Azo dyes prevent hydrocarbon-induced leukemia in the rat
(Sudan III/Sudan IV/7,12-dimethylbenz[a]anthracene/7,8,12-trimethylbenz[a]anthracene/menadione reductase)

CHARLES B. HUGGINS*, NORIFUMI UEDA*, AND ANNA RUSSO†

* Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Illinois 60637; and † Laboratorio di Ricerca Cancerologica "Charles Huggins," 00045 Genzano di Roma, Italy

Contributed by Charles B. Huggins, June 23, 1978

ABSTRACT A set of intravenous injections of 7,8,12-trimethylbenz[a]anthracene (Tyrone-zo) in rats caused marked and impressive damage to rat’s adrenals and liver, whereas protein concentration was unchanged. A single feeding of 1 mg of 7,12-dimethylbenz[a]anthracene, but 50% of the survivors developed leukemia; unprotected rats succumbed in 1–3 days. Sudan III was not carcinogenic under the experimental conditions.

In the experiments now to be described it was found that a set of multiple intravenous injections of 7,12,13-trimethylbenz[a]anthracene (7,8,12-TMBA) at weekly intervals consistently elicited leukemia in adult female Long–Evans rats in more than 75% of the animals at risk within 8 weeks. Thus, we have produced hydrocarbon-induced leuemagenesis at a high frequency in these rats.

In the present work it was found that a single feeding of a small quantity (1 mg) of certain azo dyes prior to the intravenous injection of powerful hydrocarbons (i) resulted in a profound decrease in the incidence of leukemia caused by 7,8,12-TMBA and (ii) prevented fatal toxicity in rats injected with a massive amount of 7,12-dimethylbenz[a]anthracene (7,12-DMBA). Azo dyes which were highly effective in preventing hydrocarbon-induced leukemia (3) and preventing toxicity to azo dyes were: 1,2,3,4,5,6-hexahydro-1,3,5-trinitrobenzene (Sudan III) and 1-(o-phenylazo)2-naphthol (Sudan III) and 1-(o-phenylazo)-2-naphthol (Sudan IV). The prevention of leukemia by azo dyes is selective.

In the Long–Evans rat, a set of intravenous injections (pulse-doses) of large but tolerable doses of 7,12-DMBA (1) or 7,8,12-TMBA (2) at biweekly intervals elicits predominantly two sorts of cancers—mammary carcinoma and leukemia. In male rats, leukemia occurs at a high frequency and mammary cancer, to a lesser extent; in females, both mammary cancer and leukemia are elicited in profusion. The high incidence of leukemia by 7,8,12-TMBA requires multiple pulse-doses (2); a single intravenous injection seldom elicits leukemia. The minimal number of intravenous injections necessary to cause leukemia in high yield is four; a high incidence of leukemia (more than 75% of the animals at risk) was found (2) before 100 days in 10 consecutive series of rats given four intravenous injections of 7,8,12-TMBA, 35 mg/kg every 14 days.

In addition to producing mammary carcinoma and leukemia in an impressive manner (1–4), 7,12-DMBA, 7,8,12-TMBA, and a small group of closely related congeners (5) selectively cause necrosis (6) of two entire zones in the interior of rat’s adrenal gland whereas adjacent layers in the adrenal gland are unjured; massive adrenal hemorrhage ensues and it is first apparent after 33 hr. It has been found that hormone-dependent hydrocarbon-caused corticolytic with adrenal apoplexy of this special sort can be prevented by any one of a large number of cyclic compounds (7, 8) provided that the protector is given at least 2 hr prior to the corticolytic hydrocarbon. The protective hydrocarbons influence the synthesis of a soluble drug-metabolizing enzyme, menadione reductase (EC 1.6.99.2), in adipose tissue (9) and in the liver (8, 10).

The induction of mammary carcinoma was inhibited (11) to a considerable extent when any of six polycyclic aromatic hydrocarbons was fed during a period of time that overlapped multiple injections of optimal amounts of 7,12-DMBA, which in unprotected controls, elicited large numbers of cancers of the breast. The prevention of 7,12-DMBA-induced adrenal hemorrhage in the rat is a useful preliminary screen for compounds that can suppress hydrocarbon-induced cancer.

Tumor prothylaxis by protective aromatic hydrocarbons consisted of (i) increased yields of neoplasms with respect to their incidence in unprotected controls, (ii) delay in the appearance of the cancers, and (iii) their total suppression in a proportion of the protected animals.

Series of aromatic azo and ethylene derivatives (10) were investigated for their ability to induce protection against both adrenal injury and mammary cancer induced by 7,12-DMBA. Of the compounds that were investigated, Sudan III was the most effective in (i) induction of menadione reductase, (ii) prevention of adrenal necrosis and hemorrhage, and (iii) prevention of cancer of the breast.

Large doses of 7,12-DMBA kill rats within a few days (12). A small amount of any of five hydrocarbons given before a massive dose of 7,12-DMBA resulted in the indefinite survival of rats (more than 2 mo) without specific injuries to adrenal gland and tissues. Protection of life against toxicity of large doses of 7,12-DMBA by pretreatment with small doses of aromatics required time (ca 24 hr) for its induction; under stated conditions ethionine blocked protection (12).

MATERIALS AND METHODS

Chemical. 7,12-DMBA, mp 122°–123°C, (Eastman Organic Chemicals, Rochester, NY) was refined by Florisil (Fisher) chromatography and recrystallized from acetone/ethanol. 7,8,12-TMBA, mp 127°–128°C, was synthesized by the method of Bachmann and Chemerda (13). Fat emulsions (15% lipid) of these compounds containing 0.5% (wt/wt) of 7,12-DMBA were prepared by standard methods. The compounds were injected intraperitoneally in a volume of 5 ml/kg body weight. The use of menadione reductase (EC 1.6.99.2) was studied in vitro by the method of Sjöberg (14) and in vivo by the method of Sjöberg (15). The incidence of mammary carcinomas was determined by the method of Breslow (16). The tests of the tumors were determined by the method of van der Meulen (17). The results were expressed as incidence (%) in each test. All results are represented as the mean ± SE of three experiments. The significance of the differences was determined by the Student’s t-test (18). The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: 7,12-DMBA, 7,12-dimethylbenz[a]anthracene; 7,8,12-TMBA, 7,8,12-trimethylbenz[a]anthracene.
or 7,8,12-TMBA were prepared by the method of Schurr (14).

Sudan III, mp 202.5°–204.5°C (Eastman Organic Chemicals, Rochester, NY) and Sudan IV, mp 191°–193°C, (National Aniline Company, New York, NY) were recrystallized from chloroform/ethanol. The azo dyes were dissolved in sesame oil.

Biological. Young adult Long-Evans rats were studied exclusively. This is a heterozygous strain which has been randomized inter se in our colony for 18 yr. All of the females had one or more estrus cycles before selection for the experiment. The animals were housed in stainless steel cages in air-conditioned rooms at 25° ± 1°C. They were fed a commercial ration (Rockland Mouse/Rat Diet, Teklad, Inc., Monmouth, IL) with tap water ad lib.

Compounds dissolves in sesame oil were administered by gastric instillation (3) to rats under light ether anesthesia; emulsions of hydrocarbons were injected into a caudal vein.

The diagnosis of leukemia was made by histologic methods on samples of liver and spleen obtained at surgical operation. Biopsy of spleen and liver was performed under ether anesthesia with aseptic precautions. A transverse incision (1 cm) was made in the epigastrium. The spleen was delivered through the incision by gentle traction and nodules were biopsied. For histology, a sample (50–75 mg) was excised cleanly with scissors from the margin of the liver; generally hemostasis was unnecessary. The incision was closed with a catgut suture and metallic skin clips.

Heparinized blood for hematological studies was obtained by cardiac puncture. Leukocytes were counted electronically (Coulter Counter, Model Z, Coulter Electronics, Inc., Hialeah, FL).

Enzyme Assay. Samples of liver (50–75 mg) obtained by hepatic biopsy were weighed on a torsion balance and homogenized in 2 ml of ice-cold 0.5 M NaCl containing 3 mM NaHCO3 in a Polytron homogenizer for three 10-second bursts at the highest setting. Homogenates were centrifuged at 12,000 X g for 15 min at 2°C. The enzyme assays were performed on supernatant solutions.

The aqueous reaction mixture contained 83 mM Tris-HCl (pH 7.4), 0.1 mM menadione, and 0.1 mM NADH. Samples (3-ml) of the reaction mixture were placed in plastic cuvettes of 1-cm light path; 0.01 ml of homogenate was added to start the reaction. The initial rate of oxidation of NADH at 25°C was measured for 1 min in a spectrophotometer; the optimal enzyme concentration yielded an absorbance change of 0.015–0.025 A units per mg/min at 340 nm. One unit of menadione reductase is defined as that activity which oxidized 1 μmol of NADH/min per 100 mg (wet weight) of tissue under the stated conditions.

Protein Determination. The concentration of protein in the supernatant solution was measured by the method of Lowry et al. (15).

RESULTS

Induction of Leukemia. The most common type of leukemia was stem-cell erythroleukemia. In this disease the liver was granular, pitted, fragile, and dark red in color; it attained huge size, often more than 10% of the body weight. In erythroleukemia, the leukemia cells grew in the hepatic sinusoids, and mitoses were common in clumps of cells that often were associated with erythroblasts and normoblasts. Nodules of leukemia cells were frequent in the spleen. In myelocytic and lymphocytic leukemias the spleen was big whereas the liver was not greatly enlarged. The leukemia cells grew in the portal triangles rather than in the hepatic sinusoids.

A group of 20 female rats was given a set of four intravenous injections of 7,8,12-TMBA, 35 mg/kg at biweekly intervals starting at age 50 days. The first injection is denoted day 0. The diagnosis of leukemia was based on histologic evidence obtained by biopsy of liver and spleen which was performed on days 50–54 and at biweekly intervals thereafter. There was one fatality which occurred on day 26. Leukemia was detected in 14 rats (74%) on day 54 (Fig. 1). In the entire series of 19 rats at risk, leukemia was found in 18 animals before day 112. The histological classification of the leukemias was: erythroleukemia, 17; myelocytic leukemia, 1.

Prevention of Leukopenia. Cardiac blood for hematological studies was obtained by cardiac puncture for leukocyte counts. At age 50 days (day 0), two groups of female rats were given an intravenous injection of 7,8,12-TMBA, 35 mg/kg; each group comprised 16 animals. One group was fed 1 mg of Sudan III 24 hr prior to the intravenous injection, whereas another group, designated unprotected, was fed 1 ml of sesame oil devoid of azo dye.

In the unprotected group (Fig. 2) injected with 7,8,12-TMBA, there was a pronounced leukopenia with the greatest depression on day 3; subsequently there was a return in the number of leu-
kocytes to the preinjection level found on day 0. In the group whose members had been fed 1 mg of Sudan III prior to an injection of 7,8,12-TMBA, there were no significant changes in the leukocyte counts relative to preinjection values on day 0.

**Sudan III-Induced Menadione Reductase.** A group of eight female rats, age 50 days, was subjected to hepatic biopsy; the concentrations of menadione reductase and protein were determined on the samples of liver that were obtained. One hour after the operation, each rat was fed 1 mg of Sudan III dissolved in 1 ml of sesame oil. The procedure, hepatic biopsy and subsequent feeding of 1 mg of Sudan III, was repeated at 24-hr intervals.

The results of the assays are presented in Fig. 3. The repeated daily feeding of 1 mg of Sudan III for 4 days resulted in a pronounced and progressive increase in the concentration of menadione reductase in the liver, whereas the levels of protein were not altered significantly.

### Table 1. Incidence of leukemia and mammary cancer in female rats fed azo dyes and injected with 7,8,12-TMBA

<table>
<thead>
<tr>
<th>Group</th>
<th>Azo dye</th>
<th>No. of rats</th>
<th>Incidence of</th>
<th>Mammary cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At risk</td>
<td>Leukemia</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Sudan III</td>
<td>15</td>
<td>3</td>
<td>9 (20%)</td>
</tr>
<tr>
<td>II</td>
<td>Sudan IV</td>
<td>14</td>
<td>3</td>
<td>8 (21%)</td>
</tr>
<tr>
<td>III</td>
<td>None; controls</td>
<td>14</td>
<td>13</td>
<td>6 (93%)</td>
</tr>
</tbody>
</table>

Three groups of female rats were given a set of four intravenous injections of 7,8,12-TMBA, 35 mg/kg, at biweekly intervals starting at age 50 days. Groups I and II were fed 1 mg of an azo dye in 1 ml of sesame oil 24 hr prior to every intravenous injection; controls were fed 1 ml of sesame oil devoid of azo dye.

### Table 2. Incidence of leukemia in male rats fed Sudan III and injected with 7,8,12-TMBA

<table>
<thead>
<tr>
<th>Group</th>
<th>Azo dye</th>
<th>At risk</th>
<th>Incidence of leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sudan III, 1 mg</td>
<td>16</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>II</td>
<td>Sudan III, 10 mg</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>None; controls</td>
<td>13</td>
<td>12 (92%)</td>
</tr>
</tbody>
</table>

Three groups of male rats were given a set of four intravenous injections of 7,8,12-TMBA, 35 mg/kg at biweekly intervals starting at age 50 days. Animals in two of the groups were fed azo dyes 24 hr before every intravenous injection whereas control rats were fed 1 ml of sesame oil. The animals were observed for 100 days. The results are shown in Table 1. Group I: each rat was fed 1 mg of Sudan III prior to every intravenous injection; the incidence of neoplasms was leukemia 20% and mammary cancer 60%. Group II: each rat was fed 1 mg of Sudan IV prior to every intravenous injection; the incidence of neoplasms was leukemia 21% and mammary cancer 57%. Group III, controls: each rat was fed 1 ml of sesame oil prior to every intravenous injection. The incidence of neoplasms was leukemia 93% and mammary cancer 43%.

### Prevention of Leukemia. (1) Females. Three groups of rats were given a set of four intravenous injections of 7,8,12-TMBA, 35 mg/kg at biweekly intervals starting at age 50 days. Animals in two of the groups were fed azo dyes 24 hr before every intravenous injection whereas control rats were fed 1 ml of sesame oil. The animals were observed for 100 days. The results are shown in Table 1. Group I: each rat was fed 1 mg of Sudan III prior to every intravenous injection; the incidence of neoplasms was leukemia 20% and mammary cancer 60%. Group II: each rat was fed 1 mg of Sudan IV prior to every intravenous injection; the incidence of neoplasms was leukemia 21% and mammary cancer 57%. Group III, controls: each rat was fed 1 ml of sesame oil prior to every intravenous injection. The incidence of neoplasms was leukemia 93% and mammary cancer 43%.

### Order of Administration of Challenger and Protector. We investigated the influence of sequence of administration (protector/challenger or challenger/protector) on the incidence of leukemia. Two groups of female rats were given a set of four injections of 7,8,12-TMBA, 35 mg/kg, at biweekly intervals starting at age 50 days. Groups I and II were fed 1 mg of an azo dye in 1 ml of sesame oil 24 hr prior to every intravenous injection; controls were fed 1 ml of sesame oil devoid of azo dye.

### Table 3. Influence of the order of administration of Sudan III and 7,8,12-TMBA on the incidence of leukemia

<table>
<thead>
<tr>
<th>Sequence of administration</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sudan III/7,8,12-TMBA</td>
</tr>
<tr>
<td>II</td>
<td>7,8,12-TMBA/Sudan III</td>
</tr>
</tbody>
</table>

Groups of female rats were given a set of four intravenous injections of 7,8,12-TMBA, 35 mg/kg, and in addition four feedings of Sudan III, 1 mg. The order of administration differed. Group I: Sudan III was fed 24 hr before each injection of 7,8,12-TMBA. Group II: 7,8,12-TMBA was injected 24 hr before each feeding of Sudan III.
intrapavenous injections of 7,8,12-TMBA, 35 mg/kg at biweekly intervals starting at age 50 days; both groups were fed 1 mg of Sudan III on four occasions but the sequence of administration differed. The results are given in Table 3. Group I: each rat was fed 1 mg of Sudan III 24 hr prior to every intravenous injection; the incidence of leukemia was 13%. Group II: each rat was injected with 7,8,12-TMBA 24 hr before being fed 1 mg of Sudan III; the incidence of leukemia was 80%.

Prevention of Fatal Toxicity Caused by 7,12-DMBA. The dosage (6) of 7,12-DMBA that causes the death of half of a group of rats (LD₅₀) within 21 days after a single intravenous injection is 47.5 mg/kg. A group of 16 female rats, age 38 days, was given a single intravenous injection of 7,12-DMBA, 75 mg/kg; all of the animals succumbed in 1–3 days. An identical group of female rats was given a single feeding of 1 mg of Sudan III by gastric instillation 24 hr prior to an intravenous injection of 7,12-DMBA, 75 mg/kg; there were no deaths. The procedure (Sudan III-feeding and subsequent 7,12-DMBA injection), was repeated thrice at intervals of 21 days. There were no deaths in this group but leukemia developed in 5 of 16 rats; leukemia was detected in 60 to 74 days, mean 63.5 ± 6.5 days.

Failure of Sudan III to Elicit Tumors. Sudan III was tested for possible carcinogenicity in two experiments. (i) A solution of Sudan III, 0.5% (wt/vol), in sesame oil was prepared; 0.5 ml of the solution was injected in thigh muscle of both legs of eight rats, age 27 days; the animals were observed for 9 mo. At necropsy on day 276 a residual depot of red dye-colored oil was found at every injection site but no tumors were present. (ii) A solution of Sudan III, 0.1% (wt/vol) in sesame oil was prepared. A group of 16 female rats was fed, by gastric instillation, 1 ml of the solution five times each week for 25 weeks. At necropsy there were no tumors in the liver or elsewhere.

DISCUSSION

In young female Long–Evans rats, a series of four intravenous injections of 7,8,12-TMBA, 35 mg/kg at biweekly intervals, elicited leukemia consistently, rapidly, and in high yields. The production of leukemia in this way is exceedingly simple and the celerity of emergence of the leukemia is dramatic.

The incidence of 7,8,12-TMBA-induced leukemia in Long–Evans rats was profoundly reduced by feeding a small dose (1 mg) of Sudan III or Sudan IV in one sitting before large doses of 7,12-DMBA or 7,8,12-TMBA, whereas mammary cancer was not prevented. Hydrocarbon-induced leukemia in these rats was completely suppressed by a larger dose (10 mg) of Sudan III on two occasions prior to each leukemogenic dose of 7,8,12-TMBA. The daily feeding of Sudan III to rats induces cumulative amounts of menadione reductase in liver.

Richardson and Cunningham (16) fed rats 3’-methyl-4-dimethylaminoazobenzene and liver tumors arose; fewer neoplasms of the liver developed in companion groups that also received small doses of the strong carcinogen, 3-methylcholanthrene. Richardson et al. (17) and Miller et al. (18) found that the induction of hepatic tumors by 3-methyl-4-dimethylaminoazobenzene was inhibited when one of the following aromatic hydrocarbons was added to the diet: 3-methylcholanthrene, benzo[a]pyrene, benzo[a]anthracene, or dibenz[a,h]anthracene.

In the Richardson–Cunningham effect (16) a minute amount of a carcinogenic polycyclic hydrocarbon inhibited the formation of hepatic tumors in rats continuously fed a carcinogenic azo dye in the ration. In our experiments a single feeding of a small quantity of an azo dye effectively prevented hydrocarbon-induced leukemogenesis. It is noteworthy that our sample of Sudan III was not carcinogenic.

Two methods are available in the rat for the rapid screening of compounds that will prevent hydrocarbon-induced cancer: (i) the prevention of adrenal apoplexy (6, 11), and (ii) protection of the rat from fatal toxicity from massive doses of hydrocarbons as described in the present study.

We thank Dr. John Pataki, The Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, IL, for synthesis of 7,8,12-TMBA and Dr. Paul E. Schurr, The Upjohn Company, Kalamazoo, MI, for preparation of lipid emulsions. This work was supported by grants from the American Cancer Society and The Jane Coffin Childs Memorial Fund for Medical Research and by U.S. Public Health Service Grant CA11603-09 awarded by The National Cancer Institute, Department of Health, Education, and Welfare.