Systemic Two-Stage Carcinogenesis in the Epithelium of the Forestomach of Mice Using 7,12-Dimethylbenz(a)anthracene as Initiator and the Phorbol Ester 12-O-Tetradecanoylphorbol-13-Acetate as Promoter

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ABSTRACT

In a modified two-stage carcinogenesis experiment, the effectiveness of the initiator 7,12-dimethylbenz(a)anthracene (DMBA) and the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) in the epithelium of the forestomach of the mouse has been investigated.

Fifty mice were treated intragastrically with a single dose of DMBA (50 mg/kg body weight), followed by repeated intragastric administration of TPA (10 mg/kg body weight) over a period of 35 weeks. In comparison with the corresponding control groups (no treatment, DMBA initiation only, and TPA treatment only), the initiated and promoted group clearly showed the highest tumor incidence in the target organ (45 tumor-bearing animals of 50 animals). No tumors of the forestomach were found in the untreated control group and the TPA-treated group, whereas in the DMBA-initiated group, ten animals had developed tumors of the forestomach.

In addition to the mouse skin model for two-stage carcinogenesis, the mouse forestomach appears to respond to DMBA initiation-TPA promotion. This organ provides an additional tissue with which to investigate tumor promotion and further to ascertain specific parameters of the promotion step.

INTRODUCTION

The DMBA²-phorbol ester-mediated 2-stage carcinogenesis experiment, along with its numerous modifications both at the level of initiation and the level of promotion, has proved to be a most useful model for the study and understanding of carcinogenesis. The original experimental design, as introduced by Benenbium (3) and Mottram (18), leads to the development of tumors in the mouse skin, and all of our knowledge on the initiation-promotion process stems almost exclusively from experiments in this tissue. Apart from historical reasons, this can be traced back to the fact that species other than the mouse seemed to be refractory to this experimental design (27) and that findings in skin could obviously not be extrapolated to other organs (6). These limitations have impaired the acceptance of initiation and promotion in chemical carcinogenesis since only limited combinations of agents led to duplication of the 2-stage mechanism in other organs (2). However, recent experiments involving liver (2, 7, 16, 20, 21), lung (2, 33), colon (23), and bladder (14) have unequivocally demonstrated the concept of initiation-promotion in other tissues. In previous experiments involving transmaternal (11) and diaplacentai OMBA-TPA-mediated 2-stage carcinogenesis (12, 24), we not only observed tumors in the skin but also found a broad tumor spectrum in internal organs when the animals were kept for lifelong studies (13). These findings prompted us to reinvestigate systematically the effectiveness of the initiation-promotion phenomenon in organs other than mouse skin and also to reevaluate its applicability to other species.

In order to reduce variations due to tissue differences to a minimum, we initially decided to investigate the 2-stage experiment in another epithelium, namely, the forestomach of the mouse. This epithelium, which has already been the subject of an unsuccessful 2-stage experiment (4), has recently been shown to be susceptible to croton oil promotion in N-methyl-N-nitrosoguanidine-treated rats (29). In contrast to the adjacent columnar glandular stomach, the forestomach in rodents is lined by a stratified squamous epithelium which has some features in common with the interfollicular back skin (19). Both the initiator and the promotor were administered intragastrically. It was of further interest to repeat our earlier observations that tumors developed in organs other than the primary target organ when a modified Berenblum-Mottram design was used.

MATERIALS AND METHODS

Two hundred female C57 BL/6 mice, bred under specific-pathogen-free conditions in the German Cancer Research Center, were used in the experiment.

After random distribution into 4 experimental groups (50 animals/group), the mice were maintained in Macrolon type II cages and fed with Altromin R-10 Standard (Lage/Lippe, West Germany) with water available ad libitum. DMBAs (Fluka AG, Buchs, Switzerland) was used as an initiator and TPA, kindly provided by Dr. E. Hecker, Institute of Biochemistry, was used as a promoter. Animals of Group 1 served as control animals and were left without DMBA and TPA treatment. At the age of 10 weeks, each animal of Group 2 received a single intragastric dose of 50 mg DMBA per kg body weight in 100 μl of sesame oil.

One week later, each animal of Group 3 received 10 mg TPA per kg body weight in 100 μl of sesame oil intragastrically twice per week over a period of 35 weeks. This gave a total of 700 mg TPA per kg body weight.

Animals of Group 4 were initiated with DMBA and pro-
nated with TPA according to the application scheme of Groups 2 and 3, respectively.

All substances were administered by means of a stomach tube especially manufactured for this purpose.

Two weeks after the last TPA application to Groups 3 and 4, i.e., 37 weeks after commencing the experiment, animals still alive were killed by ether anesthesia. These animals, as well as those which died in the course of the experiment, were histologically investigated. The following organs were included in the histological examination: stomach, trachea, esophagus, lung, small intestine, colon, liver, kidneys, adrenal gland, spleen, uterus, ovaries, vagina, bladder, thymus, lymph nodes, and skin.

RESULTS

**Group 1 (Control, No Treatment, n = 50).** All animals survived during the observation period of 37 weeks. Neither macroscopically nor histologically did any of the animals show signs of benign or malignant alterations in any of the organs investigated. The forestomach epithelium appeared normal (Fig. 3a).

**Group 2 (DMBA Initiation, No Promotion, n = 50).** Four animals died during the experiment. Tumors were found in the following organs: forestomach (10 papillomas at early stages of development; see Fig. 3b); small intestine (1 reticulosarcoma with metastases in the liver and the lymph nodes); colon (1 adenocarcinoma); thymus (2 thymomas); and ovaries (2 granular cell tumors). This gave a total of 15 tumor-bearing animals and a total of 16 tumors (one animal had developed 2 tumors).

**Group 3 (No Initiation, TPA Treatment, n = 50).** Six animals died during the experiment. In all animals, the forestomach, esophagus, and intestinal tract were free of tumors; however, moderate acanthosis and a pronounced hyperkeratosis were generally visible in the forestomach epithelium (Fig. 3c). One animal had developed a tumor in the anal region, 2 animals showed lymphomas (1 liver and 1 bladder), and one animal had a trabecular lung adenoma. This gave a total of 4 tumor-bearing animals and a total of 16 tumors.

**Group 4 (DMBA Initiation, TPA Promotion, n = 50).** Twenty-one animals died during the experiment. Only 3 of 50 animals were free of tumors; 22 animals had developed multiple tumors. Forty-five animals showed tumors (partly multiple) of the forestomach; epithelial tumors of the integument were seen in 11 animals. Altogether, 47 animals were tumor bearers, and a total of 65 tumors were determined (multiple tumors of the forestomach were counted as one tumor). The tumor spectrum comprising tumors in epithelial tissues as well as in several internal organs is summarized in Table 1.

**DISCUSSION**

The aim of the present study was to demonstrate that the DMBA-phorbol ester-mediated 2-stage carcinogenesis experiment is not only effective in the epidermis of mouse skin but also can be transferred successfully to other organs. To this purpose, the epithelium of the forestomach of the mouse was chosen as a target organ. Although this particular epithelium, unique to rodents, resembles the interfollicular epidermis of the mouse skin in that it represents one of the rare internal epithelia showing true orthokeratinization (Fig. 1), substantial differences exist between the 2 tissues with regard to environmental conditions, function (19), and proliferative rate (8, 19).

Using both intragastric initiation and promotion, we were able to show unequivocally that the combined application of DMBA and TPA was superior, with respect to tumor formation in the target organ.

In addition to mouse skin, there is now a further organ available for DMBA-TPA-mediated 2-stage carcinogenesis (Fig. 2). This finding not only has considerable importance for the experiment as such but also offers the possibility of checking whether results in mouse skin on the morphological, cellular, and subcellular level, thought to be crucial to the initiation-promotion process, are generally valid for all epithelia or are only specific for the primary target organ under consideration.

The forestomach is a glabrous epithelium (19). Since the role of hair follicles and sebaceous glands in skin carcinogenesis is still a matter of controversy (1, 5, 10, 15, 25, 30), the lack of these appendages in the forestomach may help to evaluate the degree of participation of cutaneous appendages in tumor formation in haired skin. Moreover, delayed promotion experiments (31, 34) in the forestomach may contribute to the understanding of initiation and give further insight into the nature of initiated cells.

The role of inflammation (26) and hyperplasia (9, 22, 28) during the promotion phase in mouse skin has been the subject of extensive studies. It can be said from our experiment that hyperplasia seems to be an accompanying event of promotion in the forestomach. However, the type of increased cellular activity differs from that in the epidermis, in that it consists of a pronounced hyperkeratosis of the upper layers rather than a visible acanthosis of the living cell layers (Fig. 3c).

We have repeatedly emphasized that the combination DMBA-TPA leads not only to tumor formation in mouse skin but also to considerable tumor formation in various internal organs (11-13, 24). This finding, which may be important in view of a possible promoting activity of TPA metabolites (2), could not definitely be confirmed in the present experiment since the conditions were obviously not suitably adjusted to minimize tumor induction by the initiator alone [compare...
tumor incidence in organs other than epithelia of Groups 2 and 4 (Table 1).

In summary, the applicability of the 2-stage experiment to the forestomach of the mouse is indicative of an evident preference of the combination DMBA-TPA for keratinizing epithelial tissues. This is substantiated by the fact that even upon systemic initiation and promotion, a high incidence of tumors of the integument is noted.

The forestomach of the mouse has no direct analog in the human organism. However, its morphological appearance is very similar to human esophageal tissue. There is a large body of evidence that the exceptionally high rate of esophageal cancer in the Netherland Antilles is due to a cocarcinogenic mechanms causally related to tumor-promoting phorbol esters (32). These are ingested by the widespread and frequent use of the plant Croton flaves as an ingredient of a popular tea-like beverage (17, 32). This finding is indicative of the role which tumor promoters may play in human cancer etiology. However, before one can speak of a universal concept of the initiation-promotion phenomenon, the experiment necessarily needs to be reproduced in a wide range of species. Current investigations in our laboratory using the rat, rabbit, and hamster tend to reinforce the concept of initiation and promotion.

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REFERENCES

Fig. 1. Comparison of the structure of the forestomach epithelium (a) and interfollicular epidermis of the back skin (b). KL, keratin layer; GL, granular layer; BL, basal lamina. H & E, × 1330.

Fig. 2. Two-stage carcinogenesis experiment in the epithelium of the forestomach. DMBA-TPA-treated forestomach (left), showing multiple papillomas; right, forestomach of untreated control. FS, forestomach; GS, glandular stomach. Two times its natural size.
Fig. 3. Two-stage carcinogenesis experiment in the epithelium of the forestomach. a, control (normal epithelium showing orthokeratinization and physiological hyperkeratosis); b, DMBA-treated animal (moderate hyperplasia and acanthosis along with preneoplastic lesions); c, TPA-treated animal (slight hyperplasia of the living cell layers and pronounced hyperkeratosis); d, DMBA-TPA-treated animal (part of a papilloma). H & E, x 133.
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