Role of the Vehicle in the Genesis of Bladder Carcinomas in Mice by the Pellet Implantation Technic

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Summary

Several compounds—arachic acid, hexaethylbenzene, hexamethylbenzene, 1-octadecanol, palmitic acid, and stearamide—were individually compressed as pellets and tested, by implantation into the mouse bladder, as possible vehicles for carcinogenicity experiments. All of these compounds were found to be associated with a 3-17% incidence of bladder carcinomas. Pellets of 23-methylcholanthrene (MC) used alone induced an incidence of 52%, whereas pellets of XAE used alone induced an incidence of 1.6%. The pellets of XAE disintegrated within 2 days following surgical introduction into the mouse bladder. When cholesterol pellets were placed in the mouse bladder and XAE was injected s.c., a 30% incidence of bladder carcinomas was observed. However, the repeated s.c. injection of XAE into mice whose bladders did not contain cholesterol pellets failed to produce any bladder carcinomas. XAE was observed to be present in the urine of mice following s.c. injection. It was suggested that the prolonged presence of the vehicle in the mouse bladder had a promoting effect on the genesis of the bladder carcinomas. Several metabolites of the essential amino acid tryptophan were quantitatively measured in the urine of mice. It was found that 3-hydroxy-L-kynurenine was present in mouse urine; this had previously been demonstrated to be associated with the production of a significant incidence of mouse bladder carcinomas when implanted in a cholesterol vehicle. It was postulated that the combination of continued excretion of this compound or some other metabolite in the urine and the protracted presence of a pellet resulted in the genesis of a low-background incidence of bladder carcinomas in mice.

Introduction

The technic, developed by Maisin and Picard (28) and modified by Jull (26) and Allen et al. (1), of testing the carcinogenicity of chemical compounds by pellet implantation into the mouse bladder, was utilized (1-9, 14-16, 18, 20, 25, 26) with the assumption that the test compound must be eluted in vivo from pellets composed of this chemical and a carrier medium. It was suggested that prolonged exposure of the bladder epithelium to focal high concentrations of the compound was obtained without hepatic metabolism of the test chemical (6, 9, 19). However, it was observed (13, 16) that a short exposure of the bladder epithelium to the test compound was followed by the subsequent development of a significant incidence of bladder carcinomas. In addition, it was found (17) that a large fraction of the test compound eluted from the pellet was absorbed through the mouse bladder and subjected to metabolism in other tissues of the test animal.

Though paraffin (1-7, 15, 18, 20, 25, 26) and cholesterol (1, 9, 14, 16, 20) have been employed most frequently as carrier media, more recently other chemicals, e.g., stearic acid (3), smooth and roughened glass beads (2), magnesium stearate, 1-hexadecanol, 1-octadecanol, and naphthalene (9), have been tested in an attempt to find a substance that would not induce bladder carcinomas. However, in spite of the great diversity in chemical nature of these media, bladder carcinomas have been observed following the implantation of any one of these media in pure form. It has been suggested (1, 2, 6) that the medium itself may have a cocarcinogenic role in the production of experimental bladder tumors by the implantation technic. The studies presented in this communication were undertaken in an attempt to ascertain the role of the carrier medium in the genesis of mouse bladder carcinomas.

Materials and Methods

GENERAL PROCEDURES. The preparation of XAE and cholesterol has been described (13, 15, 29). Arachic acid, 1-hexadecanol, hexaethylbenzene, hexamethylbenzene, 1-octadecanol, palmitic acid, stearamide, and MC were obtained from commerical sources (Eastman Organic Chemicals).

The details of the pellet preparation, operative technic, selection and care of the mice, and preparation and staining of the bladder specimens for histologic examination have been presented (13-16). Any external, thoracic, or abdominal tissues suggestive on gross examination of neoplastic growth were prepared, stained with hematoxylin and eosin, and inspected microscopically. Lesions observed in the bladder were evaluated with the criteria of Bonser and Jull (7) and Roe (30). Carcinomas that were not observed to invade muscle were classified as Stage I; those which were seen to invade muscle, as Stage II; and those which presented evidence of local metastases or serosal spread, as Stage III. Only the total incidence of carcinomas was used to assess carcinogenicity. As the carcinogenicity experiments re-
TABLE 1
SURVIVAL OF MICE FOLLOWING BLADDER IMPLANTATION AND INCIDENCE OF CHANGES IN MOUSE BLADDERS WITH IMPLANTS OF COMPOUNDS

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>Pellet Characteristics</th>
<th>Days intact in vivo</th>
<th>No. of mice given implants</th>
<th>No. of mice that died or were killed (days)</th>
<th>Average survival (days)</th>
<th>Squamous metaplasia</th>
<th>Benign tumors</th>
<th>Carcinomas</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Total</th>
<th>%</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Good</td>
<td>&gt;330</td>
<td>294</td>
<td>3</td>
<td>5</td>
<td>16</td>
<td>63</td>
<td>87</td>
<td>449</td>
<td>0</td>
<td>15</td>
<td></td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td>Arachic acid</td>
<td>Excellent</td>
<td>330</td>
<td>56</td>
<td>6</td>
<td>11</td>
<td>10</td>
<td>27</td>
<td>293</td>
<td>3</td>
<td>5</td>
<td></td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Std</td>
<td>Excellent</td>
<td>330</td>
<td>56</td>
<td>2</td>
<td>10</td>
<td>13</td>
<td>25</td>
<td>299</td>
<td>2</td>
<td>5</td>
<td></td>
<td>4</td>
<td>1</td>
<td>4</td>
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<tr>
<td>1-Hexadecanol</td>
<td>Very poor</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexaethylbenzene</td>
<td>Poor</td>
<td>330</td>
<td>56</td>
<td>5</td>
<td>11</td>
<td>17</td>
<td>33</td>
<td>304</td>
<td>3</td>
<td>3</td>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hexamethylbenzene</td>
<td>Good</td>
<td>330</td>
<td>56</td>
<td>1</td>
<td>8</td>
<td>29</td>
<td>38</td>
<td>325</td>
<td>1</td>
<td>2</td>
<td></td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1-Octadecanol</td>
<td>Good</td>
<td>&lt;40</td>
<td>56</td>
<td>5</td>
<td>9</td>
<td>25</td>
<td>39</td>
<td>308</td>
<td>0</td>
<td>7</td>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>Fair</td>
<td>&lt;330</td>
<td>56</td>
<td>6</td>
<td>12</td>
<td>10</td>
<td>28</td>
<td>294</td>
<td>3</td>
<td>4</td>
<td></td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Paraffin</td>
<td>Good</td>
<td>&gt;330</td>
<td>56</td>
<td>2</td>
<td>7</td>
<td>21</td>
<td>30</td>
<td>317</td>
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<td>2</td>
<td></td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Stearamide</td>
<td>Good</td>
<td>330</td>
<td>56</td>
<td>9</td>
<td>5</td>
<td>7</td>
<td>21</td>
<td>276</td>
<td>1</td>
<td>1</td>
<td></td>
<td>1</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>20-Methylcholanthrene</td>
<td>Good</td>
<td>&lt;2</td>
<td>96</td>
<td>5</td>
<td>0</td>
<td>19</td>
<td>44</td>
<td>318</td>
<td>0</td>
<td>4</td>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Cholesterol + XAE</td>
<td>Good</td>
<td>183</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>35</td>
<td>54</td>
<td>408</td>
<td>2</td>
<td>5</td>
<td></td>
<td>11</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cholesterol pellet in bladder + XAE s.c. (Group 1)</td>
<td>Good</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>16</td>
<td>23</td>
<td>433</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>No foreign body in bladder + XAE s.c. (Group 2)</td>
<td>Good</td>
<td>42</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>18</td>
<td>21</td>
<td>447</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No foreign body in bladder + H2O s.c. (Group 3)</td>
<td>Good</td>
<td>42</td>
<td>0</td>
<td>5</td>
<td>8</td>
<td>9</td>
<td>22</td>
<td>405</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No foreign body in bladder; no s.c. injection (Group 4)</td>
<td>Good</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>19</td>
<td>24</td>
<td>431</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results previously reported, Ref. 16.
  † Flat, flat face die; Std, standard face die.
  ‡ Results previously reported, Ref. 15.
  § XAE, 8-methyl ether of xanthurenic acid.
  ¶ H2O, aqueous phosphate buffer without XAE.
  ‖ Beginning from 1st day of s.c. injection.

ported here were conducted at the same time as those reported previously (16), statistical comparison was made with the cholesterol group of the incidence of carcinomas related to the introduction of pellets composed of different media, or related to the s.c. injection of XAE into mice bearing cholesterol pellets in their bladders. Probabilities of statistical significance were computed by the exact method for 2 x 2 tables (21).

TESTING OF MEDIA FOR BLADDER CARCINOGENICITY. The test compound was ground to a fine powder in an agate mortar and compressed into spheroidal pellets, $\frac{\frac{1}{2}}{2}$ inch in diameter and weighing 24–27 mg, with a standard face die in a Colton pellet press. In addition, pellets of arachic acid were made, $\frac{\frac{1}{2}}{2}$ inch in diameter and weighing 25–27 mg, with a flat face die. Pellets were inserted into the bladders of several groups of 56 mice each (Table 1). The animals were sacrificed 330 days following surgery. Those animals dying before this time were evaluated for the presence of bladder carcinoma only if they had survived more than 175 days following surgery.

IMPLANTATION OF PELLETS OF MC OR XAE WITHOUT ANY MEDIA. The pellets were prepared as described above and were inserted into the bladders of groups of 96 mice each (Table 1). Only animals surviving more than 175 days were evaluated for the presence of bladder carcinomas with the MC and XAE groups.

INJECTION (s.c.) OF XAE INTO MICE. Four groups of mice were studied (Table 1). Group 1 was composed of 38 mice that had pellets of cholesterol, compressed with a deep rounded cup die in a Stokes pellet press into $\frac{\frac{1}{2}}{2}$-inch-diameter, 15–25-mg spheroids, inserted into their bladders 90–100 days before the initiation of

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Footnote:
s.c. injections of XAE. Group 2 consisted of 42 mice that received s.c. injections of XAE, but had no pellets in their bladders. Group 3 (42 mice) received s.c. injections of the aqueous solvent utilized to dissolve XAE, and Group 4 (42 mice) served as untreated controls. The mice in Groups 2-4 were the same age and size as those in Group 1, and the s.c. injections into the mice in Groups 2 and 3 were carried on simultaneously with those of Group 1.

The XAE was dissolved with a few drops of 7.16 N NH$_4$OH and diluted with 2 × 10$^{-2}$ m phosphate buffer (pH 7.4) to a final concentration of 1 mg of XAE/ml of solution. The phosphate buffer containing a few drops of 7.16 N NH$_4$OH was used for Group 3. The s.c. injections of 1 ml each of XAE solution (Groups 1 and 2) and aqueous buffer (Group 3) were given 3 times each week for a period of 44 weeks (132 mg of XAE/animal in Groups 1 and 2). All animals dying before the completion of the series of injections were discarded from the experiment. Animals surviving more than 308 days after the initiation of the injections were evaluated for the presence of bladder carcinomas and other tumors.

The XAE in the injection solution was analyzed by ultraviolet spectrophotometry and paper chromatography (13, 29). Urine samples were obtained periodically from mice in Groups 1 and 2 following the injection of XAE. Paper chromatographic comparison of the urine samples with pure samples of XAE (29), followed by the elution of the spot of XAE in the urine samples and ultraviolet spectrophotometric comparison with samples of pure XAE eluted from paper chromatograms, was utilized to aid in the identification of XAE in urine.

**QUANTITATIVE ESTIMATION OF CERTAIN TRYPTOPHAN METABOLITES IN THE URINE OF MICE.** Six mice were housed together in a metal metabolism cage and quantitative 24-hr urine specimens were collected for 6 consecutive days. As the i.p. injection of L-tryptophan into rats increased the excretion of urinary tryptophan metabolites (27), at the beginning of the 7th 24-hr period, 25 mg of L-tryptophan suspended in distilled water was injected i.p. into the mice. Anthranilic acid glucuronide, o-aminohippuric acid, acetylkynurenine, and kynurenine were measured by the method of Brown and Price (11); 3-hydroxykynurenine was estimated as described by Brown (10); and indoxyl sulfate was determined by the method of Bryan (12).

**Results**

**TESTING OF MEDIA FOR BLADDER CARCINOGENICITY.** In Table 1 are presented the characteristics of the pellets of the compounds, the length of time the pellets remained intact in vivo, the number of animals subjected to the surgical implantation of pellets, the distribution of the deaths of the animals for 75-day periods beginning 175 days after surgery, the number of animals surviving a minimum of 175 days following surgery, the average survival of the animals in a group, and the incidence of microscopic changes observed in the mouse bladders. A total of 56% of the mice lived beyond 175 days. This contrasts with a survival of 35% of the mice whose bladders were implanted with cholesterol pellets (16) and 85% of the mice whose bladders were implanted with paraffin pellets (15). Many of the animals that did not live for 175 days following surgery died within 30 days owing to impaction of the pellets into the urethra. Arachic acid, hexamethylbenzene, stearamide, and 1-octadecanol were easy to pellet; palmitic acid and hexaethylbenzene were difficult to pellet; and 1-hexadecanol was very difficult to pellet and thus was not utilized in the implantation studies. 1-Octadecanol pellets disintegrated in vivo in less than 40 days and palmitic acid pellets crumbled in less than 330 days. Pellets composed of the other compounds remained intact, though decreasing in size, for at least 330 days. The average survival of the groups of animals ranged from 293 days (arachic acid) to 449 days (cholesterol). The incidence of carcinomas observed ranged from 3% for hexaethylbenzene to 17% for stearamide. When a statistical comparison was made with the incidence of bladder carcinomas reported in mice following the implantation of cholesterol pellets (16), no significant differences were noted for the media tested at this time or for paraffin, which was tested previously (15). Mammary tumors, pulmonary adenomas, lymphomas, and a few other tumors were found in a low incidence in all groups.

**IMPLANTATION OF PELLETS OF MC OR XAE WITHOUT ANY MEDIA.** MC was easy to pellet, and the pellets remained intact in vivo usually for 330 days (Table 1). On the other hand, though XAE was easy to pellet, the pellets disintegrated in less than 2 days in vivo. The exposure of the mouse bladders to XAE presented in this manner was nearly identical to the exposure following the implantation of XAE suspended in cholesterol (13). Only 1 bladder carcinoma was observed in 63 mice (1.6%) exposed to pellets of XAE alone. This incidence was not significantly different from that observed with pellets of cholesterol alone. With MC, 11 mouse bladders of the 21 examined contained carcinomas (52%). This incidence was significantly greater than that found for pellets of cholesterol alone.

**INJECTION (S.C.) OF XAE INTO MICE.** In Table 1 are presented the number of animals present in each group, the distribution of the deaths of the animals for 75-day periods beginning 175 days after the initiation of the s.c. injections, the total number of animals surviving a minimum of 308 days following the beginning of the injections, the average survival of the animals in a group, and the incidence of microscopic changes observed in the mouse bladders. The average survival for the 4 groups was comparable (405 days–447 days). Bladder carcinomas were observed only in those animals containing a cholesterol pellet in their bladders and receiving injections of XAE (Group 1). The incidence of 7 carcinomas in 23 animals (30%) compares well with that obtained following implantation of pellets composed of 20% XAE and 80% cholesterol (33%). No bladder carcinomas were observed in the animals in Groups 2-4.

Only XAE could be detected in the aqueous, buffered solution before injection. XAE was the only new compound that could be detected in the urine of mice given injections of XAE, compared with the urine of mice receiving s.c. injections of the aqueous buffer. The identity of the spot seen following paper chromatographic separation of the urine sample, after XAE injection, was identical in R$_p$ to an authentic sample of XAE (29). Following elution from the paper this spot demonstrated ultraviolet absorption characteristics indistinguishable from a spot of known XAE (13, 29).

**QUANTITATIVE ESTIMATION OF CERTAIN TRYPTOPHAN METABOLITES IN THE URINE OF MICE.** The levels of excretion of certain
George T. Bryan and Peter D. Springberg

TABLE 2
EXCRETION OF TRYPTOPHAN METABOLITES IN MOUSE URINE
BEFORE AND AFTER I.P. ADMINISTRATION OF 25 MG OF
L-TRYPTOPHAN

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>AMOUNT EXCRETED (µmoles/mouse/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before ± S.D.</td>
</tr>
<tr>
<td>Indoxyl sulfate</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>Anthranilic acid glucononide</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>α-Aminoisobutyric acid</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Acetylkynurenine</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>L-Kynurenine</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>3-Hydroxy-L-kynurenine</td>
<td>0.09 ± 0.05</td>
</tr>
</tbody>
</table>

A specific role for the vehicle in the genesis of these experimental bladder carcinomas is suggested by the data obtained following the exposure of the bladder to XAE under differing conditions. The implantation of XAE suspended in cholesterol into the mouse bladder (16) or the s.c. injection of XAE into mice whose bladders contained pellets of cholesterol was followed by the appearance of a significantly greater incidence of bladder carcinomas than occurred following the implantation of pellets of pure cholesterol (16). However, when pellets of pure XAE, which disintegrated rapidly, were placed into the bladders or when XAE was injected s.c. without the presence of cholesterol pellets in the bladders, a very low incidence of bladder carcinomas was observed. The incidence of carcinomas obtained with the test chemical and the vehicle was significantly greater than that obtained by either the test chemical or the vehicle alone, suggesting that some process other than a mere additive effect of the combination was taking place. It seems reasonable to suggest that the test chemical, eluted rapidly from the vehicle (13), acts as the initiating agent, whereas the vehicle, continuing intact in the bladder for a prolonged period of time, perhaps as a mechanical irritant, participates as the promoting agent, as defined by Friedewald and Rous (22). The promoting action of the vehicle in the process of the induction of bladder carcinomas with this technic may have important implications in the study of the causation of human or bovine (14) bladder cancer.

It is of interest to compare the incidence of carcinomas obtained with MC and XAE. Pellets of MC alone, without the addition of a vehicle, remained intact in the bladder for the full duration of the experiment and resulted in an incidence of 52% bladder carcinomas. This incidence is comparable to that observed by Bonser et al. (3) following the implantation of MC suspended in paraffin into mouse bladders. The incidence of bladder carcinomas observed with XAE and cholesterol placed in the bladder, 33% (16), or with XAE injected s.c. into mice whose bladders contained a cholesterol pellet, 30%, was not significantly different from that obtained with pellets of MC alone (P = 0.15).

Several normally occurring urinary metabolites of the essential amino acid tryptophan—3-hydroxy-L-kynurenine, 3-hydroxyanthranilic acid, xanthurenic acid, 8-hydroxyxquinolinic acid, and XAE—were found to induce a significantly greater incidence of bladder carcinomas in mice than did the cholesterol vehicle alone (16). It was determined that mice normally excrete in the urine small quantities of 1 of these compounds, 3-hydroxy-L-kynurenine, as well as several other metabolites of tryptophan. It was observed (17) that the mouse bladder permits the passage of substantial quantities of at least 2 of these metabolites, 3-hydroxy-L-kynurenine and 3-hydroxyanthranilic acid, when they are placed in the bladder suspended in a cholesterol pellet or when they are present in an aqueous solution. Thus the quantities of these metabolites that are present in the voided urine probably represent smaller quantities than the bladder mucosa is normally exposed to. It is possible that the continued urinary excretion of one or more of the tryptophan metabolites, combined with the protracted presence of any one of the variety of media that have been tested in pellet form in the bladder, may result in the development of a low-background incidence of bladder carcinomas. It would therefore seem probable that any media that might be tested for bladder carcinogenicity would be associated with a low incidence of bladder carcinomas.

**Acknowledgments**

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