Induction of Mammary Cancer after *in Vitro* Exposure to 7,12-Dimethylbenz(*a*)anthracene[1] (34486)

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Polycyclic aromatic hydrocarbons are considered to be "direct" carcinogens, requiring localization of the agents in the target tissue (1, 2). We reported earlier the induction of mammary tumors in grafts that were transplanted to the recipient hosts as soon as 10 min after intravenous injection of 7,12-dimethybenz(*a*)anthracene (7,12-DMBA) into the donor rats (3). The most convincing evidence, however, is the induction of tumors in the mammary gland exposed *in vitro* to 7,12-DMBA (4). We now reporting experiments carried out to determine the level of the carcinogen in the target tissue after *in vitro* exposure to 7,12-DMBA, and the relationship to subsequent carcinogenic response. Our results show that, although only a minute amount of 7,12-DBMA is required to initiate carcinogenesis in the mammary gland, a "critical" level of carcinogen in the target tissue must be present for tumor induction. Our data strongly suggest that the carcinogenic effect of 7,12-DMBA on the mammary gland is "direct" and not via the host.

Materials and Methods. Female Sprague-Dawley rats, 55–60 days old, were anesthetized under ether, and the right abdominal inguinal mammary gland was excised under sterile conditions. The mammary gland was incubated for 10 min at 4° in a medium containing 7,12-DMBA, the mixture being stirred constantly with a magnetic stirrer. Direct exposure to light during incubation was carefully avoided. The incubation medium was prepared freshly as follows. A known amount of 7,12-DMBA was added to 5 ml of 5% Tween-80 with constant stirring to obtain a homogeneous mixture, and 95 ml of Eagle's medium was added gradually. The final mixture formed a clear yellow homogeneous solution. All glassware was sterilized, and the solution was prepared without direct exposure to light.

Immediately after incubation, the mammary gland was removed from the 7,12-DMBA solution and was washed thoroughly in freshly prepared Robinson's solution (5). The mammary gland fat pad was then gently blotted on sterile gauze, and was reimplanted subcutaneously in the interscapular region on the back of the same rat. The rats were killed 4 months after the autologous transplantation. At the time of autopsy, mammary grafts were removed and were examined for gross appearance of tumors, and whole-mount preparations were made for microscopic examination.

The concentration of 7,12-DMBA in the mammary gland was determined quantitatively by the method described by us earlier (3). After removal, the mammary glands were weighed, frozen, and minced. The minced tissue was then saponified and extracted with spectroanalyzed heptane. Quantitative assay of 7,12-DMBA was carried out on this heptane extract with the use of an Aminco-Bowman spectrophotofluorometer. The wavelengths used for these analyses were selected to employ the maximum excitation peak and also the maximum emission peak to reduce interference by natural fluorescence. For 7,12-DMBA, the maximum excitation wavelengths is 370 mμ, and the emission wavelength is 415 mμ. The results of the determinations were calculated and expressed as micrograms of 7,12-DMBA per gram of tissue.

The effect of dosage of 7,12-DMBA on the

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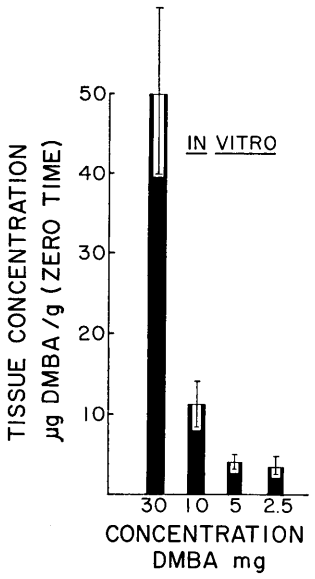

FIG. 1. The experiment consisted of four groups of five rats each. A mammary gland was excised from each female rat, incubated for 10 min at 4° in a medium containing 7,12-DMBA at different dose levels. The incubated mammary gland was then washed with Robinson's salt solution to remove the excess 7,12-DMBA, and it was then prepared for assay of the carcinogen content. In the control group, the mammary glands were incubated in the medium containing no carcinogen. In this experiment there was no measurable 7,12-DMBA in the mammary gland *in situ*. The tissue concentration of 7, 12-DMBA is expressed as micrograms of 7,12-DMBA per gram of mammary tissue.

induction of tumors in mammary glands after *in vitro* exposure to the carcinogen was studied in four experimental groups using autologous transplantation of treated mammary glands after incubation in media containing 30, 10, 5, and 2.5 mg of 7,12-DMBA and a fifth control group in which the mammary glands were incubated in media containing no carcinogen.

7,12-DMBA was purchased from Eastman Organic Chemical Co., Inc. (mp 123°). Purity of the compound was determined by a single thin-layer chromatogram in benzene:ethylene glycol monomethyl ether system (8:92).

Results. Figure 1, which shows the localization of 7,12-DMBA in the mammary gland immediately after the completion of incubation in media containing various amounts of the carcinogenic agent (zero time), clearly demonstrated that the level of 7,12-DMBA in the mammary gland is proportional to the dose of carcinogen in the solution. We also removed the host's untransplanted mammary glands (*in situ*) to determine whether they contained any 7,12-DMBA that might have been transported to them from the 7,-12-DMBA-treated mammary gland. We failed to detect any measurable amount of 7,12-DMBA in the *in situ* mammary glands examined.

The concentration of the 7,12-DMBA remaining in the medium that had contained 30 mg of 7,12-DMBA at the beginning of the experiment and the concentration of the 7,-12-DMBA in the Robinson's solution used for washing were also measured. We recovered 27.9 mg of 7,12-DMBA in the remaining 93 ml of medium and 429 µg of 7,12-DMBA in a total of 68 ml of Robinson's solution. These results clearly show that only a very small amount of 7,12-DMBA is needed for the induction of a tumor in a mammary gland.

The ability of 7,12-DMBA to induce tumors in the mammary gland after *in vitro* exposure to the carcinogen for only a short period is illustrated by the results in Table I. Six adenocarcinomas developed in nine surviving mammary grafts incubated in a medium containing 30 mg of 7,12-DMBA. The incidence of tumors in the graft decreased with the amount of 7,12-DMBA in the medium. No mammary tumors were observed in grafts exposed to a medium containing only 2.5 mg of the carcinogen. In this entire experiment, no tumors were observed in untransplanted mammary glands.

Discussion. In systemic administration of the carcinogenic agents, we believed that the localization of a carcinogen in the target tissue is a prerequisite to neoplastic transformation of the tissue. Thus the dose of a carcinogen and the duration of exposure are important factors in the induction of tumors (2). Although only a very small amount of the carcinogenic agent is needed to initiate carcinogenesis, the presence of a "critical" level of the carcinogen in the target tissue is an

TABLE I. Induction of Tumors in Mammary Glands by *in Vitro* Exposure to 7,12-Dimethylbenz-(*a*)anthracene (7,12-DMBA).[a]

Dose of 7,12-DMBA (mg)	Rats (no.)	Surviving grafts (no.)	Surviving grafts with tumors (no.)
30	10	9	6
10	10	9	4
5	10	7	1
2.5	10	8	0
Control	10	9	0

[a] A mammary gland was excised from each female rat, incubated for 10 min at 4° without exposure to light in a culture medium containing 7,12-DMBA, and then reimplanted into the interscapular region of the same rat. In the control group, mammary glands were incubated in a medium which did not contain the carcinogen. The rats bearing the autografts were killed 4 months later. Grafts and the mammary glands *in situ* were removed for whole-mount preparations. The tumors were all adenocarcinomas.

important requirement. Our results show that there is a definite relationship between the incidence of tumors and the concentration of the carcinogen in the target tissue.

The question whether transformation of a carcinogenic agent to a more active metabolite is necessarily the first step in initiating carcinogenesis is not yet settled. Although there is conclusive evidence that aromatic amines undergo *N*-hydroxylation and esterification of the *N*-hydroxyl group to form more active carcinogens (6) carcinogenic metabolites with greater activity than the parent compounds have not been found among the polycyclic aromatic hydrocarbons. An active metabolite, 7-hydroxymethyl-12-methylbenz (*a*)anthracene has been isolated from rat liver homogenates incubated with 7,12-DMBA, but available evidence does not show that this metabolite has greater carcinogenicity than its parent compound, 7,12-DMBA (7). If prior metabolism must occur in carcinogenesis induced in the mammary gland by 7,-12-DMBA, it presumably must take place in the target tissue, namely, the mammary gland, since in our experiments the carcinogen was not administered systemically. If, on the other hand, enough of the 7,12-DMBA in the mammary gland was transported to the liver or other sites for formation of new potent metabolites, then we would expect to observe tumors in mammary glands *in situ*. This clearly is not the case. Our present data strongly suggest that 7,12-DMBA itself, and not a derivative, is probably responsible for mammary tumor induction.

Summary. Rat mammary glands which were excised and incubated in a medium containing 7,12-DMBA developed tumors after their reimplantation in the interscapular region of the back. The mammary tumor incidence in the autologously transplanted graft is parallel with the concentration of the carcinogen in the graft. The results indicate that the carcinogenic effect of 7,12-DMBA on the mammary gland is "direct" and not via the host.

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