The Neonatal and Infant Age Periods as Biologic Factors Which Modify Multicarcinogenesis by Urethan

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SUMMARY

The objective of the present investigation was to determine whether various organs of mice possess the same carcinogenic response when treated repeatedly with urethan during only the infant period of life (7 days and older) or during the neonatal period (1st week of life) as well, and, if not, whether the susceptibility of older infants may be modified by priming with urethan during the newborn age period.

In the 1st part of the experiment, C57BL x C3H F1, mice were given urethan 6 times at 3-day intervals. The treatments began on the 1st, 4th, or 7th day of life. Sixty weeks later the animals were killed. Several observations were made. First, the results of the experiment further documented the multicarcinogenicity of urethan as evidenced by the development of two types of ovarian tumors, Harderian gland adenomas, hepatomas, lung adenomas, and stem-cell malignant lymphomas (30, 31) in the treated animals. The development of the ovarian tumor is of particular interest as it adds another organ to those already known to be capable of being affected by urethan. Second, it was observed that the mice when 1st treated at 7 days of age developed hepatomas (females) and malignant lymphomas in a significantly lower proportion than did the mice which were 1st treated when newborn, but they developed Harderian gland adenomas in a higher proportion. On the other hand, the induction of ovarian tumors and lung adenomas was not affected by the age differences. It became apparent, then, that carcinogenesis in various tissues does change independently from one another shortly after birth.

In the 2nd part of the experiment the animals were given 6 urethan treatments at 3-day intervals delivered in two separate triads. The 1st set of treatments began on the 1st day of life (priming), while the 2nd triad followed the 1st triad after 9, 15, or 21 days. The incidence of malignant lymphomas, hepatomas (females), and Harderian gland adenomas decreased as the interruption of treatment increased. Thus the priming of newborns with urethan was ineffective in enhancing tumor response in the older infant mice.

INTRODUCTION

Multipotential carcinogenicity of urethan has been well demonstrated not only for mice (23, 24, 25) but also for Syrian golden hamsters (28) and more recently for rats (26). In several papers the authors have indicated a greater responsiveness in newborn mice than in young adults to urethan carcinogenesis by noting a higher incidence of leukemia (5, 6, 20), lung adenomas (2), and hepatomas (1, 3, 16, 17).

The objective of the present investigation was to determine whether various organs of mice possess the same carcinogenic response when treated repeatedly with urethan only during the infant period of life (7 days and older) or during the neonatal period (1st week of life) as well, and, if not, whether the susceptibility of older infants may be modified by priming with urethan during newborn or neonatal age. An integrated study was therefore initiated to evaluate how the age at the inception of urethan treatment affects carcinogenesis in several organs or tissues, such as lungs, livers, Harderian glands, and the lymphoreticular system.

Previous papers from this laboratory indicated that newborn mice were significantly more prone to leukemogenesis by urethan when they were exposed to continuous and periodic treatments than when they were exposed to interrupted treatment (30, 31). The present paper deals with other types of tumor development in other organs and tissues under the same experimental conditions.

MATERIALS AND METHODS

Mice. The experimental animals were from the F1 generation of C57BL and C3H inbred mice, raised in our laboratory (30). Pregnant females were allocated at random to experimental groups. Their offspring were weaned at about 30 days of age, numbered, and housed in plastic cages in sets of 10. Sanicel was used as bedding. The mice were kept in a temperature-controlled laboratory at 78°F and were fed Rockland diet and given water ad libitum.

Throughout their life-span the animals were inspected once a week for external tumors and other symptoms and were weighed at 2-week intervals. The protrusion of the eye, which was the 1st clinical sign of developing Harderian gland adenoma, was recorded at each inspection. The time of appearance of the symptoms was used to estimate the average latent period of the development of Harderian gland tumors. The animals were also examined for the presence of distended abdomens as an indication of advanced hepatoma.

All the animals were sacrificed 60 weeks following the last treatment. At this time, the mice were autopsied and specimens were taken from all the internal organs, endocrine glands, and the brain. In addition, both eyes were removed with the Harderian glands. Both the control and the experimental groups were...
carefully examined at the autopsy and then histologically. The tissues were fixed in 10% formalin, processed, and stained with hematoxylin and eosin.

Urethan. White, crystalline urethan (ethyl carbamate) reagent-grade was used. A solution of 10% concentration was always made in distilled water shortly before use.

Treatment. An intraperitoneal route of application was used throughout the experiment. Urethan (10% solution) was delivered by a Hamilton microsyringe with a 30-gauge needle, 0.005 ml (0.5 mg) per gm body weight, at each injection. Leakage through the needle puncture was minimized, as previously described (30). Groups 3, 4, 5, 11, 12, and 13 received 6 injections at 3-day intervals, 1st delivered when the mice were 1, 4, or 7 days of age, respectively. Mice of Groups 6, 7, 8, 14, 15, and 16 were 1 day old when they received the 1st of 6 injections. However, the treatment in this series was interrupted following the 3rd injections for 9, 15, or 21 days, respectively. Groups 1 and 9 served as the nontreated controls, and Groups 2 and 10, which received only the 1st 3 injections, acted as the positive control for the effect of the primary treatment.

RESULTS

The experiments proceeded smoothly and satisfactorily. All the animals which died during the 1st 35 weeks of life developed only malignant lymphomas (the stem-cell type). These results have already been reported separately as an integrated part of dose-response studies and the role of neonatal age and periodicity of urethan treatment on leukemogenesis (30, 31). Only 5% of the animals died between the 35th week of life and the end of the experiment. Their deaths were randomly distributed throughout all 16 groups, and none showed any neoplasia at the autopsy. Only the mice that survived the entire duration of the experiment were evaluated for tumor response. Thus, all the control and experimental animals had the same fixed lifespan to express their neoplastic potentialities.

Ovarian Tumors. The ovaries of the treated animals were routinely studied histologically. The majority of them when inspected grossly appeared slightly enlarged (2–3 mm in diameter) and yellow-tan in color, and only a very few were markedly enlarged (up to 10 mm). Occasionally, ovaries had either hemorrhagic or clear cysts. Histologic study of semi-serial sections of the ovaries revealed the absence of follicles, corpora lutea, and ovogenesis; remarkable stromal and epithelial hyperplasia; and the presence of tumors in all treated groups (Table 1). Morphologically these neoplasias were of either stromal or epithelial origin. A few histologic patterns of these tumors are presented in Figs. 1–8. The incidence of ovarian tumors was not significantly affected by the age of the mice at the inception of treatment (Groups 3, 4, and 5). The occurrence of the stromal-cell tumors had a tendency to decrease with the increase of the interruption of treatment (Group 6 vs. Group 8). In the majority of the groups the proportion of mice with tubular adenomas was somewhat lower than the proportion of mice with the stromal type of tumors.

Harderian Gland Adenoma. The animals developed this neoplasia in a relatively high proportion. Microscopically the tumors showed either papillary structure, cystic spaces, or solid adenomatous areas, or a combination of these. Table 2 gives the incidence of these tumors for both sexes. In no instance was either sex significantly more responsive than the other. In most of the experimental groups a fraction of the animals developed bilateral neoplastic processes. While 3 urethan treatments did not result in any bilateral tumors (Group 2 males and Group 10 females), 6 such treatments caused neoplasia in only 14% (weighted average of Group 3 males and Group 11 females). However, when applications of urethan were started at 4 or 7 days of age, bilateral tumors developed on the average in 34% and 47% of the mice, respectively (Groups 4, 5, and 12, 13, males and females).

The highest overall Harderian gland tumor response was observed in the group from the series with the interrupted treatment in which the period between the priming and the secondary treatment was 9 days (Group 6 males and Group 14 females). As the secondary treatment was further delayed, the incidence of the Harderian gland tumor decreased (Groups 7, 8, 15, and 16) and the average latent period increased (Table 2, Column 9). Only 1 control male mouse developed unilateral adenoma, which was found at the autopsy.

Hepatoma. Table 2 also summarizes the incidence of hepatomas for both sexes. A majority of mice at the autopsy had livers practically "replaced" by the tumors so that it was not possible to distinguish individual hepatomas. The average weight of such livers was 5.5 gm (approximately 17% of carcass weight). Only a few of the tumor-bearing males had small, circumscribed hepatomas, and their livers weighed, on the average, 2.5 gm (7.5% of carcass weight). All the male mice developed this neoplasia even after 3 injections of urethan. It was apparent that the overwhelming dose of urethan completely masked the underlying biologic differences.

In females, the yield of hepatomas was significantly lower. After 3 urethan treatments, hepatomas developed in 13% of the animals (Group 10). When the number of treatments was increased to 6 injections, the incidence of hepatoma-bearing
females rose to 61% out of which only 8% of the mice had tumors having the same incidence as Group 10, which received only 

Similarly, the yield of hepatomas decreased in the groups in which the last 3 injections of urethan were given during the 3rd week of life, developed hepatomas in 12% of the animals, having the same incidence as Group 10, which received only primary treatment. None of the untreated females developed this type of tumor.

**Lung Adenoma.** As was anticipated, the urethan-treated population also developed the classic pulmonary adenoma. These tumors generally were small and solitary. Six urethan treatments resulted in the development of these tumors in approximately 36% of the animals regardless of the age and discontinuity of the treatment.

**DISCUSSION**

The observations made in this experiment further emphasize the multicarcinogenicity of urethan. Practically all the animals had multiple primary neoplasias at the time of the autopsy. Considering the relatively short time of the treatment, as well as the low total dosage of urethan delivered to the animals, it is obvious that both the newborn and the infant mice are quite prone to multicarcinogenesis by urethan.

The observation of the development of ovarian tumors is of interest as it adds new neoplastic lesions to the already long list of tumors that can be induced or potentiated by urethan. It is also of interest to note that previously X-irradiation (7, 8), intrasplenic grafting of ovaries (14, 15), or percutaneous applications of 9,10-dimethyl-1,2-dibenzanthracene (10, 18) were the only known factors responsible for the development of ovarian tumors. Urethan now becomes a new and quite potent member of this group. The present experiment did not reveal the existence of any significant biologic variation between newborn and infant mice regarding genesis of ovarian tumors. The absence of ovarian follicles and corpora lutea represents a common histologic denominator in all the above-mentioned situations, as well as in the case of ovarian tumorigenesis in C57BL-W/vW/v mice, which was caused by gene substitution (22). A similar morphology was observed in the ovaries of urethan-treated mice. Thus the ovarian atrophy was already found in 10-day-old C3H/1 mice which had been treated with urethan on the 1st day of life (16). The histology of the testes appeared normal in urethan-treated males. Further studies are required to establish the role of urethan in the induction and/or the development of ovarian tumors and its possible relationship to hormonal factors.

The induction of Harderian gland tumors by urethan was

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**TABLE 2**

The Effect of Age of Mice and the Periodicity of Treatment with Urethan on the Incidence of Harderian Gland, Liver, and Lung Tumors

<table>
<thead>
<tr>
<th>Group number</th>
<th>C57BL x C3H F1 mice</th>
<th>Administration of urethan</th>
<th>% of mice with</th>
<th>% of mice with</th>
<th>% of mice with</th>
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<tr>
<td></td>
<td>Sex</td>
<td>Age at start (days)</td>
<td>Effective number</td>
<td>Number of injections</td>
<td>Interruption of treatment (days)</td>
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* Of the 10% water solution, 0.005 (0.5 mg of urethan) was given/gm body weight. Each mouse received specified number of i.p. injections at 3-day intervals unless stated otherwise.

+ Age at 1st injection of urethan.
+ Survivors at the termination of experiment.
+ Period between 1st and 2nd triad of treatment exceeding periodicity of 3 days.
+ Incidence of animals in which Harderian glands of both eyes have undergone neoplastic changes; difference between this and total incidence indicates proportion of mice with monolateral involvement.
+ Average age at which first clinical signs of developing Harderian gland adenomas (protrusion of the eye in association with discoloration of the eyebrow) were observed.
reported for the 1st time by Tannenbaum and Silverstone (25) in C57BL x C3H F1 mice. Recently, Deringer (4) and Klein (13) observed a moderate yield of these tumors in urethan-treated DBA/2eBD and B6AF1/J mice, respectively. As shown in the Results section, the high responsiveness of these glands to urethan carcinogenesis has been mirrored in the high proportion of the animals having bilateral involvement, particularly when the mice were exposed to urethan during the 2nd and 3rd (Groups 5 and 13, males and females) as well as the 1st and 3rd weeks of life (Groups 6 and 14, males and females). Thus, it appears that the 3rd week of life may be the age at which Harderian glands are most amenable to neoplastic change. This idea aroused our interest in studying the morphology of these glands in newborn and infant mice (S. D. Vesselinovitch, M. Greenblatt, and N. Mihaiovich, unpublished study). It has been known that the eyelids of mice open at the end of the 2nd or the beginning of the 3rd week of life. It suffices here to mention that Harderian glands appear quiescent before the opening of the eyelids and show simple, tubuloalveolar structures. The cells are low cuboidal and the nuclei are centrally located, with a diffuse distribution of chromatin (Figs. 9, 10). However, after the eyelids have opened, the appearance of these glands changes. The cells become high columnar with densely stained nuclei located at their bases, and the cytoplasm of the free ends of the cells, facing the glandular lumens, develop a foamy or vacuolated appearance indicating active secretion (Figs. 11, 12). Thus, it is intriguing to speculate that this secretory activation of Harderian glands might be causally related to their increased tumor response, possibly due to the increased turnover of urethan.

It has already been reported that urethan acts as a potent liver carcinogen when given to newborn mice of various strains (1, 3, 13, 16, 17, 29). The adult animals, however, are much less, or are not at all, responsive (1, 3, 9, 16). It has been well known that a sex difference exists in the development of spontaneous and induced hepatomas, the females being less sensitive than the males. The same phenomenon was observed in the present experiments and by others (1, 3, 13, 16) for urethan hepatogenesis. Liebelt et al. (16) recently indicated that both the male and female C3H/l mice which developed hepatomas following neonatal urethan treatment showed signs of androgenic stimulation. This observation led the authors to conclude that the presence or absence of tumors in various tissues may depend to some extent upon the hormonal environment of the host.

Hepatomas and Harderian gland adenomas were readily induced in C57BL x C3H F1 mice by a moderate urethan treatment, but the lungs appeared to be less responsive. Neither the age nor the sex of the animals modified carcinogenesis in this organ. This fact is in keeping with other investigations which show that newborns are not more responsive to lung tumorigenesis than older infant mice when challenged by a variety of carcinogens (11, 19, 26).

The objective of previous reports from this laboratory (30, 31) and of the present investigation was to determine how the newborn and the infant age periods as biologic factors affect multicarcinogenesis by urethan. It was observed that a one-week delay of the inception of treatment with urethan changed the incidence of several types of tumors or the tumor profile. Thus, with the one-week delay the occurrence of leukemia decreased in both sexes (30), as did the development of hepatomas in females, but the incidence of Harderian gland adenomas slightly increased in both sexes. The induction of lung adenomas and ovarian tumors was not affected by the age at which treatment was begun. The sex of the animal was not observed to have an effect in either lung or Harderian gland carcinogenesis. When the animals were primed during newborn age and received their last 3 urethan injections during the 3rd, 4th, or 5th week of life, the incidence of leukemia (31), hepatomas, and Harderian gland adenomas decreased while that of lung adenomas remained the same. Thus, the initial urethan treatment during newborn age did not “sensitize” or prepare any of the tissues to respond more readily to the secondary treatment which was delivered later during infancy.

It is apparent that carcinogenesis in various tissues does change independently from one another shortly after birth. This is because urethan, as a carcinogen, may be considered to operate either directly or indirectly depending upon the tissue, since there is no reason to assume the unitarian mode or mechanism of its carcinogenic action. In addition, the cellular and metabolic immaturity [thymus (30, 31)], the status of the functional activity (Harderian glands), the hormonal environment [liver (1, 3, 13, 16)], or a hormonal imbalance (ovaries) may be the underlying factors modifying the genesis and/or the development of tumors. Therefore, the extent of the multicarcinogenicity by urethan is conditioned by the biologic factors at the time of its action and by the interplay of its effects manifested later in life.

In the last few years there has been an increasing interest in the use of newborn and infant mice as a “sensitive” and broad biologic system for demonstrating carcinogenicity (1, 2, 11, 12, 21). The presented and discussed results sufficiently substantiate the importance of the biologic background of the newborn period for the most effective leukemogenesis and hepatogenesis in mice and of the infant age period for the development of Harderian gland tumors. These ages, though, do not apparently play a significant role in lung and ovarian tumorigenesis. Thus, depending upon the type of neoplasia anticipated for a given agent, the treatment should be timed with the age period during which the biologic conditions favor neoplastic changes in that particular tissue. If this age period has been missed (and/or insufficient dose of this agent was delivered), certain carcinogenic effects of the compound may remain unrevealed.

ACKNOWLEDGMENTS

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REFERENCES

S. D. Vesselinovitch and N. Mihailovich


Fig. 1. The cortical surface of an epithelial ovarian tumor is shown. The germinal epithelium is contiguous with the series of anastomosing tubular structures. H & E, × 200.

Fig. 2. Higher magnification of the same tumor as in Fig. 1. The tubular elements are lined by a uniform low cuboidal epithelium. The intervening stroma contains a variety of cells, some of which resemble luteal cells while others are spindle-shaped. H & E, × 450.

Fig. 3. This stromal tumor shows a "folliculoid" pattern with rounded masses of cells frequently with a central lumen. H & E, × 200.

Fig. 4. Higher magnification of the same tumor as in Fig. 3. The "folliculoid" pattern is depicted. The cells are regular. H & E, × 450.
**FIG. 5.** This stromal tumor is characterized by a pronounced “whorled” arrangement of tumor cells. H & E, × 200.

**FIG. 6.** Higher magnification of the same tumor as in Fig. 5. The cells are arranged in “nests” or Zellballen. Their character is spindle-shaped. H & E, × 450.

**FIG. 7.** This stromal tumor has a uniform epithelioid pattern. Note the high frequency of mitotic figures. H & E, × 200.

**FIG. 8.** Higher magnification of the same tumor as in Fig. 7. The cells show moderate variation in nuclear size and shape while the cytoplasm is abundant but poorly delineated. H & E, × 450.
FIG. 9. Harderian gland of 10-day-old C57BL x C3H F1 mouse is presented. Note the tubuloalveolar structures within the lobes which are separated from one another by loose connective tissue. H & E, X 150.

Fig. 10. Higher magnification of the same Harderian gland as in Fig. 9. The epithelial cells are low cuboidal, and the round nuclei, containing one to three nucleoli, are centrally located. H & E, X 400.

Fig. 11. Harderian gland of 19-day-old C57BL x C3H F1 mouse is shown. The individual lobes are not conspicuous, but the tubuloalveolar structure is apparent. H & E, X 150.

Fig. 12. Higher magnification of the same Harderian gland as in Fig. 11. The epithelial cells are high columnar. The densely stained nuclei are located at the base of the cells, and the cytoplasm has a foamy or vacuolated appearance. H & E, X 400.
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