Further Evidence of Common Antigenic Properties in Chemically Induced Sarcomas of Mice

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SUMMARY

The transplantability of five methylcholanthrene-induced sarcomas of C57BL/6 mice was tested by subcutaneous inoculation of quantitated tumor cell suspensions in variously immunized syngeneic recipients. All five tumors gave evidence of resistance in recipients that were immunized and challenged with the same tumor. There was no evidence of cross-resistance between these tumors when mice were immunized with one tumor and challenged with a different tumor. However, when mice were immunized with several (up to four) of these same tumors sequentially, or with four tumors as a mixture, they developed resistance in some of the combinations tested to subsequent challenge with cells from a tumor which had not been included in the immunizing procedures. Multiple injections of normal syngeneic liver cells did not affect the resistance of recipients.

Inoculation of X-irradiated minor cells (8,000 R) also increased resistance to challenge with live cells from the same syngeneic tumor. Cross-resistance against an unrelated tumor was found in one group of mice which had been immunized with a mixture of X-irradiated cells from four of the tumors and was challenged with cells from the fifth tumor, but in one experiment with another combination of pooled X-irradiated cells from four tumors as the immunizing preparation, there was an increased frequency of takes when the mice were challenged with the fifth tumor.

These findings confirm previously reported studies in which a different series of five tumors was used. They indicate that the major changes in antigenicity in these chemically induced tumors are unique to individual tumors. They also support the hypothesis that, as part of the tumor-specific changes in antigenicity, there are multiple minor antigenic alterations which are shared by some, but not necessarily all, tumors of the same etiology in a specific strain of mice. The sum of such shared antigenic alterations can result in an immunologic reaction in the host which may be sufficient to hinder tumor growth.

INTRODUCTION

We previously reported that resistance against quantitated isotransplants of cells derived from a chemically induced tumor in C57BL/6 mice could be induced by prior immunization with a mixture of tumor cells from 4 different chemically induced tumors which, when tested individually, showed no evidence of cross-resistance to the challenge tumor (6). On the basis of those data we postulated that, as part of individually tumor-specific changes in the antigenicity of chemically induced tumors, there are multiple minor antigenic alterations which are shared by some, but not necessarily all, tumors of the same etiology in a specific strain of mice. The sum of such shared antigenic alterations can result in an immunologic reaction in the host which may be sufficient to hinder tumor growth.

The present experiments were designed to test the above hypothesis further by repetition of the previous experiment and by exposing mice to several individual tumors consecutively instead of simultaneously as in the previous study.

By using quantitated inocula of living tumor cells for each successive step in this immunization program, we hoped to relate the development of resistance to the particular tumors and the number of tumors to which the recipients had been exposed.

MATERIALS AND METHODS

Mice and Tumors

The mice used in these studies were C57BL/6 females obtained from Millerton Research Farms in Millerton, N. Y. To induce tumors, 8-week-old mice were given a single intramuscular injection of 0.01 mg of 20-methylcholanthrene (MC) in 0.1 ml of olive oil into one thigh. Tumors became palpable at the injection sites 13-18 weeks later and grew progressively thereafter. They were used for the initial series of transplantation studies 20 weeks after MC injection, at which time they were larger than 20 mm in diameter. The 5 tumors used in this series of experiments were fibrosarcomas and were histologically indistinguishable. They were arbitrarily designated by numerals VI to X. An additional tumor (XI) of this same type and history was used in one control study.

For the tumor transplantation studies, it was necessary to use mice ranging in age from six to 14 weeks at the time of the inoculation of the primary tumors.
Common Antigens of Sarcomas

**Chart 1. Flow chart of tumor transplantations in the sequential immunization experiments.** Each group started with 25 mice except the first group which began with 50 animals. MC, methylcholangthrene.

As a further control study of the possibility that four successive injections of normal cells and repeated anesthetization and surgery might have nonspecific effects on resistance to the tumor transplants, the following experiment was carried out. Two groups of 25 mice each received four consecutive subcutaneous injections of liver cells at the same intervals as were used for the successive tumor immunizations. Each consisted of 1,000,000 liver cells processed in the same manner as was used for tumor cell suspensions. In one group the subcutaneous tissues at the injection sites and the overlying skin were excised two weeks after each injection. The other group was not treated in this manner. Six weeks after the fourth liver cell injection, these mice, as well as mice that had been immunized with a single injection of 1,000,000 live Tumor XI cells, and unimmunized controls, were challenged with an injection of 500,000 cells of this same tumor.
Julius Reiner and Chester M. Southam

For immunization with tumor mixtures the procedure was essentially the same as previously reported (6). Mice were inoculated when 12-14 weeks old with a mixture of cells from 4 of the 5 tumors, omitting that tumor which would be used for the subsequent test of immunity. Because these five tumors differed in their rates of growth, the composition of the tumor cell mixtures was adjusted, on the basis of the previously determined aggressiveness of each individual tumor, so as to permit growth of each of the individual tumors in the mixture. In these immunizing mixtures, the number of cells per mouse and the number of previous passages was as follows: Tumor VI, fourth passage, 200,000 cells; Tumor VII, sixth passage, 100,000 cells; Tumor VIII, sixth passage, 500,000 cells; Tumor IX, fifth passage, 500,000 cells; Tumor X, sixth passage, 1,000,000 cells. The mice which were immunized with the same tumor which was subsequently used for challenge received four times the above number of cells of that tumor in the immunizing dose.

In parallel with some of the above immunization studies, additional groups of mice were given a single subcutaneous injection of tumor cells in suspensions which were irradiated with 8,000 R from a 200-kv X-ray source. The number of X-irradiated cells of each tumor was the same as for the live cell mixtures (see above). Such cells were morphologically intact, and when tissue cultures of these same cell lines were irradiated to the same degree, they continued to metabolize for as long as four to five weeks but did not propagate. No palpable tumors developed at the site of inoculation of such X-irradiated cells.

Tumor Challenges

The term tumor challenge is used to designate those inoculations of tumor cell suspensions by which the recipients were tested to determine whether the various immunizing procedures had resulted in resistance to syngeneic tumor growth. The preparation of cell suspensions for challenge inocula was the same as for the immunizing inocula, but the tumor from which the cell suspension was prepared had usually been through one more syngeneic passage. Because of the longer time required for the sequential immunization program, the final challenge inocula for these experiments was usually prepared from tumors which had been through one or two more passages than those used for the immunizing inoculations. Challenge inocula were injected subcutaneously on the side opposite from the preceding immunizing injection. Following challenge, mice were examined twice each week. The diameter of palpable tumors was measured by calipers and recorded.

Challenge inocula for experiments in which tumor mixtures or sequential tumor inoculations were used for the immunizations was a dose which would yield, as judged by previous transplantation experience, about 50% takes in the untreated controls, in order to provide maximum sensitivity for the detection of small degrees of cross-resistance.

RESULTS

Table 1 summarizes the results of the experiments in which mice were immunized with propagating cells of a single tumor or with live liver cells from the donors of the primary tumors. Exposure to cells of Tumors VI, VII, VIII, and IX resulted in an increased resistance to a challenge with the same tumor. This immunity was almost complete (to the challenge dose of 200,000 cells) for Tumors VIII and IX, but only partial for Tumors VI and VII. The tests with Tumor X could not be evaluated because of the low incidence of tumor growth in the controls. Mice which received injections of normal liver cells from the primary tumor donors were just as susceptible to tumor challenge as were the uninjected controls. These results show that the resistance of mice which had been inoculated with tumor cells was directed specifically against the antigentic properties of the individual tumors, not against normal cell antigens. Immunization with tumor cells from any one of the five tumors did not result in a significant resistance to transplantation of any of the four tumors with which the recipients had not had previous contact. These results indicate that each of the five tumors is antigenically unique.

Similiar results have been reported in several previous studies of chemically induced mouse tumors.

Results of the studies in which mice received the five tumors consecutively are summarized in Tables 2 and 3.

The data shown in Table 2 were obtained during the successive steps in this immunization procedure. In these studies, because the data were obtained at intermediate steps in the experimental protocol, only a few of the possible combinations and permutations of the five tumors were tested. There were no simultaneously tested groups that had been immunized previously with cells of the challenged tumor, so that evaluation of immunity is based on comparison with the unimmunized controls. Mice that were challenged with Tumor VIII after immunization with Tumor VII followed by Tumor VI showed partial resistance to Tumor VIII (tumor incidence of 41% as compared with 100% in unimmunized controls). This is a highly significant difference ($P < 0.001$). In the simultaneously tested groups that had been exposed previously to only one tumor (VII or VI), the slight decrease in tumor takes (to 88 and 84 percent) is probably not significant.

When challenged with Tumor IX, the group that had been immunized consecutively with three tumors (VII, VI, and VIII) had only 73% tumor takes as compared with 100% in the immunized controls. This difference probably indicates a significant degree of immunization ($P < 0.001$). The three groups which had been exposed to two different tumors prior to challenge with Tumor IX all had slightly fewer takes than in the controls (88, 81, 92%). These differences may indicate a low grade of protection, but they are not statistically significant.

The last column in Table 2 shows the results of challenging the several immunized groups with 1,000,000 cells of Tumor X. Consecutive immunization with Tumors VII, VI, and VIII gave significant resistance to the Tumor X challenge (43% as compared to 76% in the unimmunixed controls, $P < 0.02$). Consecutive immunization with these tumors followed by Tumor IX gave even greater resistance against Tumor X (24% takes versus 76% in the controls, $P < 0.001$). Consecutive immunization with Tumors VII, VIII, and IX resulted in a...
slight decrease in tumor takes (54% as compared with 76% in controls), but such a difference may not be significant ($P = 0.10$). The other 2 tumor combinations (VI, VIII, IX or VII, VI, IX) did not give increased resistance against Tumor X.

Table 3 shows the results of the final challenges with each of the five tumors in mice which previously had been immunized by successive transplants of each of the other 4 tumors in various combinations. At this stage of the studies, in addition to unimmunized controls, there were also groups of mice which had been immunized against the same tumor as was used for the challenge. This provided a simultaneous determination of the immunogenicity of each of the five tumors and permits comparison of the efficacy of the same or the other four tumors as immunizing agents. The cell doses used for these final challenge inocula were selected on the basis of the earlier experience and are indicated at the top of each column.

Transplantation of Tumor IX was completely prevented by prior immunization with the same tumor (group designated "+Tu IX") and was significantly inhibited by prior successive immunization with the other four tumors (group designated "-Tu IX"). Similarly with Tumor X, prior exposure to the same tumor reduced the frequency of takes to 16% as compared with 60% in the unimmunized controls. Prior exposure to the other four tumors reduced takes of Tumor X to 31% ($P < 0.05$). Tumor VII gave fewer takes in both the +Tu VII and −Tu VII groups (72 and 61% respectively) than in the unimmunized controls (84%), but these differences could readily occur by chance.

In the additional control study in which the effect of four successive injections and excisions of syngeneic liver cells was tested, there was no change in receptivity to Tumor XI transplants. Takes at 60 days after tumor transplantation were: 52% (13/25) in the unimmunized controls, 52% (13/25) in the group that had received 4 consecutive injections of liver cells without surgery, 54% (13/24) in the group that had excision after each of the 4 liver cells injections, and 22% (5/23) in mice that had been specifically immunized with cells from this tumor.

Results of the studies in which mice were immunized with a mixture of tumor cells of four of the five tumors and then challenged with the tumor which had been omitted from the immunizing mixture are presented in Table 4. These are compared with the parallel experiments in which mice were immunized and challenged with the same tumor. These experiments were made after those described above, and hence the tumors had been through one or two more syngeneic transplant passages than those used for the final challenges recorded in Table 3. In this series of experiments, additional groups of mice were immunized with X-irradiated tumor cells using either a single tumor or a mixture of four tumors as in the groups which were immunized with live cells.

In the groups of mice that received immunizing inocula of unirradiated cells from a single tumor, tumor nodules developed in 50-88 percent of the mice and were resected. All mice that received the pooled unirradiated tumor cells developed tumors which were also resected. No tumors grew from the inoculation of a single or pooled tumor which had been X-irradiated.

The groups of mice which were immunized with living cells of one tumor all showed some degree of resistance to subsequent challenge with cells of the same tumor in these experiments (groups designated +Tu VI, +Tu VII, etc, in Table 4). These results are in general agreement with those obtained in the experiment shown in Tables 1 and 3. The minor differences can probably be attributed to differences in the number of tumor cells in the challenge inocula. A similar degree of protection was afforded by immunization with X-irradiated tumor cells with the exception of Tumor X, against which no resistance was produced by X-irradiated cells of the same tumor (groups designated +Tu VIX, + Tu VIIx, etc.). In the groups which were immunized with mixtures of live tumor cells and then challenged with the tumor which was not present in the immunizing mixture, there were
**Table 2**

<table>
<thead>
<tr>
<th>Immunizing tumor cells and sequence</th>
<th>Group designation</th>
<th>Tumor used for challenge</th>
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<tbody>
<tr>
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<td>-Xa,-Xb,-IX,-VIII</td>
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<tr>
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<td>VIII 38/50 76</td>
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<td>-VI</td>
<td>VII 21/24 88</td>
</tr>
<tr>
<td>VI only</td>
<td>-VII</td>
<td>VIII 21/25 84</td>
</tr>
<tr>
<td>VII, VI</td>
<td>-Xa,-Xb,-IX</td>
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<td>+VIII</td>
<td>X 25/25 100</td>
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<tr>
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<td>-VI</td>
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</tr>
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<td>-VII</td>
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</tr>
<tr>
<td>VII, VIII</td>
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**Growth of challenge transplants of syngeneic tumor cells after prior exposure of recipients to various numbers and combinations of syngeneic tumors. Data from the intermediate steps in the sequential immunization studies.** For details see Chart 1 and Materials and Methods. Fractions show the number of mice with growth of the challenge tumor divided by the total number of mice challenged. Italics are these same fractions as percentages. *P* values are based on chi-squared analysis of indicated groups compared to their simultaneous unimmunized controls.

<sup>a</sup>*p< 0.001.<sup>b</sup>*p< 0.01.<sup>c</sup>*p< 0.02.

**Table 3**

<table>
<thead>
<tr>
<th>Immunizing tumor cells and sequence</th>
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<td>IX 1,000,000 16</td>
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**Growth of challenge transplants of syngeneic tumor cells after prior exposure of the recipients to four successive syngeneic tumors other than that used as the challenge.** For details see Chart 1 and Materials and Methods. Fractions show number of mice with growth of the challenge tumor divided by the total number of mice challenged. Italics are these same fractions as percentages. *P* values based on chi-squared analysis of indicated groups compared to their simultaneous unimmunized control.

<sup>a</sup>*p< 0.01.<sup>b</sup>*p< 0.05.
significantly fewer tumor takes (as compared to unimmunized controls) when the challenge was Tumor VIII. In the groups in which Tumor VI or IX was the challenge, there were also fewer takes, but these differences were small. Tumor VII could not be evaluated in this study because the unimmunized control mice were lost due to an error. The other combination, challenged with Tumor X, showed a slight but statistically insignificant increase in takes in the groups that had been immunized with the tumor mixture.

The groups which were immunized with the irradiated mixtures from four tumors and then challenged with Tumor VIII showed significant resistance to the tumor challenge, just as did the groups which had received live tumor cell mixtures as the immunizing inoculum. Similarly, when the challenge was Tumor IX, there were slightly fewer takes in the immunized group. However, the reverse occurred in the experiments in which the challenge was Tumor X or Tumor VI. In these two groups the frequency of takes was higher in mice that had received the mixtures of irradiated tumor cells for immunization. This increase in takes of Tumor X is probably not significant (64% in the -Tu Xx group as compared to 40% in the unimmunized controls (P < 0.1). But with Tumor VI the difference is too great to be dismissed as chance (48% in the -Tu VIx group vs 16% in the control, (P < 0.02).

DISCUSSION

All five tumors of the present series showed some evidence of tumor-specific antigenic characteristics as judged by immunogenicity in syngeneic mice. They did not show any evidence of cross-resistance when these tumors were tested individually against each other. This confirms our previous findings (6) and those of several other investigations (2-4). However, cross-resistance against some of these tumors did occur in syngeneic recipients which had been immunized previously against multiple tumors, exclusive of the one used to test resistance. Thus, the sum of the immune response to several tumors afforded resistance against a tumor with which the recipient

Table 4

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<th>Immunizing tumor cells (mixtures)</th>
<th>Tumor used for challenge</th>
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Growth of challenge transplants of syngeneic tumor cells after prior exposure of the recipients to a mixture of four syngeneic tumors, not including that used as the challenge. Comparison of live and X-irradiated cells for immunization. Fractions show the number of mice with growth of the challenge tumor divided by the total number of mice challenged. Italics are these same fractions as percentages. P values are based on chi-squared analysis of indicated groups compared to their simultaneous unimmunized controls. 

*P < 0.01.

**P < 0.02.

*P control animals lost because of technical error.
had not had prior contact. The data confirm those reported previously, using a different series of five tumors.

Immunization with as few as two MC-induced tumors was sufficient to confer resistance against a third tumor of the same etiology in one of the combinations which was tested. Cross-resistance was more often demonstrated after immunization against three or four tumors. In the sequential experiments (Table 2) all groups which had been immunized with tumor cells had fewer tumor takes of the unrelated challenge tumor than did their nonimmunized controls. These differences in most cases were not statistically significant. However, when certain combinations of tumors were used to immunize, there was a significant degree of protection against an unrelated tumor. Interpretation of these data as a summation of responses to shared tumor-specific antigenic alterations, rather than a summation of the nonspecific stimuli of the immunizing procedures, seems justified by the fact that several of the multiple combinations of tumors failed to cause cross-resistance and that multiple sequential injections of syngeneic liver cells with or without subsequent resections failed to alter the resistance of recipient mice.

The finding that X-irradiated tumors cells may be as effective in immunizing syngeneic recipients as unrelated tumor cells confirms the findings of Revesz (7).

The increased frequency of takes in two of the groups of mice that were immunized with pooled irradiated cells (groups -Tu Xx and -Tu VIx in Table 4) suggests that immunologic enhancement of tumor growth (1) may occur even in syngeneic recipients. Further observations are necessary before this interpretation is accepted, but if it be true that this experiment shows specific immunologic enhancement, it is further evidence of shared tumor specific antigenic properties among these tumors.

Comparison of the results of the cross-immunization studies by multiple tumors given sequentially (Table 3) or as a mixture (Table 4) reveals that both procedures were sometimes effective and that the sequential procedure was generally more effective than the mixture of tumors. Although the two methods did not agree as to which tumors were more susceptible to inhibition by cross-resistance, it is noteworthy that the three tumors (VIII, IX, X) judged most highly immunogenic by the conventional transplantation technique (immunization and challenge with the same tumor) were the same three which were inhibited by cross-immunization with multiple tumors by either the mixed or sequential procedure. If specific immunologic enhancement of tumor growth can occur in this syngeneic system, the consequence of immunizing with several different tumors must be the sum of the enhancement and resistance effects. This might explain the discrepancies that were found in these experiments between the sequential and the mixed immunization procedures.

The results of these experiments, and of those reported previously, again lead us to postulate that the antigenic alterations which occur in MC-induced tumors of C57BL/6 mice are multiple but finite. They probably are not common to all tumors, but some occur frequently enough to permit the use of a few such tumors for immunization against other syngeneic tumors of this type. There are perhaps only a limited number of sites within the genetic substrate of a cell which are susceptible to the antigenic alterations that arise during malignant transformation. If so, it should be possible eventually to determine the number, chemical nature, and cellular location of the antigenic alterations that occur in these experimental tumors as well as the genetic sites which are altered. We have previously discussed the manner in which such changes might arise (6).

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