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Urethan (Ethyl Car-bamate) as a Multipotential Carcinogen in BALB/C, ZB and DB Female Mice

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Abstract

1. The objective of this investigation was to test the influence of mammary cancer tissue extract on the induction of various tumors by urethan. Three strains of female mice, Balb/c, Zb and Db, were used in this experiment. 2. It was found that urethan was a multipotential carcinogen in the induction of (a) lung tumor, ovarian hemorrhagic cyst, and hemorrhagic lesions in various tissue in Balb/c mice, (b) lung tumor, hepatoma, leukemia, mammary cancer, Harderian gland tumor and hemorrhagic lesions in various tissues such as spleen, mesenteric lymphnode, liver and ovary in Zb strain, and (c) lung tumor, mediastinal lymphoma, leukemia, hepatoma and hemorrhagic lesions in the liver and ovary in Db mice. 3. Mammary cancer tissue extract seemed to have a promotive effect on the generalization of the mediastinal lymphoma induced by urethan in Db female mice.

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URETHAN (ETHYL CARBAMATE) AS A MULTIPOTENTIAL CARCINOGEN IN *BALB/C, ZB* AND *DB* FEMALE MICE

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The objective of this investigation was to test the influence of mammary cancer tissue extract on the induction of various tumors with urethan. NETTLESHIP, HENSHAW and MEYER[1] reported that ethyl carbamate (urethan) induced multiple pulmonary tumors in C3H mice exposed to roentgen rays. Since then, it has been generally accepted that urethan was one of the carinogens that produce lung adenomas in mice[2-6]. It was also demonstrated in our laboratory that urethan augmented in mice the induction of leukemia by X-rays, estrogenic hormone, or methylcholanthrene[7].

More recently, it was pointed out by TANNENBAUM and SILVERSTONE[8] that urethan was a multipotential carcinogen, capable of inducing or augmenting pulmonary adenomas, mammary carcinoma, malignant mesenchymal tumors in the interscapular fat, cystadenomas of the lacrimal gland, and blood cysts in the liver in mice.

In addition, HESTON, VLAHAKIS and DERINGER[9] reported that repeated injections of urethan induce hepatomas which were larger in size than those in the uninjected controls, although the numbers of the tumors were not shown to be greater than those in the mice receiving a single injection. LIEBELT[10] also observed a high incidence of hepatoma in Zb mice, (higher in male than female) after a single injection of urethan at birth.

Interesting findings of the studies here described include (1) development of lung tumor, mediastinal lymphoma, systemic generalized stem cell leukemia, hepatoma, hemorrhagic lesions in the liver, and enlarged hemorrhagic mesenteric lymphnode, and enlarged ovaries (polyfollicular ovaries) in an individual female mouse of Zb strain, (2) the association of lung tumor, huge ovarian hemorrhagic cyst in an individual Balb/c mouse and (3) the association of lung

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tumor, hepatoma, enlargement of ovaries and significant involvement of Peyer's patches in an individual mouse of Db strain.

EXPERIMENTAL

Mice: Three strains of female mice, Balb/c (Albino mice without mammary tumor agent), Zb (C3H without mammary tumor agent) and Db, have been used in this experiment. They were fed on Purin Laboratory chow and given water *ad libitum*. At this mouse colony, 62 Balb/c, 56 Zb, and 97 Db female mice, all untreated, were autopsied during the observation of the experimental animals (1957—1959). According to the data shown in Table 1, Balb/c female mice developed lung tumor (5%), leukemia (25%), mammary cancer (11%) and hepatoma (3%) spontaneously at an average age of 670 days. The Zb female mice developed mammary cancer (2%), leukemia (5%), and lung tumor (2%) spontaneously at an average age of 442 days. It was noted that lung tumor (1%), leukemia (12%), and mammary cancer (7%) developed spontaneously at an average age of 564 days in Db female mice.

Table 1. Incidence of the spontaneous tumors in Balb/c, Zb and Db female mice.

Strain of mice	Number of animal	Lung tumor	Leukemia	MaCa	Hepatoma	Average age (day)
Balb/c	62	3 (5%)	15 (25%)	7 (11%)	2 (3%)	670
Zb	56	1 (2%)	3 (5%)	1 (2%)	0	442
Db	97	1 (1%)	12 (12%)	7 (7%)	0	564

Urethan injection: The experimental animals were injected intraperitoneally with urethan in 10 per cent aqueous solution, in the dosage of 1 mg/g of body weight, every 4 days for 11 times, starting at weaning age. All animals were observed for about 18 months after treatment and many were sacrificed when they were near death during the course of the experiment. Sections of varioues tissues were made for microscopic study.

Mammary cancer extract: As a source of mammary cancer tissue, spontaneous mammary cancer in C3H, Dba, Stoli, C58, Db F_1 hybrid, and NH strains and Db, Fn Mca-induced mammary cancer were used. The extracts of the mammary cancer tissues were made as follows: Mammary tumors were removed from the animals aseptically. A twenty per cent suspension of mammary cancer cells in 0.9%saline solution was made, using a homogenizer. The cell suspension was centrifuged twice at speed of 2,300 r. p. m. for twenty minutes at 0°C. The fat layer was then removed with a pipette and the supernatant thus treated was filtered through a porcelain filter (# 03). This filtrate was stored at −70°C, and was used for inoculation.

Treatment: We set up three groups of mice as follows:

(1) The first group received injections of urethan (every 4 days for 11 times) starting at weaning age, 5 weeks old.

(2) The second group received urethan injections followed by a single injection of mammary cancer extract. (0.5 ml. intraperitoneally, with the last injection of urethan: between 8 and 9 weeks of age).

(3) The third group received a single injection of mammary cancer extract: (0.5 ml. intraperitoneally, between 8 and 9 weeks of age).

Females of each of three different strains of mice were divided into three groups and were treated in the manner outlined above.

Experiment 1. Induction of tumors in Balb/c female mice by urethan:

When Balb/c female mice were injected with mammary cancer extract at 9 weeks of age, lung tumor (7/27: 26%) and ovarian cyst (1/27: 4%) were noticed. However, when urethan was administered to the animals, (a) lung tumor (29/29: 100%) and (b) ovarian cyst (4/29: 14%) were observed in association with hemorrhage in various organs including ovary, uterus, lung and spleen, as shown in Table 2 and Fig. 1. When the urethan treatment was followed by the injection of mammary cancer extract, the incidence of tumor was almost the same as that in the group injected with urethan alone. Lung tumor (26/32: 81%) and ovarian cyst (6/32: 19%) were observed, in association of hemorrhagic lesions in various organs. In addition, two leukemias including mediastinal lymphoma, mammary cancer and hepatoma were noted in the 32 experimental animals.

Table 2. Induction of various tumors in Balb/c female mice by urethan.

Group	Number of mice	Lung tumor	Leukemia (ML)	MaCa	Hepatoma	Average age (day)
Urethan	29	29(100%)	0 (0)	0	0	469
Urethan and MaCa Extr.	32	26 (81%)	1(3%) (2)(6%)	1 (3%)	1 (3%)	441
MaCa Extr.	27	7 (26%)	0 (0)	0	0	533

From these data, urethan appeared to induce not only lung tumors but also ovarian hemorrhagic cysts and numerous other hemorrhagic lesions including hemorrhage in uterine wall. (Fig. 2.). However, mammary cancer extract did not seem to produce any additional effects upon the urethan induced tumors in Balb/c strain of mice.

Experiment 2. Induction of tumors in Zb female mice by urethan.

When Zb female mice were treated with mammary cancer extract alone at 9 weeks of age, one leukemia (4%), and two mediastinal tumors (7%) developed in 27 experimental animals. However, neither lung tumors nor hepatomas were

found in this group. On the other hand, when urethan was injected intraperitoneally, lung tumor (20/28: 71%), leukemia (6/28: 21%), including mediastinal lymphoma (6/28: 21%), mammary tumor (1/28: 4%) and hepatoma (5/28: 18%) were observed. When mammary cancer extract was added to the urethan treatment, the incidence of various tumors above described appeared to be almost same as in the group injected with urethan alone. The percentage of lung tumor was 72 per cent (18/25), leukemia 16 per cent (4/25), mediastinal lymphoma 20 per cent (5/25), mammary cancer 8 per cent (2/25) and hepatoma 32 per cent (8/25). Throughout these three groups, hemorrhagic lesions in various tissues such as liver, mesenteric lymphnodes, lungs were observed.

In this particular strain of mice (Zb mice) thus treated, it should be emphasized that various tumors such as lung tumor, hepatoma, Harderian gland tumor and leukemia or mammary tumor were observed simultaneously in one and the same animal as shown in Table 3 and Figs. 3, 4.

Table 3. Induction of various tumors in Zb female mice by urethan.

Group	Number of mice	Lung tumor	Leukemia (ML)	MaCa	Hepatoma	Average age (day)
Urethan	28	20 (71%)	6(21%) (6)(21%)	1 (4%)	5 (18%)	430
Urethan and MaCa Extr.	25	18 (72%)	4(16%) (5)(20%)	2 (8%)	8 (32%)	470
MaCa Extr.	27	0	1(4%) (2)(7%)	0	0	474

The pattern of the association of various tumors in an individual animal seems to be fairly uniform in this experiment. Therefore, it appeared likely that urethan was a multipotential carcinogen in this strain of mice.

Experiment 3. Induction of tumors in Db female mice by urethan.

As shown in Table 4, urethan induced not only lung tumor but also mediastinal lymphoma, hepatoma and hemorrhage in the liver in Db mice. If mammary cancer extract was injected into the mice after urethan treatment, mediastinal lymphoma appeared to generalize, subsequently resulting in leuke-

Fig. 1. Balb/c #49 female: Urethan treatment
(1) Lung tumor. (2) Ovarian hemorrhagic cyst.

Fig. 2. Balb/c #5931 female: Urethan treatment
Hemorrhage in the uterine wall and ovaries.

Fig. 3. Zb #1107 female: Urethan treatment
(1) Leukemia (stem-cell). (2) Mediastinal lymphoma. (3) Hepatoma.
(4) Hemorrhagic mesenteric lymphnode. (5) Enlarged Peyer's patches.
(6) Hemorrhagic lesions in liver, spleen. (7) Lung tumor.

Fig. 4. Zb #1128 female: Urethan treatment
(1) Lung tumor. (2) Mammary tumor. (3) Enlarged cervical lymphnodes and spleen.
(4) Numerous enlarged Peyer's patches. (5) Ovarian hemorrhagic cyst.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

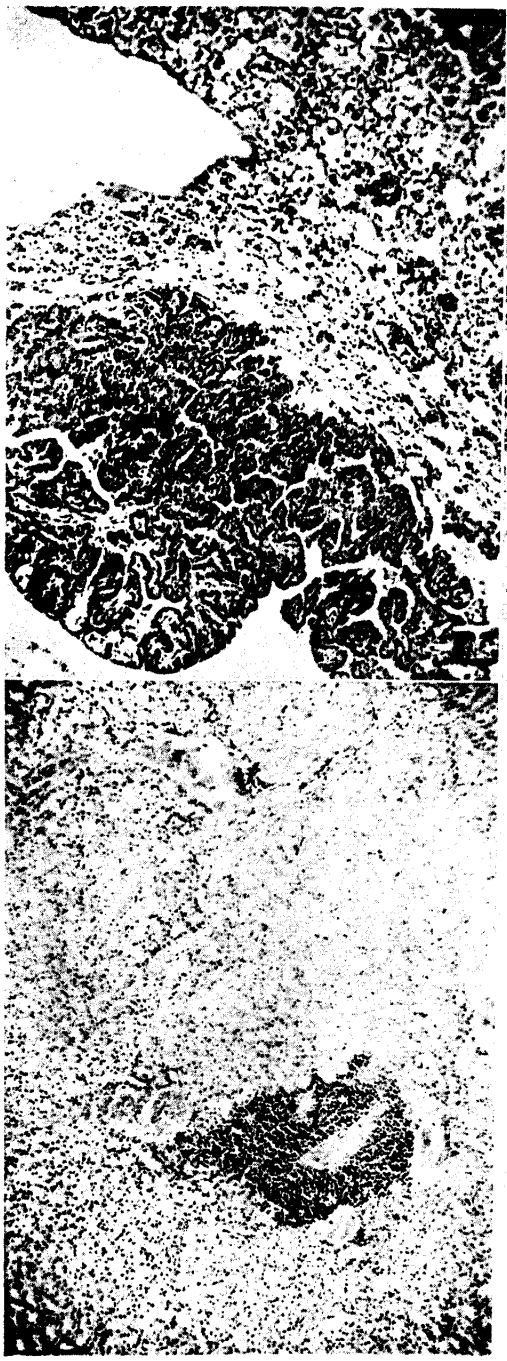

Fig. 6

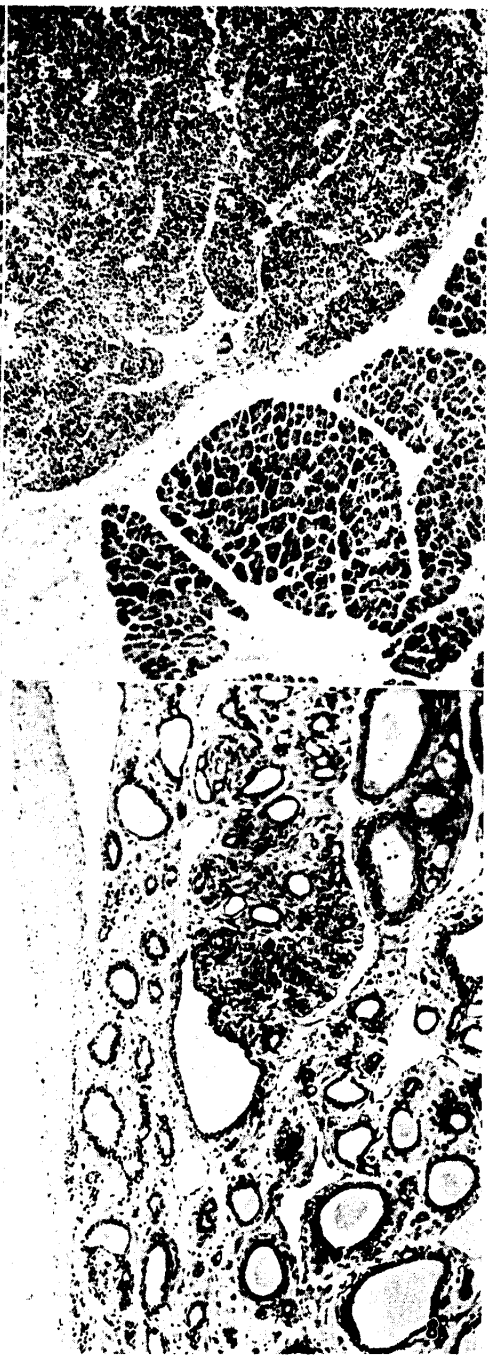

Fig. 7

Fig. 8

mia. Mammary cancer extract injected seemed to have a stimulative effect upon the female endocrine organs.

Table 4. Induction of various tumors in Db female mice by urethan.

Group	Number of mice	Lung tumor	Leukemia (ML)	MaCa	Hepatoma	Average age (day)
Urethan	25	11 (44%)	0 (7) (28%)	2 (8%)	5 (20%)	364
Urethan and MaCa Extr.	34	11 (32%)	11 (32%) (13) (38%)	9 (26%)	2 (6%)	382
MaCa Extr.	26	0	1 (4%) (0)	8 (31%)	0	428

The results may be described as follows: (1) Lung tumor: When mammary cancer extract was injected in Db female mice, no lung tumor developed. However, if either urethan alone or urethan combined with mammary cancer extract were administered intraperitoneally, lung tumor developed in 44 per cent (11/25) in the former group and in 32 per cent (11/34) in the latter group. (2) Leukemia and mediastinal lymphoma: When mammary cancer extract was injected into the animal, only one out of the 26 mice developed leukemia. Mediastinal tumor was found in none of them. However, when urethan was administered intraperitoneally to the same strain of mice, mediastinal lymphoma was noted in as high as 28 per cent (7/25), but no leukemia was detected. If mammary cancer extract was injected after the urethan treatment, the incidence of mediastinal lymphoma was 38 per cent (13/34), and the incidence of leukemia was 11 out of 34 animals, or an incidence of 32 per cent. These results suggested that the mammary cancer extract had a synergistic action with urethan in the induction of leukemia in Db mice. (3) Mammary cancer: When urethan was administered to the Db female mice, mammary cancer developed in two out of 25 mice (8%), but when mammary cancer extract alone or mammary cancer extract and urethan were given to the mice, mammary cancer developed in 31 per cent (8/26) and in 26 per cent (9/34), respectively. From these data, it seemed likely that mammary cancer extract had a promotive effect on the development of mammary cancer, regardless of the administration of

Fig. 5. Papillary adenocarcinoma of the lung in Zb # 1107 female treated with urethan. Note marked hemorrhage in alveolar lumen. Hematoxylin and eosin. ×80

Fig. 6. Infiltration of leukemic cells in the lymphnode of Zb # 1107 female treated with urethan. Hematoxylin and eosin. ×110.

Fig. 7. Infiltration of leukemic cells in the areas surrounding the blood vessels and difuse hemorrhage in both tubules and capillaries in the kidney of Zb # 1107 female treated with urethan. Hematoxylin and eosin. ×80.

Fig. 8. Cystadenomatous lesion in mammary tumor of Zb # 1128 female treated with urethan. Hematoxylin and eosin. ×90.

urethan to the mice. (4) Hepatoma : The injection of mammary cancer extract did not seem to influence the occurrence of spontaneous hepatoma (0/28 : 0%). If mammary cancer extract were injected with urethan treatment, the incidence of hapatoma was 6 per cent (2/34), in contrast to the group injected with urethan alone, in which the hepatoma developed in 5 out of the 25 animals (20%). These data suggest the possibility that urethan may accelerate the induction of hepatomas and mammary cancer extract may have a suppressive effect on the induction of hepatoma. Further investigation will be needed for the confirmation of these interesting results. (5) Hemorrhagic lesions : When either urethan or urethan combined with mammary cancer extract was injected into the mice, hemorrhagic lesions, mainly in the liver tissue occurred in 11 out of 25 and 10 out of 34 animals, respectively, whereas no hemorrhagic lesions were found in the group injected with mammary cancer extract alone.

DISCUSSION

Our experiment indicated that urethan was a multipotential carcinogen to the female in Balb/c, Zb and Db strain of mice.

TANNENBAUM and SILVERSTONE[8] had demonstrated previously that urethan was a multipotential carcinogen, capable of inducing pulmonary adenomas, mammary carcinomas, malignant mesenchymal tumors in interscapulary fat, cystadenomas of the lacrimal gland and blood cysts in the liver in C57 Black x C3H F_1, Dba, and C3H mice. In their work, it was speculated that urethan would be carcinogenic for other tissues and for other species. The following changes were observed in our experiment : hemorrhagic lesions in the uterine wall, ovarian hemorrhagic cyst and lung tumor in Balb/c ; lung tumor, hepatoma, leukemia, ovarian hemorrhagic cyst, mammary cancer, Harderian gland tumor, and hemorrhagic lesions of various tissue such as spleen, mesenteric lymphnode, liver, ovary in Zb mice ; and lung tumor, mediastinal lymphoma, leukemia, hepatoma, and hemorrhagic lesions in the liver and ovary in Db mice.

Since the report of NETTLESHIP, HENSHAW and MEYER (1943)[1], (1944)[11], urethan had been considered as a lung specific carcinogen. JAFFE (1944)[12], (1947)[13], LARSEN and HESTON (1945)[14] and LARSEN[15] later confirmed their results. However, NOBLE and MILLAR (1948)[16] noticed lymphosarcoma in Swiss albino mice, which showed extensive spread to gut, liver and kidneys, three or four months after a single subcutaneous administration of 25 per cent urethan in a 5 per cent solution of zinc acetate (0.1 ml/gm). He also found a malignant hemangioendothelioma in his experiment. SINCLAIR (1949)[17] found adenomatous lesions in C3H mice besides lung tumors. Since then, it has been speculated that urethan might be a carcinogen in tissues other than lung. SALAMAN and

ROE (1953)[18], GRAFFI, VLANYNCK, HOFFMAN and SCHULZ (1953)[19] demonstrated that skin tumor was induced by urethan and croton oil, concluding that urethan was an initiator for mouse skin tumor. When BERENBLUM and HARAN (1955)[20] tried to confirm these results regarding the initiating action of urethan, they noticed lesions of the liver, resembling hemangiomas, but proving histologically to be hemorrhages with necrosis of liver parenchyma.

In 1957, KIRSCHBAUM and his associates[21] found that urethan had a potentiating effect on the induction of mouse leukemia by X-rays. Later, we (1958)[7] reported that urethan augmented the induction of leukemia in mice by X-rays, estrogenic hormone, and methylcholanthrene, respectively. When the influence of urethan on the high incidence of spontaneous leukemia of presumably viral etiology in such strains as AKR and C58 was studied in our laboratory (1961)[22], it was demonstrated that the onset of leukemia was slightly accelerated by this drug.

In the present experiment, we also observed various locations of hemorrhagic lesion: in the tissue of ovary, uterus, lung, spleen, and thymus in Balb/c female mice; and liver, lung, mesenteric lymphnode, kidney, abdominal cavity, liver, lung and intestine in Db and Zb female mice. These hemorrhagic lesions have been described by HESTON, VLAHAKIS and DERINGER (liver)[9], TANNENBAUM and SILVERSTONE (liver)[8], KIRSCHBAUM and BELL and GORDON (liver)[23], ROE and SALAMAN (hepatic tumors)[24], NOBLE and MILLAR (a slower growing tumor behind the humerus)[16], KAWAMOTO, KIRSCHBAUM, IBANEZ, TRENTIN and TAYLOR (liver, intestines, pancreas)[22] and DOLJANSKI and ROSIN (liver)[25]. TANNENBAUM and SILVERSTONE[8] have pointed out that the blood cysts in the livers of urethan-treated mice were the results of vascular injury and that they were not neoplasia. BERENBLUM and HARAN[20] seemed to agree with TANNENBAUM[8].

It was of interest that a significantly higher incidence of hepatomas was found in the Zb and Db female mice treated by urethan, but not in Balb/c female mice. HESTON, VLAHAKIS and DERINGER[9] reported a high incidence of hepatomas in C3H males that received 8 injections of 20 mg urethan each. However, the occurrence was not shown to be greater than that in the males receiving one injection. According to LIEBELT and his associates[10], a single injection of urethan at birth induced a high incidence of hepatomas and leukemias in Zb mice. From these data, urethan was obviously carcinogenic in the liver tissue in mice. However, it did not seem to act as carcinogen in the liver of Balb/c female mice. The induction of hepatomas by urethan may depend upon the susceptibility of strain of mice.

When Db female mice were injected with urethan, seven mediastinal lymphomas but no leukemias were found in 25 female mice. However, if mammary cancer extract was added to the urethan treatment, then not only mediastinal

lymphomas (13 out of 34 mice), but also leukemias (11 out of 34 mice) developed. On the other hand, when mammary cancer extract alone was administered to the mice, no mediastinal lymphoma and only one leukemia developed among 28 experimental animals. These data suggested that urethan induced mediastinal lymphomas in Db mice and that the mammary cancer extract aided in the generalization of the mediastinal lymphoma. Considering our previous results[7] that suggested a synergistic effect of urethan and estrogenic hormone on the induction of leukemia in castrated C57 Black mice, the data in the present experiment seemed to indicate need for further investigation of the relationship between sex hormone and urethan.

When either urethan alone or urethan combined with mammary cancer extract was injected to Zb female mice, leukemias developed in 21 per cent and 16 per cent, respectively, whereas only one leukemia developed in 27 animals (4%) in the group injected with mammary cancer extract alone. Urethan appeared to be a leukemogen in the Zb strain of mice in this experiment.

Studies have been made on the induction of tumors by various tumor viruses and enhancement by chemical agents: According to ROUS *et al.*[26,27], it was demonstrated that significantly numerous warts were induced in rabbits when treated with Shope papilloma virus followed by painting with tar, although the incidence was low or negligible in the animals either treated with tar alone or injected with the virus alone. AHLSTROM and ANDREWES[28] also demonstrated enhancement of the Shope fibroma virus-induced tumor by applying either additional tar or carcinogenic hydrocarbons.

Along the same line, it was reported by DURAN-REYNALS[29] that methylcholanthrene induced either benign or malignant local tumors in cortisone-treated mice, when vaccinia-virus was injected intradermally later. However, only local lesions were noted when animals thus treated were injected with the vaccinia-virus alone.

More recently, ROWSON *et al*[30]. inoculated newborn mice with polyoma virus, followed by the dermal application of 9 : 10-dimethyl-1, 2-benzanthracene (DMBA) or 3, 4-benzpyrene (BP) with the subsequent administration of tumor promoting factor (croton oil). He demonstrated that mice inoculated with polyoma virus when newly born and later treated by application to the skin of carcinogenic or tumor-promoting agents developed significantly more polyoma-type tumors than mice which received the virus alone.

When our experiment was initiated, serological test for polyoma virus to the experimental animals was not conducted. Therefore, it was unfortunate that the possibility of contamination with polyoma virus could not be ruled out in our experiment. However, neither parotid tumors nor kidney tumors which were most common in polyoma type tumors were observed in our experiment.

SUMMARY

1. The objective of this investigation was to test the influence of mammary cancer tissue extract on the induction of various tumors by urethan. Three strains of female mice, Balb/c, Zb and Db, were used in this experiment.

2. It was found that urethan was a multipotential carcinogen in the induction of (a) lung tumor, ovarian hemorrhagic cyst, and hemorrhagic lesions in various tissue in Balb/c mice, (b) lung tumor, hepatoma, leukemia, mammary cancer, Harderian gland tumor and hemorrhagic lesions in various tissues such as spleen, mesenteric lymphnode, liver and ovary in Zb strain, and (c) lung tumor, mediastinal lymphoma, leukemia, hepatoma and hemorrhagic lesions in the liver and ovary in Db mice.

3. Mammary cancer tissue extract seemed to have a promotive effect on the generalization of the mediastinal lymphoma induced by urethan in Db female mice.

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