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Differential Carcinogenic Effects of Intraperitoneal Initiation with 7,12-Dimethylbenz(a)anthracene or Urethane and Topical Promotion with 12-O-Tetradecanoylphorbol-13-acetate in Skin and Internal Tissues of Female SENCAR and BALB/c Mice

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Groups of female SENCAR or BALB/c mice were initiated once intraperitoneally with 300 μg/mouse of 7,12-dimethylbenz(a)anthracene (DMBA) or 20 mg/mouse of urethane at 7 weeks of age. Beginning one week later, mice received topically applied acetone or 12-O-tetradecanoylphorbol-13-acetate (TPA), once weekly, at 2.5 μg/mouse for weeks 1 through 6 and 1.25 μg/mouse for weeks 7 through 52. The skin lesions were evaluated clinically. A complete necropsy was performed on all mice at week 52. SENCAR mice exposed to DMBA/TPA and urethane/TPA had more skin tumors than SENCAR mice exposed to DMBA or urethane alone and more than BALB/c mice in any treatment group. Of all skin carcinomas diagnosed histologically in DMBA/TPA-exposed mice, less than one-third had been identified clinically while the mice were alive. Most of the carcinomas arose within papillomas. BALB/c mice developed more vascular and uterine tumors than did SENCAR mice injected with DMBA and more lung and vascular tumors than did SENCAR mice injected with urethane. TPA exposure after treatment with either initiator had no significant effect on internal tumor development in either SENCAR or BALB/c mice.

Introduction

SENCAR mice were originally bred for susceptibility to two-stage carcinogenesis in mouse skin (*1*). It has been demonstrated that these mice are exceptionally more sensitive to skin carcinogenesis by chemicals (*2–4*) and by ultraviolet irradiation (*5*) than most other strains or stocks of mice, and that SENCAR mice have a population of spontaneously initiated cells (*3*), as well as dark cells (*2*), which are possible targets of carcinogens. An earlier study of two-stage carcinogenesis in SENCAR and BALB/c mice has shown that SENCAR mice are considerably more sensitive than BALB/c mice (*3*) to two-stage carcinogenesis. The aim of this current study was to evaluate the differential carcinogenic effects of 7,12-dimethylbenz(a)anthracene (DMBA) and urethane on internal tumor development in female SENCAR and BALB/c mice and to observe the effects of a skin tumor promoter, topically applied 12-O-tetradecanoylphorbol-13-acetate (TPA), on development of both epidermal and nonepidermal neoplasms.

Materials and Methods

Animals

Two hundred female SENCAR mice (Oak Ridge National Laboratories, Oak Ridge, TN) and two hundred female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were received and held until they were 7 weeks of age, at which time they were

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Table 1. Experimental design: the carcinogenicity of DMBA, urethane, and TPA in SENCAR and BALB/c mice.

Group	Strain or stock	No. of mice intitiated	Initiator[a]	Promoter[b]
1	SENCAR	15	0.25 mL DMSO/SSV	Acetone
2		15	0.25 mL DMSO/SSV	TPA
3		15	0.25 mL Saline	Acetone
4		15	0.25 mL Saline	TPA
5		30	DMBA (300 μg in 0.25 mL DMSO/SSV)	Acetone
6		30	"	TPA
7		30	Urethane (20 mg in 0.25 mL saline)	Acetone
8		30	"	TPA
9	BALB/c	15	0.25 mL DMSO/SSV	Acetone
10		15	0.25 mL DMSO/SSV	TPA
11		15	0.25 mL Saline	Acetone
12		15	0.25 mL Saline	TPA
13		30	DMBA (300 μg in 0.25 mL DMSO/SSV)	Acetone
14		30	"	TPA
15		30	Urethane (20 mg in 0.25 mL saline)	Acetone
16		30	"	TPA

[a] Once intraperitoneally at 7 weeks of age.
[b] Once per week, 0.2 mL topically, 2.5 μg of TPA from weeks 1 to 6 and 1.25 μg of TPA from weeks 7 to 52.

initiated. All animals were housed 5 per 7 in. × 11.5 in. polycarbonate cage on 1/8 in. corn cob bedding, according to strain and treatment. Water and Purina Lab Chow were available *ad libitum* throughout the experiment. All water bottles and cages were changed and sanitized twice per week, the adequacy of food and water was checked daily, and death checks were performed twice daily.

Chemicals

Initiators. DMBA was purchased from Eastman Organic (Rochester, NY) and prepared as a solution of 1,200 μg/mL in a dimethyl sulfoxide (DMSO) steroid suspending vehicle (SSV) (10%/90%). The DMBA was first dissolved in the appropriate volume of DMSO (Pierce Chemicals, Rockford, IL) and then diluted with nine times the volume of SSV (National Cancer Institute [NCI], Cancer Chemotherapy Branch). Urethane purchased from Matheson, Coleman and Bell (Norwood, OH) was prepared as a solution of 80 mg/mL in sterile saline.

Working solutions of both carcinogens were prepared in yellow light under a hood, contained in amber multidose serum vials, and administered immediately after preparation to the animals. DMBA stocks used for dose preparations were stored at –20°C over Drierite in amber glass vials; urethane stocks used for dose preparations were stored at room temperature in foil-wrapped vessels.

Promoter. TPA purchased from CCR Inc. (Eden Prairie, MN) was prepared as a 12.5 μg/mL or 6.25 μg/mL solution in acetone (Baker Chemical, Reagent Grade) depending on the dose to be given. Stock solutions of TPA were kept in tightly stoppered glass vessels wrapped in foil and kept refrigerated between uses. Stocks of TPA were stored in the dark at –20°C over Drierite.

Preparation of Animals and Methods of Administration

Mice receiving single intraperitoneal (IP) injections of DMBA (300 μg) or urethane (20 mg) as initiators were

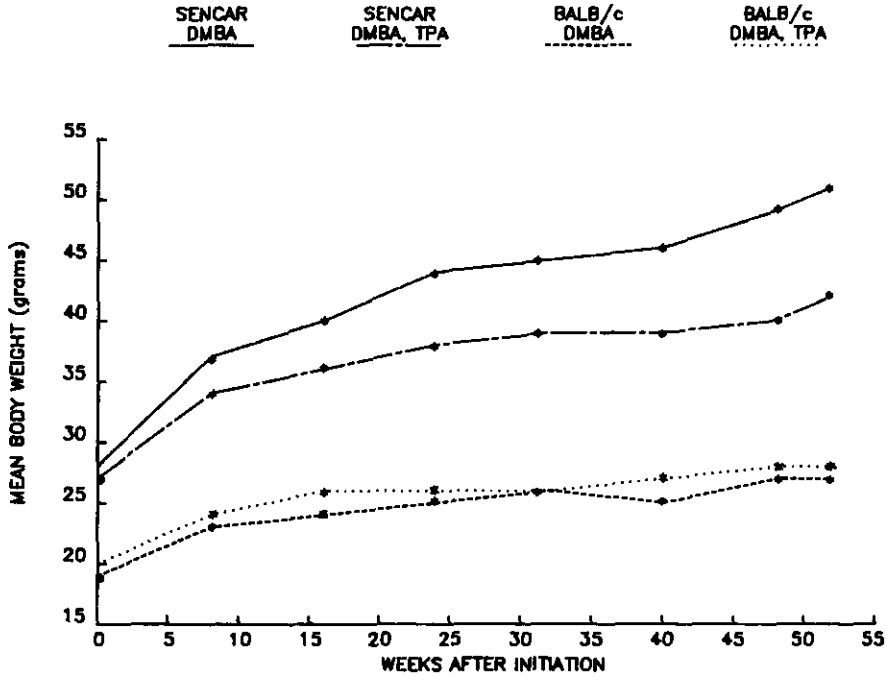

FIGURE 1. Growth of female SENCAR and BALB/c mice exposed to DMBA and TPA.

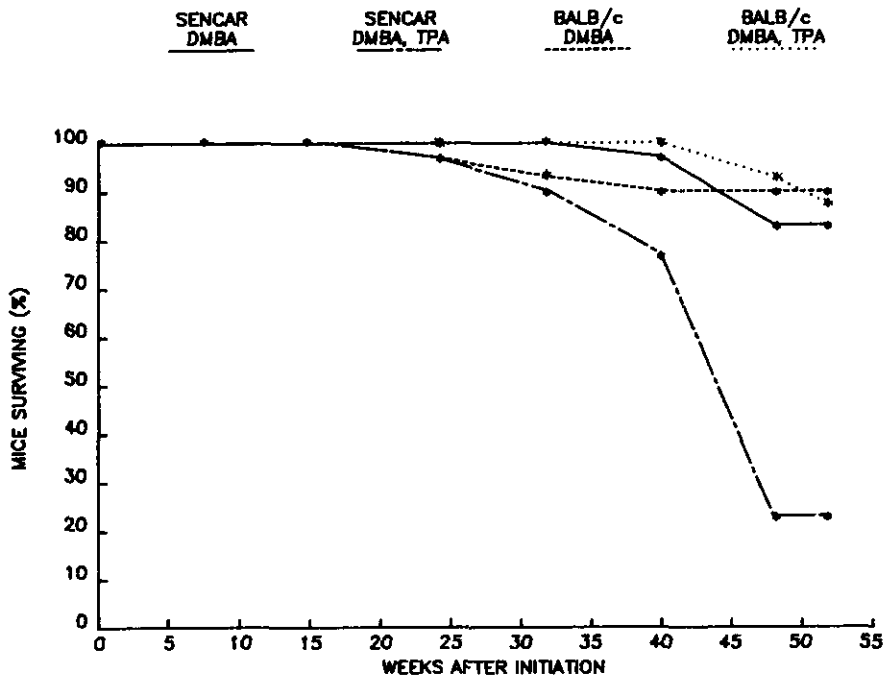

FIGURE 2. Survival of female SENCAR and BALB/c mice exposed to DMBA and TPA.

treated at 0 experimental time (7 weeks of age). DMBA (1200 μg/mL in DMSO/SSV), urethane (80 mg/mL in saline), or the appropriate vehicle were given under a portable charcoal-filtered lab hood by injecting 0.25 mL/mouse using 1-mL tuberculin syringes attached to appropriate-sized hypodermic needles.

TPA solutions (12.5 μg/mL or 6.25 μg/mL in acetone) or acetone vehicle were applied topically to appropriate groups at 0.2 mL, once weekly, from the first week after initiation through the 52nd experimental week. All mice were shaved biweekly at least 24 hr before promotion in a standardized area to expose the back skin to topically applied chemical treatments. Solutions were applied from Eppendorf multipipettes delivering 0.2 mL

Table 2. Tumor incidence in groups of 15 or 30 female SENCAR or BALB/c mice 52 weeks after intraperitoneal DMBA initiation and with topical TPA promotion.

	No. of SENCAR mice affected (% of treatment group)				No. of BALB/c mice affected (% of treatment group)			
Tumor type or tissue	DSMO/ SSV/ acetone[a]	DMSO/ SSV/ TPA[a]	DMBA/ acetone[b]	DMBA/ TPA[b]	DMSO/ SSV/ acetone[a]	DMSO/ SSV/ TPA[a]	DMBA/ acetone[b]	DMBA/ TPA[b]
Skin	0	7 (47)	0	23 (76)	0	0	0	0
Papilloma	0	7 (47)	0	18 (60)*[a]*	0	0	0	0
Cumulative no. per initiated mouse	0	0.8	0.1	4.2	0	0	0	0.03
Carcinoma	0	1 (7)	0	17 (56)*[a]*	0	0	0	0
Sarcoma	0	0	0	2 (6)	0	0	1 (3)	1 (3)
Lung	0	0	11 (36)	7 (23)	1	0	10 (33)	14 (46)
Ovary	0	0	4 (13)	2 (6)	0	0	4 (13)	2 (6)
Blood vessels	0	0	5 (16)	2 (6)	0	0	15 (50)*	20 (67)*
Uterine polypoid adenoma	0	0	1 (3)	0	1	0	5 (16)	6 (20)*
Lymphoma	0	0	3 (10)	2 (6)	0	0	0	2 (6)
Mammary gland carcinoma	0	1 (7)	2 (6)	2 (6)	0	0	0	0
Intestine	0	0	1 (3)	1 (3)	0	0	0	0
Histiocytic sarcoma	1 (7)	0	0	1 (3)	0	0	0	0
Any tumor	1 (7)	7 (47)	20 (67)	26 (86)	2/15 (13)	0	14 (80)	26 (86)

[a] 15 mice.
[b] 30 mice.
* $p < 0.05$ vs. same group for other mouse strain.

Table 3. Tumor incidence in female SENCAR and BALB/c mice 52 weeks after intraperitoneal urethane initiation and/or topical TPA promotion.

Tumor type or tissue	No. of SENCAR mice with tumors (% of treatment group)				No. of BALB/c mice with tumors (% of treatment group)			
	Saline/ acetone	Saline/ TPA (n = 15)[a]	Urethane/ acetone (n = 29)[a]	Urethane/ TPA (n = 30)[a]	Saline/ acetone	Saline/ TPA (n = 15)[a]	Urethane/ acetone (n = 30)[a]	Urethane/ TPA (n = 30)[a]
Skin	0	1 (7)	1 (3)	20 (67)[c]*	0	0	0	1 (3)
Papillomas	0	1 (7)	0	15 (50)[c]*	0	0	0	1
Cumulative no. per initiated mouse	0	0.4	0	1.9	0	0	0	0.03
Carcinomas	0	0	1 (3)	12 (40)[c]*	0	0	0	0
Lung	1 (7)	1 (7)	18 (62)	24 (80)	2 (13)	2 (13)	26 (92)[a,b]	29 (100)[c]
Blood vessels	0	0	2 (6)	1 (3)	0	0	9 (30)[c]*	7 (23)[c]*
Hemangioma	0	0	1	1	0	0	2	5
Hemangiosarcoma	0	0	1	0	0	0	7	2
Hematopoietic system	0	0	3 (10)	1 (3)	0	1 (7)	0	0
Forestomach	0	0	2 (6)	0	0	0	0	0
Uterus	0							
Leiomyosarcoma	0	0	1 (3)	0	0	0	0	0
Polypoid adenoma	0	1 (7)	0	0	0	3 (20)	3 (10)	3 (10)
Histiocytic sarcoma	0	2 (13)	0	0	0	0	0	0
Any tumor	1 (7)	5 (33)	23 (79)	24 (80)	2 (13)	5 (36)	26 (87)	29 (97)

[a] Number of mice examined.
[b] From 28 lungs available for histology.
[c] From 29 lungs available for histology.
*$p < 0.05$ vs. same group for other mouse strain.

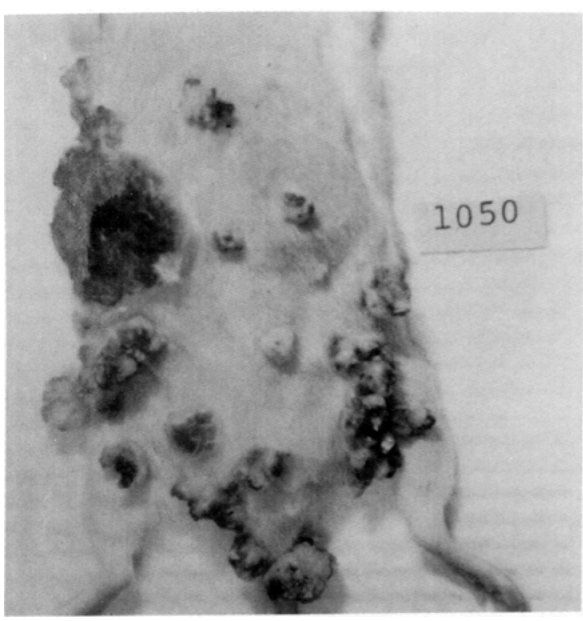

FIGURE 3. Multiple skin papillomas and one carcinoma in a SENCAR mouse initiated with DMBA and promoted by TPA.

of solution. Disposable tips were changed between groups receiving TPA or vehicle. The concentration of TPA stocks used from weeks 1 through 6 was 12.5 μg/mL; 6.25 μg/mL was given from weeks 7 through 52. Using these stocks resulted in doses of 2.5 μg/mouse or 1.25 μg/mouse per week, respectively. The doses were lowered when toxic skin reactions to TPA, which had previously been shown to be progressive, were observed.

Experimental Design

Table 1 summarizes the designation, composition, and treatment for each group within this study. Mice were weighed monthly, and papilloma counts were performed biweekly as previously described (*3*).

Pathology

Necropsies were performed on all animals found dead, on moribund mice euthanized during the study, and on mice killed at termination (52 weeks) unless precluded by autolysis or cannibalization. The following tissues were routinely preserved in 10% formalin: skin, including all malignant and benign tumors; any TPA-induced skin lesions; and backskin, spleen, pancreas, lungs, heart, thymus, trachea, larynx, thyroid, lung tumors, kidneys and adrenals, liver, ovaries and uterus, brain, pituitary, and grossly abnormal lymph nodes and other lesions. Avidin-biotin peroxidase-complex (ABC) immunocytochemistry (*6*) was performed to localize keratin in selected trypsinized skin tumors, surfactant apoprotein (SAP) and Clara cell antigen (CCA) in lung tumors by using rabbit antibodies to human keratin (DAKO Corp., Santa Barbara, CA), specific antibodies to SAP and CCA provided by Dr. G. Singh (*6*), and the Vectastain kit (Vector Laboratories, Burlingame, CA).

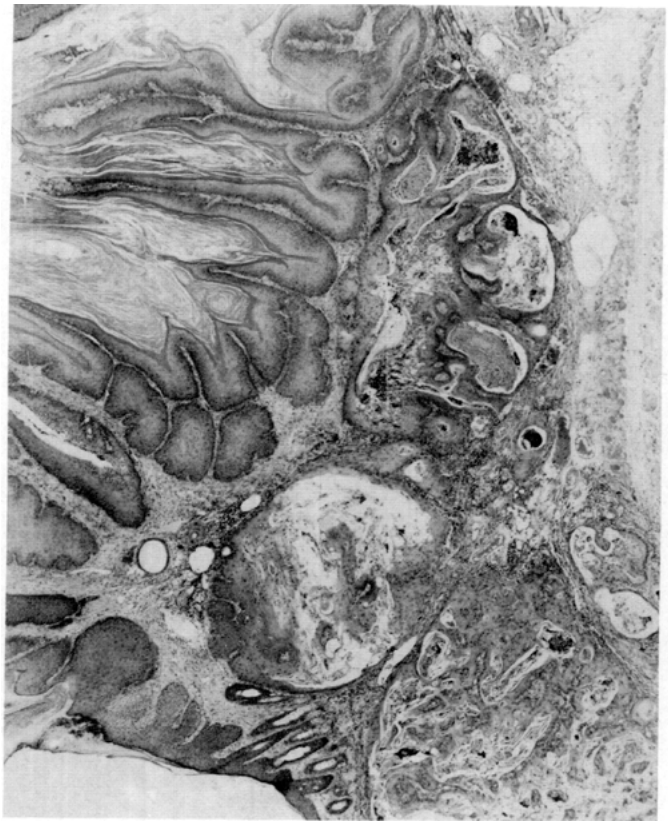

FIGURE 4. Squamous cell carcinoma arising within base of papilloma after exposure to DMBA and TPA. Hematoxylin and eosin, × 25.

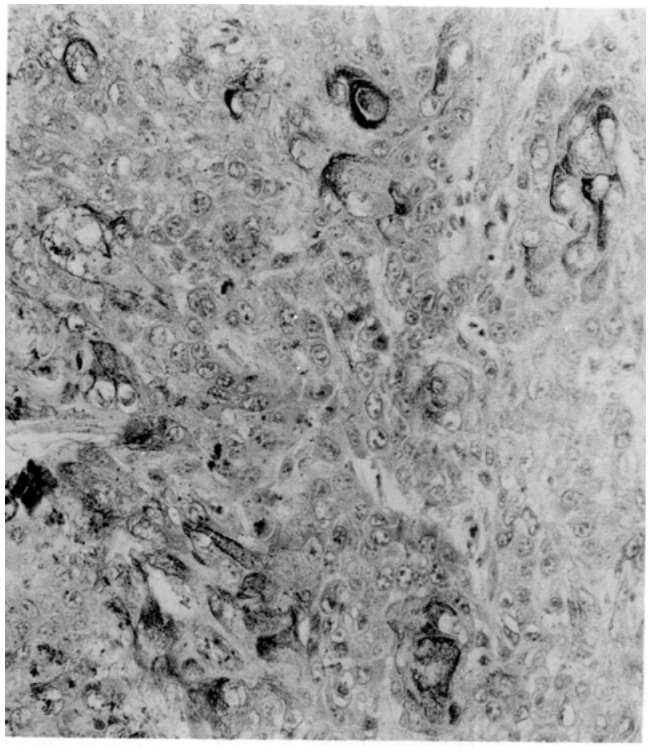

FIGURE 5. Keratin in poorly differentiated squamous cell carcinoma after trypsin digestion of tissue section. ABC immunoperoxidase and hematoxylin, × 250.

Results

Body Weight and Survival

The growth of female SENCAR mice in all groups was similar except for mice exposed to DMBA/TPA. The latter mice gained weight at a considerably slower rate than did other SENCAR mice (Fig. 1). BALB/c mice of all groups gained weight at the same rate (Fig. 1).

SENCAR mice exposed to DMBA/TPA also had the worst survival rate (Fig. 2), since only 23% of the mice survived to the end of the experiment, whereas 63% of the SENCAR mice receiving both urethane and TPA survived, and 77% to 100% of the SENCAR mice in other groups survived. From 87% to 100% of the BALB/c mice were alive at 52 weeks (Fig. 2).

Tumors in Control Mice

At 52 weeks after treatment with the initiator vehicle (DMSO/SSV or saline) and promoter (TPA) or promoter vehicle (acetone) (59 weeks of age) skin and other tumors were found in SENCAR or BALB/c mice (Tables

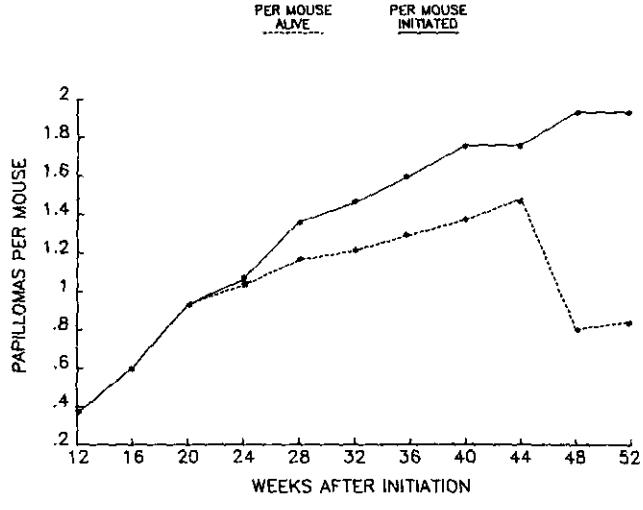

FIGURE 6. Skin papillomas in female SENCAR mice initiated by 20 μg of urethane intraperitoneally at 7 weeks of age and promoted topically for 1–6 weeks later with TPA at 2.5 μg per mouse and then 1.25 μg per mouse for weeks 7–52.

Table 4. A comparison of gross and microscopic pulmonary lesions in female SENCAR and BALB/c mice 52 weeks after urethane initiation and topical TPA promotion.

	SENCAR			BALB/c		
	Saline/ acetone (n = 15)[a]	Urethane/ acetone (n = 29)[a]	Urethane/ TPA (n = 30)[a]	Saline/ acetone (n = 15)[a]	Urethane/ acetone (n = 30)[a]	Urethane/ TPA (n = 30)[a]
Lung tumors, grossly visible	7 (47)	17 (59)	24 (80)	1 (7)	26 (92)	26 (92)
Lung tumors, microscopic confirmation	1 (7)	18 (62)	24 (80)	2 (13)	26 (92)	29 (100)
Grossly visible lung nodules per mouse initiated[b]	1.3 ± 0.57	3.3 ± 0.79	4.3 ± 0.79	0.07 ± 0.07	4.5 ± 0.77	3.4 ± 0.43
Microscopic confirmation—lung tumors per mouse initiated[b]	0.07 ± 0.07	2.1 ± 0.52	2.7 ± 0.5	0.13 ± 0.09	4.6 ± 0.71	4.4 ± 0.39

[a] Number of mice examined.
[b] Mean ± SEM.

Table 5. Histologic patterns of lung tumors in female SENCAR and BALB/c mice initiated with urethane and topical TPA promotion.

	Number of tumors (% of treatment group)	
Tumor type	SENCAR	BALB/c[a]
All tumors	145 (100)	259 (100)
Alveolar, solid or mixed	68 (47)	79 (31)
Solid/tubular or tubular	32 (22)	71 (27)
Tubular/papillary or papillary	45 (31)	109 (42)

[a] Urethane/acetone and urethane/TPA groups combined.

2 and 3). An increased incidence of skin tumors was associated with TPA promotion in SENCAR mice after DMSO/SSV vehicle initiation. In contrast, only a few skin neoplasms were associated with TPA promotion following saline vehicle initiation.

DMBA Initiation and TPA Promotion

DMBA/TPA-treated SENCAR mice that died early in the study had many skin tumors as previously reported (*3*) (Table 2). The occurrence of skin (Fig. 3), vascular, and uterine tumors was strain-related (Table 2). In the DMBA/TPA-treated SENCAR mice, 17 mice had 37 squamous cell carcinomas of the skin (10 seen grossly), 3 of which metastasized to lymph nodes and 2 to lungs. Several carcinomas were seen to arise within papillomas (Fig. 4). Keratin was found in well and poorly differentiated carcinomas (Fig. 5). The vascular tumors were usually small hemangiomas and were found in 35 of 60 BALB/c mice and in 7 of 60 SENCAR mice exposed to DMBA. In BALB/c mice, 57 vascular tumors were found in 35 mice. These tumors usually involved the serosa of intestines (18 mice), uterus (23), mesentery (5), and other abdominal tissues (11). Uterine polypoid adenomas were found more frequently in BALB/c mice (11/60) than in SENCAR mice (1/60). Few skin tumors were found in BALB/c mice. Other tumors were found in similar incidences in both strains, although mammary and intestinal tumors were found only in SENCAR mice. Control mice had few tumors of the skin, blood vessels, and uterus. Lung tumors (adenomas or adenocarcinomas) occurred in higher incidence, whether confirmed microscopically (14/30 vs. 10/30) or counted visually (17/30 vs. 11/30), and in greater multiplicity (2.0 vs. 0.5), in BALB/c mice exposed to DMBA/TPA than in BALB/c mice given DMBA alone. SENCAR mice showed no differences between these treatment groups.

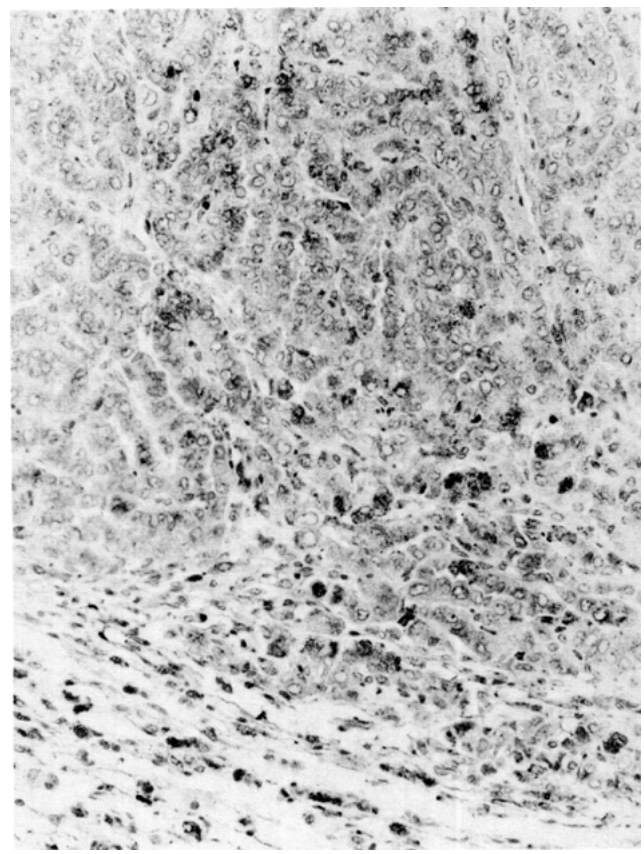

FIGURE 7. Surfactant apoprotein in normal alveolar Type II cells (bottom of figure) and in some tumor cells in solid-tubular pulmonary adenoma induced by urethane. ABC immunoperoxidase and hematoxylin, × 250.

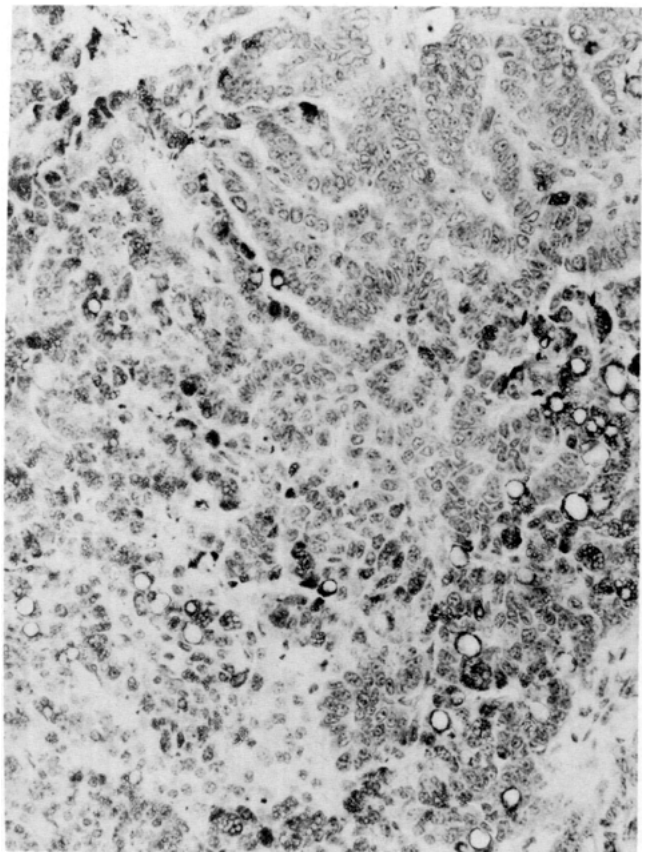

FIGURE 8. Surfactant apoprotein in vacuolated tumor cells in solid areas of pulmonary adenoma and not in tubular focus. ABC immunoperoxidase and hematoxylin, × 250.

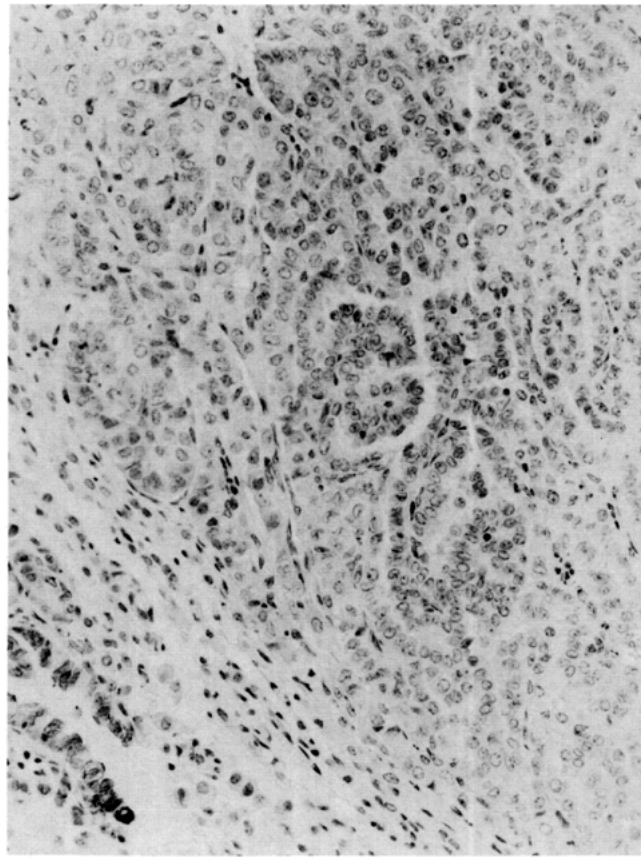

FIGURE 9. Clara cell antigen in normal Clara cells in bronchiole (lower left) but not in tubulopapillary pulmonary adenoma. ABC immunoperoxidase and hematoxylin, × 250.

Urethane Initiation and TPA Promotion

In the urethane experiments, skin tumors were found primarily in SENCAR mice given urethane and TPA (Fig. 6). Twelve mice had squamous cell carcinomas, confirmed histologically, of which seven had been observed clinically. Metastasis occurred in 5 of the 12 mice with squamous cell carcinoma; 4 had metastases to lymph nodes and 2 to lungs. Vascular tumors were significantly more common in BALB/c mice (16/60) than in SENCAR mice (3/59). Most were hemangiosarcomas of the uterus and peritoneal surfaces. Uterine polypoid adenomas occurred primarily in BALB/c mice (6/60). The occurrence of lung tumors noted by gross inspection was subjected to histologic confirmation (Table 4). The number of microscopically visible lung tumors could have actually been more numerous since only gross lesions were sectioned. The many gross nodules in saline-treated SENCAR mice were found histologically to be focal, chronic inflammatory lesions probably caused by chronic Sendai infection, although these histological lesions were also seen in treated mice. Histologically, tumors were identified as alveolar, solid, tubular, or papillary adenomas and adenocarcinomas (Table 5). Distribution of tumor types was not affected by treatment, although more papillary tumors were seen in BALB/c mice.

Selected representative lung tumors were evaluated for the presence of pulmonary antigens. Immunocytochemically, SAP was found in the cytoplasm of many tumor cells of alveolar and solid lung tumors (Figs. 7 and 8) but in few cells of tubular and papillary tumors. Rarely, tumor cell nuclei of papillary tumors contained the antigen. CCA was never found in any lung tumors, not even in papillary tumors (Fig. 9).

TPA application alone after using either of the two vehicles caused a few skin papillomas and carcinomas in SENCAR mice by 52 weeks (Tables 2 and 3).

Discussion

SENCAR mice were much more susceptible to two-stage induction of skin tumors than were BALB/c mice as previously reported (*3*) but developed fewer carcinogen-initiated vascular and uterine tumors than did BALB/c mice. The increased susceptibility of BALB/c mice to vascular tumors has been previously reported

(*7–9*). The decreased susceptibility to these induced tumors in SENCAR mice cannot be readily explained. It was shown that metabolism of some carcinogens in selected tissues does not differ significantly in these mice (*1*), but their increased susceptibility to development of skin tumors, however, may be related to the dark cell population (*2*). Strains and stocks of mice generally differ in their susceptibility to carcinogens because of differences in genetics, pharmacokinetics, background tumor incidences, and other factors. The glomerulonephritis and associated immunological lesions in our SENCAR mice alter their responses to initiated cells, preneoplastic lesions, or tumors (*10*). Skin tumors induced by ultraviolet (UV) light have been found to be associated with immunological dysfunction that may be related to skin carcinogensis by chemicals (*11*). Since SENCAR mice are also more susceptible to UV skin carcinogenesis than other mouse strains, similar immunologic mechanisms may act in SENCAR mice. Internal tumor development in these mice may not, however, depend so much on immunological changes (*12,13*).

No significant increase in lung carcinogenesis was seen in either SENCAR mice initiated with urethane and promoted with TPA or in BALB/c mice. It has been reported that phorbol promotes lung tumors when initiated with dimethylnitrosamine (*14*). We may have expected a greater tumor incidence and multiplicity in urethane/TPA-treated mice, but they died earlier than the SENCAR mice treated only with urethane. Also, Sendai virus infection, suggested in these mice by pulmonary inflammation, has been shown to reduce induced lung tumor incidence (*15*) and may have affected the outcome of our experiment. Selected urethane-induced lung tumors were usually immunoreactive for SAP, a marker for alveolar Type II cells (*6*). Clara cell antigen was never detected in any lung tumors, even in papillary tumors, which have been suggested to be of Clara cell origin (*16*).

The increased number of skin carcinomas seen histologically over those found by clinical examination is in agreement with the poor gross/microscopic correlation seen in our other studies. In two other studies with topical DMBA and TPA, we found 24 carcinomas in 16 mice histologically, whereas only 6 were found clinically in six mice. The ratio of three to four carcinomas found histologically to every one identified by clinical examination appears to be consistent in our three studies. It has been previously described (*17*) that squamous cell carcinomas of the mouse skin arise within papillomas. Thus, some of the early carcinomas arising within papillomas are obviously seen clinically as papillomas. Other malignant lesions, however, can be mistaken for papillomas. It would be prudent to section all skin tumors in studies when carcinoma development is a critical endpoint.

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