Metabolism of Aflatoxin B₁ in the Upper Airways of the Rabbit: Role of the Nonciliated Tracheal Epithelial Cell

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ABSTRACT

Short-term tracheal explant cultures from the rabbit were used to study the metabolism of the carcinogen aflatoxin B₁ (AFB₁) and to determine the cell types that are susceptible to damage by AFB₁ and their relative contents of monooxygenase enzymes. Tracheas were cultured in serum-free medium for 0.5-24 h with 0.7 μM [³⁵C]AFB₁, and metabolism was measured by determining the level of binding of the carcinogen to DNA and by the release of metabolites into the medium. The binding of aflatoxin B₁ was time dependent and appeared to peak at 12 h in culture. In addition, the metabolites aflatoxicol, aflatoxin M₁, and aflatoxin Q₁ were produced by the explants. Ultrastructural examination of cultured tracheal explants showed degenerative changes exclusively in nonciliated secretory cells after 4 h in culture. Extensive nonciliated secretory cell necrosis was evident by 12 h. Ciliated cells did not show degenerative changes until 12 h and appeared more viable after 24-h exposure to AFB₁ relative to the nonciliated cells. Tracheal sections stained to demonstrate rabbit lung cytochrome P-450, Forms 2 and 5, and cytochrome P-450 reduced nicotinamide adenine dinucleotide phosphate reductase by an immunoperoxidase technique showed intense staining selectively within nonciliated cells. In total, the data revealed that: (a) rabbit tracheal explants are able to metabolize aflatoxin B₁; (b) the nonciliated secretory cell population in this tissue is the target cell for cytotoxicity of this carcinogen; and (c) as is the case in the more distal airways, the nonciliated epithelial cells appear to have a high content of components of the pulmonary cytochrome P-450 monooxygenase system, which may be an important factor in the susceptibility of these cells and this region of the airways to suspected airborne carcinogens.

INTRODUCTION

AFB₁ is a carcinogenic mycotoxin produced by strains of Aspergillus flavus. Although AFB₁ is a potent hepatocarcinogen in a wide variety of animal species and is likely to be an important etiological agent of human liver cancer (1, 2), there is some evidence that this toxin may play a role in human respiratory cancer. Agricultural surveys have demonstrated that AFB₁ is frequently present in airborne, respirable grain dusts at levels far exceeding those found in contaminated food products (3) and may, therefore, represent an occupational hazard to those exposed. The relationship between occupational exposures and the incidence of respiratory tumors is uncertain. Recently, however, in an epidemiological study of Dutch peanut-processing workers exposed to AFB₁-contaminated dusts, a significant increase in mortalities for total cancer and respiratory cancer was observed in the AFB₁-exposed group relative to their unexposed cohorts (4). It was established that the level of occupational exposure of these workers was approximately 0.04–2.5 μg of aflatoxin per wk based on a respiration rate of 1 m³/h (5).

Recent biochemical studies conducted in our laboratory (6) and by others (7–9) indicate that AFB₁ is metabolically activated by either isolated respiratory tract cells or organized epithelium derived from the respiratory tract of rodents and humans. The metabolic capability of such tissues is often measured by (a) the profile of AFB₁ metabolites that accumulate in the culture medium; (b) extent of binding of AFB₁ to cellular macromolecules such as DNA; and (c) pathological alterations in cells of the respiratory epithelium.

It has been previously shown that, at least in the lower airways of rodents, components of cytochrome P-450 monooxygenase, the enzyme system most important in xenobiotic metabolism, are localized primarily in the nonciliated bronchiolar epithelial (Clara) cells (10, 11). Accordingly, it is this cell type which appears to be the most prevalent target for the toxic action of chemicals requiring metabolic activation in distal respiratory tissues, such as carbon tetrachloride (12), 4-ipomeanol (13), and 3-methylfuran (14). At this time, however, few data are available on the cellular relationships with respect to carcinogen metabolism in the upper airways, i.e., the trachea and larger bronchi. In this paper, we report biochemical and morphological evidence that the nonciliated tracheal epithelial cell, the Clara cell "equivalent" in the upper airways, is the cellular locus of cytochrome P-450-mediated AFB₁ metabolism.

MATERIALS AND METHODS

Animals. Male New Zealand white rabbits weighing about 2.8 kg, obtained from Dutchland, Inc. (Denver, PA), were used for this study.

Chemicals. Ring-labeled [³⁵C]AFB₁ was prepared from cultures of Aspergillus parasiticus, American Type Culture Collection 15517, as described (15). Purity of this compound, which was greater than 98%, was assessed as described earlier (6).

Isolation and Culture of Explants. Animals were anesthetized using sodium pentobarbital (1.5 ml, 100 mg i.p.) and exsanguinated by severing the brachial arteries. Trachea were removed and cultured according to previously published methods (6). [³⁵C]AFB₁ (0.7 μM; 0.14 μCi) was added to the medium in a volume of 12 μl of ethanol; the final ethanol concentration was 1%. Control cultures received 12 μl of ethanol containing no [³⁵C]AFB₁. Cultures were then incubated at various time intervals at 37°C in covered Petri dishes in an atmosphere of O₂:CO₂ (95:5) and a relative humidity of approximately 100%. At the end of the various time intervals, the explants were removed from cultures, and a thin (approximately 2 mm) section was removed from the midsection of each trachea and placed in diluted Karnovsky's fixative (16). The culture medium and the remaining portions of each trachea were frozen in liquid nitrogen and stored at −80°C until analysis.

Analysis of AFB₁ Metabolism. The culture medium was extracted 3 times with equal volumes of chloroform:acetoniclorite (9:1, v/v). These extracts were then transferred to small vials, and known amounts of unlabeled AFL, AFB₁, AFM₁, and AFQ₁ were added to identify AFB₁ metabolites in the extracts by coelution via high-pressure liquid chromatography. The residues were then evaporated in a gentle stream of air.

Received 9/9/85; revised 3/20/86; accepted 4/22/86.

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Supported in part by USPHS Grant ES03591 and a grant from the American Lung Association of California. Paper 3134 of the Utah Agricultural Experiment Station.

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The abbreviations used are: AFL, aflatoxin B₁; AFB₁, aflatoxicol; AFQ₁, aflatoxin Q₁; AFM₁, aflatoxin M₁.
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nitrogen, then reconstituted in 150 μl of 20% acetonitrile (v/v) in water, and injected directly into a Model 5000 high-pressure liquid chromatograph (Varian Assoc., Sunnyvale, CA) equipped with a Zorbax octadecyl silicate column (4.6 mm x 25 cm). The samples were eluted at 27°C with an isocratic mixture of 28% acetonitrile (v/v) in water at a flow rate of 1 ml/min. The eluates were monitored at 345 nm (Var-Chrom; Varian Associates), and the AFB1 metabolites were quantitated by collecting 0.5-ml fractions directly into scintillation vials, followed by counting (Packard Tri-Carb Model 2660).

DNA Purification and Analysis. The extent of total binding of AFB1 to tracheal DNA was quantitated according to previously published methods (6).

Electron Microscopy. Cultured tracheas fixed at indicated time points were postfixed with osmium tetroxide, dehydrated in a graded concentration series of ethanol and propylene oxide, and embedded in araldite using standard techniques (16). One-μm light microscopic sections were examined to ensure preservation of epithelial structures in culture and to select regions with good cross-sectional orientation for ultrastructural studies. Thin (90–100 nm) sections were made with a Sorvall MT2-B ultramicrotome and diamond knives. These were mounted on formvar-coated, single-slotted grids and stained with uranyl acetate and lead citrate. Specimens were viewed on a Zeiss EM 10 electron microscope. For cell population density determinations, a montage of low magnification (×2000) prints of all epithelial cells present in a section was constructed. This allowed 200-300 cells to be counted for each specimen. Cells were characterized as ciliated, nonciliated secretory, or basal based on presence or absence of cilia and apical basal bodies, nuclear morphology, cytoplasmic constituents, and staining density using criteria previously described (17). Only cells which had a nuclear profile present in the section were included in cell counts to minimize the effects of cell size on population density determinations (18). Cells which had condensed, shrunken nuclei with granular, densely staining aggregates of chromatin were classified as necrotic as were cells with extensively swollen, lucent cytoplasm containing fragmented organelles. Population densities were calculated as the percentage of total population represented by individual cell types. One block from each replicate culture at each time point was counted. Sections for each time point from control cultures were also counted.

Immunocytochemistry. Antibodies to purified rabbit lung cytochrome P-450, Forms 2 and 5, and NADPH cytochrome P-450 reductase were produced in goats and previously characterized (19). These antibodies were applied to paraffin sections of rabbit trachea that had been fixed by intratracheal infusion of 1% paraformaldehyde for 1 h. Antibodies were visualized by the avidin-biotin technique using biotinylated mouse anti-goat IgG and horseradish peroxidase-conjugated avidin (20). Antibody deletion and normal goat serum-treated specimens were used as controls.

RESULTS

As presented in Fig. 1, high-pressure liquid chromatography analysis of the extracted medium from one of the 24-h cultures revealed four resolvable peaks corresponding to the metabolites AFM1, AFQ, and AFL in addition to the unretracted parent compound, AFB1. The distributions of these metabolites were 7.1, 1.7, and 2.9%, respectively, of the activity injected into the chromatograph. Parent AFB1 accounted for a considerable portion (approximately 25%) of the activity extractable from the medium. There were also some unidentified peaks in the midst of the metabolite peaks. The identities of these peaks, some of which represented nearly 5% of the total activity of the extract, were not pursued. Metabolism of AFB1 by explant cultures resulted in alklylation of tracheal DNA (Fig. 2). Total DNA binding was essentially linear up to 12 h, when it reached a plateau.

Using antibodies specific for rabbit lung cytochrome P-450 Isozymes 2 and 5 and NADPH cytochrome P-450 reductase, paraffin sections of rabbit trachea fixed by intratracheal infusion of paraformaldehyde were stained to determine the relative distribution of this enzyme in the rabbit upper airways. The reaction product was diffusely evident in the region of cilia and microvilli on the mucosal surface but was only present over the cytoplasmic region of nonciliated cells where ultrastructural studies show smooth endoplasmic reticulum to be concentrated (Fig. 3, B to D). Control sections did not contain reaction product (Fig. 3A).

Ultrastructural analyses were performed on sections of explants in order to determine which cell types were susceptible to the effects of AFB1 in culture. Luminal surfaces of the rabbit trachea are populated primarily by nonciliated and ciliated epithelial cells (17). Generally, AFB1 treatment appeared to affect the nonciliated cells to a greater degree than the ciliated cell population. Only mild degenerative changes were evident in untreated cultures after 24 h. These included cytoplasmic vacuole formation in both ciliated and nonciliated cells (Figs. 4A and 5A). Most vacuoles were empty, but some contained membranous debris.
cytochrome P-450 NADPH reducéase (D). Reaction product is concentrated on chrome P-450 isozyme Form 2 (B); cytochrome P-450 isozyme Form 5 (C); and remaining nonciliated cells had irregular inclusions of electron-dense homogeneous material containing vacuolated membranous structures and condensed pyknotic nuclei. Intraepithelial clumps of irregular, fragmented electron-dense material were interpreted to represent lytic cells. Most of the ciliated cells at this time point had large cytoplasmic inclusions of membranous material. Mitochondrial changes in ciliated cells were not appreciably different from those in the 4- and 12-h cultures.

The relative specificity of AFB, toward the nonciliated cell population is borne out by the differential cell count presented in Table 1. The relative proportion of each cell type in control cultures is similar to that previously reported for the rabbit trachea (17). No significant alterations in the relative population densities occurred in control cultures through the 24 h in culture. Little change in population densities occurred in the 0.5-, 1-, and 4-h cultures. At 12 and 24 h, there was marked decrease in nonciliated cells from 25–30% of the population to 9%. No change in the proportion of ciliated cells was apparent, but there was a slight increase in the apparent basal cell population.

DISCUSSION

This study was designed to investigate the metabolism of a model carcinogen in the upper airways of the rabbit and to definitively establish the cellular relationships with respect to components of the cytochrome P-450 isozyme system in this region of the respiratory tract. The tracheal explant culture is ideally suited for these studies as it enables the exposure of several cell types in an organized tissue to the carcinogen, and it is amenable to the observation of histopathological changes in the tissue after exposure. In addition, the inclusion of serum for growth is not needed in these short-term cultures. Some cytochrome P-450-mediated reactions have been shown to be altered by the addition of serum in vitro (21), as well as to diminish over time (22).

The evidence implicating AFB, as a respiratory carcinogen is tenuous. An early study showed that high doses of AFB, (0.3 mg administered intratracheally twice weekly for 3 wk) elicited tracheal carcinomas in three of six rats (23). Some epidemiological studies have also implied a degree of cause-and-effect (4, 5). Although the processes underlying AFB, metabolic activation are similar to those for known respiratory procarcinogens, the establishment of a definite relationship between AFB, exposure and respiratory carcinogenesis awaits rigorous examination.

The pattern of unconjugated AFB, metabolites formed by the rabbit tracheal explants (Fig. 1) is similar to those we recently reported for the hamster (6), except that unlike the hamster, rabbit explants convert AFB, to AFM,. However, rabbit explants formed much less AFL than hamster (2.7% versus 16%, respectively) (6). Although the metabolites AFQ, and AFM, are significantly less biologically active than the parent compound (24, 25), AFL is not considered to be a detoxification product due to its potent mutagenic (24) and carcinogenic (26) properties. Since the metabolism of AFB, is a requisite step in the production of a cytotoxic intermediate(s), the cytotoxicity of AFB, in culture may have been due, in part, to the production of AFL.

Binding of AFB, to rabbit tracheal DNA (Fig. 2) demonstrated the ability of this tissue to activate AFB, to the putative active intermediate, the AFB, 2,3-oxide (27). The overall extent of binding of AFB, to rabbit tracheal DNA is approximately 2 times that seen in hamster tracheal explant systems (6) and over 150 times that observed in cultured human bronchus...
exposed to 0.5 μM AFB₁ for 24 h (28). Whether these differences in binding are due to absolute rates of AFB₁ activation or efficiency of removal of AFB₁-DNA adducts in these tissues remains to be studied. Since DNA adduct formation is a necessary condition for tumor induction with many chemicals (29), the data in this study suggest that airway exposure to AFB₁ is a potential carcinogenesis risk to those exposed.

The relative potential for AFB₁ activation and susceptibility to AFB₁ toxicity of individual cell types in the rabbit trachea were investigated. This was approached in two ways: (a) via light microscopic and ultrastructural analyses of the explant epithelium; and (b) via immunohistological techniques to study the cellular distribution of the rabbit cytochrome P-450, Forms 2 and 5, each of which has been reported to be involved in the metabolism and/or the activation of AFB₁ (30).

Although ciliated cells were less affected by AFB₁, the luminal surfaces of these cells showed reactivity toward the anti-rabbit cytochrome P-450 Isozymes 2 and 5 and cytochrome P-450 reductase. The polyclonal antibodies used in this study were raised against purified proteins. While cross-reactivity with some unknown protein at the cell surface is possible, it seems unlikely that all three antibody preparations directed towards three immunochemically and catalytically distinct enzymes would have a similar cross-reactivity. However, the only cytoplasmic staining for cytochrome P-450-related enzymes was in the cells that were most susceptible to the cytotoxic effects of AFB₁, i.e., the nonciliated epithelial cells.

The results of both cell count (Table 1) and ultrastructural analyses (Figs. 4 and 5) indicate that the nonciliated epithelial cell types are the most dramatically affected, and hence they may be considered to be the target cell for the cytotoxicity of AFB₁ in these tissues. We have recently reported similar findings in hamster tracheal explant cultures (6). The ciliated cell population density remained largely unchanged. These cells did show treatment-associated degenerative changes at the 12- and 24-h time points, while basal cells appeared unaffected but showed increases in relative population density. While it seems logical that basal cell hyperplasia might have occurred, it is also possible that the increased basal cell population density occurred due to an absolute decrease in both secretory and, to a
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Fig. 5. Higher magnification electron micrographs of ciliated and nonciliated secretory cells from rabbit tracheal explants. Explants were incubated for the indicated time period with 0.7 \( \mu M \) AFB: A, 24-h ethanol vehicle control (\( \times 16,250 \)); B, 0.5 h (\( \times 23,750 \)); C, 1 h (\( \times 10,427 \)); D, 4 h (\( \times 5,422 \)); E, 12 h (\( \times 5,422 \)); and F, 24 h (\( \times 5,114 \)). Nonciliated cells at 0.5 and 1 h had swollen mitochondria (arrowheads). Irreversible degenerative changes are evident in nonciliated cells (NC) by 4 h in culture. See text for detailed description of changes.

Table 1 Cell counts of tracheal epithelium from rabbit tracheas cultured with 0.7 \( \mu M \) AFB.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ciliated</th>
<th>Nonciliated</th>
<th>Basal</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>All control</td>
<td>50 ± 5</td>
<td>25 ± 3</td>
<td>25 ± 3</td>
<td>0</td>
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<tr>
<td>0.5h</td>
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<td>25</td>
<td>25</td>
<td>0</td>
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<td>35</td>
<td>4</td>
</tr>
<tr>
<td>24h</td>
<td>51</td>
<td>9</td>
<td>36</td>
<td>4</td>
</tr>
</tbody>
</table>

* \( n = 7 \), one fixed immediately and 2 each from 4, 12, and 24 h.
\( \# \) Mean ± SD.
\( \ast \) \( n = 2 \).

lesser extent, ciliated cell numbers. An alternate possibility is that basal cell nuclear size increased which may have resulted in a more frequent appearance in the section.

The acute toxicity (i.e., cell death) of AFB, is closely correlated with the binding of active metabolite(s) to critical cellular proteins (31). One such metabolite, the AFB, 2,3-dihydrodiol, presumably binds to cellular proteins via the formation of Schiff bases with free amino groups (32). Additionally, the AFB, 2,3-oxide (27) should also significantly contribute to this cytotoxic protein binding. The extensive necrosis of nonciliated cells after 12 h in culture may have been responsible for the plateau in DNA alkylation seen after 12 h (Fig. 2), since necrotic cells would be less likely to activate AFB, to species which alkylate DNA. An alternative explanation for this plateau would be diminished cytochrome P-450 activity with time in culture. An unsuccessful attempt was made to qualitatively assess cytochrome P-450 levels in cultured explants from these experiments by immunohistochemistry. More experiments are needed to rigorously examine the stability of mixed-function oxidase components in explant culture.

Structurally similar nonciliated bronchiolar epithelial cells (Clara cells) have been shown to be the target cell for the action of many toxicants in the lower airways of several mammalian species (12–14). Clara cells have also been shown to possess greater quantities of cytochrome P-450 relative to other cell types in the lung (11). Significantly, the results of this study indicate that the nonciliated tracheal epithelial cell, the Clara cell "equivalent" in the upper airways, similarly appears to be primarily responsible for carcinogen metabolism in this region of the respiratory tract.

ACKNOWLEDGMENTS

The authors wish to thank Viviana Wong, Margaret Brummer, and Janalee Burbank for their assistance.

REFERENCES

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