Immunogenicity of Chemically Induced Murine Colon Cancers

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ABSTRACT

The antigenicity and immunogenicity of three colorectal carcinomas induced in BALB/c mice by 1,2-dimethylhydrazine or N-methyl-N-nitrosourea were studied. All tumors were readily transplantable. Two of these tumors metastasized when transplanted reached sufficient size. All tumors were found to be immunogenic in the strain of origin, and all tumors were shown to contain unique tumor-specific transplantation antigens in cross-protection experiments. The use of these tumors as an animal model for studies of adjuvant immunotherapy and chemoimmunotherapy is suggested.

INTRODUCTION

Since the initial demonstration by Walpole and Williams (29), in 1958 that 2,3-dimethyl-4-aminobiphenyl induced colon carcinomas in rats, gastrointestinal tumors have been induced in diverse experimental animals by a variety of agents (30). Nonetheless, very few investigators have used experimental colon carcinomas as models for human disease (3, 8, 14, 15, 21). Most studies have dealt primarily with either the induction and growth of such tumors or their sensitivity to chemotherapeutic agents. Investigations of the immunological characteristics of rodent colon tumors have rarely been undertaken (7, 13, 21). In the present work, the immunogenicity and antigen specificity of 3 chemically induced murine colon adenocarcinomas were studied in vivo, primarily in order to determine their suitability as models for the immunotherapy and chemoimmunotherapy of colorectal cancer.

In 1975, Corbett et al. (4) reported the development of 3 transplantable colon adenocarcinomas in BALB/c mice following tumor induction with DMH or N-methyl-N-nitrosourea. These tumors (identified as CT 26, CT 36, and CT 51) were noted to be readily transplantable in syngeneic hosts, eventually killing the animals. No spontaneous regressions occurred. With 2 of the 3 tumors (CT 26 and CT 51), a high percentage of mice died of widespread metastases following excision of growing tumor transplants of suitable size. Although all 3 tumors are adenocarcinomas, they differ with respect to degrees of differentiation, growth rates, transplantation characteristics, and incidence of metastasis. CT 26 is the most undifferentiated and has the fastest growth rate and the highest metastatic potential. CT 36 is a well-differentiated adenocarcinoma with a relatively slow growth rate and rarely metastasizes. CT 51 has intermediate characteristics. The variation in these parameters is analogous to individual differences found in human colorectal cancer.

Moreover, Corbett et al. (3) have reported chemotherapeutic sensitivities of these BALB/c colon tumors which are quite similar to the drug sensitivities of human colorectal carcinomas. Those single agents found most effective were the nitrosoureas, including 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea and 5-FUra. The 2 drug combinations shown to be most active were 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea plus 5-FUra (3). These authors have also conducted studies of postoperative chemotherapy used as an adjuvant to surgical excision. The successful use of these colon tumors in adjuvant chemotherapy experiments suggested that these tumors would serve as useful models for studies of adjuvant immunotherapy and chemoimmunotherapy. However, despite the attention given to these tumors, no immunological characterization has hitherto been available.

Sjögren and Steele (21) have extensively studied the immunobiology of chemically induced colon carcinomas in the rat. Using in vitro lymphocyte-dependent cytotoxicity assays, these authors showed many immunological features of DMH-induced rat colon tumors which were similar to their human counterparts. Particularly, the demonstration of common cross-reacting antigens among all colon tumors tested seemed to indicate the presence of a tissue-type-specific antigen which elicited a cellular immune response (24). However, they did not study the immunogenicity of these tumors in vivo and, therefore, whether or not these common antigens functioned as rejection-type antigens, i.e., TSTA in vivo, is not known. Since, chemically induced tumors are generally considered to have unique TSTA (17) and common or shared TSTA are generally attributed to viral etiology (20), it seemed important to extend the studies of Steele and Sjögren on the immunology of chemically induced colon carcinomas of rodents to include evaluation of tumor rejection responses in vivo.

The following studies of the immunogenicity of the 3 chemically induced BALB/c colon carcinomas originally induced by Corbett et al. were undertaken to answer 2 questions. (a) Were these tumors immunogenic in their strain of origin (i.e., did they contain TSTA)? (b) Would these tumors express common or shared TSTA related, perhaps, to common tissue-type-specific antigens of the type detected in vitro by Steele and Sjögren?

MATERIALS AND METHODS

Mice BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and Simonsen Laborato-

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4 The abbreviations used are: DMH, 1,2-dimethylhydrazine; 5-FUra, 5-fluorouracil; TSTA, tumor-specific transplantation antigen; i.r., intrarectal.

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ries, Inc., Gilroy, Calif. All experiments utilized female mice 8 to 12 weeks old.

**Tumors.** Three colon carcinomas, chemically induced in female BALB/c mice, were obtained from the Southern Research Institute, Birmingham, Ala., through the courtesy of Dr. T. H. Corbett and were maintained by serial transplantation in their strain of origin. Colon tumor 26 (CT 26) is an anaplastic carcinoma, originally induced by the i.r. injection of N-methyl-N-nitrosourea. This tumor was used between the 66th and 78th transplant generations. Colon tumor 36 (CT 36) is a well-differentiated adenocarcinoma originally induced by s.c. injections of DMH and was studied in the 50th to 58th transplantation generations. Colon tumor 51 (CT 51), a moderately well-differentiated, mucinous adenocarcinoma, was induced by s.c. injections of DMH and utilized in transplant generations 56 to 67. Control tumor BP/B/5, a fibrosarcoma, was induced in our own laboratory, in a female BALB/c mouse, by the s.c. injection of 0.1 mg of 3,4-benzo(a)pyrene dissolved in 0.1 ml of trioctanoin. BP/B/5 was used between the 4th and 8th transplant generations.

**Preparation of Tumor CellSuspensions.** For routine tumor transplantation, cell suspensions were prepared from tumors by a modification of the Snell cytosieve technique (22). For challenge of immunized animals and determination of transplantation characteristics, single-cell suspensions were prepared by a modification of the method of Hammond et al. (9). Tumor tissue was trimmed of necrotic portions and finely minced using sterile technique. In a trypsinizing flask, 1 g of tissue was mixed with 10 ml of 0.25% pronase (Calbiochem, San Diego, Calif.) in Dulbecco’s balanced salt solution and 0.3 ml of 0.04% DNase (Worthington Biochemical Corporation, Freehold, N. J.). The mixture was agitated in a shaking water bath at 37°C for 10 min. Three sequential enzyme treatments of the same tissue were performed, each time decanting the cell suspension through sterile gauze into a glass beaker in an ice bath. The resultant pooled cell suspension was centrifuged at 250 × g for 10 min at 5°C. The cell pellet was washed twice in Hanks’ balanced salt solution, pH 7.0, and finally resuspended in 5 ml of Hanks’ balanced salt solution. Viable cells were quantitated in a hemocytometer by determining the number of cells which exclude 0.5% trypan blue. Approximately 90% of the cells in these tumor cell suspensions were viable by this criterion.

**Determination of Transplantation Characteristics.** In each tumor system, with the exception of CT 36, single-cell suspensions were prepared by the enzymatic digestion method described above. The cell concentration was adjusted to 10^6 viable cells/ml. Logarithmic dilutions were then made from 10^6 to 10^3 viable cells/ml, and 0.1 ml of each dilution was injected s.c. into the right hind limbs of groups of untreated mice. The incidence of tumor development and the latency period within each group were determined. These data were used to select a dose of tumor cells for subsequent use as challenge tumor cell inocula yielding an incidence of tumor takes of 80% to 90% in untreated animals. CT 36 was transplanted by trocar implantation of 2- to 3-mm tumor fragments s.c. in the flank, since both single-cell suspensions and cytosieve preparation grew very poorly.

**Determination of Metastatic Propensity.** CT 26 was prepared by a modification of the Snell cytosieve technique (22). Numbers of cells in excess of that predetermined to result in a 100% incidence of successful tumor transplantation were inoculated as 0.1-ml aliquots s.c. into the right hind limbs of 40 female BALB/c mice. The mice were divided into 2 groups of 20 mice. The tumor-bearing limbs of the first group were amputated when tumors reached a size of 600 to 800 mg, and the second group received amputations when tumors weighed between 800 and 1000 mg. Tumor weights were computed from the formula (3):

\[ \text{Tumor wt (mg)} = \frac{a \times b^2}{2} \]

where a is the greatest diameter of the tumor and b is the diameter at right angles to it. The metastatic propensity of CT 51 was studied in a similar manner. The range of tumor weights at amputation was 300 to 500 mg or 500 to 700 mg. Each group again included 20 animals. CT 36 was transplanted s.c. into the right flanks of 20 mice as 2- to 3-mm trocar fragments. Tumors were excised when they reached 1000 to 1200 mg. Animals dying within the first week and animals with local recurrences were eliminated from the study. Thereafter, animals were followed and survival was determined. All mice were autopsied to confirm the presence of metastases.

**Immunogenicity Studies.** The presence of TSTA was investigated after the manner of Prehn and Main (17). The immunizing tumor cell suspensions were prepared from CT 26 and CT 51 tumors by a modification of the Snell cytosieve technique (22) and injected s.c., as 0.1-ml aliquots, into the right hind limbs of 2 groups of untreated animals, distal to the popliteal space. The number of viable tumors cells in each instance exceeded that predetermined to result in a 100% incidence of progressive tumor growth. The tumor-bearing extremities were amputated under fentanyl droperidol (Innovar-vet) anesthesia. To prevent the development of metastases, limbs bearing CT 26 and CT 51 tumor transplants were amputated between the 7th and 10th day following tumor transplantation, at tumor weights not exceeding 500 mg. CT 36 was transplanted by trocar implantation of 2- to 3-mm tumor fragments s.c. into the flank, which were excised between 16 and 22 days at an average tumor weight of 800 mg. Seven days after tumor excision, each group of animals was divided into subgroups of 10 to 20 mice. For CT 26 and CT 51, mice in each subgroup were challenged with logarithmically increasing numbers of viable cells from the same transplant generation of the same tumor used for immunization. Cell doses began at that cell number for each particular tumor which had been predetermined to result in an 80 to 90% tumor incidence in untreated animals. Groups of untreated control mice were challenged in a similar fashion. In the case of mice immunized to CT 36, 3 fragment sizes (1 mm, 2 to 3 mm, and 4 to 5 mm) of CT 36 were implanted into the flanks of subgroups of immune animals 7 days after excision of established CT 36 tumor transplants. In this manner, both the presence and relative strength of the TSTA for each tumor could be established. In addition to cumulative tumor incidences, mean tumor appearance times were determined.

**Determination of Antigenic Specificity.** Three separate
experiments were performed. In all 3 experiments, female BALB/c mice were divided into 5 age-matched groups of 20 to 30 animals. One group was set aside for later use as a challenge control. Mice in each of the 4 remaining groups were inoculated with one of the 4 tumors. In each case, the number of viable cells transplanted significantly exceeded the dose required for uniform tumor transplantation. The time of inoculation for each tumor varied with its respective growth rate. This variation in the timing of transplantation allowed excision of all tumors, at approximately the same time, over a 48-h period. CT 36 was transplanted 20 days prior to excision, and tumors weighed less than 800 mg when excised. BP/B/5 was transplanted 9 days prior to limb amputation, and tumors weighed less than 1000 mg at the time of amputation. CT 26 and CT 51 were transplanted 7 days prior to amputation of tumor bearing limbs, and tumors weighed less than 500 mg at the time of amputation.

In the first experiment, a 2- to 3-mm piece of CT 36 was implanted by trocar s.c., into the left flank of each animal including untreated challenge controls. All mice were challenged 7 days after tumor excision.

In the second experiment, CT 26 was used to challenge groups of mice sensitized to each of the 4 tumors by the procedure described above. All mice were challenged 7 days after surgery. The groups consisted of 30 to 40 animals and included a challenge control. At the time of challenge, each group was subdivided, one half of the mice receiving 250 viable CT 26 cells per animal and the second half receiving 750 viable CT 26 cells. Animals were inoculated s.c. in the left limb.

In the third experiment, the procedure paralleled the second experiment, but CT 51 was substituted for CT 26. Seven days after tumor excision, mice in all subgroups were challenged with 10^3 or 10^4 viable CT 51 cells.

Beginning 10 days after tumor challenge, all animals were observed at 4-day intervals. Cumulative tumor incidences and mean tumor appearance times were recorded.

Analysis of Data. Cumulative tumor incidence was expressed as the percentage of animals developing tumors. Statistical significance was computed by the Fisher exact test for χ². The percentage of protection against tumor challenge was computed from the cumulative tumor incidence by the formula:

\[
\text{% protection = } \frac{\text{tumors in controls} - \text{tumors in immunized mice}}{\text{tumors in controls}} \times 100
\]

RESULTS

Transplantation Characteristics. With the exception of CT 36, all tumors were transplanted readily from single-cell suspensions. CT 36 required a 2- to 3-mm trocar implant to establish transplants. Relatively few CT 26 cells (10^3) resulted in tumor growth in 70 to 80% of animals. The cell number required for tumors to develop in approximately 90% of recipients was found to be 2.5 × 10^2 cells. A 4-fold increase in cell number was necessary to achieve a comparable cumulative tumor incidence with CT 51, where 10^5 cells was required to produce a tumor incidence of approximately 90%. The control tumor BP/B/5 became established after inoculation of 2.5 × 10^2 viable cells.

Metastatic Propensity. The right hind limbs of mice bearing CT 26 tumor transplants were amputated at 600 to 800 mg in the first group and at 800 to 1000 mg in the second group. In the first group, 8 of 16 animals (50%) developed metastases and died. In the second group, 13 of 18 mice (72%) developed metastases and died. All of these animals succumbed to pulmonary metastases. Of those animals with metastases, median survival was approximately 7 weeks. Quite similar survival curves were noted in 3 other experiments.

CT 51 was also studied at 2 sizes, the first and second groups undergoing amputation at tumor burdens of 300 to 500 mg and 500 to 700 mg, respectively. Eleven of 18 (61%) and 17 of 19 (89%) animals developed metastases. The rate of metastasis proved to be rather variable in subsequent experiments. The metastases occurred within the aortoiliac lymph nodes, although occasional mesenteric tumor nodules were found. These lesions tended not to obstruct vital structures, and animals frequently lived beyond 10 weeks.

CT 36 rarely metastasized following s.c. transplantation, and then only to regional nodes.

Immunogenicity Studies. The results of the immunogenicity studies are depicted in Table 1. All of the tumors were found to be immunogenic in their strain of origin. CT 26 showed moderate immunogenicity. With a challenge inoculum of viable cells, CT 26 had a protection value of 73% (p < 0.001). With challenge cell numbers of 10^3 or greater, no significant difference in cumulative tumor incidence was noted, although median tumor appearance time was delayed in immunized animals as compared to control animals. CT 36 was found to be highly immunogenic with an
overall tumor incidence of 10% in immunized animals ($p < 0.001$). CT 51 was found to be weakly immunogenic in the strain of origin. With a challenge inoculum of 10³ viable cells, the cumulative tumor incidence in immunized mice was 33% (5 of 15 animals) compared to a tumor incidence of 70% (12 or 17 animals) in controls ($p = 0.03$). In addition, a consistent delay in median tumor appearance time was noted. The control tumor BP/B/5 was also highly immunogenic, inducing significant transplantation resistance even at challenge inocula considerably higher than that required to produce a 100% incidence of tumor growth in untreated mice. With a challenge inoculum, a protection value of 93% was observed ($p < 0.001$).

**Antigenic Specificity.** In the first experiment, groups of immunized mice and an untreated group of mice were challenged with CT 36. The results of this experiment are shown in Table 2. The CT 36 implants grew at closely analogous rates in all groups except those mice immunized with CT 36. In this group, 95% of the animals failed to develop progressively growing tumors ($p < 0.0001$). The cumulative tumor incidence in the challenge control group was 79%. In the groups immunized to CT 26 and CT 51, cumulative tumor incidences of 77 and 74%, respectively, were observed. In addition, no cross-reactivity was suggested by analysis of latency periods or ultimate tumor weights in these 2 groups.

In the second experiment, groups of untreated and immunized mice were challenged with CT 26. These results are depicted in Table 3. Tumor incidences in untreated controls were 96% with $2.5 \times 10^5$ cells and 100% with $7.5 \times 10^5$ cells. In the groups immunized with CT 26, cumulative tumor incidence was 15% at the lower dose and 50% at the higher dose. These differences were statistically significant in both instances ($p < 0.001$ and $p = 0.0006$, respectively). No cross-protection between the colon tumors was demonstrated. Subgroups immunized with CT 36 showed cumulative tumor incidences of 89% ($2.5 \times 10^5$ cells) and 90% ($7.5 \times 10^5$ cells). The cumulative tumor incidence in subgroups immunized with CT 51 were 75% ($2.5 \times 10^6$ cells). None of these values were statistically different than tumor incidences observed in untreated control mice or in the colon tumors and then challenged with either 10³ or 10⁶ CT 51 cells 7 days following surgical excision of growing tumor transplants

Percentage protection values were calculated as described in "Materials and Methods."
although this remained statistically significant ($p = 0.02$). Groups immunized with CT 26 showed final tumor incidences of 71% at $10^3$ cells and 85% at $10^4$ cells. These values are not statistically different when compared to untreated control mice or to mice immunized with control tumor BP/B/5. However, the tumor latency period was prolonged in both CT 26 groups. Mice immunized with CT 36 had final tumor incidences of 92% at $10^3$ cells and 100% at $10^4$ cells. Cumulative tumor incidences in mice immunized with control tumor BP/B/5 did not vary significantly from untreated mice at either $10^3$ or $10^4$ cells.

**DISCUSSION**

CT 26 and CT 51 were found to be quite amenable to transplantation from single-cell suspensions. This feature is desirable in that scaled (decreasing) numbers of cells in challenge inocula allow more accurate detection and even quantitation of weak immune responses. The most important characteristic of these tumors is the metastatic propensity of CT 26 and CT 51. After excision of an appropriately large transplant, the majority of animals eventually die of metastatic disease. This tendency to metastasize allows evaluation of adjuvant therapies in animals with minimum residual disease. Very few spontaneously metastasizing chemically induced rodent colon tumors have been described (18), and none are in serial transplantation.

The BALB/c colon tumors show weak (CT 51), moderately weak (CT 26), and moderately strong (CT 36) tumor rejection antigens in classical transplantation experiments. The cross-immunization experiments indicate that these antigens appear to be individually unique TSTA. This finding is consonant with the general observation that carcinogen-induced tumors contain individually distinct tumor-specific rejection-type antigens which do not elicit cross-resistance in vivo (5, 17, 28), although a few exceptions to this rule have been noted (18, 27, 31). However, considerable evidence has accumulated from in vitro studies that different chemically induced tumors of similar histological types may share common tissue-type-specific antigens (1, 6, 16).

Steele and Sjögren have demonstrated cross-reacting tumor-associated antigens in vitro in DMH and nitrosoquazidine-induced rat colon tumors using lymphocyte microcytotoxicity tests. Effector lymphocytes from rats bearing primary colon carcinomas or transplants of these tumors were consistently cytotoxic to multiple other chemically induced rat colon tumor target cells, but not to control tumors cells (from tumors of other histological types) or to normal cells, including normal colon mucosa cells (24, 26). Sera from tumor-bearing rats blocked the cytotoxicity of sensitized lymph node effector cells for colon carcinoma target cells. This tissue-type-specific antigenicity closely parallels the results of studies on the antigens of human colorectal neoplasms reported by the Hellströms using similar in vitro techniques (10).

In further characterizing the antigens of rat colon carcinomas, Steele and Sjögren identified embryonic specificities (23, 25). Effector lymphocytes from rats bearing colon tumors were cytotoxic to fetal colon target cells but not to other types of fetal cells or to normal adult colonic mucosa cells. Effector lymphocytes from multiparous rats were cytotoxic to target cells from different colon tumor lines but not to normal adult colon target cells. Martin et al. (13), using immunofluorescence, have identified a membrane-associated antigen common to 5 different chemically induced intestinal carcinomas and to fetal tissues of BN and Lewis rats. Fetal antigens have been identified on CT 26 and CT 51 cultured cells in our laboratory using a radioiodinated staphylococcal protein A assay for IgG antibody (2). Several fetal-type antigens are known to be associated with human colorectal neoplasms. In addition, the Hellströms have shown that lymphocytes from human colon cancer patients are specifically cytotoxic to fetal gut cells as well as to colorectal carcinoma cells (10). In spite of the well-documented tissue-type-specific and fetal antigens found among histologically similar rat colon tumors and among histologically similar human colon tumors, the biological significance of these antigens remains unclear. Common cross-reacting antigens detected in vitro by serological tests or by assays for cell-mediated immune responses frequently do not reflect tumor rejection-type immune responses in vivo (1, 6).

It is possible that our method of immunization and/or time interval between excision of the immunizing tumor and subsequent tumor challenge (i.e., 7 days) were such that latent common antigenic specificities were not detected. Common TSTA are considered to be much weaker than unique TSTA (11). Leffel and Coggin (12) have recently shown that cross-reaction between 3-methylcholanthrene-induced sarcomas is dependent upon the method of immunization as well as upon the dose of challenge tumor cells. These authors selected 2 tumors which had been shown previously to express only individually specific antigens. Mice were preimmunized with serial doses of irradiated tumor cells and then challenged with low doses of cells. Transplantation cross-resistance was then demonstrated. In our cross-immunization experiments, the challenge doses were fairly high, i.e., that cell number for each respective tumor which would result in progressive tumor growth in approximately 90% of untreated mice. In control animals, cumulative tumor incidences of 95% (CT 26), 79% (CT 36), and 84% (CT 51) were observed.

In summary, these 3 BALB/c colon carcinomas are considered excellent models for immunotherapy-chemoimmunotherapy experiments. The most important feature is the metastatic behavior of CT 26 and CT 51, allowing studies of adjuvant therapy. Extensive information on the chemotherapy of these tumors is already available; therefore, the study of combined chemoimmunotherapy would be facilitated. These tumors are all immunogenic in the strain of origin. The relatively weak immunogenicity of CT 26 and CT 51 is analogous to human colorectal neoplasms, which are probably weakly antigenic. No cross-reacting TSTA were demonstrated in transplantation rejection experiments. This finding is in contrast to the common tissue-type antigen detected in vitro by Steele and Sjögren in the rat colon tumor model. However, weaker common TSTA may have been present in our murine tumors but were not detected by the methods used in our studies.

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