Search for Common Antigenicities among Twenty-five Sarcomas Induced by Methylcholanthrene

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SUMMARY

The possibility that antigenic specificities may be shared by sarcomas independently induced in mice by methylcholanthrene was explored in 25 tumors. Two modalities of the transplantation test for antigenicity, designated as "multiple challenge" and "multiple immunization," were used. The first consisted of immunizing mice with one tumor (implantation and excision) and testing in them the rejection of the same and others when threshold cell doses were injected intradermally in different skin sites. It was observed that, while each one of the tumors was clearly rejected in mice immunized with the same tumor line, it grew regularly in mice immunized with each one of the other tumors and in controls. There appeared to be some instances of cross-immunization in a first screening of the 90 possible tests between 10 tumors, but no such cases could be reproduced for any pair of tumors. Therefore, we conclude that each of 10 tumors tested had a different antigenic type. Fifteen tumors were studied in multiple immunization experiments. These consisted of inducing immunity simultaneously with four tumors and challenging this immunity with a single, different one. No stable cross-protection was revealed in 13 out of 14 such tests. One tumor pool was selected, however, which cross-immunized against a different tumor in three out of three tests. It was investigated whether this pool might also protect against four other tumors, with negative results. This study shows the extreme rarity of either totally or partially shared antigenic components between methylcholanthrene-induced tumors, as demonstrated by rejection of tumor cell inocula.

INTRODUCTION

The basis for the diversity of the specific transplantation antigens of chemically induced tumors is still a matter of speculation. When sarcomas induced in mice by oncogenic hydrocarbons are tested for immunogenicity within an isogenic strain, the usual finding is that each one immunizes against itself but not against others. Each new tumor induced is therefore expected to have a new antigenic individuality.

However, cross-reactivity between independently induced tumors has occasionally been reported (2, 5, 6, 8-10, 12, 13). The notion that antigenic determinants may be repeated in different tumors opens the possibility of antigenic typing.

The observations of cross-reactivity have been occasional, and no systematic attempts to reproduce them have so far been reported. The frequency of these observations suggests that cross-reactions will be detected only rarely in random tests. It has not yet been established whether that frequency reflects mere chance variability in test systems, or occasionally shared antigenic components within an undetectable number of possibilities, or a limited number of yet unidentified antigenic tumor types.

The studies reported here were designed: (a) to screen systematically a series of independently induced tumors for instances of reproducible cross-immunization between them; (b) to explore the practical possibilities of typing tumor samples by their antigenicities; and (c) to see whether pools of 4 tumors might reproducibly elicit immunity against tumors not included in the pool.

MATERIALS AND METHODS

Animals and Tumors. The 25 tumors studied (designated by Capitals A through Z) were induced, transplanted, and tested in (C57BL/6JNclcr X BALB/cAnlcr) F1 female mice 1 to 4 months old. The isogenicity of these animals was tested by skin transplantation, and no rejections were observed either in 1st or 2nd set grafts.

Millipore discs, 6 mm in diameter, embedded with 5% MCA2 in paraffin were implanted dorsally in the s. c. tissue. Three months afterwards, nodules could be palpated, and they were allowed to grow to an average diameter of 15 mm and then transplanted. Histological sections of these tumors showed them to be pleomorphic sarcomas and rhabdomyosarcomas. All grafts for propagation of the tumor lines were performed by chopping nonnecrotic tissue in PBS, containing 100 units penicillin and 100 mg streptomycin/ml, and implanting the pieces s. c. with a trocar.

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2 The abbreviations used are: MCA, 3-methylcholanthrene; PBS, phosphate-buffered 0.9% NaCl solution; TG, transplant generation.
Tests for Antigenicity and Cross-reactivity. The tests consisted of immunizing groups of mice with either 1 or 4 tumors and challenging that immunity with various intradermal inocula of tumor cell suspensions.

The immunization was performed as follows. When 1 tumor was used, 2 or 3 pieces, 1 to 2 cm mm, were placed under the dorsal skin with a trocar. When 4 tumors were used, a 13-gauge trocar was loaded with pieces of each of the tumors, separated from each other by 3 paraffin plugs punched with the same trocar from a Petri dish containing an 8-mm-thick layer of soft, solid paraffin. Care was taken, while inoculating the mice, to withdraw only the cannula, keeping mouse and stylet fixed, so as to maintain the row position of paraffin and tumor pieces under the skin. The tumors elicited by such inocula were long, s.c., lobulated strips, in which the individual growth of most pieces could be palpated. When the largest tumors attained an average diameter of about 10 mm, all were ligated within a pocket of skin (single tumors) or excised (multiple tumors). Control animals were similarly implanted with muscle or with muscle, thymus, and spleen fragments of isogeneic males plus paraffin plugs. Simulated operations, either ligation of a skin pocket or excision of the implanted tissue, were performed in them, imitating what was being done with the corresponding experimental groups. Each control inoculum contained tissues from 4 animals of different ages.

The challenge, performed 1 to 3 weeks later, consisted of intradermal inocula of single cell suspensions containing 9 to $35 \times 10^4$ trypan blue-unstained cells in 0.05 to 0.1 ml of PBS. Tumor cells were dispersed by dissociating the chopped tumors for 15-min intervals with 0.25% trypsin or Pronase and were decanted and washed 3 times in 0.9% NaCl solution. Small samples were mixed with 3% trypan blue, and the unstained cells were counted with a hemocytometer. The rest were then resuspended in PBS plus antibiotics at the desired concentration.

Experimental Design

Multiple Challenge Experiments. Animals were sensitized with 1 tumor, and the immunity elicited was challenged with the same and various others at different skin sites. Ten tumors (A through J) were used in these experiments. For large numbers of cross tests, 4 to 10 separate groups of about 18 mice each were immunized with different tumors (each group with 1 tumor) and, simultaneously, a control group was inoculated with muscle. Each mouse received up to 10 challenges of different tumors in different sites of the skin (flanks, limbs, chest, and abdomen). The same dose of each tumor suspension was injected in the same site of all animals. The cages of experimental and control mice were inoculated alternately. In this way, each experiment showed not only the growth of each of the tumor lines in animals immunized with one, but also the growths elicited by the same cell suspension in various groups of animals, each immunized with a different line. The results of 20 to 100 tests could therefore be simultaneously evaluated.

All results were in the first instance obtained from tumors between the 1st and 4th TG. Attempts to reproduce results of the first tests were done with the same lines before their 6th TG or with tumors stored frozen in a nitrogen bank at their 1st to 4th TG.

Multiple Immunization Experiments. Experimental animals were sensitized simultaneously with 4 tumors, as described, and challenged on the right flank with a tumor not represented in the immunizing inoculum. Fifteen tumors (K through Z) were used in these experiments. For insurance that each individual tumor in the inoculum had effectively immunized against its own antigens, each of the tumors was also injected into the left flanks of mice immunized with combinations that included the challenge tumor, as well as into control animals.

All immunizations were induced with primary tumors and tested with their 1st TG. Tests to reproduce the first results could be performed within 1 more TG, since each primary tumor had been preserved frozen in a nitrogen bank.

Evaluation. When tumors elicited by 1 suspension attained an average diameter of 5 to 10 mm, the 3 longest diameters of each tumor that were perpendicular to each other were measured with a millimeter scale. In a few cases, measurements were done when size was even smaller, or could not be done at all, because tumors growing faster in other sites were already killing the animals. For a given group, the average size equals

$$\frac{\sum n \left(D_1 + D_2 + D_3 \right)}{3}$$

where $D_1$, $D_2$, and $D_3$ are the longest diameters of each tumor that were perpendicular to each other and $n$ the number of mice in the group.

Results are expressed in 2 ways: average tumor size (including zeros) per group (Table 1) and percentage of tumor takes (number of tumors/number of mice) per group (Tables 2 and 3). "Antigenicity Ratio" in Tables 2 and 3 is the ratio between average tumor size in control and experimental groups. Statistical significance was determined by the Mann-Whitney U-test.

RESULTS

Multiple Challenge Experiments

The intradermal inoculation of challenge tumors produced localized growths and permitted the identification and measurement of up to 8 distinct growths in animals inoculated with 10 tumors. Tests measuring the growth of a tumor in animals immunized with the very same tumor or with others are referred to as "direct tests" or "cross-tests," respectively.

Direct Tests. The growth of all tumors (except E) was strongly inhibited in animals pretreated with the same tumor. However, repetition of the test E-E (immunization with E, challenge with E) with TG 5 and TG 6 showed a clear inhibition of the challenge inoculum. In no case was there significant evidence of growth acceleration in direct tests.

Cross-tests. The 90 possible combinations in which the growth of a tumor was measured in mice immunized with any one of the other 9 were explored under identical conditions.
Table 1

Summary and results of tests for cross-reactivity between 10 MCA-induced tumor lines

Results are expressed as average diameter in mm of each tumor line in a group of mice. Cross-rules separate results from different experiments. Growths in experimental and control groups are only to be compared within the same experiment.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Challenge</th>
<th>Immunization</th>
<th>Challenge</th>
<th>Immunization</th>
<th>Challenge</th>
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<tr>
<td>Tumor TG of mice</td>
<td>Tumor TG of mice</td>
<td>Tumor TG of mice</td>
<td>Tumor TG of mice</td>
<td>Tumor TG of mice</td>
<td>Tumor TG of mice</td>
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<tr>
<td>A</td>
<td>2 26</td>
<td>1.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3 20</td>
<td>28.8</td>
<td>34.8</td>
</tr>
<tr>
<td>B</td>
<td>2 28</td>
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<td>0.13</td>
<td>6.48</td>
<td>7.11</td>
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<tr>
<td>C</td>
<td>1 25</td>
<td>10.00</td>
<td>8.87</td>
<td>2.60</td>
<td>7.56</td>
</tr>
<tr>
<td>D</td>
<td>1 20</td>
<td>10.00</td>
<td>8.35</td>
<td>7.05</td>
<td>6.75</td>
</tr>
<tr>
<td>&amp;e Muscle</td>
<td>26</td>
<td>9.34</td>
<td>9.28</td>
<td>7.19</td>
<td>8.65</td>
</tr>
<tr>
<td>E</td>
<td>2 20</td>
<td>10.30</td>
<td>11.15</td>
<td>3.85</td>
<td>12.80</td>
</tr>
<tr>
<td>F</td>
<td>2 19</td>
<td>12.56</td>
<td>15.06</td>
<td>4.00</td>
<td>12.58</td>
</tr>
<tr>
<td>G</td>
<td>2 19</td>
<td>11.42</td>
<td>15.77</td>
<td>4.10</td>
<td>9.58</td>
</tr>
<tr>
<td>H</td>
<td>2 18</td>
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<td>13.61</td>
<td>5.78</td>
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</tr>
<tr>
<td>I</td>
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<td>18</td>
<td>9.16</td>
<td>2.71</td>
<td>5.86</td>
<td>8.40</td>
</tr>
</tbody>
</table>

<sup>a</sup> The number of mice was in some tests reduced in 1 or 2 because of deaths. Larger variations are presented in italics under the results.

<sup>b</sup> Expressed as number of trypan blue-unstained cells injected (X 10<sup>6</sup>).

<sup>c</sup> TP, trocar piece.

<sup>d</sup> Numbers in bold-face type indicate significant growth inhibition.

<sup>e</sup> Underlined numbers indicate significant growth acceleration. (p values are in both under 0.01 in 2-tailed tests)

(same animals) as the direct tests. As seen in Table 1, the growths elicited by uniform doses of a tumor cell suspension in the various groups immunized with unrelated tumors or muscle were quite uniform, and usually differed markedly from the growths in the corresponding direct test.

Growth Inhibition. In 9 of the cross-tests, the inhibition of tumor growth in immunized animals as compared with controls was so great as to have occurred by chance less than once in the 90 combinations tried. Thus, the growth of Tumor C was significantly inhibited in mice pretreated with 6 of the 9 unrelated tumors as compared with controls and similar inhibition was found in Tests G-I, J-G, and J-E (p < 0.01).

Growth Stimulation. Tumors E and G grew consistently better in many cross-immunized groups than in controls (p < 0.01). The same happened with Tumor B, although the differences were less significant. As seen in Table 1, these tumors had poorer growths in 6 different control groups than in tumor-immunized groups.

Reproducibility. As the chance of finding several “statistically significant” differences is obviously increased when large numbers of tests are performed, all direct and cross tests were repeated at least once with the same tumor lines. The results obtained in 9 direct tests and in most negative cross-tests were consistent with those first obtained. None of the instances of cross-immunization, however, could be constantly reproduced. In the 2nd scanning, again 9 borderline or significant instances were found, but the tumor pairs involved were different from those in the first testing. (Results were 90% reproducible in direct tests, 90% reproducible in negative cross-tests, and 0% reproducible in positive cross-tests.)

Multiple Immunization Experiments

Not all the tumors in the “immunizing rows” were uniformly of macroscopic size when excised. The individual immunizing capacity of each tumor fragment was demonstrated in 9 out of 14 experiments. In these, a significant resistance was shown against tumors included in the immunizing inoculum when these were later injected into the left flanks of the animals (Table 2). It is not clear whether the remaining 5 tumors failed to immunize by lack of growth, weak antigenicity, or antigenic competition of other tumors in the row.

No cross-immunization against tumors not included in the immunizing inoculum was observed in 13 out of 14 experi-
Tumors used simultaneously for immunization

<table>
<thead>
<tr>
<th>Challenge with tumors included in immunizing implant (left flank)</th>
<th>Challenge with tumors absent in immunizing implant (right flank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>Tumors/mice in experiments</td>
</tr>
<tr>
<td>L + M + O + P</td>
<td>10/16 (62%)</td>
</tr>
<tr>
<td>K + M + O + P</td>
<td>10/16 (62%)</td>
</tr>
<tr>
<td>K + L + O + P</td>
<td>L 11/18 (61%)</td>
</tr>
<tr>
<td>K + L + M + P</td>
<td>M 1/16 (6%)</td>
</tr>
<tr>
<td>K + L + M + O</td>
<td>O 11/14 (79%)</td>
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<tr>
<td>R + S + T + U</td>
<td>U 1/14 (7%)</td>
</tr>
<tr>
<td>R + S + T + U</td>
<td>Q</td>
</tr>
<tr>
<td>Q + R + S + T + U</td>
<td>R 7/15 (47%)</td>
</tr>
<tr>
<td>Q + R + S + T + U</td>
<td>S 1/10 (10%)</td>
</tr>
<tr>
<td>Q + R + S + U</td>
<td>T 5/12 (42%)</td>
</tr>
<tr>
<td>W + X + Y + Z</td>
<td>Z 6/17 (35%)</td>
</tr>
<tr>
<td>V + X + Y + Z</td>
<td>V 8/17 (47%)</td>
</tr>
<tr>
<td>V + W + Y + Z</td>
<td>W 5/14 (36%)</td>
</tr>
<tr>
<td>V + W + X + Z</td>
<td>X 3/18 (17%)</td>
</tr>
<tr>
<td>V + W + X + Y</td>
<td>Y 0/14 (0%)</td>
</tr>
</tbody>
</table>

DISCUSSION

No stable cross-reactivity was observed by transplantation technique among 10 antigenic MCA-induced sarcomas in tests designed to explore all combinations and permutations among a series of immunizing and test tumors. In 13 out of 14 experiments, no stable cross-protection against 1 tumor was obtained by pretreatment with 4 different ones. In one instance, however, this was achieved with a fair reproducibility, but the tumor combination which cross-immunized against one failed to cross-immunize against 4 other unrelated tumors.

The sporadic findings of cross-reactivity by different authors have suggested that the number of antigenic variations concomitant with tumor induction by a chemical might be limited (4, 7, 8). The fact that our tests explored systematically all pair combinations and permutations between 10 tumors allows us to conclude with certainty that, if the variations are due to a single antigen for a given tumor, such number is more than 10. If the number is between 10 and 100, this study should have detected shared specificities with a probability ranging from 100 to 10%. This finding argues against the practical possibility of typing these tumors by their antigens.

These considerations would only be valid if the antigenic changes consisted of 1 antigen/tumor. Reiner and Southam (9, 10) have proposed that there may be minor and multiple alterations in a given tumor. This is supported by experiments which apparently show that cross-immunization may be...
induced by tumor mixtures the components of which do not cross-react individually with the test tumor. In 6 out of 10 experiments reported (9, 10), this was the case. Our results do not quite coincide with theirs. Although both systems are similar, some technical conditions differ between them. In the immunizing procedures, we have used rows of tumor fragments instead of mixtures of trypsin-treated cells. In every experiment, we performed simultaneous sham inoculations and excisions in control mice, and our animals received 2 challenge inocula instead of 1. However, our doses were higher and, in some cases, perhaps, not as sensitive to the recipient's resistance.

Both the studies of Reiner and Southam and ours show that tests suggesting cross-immunization are difficult to reproduce. With the same tumor combinations, sequential and simultaneous immunization procedures (10) gave almost opposite results in terms of the final rejection of the challenge tumor. We also were unable to reproduce 9 positive or borderline tests in the "multiple challenge experiments" and the combination QRSU-T in the "multiple immunization experiments." If cross-reacting antigens are present in a pair of tumors showing a positive test, then one might expect the reciprocal test (E-J for J-E, for example) to be positive also. The lack of this reciprocity further argues against cross-reacting specificities.

We did, however, reproduce the results of Test QSTU-R. This selected pool had, at most, a narrow spectrum of cross-reactivity, since it protected against only 1 of the 5 tumors tested. Contamination of one tumor by cells of another tumor was very unlikely, since all lines were carefully handled. Exogenous contamination cannot be ruled out, since transplantable tumors may carry foreign antigens (11).

The absence of cross-reactivity, as tested by cell rejection, does not mean that, with other methods, common antigens may be detected in these tumors. This seems to be the case with some serological and in vitro methods (3).

Acceleration of tumor growth in animals immunized with one or various tumors was observed in several tests. This observation agrees with previous reports that this phenomenon depends on a low dose of test tumor cells (1) and has a sporadic character in syngeneic systems (10). Besides, in our series of tests, it seemed to be determined by some characteristic of the challenge inoculum rather than by the immunized animals. It was observed rather constantly in different groups of tumor-immunized animals challenged with the same cells. We may add that all attempts to reproduce "statistically significant" acceleration of tumor growth with the same tumor lines have thus far failed. In cross-tests, the "antigenicity ratios" clustered around 1 (no cross-reactivity) in a symmetric pattern resembling a normal distribution. However, extreme values of significant inhibition or stimulation \( (p < 0.01) \) were more than would be expected by chance \( (X^2 = 73, p < 0.001) \). This was reproduced for the sample as a whole, but not for any individual tumor in it. There may be biological effects by which tumor growth is facilitated or delayed in animals pretreated with tumor tissue, but we can not ascertain whether these effects are immunological or not.

These studies show the remarkably high degree of antigenic individuality of sarcomas induced by MCA as detected by cell rejection. We conclude that attempts to produce cross-protection on the basis of combination or typing of specificities are probably beyond practical reach.

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I extend my sincere thanks and recognition to Dr. R. T. Prehn for permanent support and encouragement during this work. I am also indebted to Dr. Gerald Bartlett for helpful discussion of this paper, and to Dr. Jerrold Zar for providing a program to compute the Mann-Whitney U-test. The technical assistance of Miss JoAnn Gates, Miss Pat Dennis, Mr. Terry O. Wirth, and Mr. William Lau is gratefully acknowledged.

ADDENDUM

Subsequent experiments showed that growth of Tumor R was markedly inhibited by either the immunity to Tumors S, U, or S + U. Therefore, this tumor does not constitute an example of the phenomenon described by Reiner and Southam but may, however, be an example of a widely cross-reacting tumor.

REFERENCES

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