Promotion of Liver Cancer Development by Brief Exposure to Dietary 2-Acetylaminofluorene plus Partial Hepatectomy or Carbon Tetrachloride

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ABSTRACT

Adult male Fischer rats were exposed to a necrogenic dose (200 mg/kg) of diethylnitrosamine or to nonnecrogenic doses of N-methyl-N-nitrosourea, 1,2-dimethylhydrazine, or benzo(a)pyrene following partial hepatectomy or sham hepatectomy. This treatment by itself led to no hepatocellular carcinomas by 8 to 18 months, except in animals given N-methyl-N-nitrosourea, which showed a 30% incidence by 12 months. With each treatment regimen, exposure to dietary 2-acetylaminofluorene for 2 weeks coupled with partial hepatectomy or the administration of a necrogenic dose of CCl₄, was associated with an incidence of 68 to 94% of cancer at 8, 12, or 18 months, depending upon the initiating carcinogen used. Appropriate controls showed either no hepatocellular carcinoma or a much lower incidence. It is concluded that the 2-week exposure to dietary 2-acetylaminofluorene plus partial hepatectomy or the administration of CCl₄ has a strong promoting effect on liver carcinogenesis with four different chemical carcinogens.

INTRODUCTION

It has been established that most chemical carcinogens have at least 2 effects during the multistep process of cancer development in several systems, an initiating effect and a subsequent promoting effect. These 2 phenomena are discrete and separable and can be induced by different agents or manipulations. Since the important discovery of agents, such as croton oil, that do not usually initiate but are very effective after treatment in several systems, an initiating effect and a subsequent promoting effect, it seemed appropriate to inhibit inhibitory actions of carcinogens on cell proliferation, are induced by a single exposure to an initiating dose of one of several carcinogens (16–18). Four chemicals were selected as initiating carcinogens. DENA,7 MNU, and 1,2-DMH were selected for long-term follow-up because of their use in a variety of other studies on initiation and on the biology and biochemistry of nodules. Also, since MNU, 1,2-DMH, and B(a)P are not normally carcinogenic for liver and induce nodules or cancer under special circumstances (5, 18, 20), it seemed appropriate to include them in this study. The resistant hepatocytes are selectively stimulated to develop into nodules by a 2-week exposure to dietary 2-AAF plus a stimulus for cell proliferation such as PH or a necrogenic dose of CCl₄.

With this model, many animals have been followed for over a year, and it has become evident that the brief exposure to the selection procedure, dietary 2-AAF plus PH or CCl₄, is associated with the ultimate development of a high incidence of liver cancer. This promoting effect after initiation with several different initiating carcinogens is the subject of this paper.

MATERIALS AND METHODS

Animals and Materials. Adult male Fischer 344 rats weighing 150 to 200 g (Canadian Breeding Farms and Laboratories, St. Constante, Quebec, Canada, and Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used. The animals were maintained on a high-protein (24%) basal diet (Bio-Serv, Inc., Frenchtown, N. J.) or basal diet containing 0.02% 2-AAF (17). The animals were housed 2 per stainless steel wire-bottomed cage with food and water available ad libitum. The environmental temperature was kept constant at 24°C with an alternating 12-hr light-dark cycle maintained at the Division of Laboratory Animal Sciences, University of Toronto.

MNU, obtained from K and K Fine Chemicals, ICN Pharmaceuticals, Inc., Cleveland, Ohio, was recrystallized from ethyl acetate and hexane before use. DENA was obtained from Eastman Kodak Co., Rochester, N. Y.; CCl₄ was obtained from Fisher Scientific Company, Fairlawn, N. J.; B(a)P was obtained from Sigma Chemical Co., St. Louis, Mo.; and 1,2-DMH was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Experimental Regimens. In the experiments with DENA as the postinitiation steps in cancer development in experimental animals, it would appear that the promoting actions of carcinogens might be an interesting and important topic for study.

A model for liver carcinogenesis, the "resistant hepatocyte model," is being developed in our laboratory for the detailed sequential biological and biochemical analysis of cancer development (9–11). In this model, altered hepatocytes, resistant to inhibitory actions of carcinogens on cell proliferation, are induced by a single exposure to an initiating dose of one of several carcinogens (16–18). Four chemicals were selected as initiating carcinogens. DENA,7 MNU, and 1,2-DMH were selected for long-term follow-up because of their use in a variety of other studies on initiation and on the biology and biochemistry of nodules. Also, since MNU, 1,2-DMH, and B(a)P are not normally carcinogenic for liver and induce nodules or cancer under special circumstances (5, 18, 20), it seemed appropriate to include them in this study. The resistant hepatocytes are selectively stimulated to develop into nodules by a 2-week exposure to dietary 2-AAF plus a stimulus for cell proliferation such as PH or a necrogenic dose of CCl₄.

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After a 2-week recovery period from the necrosis, the animals were placed on the 0.02% 2-AAF diet for 2 weeks (Weeks 3 and 4). At the end of Week 3, 67% PH or SH was performed. The animals were returned to the basal diet at the end of Week 4 and were maintained on this diet until the end of the experiment. Most of the animals were sacrificed at 8 months (Table 1, Experiments 1 and 2), while one group was kept until 10 months.

In the experiments with the other initiating carcinogens, MNU, B(a)P, and 1,2-DMH, which are not necrogenic for the liver in our studies in the doses used, 67% PH or SH was performed prior to the administration of the carcinogen to allow initiation (2-4, 18). MNU, 40 mg/kg body weight i.p. as a 5-mg/ml solution in 10 mM sodium citrate buffer, pH 6.0, was administered at 4 or 18 hr post PH; B(a)P, 200 mg/kg body weight i.g. as a 100-mg/ml solution in corn oil was administered at 12 hr post PH; and 1,2-DMH, 100 mg/kg body weight i.p. as a 100 mg/ml solution in 0.9% NaCl solution was administered at 12 hr post PH. After a 2-week recovery period, the animals were placed on the 0.02% 2-AAF diet for 2 weeks (Weeks 3 and 4). At the end of Week 3, the animals were given CCI4, 2 ml/kg body weight, i.g. as a 1:1 dilution in corn oil. At the completion of the selection at the end of Week 4, the animals were placed on a basal diet and maintained on this diet for 12 or 18 months, depending upon the carcinogen. The animals were observed carefully and weighed weekly. When 2 or more animals showed a loss of weight of 10 g or more per week, the animals in the entire experiment with a particular carcinogen including the controls were sacrificed.

The livers were removed and weighed, and representative sections were taken from each lobe for fixation in 10% buffered formalin. A complete postmortem examination of the animal including external examination and inspection of all the viscera was performed. In addition to the sections of every suspicious gross lesion, thin pieces of lung including any suspicious lesion were fixed. The tissue was processed for routine histological examination using hematoxylin and eosin stains. The diagnostic criteria for hepatocellular carcinoma and hyperplastic nodule were described previously (7, 8, 17).

RESULTS

The treatment with DENA, MNU, B(a)P, or 1,2-DMH was associated with virtually no mortality. The effects of exposure to dietary 2-AAF plus PH were variable and unpredictable. In some experiments, virtually all animals sustained this well, and no more than 10% mortality was observed. In other experiments, the mortality within 2 weeks after termination of the dietary 2-AAF was as high as 50%. The basis for this variability is not understood. The animals treated with dietary 2-AAF plus CCI4 fared much better. This corresponds to our general experience that very few animals die within a few weeks after this selection procedure.

Of the animals alive by 2 or 3 weeks after discontinuation of the dietary 2-AAF, almost every one remained healthy looking for many months, and the mortality was less than 10%. Only between 7 and 8 months with DENA, 10 to 12 months with MNU, and 17 to 18 months with B(a)P and 1,2-DMH did the animals begin to lose weight and appear sickly. All animals with any particular carcinogen and in any particular experiments were sacrificed at the same time.

As seen in Table 1, 68 to 77% of the surviving animals given DENA and selected by 2-AAF plus PH had hepatocellular carcinomas at 8 or 10 months. This is in contrast to the absence of liver cancer in the animals given the same dose of DENA but not selected with 2-AAF plus PH or given 0.9% NaCl solution in place of DENA and selected. When PH was omitted from the routine selection procedure, 45% of the animals showed liver cancer. It should be noted that the animals in this group retained all of the liver initially exposed to the DENA, while the animals in the 3 complete experimental groups had 67% of the DENA-exposed liver removed. If a correction is made in these results by subtracting from the total the positive animals in which cancer was found in the 2 lobes surgically removed in the other experimental groups, the median and left lateral lobes, the cancer incidence falls to 18% (2 of 11). Unlike the animals in the 3 experimental groups, no metastatic liver carcinoma was seen in the animals selected with only dietary 2-AAF. The overall survival rates of animals receiving the complete experimental regimen were 60 to 70%. In those selected by 2-AAF plus SH, 90% survived at least for 8 months.

Many of the animals initiated with DENA had hyperplastic nodules regardless of their subsequent treatment (Table 1). As noted in Table 1, the nodules were fewer and smaller when treatment with dietary 2-AAF plus PH was omitted. Whether

<table>
<thead>
<tr>
<th>Initial treatment</th>
<th>Experiment no.</th>
<th>Selection procedure</th>
<th>Hyperplastic nodules*</th>
<th>8 mos.</th>
<th>10 mos.</th>
<th>Metastatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENA-200[^d]</td>
<td>1</td>
<td>2-AAF + PH</td>
<td>22/22 (100)[^d]</td>
<td>15/22 (68)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2-AAF + PH</td>
<td>14/14 (100)[^c]</td>
<td>10/14 (71)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2-AAF + PH</td>
<td>8/8 (100)[^f]</td>
<td>6/8 (75)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DENA-200</td>
<td>1</td>
<td>2-AAF + SH</td>
<td>11/11 (100)[^g]</td>
<td>5/11 (45)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DENA-200</td>
<td>1</td>
<td>Basal diet + PH</td>
<td>16/17 (88)[^a]</td>
<td>0/17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Basal diet + PH</td>
<td>30/50 (60)[^b]</td>
<td>0/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Basal diet + PH</td>
<td>20/30 (67)[^e]</td>
<td>0/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9% NaCl solution</td>
<td>1</td>
<td>2-AAF + PH</td>
<td>0/10</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2-AAF + PH</td>
<td>0/20</td>
<td>0/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2-AAF + PH</td>
<td>0/30</td>
<td>0/30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[^a] Each visible nodule was examined histologically.
[^b] DENA-200, single dose of 200 mg/kg body weight.
[^c] Numbers in parentheses, percentage of incidences.
[^d] Each animal had between 5 and 15 nodules per liver, measuring from 2 to 8 mm in diameter.
such animals might have developed liver cancer if allowed to live out their normal life span is an interesting question but without an answer at this time.

As recorded in Table 2, the animals given PH plus B(a)P or 1,2-DMH showed a high incidence of hepatocellular carcinoma, including some with pulmonary metastases. None of the animals in any of the control groups for these 2 carcinogens had any evidence of liver cancer. Some animals in some of the control groups did contain hyperplastic nodules in their livers. However, invariably, these were far fewer in number than those present in the animals given B(a)P or 1,2-DMH plus the routine selection procedure.

With MNU, the animals receiving MNU at 18 hr after PH and selected showed a 71% incidence of liver cancer, while those given MNU at 4 hr or in which SH was substituted for PH had no liver cancer and very few hyperplastic nodules. An additional interesting finding was the presence of liver cancer in 3 of 10 animals given PH plus MNU at 18 hr but not selected. These animals also had a high incidence of hyperplastic nodules. It should be noted that many of the animals treated with MNU under all regimens had epidermoid carcinomas in the skin, various types of lymphomas, and a scattering of neoplasms of other organs. This was not seen in any other groups of animals given DENA, B(a)P, or 1,2-DMH with or without dietary 2-AAF. The animals receiving MNU at 18 hr after PH had a survival rate of about 40% regardless of the selection procedure, while those receiving MNU at 4 hr showed a 70% survival.

**DISCUSSION**

It is evident from the results of this study that a 2-week period of exposure to dietary 2-AAF plus PH or CCl₄ has a strong promoting effect on liver cancer development after a single dose of DENA, B(a)P, or 1,2-DMH. A similar effect is also evident with MNU as the initiating carcinogen, but in this group, a considerable number of animals (30%) developed liver cancer without any imposed selection pressure. It is noteworthy that MNU was the only initiating carcinogen associated with a spectrum of malignant neoplasms in several other sites in addition to the liver. It is worthy of emphasis that no animals subjected to only the brief selection pressure, the 2-week exposure to dietary 2-AAF plus PH or CCl₄, have developed hepatocellular carcinoma by 8, 10, 12, or 18 months. Also, this treatment without a previous initiating dose of DENA, B(a)P, 1,2-DMH, or MNU has resulted in very few if any early foci or nodules of altered hepatocytes (16–18). Thus, by criteria currently available, the dietary 2-AAF plus PH or CCl₄ does not appear to initiate liver carcinogenesis to a significant degree. However, due caution is still indicated before this conclusion can be accepted unequivocally. Experiments in which the order of administration of "initiating dose of carcinogen" and "selection procedures" is reversed might throw further light on this important aspect.

The observations with MNU and liver neoplasms without selection confirm in general those of Craddock and Frei (5) and of Kaufmann et al. (13), although the experimental design used in our study was somewhat different. The relationship between the time of administration of the MNU and the occurrence of liver cancer 1 year later emphasizes again the importance of an early round of cell proliferation in the development of cancer with carcinogens such as MNU (2–6, 10, 13) that do not induce liver cell necrosis and thus do not stimulate regenerative cell proliferation. It should be pointed out that Craddock and Frei (5) found so-called "adenomata" in their animals treated with MNU but apparently no hepatocellular carcinomata. It is impossible to distinguish adenomata from hyperplastic nodules by any criteria currently known (8).

The dietary 2-AAF for 2 weeks plus PH or CCl₄ is associated with the rapid and quite synchronous emergence of hyperplastic nodules that become grossly visible by 1 or 2 weeks following the imposition of the strong mitogenic stimulus (PH or CCl₄) (17). After the termination of this selection procedure, any oval cell or ductular proliferation disappears almost entirely, and the liver surrounding the nodules shows very few if any persistent obvious pathological changes (17). Of the nodules, the large majority undergo remodeling and blend imperceptibly with the surrounding liver (8, 17). A few persist, and "nodules within nodules" including an occasional cancer can

<table>
<thead>
<tr>
<th>Initial treatment</th>
<th>Selection procedure</th>
<th>Hyperplastic nodules</th>
<th>12 mos.</th>
<th>18 mos.</th>
<th>Pulmonary metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH + MNU (18 hr)</td>
<td>2-AAF + CCl₄</td>
<td>6/7 (86)</td>
<td>5/7 (71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + MNU (18 hr)</td>
<td>Basal diet + corn oil</td>
<td>8/10 (80)</td>
<td>3/10 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH + MNU (18 hr)</td>
<td>2-AAF + CCl₄</td>
<td>0/6</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + MNU (4 hr)</td>
<td>2-AAF + CCl₄</td>
<td>1/14 (7)</td>
<td>0/14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + B(a)P (12 hr)</td>
<td>2-AAF + CCl₄</td>
<td>16/17 (94)</td>
<td>14/17 (82)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH + B(a)P (12 hr)</td>
<td>2-AAF + CCl₄</td>
<td>1/6 (17)</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + B(a)P (12 hr)</td>
<td>Basal diet + CCl₄</td>
<td>1/8 (13)</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + 1,2-DMH (12 hr)</td>
<td>2-AAF + CCl₄</td>
<td>11/12 (92)</td>
<td>8/12 (67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH + 1,2-DMH (12 hr)</td>
<td>2-AAF + CCl₄</td>
<td>11/12 (92)</td>
<td>0/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + 1,2-DMH (12 hr)</td>
<td>Basal diet + CCl₄</td>
<td>4/8 (50)</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + corn oil</td>
<td>2-AAF + CCl₄</td>
<td>3/8 (38)</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH + 0.9% NaCl solution</td>
<td>2-AAF + CCl₄</td>
<td>0/8</td>
<td>0/8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Time after PH at which the agent was administered. MNU was administered at a dose of 40 mg/kg body weight;

B(a)P, at 200 mg/kg, and 1,2-DMH, at 100 mg/kg.

* Numbers in parentheses, percentage of incidences.
be seen inside such persistent lesions. Thus, the rapid expansion of carcinogen-induced altered cells is the obvious immediate consequence of the imposition of the selection pressure by the dietary 2-AAF plus PH or CCl₄. Therefore, one hypothesis that must be entertained is that this expansion per se may be sufficient to set in motion a sequence of events associated with the further evolution in cancer without any further need for experimental manipulations. Whether the dietary 2-AAF plus PH or CCl₄ induces additional, more subtle changes on the nodular population as it proliferates and/or on the surrounding liver remains to be explored by further appropriate experimentation.

There is an apparent similarity between the findings in the liver and in the skin during chemical carcinogenesis. Promoters or promoting environments for the skin induce focal proliferations, called papillomas, a few of which may "spontaneously" show a further cellular evolution to cancer (12, 14, 19). Whether this similarity extends to mechanisms is quite unknown and becomes an interesting challenge for further study.

REFERENCES

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