The Importance of Synergy between Weak Carcinogens in the Induction of Bladder Cancer in Experimental Animals and Humans

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Summary

It is now well established that the interaction of multiple environmental factors may increase the incidence of some human cancers more than exposure to a single carcinogen. With an in vivo experimental rat model, we have demonstrated a synergistic effect in bladder carcinogenesis between a subcarcinogenic dose of the strong bladder carcinogen, N-methyl-N-nitrosourea, and saccharin- or cyclamate-containing diets. If these artificial sweeteners are capable of interacting with other environmental bladder carcinogens, their potential for increasing the incidence of human bladder cancer is greater than many more potent chemical carcinogens, because of their wide distribution as food additives to all sections of the population. Retrospective epidemiology shows no evidence of such risk from saccharin at current levels of consumption. No comparable studies are available for cyclamate, which was consumed in greater quantities but for relatively few years. It is emphasized that it is possible for interaction between multiple factors to contribute to the incidence of human bladder cancer as it does in other human organs and in other animal species.

Introduction

Carcinogenesis is a multistep process, involving, at the very least, initiation, promotion, and propagation of tumor growth (4, 5, 11, 19, 20). In the human situation, induction of bladder cancer may occur following exposure to large or repeated doses of a single potent carcinogen, such as 2-naphthylamine (12). It is probable, however, that far more tumors are produced by the sequential exposure either to low doses of 2 or more weak environmental carcinogens, or to both weak carcinogens and promoting factors, which may or may not be weak carcinogens in their own right. In humans, for example, induction of carcinoma of the lung or of the large intestine requires some 4 or 5 separate mutational events for the production of an actively growing tumor (11, 38). In the induction of lung tumors, asbestos can act as a cocarcinogen. The relative risk ratio of asbestos workers who smoke compared with that of nonsmokers, or of heavy smokers in other occupations (26, 41), shows that asbestos fibers in the lung can promote tumor growth from dormant cells in which neoplastic change has been initiated by the carcinogens in tobacco smoke. There is evidence from animal models that bladder cancer also can be induced either by multiple doses of strong carcinogens or by a subcarcinogenic dose of a known carcinogen followed by repeated doses of other weak carcinogens or cocarcinogenic stimuli.

In this report, we present evidence from one such animal model, in which a subcarcinogenic dose of the nitrosamide MNU was used to initiate neoplastic change in the urothelium, and either of the artificial sweeteners, sodium saccharin or sodium cyclamate, was used to promote tumor growth. For comparison, the tumor incidence produced by the strong human bladder carcinogen, 2-naphthylamine, is considered. The results are discussed in the context of work from other laboratories that also demonstrates that these compounds have a cocarcinogenic potential in animal models. The possible significance for humans of such synergy is considered.

Materials and Methods

Specific pathogen-free Wistar rats, 6 to 8 weeks old at the start of the experiment, were used throughout and were divided into 4 main experimental lots.

Lot A: Untreated Controls. Fifty-five male and 50 female rats were maintained on standard 41B pelleted diet for the duration of the experiment, and all surviving animals were killed at 2 years.

Lot B: Sweetener-fed Animals. Two groups were given sodium saccharin (Fisons Ltd., Loughborough, England) p.o. The 1st group, of 75 male and 50 female rats, was maintained on standard 41B diet and received the saccharin in drinking water so that the intake averaged 2 g saccharin per kg body weight per day. The 2nd group, of 75 males and 75 females, was fed pellets of 41B diet into which the saccharin had been incorporated by E. Dixon & Co., Ware, England. Their saccharin intake averaged 4 g per kg body weight per day.

Two further groups of 95 and 150 rats, containing approximately equal numbers of male and female rats for each group, were fed pellets of 41B diet into which sodium cycla-
R. M. Hicks and J. Chowaniec

Table 1
Bladder tumor incidence in rats fed artificial sweeteners, with and without pretreatment with MNU, and tumor incidence after 2-naphthylamine treatment

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Sweetener (dose level in g/kg body wt/day)</th>
<th>No. of animals at start of experiment</th>
<th>No. of bladders at histology</th>
<th>No. of bladder tumors</th>
<th>Tumor incidence (%)</th>
<th>Time 1st tumor was observed (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control untreated males and females</td>
<td></td>
<td>105</td>
<td>98</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>B. Sweetener-fed males and females</td>
<td>Saccharin</td>
<td>2.0</td>
<td>125</td>
<td>115</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cyclamate</td>
<td>1.0</td>
<td>95</td>
<td>84</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>150</td>
<td>144</td>
<td>2</td>
<td>&lt;2% 87</td>
<td></td>
</tr>
<tr>
<td>C. 1.5 mg MNU-treated females only</td>
<td>Normal diet</td>
<td></td>
<td>150</td>
<td>124</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saccharin-fed</td>
<td>2.0</td>
<td>50</td>
<td>49</td>
<td>23</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>50</td>
<td>47</td>
<td>27</td>
<td>57</td>
<td>50% 8</td>
</tr>
<tr>
<td></td>
<td>Cyclamate-fed</td>
<td>1.0</td>
<td>30</td>
<td>24</td>
<td>14</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>50</td>
<td>45</td>
<td>20</td>
<td>44</td>
<td>50% 8</td>
</tr>
<tr>
<td>D. 2-Naphthylamine-treated females only (300 mg/kg body wt/wk)</td>
<td></td>
<td>25</td>
<td>17</td>
<td>5</td>
<td>29</td>
<td>57</td>
</tr>
</tbody>
</table>

(3 still alive)
determine whether saccharin and cyclamate are, or are not, weak carcinogens is another matter. It can be calculated that, to detect an increased tumor incidence of 1% in a statistically significant manner in animals in which there is no spontaneous tumor incidence, a minimum group size of 455 animals is required; with a group size of 100, it is not possible to detect with certainty less than a 4.5% tumor incidence (32). Thus the tumor incidence recorded in Group B in Table 1 is not statistically significant, even though no tumors were found in Control Group A. Indeed, no spontaneous bladder tumors have been found in our Wistar rat colony over the past 12 years, although 3 have occurred in 2 years in BD rats.

Although many of the tumor-bearing bladders had areas of mineralization within or below the urothelium or contained free-lying calculi, and although calculi are known to have some cocarcinogenic effects in the rat bladder (13), calculi could not entirely account for the powerful promoting action of saccharin- or cyclamate-containing diets on the MNU-treated bladders. This is discussed in more detail elsewhere (22, 25).

Discussion

Previous studies showed that 4 intravesicular doses of MNU produced tumors in all rats treated (23). It is thus a powerful bladder carcinogen when applied directly to the urothelium. In these experiments, MNU was used to simulate environmental exposure to a low dose of a potent bladder carcinogen. As well as MNU, 4 other N-nitroso compounds, the nitrosamines dibutylnitrosamine, butyl-4-hydroxybutylnitrosamine, nitrosomethylhydroxycyclamine, and ethyl-4-hydroxybutylnitrosamine, have been identified as bladder carcinogens in animals (14, 15, 27, 30). As yet, no nitrosamine has been shown to be carcinogenic for humans, although, as a class, they are widely regarded as a human carcinogenic hazard (31). Small amounts of nitrosamines can undoubtedly be produced in human urine during transient bouts of bacterial cystitis (6, 25a) and the use of MNU in these synergy experiments is thus entirely relevant. A single, initiating dose of 1.5 mg MNU, although not carcinogenic in the 2-year duration of the present experiment, was promoted to tumor formation in about 50% of the animals by maintenance on a saccharin- or cyclamate-containing diet.

Work from other laboratories suggests that these sweeteners may be weak tumor initiators. Thus Salaman and Roe (40) used saccharin as the initiator and croton oil as the promoter in mouse skin painting tests, and found the tumor incidence rose from 19% (7 of 36) with croton oil alone, to 70% (14 of 20) when saccharin was used to initiate carcinogenesis. In pellet implantation tests in the mouse bladder, Allen et al. (1) found that inclusion of saccharin in a cholesterol pellet increased the tumor incidence to 30% (4 of 13) from 4% (1 of 24) with the pellet alone. These experiments were repeated by Bryan et al. (7, 8) with both saccharin and cyclamate, and they reported a tumor incidence of 49% (64 of 130) with saccharin and 70% (75 of 107) with cyclamate, compared with 12% (13 of 103) with cholesterol pellets alone.

These studies suggest that saccharin and cyclamate are weak initiators, while our investigations show them to be powerful promoters of tumor growth. Such compounds might be expected to perform as weak solitary carcinogens, but, as already indicated, very large numbers of animals would be required to prove this unequivocally. Two other groups of investigators who have tested these compounds as solitary carcinogens also found tumors in cyclamate-fed rats, but the numbers used per group were insufficient for the results to be regarded as statistically significant for any 1 experiment (Table 2). It is notable, however, that all tumors were found in cyclamate-fed rats and not in the control animals. The evidence of the carcinogenic potential of saccharin is equivocal. There is no evidence that saccharin increases the bladder tumor incidence in rats at dose levels up to 2.5 g per kg body weight per day (29, 34, 42), but at dose levels in excess of 2.5 g per kg body weight per day (about 5% of the diet), some tumors are found in the rat bladder (Table 3).

Further evidence that these compounds may be very weak solitary carcinogens, capable both of initiating and of promoting tumor growth, is suggested by the work of Oser et al. (37) in which a 10:1 cyclamate:saccharin mixture was fed to rats (Table 4). At the highest dose rate of the mixture of 2.5 g per kg body weight per day, a 17% (12 of 70) bladder cancer incidence was recorded and papillary hyperplasia or other proliferative changes in the urothelium were found in an additional 26% (18 of 70) of the sweetener-fed animals.

Whether or not a compound that has been shown to be weakly carcinogenic or cocarcinogenic in an animal species when used at high dose levels is a potential hazard for humans depends on many factors. Among these are the size of the human population exposed, the dose level involved, and the timing and duration of that exposure. Relatively short periods of exposure to high doses of a strong carcinogen such as 2-naphthylamine can be highly effective in causing bladder cancer in humans. The manufacture of this

### Table 2

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Reference</th>
<th>Cyclamate content of diet (%)</th>
<th>Tumor incidence (no. of rats with bladder tumors/no. treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schmähl</td>
<td>42</td>
<td>2, 5</td>
<td>0/57</td>
</tr>
<tr>
<td>Hicks and Chowaniec</td>
<td>This study</td>
<td>2, 4</td>
<td>0/96</td>
</tr>
<tr>
<td>Friedman et al.</td>
<td>21</td>
<td>0.4, 2, and 10</td>
<td>0/19</td>
</tr>
</tbody>
</table>

Plus 5 papillomas
Table 3

Bladder tumor incidence in rats fed sodium saccharin

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Tumor incidence (no. of rats with bladder tumors/no. treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (g/kg body wt/day)</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>Low-dose studies (below 2.5 g/kg body wt/day)</td>
<td></td>
</tr>
<tr>
<td>Munro et al.</td>
<td>34</td>
</tr>
<tr>
<td>Hicks and Chowaniec</td>
<td>This study</td>
</tr>
<tr>
<td>Lessel</td>
<td>29</td>
</tr>
<tr>
<td>Schmäßl</td>
<td>42</td>
</tr>
<tr>
<td>High-dose studies (above 2.5 g/kg body wt/day)</td>
<td></td>
</tr>
<tr>
<td>Hicks and Chowaniec</td>
<td>This study</td>
</tr>
<tr>
<td>Lessel</td>
<td>29</td>
</tr>
<tr>
<td>Nees et al.</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 4

Carcinogenic effect in rats of a 10:1 cyclamate:saccharin mixture

This table is a summary of information in tables and “Results” section of Ref. 37.

<table>
<thead>
<tr>
<th>Dose (g/kg body wt/day)</th>
<th>Incidence of bladder changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carcinoma</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.12</td>
</tr>
<tr>
<td>2.5</td>
<td>12 of 70 (17)*</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentages.

compound largely ceased in the United Kingdom by 1957, but cases of 2-naphthylamine-induced bladder cancer are still being diagnosed today. The number of people at risk, however, is relatively small in terms of percentage of the population, because exposure was almost entirely limited to industrial use of this chemical and the products into which it was incorporated.

If, on the other hand, a weak carcinogen were to be used as a food additive, it could reach a wide cross-section of the population and all age groups. Table 5 shows some of the products into which cyclamate was incorporated before it was withdrawn from the "generally regarded as safe" list in the United States in 1969. It would appear that cyclamate must have been consumed unwittingly by almost the entire population (not only those on a low-calorie diet for obesity or other health problems). The same is probably still true for saccharin. If artificial sweeteners were shown to be weakly carcinogenic for humans, the potential hazard as judged by size of population exposed and by likely duration of exposure is thus high. However, the dose levels of sweeteners consumed by humans are a small fraction of those used in the animal experiments reported here. Saccharin consumption in the United Kingdom has been estimated by Armstrong and Doll (2, 3) and is quoted for the United States by Bungard (10) and Kramers (28). Estimates of cyclamate consumption are somewhat variable, but it is 10 times less effective as a sweetener than saccharin, and most estimates, whether made from tonnage manufactured or individual consumption figures, indicate that approximately 10 times more cyclamate than saccharin was consumed in the 7 years between 1962 and 1969 when it was in general use (17, 18). In the animal experiments reported here, dose levels of saccharin per kg body weight used were up to 250 to 500 times greater than the maximum daily human consumption, but dose levels of cyclamate were only 25 to 50 times greater than the probable maximum human consumption. This may be regarded as indicating the current saccharin consumption to be at a "safe" level for humans, although this is not an easy calculation to make (9, 32). However, retrospective epidemiological studies in the United Kingdom over the 37 years in which saccharin has been used in quantity do not show any positive correlation between the rise in bladder cancer incidence and the rise in saccharin consumption, either in the general population or in diabetics (2, 3). It was concluded that the "data cannot exclude the possibility of a weak carcinogenic effect of saccharin on the human bladder. . . . So far as they go, the data are reassuring and encourage the belief that saccharin is not carcinogenic to man in the amount commonly used by diabetics" (3). Whether or not a 30- to 50-fold difference between the minimum effective dose level in animals and the consumption of cyclamate by humans before 1969 did or did not represent a "safe" level is unlikely ever to be known. As was pointed out by the Temporary Committee for the review of data on the carcinogenicity of cyclamate, "cyclamate has been available for consumption and the exposed cohorts have been observed for too few years to expect to see a measurable impact on the risk of human bladder cancer" (35). In this discussion, we have used cyclamate and saccharin, in contrast to the strong bladder carcinogen 2-naphthylamine, to illustrate a few of the problems experienced in testing and evaluating the potential risk of exposing the general population over a long period of time to low doses of a putative weak carcinogen. In particular, we wish to emphasise that interaction between weak carcinogens or promoting agents and other identified or unidentified environmental bladder carcinogens may play an important role in the induction of human bladder cancer. The action of asbestos in promoting lung cancer in smokers has already
been cited as one example of synergy in the human situation, and among others are the combined action of alcohol and smoking for cancer of the mouth and pharynx (39) and of radiation and smoking for lung cancer (43). Evidence presented here shows synergy of action between a cyclamate- or saccharin-containing diet and subcarcinogenic doses of the N-nitrosamide, MNU, for bladder carcinogenesis in rats. Whether or not artificial sweeteners have a comparable synergistic action in humans with known bladder carcinogens, or with putative environmental carcinogens such as nitrosamines, is not yet established, but, because of the relative levels of consumption, judging from animal experiments, any risk must be greater with cyclamate than with saccharin.

The purpose of this report is to consider synergy and the possible effects of weak environmental bladder carcinogens in humans, not to evaluate the risk:benefit ratio of the possible reintroduction of cyclamate onto the market. Accordingly, no account has been taken here of the possible value of using cyclamates as well as saccharin for the management of patients with diabetes and for the control of obesity and associated cardiovascular problems. The wider implications of restricting the use of artificial sweeteners have been considered by several independent groups (16-18), as well as by the manufacturing industries concerned.

References

Fig. 1. Part of the bladder from a male Wistar rat fed 4 g saccharin per kg body weight per day. A papillary plus infiltrating transitional cell tumor is shown, in which proliferation of epithelial cells into the supporting stromal cores gives a nodular appearance. There is early invasion into the submucosa of the bladder wall. H & E, × 69.

Fig. 2. Part of the bladder from a female Wistar rat after 1 year of treatment with 2-naphthylamine. In this animal, there were multiple papillary transitional cell tumors, with some areas of squamous metaplasia on the urinary surface of the urothelium. The invasive downgrowths had a predominantly adenomatous growth pattern with some areas of mucous metaplasia. H & E, × 92.
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