Urethan and X-ray Effects on Mice of a Tumor-resistant Strain, X/Gf1

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SUMMARY

Inbred X/Gf mice with an extremely low incidence of spontaneous neoplasms were tested for their response to urethan and X-irradiation. Newborn, infant, and adult mice of both sexes were treated weekly with various doses of urethan [0.5 mg/g body weight (7 times) for newborn mice; 1.0 mg/g body weight (12 times) for infant and adult mice applied i.p., and 24 mg/mouse/week applied cutaneously]; with X-irradiation ranging from 300 rads (1 time) to 80 rads (10 times) and with combined treatments [urethan, 1.0 mg/g body weight (4 to 12 times) and X-irradiation, 75 to 150 rads (4 times)].

The incidence of neoplasms was very low in all age groups regardless of the mode of treatment, and it reached 3.4% for the total of 1023 mice. The only statistically significant difference was noticed between the group subjected to combined treatments [urethan, 1.0 mg/g body weight (4 to 12 times) and X-irradiation, 75 to 150 rads (4 times)] and pooled data on all experimental groups (p < 0.05 for the "both-tail" test). Differences between other modes of treatment and between pooled data for both sexes were not statistically significant. Among 38 recorded neoplasms, 18 were thymomas of the lymphoblastic type, 13 were thymomas of the lymphocytic type, 3 were reticulum cell sarcomas, and 4 were mammary tumors.

The very low incidence of neoplasms induced by urethan and X-irradiation in X/Gf mice is explained on the basis of (a) their high endogenous immune competence, (b) the absence of leukemia viruses, and (c) the very low incidence of mammary tumor virus.

INTRODUCTION

For the past several years, investigations in this laboratory have been concerned with the detection of constitutional or inherent factors that may be involved in the development of malignant neoplasms, either spontaneously or after application of external oncogenic agents. Investigations have been performed on X/Gf mice. These mice have been inbred in this laboratory since 1953 and have produced no malignant neoplasms spontaneously. In 3 instances, mammary fibroadenomas have developed in females over 1 year of age. Because of the extremely low incidence of spontaneous neoplasms, X/Gf mice constitute an ideal test system for a study of their response to external oncogenic agents.

Previous experiments with young adult and mature X/Gf mice of both sexes that were treated with urethan (ethyl carbamate), either alone or in combination with X-rays, revealed that about 4% of the mice developed either solid tumors or lymphomas at the age of about 1 year or older. Electron microscopic studies disclosed virus particles in 1 of the mammary tumors that appeared in a urethan-treated female. These virus particles represented the characteristic types A and B, according to the classification of Bernhard. Electron microscopic studies also revealed cytoplasmic and intracisternal type A virus particles and unusual ultrastructures in a thymic lymphoma that appeared in an X-ray- and urethan-treated X/Gf male. The occurrence of virus particles of type A in a thymic lymphoma, instead of the leukemia type C particles usually noted in lymphomas and leukemias, was of significant interest. The question was raised whether some of the X/Gf mice carry latent MTV-type particles that can be "activated" in female mammary glands by treatment with urethan alone or by urethan combined with X-irradiation.

To shed light on these intriguing observations, we carried out a series of experiments on X/Gf mice. In each experiment, X/Gf mice of different ages were used. The mice were treated with urethan alone, urethan and X-rays, or X-rays alone. The results of these treatments are reported in this communication. Electron microscopic observations made on the induced neoplasms are described in the subsequent paper.

MATERIALS AND METHODS

Mice

X/Gf mice of both sexes, varying in age from newborn to infant to adult were used. The mice were kept in stainless steel cages in air-conditioned animal quarters with the temperature regulated to 75°F. They were provided with Rockland diet pellets and water ad libitum. Occasionally, they received bread soaked in milk.

Treatment

Urethan i.p. Injections. Urethan (ethyl carbamate), reagent grade, was dissolved in double-distilled water. This solution was prepared for each day of treatment. A dose of 0.5 mg/g
body weight was used for newborn mice, and 1.0 mg/g body weight was used for infant and adult mice. The urethan solution was injected i.p. with the use of a microsyringe and a 30-gauge needle. Suckling mice were separated from their mothers for the time of treatment. To avoid cannibalism, they were not returned to their nursing mothers until after they had recovered from the anesthetic effect of the urethan. Each mouse received 4, 7, or 12 urethan injections i.p. at weekly intervals. Suckling mice were separated from their mothers at weaning age; males and females were placed in separate cages.

**Urethan Cutaneous Applications.** Two series of experiments were carried out. In one series, adult mice (3 months of age) were used and, in the other, suckling mice (12 to 14 days of age) were used. Twice a week each mouse received 3 drops of a 20% solution of urethan in pure acetone (12 mg/dose). Drops of this solution were delivered from a calibrated pipet to the interscapular region. The procedure was essentially the same as that used by Tannenbaum and Silverstone (43) in their studies on several strains of mice. Application of urethan continued during the entire life of the mice. Control mice were similarly treated with pure acetone.

**X-irradiation.** Groups of mice of both sexes and of various ages were exposed to different doses of X-irradiation. The same physical factors and experimental design were used as in previous experiments. A General Electric Maximal X-ray machine operating at 200 kV peak and 15 ma was used. The incident beam was filtered through 0.5-mm Cu and 1.0-mm Al; the half-value layer equaled 1.1-mm Cu. Measurements of the dose rate were made with a Victoreen ionization chamber placed in the abdominal cavity of a mature dead mouse situated in 1 of the 25 compartments of a specially designed plastic box, described and illustrated in previous publications (20, 39). This arrangement permitted the determination of the absorbed X-ray dose in rats. An average dose rate of 16.2 rads/min (±5%) was obtained with the X-ray source, which was 86.5 cm distant from the middle of the body of the mouse. For an even distribution of the X-ray beam, the plastic box containing the mice was placed on a rotating table at 3 rpm. The mice were given whole-body irradiation at weekly intervals. Doses (in rads) of 300 (1 time), 100 (3 times), 75 (4 times), 150 (4 times), 50 (6 times), and 80 (10 times) were used in the experiments. In combined treatments, the urethan dose was administered i.p. after the completion of the radiation exposures.

**Criteria**

Experimental and control mice were allowed to live their life-spans. However, any mouse that developed a bulging chest or a distended abdomen was killed in ether, and a thorough autopsy was performed. The weight of the spleen was taken routinely, and each enlarged organ was subjected to microscopic studies. Cross-sections of the tissue were fixed in either Bouin’s or Zenker’s solution and were processed by means of standard procedures. Paraffin sections were stained with hematoxylin and eosin and examined by light microscopy. In suitable instances, sections were also fixed for electron microscopic studies. Data on the incidence of neoplasms were tabulated and evaluated statistically.

For each series of experiments, approximately the same number of control X/Gf mice of both sexes were kept in the same animal quarters for the same period of time as the experimental mice and were allowed to live through their life-span. None of the control mice developed a malignant neoplasm, as shown at autopsy.

**RESULTS**

The results obtained from 15 experiments with 1129 X/Gf mice of both sexes and various ages are recorded in Tables 1, 2, and 3. Experiments performed on newborn, infant, and adult mice treated either with urethan alone, with X-irradiation alone, or with the combination of both agents are summarized in Table 1. The incidence of lymphomas and mammary tumors is recorded separately for males and females, as well as for different modes of treatment and for different ages. The frequency of neoplasms that developed in different experimental groups was compared by χ² test. The minimal sample size for the outcome of 3.4% at a 95% confidence level corresponds to \(N > 1000\). The value of the total in Table 1, where \(N = 1023\), was compared with pooled data on different types of treatment. The difference between the group treated with urethan combined with X-irradiation and the total is significant (\(p < 0.05\) for both-tail test). Differences between the other 2 groups (treated with urethan alone and X-irradiation alone) and the total, and between pooled data for both sexes, are not significant (\(p > 0.05\)).

Among infant and adult X/Gf mice treated cutaneously with urethan dissolved in acetone, 3.7 to 4.2% developed neoplasms (Table 2). Among mice of the control group, treated cutaneously with acetone only, none developed a neoplasm.

Table 3 lists types of neoplasms that developed in X/Gf mice after various modes of treatment. Among 38 recorded neoplasms, 18 were thymomas of the lymphoblastic type (Fig. 2), 13 were thymomas of the lymphocytic type (Fig. 1), 3 were reticulum cell sarcomas (Fig. 4), and 4 were mammary tumors (Fig. 3). Thymomas of the lymphoblastic type prevailed among males (incidence, males/females: 13/5), and among mice treated at age 1 day to 4 weeks (incidence, lymphoblastic/lymphocytic type: 15/5). Thymomas of the lymphocytic type predominated in females (incidence, males/females: 3/10) and in the groups treated at age 30 days and later (incidence, lymphocytic/lymphoblastic type: 8/3). Reticulum cell sarcoma was noticed only in the group of mice treated at ages ranging from 1 day to 5 weeks; mammary tumors developed in females treated at the age of 6 weeks and later.

Spleen enlargement accompanied thymomas in the majority of cases. The spleen weight in thymomas of the lymphoblastic type was 444.4 ± 299.4 mg, in thymomas of the lymphocytic type it was 443.5 ± 326.4 mg, and in reticulum cell sarcoma it was 853.4 ± 427.9 mg, as compared with the spleen weight of 122.3 ± 37.2 mg in females with mammary tumors. The survival rate was 301.6 ± 70.6 days for mice with lymphoblastic lymphomas, 294.8 ± 65.9 days for mice with lymphocytic lymphomas, 335.0 ± 26.5 days for mice with
Oncogenesis in Tumor-resistant Mice

Table 1
Incidence of neoplasms in X/Gf mice treated with urethan and X-irradiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urethan i.p. (mg/g/wk)</th>
<th>X-irradiation (rads/wk)</th>
<th>Incidence of lymphomas</th>
<th>Incidence of mammary tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute no.</td>
<td>%</td>
<td>Confidence intervals of unaffected mice</td>
<td>Absolute no.</td>
</tr>
<tr>
<td>Experiment</td>
<td>Age at 1st treatment</td>
<td>No. of treated mice</td>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 day</td>
<td>38</td>
<td>M</td>
<td>0.5 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44</td>
<td>F</td>
<td>0.5 (7)</td>
</tr>
<tr>
<td>2</td>
<td>9–11 days</td>
<td>48</td>
<td>M</td>
<td>1.0 (12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42</td>
<td>F</td>
<td>1.0 (12)</td>
</tr>
<tr>
<td>3–11</td>
<td>3–5 wk</td>
<td>25</td>
<td>M</td>
<td>1.0 (12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>F</td>
<td>1.0 (12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>220</td>
<td>M</td>
<td>300 (1–80) (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>203</td>
<td>F</td>
<td>300 (1–80) (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97</td>
<td>M</td>
<td>1.0 (4–12) 75 (4–150)</td>
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<tr>
<td></td>
<td></td>
<td>111</td>
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<td>1.0 (4–12) 75 (4–150)</td>
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<td>12, 13</td>
<td>6–9 wk</td>
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<td>85</td>
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<td></td>
<td></td>
<td>45</td>
<td>F</td>
<td>1.0 (12) 100 (3)</td>
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<tr>
<td>1–13</td>
<td>1 day – 9 wk</td>
<td>257</td>
<td>M and F</td>
<td>1.0 (7–12)</td>
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<tr>
<td></td>
<td></td>
<td>513</td>
<td>M and F</td>
<td>300 (1–80) (10)</td>
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<td>468</td>
<td>M</td>
<td>1.0 (4–12) 75 (4–100)</td>
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<td></td>
<td>555</td>
<td>F</td>
<td>300 (1–80) (10)</td>
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<td></td>
<td></td>
<td>1023</td>
<td>M and F</td>
<td>1.0 (4–12) 75 (4–100)</td>
</tr>
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</table>

The minimal sample size for the outcome of 3.4% at 95% confidence level of \( \pi \), corresponds to \( N > 1000 \). The value of the total, where \( N = 1023 \), was used for the \( \chi^2 \) test with known \( E \). The difference between the group treated with urethan plus X-irradiation and the total value is significant (\( p < 0.05 \) for both-tail test); differences between other groups of treatment and the total and between pooled data for both sexes are not significant (\( p > 0.05 \)).

b The 95% confidence intervals were calculated

\[
\pi = P \pm 1.96 \sqrt{\frac{P(1-P)}{N}}
\]

where \( P \) is the portion of mice without developed neoplasm.

c Numbers in parentheses, number of treatments given per week.

Reticulum cell sarcoma, and 331.5 ± 82.7 days for mice with mammary tumors.

DISCUSSION

The results obtained from this study are discussed with respect to (a) oncogenesis in X/Gf mice induced by urethan alone, urethan plus X-rays (and X-rays alone); (b) viral oncogenesis; and (c) constitutional factors playing a role in oncogenesis of X/Gf mice.

With respect to the effects of urethan per se, it has been proven that this agent has induced a variety of neoplasms in various animal species and is now referred to as a "multipotential" neoplastic agent (43). It was found that urethan induces neoplastic changes in the skin (3, 38) in mammary glands (43), lymphatic tissues (9, 29, 43), the liver (30, 31, 33), and other organs. Urethan also exerts a potentiating effect on X-ray leukemogenesis (4, 2, 9, 29, 32, 44, 49), and it exerts greater effects on newborn and infant mice than on adult mice (7, 8, 10, 29, 31, 34, 37, 45, 46, 48, 49). The mechanism involved in urethan carcinogenesis is not yet clear (35).

In all of those experiments, mice that possessed a certain degree of susceptibility to the development of a specific type of neoplasm were used. For example, mice that usually produce mammary tumors spontaneously produced this type of neoplasm to a significantly greater extent after treatment with urethan. This fact holds true for other types of neoplasms, as referred to above.

In contrast, the present experiments were performed on X/Gf mice that do not produce malignant neoplasms spontaneously. The application of urethan to newborn or adult X/Gf mice induced neoplasms averaging only 2.7% (Table 1) as compared to 80 to 90% in susceptible mice treated similarly.

X-irradiation as well as other types of ionizing radiation proved to induce a variety of neoplasms in a high percentage of various animal species (6, 11, 12, 25, 26, 28, 47). The results obtained in this study show that only 2.1% of
groups irradiated with various doses ranging from 300 rads (1.06 to 3.33%).

In experiments reported in the literature to which references have been cited, mice have been used that carry an oncogenic virus. Recent studies have shown that X-irradiation releases the virus particles from infected cells (22, 23). Thus, presumably, the potentiating effects of X-ray or urethan may act through the release of the endogenous virus. In support of this interpretation, reference is made to the presence of the Incidence of neoplasms of type Reticulum cell sarcoma Thymoma lymphoblastic Thymoma lymphocytic Mammary carcinoma

<table>
<thead>
<tr>
<th>Experiment</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
</tr>
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<tbody>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3–11</td>
<td>0</td>
<td>2</td>
<td>9</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>12, 13</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
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<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total (each sex)</td>
<td>1</td>
<td>2</td>
<td>13</td>
<td>5</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Total (combined)</td>
<td>3 (0.27%)c</td>
<td>18 (1.59%)</td>
<td>13 (1.15%)</td>
<td>4 (0.35%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Approximate age of mice and mode of treatment are specified in Table 1 (Experiments 1 to 13) and Table 2 (Experiments 14 and 15).

b The incidence is given as the absolute number.

c Numbers in parentheses, percentage of the total.

X-irradiated X/Gf mice developed neoplasms (Table 1). In contrast, among mice of a susceptible strain (similarly treated) as high as 80 to 90% developed neoplasms, particularly thymic lymphomas (27).

As mentioned above, X-irradiation exerted a potentiating effect on urethan leukemogenesis. In all of these experiments, as in those with urethan alone, mice susceptible to the development of spontaneous neoplasms were used. In this study, the combined treatments reached the highest incidence of neoplasms (5.5%) compared with treatment with urethan or X-rays alone (2.1 and 2.7%) (Table 1). The data on X-irradiation alone represent pooled results from several groups irradiated with various doses ranging from 300 rads (1 time) to 80 rads (10 times). Differences in the incidence of neoplasms between individual groups are not significant (range of 1.06 to 3.33%).

In experiments reported in the literature to which references have been cited, mice have been used that carry an oncogenic virus. Recent studies have shown that X-irradiation releases the virus particles from infected cells (22, 23). Thus, presumably, the potentiating effects of X-ray or urethan may act through the release of the endogenous virus. In support of this interpretation, reference is made to the presence of the MTV2 in 1 X/Gf mouse that had been treated with urethan (18), and in 2 mammary tumors, included in the present study, that had developed in X/Gf females treated with X-rays and urethan. This is in agreement with most recent findings that a small percentage of X/Gf mice is susceptible to MTV.

Conversely, no murine leukemia virus (type C) had been disclosed in lymphomas that developed in X/Gf mice treated with urethan alone or in combination with X-rays. This is also in accord with most recent tests on 60 control X/Gf mice of both sexes which revealed that X/Gf mice do not possess the murine leukemia virus antigens in their milk (R. Nowinski, personal communication). X/Gf mice proved also to be resistant to the polyoma virus (20) to Friend leukemia virus (17), and to FBJ osteosarcoma virus (M. Finkel, personal communication).

With respect to endogenous or constitutional properties, it was revealed that X/Gf mice are resistant to radiation carcinogenesis (13, 16) and have high immune responses against sheep red cells (41), pronounced splenic phagocytic activity (42), high levels of antibody against MTV in the blood serum (1), and an increased tendency to spontaneous amyloidosis (39). The fact that MTV particles were noted electron microscopically in 3 mammary tumors and, curiously, in type A particles in 1 thymic lymphoma that had developed after urethan and X-ray treatments indicates that some of the X/Gf mice carry MTV particles in a latent state. Urethan and X-rays, both known as immunosuppressive agents (36), probably reduced the endogenous immunological competence in X/Gf mice, thus providing a more favorable medium for viral oncogenesis. This interpretation is in accord with recent findings that X-irradiation prompted leukemogenesis by depressing the immune mechanism (24) and by viral release from infected cells (22).

On the basis of the experimental results reported in this as well as in previous communications, the following 2 basic factors may be considered in attempts to explain the resistance of the X/Gf mice to the development of spontaneous neoplasms and the low incidence of neoplasms in the X-ray- and urethan-treated mice: (a) their immune competence and (b) the absence of an apparent oncogenic virus as revealed by electron microscopy. According to the concept of a viral origin of neoplasms, the virus may integrate into the cell and become

\[ MTV \]

\[ \text{The abbreviation used is: MTV, mammary tumor virus.} \]
ACKNOWLEDGMENTS

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ADDITIONAL

After submission of this manuscript, the author discovered a relevant paper by Schrodt and Foreman (40). In this paper, the authors report observations of dense bodies in a variety of tumor cells and pathological conditions.

REFERENCES


Fig. 1. Lymphoma of lymphocytic type that developed in a female X/Gf mouse treated with 12 injections of urethan and X-irradiation (100 rads, 3 times). H & E, X 350.
Fig. 2. Lymphoma of lymphoblastic type that developed in a male X/Gf mouse treated with 4 injections of urethan and X-irradiation (150 rads, 4 times). H & E, X 350.
Fig. 3. Mammary carcinoma that developed in female X/Gf mouse treated cutaneously with 732 mg of urethan. H & E, X 350.
Fig. 4. Reticulum cell sarcoma that developed in a male X/Gf mouse treated with 7 injections of urethan at newborn age. H & E, X 350.
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