Specific induction of erythroleukemia and myelogenous leukemia in Sprague–Dawley rats

(N-nitroso-N-methyleurea/7,8,12-trimethylbenz[a]anthracene/mammary carcinoma/ear duct carcinoma)

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ABSTRACT The experimental induction of leukemias of two sorts by two powerful chemical carcinogens was investigated in rats of a single strain. In Sprague–Dawley rats a series of intravenous injections of N-nitroso-N-methyleurea selectively elicited myelogenous leukemia in high yields, whereas erythroleukemia was not evoked. Conversely, a set of intravenous injections of 7,8,12-trimethylbenz[a]anthracene specifically elicited erythroleukemia in high incidence in the rats, whereas myelogenous leukemia was not produced.

In the experiment now to be described sets of massive but generally tolerable doses of two powerful carcinogens, N-nitroso-N-methyleurea (NMU) and 7,8,12-trimethylbenz[a]anthracene (Me3BA), were given by intravenous (i.v.) injection to albino rats of the Sprague–Dawley (S-D) strain. Throughout the experiment each group of rats received multiple doses of a single carcinogen—"one rat, one compound." It was found out that Me3BA preferentially elicited erythroleukemia (EL), whereas NMU selectively elicited myelogenous leukemia (ML).

The mammary acini of young female S-D rats are foremost among cells of living creatures in their susceptibility to the induction of cancer by chemical carcinogens or irradiation (1). In an earlier experiment (2) a single injection of NMU or Me3BA was given to young rats. (i) Fourteen female S-D rats, age 25 days, were given one i.v. injection of NMU, 35 mg/kg, and observed for 5 months thereafter; mammary cancer arose in 11 rats (79%), whereas other neoplasms did not appear. (ii) Twelve female S-D rats, age 28 days, were given one i.v. injection of Me3BA, 35 mg/kg, and observed for 150 days thereafter; mammary cancer was observed in 11 rats (92%) and EL in 1 rat.

Shay et al. (3) found that the gastric instillation of 3-methylcholanthrene, 2 mg daily, for many months was leukemogenic for rats of the Wistar strain but the incidence of leukemia was low; six cases of lymphatic leukemia and two of ML were detected in large groups of rats at risk. Transfer of ML (4) to other rats was obtained readily by intraperitoneal (i.p.) injection of blood from rats with ML into newborn Wistar rats. The i.p. inoculation of blood from donors with ML evoked ML that grew in pigmented form in newborn rats.

Hartmann et al. (5) fed a diet containing 2-acetylaminophenanthrene to albino rats of the Holtzman strain for 2–4 months. The carcinogenic diet elicited three sorts of neoplasms: leukemia, mammary carcinoma, and ear duct cancer. In a series of 56 leukemias elicited in rats by 2-acetylaminophenanthrene, there was one animal with ML.

Odashima (6) reported the high incidence of EL in rats continuously receiving N-nitroso-N-butylurea in their drinking water for 4 months. Uenaka et al. (7) studied 34 cases of leukemia of various sorts that developed in rats of the Long–Evans (L–E) strain that were given N-nitroso-N-butylurea in their drinking water; 9 of the leukemias (26%) were classified as peroxidase-positive ML. Zeller and Schmähl (8) elicited leukemia in rats by i.v. injections of N-nitroso-N-ethyleurea, 15 mg/kg, at weekly intervals for 15 weeks and found that 15 (16%) of 91 leukemias were myelogenous. In the experiments of Gulino et al. (9) three i.v. injections of NMU, 50 mg/kg, were given to rats at intervals of 4 weeks; mammary adenocarcinoma developed in intact S-D females in high incidence (73%) and metastases to bone marrow and spleen were evident, whereas primary neoplasms, other than mammary cancer, were not observed.

Prigozhina (10) fed 7,12-dimethylbenz[a]anthracene (Me3BA), 1–2 mg semiquarterly, to rats of the Wistar strain and found that leukemia, mammary cancer, and ear duct cancer appeared in the recipients; the incidence of leukemia was 47% of the animals at risk. The leukemia was designated leukemia–erythroleukemia and its gross anatomical features were remarkably uniform in the group of leukemic rats; most notable was the development of a huge liver with infiltration of the hepatic sinusoids by leukemic cells.

In the experiments of Huggins et al. (11) a series of four i.v. injections of Me3BA (35 mg/kg) at biweekly intervals induced EL rapidly and consistently in rats of the L–E strain. Huggins et al. (12) found that a series of eight feedings of Me3BA given biweekly by gastric intubation to juvenile rats of the L–E strain elicited leukemia reproducibly and in high yields: females, 82%; males, 70%. Studied histologically were 56 consecutive cases of leukemia, and these were classified: stem-cell EL; 55; ML, 1. Newborn L–E rats were given subcutaneous (s.c.) injections of whole blood from donors with EL; sarcomas were elicited at the site of injection. The erythroasrascomas grew rapidly as large, firm, grapelike masses, which rapidly coalesced and became fixed as they infiltrated skin and muscle. It was found (12) that the erythroasrascomas induced in newborn rats by blood of rats with advanced EL are hormone dependent.

MATERIALS AND METHODS

Chemicals. NMU, mp 122–123.5°C, was synthesized (2) and stored in a desiccator in a refrigerator at 4°C. Prior to i.v. injection, NMU (0.5 g) was dissolved in saline (100 ml).

Me3BA, mp 127–128°C, was synthesized by the method of Bachmann and Chemerda (13). Sterile fat emulsions (15% vol/vol) lipid containing 0.5% Me3BA were prepared by the

Abbreviations: EL, erythroleukemia; ML, myelogenous leukemia; i.v., intravenous; s.c., subcutaneous; S-D, Sprague–Dawley; L–E, Long–Evans; Me3BA, 7,8,12-trimethylbenz[a]anthracene; Me3BA, 7,12-dimethylbenz[a]anthracene; NMU, N-nitroso-N-methyleurea; i.p., intraperitoneal.

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method of Schurr (14); stored in a refrigerator at 4°C, the emulsions have remained stable and sterile and carcinogenic for more than 12 yr.

**Biological Materials.** Heterozygous S–D rats were obtained from Harlan/Sprague–Dawley (Madison, WI) and housed 10–15 rats per group in large stainless steel cages in air-conditioned rooms at 25 ± 1°C. The rats were randomly bred among themselves; they were fed a commercial ration [Rockland mouse/rat diet (Teklad, Monmouth, IL)] with tap water ad lib. NMU or Me3BA was injected in one of the caudal veins; the day of the first injection is designated day 0. All surgical operations were performed with ether anesthesia and with aseptic precautions. Once each week the rectal temperature of each rat was measured with a thermocouple (Thermistor thermometer, YSI model 43, Yellow Springs Instrument, Yellow Springs, OH).

**Hematology.** By cardiac puncture using a hypodermic needle (25 gauge; ½ inch), blood was obtained in a syringe moistened with heparin. While the animal was under brief ether anesthesia, the point of maximum cardiac impulse was located on the left at the inferior part of the sternum and here the needle was inserted into the heart. The blood was examined at intervals of 2–7 days; 0.1–0.2 ml of blood was drawn on each occasion. With the aforementioned technique venous blood flows freely into the syringe without foaming or hemolysis.

The blood cells were counted electronically [Coulter Counter, model ZB1 (Coulter Electronics, Hialeah, FL)]. Blood films were stained by the Giemsa technique and by histochemical methods for peroxidase (15) and alkaline phosphatase (16).

**Buffycrit.** The method of Huggins and Sugiyama (1, 17) was used to measure strata of packed leukocytes, platelets, and erythrocytes (Fig. 1). Glass capillary tubing (Owens-Illinois, Vineland, NJ) was cut with a diamond saw to make standard buffycrit tubes with the following characteristics: length, 75 mm; external diameter, 0.7–1 mm; wall thickness, 0.25 mm; capacity, 12 ± 1 µl. The standard buffycrit tube was filled by capillarity and centrifuged at 12,000 × g for 5 min in an Adams microhematocrit centrifuge (Clay-Adams, Parsippany, NJ). The lengths of the packed strata were measured with an ocular micrometer (×35) and an Adams microhematocrit reader.

**RESULTS**

Blood obtained by cardiac puncture from rats briefly anesthetized with ether was examined at intervals of 2–7 days, beginning 14–28 days after the final i.v. injection of carcinogen; dark venous blood was always obtained by cardiac puncture. The method is simple and safe; there was no mortality in more than 2,000 rats subjected to this procedure.

The rectal temperature of disease-free rats in our colony was 38.2 ± 0.5°C. It was a premortal sign when the rectal temperature dropped below 35.5°C.

**Toxicity of NMU.** The toxic effect of a single i.v. injection of NMU, 35 mg/kg, was investigated in S–D female rats injected at age 50 days (day 0).

There was no mortality or significant loss of body weight. The principal abnormalities (Table 1) were hematologic: (i) a slight decrease in hematocrit; (ii) decrease in leukocyte count on days 3–10 due to a decrease in the number of lymphocytes. There were no significant changes in packed platelet mass.

**Leukemia in Rats Injected with NMU.** Studied histologically were 28 consecutive cases of leukemia induced by a series of five i.v. injections of NMU at biweekly intervals in ovariec-tomized rats. The leukemias were classified according to morphologic criteria: ML, 26; lymphatic leukemia, 1; reticulum-cell sarcoma of spleen with metastases, 1. EL was not observed.

**NMU-Induced Neoplastic Disease.** Male S–D rats were given a series of five i.v. injections of NMU, 35 mg/kg of body weight, at biweekly intervals. The first dose was injected at age 49 days (day 0). The blood, obtained by cardiac puncture, was examined at intervals of 7 days beginning on day 78. Twenty rats were started in the experiment and there were 3 early deaths from hepatic necrosis and aplastic anemia. The experiment terminated on day 153.

Neoplastic disease of three principal sorts was found in the group of 17 effective survivors: (i) mammary carcinoma, 17 rats

**Table 1. Hematology of S–D rats given a single injection of NMU**

<table>
<thead>
<tr>
<th>Day</th>
<th>Body weight, g</th>
<th>Hematocrit, %</th>
<th>Leukocytes/mm³</th>
<th>Packed platelets, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>163</td>
<td>35.9 ± 0.3</td>
<td>7,332 ± 569</td>
<td>0.72</td>
</tr>
<tr>
<td>3</td>
<td>165</td>
<td>33.6 ± 0.9</td>
<td>3,100 ± 342</td>
<td>0.79</td>
</tr>
<tr>
<td>5</td>
<td>170</td>
<td>33.1 ± 0.5</td>
<td>2,967 ± 670</td>
<td>0.80</td>
</tr>
<tr>
<td>7</td>
<td>171</td>
<td>34.3 ± 1.5</td>
<td>3,360 ± 495</td>
<td>0.65</td>
</tr>
<tr>
<td>10</td>
<td>176</td>
<td>34.5 ± 0.9</td>
<td>3,830 ± 382</td>
<td>0.64</td>
</tr>
<tr>
<td>14</td>
<td>190</td>
<td>34.2 ± 0.9</td>
<td>4,960 ± 220</td>
<td>0.93</td>
</tr>
<tr>
<td>17</td>
<td>195</td>
<td>34.4 ± 1.1</td>
<td>5,616 ± 780</td>
<td>0.90</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>38.6 ± 0.5</td>
<td>7,152 ± 432</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Female S–D rats were injected i.v. with NMU, 35 mg/kg, at age 50 days (day 0). Mean values are given ± SEM; n = 8.
(100%) with time of detection 82.4 ± 2.7 days; (ii) ML, 10 rats (59%) with time of detection 94.8 ± 4.5 days; (iii) ear duct carcinoma, 7 rats (41%) with time of detection 113.6 ± 3.8 days. In addition to the foregoing the following neoplasms were found: thymoma, 1; fibrosarcoma of mesentery, 1. EL was not observed.

The development of ML was observed in increases in the leukocyte count (Table 2) and in the packed leukocytes in the stratum B of the buffycoat tubes (Fig. 1).

Seven litters of allogeneic S-D rats, age 1–3 days, were inoculated s.c. in the scruff of the neck with blood (0.1 ml) of a male rat with advanced ML. Each litter consisted of 10 newborns. There were 5 donors; the leukocyte counts of the bloods from the donors were 47,700–227,600 per mm³. The day of inoculation of leukemic blood is denoted day 0. Small masses of tumor became palpable at the injection site after day 30. Many of the recipients developed paralysis of the lower extremities.

Necropsy was performed on day 30–50. It was found that the blood from each of the donors had elicited alkaline phosphatase-positive sarcoma at the injection site. The myelosarcomas were green, flat, and soft; they infiltrated adjacent musculature and compressed the spinal cord. There were 14 rats that developed paraplegia.

**Characteristics of NMU-Induced ML.** The following criteria of ML in rats were well defined: (i) The first physical sign of ML was splenomegaly. The spleen grew to a huge size; it was smooth, free from nodules, and readily palpable. In 11 disease-free normal uninjected female S-D rats, the spleen weighed 0.18–0.26 g/100 g of body weight. In 12 cases of ML the spleens weighed 1.1–1.7 g/100 g. (ii) The leukocyte counts were 47,700–441,000 per mm³. (iii) In all cases there was a typical hemogram consisting of abnormal promyelocytes and myelocytes containing peroxidase granules and alkaline phosphatase (Fig. 2A). (iv) There was an increase (above 0.2%) of packed leukocytes in the buffycoat (Table 2). (c) Marrow and lymph nodes were green due to an increase of verdoperoxidase (18). (vi) Thin marrow-containing bones were green; the green coloration due to chloroleukemia was evident in the occiput and parietal bones of the cranium. (vii) There was characteristic localization of ML in the periportal region of the hepatic lobule (Fig. 2B). (viii) There was characteristic localization of ML in the interstitial region surrounding the proximal convoluted tubules of the kidney. (ix) There was growth of characteristic soft green myelosarcoma in allogeneic newborns at the site of s.c. inoculation of whole blood from five donors with ML.

**Me3BA-Induced Neoplastic Disease.** (a) A group of female S-D rats was subjected to ovariecotmy at age 50 days followed by a series of four i.v. injections of Me3BA at biweekly intervals.

<table>
<thead>
<tr>
<th>Day</th>
<th>Body weight, g</th>
<th>Hematocrit, %</th>
<th>Packed leukocytes, %</th>
<th>Leukocytes/mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>78</td>
<td>341</td>
<td>41.5</td>
<td>0.04</td>
<td>7,611</td>
</tr>
<tr>
<td>85</td>
<td>344</td>
<td>40.5</td>
<td>0.20</td>
<td>13,400</td>
</tr>
<tr>
<td>97</td>
<td>338</td>
<td>42.5</td>
<td>0.20</td>
<td>12,800</td>
</tr>
<tr>
<td>105</td>
<td>341</td>
<td>35.5</td>
<td>1.44</td>
<td>43,000</td>
</tr>
<tr>
<td>107</td>
<td>331</td>
<td>39.5</td>
<td>2.16</td>
<td>58,000</td>
</tr>
<tr>
<td>112</td>
<td>313</td>
<td>35.5</td>
<td>4.40</td>
<td>115,150</td>
</tr>
<tr>
<td>114</td>
<td>308</td>
<td>35.0</td>
<td>6.0</td>
<td>164,000</td>
</tr>
<tr>
<td>119</td>
<td>294</td>
<td>20.5</td>
<td>12.0</td>
<td>227,600</td>
</tr>
</tbody>
</table>

A male S-D rat was injected i.v. with NMU, at 35 mg/kg, on five occasions at biweekly intervals starting at age 49 days (day 0). Blood, 0.1 ml, was obtained from the lightly ether-anesthetized rat by cardiac puncture.

The first dose of Me3BA, 35 mg/kg, was injected at age 51 days (day 0); each subsequent dose consisted of 5 mg of Me3BA. Fifteen rats started in the experiment and there were 5 early deaths on days 24–45. The first examination of the blood was performed on day 53 and it was followed on the same day by liver biopsy. The experiment terminated on day 68.

Neoplastic disease of two sorts was found in the group of 10 effective survivors: (i) EL, 9 rats (90%), was detected on day 53; (ii) mammary carcinoma, 1 rat (10%), was detected on day 63. ML was not elicited.

(b) A group of male S-D rats was given a series of four i.v. injections of Me3BA at biweekly intervals. The first dose of Me3BA, 35 mg/kg, was injected at age 51 days (day 0); each subsequent dose consisted of 5 mg of Me3BA. Fifteen rats started in the experiment and there were 3 early deaths on days 47–53. The first examination of blood and liver biopsy specimens was performed on day 53. The experiment terminated on day 68.

Neoplastic disease of a single sort was found in the group of 12 effective survivors: EL, 6 rats (50%), was detected on days 46–61.
Characteristics of EL. The most striking anatomical findings in S–D rats with EL were found in liver and spleen. In early stages, EL was distributed at random in the sinusoids of a hepatic lobule without specific geometric localization in the periporal region. In an advanced stage the liver was huge, dark red, pitted, and friable, and the hepatic edge was rounded. In advanced stages of EL the hepatic sinusoids became extensively infiltrated until the sinusoidal endothelium was replaced by cells of EL. In EL, the spleen contained strikingly impressive large white nodules. In nine cases of far-advanced EL, on day 68 the leukocyte count in cardiac blood was 9,876 ± 650 per mm³.

DISCUSSION

The carcinogens studied in this experiment have widely diverse chemical properties. Me₂BA is a lipid-soluble, water-insoluble polycyclic aromatic hydrocarbon; NMU is water-soluble and acyclic. Me₂BA participates in donor-acceptor complexes (1, 19) as an electron donor.

In their ability to evoke cancer in rodents, Me₂BA, Me₃BA, and a small number of congeners exceed by a factor of 2 or more other carcinogenic hydrocarbons (20). Me₂BA and Me₃BA are DNA reagents (21, 22) that damage cells during mitosis, whereas neighboring cells in meiosis are spared from injury. In male S–D rats a series of five i.v. injections of NMU selectively elicited in high incidence a triad of neoplasms consisting of mammary carcinoma, squamous carcinoma of the peri-auricular sebaceous glands, and ML. EL was not observed.

In a group of 12 male S–D rats, a series of four i.v. injections of Me₂BA preferentially elicited a single sort of neoplasm; EL was evoked in 6 animals. ML was not induced.

In a group of 10 ovariectomized S–D females, a series of i.v. injections elicited EL in 9 rats (90%) and mammary carcinoma in one animal; ML was not elicited.

The mammary cancers and ear duct cancers induced preferentially by NMU in intact female S–D rats resemble in histological appearance the same class of neoplasms selectively evoked by Me₂BA, but the types of leukemia elicited by these carcinogens are quite different. It would appear that Me₂BA specifically induces leukemia in primitive hemopoietic cells committed to erythropoiesis, whereas the blood-forming cells susceptible to induction to leukemia by NMU are committed to leukopoiesis.

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