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ACQUIRED IMMUNITY TO EHRlich ASCITES TUMOR IN MICE AFTER TREATMENT WITH FRESH OR SONIZED TUMOR CELLS AT VARIOUS AGES*

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Tumor immunity has been attributed to incompatibility between tumor antigens and the host. This incompatibility is due to strain-specific tumor antigens, as well as tumor-specific antigens. These antigens are unstable in some cases and undetectable after several generations of transplantation (1) or after storage of the tumors at low temperature (2).

There are, at present, a few established tumors in this category; e.g., Ehrlich ascites tumor, which is not only lethal to all strains of mice, but also transplantable in rate for many generations (3). Furthermore, induction of immunity against a spontaneous tumor is difficult to achieve in animals of the strain in which the tumor originates. This is because the antigens that these tumors contain, although strain-specific, are not tumor-specific. In the case of the Ehrlich tumor, even the isoantigens appear to be destroyed or masked in transplantation, so that many efforts have been unsuccessful in the induction of immunity to this tumor.

Recently there have been two reports of the successful development of immunity after treatment of mice with X-irradiated tumor cells (4, 5). The immunity appeared to be more definite in Swiss mice than in the C57BL strain, indicating possible influence of isoantigens of the treated tumor cells in the induction of immunity in the recipients, as genetic purity is not characteristic of Swiss mice.

The experiments reported in the present paper were carried out in order to detect any role that isoantigens may play in immunity to the Ehrlich tumor, and to determine whether the effects of sonic vibrations on tumor cells are similar to those of X radiation. Mice at differing age levels were used in order to reveal effects of age on the pathogenicity of the tumor and on the acquisition of immunity.

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Acquired Immunity to Ehrlich Ascites Tumor in Mice After Treatment with Fresh or Sonized Tumor Cells at Various Ages

MATERIALS AND METHODS

Two strains of mice, CF1 and C57BL/Ka, were used in the experiments. The former are a stock of Swiss mice bred in this laboratory for many years. The latter were kindly offered by Dr. H. Kaplan's laboratory.

The Ehrlich ascites tumor has been propagated in CF1 mice at this laboratory for several years. Tumor cells removed from fresh ascites of tumor-bearing mice were centrifuged at 600g several times, and were then resuspended in a 5-fold volume of Tryode's solution. Finally the cell suspension was put into plastic tubes and was subjected to sonic vibration at 10 KC, 1.5 A, 6 to 8 times, for 1-minute periods with 4-minute intervals.

The sonized cell suspensions were injected intravenously or intraperitoneally into mice at various ages (Table 1). The intravenous injections were performed in newborn mice through an orbital branch of the anterior facial vein (6), in sucklings through the femoral vein, and in adults through the caudal vein.

Lymphoid cells teased from spleens and lymph nodes of normal adult GF1 mice were prepared for the immunization of animals in the same manner, but without the use of sonic vibration.

The mice pretreated with a single application of these materials were challenged with an intraperitoneal injection of Ehrlich tumor cells 4 weeks after the immunization. The animals surviving from the first challenge were tested for immunity again by second and third injections, at 4-week intervals. So that the pathogenicity of the tumor cells would be the same for every challenge, fresh ascites diluted 1:3 with Tyrode's solution was separately poured into more than 100 tubes and kept at -70°C. with dry ice, and 0.2 ml of ascites restored from this stock was injected intraperitoneally into every mouse, whether immunized or control.

For histological examination, suitable organs were removed at autopsy from the mice killed by the tumor. After fixation of the organs with formalin, the tissues were sectioned in paraffin blocks and stained with hematoxylin and eosin.

RESULTS

Pathogenicity of the fresh or restored tumor cells to mice at various ages.

All adult mice were killed by progressive ascites tumor, with an average survival time of 17.5 days after intraperitoneal injection of fresh tumor cells. Restored cells showed almost the same pathogenic activity as did fresh cells. The slight difference in activity between these two types of cells, as shown in Table 1, might be
Table 1. Treatment of Mice at Various Ages with Fresh or Sonized Ehrlich Tumor Cells.

<table>
<thead>
<tr>
<th>Age at treatment</th>
<th>Injected material</th>
<th>Route of inject.</th>
<th>Amount (ml)</th>
<th>Total No. of mice</th>
<th>No. of deaths</th>
<th>Average survival (days)</th>
<th>Type of disease</th>
<th>No. surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>Fresh E.C.</td>
<td>iv</td>
<td>0.1</td>
<td>22</td>
<td>0</td>
<td>17.4</td>
<td>A</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>0.1</td>
<td>20</td>
<td>20</td>
<td>19.4</td>
<td>L</td>
<td>4</td>
</tr>
<tr>
<td>Suckling</td>
<td>Fresh</td>
<td>iv</td>
<td>0.03</td>
<td>14</td>
<td>10</td>
<td>14.0</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>0.03</td>
<td>10</td>
<td>10</td>
<td>13.3</td>
<td>L**</td>
<td>0</td>
</tr>
<tr>
<td>Newborn</td>
<td></td>
<td>iv</td>
<td>0.01</td>
<td>18</td>
<td>18</td>
<td>12.3</td>
<td>L**</td>
<td>0</td>
</tr>
<tr>
<td>Adult</td>
<td>Sonized E.C.</td>
<td>ip</td>
<td>0.5</td>
<td>28</td>
<td>6</td>
<td>20.3</td>
<td>A</td>
<td>22</td>
</tr>
<tr>
<td>Suckling</td>
<td></td>
<td>ip</td>
<td>0.2</td>
<td>15</td>
<td>13</td>
<td>15.2</td>
<td>A</td>
<td>2</td>
</tr>
<tr>
<td>2–3 days</td>
<td></td>
<td>iv</td>
<td>0.1</td>
<td>28</td>
<td>25</td>
<td>0.5</td>
<td>S</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>0.1</td>
<td>32</td>
<td>16</td>
<td>11.7</td>
<td>L+A**</td>
<td>16</td>
</tr>
<tr>
<td>12 hrs</td>
<td></td>
<td>iv</td>
<td>0.05</td>
<td>67</td>
<td>67</td>
<td>0.5</td>
<td>S</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>0.05</td>
<td>66</td>
<td>40</td>
<td>11.7</td>
<td>L**</td>
<td>26</td>
</tr>
</tbody>
</table>

# A (ascites tumor), L (lung tumor), S (shock).
* Many of the mice also developed subcutaneous tumors at the site of injection.
** Some of the mice died from shock.

due to differences in the sources of the ascites and in the numbers of active tumor cells.

The injection appeared to be more intensively toxic to newborn mice. The average survival after treatment was 11.3 days for newborn, but 17.4 days for adults. The contrast is significant, especially since the number of tumor cells injected into newborn was smaller, being in proportion to body weight. The difference in the histological findings was even more significant. Examination of the newborn at autopsy showed abundant tumors in the lung. There were no macroscopic lesions outside the lung, but histological examination demonstrated invasion of tumor cells into the peritoneum, pancreas, liver, and kidneys. Intravenous injection of fresh tumor cells into adult mice did not result in either symptoms of illness or microscopically demonstrable lesions, but was occasionally followed by the development of small subcutaneous indurations at the site of injection. A similar injection of a reduced amount of tumor cells into newborn resulted in all treated animals dying from lung tumors. Besides the lung tumors, small invasions of tumor cells were demonstrated microscopically in the liver and kidneys.

The pathogenicity of the tumor to suckling mice was intermediate between that to adult and newborn. Average survival time of mice receiving the tumor at ages of 12 to 14 days were 14.0 days after intraperitoneal injection and 19.4 days after...
intravenous injection. Intraperitoneal injection was followed by death from progressive ascites, and intravenous injection by death from lung tumors. Large tumors were also produced at the site of the intravenous injection, but histological examination showed that it was the lung tumors that were responsible for the death of the mice.

In these experiments, young mice appeared to be more sensitive than old mice to the Ehrlich tumor, and mice of any age were more sensitive to the tumor cells injected intraperitoneally than intravenously. Furthermore, it was demonstrated that the most susceptible tissue in mice changes from the lung to the peritoneum with increasing age.

**Toxicity of sonized tumor ascites.**

After application of sonic vibration to ascites for one minute, cells were observed to aggregate, but only a small number of the cells were disrupted. Application of the vibration for five one-minute periods resulted in almost complete destruction of the cells, only cell fragments being demonstrated under the microscope. Intraperitoneal injection of the fragments, however, sometimes induced progressive tumors in adult mice, although the survival time of 6 mice dying of such tumors was markedly prolonged, as table 1 shows.

<table>
<thead>
<tr>
<th>Strain of donor</th>
<th>Material</th>
<th>Preparation</th>
<th>Route of inject.</th>
<th>Strain of recipient</th>
<th>Age at immuniz.</th>
<th>No. of mice at first challenge</th>
<th>Deaths after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFl</td>
<td>Ehrlich</td>
<td>Fresh</td>
<td>iv</td>
<td>CFl</td>
<td>Adult Suckling</td>
<td>22</td>
<td>16 (14.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>16 (14.0)</td>
</tr>
</tbody>
</table>

| CFl            | Ehrlich  | Sonized     | ip               | CFl                 | Adult Newborn  | 16 (15.9)                   |
|                |          |             |                  |                     |                | 23 (16.5)                   |

| CFl            | Ehrlich  | Fresh       | ip               | C57BL               | Adult Suckling | 2                           |
|                |          |             |                  |                     |                | 6 (15.0)                    |

| CFl            | Lymphoid | Fresh       | ip               | C57BL               | 14 days        | 2                           |
|                |          |             |                  |                     |                | 12 (18.2)                   |

* Numbers in parenthesis are average survival (in days) for mice that died.

After 6 to 8 one-minute periods of vibration, the suspension appeared non-pathogenic to adult mice, but still toxic to young mice. Newborn receiving intra-
peritoneal injections of the suspension were eventually killed by ascitic tumors. Among mice receiving such injections at the age of 2 or 3 days, many died of lung tumors and some died of ascites. In this group of mice, deaths due to lung tumors occurred within an average of 13 days after the injection, and deaths due to ascites after an average of 18 days.

Intravenous injection of the sonized ascites into newborn mice was followed by death due to shock. All treated mice became less active immediately after the injection, and died within 12 hours. Similar injections into 2- or 3-day-old mice were also followed by death due to shock, though some of the mice survived as long as 4 days. Death due to shock also occurred in some of the mice after intraperitoneal injection of the suspension.

Immunity in CF1 mice pretreated with fresh or sonized tumor ascites from other CF1 mice.

All CF1 mice surviving from previous injection of sonized CF1 ascites were killed by the first challenge injection of the tumor. No prolonged survival time was observed in these groups of mice; indeed, the pretreatment seemed to enhance the growth of the tumor. In contrast, some of the mice pretreated by intravenous injection of fresh tumor cells showed resistance to one or more challenges. Evidently induction of immunity in adult CF1 mice by means of CF1 tumor antigens is possible to some extent only with living tumor cells, and not with sonized cells or cell fragments.

Immunity induced in C57BL mice by pretreatment with CF1 tumor.

All C57BL mice surviving from intravenous injection of fresh CF1 tumor cells were resistant to challenge injections. In some mice, the first challenge induced a slight accumulation of ascites, which appeared on the 7th day of the challenge, reached a maximum on the 12th day, and disappeared after that. No accumulation of ascites was seen after the second or third injection.

About half of the mice surviving from injection of sonized CF1 tumor were resistant to the first challenge. Some of the mice that were resistant to the first challenge were killed by the second challenge.

Immunity in C57BL mice pretreated with lymphoid cells from CF1 mice.

Lymphoid cells from CF1 mice were also effective in furnishing C57BL mice with immunity. Such pretreatment was almost fully effective for 14-day-olds, less
effective for 7-day-olds, and ineffective for 2-day-olds. The survival time of the mice dying in these three groups seemed to be prolonged to some extent, showing induction of incomplete immunity.

**DISCUSSION**

Many studies have shown that induction of tumor immunity in mice of the strain in which the tumor originated was possible for newly induced tumors, but was extremely difficult for spontaneous tumors. The difficulty has been considered to be due to lack of antigenicity or to masked antigens in the tumors. Transplantation of X-ray-inactivated tumor cells was successful in detecting the isoantigenicity of the tumors (7, 8). X-irradiated tumor cells have also been used successfully for induction of immunity to Ehrlich ascites tumor (4, 5). Irradiation is known to reduce the activity of the tumor cells (9) and to induce the development of slow-growing cells considered responsible for the induction of tumor immunity (5). It is well known that strangulation or resection of tumors is able to induce immunity in tumor-bearing animals. Immunity has sometimes been observed in animals receiving inadequate implantations of tumors. Slow-growing cells may also play a role in the induction of these latter types of immunity.

The experiments reported in this paper were successful in producing immunity to Ehrlich tumor by pretreatment of homologous animals with fresh or sonized tumor cells. Repeated applications of sonic vibration reduced the activity of tumor cells markedly, but the sonized ascites still contained living cells, since injection of the materials induced progressive ascites or lung tumors in newborn animals. Presumably the immunity induced after injection of sonized ascites may be attributed to slow-growing cells. The immunity also was produced even in homologous animals to some extent, when they were pretreated with a large quantity of fresh tumor cells through an inadequate route for transplantation of the tumor. These experiments suggest importance of living tumor cells in the sensitizing materials. Why living cells are indispensable to the acquisition of tumor immunity, whereas pretreatment with dead cell materials enhances tumor growth (10), remains unexplained. It should also be pointed out that the induced immunity may be relatively nonspecific (11). These problems are analogous to those presented by some bacterial immunities.

In any event, injection of large amounts of living cells seems to be essential to the induction of tumor immunity, and this principle was taken into account in the present study through the use of tumor cells of low virulence, obtained by means of sonic vibration, or by administering the cells by an inadequate route, namely
intravenous injection in the case of adult mice. This study failed to demonstrate iso-immunity in CF1 mice, although the possibility of achieving such immunity through the intravenous injection of tumor cells showed some promise. In contrast, the iso-antigenicity of CF1 tumors was established in C57BL mice, which exhibited rather definite immunity after injection of fresh or sonized tumors. Pretreatment of C57BL mice with large amounts of CF1 lymphoid cells was also effective in inducing tumor immunity, but not in very young mice. Presumably the nonspecific pathogenicity of the Ehrlich tumor may be due to rapid growth rather than to lack of isoantigenicity.

Furthermore, the present study has demonstrated a specific susceptibility of young mice to the Ehrlich tumor. Newborn mice developed significant lung lesions after either intravenous or intraperitoneal injection of the tumor. The high susceptibility of the lung in the newborn may extend to other tumors.

**SUMMARY**

Homologous immunity to Ehrlich ascites tumor was induced in C57BL mice after treatment with CF1 tumor or lymphoid cells. The induction of tumor immunity was considered to result from long-lasting antigenicity in CF1 cells of low virulence. A possibility of producing iso-immunity in CF1 mice was shown to exist after intravenous injection of large amount of CF1 tumor cells. Finally, a high sensitivity to the Ehrlich tumor was demonstrated in the lungs of young mice.

**REFERENCES**