Interaction of Aflatoxins $B_1$ and $G_1$ with Tissues of the Rat

William Lijinsky, Kyu Y. Lee, and Clifford H. Gallagher

Division of Oncology, Institute for Medical Research, The Chicago Medical School, Chicago, Illinois 60612

SUMMARY

The incorporation of tritium-labeled aflatoxins $B_1$ and $G_1$ into DNA, RNA, and soluble protein of liver, kidney, spleen, and intestine of rats has been examined. The variation of the amount of incorporation of labeled compound with time has been followed by isolation of nucleic acids and proteins from animals killed 1 hr, 6 hr, 18 hr, 1 week, 4 weeks, and 8 weeks after being given a single i.p. injection of the labeled aflatoxins. Maximal incorporation of radioactivity into all 3 components of the 4 organs was found between 6 and 18 hr after injection. With both aflatoxins, a progressive decline in the incorporation was found between 6 and 18 hr and between 18 hr and 8 weeks. The highest specific activity with aflatoxin $B_1$ was in the liver protein at 6 hr, which contained 10% of the injected radioactivity; with aflatoxin $G_1$, the highest specific activity was in liver RNA at 18 hr. Labeling of kidney and spleen protein was particularly persistent with both aflatoxins. The radioactivity appeared to be covalently bound to protein. It was not possible to correlate the binding of the 2 aflatoxins to DNA, RNA, or protein of the various organs with the induction of tumors by the aflatoxins.

INTRODUCTION

One approach to the exploration of the carcinogenic action of the aflatoxins is the study of their interaction with the macromolecular components of various organs and tissues of a susceptible species and correlation of the results with the carcinogenic effect. Such studies have been useful in the case of the nitrosamines and other types of carcinogens, without, however, yielding conclusive results.

The biological effects of aflatoxins have been extensively examined, but there have been few reports of their biochemistry. Clifford and Rees (3) and Sporn et al. (9) have reported interaction of aflatoxins with DNA in nonbiological systems and Wogan et al. (10) have studied the distribution and metabolism of aflatoxin $B_1$ with the $^{14}C$-labeled compound. The experiments to be described here were carried out in conjunction with long-term feeding tests of aflatoxins $B_1$ and $G_1$ in which both compounds were found to be potent carcinogens for our strain of rats (1).

Because of the difficulty of preparing adequate quantities of $^{14}C$-labeled aflatoxins, it was decided to conduct the present experiments with aflatoxins labeled with tritium by exchange. The first attempt yielded aflatoxin $B_1$ of reasonably high specific activity and acceptable radiochemical purity, but the aflatoxin $G_1$ had low specific activity and the radiochemical purity left something to be desired. The results of the in vivo studies with this material were presented in a preliminary report (5), but were not very reliable. More recently, samples of both aflatoxins with high specific activity were prepared (6), and administered to rats, and the nucleic acids and soluble protein were isolated from several organs.

MATERIALS AND METHODS

The labeled aflatoxins $G_1$ and $B_1$ were prepared by exchange with tritium oxide in dimethylformamide at 70° in the presence of platinum as catalyst. The mixture was stirred during 24 hr, and labile tritium was then removed by evaporation with methanol (these procedures were carried out by New England Nuclear Corp., Boston, Mass.). Exposure of 25 mg of aflatoxins $B_1$ and $G_1$ yielded crude materials containing 2 to 5 Ci of tritium. Analysis of the products revealed that much of the radioactivity was contained in decomposition products and that only about 20% were chloroform-soluble aflatoxins, these containing about the same proportion of the radioactivity. Repeated chromatography of the 2 products on silica gel TLC plates removed most of the impurities (together with a good deal of the radioactivity), and, finally, fractions were obtained which were sufficiently pure to be recrystallized with the respective unlabeled aflatoxins. Several recrystallizations from chloroform-methanol yielded compounds of constant specific activity; analysis by TLC (7) showed that more than 80% of the radioactivity applied as crystalline aflatoxins was in the fluorescent band (82% for $B_1$, 86% for $G_1$). The respective specific activities of the 2 crystalline compounds were 750 $\mu$Ci/mg of $B_1$ and 325 $\mu$Ci/mg of $G_1$. The

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2Present address: Eppley Institute for Research in Cancer, University of Nebraska College of Medicine, Omaha, Nebraska 68105.
3Recipient of a Research Career Development Award, USPHS.
4Recipient of a Research Career Development Award, USPHS.

5The abbreviation used is: TLC, thin-layer chromatography.
preparation and purification of similar samples of the tritium-labeled aflatoxins have been described in detail (6).

Subsequent studies of the labeled aflatoxins have shown that partial decomposition takes place during thin-layer chromatography; repeated chromatography of a homogeneous fluorescent aflatoxin-$^3$H zone leads to continuous production of radioactive material that appears outside the fluorescent zone on the TLC plate. This indicates that the purity of the aflatoxins was higher than the above figures. There is, of course, a high probability that similar decomposition of the highly labeled aflatoxin molecules occurs during the animal treatments, but errors on this account in the present studies will be similar to errors in investigations which have used other chemicals labeled at high specific activity.

Groups of female MRC rats were treated with a single i.p. injection of a solution of 1 of the labeled aflatoxins in 50% aqueous dimethylformamide. Fourteen animals were each given injections of 0.5 ml of solution containing 0.9 mg of aflatoxin $B_1$ (650 $\mu$Ci). A 2nd group of 14 animals was treated with 0.3 ml of solution containing 1.25 mg of aflatoxin $G_1$ (410 $\mu$Ci). The dose injected was determined by spectrometric assay and radioassay of the solution injected.

Two of the treated rats of each group were killed at each of the following times after treatment: 1 hr, 6 hr, 18 hr, 1 week, 4 weeks, and 8 weeks. The liver, kidneys, spleen, and small intestines were dropped immediately into liquid nitrogen. The tissues were then homogenized, and DNA, RNA, and soluble protein were isolated by a modification of a method of Kirby and Cook (4), which has been described in detail (8). In most cases, sufficient nucleic acid was obtained, even from spleen and kidney, for radioassay.

Because the nucleic acids, particularly DNA, frequently contained extraneous material (probably carbohydrate), it was felt that specific activities based on weighed quantities of the nucleic acids would be imprecise. To obviate this difficulty, the estimations were based on absorbance determinations with standard values of 1.0 A equaling 40 $\mu$g DNA/ml or 46 $\mu$g RNA/ml at the absorbance maximum near to 260 $\mu$m (absorption spectra were taken on an automatically recording spectrophotometer, Cary Model 15). Approximately 1 mg (in some cases, however, much less was available) of the nucleic acid was dissolved in 5.0 ml of distilled water in a counting vial by warming at 60°C for a few minutes. A 1.0-ml aliquot of the solution was withdrawn and diluted with 4.0 ml of water; the absorption spectrum of this solution from 320 to 240 $\mu$m was taken. The remaining solution in the vial was evaporated to dryness in a stream of nitrogen. 20 drops of NCS solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill.) were added together with 2.0 ml ethanol and 13 ml scintillation mixture (5 g PPO + 0.3 g POPOP/liter of toluene), and the solution was counted in a Packard Tri-Carb liquid scintillation spectrometer. Appropriate corrections were made for quenching (with an internal toluene-$^3$H standard), and the specific activity of the nucleic acid was calculated. The protein was assayed by dissolving 2 to 5 mg of protein in 20 drops of solubilizer and proceeding as described.

RESULTS

The specific activities of DNA, RNA, and protein isolated from liver, kidney, spleen, and intestines at different times after treatment of rats with tritium-labeled aflatoxin $B_1$ are shown graphically in Chart 1. The corresponding figures for the rats treated with aflatoxin $G_1$ are shown in Chart 2. In both cases, the maximum incorporation appeared to take place between 6 and 18 hr after injection of the labeled aflatoxin. Also, incorporation in DNA, RNA, and protein of the liver was considerably greater than in the macromolecular components of the other organs, but the specific activity of these components of the liver fell off much more rapidly than in the kidney or the spleen. The incorporation of aflatoxin $G_1$ into DNA and RNA of the liver was considerably higher than that of aflatoxin $B_1$, whereas the incorporation into soluble protein was about the same.

Some estimation can be made of the proportion of the dose of radioactivity incorporated in the macromolecules of...
the organs examined. Assuming average organ weights per rat of 8 g liver, 1.2 g kidney, and 0.3 g spleen (the figures for the intestine are not meaningful, since only an arbitrary 10-cm section of small intestine was examined), the figures are as follows. Aflatoxin B₁ at 6 hr: 10.2% was bound, of which 10% was in the liver, almost all to protein, 0.009% to nucleic acids; 0.17% was in the kidney, almost all to protein; and 0.02% was in the spleen. Aflatoxin G₁ at 18 hr: 2.85% was bound, of which 2.63% was in the liver, 2.4% to protein, 0.19% to RNA, and 0.04% to DNA; 0.2% was in the kidney, of which 0.006% was to nucleic acids; and 0.014% was in the spleen, of which 0.001% was to nucleic acids. It is not known what weight of aflatoxins or their metabolites these figures represent, since the loss of tritium in covalent binding of the aflatoxins cannot be estimated, but is probably significant.

For determination of whether the high specific activity of the protein might be due partially to adsorption of labeled material, a representative protein sample was dialyzed. The liver protein of animals treated with aflatoxin B₁ 6 hr previously (207 mg, 81,000 dpm/mg) was suspended in 20 ml water and dialyzed for 50 hr against 200 ml methanol. The dialysate was evaporated to dryness, the residue was dissolved in 5 ml ethanol, and an aliquot was counted. The total radioactivity was 177,000 dpm. The same protein suspension was then dialyzed for 78 hr against 300 ml water, and the residue after evaporation of the dialysate to dryness was also assayed for radioactivity. The total activity in the dialysate was only 1.5% of that in the protein sample.

Tissues of the animals killed were fixed for histological examination. Details of the pathological examination will be given elsewhere, but the only lesions discernible (by light microscopy) were severe necrosis in the liver, observed at 18 hr and persisting to the 4th week after treatment. The other organs appeared normal.

We have attempted to determine the nature of the aflatoxin moiety bound to nucleic acids by hydrolysis and chromatography of the product. Such experiments have indicated that the labeled material in the "bound" aflatoxin is destroyed during hydrolysis. In a typical experiment, 1.2 mg of rat liver RNA isolated 6 hr after treatment with aflatoxin G₁ and containing 16,500 dpm were dissolved in 2 ml 0.1 N HCl and warmed 4 hr at 60°, followed by 2 hr at 100°. The solution was evaporated to dryness at room temperature in a stream of nitrogen, with addition of ethanol to facilitate evaporation. The residue was dissolved in a small volume of ethanol and chromatographed on a TLC plate of silica gel (5 x 20 cm), with a mixture of butanol and acetone containing a little acetic acid as solvent (This mixture has been suitable for separating hydrolysis products of nucleic acids). There were 2 fluorescent bands at the solvent front and an absorbing band near the origin; the remainder of the chromatogram was colorless and non-fluorescent. The absorbing and fluorescent bands were scraped from the plate, and the remainder was divided arbitrarily into 4. The material in each of the 7 bands was eluted with 5 ml ethanol; 1 ml of each was evaporated to dryness, dissolved in scintillation mixture, and counted. While recovery of radioactivity was complete (100% of the amount in the RNA hydrolyzed), there was no concentration of activity in any 1 band which would signify the presence of a single compound. Instead, each of the bands except the origin was radioactive and contained between 9 and 27% of the total radioactivity.

DISCUSSION

Aflatoxins B₁ and G₁ were incorporated into both RNA and DNA of the liver; this incorporation was maximal between 6 and 18 hr after the injection. The incorporation of aflatoxin G₁ was especially high. However, the extent of incorporation dropped off sharply between 18 hr and 1 week, probably owing to excretion of the bound aflatoxin or its metabolites. It was only in the extent of this initial interaction that the liver differed from kidney, spleen, and intestine, for there was also appreciable incorporation of the labeled aflatoxins into the nucleic acids of these organs.

There was no difference between the persistence of the binding of the aflatoxins (or their metabolites) to the nucleic acids of the liver and to those of the other organs. No correlation can be made with the established fact that these aflatoxins produce tumors in the liver, but not in the other 3 organs [except occasionally in the kidney with aflatoxin G₁ (1)]. There is also no correlation with the higher carcinogenic potency of aflatoxin B₁ compared with G₁ (1). One feature of these results is the very high binding to soluble protein shown by both aflatoxins. This correlates with autoradiographic examination of the liver and kidneys, which reveal a much greater density of radioactivity in the cytoplasm than in the nuclei (5). Further, the protein binding appears to correspond to that of the nucleic acids and is equally persistent. After 2 months, the binding of aflatoxin G₁ to protein in the kidney is more than one-third of its maximum value. At this time, the kidney protein is much more highly labeled than liver protein. Also, spleen protein remains strongly labeled, while the labeling of nucleic acids is dropping off rapidly. This binding of aflatoxin to soluble protein is not due to adsorption, since dialysis of a sample of protein for a week against aqueous methanol, methanol, and water removed less than 1.5% of the radioactivity present; the binding is probably covalent.

It has not yet been possible to determine the nature of the moiety of either aflatoxin (or the metabolites) bound to nucleic acids. Attempts to work up the hydrolysates of the nucleic acids by ion exchange chromatography (as had been the custom in the experiments with labeled nitrosamines) have led to loss of much of the radioactivity, either by destruction of the labeled compound or by retention on the column. Separation of the products of hydrolysis by chromatography on paper or TLC plates has not led to isolation and identification of a compound derived from either aflatoxin.

It would be difficult to deduce from the results presented here that a connection exists between interaction of the aflatoxins or their metabolites with DNA, RNA, or protein per se and carcinogenesis. It has been shown (2) that single injections of aflatoxin into rats does give rise to a high incidence of liver tumors, which is not true of nitrosamines.
It is possible that a relation does exist between tumor development in a particular organ and interaction of aflatoxin or its metabolites with some component of the nucleic acids or proteins, rather than with the rough preparations described here. The demonstration of this requires much more extensive work than has been done, and experiments in this direction are in progress.

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