Immunogenic Strength of Sarcomas Induced by Methylcholanthrene in Millipore Filter Diffusion Chambers

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SUMMARY

Normal BALB/c fibroblasts enclosed in two-compartment diffusion chambers with a methylcholanthrene (MCA) disc were left in the peritoneal cavity of BALB/c mice or were incubated in vitro for 4 to 24 weeks while protected from the immunoselective process. This protection was demonstrated by the fact that cells of a BALB/c sarcoma induced by MCA, which were immunogenic when injected s.c., were unable to sensitize when grown for 2 weeks within 0.22-μm filter diffusion chambers placed in the peritoneal cavity of syngeneic mice. The chambers with the fibroblasts exposed to MCA were recovered and opened at 4-week intervals; their contents were implanted s.c. into immunodepressed syngeneic or semisyngeneic mice. In 41 to 63% of the cases, the in vivo and in vitro diffusion chamber cultures gave rise to sarcomas. When the diffusion chambers were made with 1.2-μm lymphocyte-permeable filters and were kept in vivo, only 10% of the cultures produced tumors. Fifty tumors were tested for immunogenicity by transplantation methods and 45 were found to be immunogenic. The immunogenic strength, however, showed a large variability without any correlation with the latent period, length of exposure to the carcinogen, or growth rate. We concluded that an immunological control operates during MCA carcinogenesis but that other factors influence the immunogenic strength of these sarcomas.

INTRODUCTION

Tumor-associated antigenicity seems to occur early during chemical carcinogenesis as well as during viral carcinogenesis (5, 8, 15). However, it is not known whether all tumors are immunogenic since their inception; nor is it known how their immunogenicity can be modified during the subsequent growth. In fact, a large variability in immunogenic strength among MCA-induced sarcomas has been repeatedly reported, together with a decline in immunogenic strength with an increased latent period (2, 7, 13). If the immunosurveillance of the host is the only or major explanation of these findings, sarcomas elicited in an immunologically free environment should display similar antigenic properties.

We tested this hypothesis with the aid of the DC cultures. In that study (12), mouse fibroblasts grew within cell-impermeable chambers while exposed to MCA, either when the DC's were implanted in the peritoneum of mice or when they were placed in vitro. Thus the neoplastic transformation may occur in vivo in the absence of the host cell-mediated immunological pressure, whereas the in vitro DC cultures provided an absolutely immunologically free environment. Therefore, tumors obtained in this system should display their original cell surface immunogenicity.

MATERIALS AND METHODS

The construction of the 2-compartment DC's and their use for cultivating cells in vivo and in vitro have been described (11). In the 1st 3 experiments, the outer Millipore filters of the DC's had a porosity of 0.22 μm, which does not allow passage of lymphocytes (16) while, in the last experiment, lymphocyte-permeable 1.2-μm filters were used. The internal filter, on which the cells grew, had a porosity of 1.2 μm in all of the experimental groups.

DC Culture of Antigenic Sarcoma Cells. Cells obtained by trypsinization from an immunogenic sarcoma induced by MCA in a BALB/c female and maintained in syngeneic mice for 4 generations were grown in the larger space of the 2-compartment DC's. Twenty-three DC's were filled with 1.6 X 10⁶ tumor cells per chamber suspended in 0.05 ml of minimum essential medium plus 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) with antibiotics, while 16 DC's were similarly loaded with normal muscle cells obtained from the sarcoma-bearing animals. The smaller compartment of each DC contained a disc of paraffin to reproduce the same conditions of the chambers used in the experiments described below in which normal fibroblasts were exposed to MCA-embedded paraffin discs. The cells, either neoplastic or normal, were left growing for 2 weeks in the peritoneal cavity of 2-month-old BALB/c males; then the DC's were removed by laparotomy. The same tumor used to fill the DC's was implanted s.c. by trocar in 12 BALB/c male mice and was excised 2 weeks later when it had reached 5 to 8 mm in diameter. After 8 days, mice of the 3 groups were challenged by s.c. injection in the flank with 10⁵ cells of the same sarcoma.

Tumor Induction by MCA in DC Cultures. Muscle cells obtained by trypsinization from newborn BALB/c mice were cultured in DC's that contained 1% (30 μg) MCA discs in the...
thinner compartment. The chambers were inserted in the peritoneal cavity of 2-month-old BALB/c male mice (Group 1). After 4, 8, 12, 16, 20, and 24 weeks of culture, groups of 8 to 12 DC’s were removed, and the MCA was discarded. The internal filter of each DC (i.e., filters that had not been in contact with the outside peritoneal cells) was implanted, along with the overlying cells, into the flanks of immunodepressed, 4-month-old C3H X BALB/c F1 (hereafter called C3C) mice. When a tumor appeared in a mouse, we transplanted it by trocar in 5 C3C and 5 BALB/c males in order to ascertain the genotype. Only tumors that grew in both the hybrid and parental strains, i.e., BALB/c tumors, were considered to have been induced by MCA within the DC environment. A total of 32 sarcomas were obtained in this experiment, and 25 were used for immunogenicity studies. By adopting the same procedure of tumor induction in an experiment, the details of which were reported previously (12), we kept similar DC’s either in the peritoneal cavity of mice or in vitro in plastic dishes. Eleven sarcomas obtained from in vivo Group 2 and 12 sarcomas from in vitro Group 3 were examined for immunogenicity. In vivo Groups 1 and 2 were considered separately, since they had been done at different times and in different laboratories, and since some important modifications in the evaluation of tumor immunogenicity had been introduced in Group 1 to improve the sensitivity of the method previously used (see below). To determine the growth rate of 25 Group 1 tumors, we transplanted 10^5 cells from each into the flanks of 18 to 20 unconditioned, syngeneic BALB/c male mice, 2 to 4 months of age. The mean tumor diameter was recorded each week for 4 to 6 weeks. The growth rate of these tumors was evaluated according to Bartlett (3) as (mean tumor diameter at last week — mean tumor diameter at 1st week)/ no. of elapsed weeks. This ratio gives the increase of the tumor diameter in mm/week; the highest values indicated the more rapidly growing sarcomas. The histology of 17 tumors was examined.

Tumor Induction by MCA in Cell-permeable DC’s. In this experiment, 3 groups of 12 cell-permeable, 2-compartment DC’s were filled with BALB/c newborn muscle cells, exposed to MCA, and kept for 4, 8, and 12 weeks, respectively, in the peritoneum of BALB/c male mice. The internal filters were then implanted into immunodepressed C3C mice for a test of tumor production, as described above. The resulting tumors were then tested for immunogenicity.

In all experiments of tumor induction by MCA, the assay mice were observed for 40 weeks before they were considered negative for tumor development.

Immunodepression. Adult C3C male mice were thymectomized by pipet suction. Two weeks later they received 450 R, and 48 to 72 hr afterward the mice were given implants of filters obtained from the various DC cultures. Whole-body irradiation was performed in a rotating plastic holder with a source of γ-rays (Gammatron-3; Siemens Corp., Iselin, N. J.); the irradiation characteristics included a 60-mm tube-to-surface distance, with an exposure time of 6 min and 59 sec.

Immunogenicity Tests. The immunogenic strength of the tumors was measured by the protection that their growth elicited upon a challenge by the same tumor. However, several variations were introduced in the subsequent experiment of in vivo DC tumor induction (Group 1) as compared to Groups 2 and 3, in which in vivo and in vitro DC cultures were performed. In the latter case (Groups 2 and 3), the challenge was done immediately after the surgical removal of the growing neoplasm by s.c. implantation of 1- to 2-mm pieces of the same tumor in 12 to 16 immune and 12 to 16 control mice whereas, in the other in vivo experiment (Group 1), the challenge was carried out with 10^5 tumor cells 8 days after the excision of the sensitizing neoplasm in 20 immune and 20 control mice for each sarcoma. In both experiments, the control mice received normal muscle and liver tissues instead of tumor tissue; then the animals were sham operated by the removal of an area of skin comparable to that removed with the tumors in the immune mice. In addition, the immunogenicity was evaluated as the ratio between average tumor diameters measured 3 weeks after challenge in control and immune mice in Groups 2 and 3; mice without tumors were scored as zero diameter. In Group 1, the χ^2 value was obtained from the difference in number of takes between immune and control mice. In the 1st instance, values above 1.2 reflected immunogenicity, since previous studies showed that ratios of 1.2 or less are not as a rule statistically significant. In the latter instance, a χ^2 greater than 3.8 indicated significant protection at the 5% level.

RESULTS

Immunogenicity of Sarcoma Cells in DC’s. No significant antitumor protection was elicited by growing, immunogenic, neoplastic cells in DC’s inserted i.p., compared with normal cells kept in DC’s for an equal period of time. In fact, the challenge of 10^5 tumor cells produced sarcomas in 19 of 23 mice that bore DC’s with tumor cells and in 15 of 16 mice with DC’s that contained normal cells. This difference was not significant. Conversely, a complete rejection was obtained when 12 mice were immunized with the same tumor cells s.c. by the standard growth-excision method.

Tumor Production in DC Cultures. The frequency of neoplastic transformation of BALB/c cells growing in 0.22-μm DC’s in vivo and exposed to 1% MCA (Group 1) is shown in Table 1. There was no increase in tumor frequency with the increasing length of carcinogenic treatment, but the time required for nodule formation in assay mice tended to be shorter in the groups exposed for the longest time to MCA. These data are of the same magnitude as those previously reported for Groups 2 and 3 (12) although, in that case, a linear increase of tumor incidence with increasing length of treatment was evident. When the DC’s were made permeable to host lymphocytes, there was a significantly lower incidence of neoplastic transformation (Table 1).

Among the tumors obtained in Group 1, 10 were classified as well-differentiated fibrosarcomas and 7 were classified as anaplastic. There was a tendency toward the undifferentiated types among the sarcomas with the longest exposure to MCA and with a shorter outgrowth period.

Immunogenicity. In the in vivo and in vitro DC experiment (Groups 2 and 3), for which a less sensitive method was adopted to measure the immunogenicity, we found highly immunogenic, low immunogenic, and nonimmunogenic sar-
Table 1

<table>
<thead>
<tr>
<th>Length of exposure to MCA (wk)</th>
<th>Porosity of the DC's (µm)</th>
<th>No. of mice with tumors/no. of mice transplanted</th>
<th>Latent period (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.22</td>
<td>5/10</td>
<td>8.8 ± 1.9^c</td>
</tr>
<tr>
<td>8</td>
<td>0.22</td>
<td>5/12</td>
<td>9.8 ± 2.9</td>
</tr>
<tr>
<td>12</td>
<td>0.22</td>
<td>7/11</td>
<td>5.6 ± 1.2</td>
</tr>
<tr>
<td>16</td>
<td>1.20</td>
<td>6/10</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>20</td>
<td>1.20</td>
<td>5/9</td>
<td>8.4 ± 2.4</td>
</tr>
<tr>
<td>24</td>
<td>1.20</td>
<td>4/8</td>
<td>5.2 ± 3.5</td>
</tr>
</tbody>
</table>

^a Each immunodepressed mouse received the content of a single DC.
^b The time required for the DC content to produce a palpable nodule upon implantation into immunodepressed assay mice.
^c Mean ± S.E.
^d p < 0.01, compared to the similar group with 0.22-µm porosity.
^e Not significant.
^f p < 0.02.

Comas in each situation (Chart 1). Also, no relationship was noted between immunogenic strength and time of exposure to MCA in DC's. In the 2nd experiment of tumor induction by MCA in DC's i.p. (Group 1), all sarcomas were immunogenic (Chart 2); but also in this experiment there was a large variability in the immunogenicity index ranged from 5.4 to 36.0. Again, no correlation was found between immunogenic strength and length of treatment with the carcinogen in DC cultures. These sarcomas (Group 1) showed no correlation between immunogenicity and tumor growth rate (Chart 3) or histological types. Chart 4 shows that the high or low immunogenic strength of sarcomas was without any clear relationship to the total latency, calculated by adding the time of cultivation in DC's to the time required for the tumor to become palpable in the assay mice.

The immunogenic indices of the 3 tumors obtained in lymphocyte-permeable chambers were 17.3, 23.9, and 24.9, respectively.

**DISCUSSION**

In order to evaluate the antigenicity of the tumors induced in DC's, it should be proved that these environments are actually protected from the cellular immunological pressure of the host. The point is tenable in view of previous findings showing that normal and tumor tissues are not destroyed by an established immunity when they are enclosed within a Millipore filter chamber (1, 3). We found that antigenic tumor cells growing in DC's could not immunize the host, whereas similar cells injected s.c. effectively gave rise to an antitumor immunity. These data are at variance with those of Natale et al. (9), who elicited a partial protection against a s.c. challenge by growing tumor cells within 0.45-µm filter DC's. This might be due to the different way in which the chambers were constructed since, in their experiment, 0.45-µm filters were glued directly to the Lucite discs while, in our study, 0.22-µm filters were glued by means of precut plastic adhesive tape forms (4). Thus, both the afferent and efferent limbs of the cellular immune response are significantly prevented by our DC system.

Sarcomas induced in DC's in vitro, i.e., in an environment...
Immunogenicity of Tumors Induced by MCA

Chart 2. Immunogenicity and time of exposure to MCA in 25 sarcomas induced in DC's in vivo (Group 1). - - - -, value of the threshold immunogenicity. Immunogenicity index was the $x^2$ value as obtained from the difference in takes between immune and control mice after a challenge of $10^5$ tumor cells.

Completely free of any immunological influence, showed an immunogenic feature similar to that of their in vivo counterparts, since tumors of high, low, or undetectable immunogenicity appeared in both situations. In the other experiment, in which only in vivo groups were done, all sarcomas were immunogenic. We feel that this discrepancy can be attributed to the 2 different methods adopted for the immunogenicity tests. We conclude, therefore, that the great majority of sarcomas produced in an environment protected from cellular immunity are immunogenic since their early appearance.

No inverse correlation was found between immunogenic strength and latency of the tumors induced in DC's, contrary to what was reported repeatedly for the sarcomas elicited during s.c. carcinogenesis (2, 7, 13). This is in keeping with the theory that immunoselection is responsible for the progressive decline in immunogenic strength that takes place when tumors are induced in an immunologically exposed tissue of the host. In the experiment in which DC's permeable to the host peritoneal cells were used, few tumors were elicited, seemingly due to progressive killing of the arising neoplastic, immunogenic cells within DC's. These findings also gave an evaluation of the extent to which the immunosurveillance might eliminate neoplastic cells. In fact, the difference in tumor frequency between DC cultures exposed or not exposed to host immunity was 40 to 50%, and may be ascribed to the killing effect of the immune lymphocytes which could enter.
the DC’s made with 1.2-μm filters (16). If so, immunosurveillance can be considered only 1 of many possible mechanisms aimed to prevent tumor growth, as suggested by Prehn and Lappé (14). Three tumors were obtained when permeable DC’s were adopted; they were immunogenic, indicating that once escaped to an early destruction, neoplastic cells can maintain their immunogenicity even in the face of a potential immune response. The presence of blocking antibodies can be relevant in this escape mechanism (6). An alternative explanation for the lower tumor incidence in the lymphocyte-permeable chambers might lie in a reduction of the MCA dose on the fibroblasts due to the increase of the cell population in the DC’s caused by the incoming peritoneal cells and to the easier diffusion of the carcinogen to the surrounding tissues through the larger pores of the 1.2-μm filters.

A large variability in immunogenic strength was found among sarcomas apparently induced under the same conditions. Several tumors displayed a relatively low antigenic strength which, in our DC system, cannot be explained in terms of immunoselection. Among other factors that could be responsible for this feature are the length of carcinogenic treatment and the histological characteristics of the target cells. However, the immunogenic strength was not related to the length of the carcinogenic treatment. It seems, therefore, that the tumor-specific transplantation antigens are originally determined during the early process of transformation, and no modification can be obtained by further treatment with the same carcinogen. The use of primary cultures of newborn muscle cells provided a histologically homogeneous population of cells exposed to the carcinogen. However, the possibility that the cell cycle, the rate of DNA synthesis, and other unknown factors might interfere with the antigenic expression of the resulting tumors cannot be ignored at this time.

In conclusion, this study supports the hypothesis that an immunological control operates during MCA oncogenesis in mice. Since, in the absence of such a control, i.e., in the DC cultures tumors may occur with different levels of immunogenicity, possibly other factors are involved in determining the original immunogenic strength of these tumors.

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REFERENCES

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