Induction of Leukemia and Ovarian Tumors in Mice by Pulse-Doses of Polycyclic Aromatic Hydrocarbons

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SUMMARY

Pulse-doses of 7,12-dimethylbenz[*a*]anthracene and 7,8,12-trimethylbenz[*a*]anthracene share a remarkable ability to damage selectively endocrine glands and targets of hormones in female rodents. Both compounds depress synthesis of DNA in ileum and spleen.

In CF-1 mice, 7,12-dimethylbenz[*a*]anthracene and 7,8,12-trimethylbenz[*a*]anthracene severely injured the ovary and elicited ovarian tumors and leukemia, whereas the adrenal was uninjured by these compounds. 3-Methylcholanthrene and benzo[*a*]pyrene induced leukemia, but ovarian tumors did not develop.

In adult rats, a pulse-dose of 7,8,12-trimethylbenz[*a*]anthracene caused adrenal apoplexy but did not evoke ovarian tumors.

INTRODUCTION

In this paper it will be shown that an intravenous injection (pulse-dose) of certain polynuclear aromatic hydrocarbons elicits leukemias and ovarian tumors in mice and that multiple pulse-doses are more efficient than a single shot in this regard. Pulse-doses of the carcinogenic aromatics profoundly depress the synthesis of DNA.

It is well known that mice have a propensity to develop ovarian tumors following a variety of experimental procedures. These include total-body irradiation (1, 2), transplantation to spleen (3) or other sites (4, 5), administration of hydrocarbons (6), and treatment with progestational steroids (7), especially 19-norprogesterone and progesterone. It has been postulated (8) that secondary imbalance in the amount of hypophyseal hormones resulting from primary ovarian injury is the common factor which leads to tumors of the ovary.

Morton and Mider (9, 10) discovered that polynuclear aromatics are powerful agents in eliciting murine leukemia. Repeatedly painting the skin of mice with 3-MC[2] or 7,12-DMBA (11) results in a high yield of leukemias. In the experiments of Morton and Mider (10) the repeated application of 3-MC to the skin of *dba* strain mice elicited leukemia in 98.7% in 87.7 ± 12.4 days. Haran-Ghera *et al.* (12) found that multiple feedings of 7,12-DMBA were more effective than a single meal in the induction of lymphogenous tumors in mice.

Howell, Marchant, and Orr (6) discovered that 7,12-DMBA elicited ovarian tumors in mice. At fortnightly intervals they applied a solution of 7,12-DMBA to the skin of virgin IF strain mice; the incidence of tumors was: breast, 74%, and ovary, 60%. The earliest ovarian tumors were observed after 4 months, and all of them were of the granulosa cell type.

Biancifiori *et al.* (13) fed carcinogens to groups of C_3H mice: 7,12-DMBA and

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[2] The abbreviations are: BA, benz[*a*]anthracene; BP, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; DBA, dibenz[*a,h*]anthracene; 7,12-DMBA, 7,12-dimethylbenz[*a*]anthracene; 7,8,12-TMBA, 7,8,12-trimethylbenz[*a*]anthracene.

3-MC elicited ovarian tumors in high yield, and all were of the granulosa cell type; DBA and BP evoked ovarian tumors in low yield, and all were luteomas.

It has been shown that the rat is unusually vulnerable (14) to 7,12-DMBA compared with mouse; for this reason, in the present work large amounts of 7,12-DMBA were injected into mice.

Moreover, in the adult rat, 7,12-DMBA (15) and very closely related congeners (16) exert a remarkable effect in their ability to bring about selective destruction of the middle layer of the adrenal cortex; this phenomenon does not occur in infant rats or in mice.

MATERIAL AND METHODS

Virgin female CF-1 mice (Carworth Farms, Inc., Kalamazoo, Michigan) and virgin female Sprague-Dawley rats (Sprague-Dawley, Inc., Madison, Wisconsin) were housed in metal cages in air-conditioned rooms at 25 ± 1°, fed a commercial ration (Rockland Mouse/Rat Diet, Teklad, Inc., Monmouth, Illinois), and given water *ad libitum*. The vaginal smear of each animal was examined daily.

The hydrocarbons were purified by Florisil chromatography and recrystallized from acetone-ethanol. Lipid emulsions of individual hydrocarbons were prepared in the following concentrations (w/w): 0.5% 7,12-DMBA, and 0.25% BA, BP, 3-MC, or 7,8,12-TMBA. The emulsions were injected intravenously and the day of the first pulse-dose is designated day 0.

Leukocyte count, differential cell count, hemoglobin, and hematocrit were determined on heparinized cardiac blood on one or two occasions in every animal. Sick mice were kept in individual cages. Necropsy was performed on each animal; the organs were weighed on a torsion balance and tissues were prepared for histological examination.

Toxicity

The dose causing death of one-half the rats in 21 days (LD_{50}) was determined by the probit method of Gaddum (17) after a single pulse-dose of aromatics.

In studies of adrenal necrosis, the hydrocarbon was given to female Sprague-Dawley rats at age 50 days and the adrenal glands were harvested at age 53 days; the content of hemoglobin in adrenal was determined (15).

Incorporation of Tritium in DNA

Tritium-labeled thymidine (Schwarz BioResearch, Inc., Orangeburg, New York), specific activity of 3 C/mmole, was diluted with aqueous sodium chloride (0.9%), and 1 ml of the solution (corresponding to approximately 0.5 μC/g of body weight) was injected in a caudal vein.

A pulse-dose of a hydrocarbon was injected at −24 hr, and tritiated thymidine at 0 hr; the animals were killed at +1 hr. Segments of terminal ileum and spleen were weighed and homogenized for 3 min in 5 ml of ice-cold 6% trichloracetic acid containing 0.5% of thymidine. The homogenates were maintained at 4° or lower; centrifugation was performed for 10 min. The dry residue was prepared as follows.

1. Transfer homogenate to a weighed, round-bottom tube (16 × 100 mm); centrifuge; aspirate and discard supernatant.
2. Suspend precipitate in 1 ml of 0.2 N NH_4OH. Add 4 ml of 10% trichloracetic acid. Centrifuge.
3. Wash precipitate with 5 ml of 6% trichloracetic acid. Centrifuge.
4. Wash precipitate with 5 ml of a solution of 0.1 M potassium acetate in 95% ethanol. Centrifuge.
5. Wash with 5 ml of a mixture (ethanol, 75 ml, plus ether, 25 ml). Centrifuge.
6. Wash with ether. Centrifuge. Store in an evacuated desiccator.

The tissues were analyzed for tritium by the combustion procedure of Jacobson *et al.* (18). The results are expressed as disintegrations per minute per milligram of washed, acid-insoluble, fat-free dry tissue and designated *incorporation*.

RESULTS

Toxicity

Very curious species differences were observed in toxicity of 7,12-DMBA and

TABLE 1

Toxicity of a pulse-dose of aromatics in rat and mouse

LD_{50} is given for female rats of the Long-Evans strain, age 29 days, and for CF-1 mice, age 53 days.

Hydrocarbon	LD_{50}	
	Rat	Mouse
	mg/kg	
BA	>200	>200
7,12-DMBA	60	>200
7,8,12-TMBA	125	50

7,8,12-TMBA (Table 1). The rat is vulnerable to moderate doses of 7,12-DMBA, whereas the mouse is very resistant. 7,8,12-TMBA is very toxic to the mouse, but the rat is considerably more resistant. Pulse-doses of BA, 200 mg/kg, were not toxic for rat or mouse.

The effects of the hydrocarbons on adrenal glands were studied. A single pulse-dose of 7,12-DMBA, 30 mg/kg, or of 7,8,12-TMBA, 45 mg/kg, selectively destroyed zona fasciculata and zona reticularis in each of 10 rats, and adrenal apoplexy occurred. A pulse-dose of 7,8,12-TMBA, 30 mg/kg, caused adrenal hemorrhage in only 2 of 10 rats. Earlier (16) it was found that selective destruction of adrenal occurred after a single feeding of 5 mg of 7-hydroxymethyl-12-methylbenz[*a*]anthracene; this compound is the most potent of the adrenocorticolytic hydrocarbons (19).

Incorporation of Tritium in Tissues

Tritiated thymidine was injected intravenously subsequent to a pulse-dose of a lipid emulsion of a hydrocarbon; the control animals received an injection of the emulsified lipid devoid of hydrocarbons. Incorporation of tritium was measured in spleen and ileum of rat and mouse (Table 2). The percentages refer to values obtained in tissues of control animals.

Benz[a]anthracene. Pulse-doses of this hydrocarbon prior to tritiated thymidine resulted in a slight increase or no change in incorporation of tritium in the spleen or ileum.

7,12-DMBA. In the rat, a pulse-dose of this hydrocarbon caused severe diarrhea; in the mouse, feces were formed and solid. Incorporation of tritium in ileum of rat was severely depressed to 4%, whereas in mouse it was 57% of controls. Incorporation of tritium was severely depressed in spleen of both mouse (13%) and rat (2.5%).

7,8,12-TMBA. A pulse-dose of this hydrocarbon did not cause diarrhea; in

TABLE 2

Concentration of radioactivity in rats and mice injected with hydrocarbons followed by tritiated thymidine

Sprague-Dawley female rats, approximately 50 g, age 25 days, and CF-1 female mice, approximately 25 g, age 60 days, received a lipid emulsion of the hydrocarbon (100 mg/kg intravenously) at −24 hr; controls received the emulsion devoid of hydrocarbon. At 0 hr, tritiated thymidine (specific activity, 3 C/mmole), 0.5 μC/g, was injected intravenously. The animals were killed at +1 hr. There were four animals in each group. Mean values are given for disintegrations per minute per milligram of washed, acid-insoluble, fat-free dry residue.

Treatment with hydrocarbon	Rat		Mouse	
	Spleen	Ileum	Spleen	Ileum
	dpm/mg		*dpm/mg*	
Controls	7,745	4,866	7,239	9,679
	(100%)	(100%)	(100%)	(100%)
BA	11,800	9,796	6,682	10,679
	(152%)	(201%)	(92%)	(110%)
7,12-DMBA	194	195	945	5,545
	(2.5%)	(4%)	(13%)	(57%)
7,8,12-TMBA	408	2,341	307	2,532
	(5%)	(48%)	(4%)	(26%)

ileum there was a moderate decrease in incorporation of tritium, more severe in mouse (26%) than in rat (48%). Synthesis of DNA was severely depressed in spleen of both rat and mouse (4%).

Characteristics of Mouse Leukemia

In 100 consecutive cases of mouse leukemia elicited by 7,12-DMBA, it was found that the disease manifested itself in four principal presenting syndromes: (a) dyspnea with bilateral exophthalmos and venous congestion due to thymoma, 71%; (b) prominent lymph nodes, which were easily palpable, 18% (the lymph nodes of normal mice cannot be palpated in living animals); (c) protuberant abdomen due to enlargement of liver and spleen, 9%; and (d) persistent loss of weight (a nonspecific sign), 2%. The first leukemias in our mice were detected *in vivo* in two cases on day 57.

Hematology

Female CF-1 mice, age 28 days, received a pulse-dose of 7,12-DMBA, 100 mg/kg; control mates were not injected. The animals, in groups of four, were killed at intervals of 1–3 days. Soon after the injection, in hydrocarbon-treated mice, there was a decrease in the number of leukocytes in venous blood (Fig. 1) and in weight of the spleen, followed by recovery to normal values. Leukopenia was most severe on day 1, and the weight of the spleen was lowest on days 3–5.

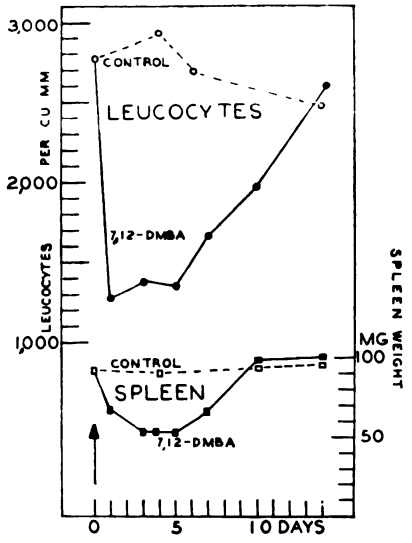

FIG. 1. *Leukopenia caused by 7,12-DMBA*

A pulse-dose of 7,12-DMBA, 1 mg (100 mg/kg), was given to female CF-1 strain mice at age 28 days (day 0); controls were uninjected. Leukocyte counts were made on venous blood, and the spleen was weighed at intervals of 1–3 days. Each point is the mean value from a group of four mice.

Ovarian Function

Vaginal cytology was studied for 3 months in 84 adult mice which received three pulse-doses of 7,12-DMBA, 80 mg/kg, at age 108, 111, and 114 days. The incidence of estrus declined after the first injection (Fig. 2), and on day 11 no mouse had the cornified cells which are vaginal characteristics of estrus. A slow recovery of ovarian function occurred soon thereafter, and on day 70 a peak incidence

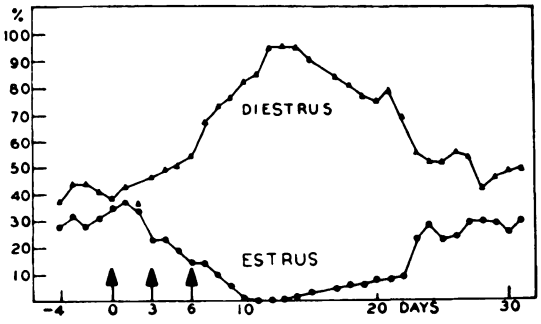

FIG. 2. *Percentage of estrus and diestrus in vaginal smears of a group of 87 CF-1 mice given pulse-doses of 7,12-DMBA (80 mg/kg) at age 108, 111, and 114 days (days 0, 3, and 6)*

of estrus (49%) again was found. After day 70, the mice gradually lost estrus again, and metestrus (leukocytes and cornified cells) slowly replaced estrus. The vaginal smear always was very irregular in mice which had received 7,12-DMBA, with prolonged periods of estrus or diestrus lasting for weeks.

Mice which developed an ovarian tumor usually showed an abundance of cornified epithelial cells in the vaginal smear, but the presence of estrus was not invariable, since some mice with granulosa cell tumors of ovary exhibited metestrus. The uterus

FIG. 3. *Ovarian tumor in the ear of an untreated CF-1 mouse 15 months after autotransplantation of both ovaries*

was invariably large and hyperplastic, sometimes cystic, in mice with ovarian tumors in these experiments.

Tumors in Control Mice

There were 15 mice with tumors in a group of 103 untreated virgin female mice observed for 17 months. The tumors were: leukemia, 7; mammary cancer, 4; ovarian tumor, 1; pulmonary adenoma, 1; skin, 1; and sarcoma of muscle, 1.

Tumors Elicited in Mice by a Single Pulse-Dose of Hydrocarbons

A single massive pulse-dose (240 mg/kg) of 3-MC or 7,12-DMBA was injected into CF-1 female mice, age 50 days. 3-MC elicited predominantly pulmonary adenomas (27%), and this is attributed to deposition of crystals of the hydrocarbon in the lung. 7,12-DMBA evoked both leukemia (32%) and ovarian tumors (32%) in moderate yield (Table 3).

Six female mice, age 130 days, were given a single pulse-dose of 7,8,12-TMBA, 20–40 mg/kg, and observed for 6 months thereafter. Tumors were found in all of the animals. Ovarian tumors (three mice) and leukemia (three mice) were found 186–269 days after the injection. It is noteworthy that 7,12-DMBA and 7,8,12-TMBA evoked ovarian tumors whereas 3-MC failed to do so.

Tumors Elicited by Multiple Pulse-Doses of Hydrocarbons

Young adult female mice were injected with three pulse-doses of hydrocarbon, 80 mg/kg, at age 108, 111, and 114 days; control mice received no injections.

The ovaries of mice were always abnormal subsequent to the injections of 7,12-DMBA. This finding is in great contrast to what was observed in the gonads of mice injected with 3-MC or BP, where the ovaries often were normal.

TABLE 3

Incidence of tumors following a single massive pulse-dose of hydrocarbon

Female CF-1 mice, age 50 days, were given a lipid emulsion of 3-MC or 7,12-DMBA, 240 mg/kg intravenously. The animals were killed on day 136.

Hydrocarbon	No. of mice			Neoplasms				
	Original	Effective	With tumors	Leukemia	Ovary	Breast	Lung	Other
3-MC	35	26	9 (35%)	2 (8%)	0	1 (4%)	7 (27%)	0
7,12-DMBA	39	31	12 (39%)	10 (32%)	10 (32%)	0	2 (6%)	1[a]

[a] Endometriosis.

TABLE 4

Incidence of tumors following three pulse-doses of hydrocarbons

Female CF-1 mice were given a lipid emulsion containing BP, 3-MC, or 7,12-DMBA, 80 mg/kg intravenously, at age 108, 111, and 114 days; control mice were not injected. Ovarian tumors are classified *big* when visible in the gross, and *small* when found on microscopic examination.

Hydrocarbon	Duration of experiment	No. of mice		Neoplasms				
					Ovary			
		Total	With tumors	Leukemia	Total	Big	Small	Breast
	days							
7,12-DMBA	70	32	16 (50%)	8 (25%)	9 (28%)	1	8	0
7,12-DMBA	100	31	18 (58%)	8 (26%)	13 (42%)	0	13	0
7,12-DMBA	136	25	19 (76%)	10 (40%)	9 (36%)	3	6	0
BP	174	57	7 (12%)	7 (12%)	0			0
3-MC	174	59	19 (32%)	18 (31%)	0			1
None; control	341	43	1 (2.3%)	0	0			1

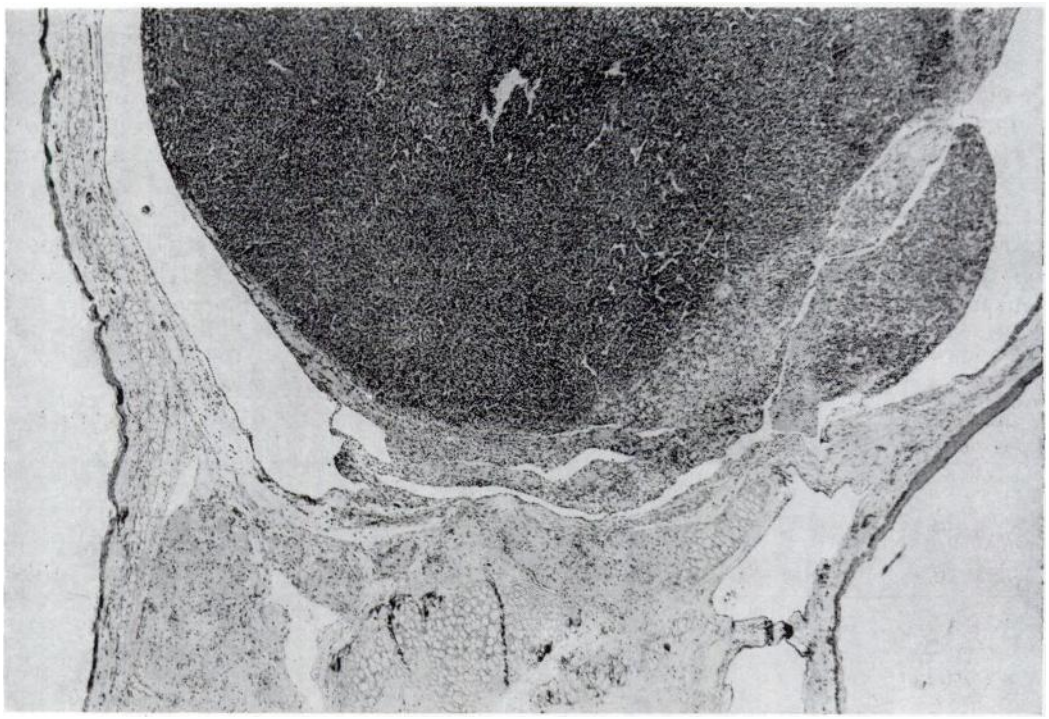

FIG. 4. *Granulosa cell tumor of ovary in the ear of the mouse shown in Fig. 3* (×75)

Ovarian tumors were found in mice given 7,12-DMBA (Table 4), but not in those which had received BP or 3-MC. The tumors were round or oval, yellow in color, with dilated blood vessels and hemorrhagic spots. Frequently they grew to a size greater than 1 cm in diameter, and in these mice the contralateral ovary was small and atrophic. All of the ovarian tumors were composed of basophilic cells resembling the cells which line the ovarian follicle.

R. T. Hill (20) has studied the physiology of ovaries transplanted to the ear of rodents. We found that tumors of granulosa cells were elicited in untreated female mice in which both ovaries had been transplanted to a subcutaneous pocket in their own ears (Figs. 3 and 4); two of six mice of this sort observed for 17 months developed ovarian tumors in the ear transplants.

It was of interest that tumors of the ovary in its normal site, some big and others of microscopic size, were found in 28% of mice within 70 days after the first pulse-dose of 7,12-DMBA (Fig. 4).

DISCUSSION

In female rats under simple conditions, a pulse-dose of large but tolerable amounts of 7,12-DMBA evokes mammary tumors (21), benign and malignant in every animal; ovarian tumors have not been observed in rats treated in this way. The number of mammary cancers per rat is vastly enhanced (22) in animals which are given a series of pulse-doses at short intervals, and these sets of injections evoke a profusion of tumors. In the male rat, by rearrangement of the experimental parameters, still very simple, leukemia is elicited in very high yield (23).

In the female mouse, because of its tolerance of 7,12-DMBA, the same large doses used in the aforementioned experiments were employed despite disparity in the size of the species. In our studies there was high incidence of ovarian tumors and leukemia, but mammary cancer was not common although very large amounts of hydrocarbons had been injected.

The biological effects elicited in living creatures by 7,12-DMBA and 7,8,12-TMBA are qualitatively indistinguishable, striking in character, and specific in nature, but the compounds differ in the quantities required to elicit toxic and neoplastic effects in the animal body. Both compounds have a remarkable ability to attack endocrine glands and targets of hormones as well. In brief, in Sprague-Dawley rats, both 7,12-DMBA and 7,8,12-TMBA evoke adrenal necrosis and induce mammary cancer and leukemia whereas the ovary is little affected and ovarian tumors have not been observed. In CF-1 mice, both 7,12-DMBA and 7,8,12-TMBA cause severe damage to the ovary whereas the adrenal is spared from significant injury. The compounds elicit tumors of ovary and leukemia in all strains of mice, and mammary cancer in high yield in some of the stocks.

In animals injected with tritiated thymidine, the incorporation of radioactivity in washed, acid-insoluble, fat-free dry residue of tissues from which volatile material has been evacuated is an index of synthesis of DNA. Pulse-doses of 7,12-DMBA and 7,8,12-TMBA cause depression of synthesis of DNA in both spleen and ileum of rodents. Leukemia frequently develops thereafter, but tumors of ileum are very rare. Nonspecific depression of DNA synthesis, even that of very great magnitude, is insufficient to elicit cancer.

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