josephy et al.,
1990; Lourca-Pina et al., 1998). The hepatoprotective drug
YH509 has been shown to inhibit the mutagenicity of benzo-
(a)pyrene diol epoxide (BPDE) in Salmonella as well as reduce
DNA adduct yields derived from BPDE (Surh et al., 1996).

In the present investigation, the mutagenic effects of two
well known genotoxins were evaluated in Salmonella. AFB1,
and AAF were chosen for the study because a great deal is
already known about their interactions with DNA individually
but not in combination. AFB1-8,9-epoxide enhanced, in a con-
centration-dependent manner, the mutagenicity of N-AcO-
AAF in frameshift allele hisD3052 of Salmonella strains TA98
and TA1538, whereas N-AcO-AAF inhibited the mutagenicity
of AFB1-8,9-epoxide in the base substitution allele hisG46
(strain TA100) in a concentration-dependent manner.

MATERIALS AND METHODS

Bacterial strains and chemicals. N-acetoxy-acetylaminofluorene
(N-AcO-AAF) was obtained from Chemsys (Lenexa, KS) and aflatoxin B1,
was purchased from Sigma (St. Louis, MO). Stock solutions of these genotoxins
(37°C), followed by treatment with increasing amounts of
N-AFB 1-8,9-epoxide (17.3 ng/plate) was added to 0.1 ml of overnight culture of
strain TA100) in a concentration-dependent manner.

et al (a)pyrene mutagenicity has also been reported (Josephy
strain TA98 along with 0.5 ml of sodium phosphate buffer, pH 7.4, to give a final concentration of 0.6
μM of genotoxin. After incubation for 15 min (37°C), increasing amounts of
AFB1-8,9-epoxide (0, 8.6, 25.9, and 43.1 ng/plate) were subsequently added
and incubation continued for another 15 min. Following the dual genotoxin
treatments, samples were mixed with 2 ml of molten top agar and spread over
the surface of minimal agar plates. The number of background (spontaneous)-
revertant colonies were subtracted from the responses of the individual and con-
secutive genotoxin exposures.

Pretreatment of cells with increasing amounts of the first genotoxin
followed by a fixed amount of the second. A concentration-dependent en-
hancing effect of AFB1-8,9-epoxide on N-AcO-AAF-induced mutagenicity
was observed when increasing amounts of AFB1-8,9-epoxide (1.68–431 ng/
plate) were added to 0.1 ml of strain TA98 culture along with 0.5 ml of 0.2 M
sodium phosphate and incubation for 15 min (37°C), followed by addition of
N-AcO-AAF (0.25 μg/plate) and incubation for an additional 15 min. A
control experiment in which 0.25 μg/plate of N-AcO-AAF was used to treat
bacteria in the absence of added AFB1-8,9-epoxide was also performed.

A concentration-dependent inhibitory effect of N-AcO-AAF on AFB1-in-
duced mutagenicity was achieved when 0.1 ml of an overnight culture of strain
TA100 was mixed with 0.5 ml of 0.2 M sodium phosphate buffer and
pretreated with increasing amounts of N-AcO-AAF (0.1–1000 ng/plate) and
incubated for 15 min (37°C). Following this first treatment, AFB1-8,9-epoxide
(43 ng/plate) was added and incubation continued for an additional 15 min. A
color control experiment was also performed in which only 43 ng/plate of AFB1-
8,9-epoxide was used to treat bacteria in the absence of added N-AcO-AAF.
The mixtures were added to 2 ml of molten top agar and poured onto minimum
agar plates as described above.

RESULTS

Enhancing Effect of AFB1 on the Mutagenicity of N-AcO-
AAF in Frameshift Strain TA98

To determine the theoretical sum of revertants caused by
each individual component of the consecutive pairs, the num-number of revertants generated by the fixed amounts of AFB1-8,9-
epoxide alone was determined and added to the number of revertants generated by each concentration for N-AcO-AAF
alone. This theoretical additive value of revertants for the two

genotoxins in combination was then compared to the experi-
mental number of revertants generated by the combination.

Pretreatment of frameshift strain TA98 with a fixed amount of
AFB1-8,9-epoxide (17.3 ng/plate, Fig. 1A) enhanced the
mutagenic potency of N-AcO-AAF ~3-fold above the theoretical
additive effect. AFB1-8,9-epoxide also enhanced the mutagen-
icity of N-AcO-AAF in frameshift strain TA1538 (Δ uvrB,
-pKM101) ~2-fold above the theoretical additive effect (Fig.
1B). The number of revertants generated in this strain was
much lower than in strain TA98 (Δ uvrB, pKM101), con-
sistent with its lack of an inducible SOS repair response. The
existence of pKM101 in strain TA98 may explain the higher
number of revertants generated as compared to strain TA1538.
However, the enhancement of the mutagenic potency of
N-AcO-AAF by pretreatment of both strains with AFB1
appeared similar (~2–3-fold) regardless of Salmonella strain.
Plasmid pKM101 has been found to increase chemical muta-
genicity in both \textit{E. coli} and \textit{Salmonella} (McCann et al., 1975; Shanabruch and Walker, et al., 1980; Walker and Dobson, 1997). Figure 2 shows the concentration-dependence of AFB$_1$-8,9-epoxide on the mutagenic enhancement of N-AcO-AAF after pretreatment of bacteria with the indicated amounts of AFB$_1$-8,9-epoxide followed by 0.25 mg/plate N-AcO-AAF (the amount used to generate the greatest number of revertants on the N-AcO-AAF standard curve). Pretreating bacteria with increasing concentrations of AFB$_1$-8,9-epoxide alone (up to 431 ng/plate) did not produce significant numbers of revertants in frameshift strain TA98 as shown in Figure 2 (black columns) indicating that the enhancement was due to an increase in N-AcO-AAF mutagenic potency, not to additive effects of the genotoxins in combination.

\textbf{Effect of N-AcO-AAF on the Mutagenicity of AFB$_1$}

The mutagenicity of AFB$_1$-8,9-epoxide (Fig. 3) was inhibited when bacteria were pretreated with N-AcO-AAF (0.1 \mu g/plate). The degree of inhibition was 61.5\% for AFB$_1$-8,9-epoxide at the highest concentration used (43 ng/plate). Figure 4 describes the concentration-dependence of this inhibitory effect by varying the amounts of N-AcO-AAF (0.0001–1 \mu g/plate) used to pre-treat the bacteria followed by a fixed amount of AFB$_1$ (43 ng/plate). Figure 4A gives the total number of revertants formed following dual treatment (white columns). At a concentration level of 1 ng/plate N-AcO-AAF, the total number of revertants had decreased to 44\% of that induced by AFB$_1$-8,9-epoxide alone. In a separate experiment N-AcO-AAF in the higher amounts (0.001–1 \mu g/plate), particularly the highest concentration, produced by itself a concentration-response relationship in the base substitution strain TA100 (Fig. 4A, black columns). The number of revertants for each concentration of N-AcO-AAF alone was subtracted from the total revertants caused by the consecutive exposure to N-AcO-AAF and AFB$_1$-8,9-epoxide (Fig. 4A) to produce the net number of revertants due solely to AFB$_1$-8,9-epoxide (Fig. 4B). Therefore, the increases in total revertants found when

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Enhancing effect of AFB$_1$-8,9-epoxide on the mutagenicity (revertants per plate) of N-AcO-AAF in frameshift strains TA98 (A) and TA1538 (B) of \textit{Salmonella}. Shown in each graph are the concentration-response curves of N-AcO-AAF alone (\textcircled{□}), experimental values found after pretreatment of bacteria with a fixed amount of AFB$_1$-8,9-epoxide (17.3 ng/plate) followed by increasing amounts of N-AcO-AAF (\textcircled{A}), and the sum of revertants expected from the individual components of the binary combination (additive effect) (\textcircled{C}). All values were corrected for spontaneous revertants and are therefore considered net revertants. Error bars represent the SD of 3 plates per data point.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Concentration-dependent enhancement of AFB$_1$-8,9-epoxide on the mutagenicity (revertants per plate) of N-AcO-AAF in frameshift strain TA98 of \textit{Salmonella}. Bacteria were pretreated with the indicated amounts of AFB$_1$-8,9-epoxide followed by a fixed amount (0.25 \mu g/plate) of N-AcO-AAF (white columns). Revertants generated from treatment with AFB$_1$-8,9-epoxide alone are represented by the black columns. Each value represents the mean of 2 plates and are background corrected.}
\end{figure}
N-AcO-AAF levels increased from 10–1000 ng/plate (Fig. 4A) were due to the contribution of N-AcO-AAF-induced revertants in this strain. Revertants induced solely by AFB₁-8,9-epoxide had been maximally reduced following pretreatment with 1 ng N-AcO-AAF/plate to 40% of the amount generated in the absence of added N-AcO-AAF. N-AcO-AAF levels 10³ and 100³ greater than this amount did not cause a further inhibition. However, at 1 μg/plate, N-AcO-AAF appeared to completely inhibit AFB₁-8,9-epoxide induced mutagenicity.

**DISCUSSION**

Most mutagenicity studies of combinations of chemicals have involved interactions between a nonmutagen and mutagen that either enhances or inhibits the potency of the mutagen (Crebelli et al., 1991; Kawalek and Andrews, 1981; Ogawa et al., 1985, 1987; Whong et al., 1989; Yoshida et al., 1979). This study examined the mutual interactions of two well known mutagens, N-AcO-AAF and AFB₁-8,9-epoxide, in two Salmonella tester strains. The frameshift allele (hisD₃₀⁵₂; strain TA98) is sensitive to reversion by N-AcO-AAF while the base substitution allele (hisG₄⁶; strain TA100) is sensitive to AFB₁-8,9-epoxide. Therefore, base-substitution strain TA100 was selected to evaluate what effect pretreatment with N-AcO-AAF had on the mutagenicity of AFB₁-8,9-epoxide. Frameshift strain TA98 was selected to study the effect of pretreatment with AFB₁-8,9-epoxide on the mutagenicity of N-AcO-AAF.

Both N-AcO-AAF and AFB₁-8,9-epoxide are direct acting mutagens that do not require metabolic activation. The half life, of AFB₁-8,9-epoxide in an aqueous medium is approximately 1 second (Johnson and Guengerich, 1997); that of N-AcO-AAF is more than one h (Miller et al., 1966). In the case where AFB₁-8,9-epoxide is added to the cultures first, no active genotoxin remains after 15 min exposure, the time when the second genotoxin, N-AcO-AAF, is added. In the reverse case where N-AcO-AAF is added to the cultures first, some genotoxin may still be present when the second genotoxin is added. However, removing any unreacted first genotoxin remaining in the medium before adding the second did not have a significant effect on the magnitude of enhancement or inhibition. It is unlikely, therefore, that the modulation of mutagenicity seen in this study is due to reaction between AFB₁-8,9-epoxide and N-AcO-AAF.
exopoxide and N-AcO-AAF before either genotoxin could react with the bacterial DNA.

It was shown in this study that combinations of AFB1-8,9-epoxide and N-AcO-AAF, added sequentially at low concentrations (in either order), caused nonadditive mutagenic effects in Salmonella. Both enhanced (pretreatment of TA98 with AFB1-8,9-epoxide followed by N-AcO-AAF) or reduced (pretreatment of TA100 with N-AcO-AAF followed by AFB1-8,9-epoxide) mutagenic effects were observed.

Several factors may account for the concentration-dependent enhancing effect of AFB1-8,9-epoxide on the mutagenicity of N-AcO-AAF in strain TA98; one may be a strong SOS-inducing signal generated by AFB1-8,9-epoxide-derived DNA damage. The mutagenic potency of AFB1-8,9-epoxide in both E. coli and S. typhimurium is MucAB-dependent (Foster et al., 1988; Urios et al., 1994). MucAB induces primarily a –2 frameshift mutation in E. coli following treatment with N-AcO-AAF (Janel-Bintz et al., 1994). Thus, in the TA98 strain that contains plasmid pKM101, consecutive treatment with AFB1-8,9-epoxide and N-AcO-AAF may enhance either transcriptional or post-translational regulation of MucAB to a greater extent than that caused by either AFB1-8,9-epoxide or N-AcO-AAF treatment alone. Furthermore, pretreatment of bacteria with AFB1-8,9-epoxide may be stimulating SOS-responsive genes prior to treatment with N-AcO-AAF. It was shown previously that Lex A protein begins to be cleaved 1 min following exposure to ultraviolet radiation (Little, 1983), thereby initiating an SOS-response min following genotoxic insult. Another factor that could account for the enhancing effect might be an increased frequency of complex frameshift mutations as a result of consecutive exposure to AFB1-8,9-epoxide and N-AcO-AAF. The simultaneous presence of both Gua-N7-AFB1 adducts and Gua-C8-AAF adducts (not on the same purine molecule) in genomic DNA might cause alterations in DNA conformation that do not exist when the bacteria are treated with N-AcO-AAF alone. The nature of the conformational changes in DNA might initiate the formation of complex damage, thereby varying the mutational spectrum normally associated with the individual genotoxin. The importance of mutational type and specificity in the spectrum of mutations caused by the binary combination of AFB1-8,9-epoxide and N-AcO-AAF in the hisD3052 allele has yet to be determined.

In addition, the present study shows that AFB1-8,9-epoxide also appears to enhance the mutagenicity of N-AcO-AAF in strain TA1538 (which does not contain pKM101), although the mutagenic potency was ~4-fold less than observed in the pKM101-containing strain (TA98). One explanation for this result may be the existence of MucAB-independent pathways leading to –2 frameshift mutations at alternating CG sequences such as that previously observed in the AAF-inducible npf-pathway in E. coli (Maenhaut-Michel et al., 1992). The observation that N-AcO-AAF alone is mutagenic in strain TA1538 (Fig. 1) suggests the existence of a MucAB-independent mutagenic pathway. Alternatively, proteins encoded by UmuD/CST or SamAB genes, found either on the bacterial chromosome or 60-MDa cryptic plasmid in Salmonella (Woodgate and Sefwick, 1992), may be responsible for the mutagenic enhancement observed in the pKM101-deficient strain. Regardless of the mechanism, pretreatment of bacteria containing hisD3052 alleles with AFB1-8,9-epoxide enhances the putative frameshift mutagenicity of N-AcO-AAF by both MucAB-dependent and MucAB-independent pathways.

One possible mechanism to explain the concentration-dependent inhibitory effect of N-AcO-AAF on the mutagenicity of AFB1-8,9-epoxide in base-substitution strain TA100 could be that prior adduction of Salmonella genomic DNA by N-AcO-AAF reduced the number of binding sites available for reaction with AFB1-8,9-epoxide. Such interactions between N-AcO-AAF and AFB1-8,9-epoxide at the level of DNA covalent binding were investigated in experiments described by Ross and coworkers (1999). Briefly, the presence of Gua-C8-AAF adducts appeared to reduce the susceptibility of a second guanine to adduction by AFB1-8,9-epoxide in in vitro experiments. This inhibition was attributed to the initial AAF adduct because of steric interference and/or conformational alterations in the DNA polymer that precluded reaction. Conversely, it was found that initial modification of DNA by AFB1-8,9-epoxide did not alter subsequent binding of N-AcO-AAF.

Classes of genotoxins that interact with DNA by intercalation may have altered mutagenic responses in cells that contain preexisting adducts, which can severely distort normal DNA conformation. The results presented here suggest that consecutive exposure to genotoxins may not always give rise to additive mutagenic effects, particularly if the mixture contains different classes of genotoxins. Continuing work is investigating whether the nonadditive effects observed here are class-specific or compound-specific and are determining quantitatively the amount of each genotoxin binding to the DNA. Further, when significant nonadditive effects by combinations of genotoxins occur, the mutational spectra and SOS response derived from these mixtures will be determined.

ACKNOWLEDGMENTS

The authors thank Claudia Sofis and Elizabeth Valenzi from UC Bimolecular Minority Science Programs for their help with the mutagenicity assay. This work was supported by U.S. Environmental Protection Agency Grant No. R82580.

REFERENCES


