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THE DISTRIBUTION AND INTRACELLULAR TRANSLOCATION OF AFLATOXIN B_1 IN ISOLATED HEPATOCYTES

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The distribution and intracellular translocation of AFB_1 in various subcellular fractions was investigated in isolated hepatocytes by pulse-chase experiments. After labeling the hepatocytes with $[^3H]$-AFB_1 (14.5 nM) for 15 min, the highest concentration of $[^3H]$-AFB_1 was found in the cytosolic fraction where 66% was bound noncovalently and 1.5% covalently. The lowest concentration of $[^3H]$-AFB_1 was found in the nuclear fraction; 36% and 4.0% were bound noncovalently and covalently respectively. When the $[^3H]$-AFB_1 loaded cells were chased with unlabeled AFB_1 (1 μM), the radioactivity of $[^3H]$-AFB_1 in the cell lysate and cytosolic fraction decreased in time with an apparent rate of elimination ($t1/2$) of 92 min and 66 min, respectively. The levels of covalently bound AFB_1 increased with time and reached a maximum at 60 min in nuclei (270%), and at 120 min in mitochondria (220%) and cytosol (420%) as compared to the zero time. Only in the microsomal fraction was there no significant increase with time in covalently bound AFB_1. These results suggest that the toxin after activation by the microsomal mixed function oxidases was either detoxified or transported to other cellular organelles where covalent binding of macromolecules occurred.

It has been recognized that Aflatoxin B_1 (AFB_1) produced by strains of aspergillus flavus is hepatotoxic and carcinogenic in laboratory and domestic animals (1-3). Microsomal activation of AFB_1 into a reactive epoxide, AFB_1-2,3-epoxide, and the subsequent interaction of the epoxide with cellular DNA, RNA and proteins has been implicated in the mechanism of acute toxicity and carcinogenesis (4-10). Although AFB_1 is known to be selectively taken up by the liver, the temporal sequence of cytoplasmic-nucleo translocation of AFB_1 is still unclear. The problem of subcellular distribution of AFB_1 has been approached by autoradiography, fluorescence microscopy, immunoperoxidase localization and isotopic labeling of animals in vivo (11-16). Quantitative and kinetic evaluations with these techniques has been difficult due to

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Abbreviations: AFB_1, Aflatoxin B_1; SDS, sodium dodecyl sulfate; TCA, trichloracetic acid.

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differences in rates of absorption, tissue distribution, and elimination associated with intact animals. We described recently an in vitro system using isolated rat hepatocytes for studying the kinetics of AFB_1 uptake and binding, and the effect of several microsomal enzyme inhibitors on AFB_1-induced cytotoxicity and AFB_1-inhibited RNA synthesis (17,18). In this paper, we report on the distribution and translocation of AFB_1 in various subcellular fractions using isolated hepatocytes in vitro. Chasing the [^{3}H]-AFB_1-loaded hepatocytes with unlabeled AFB_1 allows the establishment of the temporal sequence of AFB_1 translocation and quantitative comparison of covalently and non-covalently bound forms of AFB_1 associated with cellular macromolecules. With this approach systemic and vascular effects inherent in whole animal studies are avoided.

Materials and Methods

[^{3}H]-Aflatoxin B_1 (20 Ci/mmole) was obtained from Moravek Biochemical, Inc. Other chemicals were purchased from sources as previously described (17). Rats (250-300g, adult male Wistar) were obtained from West Jersey Farm Division of the Parco Scientific Co. and were fed water and Ziegler laboratory chow ad lib. Hepatocyte isolation and conditions of incubation were similar to reports published previously from this laboratory (17-19). In pulse-chase experiments, the hepatocytes were first incubated with [^{3}H]-AFB_1 (14.5 nM) for 15 min. After removing the [^{3}H]-AFB_1 from the medium, the cells were washed and re-incubated in fresh medium containing 1 µM unlabeled AFB_1 for a period up to 120 min. At indicated times, the cellular contents of [^{3}H]-AFB_1 and bound AFB_1 were determined as previously described (17).

To isolate various subcellular fractions, hepatocytes ($16\text{-}22 \times 10^6$ cell) were packed by centrifuging at 30g for 10 min at 4° and washed once with Swim's S-77 medium. The cells were suspended in 5 ml of isolation buffer containing 0.25 M sucrose/1 mM EDTA/2 mM Tricine, pH 7.6 and sonicated for 45 sec at 45 watts in three 15 sec bursts at 4° in a Sonifier cell disruptor (Heat-Systems, Model W 185). The volume was then brought to 10 ml, centrifuged for 10 min at 400g, then the supernatant for 10 min at 10,000g and again for 60 min at 105,000g to obtain nuclei, mitochondria, and microsomes. The 105,000g supernatant was taken as the cytosolic fraction. The nuclear and mitochondrial fractions were washed and resuspended in isolation buffer. The protein content of each fraction was determined by the procedure of Lowry et al. (20).

To determine the total content of [^{3}H]-AFB_1 of the subcellular fraction, duplicated samples (100 µl) were added directly to counting vials. The total bound AFB_1, was determined by filtering samples in a Millipore apparatus, washed twice with isolation buffer and followed by addition of 10 ml cold 10% trichloroacetic acid (TCA); the TCA-insoluble materials collected on glass fiber discs (Whatman GF/C) were transferred to counting vials. To determine the covalently bound AFB_1, samples were filtered and washed with the isolation buffer containing 0.1% sodium dodecyl sulfate (SDS) followed by TCA; the materials collected on the filter disc were transferred to counting vials.

The noncovalent bound AFB_1 was the difference between total bound and covalently bound AFB_1.

To determine $[^3H]$-AFB_1 radioactivity, 1.0 ml of NCS solubilizer was added to the counting vials. After standing at room temperature over night, 15 ml OCS counting solution was added and radioactivity determined in Beckman LS-3100 liquid scintillation system. Cpm were computed to dpm using external standardization. The counting error was 5% or less.

Results and Discussion

As depicted in Fig. 1, uptake and binding of AFB_1 occurred rapidly during the first 30 min of incubation. With further incubation of the hepatocytes in the presence of $[^3H]$-AFB_1 (14.5 nM), gradual increases were seen. However, when the $[^3H]$-AFB_1 in the medium was replaced with unlabeled AFB_1 (1 μM), the intracellular content and bound $[^3H]$-AFB_1 exhibited a time-dependent decrease suggesting that AFB_1 was effectively metabolized and eliminated from the hepatocytes. The time-dependent decrease of intracellular $[^3H]$-AFB_1 also suggest that the exchange of extracellular unlabeled AFB_1 with intracellular $[^3H]$-AFB_1 was at a minimum, otherwise a rapid biphasc decrease of $[^3H]$-AFB_1 at 30 min should be observed. The distribution of $[^3H]$-AFB_1 in the

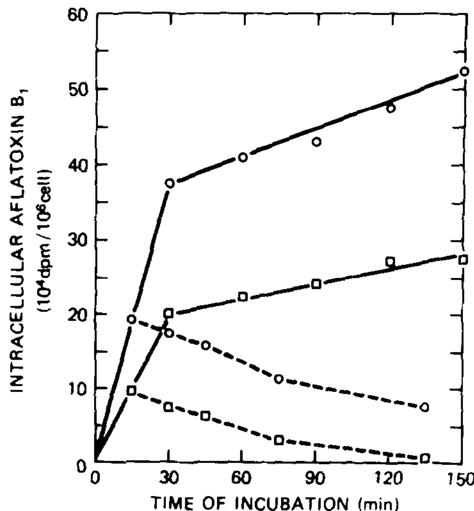

Figure 1. Time-dependent uptake, binding and release of $[^3H]$-AFB_1 in isolated hepatocytes. The cells were incubated with $[^3H]$-AFB_1 (14.5 nM) as described in the methods. At indicated times, the radioactivity of total cellular (-o-) and bound (-▫-) AFB_1 was determined. At 15 min $[^3H]$-AFB_1 present in the medium was removed and replaced with unlabeled AFB_1 (1 μM) in some experiments. The decrease of radioactivity of cellular (--o--) and bound (--▫--) AFB_1 was determined as described under "Materials and Methods". Data presented are average of 5-6 experiments.

Table 1. Distribution of $[^3H]$-AFB_1 in Subcellular Fractions and Relative Content of Bound AFB_1[a]

Subcellular Fractions	Cellular Content of		Relative Content of Bound-AFB_1[b]	
	Protein ($mg/10^8$cell)	Radioactivity ($10^{-6}dpm/10^8$cell)	Non-Covalent Bound (%)	Covalent-Bound (%)
	(Mean ± S.E.M.)			
Cell lysate	165 ± 12	19.0 ± 1.6	46.4 ± 2.0	2.3 ± 0.4
Nuclei	8 ± 1	0.6 ± 0.1	36.0 ± 4.0	4.9 ± 0.4
Mitochondria	26 ± 3	2.2 ± 0.2	43.5 ± 6.0	4.5 ± 0.5
Microsomes	35 ± 3	3.6 ± 0.5	39.0 ± 4.2	4.8 ± 0.6
Cytosol	82 ± 5	12.0 ± 0.6	66.0 ± 4.3	1.5 ± 0.2

[a]The concentration of $[^3H]$-AFB_1 in the medium was 14.5 nM and hepatocytes were incubated for 15 min before the isolation of various subcellular fractions. Data presented are mean ± S.E.M. from nine experiments.

[b]The relative content of bound-AFB_1 was calculated as percent of total $[^3H]$-AFB_1 radioactivity in the subcellular fraction.

various subcellular fractions was determined at the end of 15 min pulsing and showed that the amounts found in the nuclei, mitochondria, microsomes, and cytosol were 3.2%, 11.6%, 18.9% and 63% of the total radioactivity in the cell lysate (Table 1). The recovery of protein and $[^3H]$-AFB_1 radioactivity was 92% and 97% respectively suggesting that there were no excessive losses during subcellular fractionation. On examination of the different forms of bound AFB_1, the nuclei, mitochondria and microsomes contained similar amounts of both noncovalently and covalently bound AFB_1 whereas the cytosolic fraction contained the highest amounts of noncovalently bound AFB_1 (66% of total intracellular AFB_1) and lowest amount of covalently bound AFB_1 (1.5%). These results suggest that 15 min of incubation of hepatocytes with $[^3H]$-AFB_1 (14.5 nM) was sufficient to label the various subcellular fractions so that chasing with unlabeled AFB_1 would yield detectable amounts of radioactivity required for the study.

Table 2 presents the changes of $[^3H]$-AFB_1 radioactivity present in the various subcellular fraction during chasing with 1 μM unlabeled AFB_1. In

Table 2. Distribution of [^{3}H]-AFB$_1$ in Subcellular Fractions During Chasing With Unlabeled AFB$_1$[a]

Time of Chasing (min)	Number of Experiments (N)	Nuclei	Mitochondria	Microsomes	Cytosol
		(10^{-6} dpm/10^8 cell)			
0	9	0.6 ± 0.1	2.2 ± 0.2	3.6 ± 0.5	12.0 ± 0.6
15	4	0.6 ± 0.1	1.9 ± 0.2	3.2 ± 0.6	10.2 ± 1.4
30	6	0.6 ± 0.1	1.9 ± 0.3	2.4 ± 0.6	9.5 ± 1.0
60	4	0.5 ± 0.1	2.3 ± 0.3	2.0 ± 0.9	6.1 ± 0.9*
120	4	0.5 ± 0.1	1.7 ± 0.3	2.2 ± 0.4	3.5 ± 1.1*

[a]Hepatocytes were incubated 15 min with [^{3}H]-AFB$_1$ (14.5 nm). After removing [^{3}H]-AFB$_1$ from the medium, cells were reincubated in fresh medium containing unlabelled AFB$_1$ (1 μM). At times indicated, cellular fractions isolated and radioactivity determined. Data presented are mean ± S.E.M.

*Indicates p values < 0.05 compared to zero time.

the nuclei and mitochondria, the amounts of [^{3}H]-AFB$_1$ were not altered throughout the 120 min, while the microsomal fraction showed a 34% decrease of radioactivity at 30 min. The cytosolic fraction, however, exhibited a time-dependent decrease of [^{3}H]-AFB$_1$ similar to the cell lysate (Fig. 1). The calculated rate of AFB$_1$ elimination (t1/2) in the cell lysate and the cytosolic fraction was 92 min and 66 min, respectively (Fig. 2). The faster rate of AFB$_1$ turnover exhibited by the cytosolic fraction can be explained by the fact that the water-soluble polar metabolites, were detoxified and removed rapidly from the cytosol (3). The turnover rate of bound AFB$_1$ calculated from data shown in Fig. 1 was 39 min (Fig. 2) which indicated a rapid removal of bound AFB$_1$ from the cytosol. Examination of the non-covalently bound AFB$_1$ revealed a significant decrease of the relative content of bound AFB$_1$ in the cytosol at 15 min of chasing (68% to 38%) while the bound AFB$_1$ in the nucleus increased from 41% to 62% (data not shown). These results support the possibility that an AFB$_1$ binding protein exists in the liver cytosol (21) which may be responsible for the cytoplasmic-nucleo translocation of the toxin.

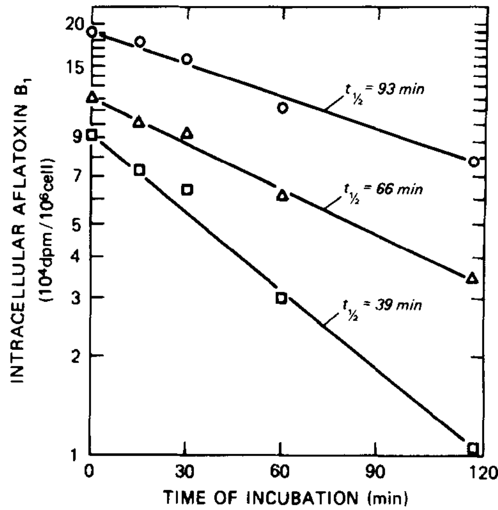

Figure 2. The turn-over rate of intracellular [^{3}H]-AFB_1. The experimental conditions were similar to Fig. 1 except that the radioactivity of cytosolic AFB_1 (▲) was determined in addition to the total cellular (○) and bound (□) [^{3}H]-AFB_1.

Since it is known that the nuclear DNA, mitochondria DNA, cellular RNAs and proteins are targets of AFB_1 modification (4-9, 22, 23), the levels of covalently bound AFB_1 present in the subcellular fractions were investigated. Table 3 presents the relative contents of covalently bound AFB_1 in cell lysate and various subcellular fractions during chasing. The levels of

Table 3. Relative Content of Covalent-bound [^{3}H]-AFB_1 in Various Subcellular Fractions During Chasing With Unlabelled AFB_1[a]

Time of Chasing (min)	Number of Experiments (N)	Cell Lysate	Subcellular Fractions			
			Nuclei	Mitochondria	Microsomes	Cytosol
		(% of total radioactivity in fraction)				
0	9	2.3 ± 0.4	4.9 ± 0.4	4.5 ± 0.5	4.8 ± 0.6	1.5 ± 0.2
15	4	2.7 ± 0.6	4.8 ± 1.8	5.1 ± 0.6	5.1 ± 0.5	1.7 ± 0.4
30	6	3.4 ± 0.6	4.9 ± 0.8	5.3 ± 0.8	5.3 ± 0.8	2.5 ± 0.6
60	4	5.0 ± 0.8*	13.4 ± 2.2*	8.5 ± 2.7*	6.7 ± 0.9	4.6 ± 0.6*
120	4	8.6 ± 2.4*	12.8 ± 5.0*	10.1 ± 1.9*	6.0 ± 1.5	6.5 ± 1.9*

[a]Incubation, isolation and assay conditions were similar to Tables 1 and 2.
*Indicates $p < 0.05$.

covalently bound AFB_1 in cell lysate and cytosolic fraction rose significantly in time; 4 fold enhancements were seen at the end of 120 min chasing. The microsomal fraction showed a slight increase over the initial level. The nuclei and mitochondria, however, exhibited different time periods for maximum covalent binding. In the nucleus, 273% increase occurred between 30 min and 60 min whereas in the mitochondria 224% enhancement was seen at 120 min. These data were in agreement with *in vivo* findings that nuclear and mitochondrial DNA were the preferred target for AFB_1 action.

In the present study, we have conducted pulse-chase experiments in isolated hepatocytes. The findings suggest that AFB_1 after entering into the cell, may be translocated to microsomes for biotransformation facilitated by a cytoplasmic binding protein; the majority of the AFB_1-2,3-epoxide so formed is detoxified and rapidly removed from the cell whereas a portion of the epoxide is translocated to various subcellular sites where covalent binding takes place; initially, a limited amount of cellular macromolecules of the endoplasmic reticulum is covalently modified,followed by a time-dependent modification of nuclear, mitochondrial, and cytosolic macromolecules.

Since animals differ greatly in their susceptibility to the biological effects of AFB_1, it is possible that tissues of different animal species have different cellular transport systems, activating and detoxication enzymes, and competitive binding characteristics to various cellular macromolecules (1-3, 24-29). Earlier studies as well as data presented in this paper suggest the presence of an AFB_1 binding protein (17, 18, 21), whether it binds to the parent toxin or the activated metabolite is not known. Further study on the protein in distinguishing from other known carcinogen binding proteins (30-34) and its role in AFB_1 translocation in the target tissue will offer a better understanding of AFB_1 toxigenesis and carcinogensis.

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