Effect of an Extract of UV-irradiated Linolenic Acid on Azo Dye Carcinogenesis

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SUMMARY

One to four intraperitoneal injections of water extract of UV-irradiated linolenic acid were given to male Osborne-Mendel rats. Injections were at monthly intervals with graded doses (120 to 600 thiobarbituric acid units/kg/dose) of the extract. Some of these rats received a subcarcinogenic dose (0.12%) of p-dimethylaminoazobenzene (DAB) in powdered Purina chow diet from the day of first injection for four months. The experiments were terminated two months later. Four hundred units or more of UV-extract alone were lethal within 72 hours, lower dosage of the UV-extract produced chronic toxicity, with swelling of the liver, fusion of its lobes, and adhesions to surrounding structures. DAB in the diet for 4 months and 3 or 4 injections of UV-extract caused extensive cholangiofibrosis of the liver in all the animals and hepatoma nodules in 25% of the rats. Activity of some oxidases, dehydrogenases, and phosphatases of the livers of these rats was 25 to 40% lower than that of the comparable enzymes in control rats.

INTRODUCTION

Polyunsaturated fatty acids are extremely susceptible to oxidation, and the oxidized products inhibit bacterial growth and are toxic to mammalian systems (2, 13, 19). Oleic, linoleic, linolenic, and arachidonic acids are the predominant unsaturated fatty acids of biologic interest. A number of environmental factors such as heat, light, and ionizing radiation are capable of influencing the rate and course of oxidation of these fatty acids.

Oxidized products which are released after incubation of animal tissues and detected by the thioarbituric acid (TBA) test are considered to arise from the autoxidation of lipids present in these tissues (2). Accordingly, methyl linolenate or methyl arachidonate, after irradiation with ultraviolet light, gave water-soluble products capable of reacting with the TBA reagent to form a characteristic pink coloration. The water-soluble products were found to inhibit succinoxidase, cytochrome oxidase, and cholinesterase activities of mammalian liver (19).

Recently, it was shown that the aqueous extracts of the ultraviolet-irradiated linolenic acid contain peroxides and carbonyl compounds. The latter react with the TBA reagent to form a pink coloration (14, 15). These extracts, when injected into mice, induce hyperplastic changes in the liver and skin of these animals (22, 24). Since the oxidation products of the unsaturated fatty acids can elicit inflammatory changes in the liver of mice (23) and rabbits (11), the effect of these products on hepatocarcinogenesis in the rat was examined.

This paper deals with lesions induced in rats when aqueous extracts of UV-irradiated linolenic acid were given alone or in combination with a subcarcinogenic dose of p-dimethylaminoazobenzene (DAB).

MATERIALS AND METHODS

Male Osborne-Mendel rats, about 3 months old and weighing approximately 300 gm, were used in all the experiments. The number of rats used, the general plan of diet, the i.p. injections, and the time of sacrifice for the experimental animals are given in Table 1. The experimental animals were fed powdered Purina laboratory chow containing 0.12% DAB in 5% corn oil for 4 months and, after this, chow alone for the next two months when the experiment was terminated. The animals received i.p. injections of 1 ml of water extract of UV-irradiated linolenic acid (UV-extract) on the day they started to receive the DAB diet. Two, three, or four injections of UV-extract were given at monthly intervals during the time these animals were on the DAB diet. A group of animals fed the carcinogenic diet but not given any UV-extract served as DAB controls.

Other control groups were rats receiving 1 to 4 i.p. injections, 120 units/kg of UV-extract alone and no DAB in the diet, those receiving only one injection of 300, 350, 400, or 600 units/kg of UV-extract alone, and those receiving 1 to 4 injections of an extract of nonirradiated linolenic acid and DAB in the diet. The injection and sacrifice schedule of these rats was the same as shown in Table 1, except for 2 groups receiving 400 and 600 units/kg of UV-extract. They showed symptoms of acute toxicity and died prematurely. Some rats were given i.p. injections of 2 or 4 ml of 3% H₂O₂ twice a week for 5 weeks and sacrificed at the end of that period.

Linolenic acid (5 gm) was irradiated with ultraviolet light for 18 hours as described previously (22); it was then extracted...
RESULTS

available purity from commercial sources, and linolenic acid by Appelmans et al. (1); the inorganic phosphate released was estimated by the procedure of Fiske and SubbaRow (3). Iso-
citric dehydrogenase activity was assayed by the procedure of Hogeboom and Schneider (5). Uricase activity was determined spectrophotometrically (16), and a millimolar absorptivity of 12.2 for uric acid at 292 m\(\mu\) (9) was employed in the calculation of results.

All chemicals used in this investigation were of the highest available purity from commercial sources, and linolenic acid (99% purity) was purchased from the Hormel Institute.

**Table 1**

<table>
<thead>
<tr>
<th>Number of rats</th>
<th>Time in months</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>S S S S</td>
</tr>
<tr>
<td>6</td>
<td>FA FA FA</td>
</tr>
<tr>
<td>6</td>
<td>FA FA FA FA</td>
</tr>
<tr>
<td>6</td>
<td>FA FA FA FA</td>
</tr>
<tr>
<td>12</td>
<td>FA* FA* FA*</td>
</tr>
</tbody>
</table>

Time relation of p-dimethylaminoazobenzene (DAB) feeding, injection of irradiated fatty acid extract (FA), 120 units/kg, and sacrifice (S). DAB (0.12%) in lab chow was given during months 0—4; lab chow alone was given during months 5 and 6.

with distilled water and concentrated by lyophilization. On thawing, the amount of the oxidation products in the extract was measured by employing the thiobarbituric acid (TBA) test. To an appropriate dilution of the extract made to 1 ml with water, 2 ml of TBA reagent (22) were added, and the mixture was heated for 20 minutes in a boiling water bath. The intensity of the pink color that developed in the mixture was measured in a spectrophotometer at 532 m\(\mu\) against a reagent blank. TBA units in a sample equal its optical density reading. The TBA activity was defined as the absorbance units per ml at 532 m\(\mu\) for the entire undiluted extract. The extract usually showed TBA activity of approximately 50 units per ml at 532 m\(\mu\). A similar procedure was employed to obtain an extract of nonirradiated linolenic acid which had a TBA activity of approximately 1 unit per ml.

During the course of the experiment, animals were killed with ether, necropsies were performed, and livers and other organs were examined for possible lesions. Representative samples of liver were kept in a Petri dish chilled in crushed ice until the tissues were utilized for enzymic studies. Other samples of organs utilized for histologic study were fixed in Zenker's acetic fluid, embedded in paraffin, and stained with hematoxylin and eosin.

Tissues were weighed and homogenized in 0.25 M sucrose solution for the enzyme assays. The homogenates were prepared in a smooth-walled glass tube fitted with a Teflon pestle. Succinoxidase activity was assayed manometrically (17). Glu-
tamic dehydrogenase activity was assayed spectrophotomet-
ically at 340 m\(\mu\) by measuring the rate of reduction of di-
phosphopyridine nucleotide in the presence of L-glutamic acid (6). Glucose-6-phosphatase activity was estimated as described by Appelmans et al. (1); the inorganic phosphate released was estimated by the procedure of Fiske and SubbaRow (3). Iso-
citric dehydrogenase activity was assayed by the procedure of Hogeboom and Schneider (5). Uricase activity was determined spectrophotometrically (16), and a millimolar absorptivity of 12.2 for uric acid at 292 m\(\mu\) (9) was employed in the calculation of results.

All chemicals used in this investigation were of the highest available purity from commercial sources, and linolenic acid (99% purity) was purchased from the Hormel Institute.

**RESULTS**

The incidence and extent of pathologic lesions in the experi-
mental animals is summarized in Table 2. For convenience, groups with slightly different treatments but having similar lesions are pooled together. In the rats receiving injections of UV-extract alone, there were signs of toxicity, and the degree of toxicity was roughly proportional to the number of TBA units/kg/injection and to the total number of such injections. A single injection of 600 or 400 TBA units/kg showed acute toxicity, and 70 and 30% of the rats from the respective groups died within 72 hours after the injection. In these rats, the blood vessels were distended and the lungs were congested, and there were internal hemorrhages and bloody fluid in the peritoneal cavity. All rats receiving a single injection of 300 units/kg or multiple injections of 120 units/kg lived for 30 days or longer after the last injection and showed symptoms of chronic toxicity. There were occasional internal hemorrhages, adhesions in the peritoneal cavity, swelling and fusion of the liver lobes and rounding of their margins, and thickening of the liver capsule. An example of the thickening of the liver capsule from a rat 35 days after a single injection of 300 units/kg of UV-extract is illustrated in Fig. 1. An example of the fusion of the liver lobes from a rat 75 days after the second injection of 120 units/kg of UV-extract is shown in Fig. 2. The rats receiving i.p. injections of 3% \(\text{H}_2\text{O}_2\) showed liver lesions similar to but much milder than the lesions ob-
erved in livers of rats receiving a single injection of 120 units/
kg of UV-extract. The majority of rats receiving injections of UV-extract or 3% \(\text{H}_2\text{O}_2\) showed a chronic nephritis with moderate to marked dilation of convoluted tubules and formation of hyalin casts (Fig. 3).

The combination treatment of DAB in the diet and one or two i.p. injections of 120 units/kg induced fusion of the liver lobes, occasional focal necrosis, and patchy lymphatic infiltr-
atation in portal areas. The liver lobes of the rats given 3 or 4 injections and killed at the end of the 5th or 6th month had grossly visible gray areas varying from 50 to 300 sq mm. The microscopic examination of these areas revealed cholangiofi-
brrosis (Fig. 4) and corresponded more or less with the gray areas observed with the naked eye. Many such areas were found in each liver. Higher magnification of this lesion (Fig. 5) showed numerous irregular deeply staining bile ducts sur-
rounded by proliferating fibroblasts and a few lymphocytes. Two of these livers showed microscopic hepatoma nodules (Fig. 6).

The livers of the rats receiving DAB alone showed occasional large groups of cells with clear or feathery cytoplasm, often faintly basophilic. Usually, adjacent portions of more than one liver lobule were involved (Fig. 7) (12).

The group of rats on 350 units/kg injections and DAB in the diet began to show symptoms of toxicity after the 3rd i.p. injection of UV-extract. These rats were sluggish; the abdomen was distended and the whiskers were reddened. The 4th injec-
tion of UV-extract was withheld from these rats. At necropsy these rats showed an enormously distended gastrointestinal tract that was packed solid with fecal material, and some bloody fluid was observed in the peritoneal cavity. The livers were usually swollen and fused into a mass. Histologically, the livers showed thickening of the capsule, fibrous adhesions, and hydropic swelling of hepatic cells, but no proliferative changes. The lungs were congested, the spleens were rough and dark, and there were adhesions all over the peritoneal cavity.
Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Congestion &amp; hemorrhage of lungs &amp; viscera</th>
<th>Ascites fluid</th>
<th>Thickening of capsule, fusion of lobes</th>
<th>Cholangiofibrosis</th>
<th>Hepatoma</th>
<th>Interstitial adhesions</th>
<th>Bowel obstruction</th>
<th>Nephritis</th>
<th>Remarks</th>
</tr>
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<tbody>
<tr>
<td>600 units/kg</td>
<td>10</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>7 died within 72 hours, others within 7 days</td>
</tr>
<tr>
<td>400 units/kg</td>
<td>10</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>3 died within 72 hours, others within 14 days</td>
</tr>
<tr>
<td>300 or 350 units/kg</td>
<td>9</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Killed 30 to 60 days after the treatment</td>
</tr>
<tr>
<td>120 units/kg, 1–4 doses</td>
<td>12</td>
<td>±</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Killed 30 days or more after last dose</td>
</tr>
<tr>
<td>3% H₂O₂, 3–6 doses</td>
<td>6</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Killed after 5 weeks from start</td>
</tr>
<tr>
<td>120 units/kg, 1 &amp; 2 doses + DAB</td>
<td>12</td>
<td>+</td>
<td></td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Killed as scheduled in Table 1</td>
</tr>
<tr>
<td>120 units/kg, 3 &amp; 4 doses + DAB</td>
<td>12</td>
<td>+</td>
<td>++</td>
<td>8</td>
<td>2</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>Killed as scheduled in Table 1</td>
</tr>
<tr>
<td>350 units/kg, 3 doses + DAB</td>
<td>12</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td>Killed as scheduled in Table 1</td>
</tr>
</tbody>
</table>

Incidences and extent of lesions in groups of experimental rats. Lesions are graded from + the least to ++++ the most on the basis of extent and severity. Numbers in lesion columns give number of cases.

aThiobarbituric acid units.
bpdimethylaminoazobenzene.

Studies were also conducted to examine changes in enzymic activities of these livers. The enzymic systems selected were those associated with the organelles of cytoplasmic origin. Table 3 shows mean values with S.D. in percent of controls in enzymic activity of the livers of experimental rats. In the animals receiving DAB and UV-extract, the livers that developed cholangiofibrosis and/or hepatomas showed significant loss (P less than 0.01) in all but two enzymes studied, while livers with no visible lesions showed significant loss only in glucose-6-phosphatase and isocitric dehydrogenase. The enzymic activity of the livers of rats receiving UV-extract alone showed no significant differences from the control values except in the case of acid phosphatase, which was increased. Similarly, enzymic activity of the livers of rats given DAB alone or extract of nonirradiated fatty acid showed no significant differences from the control values.

DISCUSSION

The data indicate that a subcarcinogenic dose of DAB, when combined with the administration of certain concentrations of UV-extract, induces cholangiofibrosis in the liver and hepatoma nodules. Neither of these treatments when employed alone induced these lesions. When administration of the extract was calculated in terms of TBA units/kg given in conjunction with a subcarcinogenic dose of DAB, it was found that a small amount of the UV-extract had no demonstrable effect either on bile duct proliferation or carcinogenesis, but a larger amount of the UV-extract induced cholangiofibrosis, and a still larger amount of the extract induced hepatomas. A further increase in the administration of UV-extract led to such a degree of toxicity, that normal growth was prevented and the rats died early.

The lesions induced in the livers of rats by UV-extract alone were similar to those reported in the rabbit liver after intravenous injection of methyl linoleate hydroperoxide (11). These lesions were similar but more severe than lesions induced with intraperitoneal injections of H₂O₂ in the experiments described in this paper.

The exact nature of the UV-extract which we used is not known. This extract has pronounced thiobarbituric acid activity, and the degree of toxicity of the extract has been demon-

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strated to be roughly proportional to the level of TBA activity administered. Irradiated linolenic acid has been found to contain carbonyl compounds and peroxides (15). Kohn and Liversedge (10) and Thiele et al. (21) have reported the production of TBA-reacting substances by incubation of mitochondrial and microsomal fractions of various tissues, but the incubation of hepatoma tissue did not yield similar substances in their experiments. This reaction is retarded by ascorbic acid, α-tocopherol, and many other drugs (10) and enhanced by hematin (20) and ferrous ions (7). These endogenous TBA-reacting substances lead to swelling and lysis of liver mitochondria (4, 7, 8) under certain circumstances. The evidence presented here supports the opinion that the damage caused by UV-extract may be similar to that caused by endogenous TBA-reactive substances, in the studies of mitochondrial fractions of liver cells.

Since hepatoma tissue contains no chemical precursors capable of inducing TBA-reactive substances (21) and has low oxidase activity (18), one could argue that these deficiencies may have blocked one oxidation reduction pathway and forced the cells to take another and thus induce neoplastic changes. However, any dose of UV-extract alone did not induce any cell proliferation, nor did 0.12% DAB alone induce cell proliferation. Thus, it is more likely that destruction of liver cells by UV-extract reduced the available number of cells for detoxification of DAB. This enabled DAB to remain in contact with hepatic cells for an extended period of time, which may have influenced the induction of proliferative changes in the hepatic cells.

REFERENCES


Figs. 1-7. All sections are from rats receiving i.p. injections (units/100 gm body wt./dose) of aqueous extracts of UV-irradiated linolenic acid. Hematoxylin & eosin.

Fig. 1. Marked fibrous thickening of liver capsule, in a rat 33 days after a single dose of 30 units of UV-extract. × 108.

Fig. 2. Partial fusion of liver lobes in a rat 75 days after one dose of 12 units of UV-extract. × 108.

Fig. 3. Chronic nephritis in a seven-month-old rat, 2.5 months after 2 doses of 12 units each of UV-extract. × 108.

Fig. 4. Extensive cholangiofibrosis in a rat receiving p-dimethylaminoazobenzene in the diet and 3 doses of 12 units each of UV-extract. × 60.

Fig. 5. Higher magnification of Fig. 4, showing marked proliferation of fibroblasts and fragmented cords of trapped liver cells. × 160.

Fig. 6. Hepatoma nodule in a rat receiving p-dimethylaminoazobenzene in the diet and 4 doses of 12 units each of UV-extract. The hepatoma cells are large and have basophilic cytoplasm and vesicular nuclei. × 135.

Fig. 7. Liver changes in a rat receiving only p-dimethylaminoazobenzene (0.12%) in the diet. There are occasional large groups of cells with clear or feathery cytoplasm, often faintly basophilic. × 135.
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