SUPPRESSION OF CELL-MEDIATED IMMUNITY THROUGH IMMUNE SPLEEN CELLS AGAINST EHRlich ASCITES TUMOR

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ABSTRACT

The adoptive transfer of immunity to Ehrlich ascites tumor was accomplished during the first two weeks in a syngeneic murine system by the intraperitoneal administration of spleen cells obtained from mice which had been hyperimmunized through three subcutaneous inoculations with the tumor. The resistance induced in this system was noted during first two weeks after tumor challenge, and thereafter there was an increased tendency for the tumor to become enhanced. Serum from mice which had previously contacted with Ehrlich ascites tumor showed enhancing effect on the subcutaneous growth of the tumor to a certain degree. Anti-tumor effect of spleen cells from various donors was examined by a simple method involving intraperitoneal injection of tumor cells and spleen cells which had been mixed and incubated for a short period of time. The results seem to indicate that suppression of tumor growth was induced by cell-mediated immunity through injected immune spleen cells, and that enhancement of tumor growth depended basically upon the ability of humoral antibodies which apparently had been produced through transferred spleen cells to suppress the development of cell-mediated immune response.

INTRODUCTION

The cells of the spleen, the regional lymphnodes, the thoracic duct lymph, and the peritoneal surface in inbred rodents have been shown by several investigators to inhibit the growth of the transplantable tumor to which the donor animal had been exposed. Old et al. reported what appears to be the first successful accomplishment of genuine adoptive transfer of immunity to a chemically induced tumor in an inbred mouse system by the intravenous administration of hyperimmune spleen cells. It has been demonstrated by many authors that pretreatment of animals with humoral antibodies directed against foreign tumor homografts efficiently suppresses the development of cell-mediated immunity detected by various methods. Lottie Kornfeld and W.W.H. Weyzen reported that peritoneal cells and spleen cells from LAF1 mice given three intraperitoneal immunizations of sheep red blood cells synthesized haemagglutinins after transfer to X-irradiated syngeneic recipients, either

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with or without a concomitant injection of antigen. Antibody formation by cells transferred with antigen resembled a secondary antibody response in intact animals. The present experiments were undertaken to study the possibility that animals which had received injection of immune spleen cells might demonstrate antagonistic effects on the subsequent tumor challenge; suppression of the tumor growth by cell-mediated immunity and enhancement of the tumor growth by suppression of cell-mediated immune response.

MATERIALS AND METHODS

Animal. Animals used throughout these experiments were about 60 days old, female SMA\(^6\) mice obtained from the Supplying Center of Laboratory Animals in this medical school. These mice demonstrated indefinite survival of intrastrain skin grafts among randomly selected animals. They were fed with a standard pellet diet and given drinking water \textit{ad libitum}.

Tumor. Tumor cells used in this study were Ehrlich hypotetraploid stock (Kaziwara 4n\(^11\)) maintained in adult female SMA mice through serial intraperitoneal transplantation at 7-day intervals in this laboratory. Fresh peritoneal fluid from a 7-day tumor-bearing mouse was harvested and cell counts were obtained.

Immunization. The female SMA mice were inoculated subcutaneously in their intrascapular regions with \(1 \times 10^5\) viable tumor cells suspended in 0.1 ml of Hanks' balanced salt solution (HBSS). Tumors grew in all mice and they were allowed to grow until 10 days after inoculation, by which time the diameter of the tumor reached to approximately 10 mm. They were, then, removed\(^12-17\) by tight ligation together with the overlying skin. On the 7th day after the ligation these mice received the 2nd inoculation in the middle portion of the back with the same dosage of tumor ascites fluid. None of the second tumors continued to grow, and they regressed completely within two weeks. On the 14th day after the 2nd inoculation, the 3rd inoculation was performed in the lower back of the mice in the same manner. The third tumors regressed.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Induction of immune spleen cell donors. EAT: Ehrlich ascites tumor.}
\end{figure}
sooner than the second. Fourteen days after the last inoculation, these mice were used as immune spleen cell donors. The schedule of immunization is shown in Fig. 1.

**Spleen cell suspension.** The spleen cell donors were killed by cervical dislocation under ether anesthesia, and splenectomy was performed under aseptic conditions. Spleens were trimmed free of fat and minced with scissors in HBSS. Spleen cell suspensions were prepared after tissue fragments were removed by filtration through four layers of Nylon gauze and 5 times of centrifugation. These procedures were carried out at 2-5°C. For the determination of viability of the cells the unstained cell count method of Schrek was employed. The viability of spleen cell suspensions used in this study was more than 90%.

**Serum.** Mice were bled from the ophthalmic venous plexus into heparinized capillary pipets. Blood from mice in each experimental group was pooled and centrifuged at 1,500 x g. The plasma was then removed and stored at −20°C.

**Experimental design.** This study was carried out to evaluate the following three effects:

1. effect of immune spleen cells on the subcutaneous growth of Ehrlich ascites tumor,
2. anti-tumor effect of various spleen cells which have been mixed with tumor cells and incubated for a short period of time, and
3. effect of immune serum on the subcutaneous growth of Ehrlich ascites tumor.

**RESULTS**

**Experiment 1. Effect of immune spleen cells on the subcutaneous growth of solid Ehrlich ascites tumor in mice**

Spleens from immunized animals and those from non-immunized animals were pooled separately, and spleen cell suspensions were prepared as described above. Each of the resulting spleen cell suspensions was brought to a final concentration of 1 x 10^8 viable spleen cells per 1.0 ml.

In the first series of experiments, 20 mice in the test group and 20 mice in the control group were used. Each animal received intraperitoneal injection of 0.2 ml of either immune or nonimmune spleen cell suspension (2 x 10^7 viable spleen cells). Seven days later, all animals were challenged subcutaneously in their back with 1 x 10^6 viable tumor cells. Test and control animals were injected alternately. The tumor cell recipients were inspected and weighed twice weekly. When a tumor was palpable, measurements of its size were made with a caliper in its long axis and in the direction perpendicular to this. The effects of immune spleen cells on the subcutaneous growth of Ehrlich ascites tumor are shown in Fig. 2.
Eleven of 20 mice which had received intraperitoneal injection of immune spleen cells 7 days prior to tumor challenge showed progressive growth of the resulting tumor, whereas only one of 20 mice which had received nonimmune spleen cells showed progressive growth. Seven mice in the test group showed tumor growth within a definite period and following regression, while 19 mice in the control group showed regression after limited growth. The regression in the former was slightly retarded than the latter. Two mice in the test

TABLE 1. Effect of Immune Spleen Cells on the Subcutaneous Growth of Solid Ehrlich Ascites Tumor (on the 7th Day after Challenge)

<table>
<thead>
<tr>
<th>Control group</th>
<th>Test group</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt.–lt. tumor weight (mg)</td>
<td>rt.–lt. tumor weight (mg)</td>
</tr>
<tr>
<td>138–128</td>
<td>10</td>
</tr>
<tr>
<td>140–122</td>
<td>18</td>
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<td>138–120</td>
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<td>142–127</td>
<td>15</td>
</tr>
<tr>
<td>139–122</td>
<td>17</td>
</tr>
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</table>

| average | 14.5 | average | 6.7 |

rt.: weight of right hind pad
injected with $1 \times 10^5$ tumor cells in 0.1 ml of HBSS
lt.: weight of left hind pad
injected with 0.1 ml of HBSS
SUPPRESSION OF CELL-MEDIATED IMMUNITY

The test group failed to develop tumor. On the other hand, none of the control group failed to bear tumor.

In the 2nd series of experiments, 10 mice in the test group and 10 mice in the control group were used. Each animal received intraperitoneal injection of $2 \times 10^7$ immune or nonimmune spleen cells. Seven days later, all these animals were injected in their right hind pads with $1 \times 10^5$ viable tumor cells, and with 0.1 ml of HBSS in their opposite pads. On the 7th day after tumor inoculation, both pads of each animal were amputated at the ankle joints, and were weighed to obtain the tumor weight. It is shown in Table 1. that the tumor weight in the test group, which had received immune spleen cells 7 days prior to tumor challenge, was significantly less than in the control group.

Experiment 2. Effect of various spleen cells on the growth of Ehrlich ascites tumor in the abdominal cavity of mice

Tumor and spleen cells, which had been mixed and incubated for a short period of time, were injected into the intraperitoneal cavity of mice. Spleen cell suspensions from various donors were prepared as described above. All of the resulting suspensions were brought to a concentration of $2 \times 10^7$ viable spleen cells per 0.1 ml of HBSS. Each of these suspensions was mixed with equal volumes of tumor ascites fluid containing $1 \times 10^5$ viable tumor cells in 0.1 ml. The mixtures were vigorously agitated to break up cell clumps and suspend all cells uniformly in HBSS. After incubation for one hour at 37°C, the mixtures were again agitated and immediately injected intraperitoneally into recipient animals. Tumor development was recorded biweekly, and the host's weight was periodically measured. Each test and control group consisted of 10 mice in these experiments.

Each of the test animals in the 1st series received intraperitoneal injection of 0.2 ml of incubated mixture containing $1 \times 10^3$ tumor cells and $2 \times 10^7$ spleen cells obtained from the immune spleen cell donors as described above. Each of the control animals received intraperitoneal injection of the same volumes of incubated mixture which contained $1 \times 10^3$ tumor cells and $2 \times 10^7$ spleen cells from normal mice.

As shown in Fig. 3, 8 mice in the control group succumbed to the mixture, while 2 mice in the test group died of ascites tumor.

Thus anti-tumor effect of immune spleen cells against Ehrlich ascites tumor was successfully shown, using the incubated mixture of tumor cells and immune spleen cells.

Spleen cell donors used for the test group in the 2nd series were animals which had received intraperitoneal injection of immune spleen cells 7 days previously (corresponding to "O" day in Fig. 2). The results are shown in Fig. 4.
†: mice died of ascites tumor

Five mice in the test group succumbed to the intraperitoneal injection of the incubated mixture, while 9 mice in the control group died of ascites tumor. Adoptive transfer of immunity had been accomplished in the donor animals.
for this test group.

Spleen cell donors used for the test group in the 3rd series were animals which had received intraperitoneal injection of immune spleen cells and had subsequently been challenged with $1 \times 10^5$ viable tumor cells 10 days before splenectomy (corresponding to "10" day in Fig. 2). The donors for the control group had been injected with nonimmune spleen cells. The results are shown in Fig. 5.

![Fig. 5. Effect of spleen cells on the growth of Ehrlich ascites tumor in the abdominal cavity.](image)

Indicated by body weight of recipient animals in the 3rd series of Experiment 2.

†: mice died of ascites tumor

Six mice in the test group and 3 mice in the control group died of ascites tumor. Anti-tumor effect of the spleen cells used in the test group was less efficient than that of the cells in the control group.

**Experiment 3. Effect of serum on the subcutaneous growth of solid Ehrlich ascites tumor in mice**

Throughout the experiments in these series, test and control groups consisted of 5 mice respectively.

Each animal of the test group in the 1st series received intraperitoneal injection of 0.2 ml of the serum which was obtained from the immune spleen cell donors. Serum for the control animals was obtained from non-immunized mice. Four hours after serum administration these animals were inoculated with $1 \times 10^5$ viable tumor cells. Growth curves of the resulting tumors are shown in Fig. 6. Enhancement of the tumor growth was marked in the test animals.
FIG. 6. Effect of serum on the subcutaneous growth of solid Ehrlich ascites tumor. Each curve indicates the average diameter of tumor in recipient animal in the 1st series of Experiment 3.

The serum donors used for the test group in the 2nd series were animals which had received intraperitoneal injection of $2 \times 10^7$ immune spleen cells 7 days before bleeding (corresponding to "O" day in Fig. 2). The serum donors for the control group had been injected with nonimmune spleen cells. As shown in Fig. 7, no significant differences were observed between the test and control groups.

The serum donors for the test animals in the 3rd series were mice that had received the immune spleen cells 7 days before bleeding, and that
had received a booster of $5 \times 10^7$ viable tumor cells subcutaneously on the day just before bleeding. Serum donors for the control animals had received non-immune spleen cells and had been injected with $5 \times 10^7$ viable tumor cells. As shown in Fig. 8, the tumor growth in the test animals was slightly enhanced.

**DISCUSSION**

The cells of the spleen obtained from immunized animals have been shown by several investigators$^1$–$^4$, $^{20}$–$^{23}$ to inhibit the growth of the specific tumor to which the donor animal has been exposed. Yoshida and Southam$^3$ have recently reported a study in an autochthonous system, in which a 20-methylcholanthrene-induced tumor and the autochthonous host's spleen were excised, mixed in the 1 : 6 to 1 : 6,000 tumor spleen cell ratio, and injected back into the original host. Delorme and Alexander$^4$ reported nearly complete inhibition of growth of the first transplant generation benzpyrene-induced tumor in inbred rats when the tumor and immune spleen cells were mixed in a 1 : 200 ratio and given subcutaneously.

In the present experiments where the effects of immune spleen cells on the subcutaneous growth of Ehrlich ascites tumor were observed, the tumor : immune spleen cell ratio was 1 : 200. Intraperitoneal injection of spleen cells was performed 7 days prior to challenge of the recipient animals with tumor cells. Inhibition of the tumor growth was observed during the first two weeks after tumor challenge (Fig. 2 and Table 1).

The experiments using incubated mixture of tumor cells and spleen cells
obtained from mice which had received immune spleen cells 7 days before harvest also showed anti-tumor effect of the donors spleen cells (Fig. 4). The tumor: spleen cell ratio employed here was 1:20,000.

These results demonstrate that adoptive transfer of immunity has been accomplished in the animals which had received immune spleen cells 7 days previously. Anti-tumor effect shown in Fig. 4 seems to be induced not directly by the injected cells but indirectly by lymphoid cells derived from or proliferated from the injected spleen cells.

As shown in Fig. 2, the tumor growth in the test animals was enhanced in the later period.

Anti-tumor effect of the spleen cells harvested from mice which had received injection of immune spleen cells and had subsequently been challenged with tumor cells 10 days prior to splenectomy was less than that of the spleen cells from control animals (Fig. 5).

These results seem to indicate that treatment with immune spleen cells prior to tumor challenge inhibits the development of an efficient cell-mediated immune response in recipient animals.

Snell et al. demonstrated that passively transferred isoantibodies inhibited the development of an efficient cell-mediated immune response in recipients of allogeneic tumor grafts, and that lymphoid cells derived from antibody treated recipients were less efficient in inhibiting the tumor growth.

Several investigators reported that peritoneal cells or spleen cells from immunized mice produced antibodies after transfer to syngeneic recipients, either with or without a concomitant injection of antigen.

Serum from the mice which had received intraperitoneal injection of the immune spleen cells 7 days before bleeding did not show an increased tendency for the tumor to become enhanced (Fig. 7). This result, however, cannot completely exclude the existence of an enhancing antibody produced through injected spleen cells.

The serum from the mice that had received injection of the immune spleen cells and that had subsequently been challenged with tumor cells on the day shortly before bleeding significantly enhanced the subcutaneous growth of Ehrlich ascites tumor as well as the serum from the immune spleen cell donors (Fig. 6 and Fig. 8).

These results strongly suggest that enhancement of the tumor growth in this study was induced by humoral antibody which had, presumably, been produced through transferred spleen cells soon after tumor inoculation.

Thus, the findings described above support the hypothesis that suppression of tumor growth is induced by cell-mediated immunity through injected immune spleen cells, and that enhancement of tumor growth depends basically upon the ability of humoral antibodies which might be produced through transferred
spleen cells to suppress the development of cell-mediated immune response. Since enhancement appears to be important for the progressive growth of incompatible tumor cells in immunologically competent hosts, it may represent a mechanism by which autochthonous tumors possessing tumor-specific antigens may develop and grow progressively to the death of the hosts.

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REFERENCES

13) Mitchison, N. A., Studies on the immunological response to foreign tumor transplants in the mouse I. The role of lymph node cells in conferring immunity by adoptive
31) Weiler, E., Delayed antibody synthesis in mice after transfer of immune peritoneal fluid cells, Immunology, 7, 197, 1964.