Effector Mechanism in Antitumor Activity of Monoclonal Antibodies Produced against an Ascitic Mouse Mammary Tumor

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ABSTRACT

Therapeutic effects of monoclonal antibodies with different immunoglobulin classes, detecting the same antigenic determinant of tumor specific antigen expressed on ascitic mouse mammary tumor MM46, were examined. With i.v. administration of γ2a, γ2b, or γ1 antibody we were able to keep a significant proportion of mice tumor free against i.p. inoculation of 5 × 10^6 MM46 cells with doses as small as 0.5 μg, but with administration of μ, γ3, or α antibody we were not able to keep mice tumor free with doses up to 5 μg. With 200 μg of antibody, however, an antitumor effect was observed even with μ or γ3 antibody, although α antibody still showed no effect at all. The therapeutic effect of γ2a was further examined in mice challenged with an increasing dose of tumor cells, and a significant effect was demonstrated against 1 × 10^6 cells with 200 μg of antibody but not against 5 × 10^6 cells.

To assess the effector cells in antitumor activity of antibody in vivo, a histological examination of the tumor cells treated with each class of antibody was carried out. The tumors treated by γ2a antibody revealed a remarkable cell infiltration consisting predominantly of mononuclear cells, whereas those treated by μ, γ3, or α antibody did not. The tumor cells treated by γ1 or γ2b antibody showed a moderate cellular reaction. Next, a Winn assay was carried out in athymic C3H/HeNu-nu/nu mice using γ2a antibody. A significant antitumor effect was observed even in those mice, indicating that T-cells are not predominant effector cells. The role of macrophages was studied in mice treated with two macrophage toxic agents, carrageenan and silica particles. These agents were shown to reduce the antitumor effect of γ2a antibody in both Winn assays and therapeutic experiments. Thus, histological examination and the blocking effect of macrophage toxic agents suggested the participation of host macrophages as effector cells in antibody-mediated tumor cell suppression in vivo.

INTRODUCTION

Tumor-specific transplantation antigen of ascitic mouse mammary tumor of C3H/He was first reported by Nishioka et al. (1) and Takeuchi et al. (2), and the antigen detected by the antiserum produced by syngeneic immunization was reported to be expressed only in ascitic mammary tumor but not in normal mammary gland of C3H/He mice. Chang et al. (3) reported, however, that this antigen was also expressed as an alloantigen of lymphoid cells of some inbred mice other than C3H/He, and this anomalous alloantigen on tumor cells was designated as MM antigen. We previously produced monoclonal antibodies against MM antigen positive tumor MM46 and reported that the MM antigen system consists of at least two antigens; i.e., the one designated MM1, which has characteristics quite similar to MM antigen of Chang et al. (3) is closely associated with or identical to Ly-6.2 alloantigen, while the other MM2 is restricted to MM antigen positive tumors so far examined (4, 5).

The effect of antiserum treatment on tumor growth has been studied by many investigators (6, 7), and the immunoglobulin class of the antibodies has been assumed to be one of the important factors affecting the results of passive tumor therapy (8, 9). Since the advent of hybridoma technology, unlimited quantities of defined specificity with a single immunoglobulin class can be produced (10). The availability of antibodies as such allowed a systematic investigation of the efficacy of the different immunoglobulin classes in the antitumor activity in vivo. The previous study (4) showed that a group of 8 antibodies detecting the same antigenic determinant on MM1 antigen was found to cover major classes of mouse immunoglobulin and that the significant antitumor effects of the antibodies examined by serological tumor neutralization assay (Winn assay) were observed with γ2a, γ2b, or γ1 antibody but not with γ3, μ, or α antibody. Furthermore, attempts have been made to analyze the mechanism of the in vivo antitumor effects by studying the in vitro cytotoxic activity of the antibodies. The present investigators demonstrated a good correlation between ADMC and in vivo antitumor effects of the antibodies with regard to immunoglobulin class and suggested an important role of macrophages as effector cells.

In the present study, antitumor effects of monoclonal antibodies with different immunoglobulin classes were further examined by therapeutic experiments. In addition, the participation of host effector cells was studied by histological examination of tumors treated by antibody, and the possible involvement of macrophages in antibody-mediated tumor cell suppression in vivo was investigated by administration of macrophage toxic agents.

MATERIALS AND METHODS

Mice. Seven- to 8-week-old C3H/HeN mice were purchased from Charles River (Atsugi, Japan) for the present experiments. C3H/HeN-nu/nu mice were kindly provided by Dr. M. Saito of the Central Institute for Experimental Animals (Kawasaki, Japan).

Ascitic Mammary Tumor. The MM1 and MM2 antigen positive MM46 tumor (4) was kindly provided by Dr. T. Tachibana of Tohoku University, Sendai, Japan and was maintained by in vivo passage in C3H/HeN mice.

Monoclonal Antibodies. Monoclonal antibodies against MM46 tumor cells were produced, and the serological analysis was described previously (4, 5). These antibodies with different immunoglobulin classes used for the present therapeutic experiments were shown to detect the same antigenic determinant of MM1 antigen by binding inhibition assay and were the same as the ones used previously for the Winn assays (4). The concentration of monoclonal antibodies in nude mouse serum was determined by single radial immunodiffusion (4). Non-immune IgG was prepared as described elsewhere (11).

Therapeutic Effect of Monoclonal Antibody. In order to compare the therapeutic effects of the monoclonal antibodies with different immunoglobulin classes, various doses of each antibody were injected i.v. into C3H/HeN mice which had been inoculated i.p. with 5 × 10^6 of MM46 cells 24 h before and then were observed for 50 days. To investigate the number of tumor cells that can be eliminated with γ2a antibody, which was the most effective in this therapeutic experiment,
 Table 1 Therapeutic effects of monoclonal antibodies with different immunoglobulin classes

<table>
<thead>
<tr>
<th>Dose of antibody (μg)</th>
<th>MM1-γ1-1</th>
<th>MM1-γ2a-1</th>
<th>MM1-γ2b-1</th>
<th>MM1-γ3-1</th>
<th>MM1-μ-1</th>
<th>MM1-α-1</th>
</tr>
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<tbody>
<tr>
<td>200</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
</tr>
<tr>
<td>50</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
</tr>
<tr>
<td>5</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
</tr>
<tr>
<td>0.5</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
</tr>
<tr>
<td>0.1</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
<td>0/5*</td>
</tr>
</tbody>
</table>

* Survival rate observed at day 50 [the number of animals surviving at day 50 (numerator) among that of animals treated (denominator)].

Table 2 Therapeutic effect of MM1-γ2a-1 antibody against increasing dose of tumor cells

<table>
<thead>
<tr>
<th>No. MM46 cells inoculated</th>
<th>Survival rate with the following dose of MM1-γ2a-1 antibody for treatment:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μg</td>
</tr>
<tr>
<td>2 x 10^5</td>
<td>0/5*</td>
</tr>
<tr>
<td>1 x 10^6</td>
<td>0/5*</td>
</tr>
<tr>
<td>5 x 10^6</td>
<td>0/5*</td>
</tr>
</tbody>
</table>

* See Footnote a of Table 1.
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Fig. 1. Histology of tumors treated with either MM1-γ2a-1, nonimmune IgG, or MM1-μ-1 antibody. Tumors treated with MM1-γ2a-1 antibody showed remarkable cell infiltration (A). A higher magnification of the tumors demonstrated that a major proportion of infiltrating cells consists of mononuclear cells, presumably macrophages (B). By contrast, almost no cellular reaction was noted in tumors treated either with nonimmune IgG (C) or MM1-μ-1 (D). H & E; A, × 120; B–D, × 250.

Fig. 2. Antitumor effect by MM1-γ2a-1 antibody studied in C3H/HeN-nu/nu mice with a serological Winn assay. Four × 10^6 MM46 tumor cells sensitized with either MMγ2a-1 (U) or nonimmune IgG (0) were inoculated s.c., and the tumor weight was measured at day 10. A significant effect of MM1-γ2a-1 antibody was observed in C3H/HeN-nu/nu (P < 0.05), although it seemed slightly less than that in C3H/HeN (P < 0.001). Each group consisted of five mice. γ2a-1, MM1-γ2a-1; N.IgG, nonimmune IgG. Bars, SD.

Macrophage Toxic Agent against in Vitro ADMC. Since the blocking effects of macrophage toxic agents against antitumor activity were demonstrated in vivo, the effect of these agents in vitro was studied in ADMC. As shown in Fig. 4, carrageenan reduced dose-dependently the macrophage-mediated cytotoxicity by MM1-γ2a-1, but silica particles did not do so up to 200 μg/ml.

DISCUSSION

The comparison of monoclonal antibodies with different immunoglobulin classes, detecting the same antigenic determinant, demonstrated that γ2a, γ2b, and γ1 antibody had remarkable therapeutic effects, whereas γ3, μ, and α antibody did not. These results were generally in accordance with those by Winn assay as we reported previously (4), although γ2a was slightly more effective than was γ1 or γ2b in Winn assay. Several groups have also described that the γ2a class is superior to other classes of immunoglobulin (12, 14), although all of the six immunoglobulin classes were not systematically examined. αs and α antibodies were reported to be ineffective and, if any, only a marginal effect was observed (12, 14, 15). The efficacy of γ subclasses other than γ2a is, however, still controversial. Significant antitumor effects were reported either with γ1 (16), γ2b (17), or γ3 (14, 15, 18, 19) antibody, while Herlyn and Koprowski (12) claimed that only γ2a was effective.

One of the reasons for these discrepancies is probably due to the difference of the target antigen, including the biochemical nature and the density on the tumor cell surface. For example, Kirch and Hämmerling (19) reported that the γ2a anti-Lyt-2 antibody was effective against ERLD leukemia but not against EL4 leukemia, which suggested that the number of antigen sites on these two leukemias are different (or that the sensitivity to immunological attack which is not associated with target antigen is different in these two leukemias). Antigenic modulation of the target antigen (20) might also influence the efficacy of an antibody, as suggested by Ritz and Schlossman (21), on common acute lymphocytic leukemia antigen of human acute lymphocytic leukemia cells. Because the present therapeutic treated control tumor was observed in mice treated with carrageenan, when compared with that in untreated or silica-treated mice. In day 0 treatment with these two macrophage toxic agents, the antitumor effect of MM1-γ2a-1 was reduced in mice treated with either carrageenan or silica particles (Table 3, Experiments 1 and 2). Day −1 treatment was also carried out, which showed that carrageenan negated the antitumor effect of the antibody, but silica did not (Table 3, Experiments 3 and 4). The experiments with MM1-γ2b-1 also showed quite similar results (data not shown).

The effect of a macrophage toxic agent was further investigated in a therapeutic experiment (Fig. 3). When mice were not treated with these agents, all the MM1-γ2a-1 treated mice survived longer than day 50 in these experiments, but this therapeutic effect was reduced in mice treated with either silica particles or carrageenan.
Table 3 Effect of macrophage toxic agent against antitumor activity of MM1-γ2a-1 antibody studied by serological Winn assay

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Date of treatment</th>
<th>Macrophage toxic agent</th>
<th>% of tumor weight</th>
<th>Tumor weight (mg)</th>
<th>Nonimmune IgG</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Day 0</td>
<td>None</td>
<td>18.1</td>
<td>22 ± 12</td>
<td>122 ± 55</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica</td>
<td>69.1</td>
<td>130 ± 54</td>
<td>188 ± 74</td>
<td>0.2 &gt; P &gt; 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrageenan</td>
<td>83.5</td>
<td>238 ± 71</td>
<td>285 ± 35</td>
<td>0.2 &gt; P &gt; 0.1</td>
</tr>
<tr>
<td>2</td>
<td>Day 0</td>
<td>None</td>
<td>5.6</td>
<td>7 ± 5</td>
<td>122 ± 55</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica</td>
<td>76.1</td>
<td>89 ± 34</td>
<td>117 ± 26</td>
<td>0.2 &gt; P &gt; 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrageenan</td>
<td>55.4</td>
<td>115 ± 23</td>
<td>208 ± 49</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>3</td>
<td>Day -1</td>
<td>None</td>
<td>26.9</td>
<td>47 ± 41</td>
<td>175 ± 72</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica</td>
<td>27.8</td>
<td>43 ± 9</td>
<td>153 ± 59</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrageenan</td>
<td>76.0</td>
<td>192 ± 70</td>
<td>253 ± 61</td>
<td>0.2 &gt; P &gt; 0.1</td>
</tr>
<tr>
<td>4</td>
<td>Day -1</td>
<td>None</td>
<td>35.3</td>
<td>61 ± 40</td>
<td>173 ± 83</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica</td>
<td>40.7</td>
<td>81 ± 42</td>
<td>199 ± 51</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrageenan</td>
<td>76.7</td>
<td>202 ± 100</td>
<td>263 ± 66</td>
<td>0.2 &gt; P &gt; 0.1</td>
</tr>
</tbody>
</table>

* Macrophage toxic agents were inoculated on day 0 in experiments 1 and 2 or on day -1 in experiments 3 and 4.

Relative tumor weight (% tumor weight) was calculated as:

\[
\% \text{ tumor weight} = \frac{\text{Geometrical mean of tumor weight treated by antibody}}{\text{Geometrical mean of tumor weight treated by nonimmune IgG}} \times 100
\]

Mean ± SD.

Weight of MM1-γ2a-1-treated tumor was statistically evaluated to compare with weight of non-immune IgG treated tumor by Student's t-test.

MECHANISM IN ANTITUMOR ACTIVITY OF ANTIBODY

A number of experiments have been performed to study the mechanisms of in vivo antitumor effects of antibody (12, 23–26). In this study, significant antitumor effects were observed in C3H/HeN-nu/nu, implying that predominant effector cells are not of T-cell lineage. One could not, however, exclude the possible participation as effectors of T-cell precursors or other cells in the nude mice. It is noted that tumor weight treated with specific antibody as well as that with nonimmune IgG in C3H/HeN-nu/nu was higher than that in C3H/HeN, although statistically it was not significant. The previous study did not show the importance of killer and natural killer cells, since in vitro antibody-dependent cytotoxicity against MM46 target cells either with mouse spleen cells or human blood lymphocytes as effector cells showed no significant lysis above the medium control (without these effector cells) (4). Several experiments have implicated macrophages as important effector cells. For instance, Shin et al. (26) demonstrated that the growth affinity with different immunoglobulin classes. Attempts are now being made to obtain class-switched mutants from the γ3 to γ1 or γ2 producer, by which the relative effectiveness among γ antibodies can be examined precisely in our tumor system.
of antibody-sensitized tumor cells transplanted into irradiated host were suppressed when mixed with macrophage, and Lan
glois et al. (23) observed the rosette formation of macrophages around tumor cells in the presence of specific antibody in vivo. Our previous study (4) showed a good correlation between the antitumor effect of antibody and ADMC with regard to imm
unoglobulin class and suggested also that macrophages may play an important role in the in vivo antitumor effect of the antibodies used.

The present results with two macrophage toxic agents, car
gagean and silica particles, showed that antitumor effects of antibody were reduced both in Winn assays and therapeutic experiments, suggesting further the involvement of macrophages as effector cells. However, inactivation of effector me
chanisms other than macrophages due to these agents still remains to be considered. In the Winn assay, blocking effects of silica were observed only in mice treated on day 0, but almost no effects on day 1 indicated that the timing of the treatment is crucial for the effects against macrophages as reported by Keller (13). The tumor growth of the carrageenan-treated control mice was found to be increased in Winn assays, which might affect the antitumor activity of the antibody. In therapeutic experi
ments, however, the survival time of the carrageenan-treated control mice was similar to that of the other control groups, suggesting that carrageenan blocked in vivo antitumor effects of the antibody.

The effects of these blocking agents were further studied in vitro ADMC. Carrageenan inhibited ADMC dose-dependently, whereas silica particles did not at a concentration up to 200 μg/ml, in contrast to the results with in vivo experiments. These findings may reflect a difference in the effector mechanisms of these two blocking agents, although both were reported to be toxic for macrophages by intracytoplasmic lysosomal discharge (27). In this connection, it is noted that Gerlier et al. (28) reported that poly-2-vinylpyridine-N-oxide was able to prevent the blocking effect of silica particles against passive serum therapy but not that of carrageenan.

Histological examination of our tumor model demonstrated the cellular reaction including macrophages in tumor cells treated with γ2a, γ2b, or γ1 antibody. It is also noted that the reaction observed with γ2a antibody is stronger than that observed with γ2b or γ1 antibody. Preliminary experiments showed that a major population of infiltrating cells was clearly stained by immunofluorescence with monoclonal AcM.1 anti
body, which is reported to react predominantly with activated macrophages (29). These results were in agreement with the report by Adams et al. (30), who demonstrated the histological examination of the tumors of mice given suppressive γ2a anti
body, in which a remarkable cellular infiltration consisting predominantly of macrophages was observed but not in the tumor cells treated with non-suppressive antibody. Thus, his
tological examination and blocking experiments with macrophages toxic agents suggested a major role of macrophages as host effector cells in antitumor activity of antibody in vivo.

Together with other reports (15–17, 19, 31), the present investigation suggested the possibility that all γ subclasses of antibodies potentially suppress tumor growth through effector cells, but at least the following factors are considered to be necessary for the antibodies to express anti-tumor activity in vivo, i.e.: (a) a sufficient number of antigenic sites on the tumor cells (32); (b) a high affinity antibody which can mediate inter
action between target tumor cells and macrophages or other effector cells; and (c) a sufficient number of appropriately activated macrophages to exert antibody-dependent cytotoxic or cytostatic activity (33, 34). Each single factor as well as certain combinations of them must be studied further in order to understand the in vivo antitumor effects of the antibody.

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