Inhibition of Spontaneous Hepatocarcinogenesis in C3H/HeN Mice by Transplanted Hepatocellular Carcinomas

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ABSTRACT

Cell suspensions of tumor fragments derived from spontaneous or chemically induced primary hepatocellular carcinomas obtained from inbred C3H/HeN mice were transplanted into young male mice of the same strain. Transplantable hepatocellular carcinomas were excised as soon as they were detected, and all recipient mice were killed at one year of age. In control C3H/HeN mice, the incidence of primary hepatocellular carcinomas was 41% (41 of 100). In mice in which there was no growth of transplantable carcinomas, whether originally given injections of tumor cell suspensions or fragments, the overall incidence of primary hepatocellular carcinomas was 49% (35 of 72) with one to six tumors per liver at time of sacrifice. Transplantable hepatocellular carcinomas were established only in mice that had received tumor fragments. In these mice, from which established transplantable hepatocellular carcinomas had been excised, the overall incidence of primary hepatocellular carcinomas was 12% (3 of 25) with one tumor being found in each of three livers. The time of appearance or excision of transplantable hepatocellular carcinoma did not affect this decrease in primary hepatocellular carcinoma incidence.

INTRODUCTION

Several reports have suggested that many tumors arising spontaneously in rodents lack antigenic determinants that would discriminate them from the tissues of the host; others have only weakly positive surface antigens (2, 3, 14). In contrast, the antigenicity or immunogenicity of chemically induced tumors in mice and rats is often substantial but varies with the carcinogen used and many other factors (4, 7, 15, 16). One of the more revealing tests of immunogenicity is that of the capacity of a tumor transplant to protect against subsequent challenge (3). The procedure is usually performed by prior immunization with killed or viable tumor tissue obtained from a culture line or continual transplantation in inbred animals. Subsequent challenge is performed with the same tumor lines after excision of the growing transplant.

Highly inbred lines of C3H mice have a genetic tendency toward high rates of spontaneous PHC formation (6, 9, 11, 19, 22). Using these genetically determined tumors, one could test the ability of any transplanted tumor tissue (17) to alter the course of the spontaneous tumorigenesis expected in the liver of the host mouse. Exposure of C3H and similar mice to chemical carcinogens can induce a population of PHC that differs from that of spontaneous origin (1, 8, 12, 13, 18, 21) and closely simulates the spectrum of tumors induced in other strains of mice (5) and rats (20). Thus, we also have the possibility of comparing the abilities of spontaneous and chemically induced tumor transplants to effect host tumorigenesis.

This report presents the results of a series of experiments aimed at testing these possibilities. The results suggest that both types of tumor transplants strongly inhibit the genesis of spontaneous PHC in the host.

MATERIALS AND METHODS

Male C3H/HeN mice (Charles River Farms, North Wilmington, Mass.) obtained at 5 weeks of age were used throughout these experiments. They were housed 6/cage under bonnets and fed a fully nutritious, semisynthetic diet (Diet 101; Bio-Serv, Inc., Frenchtown, N.J.). This diet was used to limit the possible influence of hormones, antioxidants, and other variable components of standard laboratory diets. Tests conducted for up to 18 months demonstrated that mice were free of common murine viruses.

The details of the carcinogen diets used to induce PHC have been presented elsewhere (5). In brief, 0.03% AAF and 50 ppm chloroform were pelleted in Diet 101 separately. These were administered from the age of 6 weeks to a maximum of 1 year.

As we have reported previously, an elevated serum AFP is a marker for the presence of either spontaneous or induced PHC in mouse livers (5, 6). More than 95% of livers with an aggregate diameter of grossly identifiable PHC of 0.5 cm or larger are associated with AFP elevations, while less than 2% of mice without tumors demonstrated elevations. Blood obtained monthly from each potential tumor donor was sent to Dr. S. Sell (Department of Pathology, University of California, San Diego, Calif.), who determined the AFP levels by radioimmunoassay. When the AFP level was 5 times that of controls, the mouse was killed to provide tumors early in their growth phase.

PHC for transplantation were measured and sampled for histological preparation. The method of transplantation has been described previously (5). In brief, the tumor was rapidly minced by scalpels under sterile, cool, 0.9% NaCl solution to fragments approximately 1 to 2 mm in diameter. Approximately 0.25 ml of fragments was injected into a sterile, prepared s.c. site on the mid-right flank. Six- to 7-week-old male C3H/HeN mice were used as recipients and were maintained under the same conditions as were described above. Injection at this site has proved to be almost as effective as an i.p. or i.m. injection and permits early detection and easy excision of THC. In a separate group of experiments, fragments of PHC were pressed through a stainless steel mesh, and the resultant cell suspension was collected by gentle centrifugation. Suspensions prepared from these loose pellets consisted of single cells and clumps of varying sizes. Approximately 0.25 ml of cell suspension was injected as described above.

Each mouse was palpated weekly to detect tumor growth. All recipient mice that did not demonstrate THC were sacrificed at 1 year of age. At that time, host liver and transplant sites were sampled for histological examination.

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2 The abbreviations used are: PHC, primary hepatocellular carcinoma(s); AAF, N-2-acetylaminofluorene; AFP, α-fetoprotein; THC, transplantable hepatocellular carcinoma(s); PHCs, primary hepatocellular carcinoma of the spontaneous type; PHCc, primary hepatocellular carcinoma morphologically similar to chemically induced tumors.

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When THC were definitively palpated, they were immediately excised under sterile conditions and prepared for histological review. At 1 year of age, each of these mice was killed, and the livers of all recipient mice were thoroughly examined; all grossly identifiable lesions were described, measured, and sectioned for histology. At least one additional section was obtained from each liver lobe. A PHC was defined as a grossly evident tumor (1 mm or greater) that histologically conformed to prior descriptions (8). Sections were also obtained from each pulmonary lobe, and all other organs were examined grossly for metastasis. In addition, sections were obtained from the site of THC excision to determine the presence of viable, but not visible, tumor. No viable tumor was detected. All histological sections were coded and were examined without knowledge of their origin.

RESULTS

Tumorigenesis of Untreated Mice. Many of the findings relating to detailed analysis of tumor type and other aspects are not relevant to this study and will be reported in detail elsewhere. As part of that larger study, more than 400 control mice were examined (4 groups of 100 mice) over a 3-year period. They demonstrated a remarkable consistency in the pattern of PHC development. Tumors were identifiable grossly from the 30th week of age and, with time, increased in incidence, number per mouse, and size. Approximately 10 to 15% of these mice had PHC by 36 to 40 weeks of age, and 34 to 44% had PHC at 1 year. The number of tumors per liver ranged from one to 5 at 1 year and measured from 1 to 20 mm in diameter. No deaths resulting from these tumors occurred in the first year of life.

An additional 100 control mice that were followed expressly for the currently reported experiments had an aggregate 41% incidence of PHC at 1 year of age (Table 1). All of the PHC in control mice were histologically typical of those described repeatedly for this strain.

Tumor Types. All of the PHC used for transplantation were 2 cm or less in diameter, with the majority being approximately 1 cm. The gross and histological appearances of spontaneous PHC in C3H/HeN mice have been described previously (8, 11, 13, 22). The majority of tumors in the livers of carcinogen-exposed mice could not be distinguished grossly or histologically from those in untreated mice, although a small proportion of tumors of this type demonstrated minor histological differences from the usual spontaneous PHC tumors. Therefore, whether arising in untreated or chemically treated mice, tumors with a morphology similar to that of spontaneous PHC were treated as a group of PHC of the spontaneous type and designated PHCs.

However, the gross appearance of the "chemically induced PHC" used for transplantation was markedly different from that of the spontaneous tumor types; these tumors are thus designated PHCc. The PHCc were nodular, the margins were scalloped and often invasive, and hemorrhagic and necrotic areas were evident (5). Although the histology of these tumors was like that of chemically induced PHC in other strains of mice or rats and different from spontaneous tumors (20), the difference had no apparent bearing on their effect. Further, PHC that arose in mice exposed to either AAF or chlordane demonstrated identical effects.

Injection of Tumor Cell Suspensions (Table 1). In an initial experiment, 21 mice were inoculated with cell suspensions from 14 PHC obtained from 10 mice. Approximately 80% of the cells of these suspensions were viable by the trypan blue exclusion test; however, no THC was detected in any of the recipients. Nine recipients of these suspensions demonstrated PHC at 1 year for an incidence of 43%.

Effects of THC (Table 1). The use of cell fragments prepared as rapidly as possible was relatively successful. Twenty-nine % of PHCc and 54% of PHCc gave rise to THC.

Although at sacrifice the incidence of PHC in livers of mice that had received transplants but did not demonstrate THC

### Table 1

<table>
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<tr>
<th>Injection of Tumor Cell Suspensions (Table 1)</th>
<th>Total no. of recipient mice (a)</th>
<th>No. of donor mice (b)</th>
<th>No. of donor PHC (c)</th>
<th>No. of recipients with PHC (d)</th>
<th>No. of PHC/recipient mouse (e)</th>
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<tr>
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(a) Received an injection of either 0.25 ml of tumor cell suspension or 0.25 ml of tumor fragments s.c. between 6 and 7 weeks of age.

(b) Those from which PHC used for transplantation were obtained. Multiple PHC were often obtained from a single liver.

(c) PHC in recipient mice were defined as tumor nodules that could be grossly identified, usually 1 mm in diameter or larger. Histological confirmation of the diagnosis of each lesion was obtained.

(d) Cell suspensions were prepared from spontaneous PHC obtained from untreated mice (PHCa).

(e) Mice that received cell suspensions or fragments at 6 to 7 weeks of age and were sacrificed at 1 year of age without demonstrable THC.

Numbers in parentheses, percentage.
varied from 49% in those that had received PHCs to 67% in those that had received PHCc, these differences were not statistically significant. Thus, in an aggregate of 51 mice in which no THC appeared (regardless of the type of PHC used for transplantation), 26 livers demonstrated PHC for an overall incidence of 51%. The modal number of PHC per liver was 3, and the total number of tumors was 70.

The effect of successful growth of THC on the genesis of PHC in the liver of the host was striking and consistent, regardless of the source of the PHC. Of 25 mice in which THC were identified and excised (regardless of the source or type of PHC), only 3 livers showed PHC for an overall incidence of 12%. As determined by Fisher’s exact test, this difference was highly significant ($p < 0.001$).

It can be assumed that the time of appearance of a given THC is based on a period of adaptation and subsequent growth rate. One finding of interest, therefore, was the lack of relationship between the time of excision of the THC and the eventual effect on tumorogenesis in the host. Thus, although some THC were detected and excised as early as 3 months after transplantation and others as late as 10 months, their inhibitory effect on the liver tumorogenesis of the host was the same.

**DISCUSSION**

The most striking finding of this study was the almost total suppression of spontaneous hepatocarcinogenesis in C3H/HeN mice by the prior growth of spontaneous or chemically induced THC. Neither large quantities of viable cell suspensions nor tumor fragments that failed to give rise to THC caused inhibition. This held true even in those instances in which a single PHC was the source of the fragments that gave rise to THC in one recipient and those that failed to do so in another. Thus, the successful growth of a THC seemed the major determinant in suppression of PHC development. Furthermore, it appeared that this effect was dependent only on the presence of viable THC for some period of time, regardless of growth rate. Livers of the majority of mice from which THC were excised as early as 3 to 5 months after transplantation and of those in which excision was performed at 8 months or later developed no PHCs. No viable tumor could be identified at the sites of excised THC nor at the sites of transplantation in mice without detectable THC.

At 6 months of age, between 8 and 15% of untreated mice normally demonstrate PHC, and at 10 months, this figure is approximately 20%. Thus, the effect of those THC excised before 6 months of age apparently persists for some time, and the presence of the THC cells that will become detectable at later than 6 months is equally effective.

Another finding of interest was the roughly equal effectiveness of spontaneous PHC and those of a type usually construed to be induced by chemical agents, although there were relatively few of the latter.

Several mechanisms might be responsible for the inhibition of hepatocarcinogenesis in the recipients that developed THC, including a nonspecific, generalized effect on the host or an immunological response.

The development of spontaneous PHC in C3H/HeN mice is susceptible to alterations in nutrition, general condition of the animals, and even conditions of maintenance, such as the number of mice per cage (10). However, a number of findings mitigate against inhibition having resulted from a systemic effect. At sacrifice, those mice in which THC had developed appeared to be in excellent health, demonstrated weight equal to comparable controls (including those with or without PHC), and were free of intercurrent infection or parasite infestation. Furthermore, the majority of THC were excised when they were only 1 cm in diameter, and histological examination revealed no significant necrosis or evidence of metastases.

Despite prior experimentation that suggested little or no immunogenicity of spontaneous rodent tumors and very poor immunogenicity of AAF-induced tumors (4), it remains possible that the presence of viable, growing (at different rates) THC fragments establishes an immunological recognition of normally weak antigenic components sufficient to suppress the PHC of the host. The presence of these transplanted cells and their growth taking place concurrently with the expected genesis of PHC in the host differ from the usual preimmunization protocols. Although little or no antigenicity has been demonstrated by available testing procedures (2), it is possible that sufficient antigenic determinants are present to act as an effector under these conditions. Furthermore, the unique susceptibility of the spontaneous PHC to suppression by altered environmental factors might make even a minimal immunological response inhibitory to growth.

However, in view of the lack of any tangible evidence for this process, it is reasonable to suggest yet another mechanism. Thus, it remains possible that a nonimmunological factor is elaborated by the THC, serving to suppress or delay the expression of PHC.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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