In vivo Growth Inhibition of Transplantable Marek's Disease Lymphomas by Monoclonal Antibodies against Surface Antigens on Marek's Disease Lymphoblastoid Cell Line

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Marek's disease (MD) is a lymphoproliferative disease of chickens caused by a herpesvirus [17]. Lymphoblastoid cell lines derived from MD lymphoma express MD tumor-associated surface antigens (MATSA) and other antigens [1, 2, 9, 11, 14, 15, 16, 18]. However, the importance of these antigens in the induction of the immune response against tumor cells or in the development of the MD tumor is not fully understood.

To analyze the role of the tumor cell surface antigen in the development of MD tumor, we tried to examine the effect of monoclonal antibodies against MD tumor cell surface antigens on the growth of MD tumor cells in vivo.

A transplantable MD cell line, MDCC-MSB1 clo.18 (Clo. 18) derived from MDCC-MSB1 [7], was inoculated into the LMG-line of specific-pathogen-free White Leghorn chickens. The chickens were kindly supplied from the Aburahi Laboratories, Shionogi & Co., Ltd., Shiga, Japan. Avian leukemia-derived cell line, LSCC-CU10 (CU10) [3] was also used for the experiments.

Monoclonal antibodies, 2B9, 2D8 and 2C12 were obtained from hybridomas produced by the fusion of P3-X63-Ag8.653 cells and spleen cells from BALB/c mice immunized with MDCC-MSB1 cells [8, 9]. The antibody 2B9 is specific for the MATSA on MDCC-MSB1 cells and the antibody 2D8 is reactive with the MATSA expressed on some MD lymphoma-derived cell line cells [8]. The antibody 2C12 is reactive not only with MD tumor cells but also with normal chicken thrombocytes [9]. These antibodies were obtained from the ascitic fluids of mice inoculated with the hybridomas and the undiluted ascitic fluids were used throughout the experiment. The percentages of 2B9-, 2D8- and 2C12-positive cells in cultured Clo.18 cells were 90%, less than 10% and 89% respectively, when examined by the indirect membrane fluorescent antibody test using undiluted ascitic fluids.

The experiments were designed to know the effect of monoclonal antibodies on the growth of the transplantable tumors in chickens.

In the first trial, the Clo.18 cells (1×10⁷ viable cells/bird) were inoculated subcutaneously into the wing-webs of 18 one-day-old chickens. Six of the chickens were intraperitoneally injected with the antibody 2B9 (0.1 ml/bird) at the same time and then further injected 6 times with the antibody at two-day intervals. Another 6 chickens were treated with the antibody 2D8 in the same manner. The remaining 6 chickens inoculated only with the Clo.18 cells at one day of age were kept as controls. The length (l), width (w) and height (h) of tumors at the inoculation sites were measured at 10, 14, 19, 21 and 24 days post inoculation (PI). The volume (V) of the tumors was calculated by means of the formula for the volume of a hemiellipsoid, the form most nearly approximating the shape of the tumors: \[V = 0.5236lwh\] [7]. The Student's t-test was used for evaluation of statistical difference. All the surviving chickens were killed at 24 days PI. During necropsy metastatic lesions were macroscopically checked on each organ. The results of the trial are shown in Table 1. The volume of the tumor in antibody-treated chickens was a little smaller than that of the control chickens, but statistical difference was observed only in the 2D8-treated chickens at 14 days PI (p<0.01). During experimental periods, 5 chickens in the 2B9-treated group, 2 chickens in the 2D8-treated group and 4 chickens in control group died of MD tumor. Upon necropsy, only one 2D8-treated chicken had no metastatic lesions. Other 17 chickens had metastatic lesions in various

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organs and obvious differences were not observed in the metastases between the antibody-treated chickens and the control chickens. Such results suggest that the monoclonal antibodies against MATSAs might play some effective role in tumor immunity, but not enough for the inhibition of the tumor growth.

In the second trial, 11 one-day-old chickens were inoculated with the same amount of the Clo.18 cells as in the first trial. Four of the chickens were further treated with the same amount of antibody 2C12 as described above. Other 3 chickens were treated 7 times with the double amount of antibody 2C12 (0.2 ml/bird) at two-day intervals. The remaining 4 chickens were kept as controls. At 21 days PI, the survival rate of chickens in each group was compared, and the volume of the tumor was calculated. During the experiment, 2 control chickens died at 18 days PI and the other 2 chickens died at 21 days PI. The volume of the tumor in the chickens died at 21 days PI was 24.9 cm³ and 19.0 cm³, respectively. All of the control chickens had metastatic lesions. In the 2C12-treated chickens (0.1 ml/bird at a time), one died at 14 days PI. But other 3 chickens were still alive at 21 days PI, and volume of each tumor was 13.4 cm³, 9.4 cm³ and 5.5 cm³. These 3 chickens had no metastatic lesions at necropsy. In the chickens inoculated each time with the double amount (0.2 ml) of antibody 2C12, each one of the chickens died at 17 days PI and at 19 days PI. Another chicken was still alive at 21 days PI with no metastatic lesions and the volume of the tumor in this chicken was 6.4 cm³. These results indicated that the antibody 2C12 had an inhibitory effect on the Clo.18 tumor growth in vivo, therefore, further study was carried out to confirm the effect of the antibody 2C12.

In the third trial, 22 one-day-old chickens were inoculated with the same amount of the Clo.18 cells as in the first trial. Fifteen of the chickens were further treated with antibody 2C12 in the same manner as described in the first trial. The remaining 7 chickens were kept as controls. The size of the tumor was measured at 13, 17, 20 and 23 days PI. All the surviving chickens were killed at 23 days PI. The significant differences in the volume of tumor were observed between 2C12-treated chickens and untreated control chickens at 13, 17, 20 and 23 days PI (p<0.001 or p<0.01) (Table 2). On day 23 PI, all control chickens had died but 9 2C12-treated chickens were still alive. At necropsy, all of the control chickens had metastatic lesions in various organs, whereas 2 of the 2C12-treated chickens had no metastasis. Metastatic lesions observed in other 7 2C12-treated chickens were much milder than those of the control chickens. From these results, the significant inhibition of Clo.18 tumor growth in 2C12-treated chickens was demonstrated. Although the growth of the Clo.18 tumors was often different in individual chicken [6, 7], Clo.18 tumors observed in the 2C12-treated chickens were much smaller than those of the control chickens in the third trial. Furthermore, the rate of the chickens which showed metastatic lesions was lower in 2C12-treated chickens. These observations suggest that 2C12 inhibited the growth of Clo.18 tumor to a certain extent and correlated well with our previous studies about the in vitro growth inhibition of MD lymphoma cells by 2C12 [10].

In the fourth trial, the CU10 cells (1×10⁷ viable cells/bird), which do not react with the antibody 2C12 [9], were inoculated sub-

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<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Control chickens</th>
<th>2B9-treated chickens</th>
<th>2D8-treated chickens</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.9±0.4a (6)</td>
<td>0.7±0.2 (6)</td>
<td>0.7±0.2 (6)</td>
</tr>
<tr>
<td>14</td>
<td>4.8±2.1 (6)</td>
<td>3.2±1.1 (6)</td>
<td>1.8±0.6b (6)</td>
</tr>
<tr>
<td>19</td>
<td>11.0±2.4 (6)</td>
<td>8.9±2.1 (6)</td>
<td>9.4±2.8 (6)</td>
</tr>
<tr>
<td>21</td>
<td>11.7±3.9 (4)</td>
<td>10.6±3.5 (3)</td>
<td>11.9±3.1 (6)</td>
</tr>
<tr>
<td>24</td>
<td>16.4 (2)</td>
<td>16.6 (1)</td>
<td>16.0±1.5 (4)</td>
</tr>
</tbody>
</table>

a) Mean ± standard deviation.
b) Number of the chickens measured the size of the tumor at the experimental time.
c) Significantly different from the control chickens (p<0.01).
Table 2. The effect of 2C12-treatment on the in vivo development of MDCC-MSBi clo. 18 tumor

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Control chickens</th>
<th>2C12-treated chickens</th>
<th>p value⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume of tumor (cm³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3.5±0.8⁵) (7)</td>
<td>1.5±0.8 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>17</td>
<td>7.9±2.7 (7)</td>
<td>3.9±1.3 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>20</td>
<td>13.0±3.6 (7)</td>
<td>6.4±2.5 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>23</td>
<td>26.6±9.3 (4)</td>
<td>11.1±3.9 (9)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

a) P values were determined by Student’s t-test.
b) Mean ± standard deviation.
c) Number of the chickens measured the size of the tumor at the experimental time.

d) p values were determined by Student’s t-test.

Table 3. The effect of 2C12-treatment on the in vivo development of LSCC-CU10 tumor

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Volume of tumor (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control chickens</td>
</tr>
<tr>
<td>12</td>
<td>0.8±1.0⁶) (4)</td>
</tr>
<tr>
<td>16</td>
<td>2.2±0.7 (4)</td>
</tr>
<tr>
<td>18</td>
<td>3.1±2.4 (3)</td>
</tr>
</tbody>
</table>

a) Mean ± standard deviation.
b) Number of the chickens measured the size of the tumor at the experimental time.

cutaneously into the wing-web of 15 two-day-old chickens. Seven chickens were further treated with the antibody 2C12 in the same manner as described in the first trial. The size of the tumor was measured on days 12, 16, and 18 PI. All the surviving chickens were killed at 22 days PI. In the trial, 3 out of 8 of the control chickens and 3 out of 7 of 2C12-treated chickens had tumors. The volume of the tumors is shown in Table 3. In 2C12-treated chickens, one died on day 12 PI and the other 2 died on day 18 PI. On the other hand, one control chicken died on day 16 PI and another died on day 18 PI. The remaining chicken with tumors was still alive on 22 days PI. There were no significant differences in the rate of tumor formation or the growth of the tumors between the control and the 2C12-treated chickens. If the inhibition of the Clo.18 tumor growth was caused by the reaction of the antibody 2C12 with thrombocytes in vivo, the inhibition of the CU10 tumor growth might be expected. Therefore, the effect might be caused by the reactivity of the antibody 2C12 with the tumor cells.

Previously, Masui et al. [13] reported that monoclonal antibodies against receptors for epidermal growth factor from A431 cells (a human epidermoid carcinoma cell line) inhibited tumor formation in athymic mice by A431 cells and/or another human epidermoid carcinoma. Another report suggests that the monoclonal antibody against transforming growth factor-α inhibited the human lung adenocarcinoma cell lines (A-549 and POC-9) in vitro [12]. If growth factors such as transforming growth factor α and β or their receptors such as those for epidermal growth factor are present on Clo.18 cells as suggested in the mammalian systems [4, 12, 13], the blocking of these factors or receptors by antibodies might result in the partial inhibition of tumor growth. The mechanism of in vitro growth inhibition of MD tumor cells by the antibody 2C12 [10] could also be explained by this hypothesis. However, the exact mechanism of the inhibition of MD tumor growth by the antibody 2C12 was not known. Further studies are necessary to clarify the role of thrombocyte-associated antigen present on MD tumor cells in tumor immunity of chickens.

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