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# Diaplacental Carcinogenesis: Initiation with the Carcinogens Dimethylbenzanthracene (DMBA) and Urethane during Fetal Life and Postnatal Promotion with the Phorbol Ester TPA in a Modified 2-Stage Berenblum/Mottram Experiment

Klaus Goerttler and Heinz Loehrke

Institut für Experimentelle Pathologie am Deutschen Krebsforschungszentrum Heidelberg, Im Neuenheimer Feld 280, D-6900 Heidelberg 1, Federal Republic of Germany

**Summary.** Diaplacental initiation with the carcinogens DMBA or urethane followed by topical treatment of mice of F-1 generation with the tumor promotor TPA led to the formation of benign and malignant tumors on the back skin and also in various internal organs. (This system constitutes a modified 2-stage experiment based on the early schemes of Berenblum and Mottram.) Application of either the carcinogens or the tumor promoter alone did not lead to the formation of tumors within one year. The highest skin papilloma yield was obtained with mice initiated with DMBA from the 16–19th day of fetal life. The highest total tumor yield was obtained after initiation from days 18–21st. The combination urethane/TPA also promoted the formation of tumors of the skin and other organs. The significance of this modified prenatal-postnatal initiation/promotion scheme in human pathology is discussed.

**Key words:** Carcinogenesis – Diaplacental – DMBA initiation – Urethane initiation – Phorbol ester-promotion – Skin papillomas – Other tumors.

## Introduction

The possibility of prenatal carcinogenesis has previously been discussed mainly with reference to the dystontogenetic theory of tumor formation (Cohnheim, 1877; Ribbert, 1906). With the accumulation of data on prenatal induction of malformation, interest has been increased in this area between oncology and teratology (Di Paolo and Kotin, 1966; Goerttler, 1968). The first report of experimental carcinogenesis was made by Larsen (1947), who induced lung adenomas by treatment of mice with urethane. Mohr et al. (1965) reported the successful induction of pulmonary tumors by dialkylnitrosamines in syrian golden hamsters by the trans(dia)placental route, and Druckrey and coworkers (1966) produced tumors of neural origin in rats. More recently, many more

workers have confirmed and extended these early results (for reviews see Tomatis and Mohr, 1973; Zeller, 1974; Ivankovic, 1975; Magee, 1975). This work, which was at this time primarily of experimental and theoretical interest, was shown to have relevance to human pathology by Herbst, Ulfelder and Poscanzer (1971). These authors observed vaginal adenocarcinomas in daughters of women who had received high doses of oestrogens in order to prevent spontaneous abortion in the early stages of pregnancy.

In the most experiments carried out up to now, the carcinogen, in most cases a nitroso compound, has been given as a single dose to pregnant animals (mainly rats) towards the end of the pregnancy. It has been shown that the maturing brain was particularly sensitive to this treatment. The formation of multiple tumors was also noted (e.g. gliomas next to neurilemmonas of the trigeminal nerve, and adenosarcomas of the kidney). Histologically, the types of kidney tumors induced were similar to those found in children, ranging from undifferentiated adenosarcomas (Wilms tumors) to fibromas with varying extent of adenomatous components (Goerttler, unpublished; Ivankovic, 1975).

In recent years, the possible effect of exogenous substances in inducing human tumors has become increasingly important. In addition, the observation that different substances can react in a syncarcinogenic (K.H. Bauer, 1963; Schmähl, 1970) or cocarcinogenic fashion (Hecker, 1975) led to the consideration that the fetus is even more exposed to danger from carcinogens than was previously thought: sensitive cells in different tissues could be initiated diaplacentally by small doses of a carcinogen. The stimulation of tumor growth, however, could take place postnatally through exposure to other carcinogens or tumor promoting substances.

The 2-stage experiment first developed by Berenblum and Mottram (s. Berenblum, 1975) showed that it is possible to initiate epidermal cells with a small, in itself non-carcinogenic dose of a suitable carcinogen. Subsequent treatment with a hyperplasiogenic agent (e.g. croton oil or TPA = 12-o-tetradecanoyl-phorbol-13-acetate) promoted the growth of visible tumors. Application in the reverse order was ineffected, as was the application of only one of the substances at the same dose level.

Experiments from this laboratory (Goerttler and Loehrke, 1976) have shown that it is possible to initiate epidermal cells via mothers milk and subsequently obtain a high tumor yield by postnatal treatment with TPA. In this way, it was shown that the 2-stage experiment could be carried out by transfer through the mother animal. We have now extended these studies to include diaplacental initiation with postnatal promotion. The carcinogens used were dimethylbenzanthracene (DMBA) and the weak epidermal carcinogen urethane, both in combination with the synthetic tumor promoter TPA.

## Materials and Methods

Female NMRI mice bred in the DKFZ were kept under SPF conditions and vaccinated against ectromelia at the age of eight weeks (vaccine from Institut für Tropenhygiene, München). Animals were paired at 12 weeks. Females with positive vaginal smears (spermatozoa) were removed and randomly distributed into the groups shown in Table 1.

**Table 1.** Experimental protocol (Diaplacental initiation)

| Group | Substance and form of application | Dose mg/kg body weight | Day of pregnancy | Number of pregnant animals |
|---|---|---|---|---|
| I | Control Livio®-Oil[a]-oral | (1 ml) | 14–21 | 6 |
| II | DMBA – oral | 5 × 30 mg = 150 mg | 14–18 | 19 |
| III | DMBA – oral | 5 × 15 mg = 75 mg | 14–18 | 9 |
| IV | DMBA – oral | 4 × 15 mg = 60 mg | 16–19 | 5 |
| V | DMBA – oral | 4 × 15 mg = 60 mg | 17–20 | 5 |
| VI | DMBA – oral | 4 × 15 mg = 60 mg | 18–21 | 5 |
| VII | urethane i.p. | 3 × 60 mg = 180 mg | 17–19 | 5 |
| VIII | urethane i.p. | 3 × 60 mg = 180 mg | 18–20 | 5 |

[a] Livio®-Oil is a commercially available plant oil

Newborn mice were investigated macroscopically and any malformation recorded. At the end of the sucking period the young animals were separated from the mothers and put into subgroups. Ia–VIIIa were treated between the ages of 12 and 26 weeks twice weekly with an acetone solution of the cocarcinogen TPA [1] (12-o-tetradecanoyl-phorbol-13-acetate). This gave a total of 48 applications to the back skin of the mice of 0.01 µmole TPA (0.00615 µg) in 0.1 ml acetone. Animals in subgroups Ib–VIIIb were not treated with TPA. Appearance of skin tumors was observed and recorded each week. All animals which died within the period of one year were autopsied and histologically investigated to determine cause of death and to check the presence of other tumors.

Animals were maintained singly in Macrolon type II cages and fed with Altromin-R-10 Standard, with water available ad libitum.

## Results

### *1. General*

Table 2 shows the effect of carcinogen treatment on the progress of the pregnancies and also gives the number of newborn mice assigned to the different groups. In group III, application of 150 mg DMBA/kg body weight led to abortion in 14 of the 19 pregnant animals. In group II, only one third of the animals aborted on treatment with a dose of 75 mg DMBA/kg body weight, and further reduction of the dose to 60 mg/kg body weight resulted in unbroken pregnancy for all of the treated animals (groups IV, V and VI). However, the number of newborn mice was still clearly less than the control value (12 per litter) Urethane at a dose level of 180 mg/kg body weight had an effect both on the pregnancies (one abortion in groups VII and VIII) and on the number of newborn. Malformations were not observed in any of the groups.

Animals which were only initiated with DMBA or urethane did not develop any epithelial tumors within the period of the experiment. Promotion by treatment of the mother animals with TPA led to the growth of fibroepitheliomas (papillomas) on the back skin with the same latent period as was observed

[1] The substance was generously provided by Prof. Dr. E. Hecker, Biochemisches Institut, Deutsches Krebsforschungszentrum Heidelberg

**Table 2.** Numbers of abortions and normal newborn, and assignment to different groups for treatment with TPA

| Group | Mother-animals/abortions | Normal newborn | Subgroup a with TPA | Subgroup b without TPA |
|---|---|---|---|---|
| I | 6/0 | 72 | 56 | 16 |
| II | 19/14 | 48 | 38 | 10 |
| III | 9/3 | 50 | 40 | 10 |
| IV | 5/0 | 45 | 35 | 10 |
| V | 5/0 | 44 | 34 | 10 |
| VI | 5/0 | 50 | 40 | 10 |
| VII | 5/1 | 32 | 22 | 10 |
| VIII | 5/1 | 30 | 20 | 10 |

with the young animals. The combination urethane/TPA also caused the development of some tumors.

### *2. Controls*

Untreated animals or animals treated only with TPA postnatally. (Subgroups Ia and Ib·$n=56$ or 16, Table 2.)

The 16 untreated animals, obtained from mother animals which were given Livio® oil only developed no epithelial tumors within 52 weeks. Also the treatment with TPA (subgroup Ia, $n=56$) did not produce any visible skin tumors.

### *3. Prenatal Initiation with DMBA or Urethane with no Postnatal TPA Treatment (Subgroups IIb–VIIIb, n=70, Table 2)*

None of the 70 young animals from the mice treated during pregnancy with DMBA at different dose levels (150-75-60 mg/kg body weight) developed tumors in the investigation period of 52 weeks.

### *4. Prenatal Initiation with DMBA or Urethane and Postnatal Promotion with TPA (Subgroups IIa–VIIIa, n=229, Table 2)*

Each young animal in these groups was treated a total of 48 times with TPA at a dose level of 0.01 µmole=0.00615 mg. In all subgroups, fibroepitheliomas (papillomas) developed on the back skin 5–9 weeks after commencing TPA treatment. Figures 1 and 2 show the corresponding data on the percentage of tumor-bearing animals and the number of papillomas/animal.

The combination DMBA/TPA caused formation of papillomas in 60–90% of the treated animals. In those animals which were treated with the highest dose of DMBA via the mother (subgroup IIa) a relatively high percentage had papillomas at an early stage but this did not increase to more than 70% at any stage. The next highest dose of 75 mg DMBA/kg body weight (subgroup IIIa) gave a similar percentage of tumor bearing animals, but after a slight delay

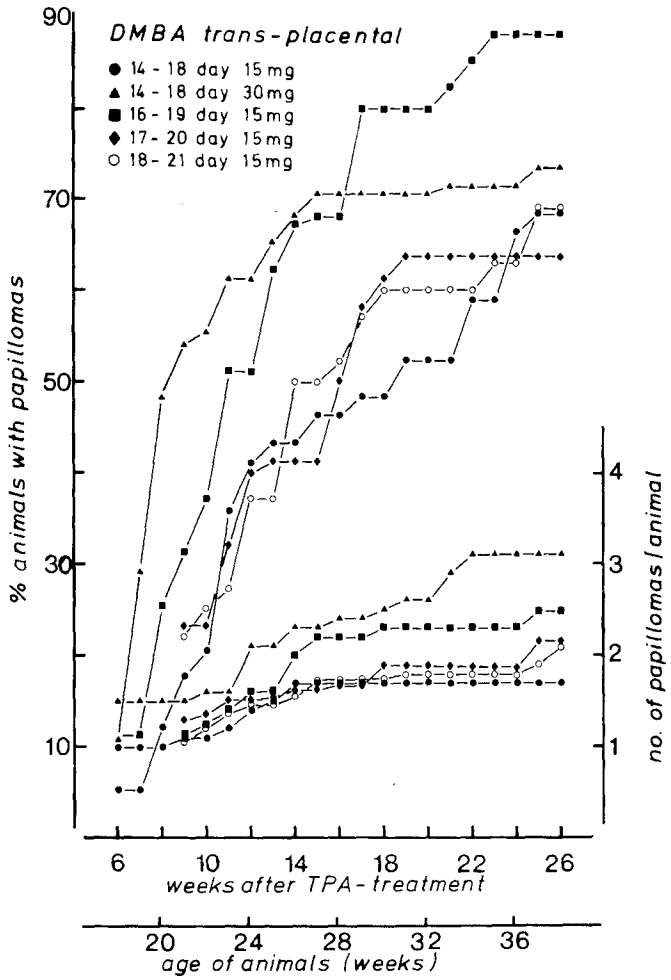


**Fig. 1.** Dia/transplacental initiation with DMBA at different times during fetal life with postnatal treatment of the F-1 generation with TPA. Percentage of papilloma bearing animals and number of papillomas per animal between the 6th and 26th weeks after commencing TPA treatment

with respect to group II. Of the groups which received 60 mg DMBA/kg body weight, those animals which were initiated diaplacentally between 16–19 days of fetal life (subgroup IVa) developed tumors earlier than those of the other groups. The percentage of tumor bearing animals was also higher in this group. The highest tumor yield was attained in the group treated with 150 mg DMBA/kg body weight (3 papillomas/animal). For the remaining subgroups (IVa, Va and VIa) which received the 60 mg dose, initiation between 16–19 days was again more active than at the other earlier or later times.

For urethane-initiated animals, the same percentage of tumor-bearing animals was reached in both groups. However, initiation from the 18–20th day led to a more rapid increase in the tumor rate and also to a higher number of tumors/animal.

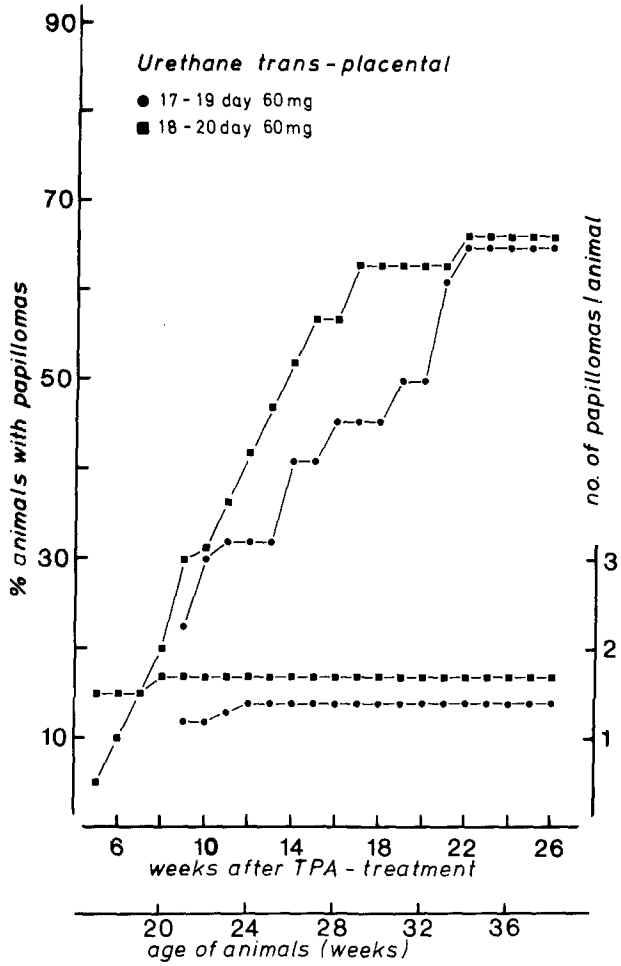


**Fig. 2.** Dia/transplacental initiation with urethane on 17–19th or 18–20th days of fetal life with postnatal TPA treatment of the F-1 generation. See also Figure 1

Statistical evaluation of the results against trend (Pfanzagl, 1968) showed that differences in the number of tumor bearing animals in the different groups were significant. At the 11th week after starting to count the tumors, the following sequence was observed in the tumor rates (highest tumor rate first):

1 = IIa
2 = IIIa
3 = IVa
4 = Va
5 = VIa

By initiation with urethane, no significant differences in the tumor rates for groups VIIa and VIIIa were observed.

**Table 3.** Number and localisation of tumors which appeared in animals died within one year after diaplacental initiation and postnatal promotion. Histopathological findings. (Organs with multiple tumors are only counted as one tumor, benign and malignant tumors in one organ is registered as two tumors)

| Sub-group | Skin | | Lungs | | Other organs | | Leucemias and Thymomas | No. of experimental animals/died animals in each group | No. of tumor bearing died animals/ tumors in each group |
|---|---|---|---|---|---|---|---|---|---|
| | benign | malignant | benign | malignant | benign | malignant | | | |
| IIa | 5 | 5 | 7 | 1 | 3 | 3 | 9 | 38/16 | 16/31 |
| IIIa | 3 | 1 | 8 | 2 | – | 3 | 5 | 40/14 | 14/22 |
| IVa | 4 | 2 | 5 | – | 1 | – | 3 | 35/10 | 9/15 |
| Va | 2 | – | 2 | 1 | – | 1 | 2 | 34/6 | 6/8 |
| VIa | 5 | 2 | 6 | 3 | 1 | 2 | 2 | 40/13 | 12/21 |
| VIIa | 1 | – | 2 | – | – | 1 | 1 | 32/5 | 3/5 |
| VIIIa | 1 | – | 1 | – | – | – | 1 | 30/4 | 3/3 |

### *5. Tumors of Animals which died During the Experimental Period of one year (n=68)*

Within this time period, no animals died which were in the control groups Ia (with TPA) and Ib (without TPA). Five nontumor bearing animals were in subgroups IIa–VIIIa. Surviving animals of the subgroups which were not treated with TPA (IIb–VIIIb) did not have any tumors.

In Table 3, data are presented on the localisation and benign or malignant character of tumors found in autopsied dead animals of subgroups IIa–VIIIa. Animals of subgroups IIa–VIa, which were treated with DMBA and TPA, showed a broad spectrum of tumors. The total numbers of tumors varied appreciably. Subgroup IIa with the highest total dose of DMBA also attained the highest number of tumors. The groups with the next highest tumor rate proved to be IVa and VIa.

With regard to the individual tumors, an undifferentiated vaginal sarcoma in subgroup IIa is particularly interesting, since on longer observation of the animal after the experimental period of one year, many of this kind were seen (Goerttler and Loehrke, unpublished results).

In addition to the tumors observed in the skin, other tumors were seen in the lung, which, with this particular mouse line tends to develop adenomas, liver (hepatocellular adenoma, carcinoma and also hemangioendothelioma) ovary (granulosa cell tumors), mammary gland, lymph nodes and thymus.

No tumors were observed after initiation with urethane alone with no subsequent promotion, whereas the combination urethane/TPA promoted the formation of tumors in the skin, lung and thymus. The total number of tumors obtained was considerably lower than after DMBA/TPA application.

## Discussion

The following results of these investigations should be emphasized:

1. It is possible to initiate cells in mice of the F-1 generation by diaplacental

administration of relatively small doses of the carcinogens DMBA and urethane. These doses are not sufficient to induce tumors during 52 weeks post natum.

2. Combination of prenatal initiation with postnatal application of TPA to the back skin led to the development of benign and malignant tumors on the skin and also in various internal organs.

3. Application of DMBA four times at 15 mg/kg body weight to the mother during days 16–19th of the pregnancy, followed by postnatal treatment of the young animals with TPA, produced more papillomas than application at days 14–18th (five times), 17–20th or 18th–21st (four times each).

4. The carcinogen urethane in combination with TPA also leads to some tumor formation in the skin but also in the lung and thymus.

An additional observation was that the phorbol ester TPA has promoting properties in organs other than the skin, which may mean that absorption of this substances into the organism takes place. Similar data on a more long range effect of TPA were obtained in previous experiments in the laboratory (Goerttler and Loehrke, 1976) and from studies using phorbol by Armuth and Berenblum (1972). The TPA could be resorbed and redistributed throughout the body via the blood vessels, when it is possible that it acts locally as a promoter at very low concentrations in other organs. As an alternative to the purely local effects of TPA a more general immune suppressive property must be considered.

The significance of these results for human medicine is obvious: It is known that the human prenatal organism is highly sensitive. In contrast to earlier ideas, the fetus is not specially protected during its intrauterine existence. Whereas embryonal development can be considered the period when defective-growth or malformation can be induced (Goerttler, 1957, 1964), the fetal period or the last third of the pregnancy can be considered to be at risk for tumor cell initiation. This period can be described as the oncogenetic determination period (Goerttler, 1968) and it might be expected that certain cross-over points exist between teratology and oncology. The initiation with relatively high doses of oestrogens described by Herbst, Ulfelder and Poskanzer (1971) took place during both the embryonal period and the fetal period after the 75th day after conception.

Our experiments show that diaplacental initiation can be achieved using carcinogen doses which on their own are not sufficient to cause termination of pregnancy. In analogy to experiments carried out on adult animals, the possibility of prenatal syncarcinogenesis must be considered in addition to the various combinations of pre- and postnatal effects of carcinogens and cocarcinogens.

A consequence of the concept of increased danger to the fetus is that it can no longer be considered that the maternal organism can tolerate "no-dangerous" doses of exogenous carcinogens. The possibility that even weak doses can led to initiation of cells and, in combination with postnatal damage, to production of tumors can no longer be denied. It should, for example, be considered that all children whose mothers smoked during pregnancy should be assigned to a high risk group. The state of health of these children should be followed and compared with that of a suitable control group whose mothers did not smoke during pregnancy.

These results also influence our conception of prenatal tumor prophylaxis. The maternal organism must be regarded more than previously as a potential source of danger for the fetus. The classical indications of prenatal defects (death of the fetus, growth defects, malformations, premature and still births) must be broadened to include "delayed–effect tumor initiation" with latent times as long as decades.

The higher tumor yield obtained after initiation on 16–19th days of fetal life should be used as the basis of further experiments with more animals to test the possibility of differential sensitivity at different times during pregnancy. The role of different carcinogens should also be studied. Previous experiments (Goerttler et al., 1970) showed that diaplacental application of aflatoxin B-1 led to an acute depression in DNA synthesis. We have recently completed a series of biological experiments on diaplacental effects of aflatoxin which will be reported elsewhere.

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