Cell Loss and Proliferation Induced by \(N\)-2-Fluorenylacetamide in the Rat Liver in Relation to Hepatoma Induction

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SUMMARY

Cell loss and replacement were examined in relation to tumor induction following various doses and exposure patterns of the carcinogen \(N\)-2-fluorenylacetamide (2-FAA) in the diet of male albino rats. In Experiment 1, the hepatic DNA was prelabeled by the injection of thymidine-\(^{3}\)H (TdR-\(^{3}\)H) into weanling animals, and the loss of the tritium activity was measured at various times after the start of the carcinogen treatment. In Experiment 2, single injections of TdR-\(^{3}\)H were given at various times during continuous exposure to the carcinogen at levels of 0.03, 0.01, and 0.003\%. The uptake of TdR-\(^{3}\)H in hepatic DNA, the parenchymal cell-labeling index, and the total hepatic DNA were determined. In Experiment 3, the incidence of hepatic carcinoma was determined for various exposure durations to 0.03, 0.01, 0.003, and 0.001\% 2-FAA. At 42 days after the start of administration of 0.03\% 2-FAA, the uptake of TdR-\(^{3}\)H in the hepatic DNA increased markedly. With 0.03\% 2-FAA, the pulse labeling index increased progressively between 42 and 120 days, while the pulse labeling index for 0.01\% 2-FAA showed no increase until 120 days. With 0.03\% 2-FAA, the prelabeled DNA decreased markedy between 42 and 72 days, while the total hepatic DNA increased by about 15 to 20\% by 72 days. The tumor data showed that a 365-day exposure to 0.01\% 2-FAA was approximately equivalent to a 112-day exposure to 0.03\% 2-FAA, i.e., an equivalent tumor yield for equal total doses. However, 28- and 56-day exposures to 0.03\% 2-FAA were far less effective than 112-day exposures to 0.01\% 2-FAA. The data suggest that, at 0.03\%, the carcinogen produced an increase in the parenchymal cell replication rate that correlated with the tumor incidence and that, when the carcinogen was stopped early enough to prevent tumors, very little of the original DNA was lost and the replication rate of the parenchymal cells was only slightly increased.

INTRODUCTION

Hepatic carcinogens produce a variety of changes in the hepatic cell populations prior to hepatoma formation (3, 11, 13, 15, 20, 22, 26, 27). Some carcinogens, such as 4-dimethylaminoazobenzene, produce histological evidence of early death of cells in the centrilobular regions, and the liver responds with an increase in the proliferation rate in the portal region (25). Other carcinogens, such as ethionine, 2-FAA, \(^{3}\)dimethylnitrosamine, and aflatoxin, do not produce clear histological evidence of early cell death but do induce cirrhotic reactions to varying degrees, which may involve the proliferation of oval cells, bile duct cells, fibroblasts, and the formation of nodules of regeneration of parenchymal cells (3, 6, 8, 11, 15, 22, 26).

In addition to producing cirrhosis, hepatic carcinogens usually produce hyperplastic parenchymal cell nodules, which are believed by some to be the precursor lesions for hepatomas (6, 11, 12). Both the cirrhotic reaction and the hyperplastic nodules are reversible to some extent if the carcinogen is stopped (22, 27). The kinetic changes, including cell death and regeneration, of the hepatic cell populations are poorly understood in relation to their quantitative effects on the induction of hyperplastic nodules and hepatomas (4, 5, 17, 19, 24–26).

The studies described here were the 1st in a series designed to measure cell killing and proliferation as a function of the dose and exposure pattern to the carcinogen 2-FAA. The carcinogen was administered continuously for various periods of time, and the cell killing, repopulation, and tumor incidence were determined.

MATERIALS AND METHODS

Charles River (CD) male albino rats, obtained as weanlings, were used in all experiments. The carcinogen 2-FAA was administered in a mash of Purina laboratory chow.

The magnitude of cell loss was measured in terms of the loss of tritium activity from the total liver DNA in rats tagged with TdR-\(^{3}\)H as weanlings (i.e., prior to the onset of 2-FAA exposure). For the purpose of tagging hepatic DNA, weanling rats (beginning at the age of 26 days) were given multiple i.p. injections of TdR-\(^{3}\)H. In most cases, a total of 80 \(\mu\)Ci was given in divided doses on each of 4 successive mornings; however, a usable level of tagging was also produced by 3 daily injections totaling 36 \(\mu\)Ci.

The rate of hepatic cell proliferation was assessed by the TdR-\(^{3}\)H autoradiographic L.I. of hepatocytes and by the \(^{3}\)The abbreviations used are: 2-FAA, \(N\)-2-fluorenylacetamide; TdR-\(^{3}\)H, thymidine-\(^{3}\)H; L.I., labeling index (percentage of labeled cells).
uptake of TdR-\(^3\)H in liver DNA. Both determinations were made in rats previously unexposed to TdR-\(^3\)H that were given an i.p. injection of TdR-\(^3\)H between 1.5 and 2.0 hr before sacrifice. In most cases, a TdR-\(^3\)H dose of 1.0 \(\mu\)Ci/g of body weight was used, but rats receiving 0.5 \(\mu\)Ci/g of body weight gave comparable results.

Sacrifice was done by ether anesthesia and cervical dislocation. The livers were removed promptly, drained of blood, and weighed. Prior to being frozen and stored in liquid nitrogen, wedges were taken for histology and autoradiography. The tissue wedges were fixed for 3 hr in Carnoy’s fluid and then were fixed in absolute ethanol. Histological sections were stained with hematoxylin and eosin and, in some cases, with Best’s carmine for glycogen. Autoradiographs were made with Kodak NTB-3 liquid emulsion and were exposed (on an average of) 4 weeks.

The liver cell population was estimated by chemical assay of the total liver DNA. Perchloric acid was used in the DNA extraction procedure (23). The frozen liver was homogenized (Omni-Mixer; Ivan Sorvall, Inc., Norwalk, Conn.) in 20 ml of cold (approximately 4°) distilled water for 1 min at 5,000 rpm and for 1 min at 14,000 rpm. An equal volume of cold 10% perchloric acid was added, and the mixture was centrifuged at 4,080 \(\times\) g for 10 min in a refrigerated centrifuge (4°). A filter trap was used for supernatant removal in order to minimize the loss of floating solids. The residue was resuspended in 75 ml of cold 5% perchloric acid and was centrifuged at 4,080 \(\times\) g for 5 min. This washing procedure was repeated first with cold 95% ethanol, then with cold ether:ethanol (3:1), and finally with cold 5% perchloric acid. DNA hydrolysis was done in 5% perchloric acid for 20 min at 90°. An 0.2-ml aliquot of the hydrolysate was used to assay the hepatic DNA by the Burton reaction (2). A tritium assay of the hydrolysate was done by liquid scintillation counting, with an efficiency of about 8%.

The autoradiographic L.I.’s were based on counts of 1 slab taken from the lower edge of the left lobe of each rat. Counts of labeled parenchymal cells were made on each autoradiograph on 20 consecutive fields of 0.04 sq mm, roughly in the center of the section. Parenchymal cell counts were made on 10 consecutive 0.01-sq mm fields also in the center of most of the sections used for counting labeled cells. In heavily cirrhotic livers, the narrow bands of oval cells and fibroblasts between nodules were excluded from the counts.

RESULTS

The 3 experiments, involving a total of 632 rats, are described below.

Experiment 1. The Persistence of Prelabeled Hepatic DNA during Exposure to 2-FAA. The DNA of weanling rats (26 to 29 days old) was labeled with TdR-\(^3\)H, and the loss of \(^3\)H from the liver DNA was determined at various times before and after the start (when rats were 55 days old) of 0.03% 2-FAA in the diet. The incidence of cirrhotic nodules was determined histologically. A total of 24 rats (6 rats/time point) were sacrificed at 55, 80, 100, and 127 days of age for an assay of the retention of \(^3\)H in the liver DNA. A total of 88 control rats (about 15 rats/time point) were sacrificed at 29, 43, 55, 80, 100, and 127 days of age for determination of body and liver weight and of total liver DNA. The latter 3 quantities are shown in Chart 1, lower graph, and they indicate that during the early weeks of carcinogen exposure the body weight was increasing. However, the total liver DNA reached a plateau by about 80 days of age, i.e., about 3 weeks after the start of the carcinogen exposure. The liver approximately doubled in weight between the end of the labeling procedure (29 days of age) and the start of the carcinogen administration. The average percentage of labeled cells 1 hr after the last TdR-\(^3\)H injection (at 29 days of age) was 8.6%, and the parenchymal and nonparenchymal cells were labeled to about the same extent.

The amount of \(^3\)H in the hepatic DNA of control rats, expressed at each point as a percentage of the average of all 4 points, is shown in Chart 1, upper graph, and was constant within experimental error during the interval. The average amount of \(^3\)H in the hepatic DNA was 0.5% of the total injected dose.

Chart 2 shows the total \(^3\)H in the liver DNA, and the total liver DNA as a percentage of the average control values versus days, with 0.03% 2-FAA. The total \(^3\)H activity in the DNA was not significantly reduced at 25 and 45 days, but a sharp drop to 29% of the control level occurred at 72 days. The total amount of liver DNA was increased relative to controls by about 15% during the 72-day 2-FAA exposure.

None of the rats on 0.03% 2-FAA had cirrhotic nodules at 25 days, while about one-third of them had small cirrhotic nodules at 45 days, and 90% (12 of 15 from both Experiments 1 and 2) had well-developed nodular cirrhosis by 72 days. Chart 2 shows a curve that represents the amount of new DNA that had appeared in the liver as a function of exposure time. The new DNA, as a percentage of the DNA in the control livers, was obtained by subtracting the TdR-\(^3\)H-DNA retention curve from the liver DNA curve in Chart 2.

Experiment 2. The Uptake of TdR-\(^3\)H in the Liver DNA and the Pulse L.I. Three dietary levels of 2-FAA (0.03, 0.01, and 0.003%) were administered for 96 days beginning at 55 days of age. At 25, 45, 72, 121, and 239 days after the start of the 2-FAA exposure, the livers of rats from each dietary group were removed promptly, drained of blood, and weighed. Prior to being frozen and stored in liquid nitrogen, wedges were taken for histology and autoradiography. Tissue wedges were fixed for 3 hr in Carnoy’s fluid and then were fixed in absolute ethanol.
Albert, Burns, Bilger, Gardner, and Troll

Chart 2. The total liver DNA (percentage of control), the retention of \(^3\)H in the liver DNA (percentage of control), the amount of new DNA (the difference between the liver DNA and TdR\(^{-3}\)H-DNA retention curves), and the incidence of nodular livers (percentage of rats with evidence of cirrhotic nodulation). The liver DNA had been tagged by i.p. injections of TdR\(^{-3}\)H, given when the rats were weanlings (26 to 39 days of age). The 2-FAA was begun when rats were 55 days old (Experiment 1).

The carcinogen, 3 rats from each dose group and 3 control rats were given i.p. injections of TdR\(^{-3}\)H, 1.0 \(\mu\)Ci/g, at 2.0 hr before sacrifice, and the uptake of \(^3\)H in the liver DNA was determined. In addition, total liver DNA, liver weight, and body weight were determined. The pulse L.I. of the parenchymal cells was determined radioautographically for dose levels of 0.03, 0.001, and 0.003\% in 6 rats at the times given above, and at 98 days for 0.03\% 2-FAA.

Body and liver weight, total liver DNA, and the uptake of TdR\(^{-3}\)H into hepatic DNA at the time of sacrifice are shown in Chart 3. The 2-FAA data are expressed as a percentage of the data obtained from control rats sacrificed at the same time. The uptake in the control rats was 0.14\% of the injected dose, corresponding to 25 and 45 days after the start of 2-FAA exposure, and was 0.10\% at 72, 121, and 239 days.

Body weight showed a dose-dependent decrease during 2-FAA exposure which reached a minimum by 72 days. There was a return toward normal after the end of 2-FAA exposure. Liver weight also declined during 2-FAA exposure as a function of dose and, at 0.03\%, rebounded above control levels after the carcinogen was discontinued. The total liver DNA remained essentially constant, except for a slight dip at 45 days, and after exposure to doses of 0.01 and 0.003\% 2-FAA. The 0.03\% 2-FAA dose produced a slow increase in total DNA which reached 120\% of that of controls by 72 days and rose to 135\% by 239 days, which was 143 days after the end of the exposure. The uptake of TdR\(^{-3}\)H in hepatic DNA showed a dose-dependent increase by 45 days that persisted even after the end of 2-FAA exposure at 96 days.

The pulse L.I. of the parenchymal cells was based on counts of a section from a slab taken from the lower edge of the left lobe of each rat. In a test group of 22 rats, the pulse L.I. in the left lobe proved to be an excellent indicator of the average L.I. of the entire liver. Five of the rats were control animals, and the rest included approximately equal numbers of rats from each of the sacrifice times at the 0.03\% 2-FAA exposure level. This selection provided comparisons at a wide range of L.I.'s. For each of the 22 rats, a ratio was obtained by dividing the L.I. of the left lobe by the mean for all 4 lobes. The agreement was excellent, since the average ratio was 1.02 (a = 0.22).

Only parenchymal cells were counted. In control livers, this meant excluding bile duct and littoral cells while, in cirrhotic livers, counts were made only within cirrhotic nodules of cells that resembled normal parenchymal cells, i.e., large cells with a relatively large amount of cytoplasm.

The pulse L.I. of the parenchymal cells is shown in Chart 4. The control rats showed a constant L.I. that ranged from 0.16 to 0.19\%. At a dose level of 0.03\% 2-FAA, the parenchymal cell L.I. was about 0.35\% at 25 and 45 days of exposure, and it rose to 0.90 and 1.14\% at 72 and 98 days, respectively. The parenchymal cell L.I. was markedly elevated at 121 days (i.e., 25 days after the end of 2-FAA exposure) to a level of 1.45\%, or 8 times the control level, but dropped to 0.44\% by 239 days.

The 0.01\% 2-FAA dose group showed no elevation in the pulse L.I. by 72 days; in fact, the L.I. value was lower than the average control level. No L.I. data were available for this group...
Hepatic Cell Loss with 2-FAA

at 98 days, but an elevation to 0.66% occurred at 121 days, followed by a decline to 0.44% at 239 days. The 0.003% 2-FAA dose group showed no appreciable change in L.I. throughout the observation period during and after the 2-FAA exposures.

Experiment 3. The Induction of Hepatic Carcinoma by Various Doses and Exposure Durations of 2-FAA in the Diet. The experiment involved a total of 260 rats in 16 treatment groups of 14 to 16 rats/group. Four 2-FAA dietary levels of 0.03, 0.01, 0.003, and 0.001% were started when rats were 59 days of age. The following 4 exposure durations were used at each dietary level: (a) 0.03% 2-FAA: 28, 56, 112, and 224 days; (b) 0.01% 2-FAA: 56, 112, 224, and 448 days; (c) 0.003% 2-FAA: 112, 224, 336, and 448 days; and (d) 0.001% 2-FAA: 112, 224, 336, and 448 days.

Autopsies were performed when the rats died or were sacrificed when moribund for histological confirmation of grossly observed liver tumors. A total of 24 carcinomas were found; of these, 21 were parenchymal cell carcinomas and 3 were biliary carcinomas.

In the 616-day observation period, no tumors occurred in the controls or in the 0.01% and 0.003% dose groups. The tumor incidences were calculated by the life table method and are shown in Chart 5 for the 0.03 and 0.01% 2-FAA groups. For 0.03% 2-FAA, the 28-day exposure did not produce any cancers, the 56-day exposure was marginally carcinogenic, and high yields occurred with 112- and 224-day exposures. In the 0.01% 2-FAA treatment groups, the 56-day exposure was noncarcinogenic, and substantial cancer yields occurred with exposures of 112 days and longer.

DISCUSSION

Prior to the onset of the cirrhotic reaction with 0.03% 2-FAA, there was clear evidence of both general toxicity and hepatotoxicity, as evidenced by a 20% body and liver weight loss. Toxicity in the form of interference by 2-FAA with protein and RNA synthesis has been reported (18). The

histological changes produced in these experiments were similar to those described by others for 2-FAA (11, 22). Variable amounts of oval cell proliferation and parenchymal cell shrinkage occurred while the development of cirrhotic nodules progressed steadily, until by 72 days the nodules comprised over 90% of the liver volume, as judged by histological sections.

Radiotoxicity associated with the dose from the β particle of tritium was unlikely. The tritium dose to the nucleus can be estimated from measured values of the activity per nucleus. It has been reported that 0.022 dpm/nucleus (in bone marrow) results in a dose rate of 6 rads/day (1). In this experiment, the average tritium activity per labeled nucleus in controls was 0.002 dpm, representing a dose rate of 0.5 rad/day, which is unlikely to produce significant lethality.

The loss of TdR-3H activity from the liver DNA of controls in this study was as slow as that reported by others (14, 21). A rapid loss of TdR-3H has been reported during exposure to 3-methyl-4-dimethylaminoazobenzene at a level of 0.05% in the diet; this result was interpreted as evidence for DNA repair synthesis (14). The findings reported here for 0.03% 2-FAA are quite different, in that there was little loss of TdR-3H from the DNA of rats tagged when weanlings in the 1st 42 days of exposure, whereas a rapid loss occurred between 42 and 72 days, as the nodular reaction progressed. The delay tends to support cell death as the dominant mode of TdR-3H DNA loss, since DNA repair should occur relatively soon after exposure to the carcinogen.

The total DNA may not reflect the actual number of cells present in the liver, because there is evidence for a downward shift in ploidy during 2-FAA carcinogenesis (16, 17). However, the maximum possible reduction of DNA per cell would be a factor of 2, and a more realistic estimate would be about 1.5.
If a reduction in DNA per cell did occur, the actual loss of original liver cells would have exceeded the amount shown in Chart 2, i.e., the DNA loss may underestimate the true cell loss.

The parenchymal cell L.I. and TdR-3H uptake followed a similar pattern, except that the increase in the former exceeded the increase in the latter by a factor of about 2. This discrepancy could possibly be explained by the occurrence of metabolic turnover of the hepatic DNA, although the constancy of the tritium level in the prelabeled DNA (Chart 1) is evidence against this explanation (21). It is also possible that the parenchymal cell L.I. increased more than the average L.I. of the whole liver; however, spot counts failed to confirm this.

The average pulse L.I. of the parenchymal cells in the 0.03% 2-FAA treatment group was about 1%, which gives a potential doubling time (if no cell loss occurred) of about 33 days (duration of DNA synthesis assumed to be 0.33 day), whereas the total DNA was increasing relative to controls with a doubling time of about 360 days (Chart 2). The magnitude of this discrepancy suggests that a considerable amount of cell loss was occurring. No evidence for an increased duration of the S phase was found, since the grain counts in treated and control livers were comparable. While it may be inferred that overall cell loss did occur, further data are required for determination of the relative magnitude of cell loss within the various subpopulations of hepatic cells. Parenchymal cells exhibit functional evidence of damage in terms of a prolonged G2 period (24), abnormal DNA (9), and abnormal glycogen (8). There is evidence of a slow release of glycogen-bound carcinogen after the removal of 2-FAA from the diet (10), which may help to explain the persistent high L.I. after the end of the carcinogen treatment (Chart 4).

The tumor response (Chart 5) follows the same general pattern observed by others (7), with the lower dose rates or shorter exposure periods resulting in a need for longer periods for tumor formation. The data suggest that dose patterns that do not cause substantial elevations in parenchymal cell L.I. are relatively nontumorigenic, for example, 0.003% 2-FAA. It also appears that the higher the 2-FAA dose level, the earlier and more pronounced is the elevation in L.I. and the shorter is the appearance time of hepatic cancers.

It appeared that the critical period of carcinogen exposure for tumor induction at dose levels of 0.03 and 0.01% 2-FAA was somewhat greater than 56 days. Shorter exposures were tumorigenically ineffective in an 88-week experiment, while longer exposures did not produce a proportionately greater decrease in tumor induction time.

Since a 112-day exposure to 0.03% 2-FAA gave a tumor yield that was equivalent to a 336-day exposure to 0.01% 2-FAA, it would be expected that a 56-day exposure to 0.03% 2-FAA should be more effective in producing tumors than an exposure of 112 days to 0.01% 2-FAA. However, the reverse was true. A 56-day exposure to 0.03% 2-FAA was sufficient to reduce the liver and body weight, to increase the pulse L.I. (Chart 4) and the uptake of TdR-3H in the hepatic DNA (Chart 3), and to cause a mild cirrhosis (Chart 2). However, it would appear (from the relatively low tumor incidence in the same exposure group) that effects occurring prior to about 56 days were reversible or contributed relatively little to tumor induction. With 96 days of exposure to 0.01% 2-FAA, the increase in pulse L.I. was delayed but was of approximately the same magnitude as that produced by 56 days of exposure to 0.03% 2-FAA. If an increase in pulse L.I. were a necessary requirement for, or an indicator of the intensity of, tumor induction (4, 5, 19), the data suggest that increases that occur after about 100 to 120 days are more likely to be associated with tumor induction than are increases that occur before that time.

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Cell Loss and Proliferation Induced by N-2-Fluorenylacetamide in the Rat Liver in Relation to Hepatoma Induction


*Cancer Res* 1972, 32:2172-2177.

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