In Vitro Demonstration of Tumor-specific Common Antigens and Embryonal Antigens in Murine Fibrosarcomas Induced by 7, 12-Dimethylbenz(a)anthracene

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SUMMARY

The cell-mediated immune response against tumor-associated antigens of s.c. fibrosarcomas induced by 7,12-dimethylbenz(a)anthracene in BALB/c mice was studied by an in vitro microtest based on 51Cr release. Eight tumors were tested at the first, fifth, and ninth transplant generation with spleen lymphocytes from BALB/c mice that were immunized against syngeneic tumors, allogeneic tumors, or embryo cells. Individual antigens were detected on fibrosarcomas at the first and fifth transplant passage, whereas they were undetectable on the eight tumors tested at the ninth passage. Lymphocytes sensitized against a pool of five C3Hf fibrosarcomas induced by 7,12-dimethylbenz(a)anthracene revealed cross-reacting antigens in one, seven, and eight out of the eight BALB/c fibrosarcomas tested at the first, fifth, and ninth transplants, respectively. Similarly, lymphocytes sensitized against embryo cells were cytotoxic on two fibrosarcomas out of the six tested at either the first or fifth passage and on six out of the seven tumors tested at the ninth passage.

INTRODUCTION

Tumor-specific transplantation antigens have been demonstrated in chemically induced tumors by inhibition of tumor growth in animals pretreated with the same tumor. The possibility that these antigens might be common for different tumors of the same histological type has been repeatedly investigated, and the results have demonstrated that cross-reactivity occurs only occasionally (3, 13, 17, 22–24). This is in contrast with the well-known fact that virus-induced tumors possess not only viral antigens but also virus-associated cellular antigens that are cross-reacting for tumors caused by the same virus.

An effort further to characterize the antigenicity of the chemically induced tumors by in vitro techniques resulted again in the demonstration of only individual antigens (1, 16, 25) or of a more or less consistent pattern of cross-reactivity (7, 8, 12). In addition, in vitro tests have provided evidence that embryonal antigens are expressed at the cell surface of chemically induced tumors (2, 4, 7). The question must be raised, therefore, as to whether the observed common antigens could be identified with embryonal ones.

The purpose of the present study was to investigate by means of an in vitro test for cell-mediated immunity whether individual and cross-reacting tumor-specific antigens and embryonal antigens were simultaneously present on the cell surface of chemically induced sarcomas and, if so, whether their interrelationship could be modified during serial transplants.

MATERIALS AND METHODS

Tumors and Immunizations. Fibrosarcomas were induced by 1 s.c. injection of 50 μg of DMBA2 in oil suspension in 10-week-old C3Hf and BALB/c male and female mice, bred in this laboratory by brother x sister mating. Sixteen BALB/c and 5 C3Hf tumors were used. The tumors were maintained in transplant in normal syngeneic animals of the same sex as the tumor donors by injecting s.c. 5 X 104 cells obtained by trypsinization and suspended in 0.1 ml of Hanks' balanced salt solution.

BALB/c mice were immunized with cells obtained by trypsinization from BALB/c or C3Hf sarcomas, from newborn C3Hf fibroblasts, and from C3Hf embryos 10 to 14 days old. The age of the embryos was selected on preliminary experimental evidence that older embryos do not elicit an antitumor immunity.

For avoidance of reactions against sex antigens, female mice were immunized with tumors from females only, while males were immunized with newborn fibroblasts or embryo cells or tumors from mice of either sex. Syngeneic and allogeneic immunization were carried out by 5 weekly injections half s.c. and half i.p.; the 1st inoculum was 2 X 106 cells and the following doses increased 2-fold. The cells were blocked with Mitomycin C (250 μg/10 X 106 cells in 2 ml of Hanks' balanced salt solution containing antibiotics) and kept for 3 hr at 37° before the inoculum.

In the syngeneic system the immunizing and target cells used in each cytotoxic test were from tumors at the same transplant generation. The tumor cells used for allogeneic immunization were from a pool of 5 tumors obtained from C3Hf females and maintained in transplant in syngeneic

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2 The abbreviation used is: DMBA, 7,12-dimethylbenz(a)anthracene.
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animals for 1 to 16 generations.

Microplate Technique for Cell-mediated Cytotoxicity. The test was carried out following a previously described technique (18). Briefly, fibrosarcoma cells or normal fibroblasts, used as target cells, were cultured in vitro for 1 week in Petri dishes in Medium 199 (Microbiological Associates, Bethesda, Md.) containing 20% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 i.u./ml; the cultures were then trypsinized and washed; each cell suspension, containing 10⁶ cells in 2 ml of Medium 199 to which were added 200 μCi of ⁵¹Cr, was incubated for 3 hr. After repeated washings, 10⁶ labeled cells were seeded in each well of Falcon plates 3040-3041 and incubated for 24 hr. Effector cells were obtained from spleens of immunized mice 7 days after the last immunization and from spleens of untreated animals and were purified of erythrocytes and granulocytes by the Ficoll-Triosol method (11). In each well containing the labeled target cells, 20 X 10⁶ lymphocytes were seeded to reach a target:effector cell ratio of 1:200, and the plates were incubated for an additional 24 hr. For each sample of lymphocytes, 14 to 16 replicates were made. The percentage of specific ⁵¹Cr release was calculated as follows:

\[
\frac{(\text{Experimental release} - \text{control release})}{(\text{total label} - \text{control release})} \times 100
\]

The control release was that obtained with lymphocytes from normal untreated animals. Statistical analysis by Student's \(t\) test of the release observed with experimental and control lymphocytes demonstrated that the tests with a specific ⁵¹Cr release equal to or higher than 20% differed from the controls significantly \((p < 0.01)\).

RESULTS

In a preliminary experiment, 16 DMBA-induced fibrosarcomas of BALB/c mice at the 1st in vivo transplant were tested in vitro for individual and cross-reacting antigenicity. Lymphocytes obtained from BALB/c mice immunized against each of the 16 tumors were specifically cytotoxic in 10 cases on the individual immunizing tumor, whereas no positive reactions were observed when the lymphocytes were tested on a tumor different from the immunizing one. In contrast, BALB/c lymphocytes sensitized against a pool of 5 C3H/DMBA-induced fibrosarcomas produced a significant specific ⁵¹Cr release in 4 cases, indicating that 25% of the tumors tested possessed cross-reacting tumor-associated antigens.

Eight of the 16 tumors, 5 positive and 3 negative for the individual antigenicity and 1 positive and 7 negative for the cross-reacting antigenicity, were selected for the experiment in which the tumors were tested at the 1st, 5th, and 9th transplant generation. Lymphocytes were sensitized against the same tumor used as target or against a pool of 5 allogeneic tumors or embryo cells or normal fibroblasts. The results of the cytotoxic assays are reported in Table 1. The autochthonous tests, \(i.e.,\) those that involved immunizing and target cells from the same tumor at the same transplant passage, were positive in 5 out of 8 cases at the 1st passage, 6 out of 8 at the 5th passage, and in none of the 8 tested at the 9th passage. On the other hand all but 1 (1 of 8) of the tests with lymphocytes sensitized against the pool of the 5 allogeneic tumors were negative on the target cells from tumors at the 1st transplant, while all but 1 (7 of 8) were positive on target cells from tumors at the 5th and all were positive (8 of 8) at the 9th transplant. The cytotoxicity of the lymphocytes sensitized against allogeneic embryos showed a similar correlation with the transplant generation of the tumors. All 3 tests on the tumors at the 1st transplant were negative, 2 tests out of 4 were positive on the cells from the 5th tumor transplant, while 6 of 7 tests on the tumor cells from the 9th transplant were positive.

The control tests were all negative, that is the antitumor or antiembryo lymphocytes were inactive on normal fibroblasts and the lymphocytes sensitized against allogeneic fibroblasts showed no specific cytotoxic activity on tumor target cells.

DISCUSSION

Our results demonstrate that both individual and cross-reacting tumor-specific antigens of DMBA-induced murine fibrosarcomas can be evidenced in vitro by studying the cell-mediated immunity with a microtest based on ⁵¹Cr release.

The detection of cross-reacting antigens may have depended not only on the sensitivity of the technique but also on the immunization system. Antigens revealed only by allogeneic immunization have been found (5, 6, 26), and the possibility of demonstrating cross-reactivity in chemically induced sarcomas when a pool of syngeneic tumors, rather than single tumors, were used to immunize has also been reported (22). We combined the 2 systems by immunizing with a pool of allogeneic tumors, and this, together with the adoption of an in vitro test, may have favored the detection of the cross-reacting antigenicity.

Contrasting results have been reported on the persistence of individual antigens of chemically induced sarcomas after serial transplant since both stability during 25 to 30 passage generations (20) and loss or decrease during early passages have been observed (10, 22). We found that individual immunogenicity decreased to the point of becoming undetectable at the 9th transplant generation, whereas with cross-reacting antigens the reverse was the case, all the tumors tested possessing common tumor specific antigens at the 9th passage. This indicates that individual antigens do not coincide with the cross-reacting ones and that they can coexist in different proportion on the primary tumors and on the successive transplants.

In addition we found that sarcoma cells were sensitive to lymphocytes that were immune against embryo cells and that this sensitivity increased with serial passage, as we had observed with the lymphocytes that were immune to allogeneic sarcomas. This seems to indicate an identity between the tumor-specific cross-reacting antigens and the embryonal antigens. Gross cellular antigens have also been reported to be present in chemically induced sarcomas (19) and the possibility that, beside embryonal antigens, virus-related antigens may contribute to the cross-reacting antigenicity of our DMBA-induced sarcomas cannot be excluded. It is unlikely, however, that the cross-reactivity can be ascribed exclusively to viral antigens because we demonstrated...
cytotoxic activity on sarcoma cells by the antiembryo lymphocytes. Mouse embryos have been shown to possess gs antigens which, however, are proved poor immunogens, unable to sensitize mice (14). In our experience, moreover, only embryos 10 to 14 days old evoked an effective immune response on sarcoma cells, and it is unlikely that only embryos of this age could elicit an antivirus immune response.

Our experiments were not designed to find out whether the individual immunogenicity and/or immunosensitivity decreased with serial passages and whether the decrease was due to a low antigen expression or to a complete modulation of the involved antigens. It has been recently reported that the phenomenon of antigenic modulation, originally described for the TL antigens (21), also takes place in other tumor-specific antigen systems (9, 15). On the other hand, individual antigens may have been affected by immunoselection which, in our experiments, could have been favored by the use of a low number of dispersed cells for transplantation. It seems unlikely that the loss of antigenicity could have been due to trypsinization, which was shown to have no detectable effect on tumor cell antigenicity (27).

Currently under way are experiments testing whether DMBA-induced sarcomas also lose immunosensitivity and whether individual antigenicity may reappear on release of tumors from the immune environment.

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