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Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B_1

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ABSTRACT **Twelve forms of human hepatic cytochrome P450 were expressed in hepatoma cells by means of recombinant vaccinia viruses. The expressed P450s were analyzed for their abilities to activate the potent hepatocarcinogen aflatoxin B_1 to metabolites having mutagenic or DNA-binding properties. Five forms, P450s IA2, IIA3, IIB7, IIIA3, and IIIA4, activated aflatoxin B_1 to mutagenic metabolites as assessed by the production of His revertants of *Salmonella typhimurium* in the Ames test. The same P450s catalyzed conversion of aflatoxin B_1 to DNA-bound derivatives as judged by an *in situ* assay in which the radiolabeled carcinogen was incubated with cells expressing the individual P450 forms. Seven other human P450s, IIC8, IIC9, IID6, IIE1, IIF1, IIIA5, and IVB1, did not significantly activate aflatoxin B_1 as measured by both the Ames test and the DNA-binding assay. Moreover, polyclonal anti-rat liver P450 antibodies that crossreact with individual human P450s IA2, IIA3, IIIA3, and IIIA4 each inhibited aflatoxin B_1 activation catalyzed by human liver S-9 extracts. Inhibition ranged from as low as 10% with antibody against IIA3 to as high as 65% with antibody against IIIA3 and IIIA4. These results establish that metabolic activation of aflatoxin B_1 in human liver involves the contribution of multiple forms of P450.**

Cytochrome P450s[†] are the principal enzymes involved in the metabolic activation of chemical carcinogens. A number of studies have been conducted in rodents that implicate certain P450 forms in the activation of specific classes of carcinogens (2, 3). Genetic evidence suggests more directly that certain forms of rodent P450s are involved in chemically induced cancer (4). The role of human P450 forms in cancer and carcinogen activation, however, is less clear. The noteworthy species differences in P450 expression make it unlikely that one can reliably extrapolate rodent carcinogen activation data to humans (5, 6). For this purpose, direct analysis of human P450 activation of procarcinogens is essential.

In recent years, a limited number of forms of P450 have been purified from human hepatic tissue (7). In some instances, these purified enzymes have been directly analyzed for catalytic activities toward various substrates by reconstituting the microsomal environment *in vitro* with additions of artificial lipid and NADPH–P450 oxidoreductase. However, at least one form of human P450, designated $P450_{NF}$,[‡] is not significantly active when reconstituted *in vitro* (11). The roles of individual P450 forms in human liver-catalyzed drug and carcinogen metabolism have generally been evaluated by correlating levels of activities with levels of specific forms of P450s in multiple human liver samples and by inhibiting these activities with use of specific anti-P450 antibodies, such as the recent examples of human P450s as metabolic activators of known carcinogens including the aflatoxins (12, 13). However, interpretation of results obtained with the use of purified enzymes and antibodies (12, 13) is limited by the lack of certainty that a particular P450 protein preparation is homogeneous and an antibody is immunospecific. For example, there are four P450s displaying high amino acid sequence similarities and at least two of these proteins comigrate in SDS-containing polyacrylamide gels (14).

The use of vaccinia virus-mediated cDNA expression (14) offers a new way to evaluate the catalytic activities of human P450s and circumvents problems inherent with P450 and antibody preparations. By expressing, in cultured cells that are virtually devoid of endogenous P450s, cDNAs encoding the major P450s expressed in human liver, it is possible to accurately define which P450 forms metabolize specific drugs or activate carcinogens. In this report, we studied the ability of 12 human P450s to metabolically activate the potent hepatocarcinogen aflatoxin B_1 (15). We determined that at least 5 distinct human P450s are capable of activating aflatoxin B_1 to mutagenic and DNA-binding metabolites. In addition, we found that antibodies generated against rodent P450s, immunorelated to 4 of these human P450s, were able to partially immunoinhibit aflatoxin B_1 activation catalyzed by extracts of two human liver specimens. Our results establish that not just one form ($P450_{NF}$; ref. 13) but rather multiple forms of human P450 are capable of activating aflatoxin B_1.

MATERIALS AND METHODS

The construction and characterization of recombinant vaccinia viruses containing the cDNAs encoding the P450 proteins IA2 (16), IIA3 (17), IIB7 (18), IIC8 (19), IIC9 (19), IIF1 (20), IIIA4 (14), IIIA5 (14), and IVB1 (21) were published. Vaccinia viruses containing the IID6 (22), IIE1 (23), and IIIA3 (11) cDNAs were constructed as described (14). When recombinant vaccinia viruses were used to infect human TK^- 143 (thymidine kinase-deficient human embryoblast) or Hep G2 (hepatoma) cells, the P450 contents of the lysates ranged from 13 to 18 pmol/mg of total cell protein. Diagnostic enzyme activities for each P450 were as follows: 7-ethoxycoumarin *O*-deethylase for IA2, IIB7, and IIF1; coumarin 7-hydroxylase for IIA3; tolbutamide hydroxylase for IIC8 and IIC9; bufuralol 1′-hydroxylase for IID6; dimethylnitrosamine *N*-demethylase for IIE1; testosterone 6β-hydroxylase for IIIA3, IIIA4, and IIIA5; and estradiol 2-hydroxylase for IVB1.

Hep G2 cells were infected with recombinant vaccinia at a multiplicity of infection of 5. Twenty-four hours after infec-

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[†]The nomenclature used in this report is that described recently (1).
[‡]$P450_{NF}$ was purified from human liver and has an amino-terminal sequence that is compatible with either the IIIA4 (8, 9) or IIIA3 (10) cDNA-deduced amino acid sequences. These proteins display 98% sequence identity and therefore antibody generated against $P450_{NF}$ would most likely crossreact with both P450 forms.

tion the cells were harvested, washed with phosphate-buffered saline, and lysed by brief sonication. Mutagenesis assays were performed as described (24, 25), with each assay mixture containing 3 mg of total lysate protein from vaccinia-infected cells. To analyze aflatoxin B_1 binding to DNA, $\approx 6 \times 10^6$ cells were infected with recombinant vaccinia virus and [^{3}H]aflatoxin B_1 (15 Ci/mmol, Moravek Biochemicals, San Francisco, CA; 1 Ci = 37 GBq) was added to 15 ml of culture medium in a 10-cm-diameter dish. Cells were harvested 24 hr after infection and were treated with proteinase K (BRL; 500 μg/ml) in 50 mM Tris/HCl, pH 8.0/5 mM EDTA/0.5% SDS for 3 hr at 37°C. Protein was removed by repeated phenol extractions and the nucleic acid was dialyzed exhaustively against 10 mM Tris/HCl, pH 8.0/1 mM EDTA. The samples were digested with RNase T_1 (10 units/ml) and RNase A (0.1 mg/ml) for 3 hr at 37°C, extracted with phenol, and again exhaustively dialyzed against 10 mM Tris/HCl, pH 8.0/1 mM EDTA. DNA concentrations were determined by monitoring absorbance at 260 nm (1 A_{260} = 50 μg/ml). Radioactivity was measured using a liquid scintillation counter. The recovery of DNA was from 220–310 μg per 6×10^6 cells.

Immunoinhibition experiments utilized a cell extract fraction prepared from two human liver specimens (M1 and M2, ref. 17) by centrifugation. Frozen liver pieces (5–10 gm) were thawed in phosphate-buffered saline at 4°C and were homogenized (10 strokes with a glass/Teflon tissue disrupter). The homogenate was centrifuged at 10,000 × *g* for 10 min and the supernatant (S-9) was used as an activation system for the Ames test using 2 μg of aflatoxin B_1 per plate. Rat P450d (26), P450e (26), P450 IIA2 (27), and P450 PCN2 (28) were purified as described and were used to elicit antibodies in rabbits. IgG fractions were purified by protein A-Sepharose chromatography.

RESULTS

Metabolic Activation of Aflatoxin B_1 by Human cDNA-Expressed P450s. The ability of individual human P450s to activate aflatoxin B_1 to mutagenic metabolites was measured by the Ames test with use of extracts of total protein from recombinant vaccinia virus-infected cells as an activation system. Five expressed P450s metabolically activated aflatoxin B_1 to mutagenic metabolites (Fig. 1). IIA3, IIIA3, and IIIA4 showed the highest activity, followed by IA2 and IIB7. IIIA5 displayed only weak activation ability. All other P450s (IIC8, IIC9, IID6, IIE1, IIF1, and IVB1) were inactive, all producing the same levels of His revertants as were found in cells infected with wild-type vaccinia virus. Human liver S-9 fraction from liver M1 (see Fig. 2) was active when concentrations of aflatoxin B_1 were low but toxic when concentrations were high (10 μg/ml) (Fig. 1). In contrast, the activations by vaccinia-expressed enzymes appeared to be unsaturable in this assay.

Human Expressed P450-Catalyzed Binding of [^{3}H]Aflatoxin B_1 to Cellular DNA. To supplement the results with bacterial cells, we examined covalent binding of aflatoxin B_1 to DNA in hepatoma cells infected with recombinant vaccinia viruses and then incubated directly with [^{3}H]aflatoxin. Measurements of DNA-bound tritium in purified DNA samples (Table 1) demonstrated that significant levels of aflatoxin B_1 bound to DNA were found only in cells infected with vaccinia virus expressing IA2, IIA3, IIB7, IIIA3, or IIIA4. Binding levels catalyzed by these enzymes were more than 20-fold higher than those obtained in cells infected with wild-type vaccinia virus. These are the same five P450 forms that displayed significant metabolic mutagen activation in the Ames test.

Immunoinhibition of the Metabolic Activation of Aflatoxin B_1 Catalyzed by Human Liver S-9 Fractions. To demonstrate that the human P450 forms identified through vaccinia virus expression were able to activate aflatoxin B_1 in human liver

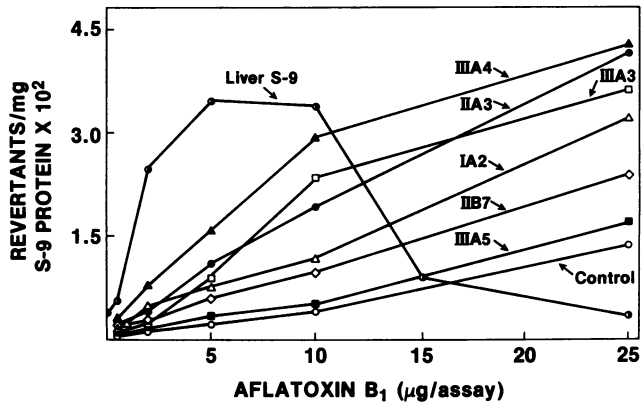

FIG. 1. Mutagen activation of aflatoxin B_1 by vaccinia-expressed human P450. Hep G2 cells were infected with vaccinia viruses containing human cDNAs. Cells were harvested 24 hr after infection and lysates were prepared by sonication. The Ames test was conducted using 3 mg of cell lysate protein from vaccinia-infected cells or human liver S-9 fraction, various amounts of aflatoxin B_1, and *Salmonella typhimurium* TA 98. Histidine revertants were scored per mg of protein. The data derived from each expressed P450 form are indicated by arrows. Control represents lysate from cells infected with wild-type vaccinia. Only those samples that yielded more revertants/mg than control are shown. Results are the averages of three determinations. The values (mean ± SD) obtained for the 10-μg/assay points are as follows: IA2, 115 ± 12; IIA3, 176 ± 15; IIB7, 86 ± 8; IIIA3, 234 ± 28; IIIA4, 295 ± 24; and IIIA5, 51 ± 8.

S-9, immunoinhibition studies were carried out. Polyclonal antibodies prepared against rat P450s were screened for their abilities to inhibit reactions catalyzed by vaccinia virus-expressed human P450 (unpublished results). Antibody against rat IIB2 was able to inhibit 7-ethoxycoumarin *O*-deethylase activity catalyzed by vaccinia-expressed IIB7; antibody against rat IA2 inhibited 7-ethoxycoumarin *O*-deethylase activity catalyzed by vaccinia virus-expressed human IA2; antibody against rat IIA2 inhibited coumarin 7-hydroxylase activity catalyzed by human IIA3; antibody against rat IIIA1 inhibited IIIA3-, IIIA4- and IIIA5-catalyzed testosterone 6β-hydroxylase activity. All activities were inhibited more than 98%. These antibodies were then tested to determine the extent of inhibition of aflatoxin B_1 activation catalyzed by two human liver S-9 samples. Three antibodies were able to significantly inhibit the S-9-catalyzed production

Table 1. *In situ* binding of aflatoxin B_1 to cellular DNA in cells infected with recombinant vaccinia expressing human P450s

Vaccinia virus	Specific binding, dpm/μg of DNA
Wild type	0.9 ± 0.2
vIA2	52.6 ± 6.2
vIIA3	39.4 ± 3.7
vIIB7	20.1 ± 1.8
vIIC8	1.0 ± 0.2
vIIC9	0.9 ± 0.2
vIID6	2.6 ± 0.3
vIIE1	1.0 ± 0.2
vIIF1	0.9 ± 0.2
vIIIA3	21.0 ± 1.6
vIIIA4	22.6 ± 2.4
vIIIA5	4.3 ± 0.7
vIVB1	1.5 ± 0.2

Hep G2 cells were grown to 90% confluence on 50-cm^2 plastic dishes and infected with recombinant vaccinia virus. [^{3}H]Aflatoxin (10 μCi) was added directly to the medium (15 ml) and cells were incubated for 24 hr. Cells were then harvested, and DNA was purified and its specific radioactivity was determined by liquid scintillation. Results are means ± SD for three experiments.

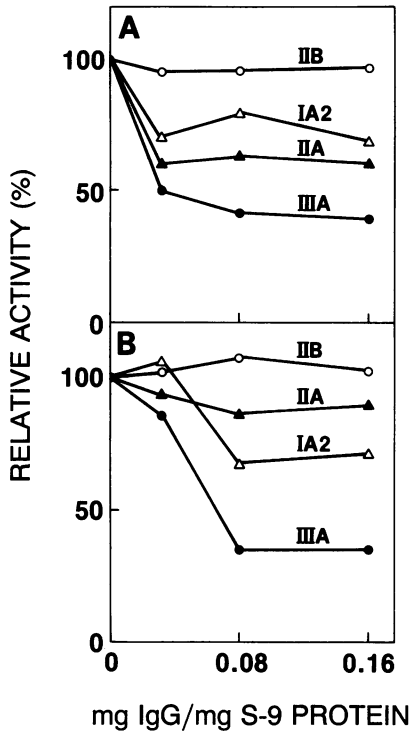

FIG. 2. Inhibition of human liver S-9-catalyzed activation of aflatoxin B_1. S-9 fractions were prepared from liver samples M1 (*A*) and M2 (*B*) and used as a source to activate aflatoxin B_1 in the Ames assay. Purified IgG prepared from sera of rabbits that had been immunized with rat P450s IIB2 (IIB), IA2 (IA2), IIA3 (IIA), and IIIA1 (IIIA) were preincubated with the S-9 fraction for 30 min at 4°C before the Ames assay was performed. Since it is still unclear how many P450s members exist in human subfamilies IIA, IIB, and IIIA, we have not labeled these immunoinhibition curves with notations for specific P450 forms (e.g., IIB7) except for IA2, since this is the only member of the IA subfamily known to be expressed in these two liver specimens. IgG from preimmune rabbits yielded no inhibition, similar to that of IIB.

of His revertants to different extents in both liver specimens (Fig. 2). Antibody against IA2 inhibited 30% and 40% and antibody against rat IIA2 inhibited 10% and 30% of aflatoxin B_1 activation by samples from livers M1 and M2, respectively. Antibody against rat IIIA1, which would be expected to inhibit all three human IIIA P450s, was the most effective, inhibiting 60% and 65% of aflatoxin B_1 activation by S-9 of livers M1 and M2, respectively (Fig. 2). Only antibody against rat IIB2 was ineffective. This was probably due to the low level or lack of expression of IIB7 in these livers (17). The variable levels of inhibition by antibodies against rat IA2, IIA2, and IIIA1 was probably due to different levels of expression of IA2, IIA3, and IIIA3/IIIA4 in each liver. In any case, these data confirm that aflatoxin B_1 activation is performed in part by these enzymes in human liver S-9.

DISCUSSION

Five forms of human P450 were able to metabolically activate aflatoxin B_1 as measured by the Ames test. All five forms also catalyzed binding of aflatoxin B_1 to DNA in whole cells. In fact, the direct binding assay appears to be more sensitive than the Ames test in identifying an aflatoxin B_1-activating P450. For example, IIB7-catalyzed production of His revertants in the Ames test was only 2-fold above control, whereas the IIB7-catalyzed binding to DNA was 20-fold over control. In contrast, seven other P450s failed to activate aflatoxin B_1 as measured by both the Ames test and the DNA-binding assay. The involvement of multiple P450 forms in aflatoxin B_1 activation was also confirmed by immunoinhibition studies in which antibodies that react with four of the five active human P450s inhibited human liver S-9-catalyzed aflatoxin B_1 activation. Only antibody that was raised against rat IIB2, that binds to human IIB7, failed to inhibit the S-9-catalyzed activation. This may have been due to a lack of significant expression of IIB P450s in the two liver samples we analyzed.

$P450_{NF}$[2] was reported to be the major P450 form activating aflatoxins in human liver (13). Our studies, using 12 cloned human P450 cDNAs, showed that five P450s, including IIIA4 and IIIA5, catalyzed aflatoxin B_1 activation. Since multiple forms are capable of activating this hepatocarcinogen, it seems unwise to attempt to monitor only $P450_{NF}$ (or IIIA3/IIIA4) expression as an indicator of human aflatoxin activation as previously suggested (13). Since P450s in the IIIA subfamily are expressed to different extents in different livers (13, 14), it is likely that in livers where either IIIA3 and IIIA4 are predominant, they would be the major enzymes contributing to aflatoxin activation. However, in livers in which the latter P450s are expressed at low levels, other P450 forms such as IA2, IIA3, and IIB7 would play major roles. Based on the results reported here, we conclude that humans have a large capacity to activate this carcinogen. These results reflect one of the notable unique characteristics of carcinogen-metabolizing enzymes and P450s, their overlapping substrate specificities.

Expression of multiple human P450s by use of vaccinia virus or another cDNA expression system should prove extremely valuable in defining the catalytic specificities of individual P450 forms. The roles of these forms in overall hepatic metabolism can be further evaluated by immunological approaches. These studies should prove valuable as a means to develop tests to define human metabolic phenotypes, to design studies in molecular epidemiology to examine the role of P450s in cancer susceptibility, and to develop human P450-based testing systems for chemicals of carcinogenic potential.

Finally, while this manuscript was under review, a report appeared in which the kinetics of production of aflatoxin B_1 8,9-epoxide was examined in human hepatic microsomes (29). The rates of generation of this active metabolite were dose-dependent, with the fraction of metabolites represented by the 8,9-epoxide increasing with decreasing dose of aflatoxin B_1. These data suggest that one or more P450 forms with high affinity for this carcinogen may be efficient at generating the active metabolite. These findings are especially significant since the aflatoxin B_1 exposure through the human diet is quite low. It should be interesting to determine which forms of human P450 have low K_m values and are proficient at production of aflatoxin B_1 8,9-epoxide.

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