Aflatoxin B1 mutagenesis, DNA binding, and adduct formation in Salmonella typhimurium

(Chemical carcinogens/mycotoxins)


Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT Salmonella typhimurium strain TM677 was mutagenized with aflatoxin B1 (AFB1) in liquid suspension culture in the presence of a rat liver postmitochondrial supernatant. Forward mutation to 8-azaguanine resistance was measured in the treated cultures and was found to increase linearly with AFB1 concentration. DNA purified from mutagenized cells was analyzed for AFB1 adduct formation by high-pressure liquid chromatography after adduct liberation. AFB1 exposures at 0.16 and 0.32 μM for 35 min produced 15 and 22 AFB1-DNA adducts per genome, respectively, and induced 8-azaguanine-resistant fractions of 4.9 × 10^{-4} and 9.6 × 10^{-4}. Approximately 76% of the AFB1 bound to DNA was chromatographically identical to 2,3-dihydro-2-(N7-guanyl)-3-hydroxy aflatoxin B1, at the two AFB1 levels used.

Aflatoxin B1 (AFB1) is one of the most potent liver toxins and carcinogens known (1), and it is also an extraordinarily powerful mutagen in bacterial cells after mammalian metabolic activation (1). Activated AFB1 is capable of binding to macromolecular cell constituents, and, in animals, the extent of binding differs as a function of hormone and species (2), with the specific binding level generally decreasing in the order DNA, RNA, protein (2). Although the specific involvement of any macromolecule as the critical target for carcinogenicity or mutagenicity has not been proved, most hypotheses implicate DNA because of its central role as the cellular repository of genetic information.

Results from several laboratories have indicated the importance of an electrophilic epoxide of AFB1 as the principal metabolite responsible for DNA binding in vitro and in vivo (2–7). It has been suggested that the epoxide is the ultimately carcinogenic metabolite of AFB1 (2, 3), and, in all systems evaluated, the major DNA adduct is 2,3-dihydro-2-(N7-guanyl)-3-hydroxy aflatoxin B1 (AFB1-N7-Gua).

This laboratory is engaged in two related lines of investigation represented in this report on AFB1 mutagenesis in Salmonella typhimurium. Our program has the dual objectives of (i) characterization and quantitation of specific molecular lesions that result when carcinogenic and mutagenic chemicals are metabolized in the presence of nucleic acids in vitro and in vivo (5, 6) and (ii) relating these molecular alterations to findings in quantitative mutagenic assays that accurately reflect the mutagenic potency of chemical compounds. The present project had the specific purpose of determining the quantitative relationship between the binding of AFB1 to Salmonella DNA, in the form of specific adducts and observed mutation to 8-azaguanine resistance (8AG8).

MATERIALS AND METHODS

Bacterial Strains. S. typhimurium strain TM677 used in this study, is a histidine-prototrophic revertant of S. typhimurium strain TA1535 into which the R factor plasmid, pKM101, was inserted (8). Bacteria were grown in Vogel–Bonner medium (9) to a cell density of approximately 85 Klett units (660-nm filter) and frozen at -80°C in 50-ml aliquots containing 10% (vol/vol) dimethyl sulfoxide.

Preparation of Cell Suspension. Fifty milliliters of frozen stock cells was thawed and diluted into 150 ml of minimal medium containing glucose (20 mg/ml), Casamino acids (2.0 mg/ml), biotin (2.24 μg/ml), and ampicillin (30 μg/ml). Cultures were grown by shaking at 130–150 rpm in four 500-ml flasks at 37°C for 250–280 min until there was an increase of 250 Klett units (660-nm filter). At that point, the cultures were immediately added to the mutagenesis reaction mixtures.

Mutagenesis Assay. The procedure used was a modification of the 8AG8 assay in S. typhimurium developed by Skopek et al. (8). The reaction mixtures for mutagenesis contained 83 mM Hepes (pH 7.4), 27.5 mM KCl, 6.4 mM MgCl₂, 1.2 mM NADP, 4.1 mM glucose 6-phosphate, 100 mM glucose, 85 μM of rat liver 9000 × g supernatant per ml [prepared from sodium phenobarbital-treated rats (10)], 250 μl of cell suspension per ml, and [3H]AFB1 [10 Ci/mmol (1 Ci = 3.7 × 10¹² becquerels)] delivered to the reaction mixture in methanol (final methanol concentration, 0.3%).

The mutagenesis procedure was started by the addition of [3H]AFB1 solution to the reaction mixture, both freshly prepared. The mixtures were shaken in the dark at 70–80 rpm at 37°C for 35 min. At that point, samples were withdrawn from each flask for the assay of induction of 8AG8 mutants and for counts of viable cells. The plating procedure was similar to the one described (8).

These reaction conditions for assaying mutation to 8AG8 differ from those described (8). The 8AG8 forward mutation assay in Salmonella as originally described involved small reaction volumes (up to 5 ml) and low cell concentrations (2.5 × 10⁹ to 1 × 10⁸ cells per ml). With a DNA content of 11 fg per Salmonella cell (11), a typical 5-ml reaction mixture with 10⁸ cells per ml would contain about 550 ng of DNA. To provide sufficient quantities of AFB1-bound DNA for adduct isolation, mutagenesis was carried out in six 62.5-ml portions at 0.16 μM AFB1, and in four 62.5-ml portions at 0.32 μM AFB1 in 500-ml flasks at a cell concentration of 7 × 10⁹/ml. Experiments showed that this increase in cell concentration and reaction mixture volume did not affect the efficiency of mutagenesis.

Abbreviations: AFB1, aflatoxin B1; AFB1-N7-Gua, 2,3-dihydro-2-(N7-guanyl)-3-hydroxy aflatoxin B1; 8AG8, 8-azaguanine resistance (resistant).

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or cell viability significantly. Reconstruction experiments similar to those described by Skopek et al. (8) were performed under the new assay conditions to exclude the possibility of experimental bias.

Analysis of AFB\textsubscript{1} Binding. Mutagenized cultures treated as described above were chilled to 0°C and centrifuged in the cold at 5000 × g for 10 min. Supernatant fluids and cell pellets were assayed for radioactivity.

Extraction of DNA was carried out by a procedure that incorporated the lysis method of Ebel-Tsipis et al. (12) and the extraction method of Marmur (13). All procedures were carried out in the dark or in tungsten light to minimize AFB\textsubscript{1} degrada
dation. Cell pellets were washed three times with 100 mM Tris, pH 6.8/100 mM EDTA/150 mM NaCl and were resuspended in 8.0 ml of the same buffer. Heat-treated RNase (80°C, 10 min) was added at 100 μg/ml and the mixture was shaken for 5 min at 37°C. Lysozyme was added at 1.0 mg/ml and the mixture was incubated for 15 min at 37°C. Sarkosyl N1-30 was added at 1.5% (vol/vol) and the mixture was shaken at 37°C for 60 min. The next addition was heat-treated Pronase (80°C, 10 min) at 2 mg/ml, and incubation was continued for 2.5 hr with shaking at 100–120 rpm at 37°C. The mixture was then brought to 1.3 M NaCl and shaken for 5 min; an equal volume of chloroform/isooamyl alcohol, 24:1 (vol/vol), was added. Shaking at 37°C was continued for at least 20 min followed by centrifugation at 8000 × g at room temperature for 10 min. The deproteinization step was repeated three more times; the DNA was then resuspended from 3 vol of ethanol at room temperature and dissolved in 2.5 ml of 15 mM NaCl/1.5 mM sodium citrate, pH 7.0. DNA, RNA, and protein were assayed as described (14–16).

Storage of DNA solutions was at 4°C.

Analysis of the AFB\textsubscript{1}–DNA Adduct. The pH of the DNA solutions was brought to 5.3 with 0.1 M potassium acetate (pH 5.0). The solutions were then heated at 100°C for 15 min, cooled rapidly in ice, and incubated with nuclease P1 (Yamasa Shoyu Co., Choshi, Japan) (approximately 10 μg/mg of DNA) at 37°C overnight. Before liquid chromatography, the solutions were deproteinized by shaking with an equal volume of CHCl\textsubscript{3}.

Portions of solutions representing 50–100 μg of DNA were analyzed by analytical reversed-phase high-pressure liquid chromatography. Nonradioactive AFB\textsubscript{1}–N\textsuperscript{7}-Gua adduct, that had been purified from DNA modified in vitro by AFB\textsubscript{1} by using rat liver microsomes, was co-injected as a retention time marker. The system consisted of a Micromeritics model 7000 liquid chromatograph equipped with a model 730 universal injector (Micromeritics Corp.) and a Waters model 440 detector which monitored UV absorbance at 254 and 365 nm (Waters Associates). After injection of a DNA hydrolysate, the μBondapak C\textsubscript{18} column (Waters Associates) was eluted isocratically with 10% methanol for 10 min, followed by a linear gradient to 80% methanol over 40 min. The column flow rate was 1 ml/min, and temperature was 60°C. Thirty-drop fractions of the detector effluent were collected and assayed by liquid scintillation counting.

A similar portion of each hydrolysate was analyzed under isocratic conditions which have been shown to provide for improved resolution of 2,3-dihydro-2,3-dihydroxyaflatoxin B\textsubscript{1} (AFB\textsubscript{1}–dihydrodiol) from AFB\textsubscript{1}–N\textsuperscript{7}-Gua (7). For this analysis, the column was eluted with 25% ethanol containing 0.001% acetic acid at 1 ml/min.

RESULTS

AFB\textsubscript{1} Mutagenesis. A linear relationship was obtained between the induced SAC\textsuperscript{R} fraction (observed fraction – control fraction) and the concentration of AFB\textsubscript{1} (Fig. 1). Virtually all cells survived the mutagenesis procedure even at the highest aflatoxin concentrations. The efficiency of mutagenesis was high; approximately 0.1% of all cells became SAC\textsuperscript{R} at 0.32 μM AFB\textsubscript{1}.

FIG. 1. SAC\textsuperscript{R} mutant induction in strain TM677 by AFB\textsubscript{1}. Unless specified, experimental conditions were as described in the text. ○, Reaction mixture at 0.16 μM AFB\textsubscript{1} consisted of six 500-ml flasks, each containing 62.5 ml of mixture; reaction at 0.32 μM AFB\textsubscript{1} consisted of four such flasks. One flask contained all components of the reaction mixture except AFB\textsubscript{1}; ○, AFB\textsubscript{1} mutagenesis in small volumes (2.5 ml) of reaction mixtures and at low cell concentration (1.7 × 10\textsuperscript{9}/ml). Percentage viability is expressed relative to control cultures without AFB\textsubscript{1}. Spontaneous SAC\textsuperscript{R} mutant frequency was 2.5 × 10\textsuperscript{-5} at the low cell concentration and 4.2 × 10\textsuperscript{-5} at high cell density.

Uptake of [\textsuperscript{3}H]AFB\textsubscript{1} by Salmonella Cells. Analysis of the cell pellets after several washing steps following mutagenesis revealed that 0.87% of AFB\textsubscript{1} radioactivity was associated with the cells. The AFB\textsubscript{1} concentration within the cell was about 13 times higher than the external concentration, an apparent accumulation of AFB\textsubscript{1} against a concentration gradient (Table 1). This phenomenon may occur by diffusion of metabolically activated AFB\textsubscript{1} into the cells followed by rapid binding to various cell constituents, thus maintaining a steep concentration gradient for the activated form. It is also possible, however, that at least part of this apparent binding was to residual microsomal constituents that may have remained attached to cell walls through the cell washing procedure. The existence of an active transport system specific for AFB\textsubscript{1} in S. typhimurium seems unlikely; it has recently been proposed, however, that many antibiotics and inhibitory analogs of the normal bacterial carbon energy sources are taken up by active transport systems normally used for the uptake of carbon energy sources in S. typhimurium (18).

Level and Nature of AFB\textsubscript{1}–DNA Adduct. In order to maintain the integrity of the AFB\textsubscript{1}–DNA adduct, all steps of the DNA isolation and purification procedures originally de-
Based on the specific cell volume of 1 x 10^{-12} cm^3 (17).}

For the isolation procedure was carried out in tungsten light or in the dark. However, the isolation procedure was carried out in tungsten light or in the dark. Also, the isolation procedure was carried out in tungsten light or in the dark. The efficiency of DNA extraction was 80-90%. (Total DNA content of the cultures was determined in samples that were lysed and treated as described above except that DNA was not isolated from them. They were deproteinized by treatment with 0.5 M HClO_4, and DNA was assayed by UV absorbance at 260 nm and by the diphenylamine color reaction.)

Because AFBI-DNA adducted DNA had not been isolated previously from bacteria, it was necessary to determine the stability of the [3H]AFB1-DNA adduct during the various steps of cell lysis and DNA isolation. Heavily adducted calf thymus DNA (1 [3H]AFB1 residue per 200 nucleotides) was subjected to the entire procedure of cell lysis by Sarkosyl, lysis, DNase, and Pronase treatment and DNA purification, and 84% of the original specific radioactivity was retained. We assume that DNA modified to a lesser extent would retain at least the same fraction of AFBI residues bound to DNA as observed here.

Table 1. Accumulation of [3H]AFB1 within cells of S. typhimurium strain TM677*

<table>
<thead>
<tr>
<th>Reaction 1</th>
<th>Reaction 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.16 μM AFB1)</td>
<td>(0.32 μM AFB1)</td>
</tr>
<tr>
<td><strong>Total cell number</strong></td>
<td><strong>Total cell volume, ml</strong></td>
</tr>
<tr>
<td>2.56 x 10^{11}</td>
<td>1.69 x 10^{11}</td>
</tr>
<tr>
<td>0.256</td>
<td>0.169</td>
</tr>
<tr>
<td><strong>Total [3H]AFB1 in cell pellet, dpm</strong></td>
<td><strong>Internal concentration: [3H]AFB1 dpm/ml packed cells</strong></td>
</tr>
<tr>
<td>1.41 x 10^{7}</td>
<td>1.68 x 10^{7}</td>
</tr>
<tr>
<td>5.50 x 10^{7}</td>
<td>9.90 x 10^{7}</td>
</tr>
<tr>
<td><strong>External concentration: [3H]AFB1 dpm/ml supernatant</strong></td>
<td><strong>Concentration factor (internal/external)</strong></td>
</tr>
<tr>
<td>4.31 x 10^{6}</td>
<td>7.72 x 10^{6}</td>
</tr>
<tr>
<td>12.7</td>
<td>12.8</td>
</tr>
</tbody>
</table>

* The numbers shown are based on data from Fig. 1 and Table 2.

† Based on specific cell volume of 1 x 10^{-12} cm^3 (17).

The principal aflatoxin-DNA adduct identified in the Salmonella DNA was AFB1-N7-Gua. Seventy percent of the radioactive DNA incorporated into each DNA sample eluted at the retention time of this adduct under two sets of chromatographic conditions (Fig. 2). The remaining radioactivity (peak II in Fig. 2), representing approximately 30% of the AFB1 bound to DNA, remains unidentified, although it is possible that this peak contains polar AFB1-deoxyribonucleotide derivatives (20). Neither the aflatoxin dihydrodiol nor the product tentatively identified (7) as the imidazole ring-opened derivative of the AFB1-N7-guanine adduct (peak II in Fig. 2) were observed in significant quantities. In our hands, the dihydrodiol is produced mainly by acidic treatment of AFB1-RNA or of AFB1-DNA that has been exposed previously to an alkaline pH [i.e., the dihydrodiol may be a facile hydrolysis product of the putative imidazole ring-opening product, 2,3-dihydro-2-(N^5-formyl-2',5',6'-tri amino-4-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B1].

**DISCUSSION**

No single line of investigation has conclusively established a cause-effect relationship between DNA adduct formation by AFB1 and mutagenesis or carcinogenesis. The research reported herein is supportive of this hypothesis, however, in view of the observed association between DNA binding levels and induction of mutation to 8AG in S. typhimurium. The present results extend previous observations in animals that indicated that the...
total extent of DNA modification by AFB\textsubscript{1} seems to parallel species or organ susceptibility to tumor formation (2, 4, 5). A noteworthy similarity between the present results and those produced previously in vitro (5, 7, 20) and in vivo (6, 7) is the fact that the major adduct between AFB\textsubscript{1} and Salmonella DNA was AFB\textsubscript{1}-N\textsubscript{7}-Gua. Our earlier findings showed that this product resulted from reaction of the electron-deficient C-2 of the β- (or exo-) epoxide of AFB\textsubscript{1} with the nucleophilic N-7 atom of guanine, which resides in a sterically accessible position in the major groove of DNA (5).

Assuming that covalently bound AFB\textsubscript{1} somehow initiates the mutagenic process, our present data may provide a first approximation of the efficiency by which covalently bound AFB\textsubscript{1} induces mutation in Salmonella. The accuracy of this estimate is dependent upon two factors which are described in detail below.

The first factor relates to our estimation of the fraction of induced mutations detected in a forward mutation assay. AFB\textsubscript{1} induces both base-pair substitution mutation and frameshift mutation in strains of Salmonella carrying the B-factor plasmid pKM101 (21, 22). Under the conditions used in our studies, approximately 9-fold more revertants are induced in the base-pair substitution strain TA100 than in the frameshift strain TA98 on the Ames His\textsuperscript{+} reversion assay (21). Although one might conclude from these data that AFB\textsubscript{1} induces 9-fold more base-pair substitution mutations than frameshifts, it must be kept in mind that the efficiency with which AFB\textsubscript{1} reacts with the specific DNA sequences in TA98 and TA100 to cause reversion may not be representative of the efficiency with which it reacts with other DNA sequences. For the arguments presented below, it is sufficient to conclude that AFB\textsubscript{1} is capable of inducing both base-pair substitution and frameshift mutations in TM677, although the relative frequency with which they occur is not known.

From a theoretical point of view, it would not be expected that all base-pair substitution mutations induced by AFB\textsubscript{1} would be observed as a phenotypic change in a forward mutation assay. Due to the degeneracy of the genetic code, it can be calculated that approximately 25% of all base-pair substitution mutations will not lead to an altered amino acid sequence in a protein (i.e., most mutations in the third base of a codon and many in the second base will not change the amino acid encoded by the original codon). Of the remaining base-pair substitution mutations that do alter amino acid sequence, some fraction of these will insert an incorrect amino acid and still give rise to a functional protein. This phenomenon has been observed by Yanofsky et al. (23) in the trpA gene in Escherichia coli. Because at present there is no way to estimate this fraction of cryptic mutations, it is assumed for the sake of argument that any mutation that inserts an incorrect amino acid of the same charge as the original may result in a functional or partially functional protein and, therefore, may not be phenotypically expressed. Considering all of the possible single-step mutations in the genetic code, it can be calculated that this class constitutes approximately 40% of the theoretically possible number of base-pair substitution mutations.

With the above theoretical points taken into consideration, it is reasonable to expect that as few as 35% of all base-pair substitution mutations induced by AFB\textsubscript{1} would be observed as a phenotypic change in a forward mutation assay (25% of the base-substitution mutations may result in no amino acid change and 40% may insert an amino acid that still results in a functional protein). We assume that most frameshift mutations induced by AFB\textsubscript{1} will be expressed in a forward mutation assay because these lesions inactivate large portions of the gene in which they occur. However, it could be postulated that some frameshifs occurring near the terminus of the gene may still allow the production of a functional gene product. Also, additions and deletions of nucleotides in multiples of three will add or delete amino acids in a protein but will leave the reading frame in mRNA intact. This type of "frameshift" mutation may also give rise to a functional protein and go undetected in a forward mutation assay.

A second important point to be addressed in attempting to relate DNA binding to mutation is the estimation of the size of the genetic target of this mutation assay. Although not all of the mutant phenotypes conferring 8AG\textsuperscript{R} are known at present, it is known (24, 25) that one mechanism for this mutation in S. typhimurium involves loss of the enzyme xanthine phosphoribosyltransferase (XPRT; EC 2.4.2.22). Only about 15% of the 8AG\textsuperscript{R} mutants induced are XPRT\textsuperscript{-} (unpublished results). On the basis of this information, it was assumed that the structural gene for XPRT represented 15% of the complete genetic target for 8AG\textsuperscript{R}. Elution data for XPRT are consistent with a molecular weight of approximately 30,000, and a protein of this size would require a DNA sequence of about 500 nucleotide pairs. Therefore, the size of the complete genetic target for 8AG\textsuperscript{R} is estimated as 5300 nucleotide pairs. This sequence of nucleotides would account for approximately 1.2 \times 10^{-3} of the Salmonella genome, assuming a total size of 4.5 \times 10^{9} nucleotide pairs per genome (19).

Preliminary results have shown that some of the mutants produced appear to overproduce purine bases, possibly as a consequence of deregulation of purine biosynthesis. These data would appear to support the suggestion that mutation at loci other than XPRT could also lead to the 8AG\textsuperscript{R} trait (unpublished results).

AFB\textsubscript{1} treatments at 0.16 and 0.32 \mu M for 35 min produced 15 and 22 adducts per genome, respectively. This means that 18 \times 10^{-3} \text{[}15(1.2 \times 10^{-3})\text{]} adducts would be expected to fall by chance in the target for 8AG\textsuperscript{R} at the 0.16 \mu M treatment and 26 \times 10^{-3} adducts for the 0.32 \mu M treatment. When these levels are compared to the induced 8AG\textsuperscript{R} fractions for the low and high AFB\textsubscript{1} treatments (0.49 \times 10^{-3} and 0.96 \times 10^{-3}, respectively), the data suggest that 27–37 adducts gave rise to one observed mutation. Thus, any chemical adduct present in the gene would have a probability of 1/27 to 1/37 of giving rise to an observed mutational event. The actual efficiency of adducts as mutagenic lesions probably is somewhat higher because the arguments developed earlier suggested that the observed mutant fraction induced by AFB\textsubscript{1} may have given substantial underestimates of the total number of mutations induced. It should be kept in mind that this calculated number of adducts per mutation is based on an estimated DNA target size that could vary from the actual size by a factor of 2 or 3. In the absence of information on exact target size, we consider that a 2- or 3-fold range in the number of adducts per mutation gives a reasonable estimate of mutagenic efficiency of DNA binding by AFB\textsubscript{1}.

The apparent excess number of total adducts relative to the number of mutations induced is not surprising. This excess could result from a low mutation efficiency of some or all AFB\textsubscript{1} adducts or from the presence of a DNA repair process competing with the fixation of premutagenic lesions. The possibility also exists that quantitatively minor adducts, representing a small percentage of the total binding, are actually the precursors to mutation and act through a more highly efficient mechanism of mutation. The mutations induced could also be the result of an error-prone repair system acting on the adducted DNA.

The possibility cannot be ruled out that the mutagenic activity of AFB\textsubscript{1} in Salmonella results from interactions with macromolecules other than DNA, especially in view of the fact...
that DNA contained only 2% of the total covalently bound AFB$_1$. The potential functional significance of non-DNA binding is unknown at present, although AFB$_1$ bound to specific proteins, such as DNA polymerases, could conceivably result in infidelity in DNA replication which ultimately might lead to mutagenesis.

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