Synergistic Antitumor Effects of BCG and Monoclonal Antibodies Capable of Inducing Antibody-dependent Cell-mediated Cytotoxicity

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Three monoclonal antibodies (MAbs) of different immunoglobulin subclasses against MH134 murine ascitic hepatoma cells, detecting the same antigenic determinant of tumor-associated antigen of the tumor cells, were tested for their ability to produce a synergistic antitumor effect with Mycobacterium bovis BCG in C3H/HeN mice. 12A2 (IgG2a) induced both antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against the tumor cells. C3H/HeN mice were inoculated ip with MH134 tumor cells (day 0), and received an ip injection of BCG (day 1) and/or 12A2 (day 5). The combined therapy with BCG and 12A2 brought about a significant prolongation of the survival period, whereas either BCG or the MAb alone exhibited poor therapeutic effectiveness. 11G2 (IgG1), inducing ADCC but not CDC against MH134 tumor cells, was shown to exhibit antitumor effects as potent as those of 12A2, when used in combination with BCG. However, 7C2 (IgM), capable of inducing CDC but not ADCC to the tumor cells, produced no apparent synergistic effect with BCG. ADCC of BCG-induced peritoneal cells was mediated by the adherent cell population of the cells and abolished by the addition of carrageenan, suggesting that the effector cells of the cytotoxicity were macrophages. Moreover, carrageenan abolished the combined antitumor effect of BCG and these MAbs in the serological Winn assay. These results suggest that activated macrophages play a major role in the synergistic antitumor effect of BCG and MAbs capable of inducing ADCC against MH134 tumor.

Key words: BCG — Monoclonal antibodies — Antibody-dependent cell-mediated cytotoxicity

The development of antitumor monoclonal antibodies (MAbs)*2 which induce antibody-dependent cell-mediated cytotoxicity (ADCC) has stimulated interest in the application of MAbs to the treatment of malignancies because of the potent and highly specific activity of ADCC against tumor cells. In vivo efficacy of ADCC induced by MAbs has been reported by several investigators.1-4 In their reports, macrophages were suggested to be

*1 To whom communications should be addressed.
*2 The abbreviations used are: ADCC, antibody-dependent cell-mediated cytotoxicity; BCG, Bacillus Calmette Guérin; CDC, complement-dependent cytotoxicity; FBS, fetal bovine serum; MAbs, monoclonal antibodies; PBS, 0.01M phosphate-buffered saline (pH 7.4) containing 0.15M NaCl and 0.01M phosphate; PEC, peritoneal exudate cells; PEMϕ, peritoneal exudate macrophages; PRC, peritoneal resident cells; PRMϕ, peritoneal resident macrophages.

effector cells of the antitumor effects, based on the findings that (1) ADCC induced by these MAbs was mediated more potently by macrophages than by lymphocytes, (2) the in vivo antitumor effects of these MAbs were significantly abolished by the treatment of hosts with carrageenan or silica, and (3) tumors treated with these MAbs showed significant infiltration of macrophages.

While the involvement of macrophages as effector cells in the in vivo antitumor effects of such MAbs has been well documented, it is still unclear whether the therapeutic efficacy would be enhanced if effector macrophages had been activated in vivo when the MAbs were administered. In our previous publication concerning the derivation of murine hybridomas secreting MAbs capable of inducing ADCC against MH134 hepatoma cells, we showed that the cytotoxicity was exhibited more potently by macrophages than by lym-
phocytes, and was significantly augmented by stimulation of effector macrophages. In this study, it was tested whether macrophage activation by *Mycobacterium bovis* BCG would augment the therapeutic effects of ADCC-inducing MAbs.

**Materials and Methods**

**Animals and Tumors** Male C3H/HeN mice were purchased from Charles River Japan, Inc., Kanagawa. Mice were used at 8–10 weeks of age. MH134 hepatoma, MM102, MM46, and MM48 mammary carcinoma, and X5563 plasmacytoma, all derived from C3H/He mice, were maintained by ip passage in syngeneic recipient mice.

**MAbs** The derivation and the nature of the syngeneic MAbs to MH134 tumor cells, i.e., 7C2, 11G2, and 12A2, were described previously. Briefly, 7C2, a MAb of the IgM isotype, induces complement-dependent cytotoxicity (CDC) but not ADCC, whereas 11G2, of the IgG1 isotype, induces ADCC but not CDC, and 12A2, of the IgG2a isotype, induces both CDC and ADCC against the tumor cells. The tumor-associated epitope(s) of MH134 tumor cells recognized by each of these 3 MAbs is closely associated or identical, and is suggested to be a part of the MM antigen. By using ammonium sulfate, these MAbs were partially purified from ascitic fluid obtained from BALB/c nu/nu mice inoculated ip with hybridomas secreting these MAbs. Ascitic fluid obtained from BALB/c nu/nu mice inoculated ip with P3-X63-Ag8-U1 murine myeloma cells was also processed similarly, and used as a source of control IgG.

**Effector Cells of ADCC** C3H/HeN mice were injected ip with 10⁷ viable units of *Mycobacterium bovis* BCG (Japan BCG Company, Tokyo) suspended in 0.5 ml of 0.9% NaCl solution. Four days after injection, peritoneal exudate cells (PEC) or peritoneal resident cells (PRC) were obtained from BALB/c nu/nu mice inoculated ip with BCG (Japan BCG Company, Tokyo). 3–6 ml of peritoneal lavage fluid, consisting of less than 5% phagocytic cells, was also processed similarly, and used as effector cells.

**Centrifugation, Adherence, and Removal of PEC or PRC** More than 92% of the resulting adherent cell population, adherent cells were removed by repeated washings of the wells, and then 100 μl of complete medium was added to each well. Approximately 60% of the cells, i.e., 2.5 × 10⁶ cells, remained as adherent cells after this procedure in the cases of both BCG-induced PEC and PRC. More than 92% of the resulting adherent cells were phagocytic mononuclear cells, as determined by phagocytosis of carbon particles. These were referred to as peritoneal exudate macrophages (PEMφ) or peritoneal resident macrophages (PRMφ), and they were used as effector cells. To obtain the nonadherent cell population of peritoneal cells, adherent cells were removed by 2 consecutive 2-hr incubations of 10⁷ cells in a 60-mm plastic dish (No. 25010, Corning, N.Y.). The resultant nonadherent cell population, containing less than 5% phagocytic cells, was also used as effector cells at a cell density of 2.5 × 10⁶ cells per 100 μl of complete medium per well.

**ADCC** One million target cells suspended in 1 ml of complete medium were labeled with 5 μCi of [5-¹⁵N]iodo-2'-deoxyuridine (Amersham, Buckinghamshire, England) in a well of a 4-well culture plate (Nunc, Roskilde, Denmark) at 37° for 4 hr in a humidified 5% CO₂ atmosphere. After incubation, nonadherent cells were removed by repeated washings of the wells, and then 100 μl of complete medium was added to each well. Approximately 60% of the cells, i.e., 2.5 × 10⁶ cells, remained as adherent cells after this procedure in the cases of both BCG-induced PEC and PRC. More than 92% of the resulting adherent cells were phagocytic mononuclear cells, as determined by phagocytosis of carbon particles. These were referred to as peritoneal exudate macrophages (PEMφ) or peritoneal resident macrophages (PRMφ), and they were used as effector cells. To obtain the nonadherent cell population of peritoneal cells, adherent cells were removed by 2 consecutive 2-hr incubations of 10⁷ cells in a 60-mm plastic dish (No. 25010, Corning, N.Y.). The resultant nonadherent cell population, containing less than 5% phagocytic cells, was also used as effector cells at a cell density of 2.5 × 10⁶ cells per 100 μl of complete medium per well.

**Effector Cells of ADCC** C3H/HeN mice were injected ip with 10⁷ viable units of *Mycobacterium bovis* BCG (Japan BCG Company, Tokyo) suspended in 0.5 ml of 0.9% NaCl solution. Four days after injection, peritoneal exudate cells (PEC) were obtained by lavaging the peritoneal cavities with PBS containing 5 units of heparin per ml (Novo Industries, Bagsvaerd, Denmark). Peritoneal resident cells (PRC) were obtained from normal C3H/HeN mice in the same manner. These cells from 3 or 4 mice were pooled, washed twice with Hanks medium (Nissui Pharmaceutical Co.) and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 units of penicillin per ml (Meiji Seika, Tokyo), 100 μg of streptomycin per ml (Meiji Seika) and 2 mM L-glutamine (Flow Laboratories, North Lyde, Australia). This medium was designated as the complete medium. To obtain the adherent cell population of peritoneal cells, 4.2 × 10⁶ PEC or PRC were added to each well of a microculture plate (No. 76-013-05, Linbro, McLean, Va.) and incubated at 37° for 2 hr in a humidified 5% CO₂ atmosphere. After incubation, nonadherent cells were removed by repeated washings of the wells, and then 100 μl of complete medium was added to each well. Approximately 60% of the cells, i.e., 2.5 × 10⁶ cells, remained as adherent cells after this procedure in the cases of both BCG-induced PEC and PRC. More than 92% of the resulting adherent cells were phagocytic mononuclear cells, as determined by phagocytosis of carbon particles. These were referred to as peritoneal exudate macrophages (PEMφ) or peritoneal resident macrophages (PRMφ), and they were used as effector cells. To obtain the nonadherent cell population of peritoneal cells, adherent cells were removed by 2 consecutive 2-hr incubations of 10⁷ cells in a 60-mm plastic dish (No. 25010, Corning, N.Y.). The resultant nonadherent cell population, containing less than 5% phagocytic cells, was also used as effector cells at a cell density of 2.5 × 10⁶ cells per 100 μl of complete medium per well.

**ADCC One million target cells suspended in 1 ml** of complete medium were labeled with 5 μCi of [5-¹⁵N]iodo-2'-deoxyuridine (Amersham, Buckinghamshire, England) in a well of a 4-well culture plate (Nunc, Roskilde, Denmark) at 37° for 4 hr in a humidified 5% CO₂ atmosphere as described previously. After labeling, the cells were washed twice. Two hundred thousand labeled cells were incubated with medium or MAbs at a concentration of 1 μg per ml at 4° for 45 min, washed once, and then 100 μl of complete medium was incubated at 37° for 24 hr in a humidified 5% CO₂ atmosphere. After incubation, 100 μl aliquots of the supernatants were counted for radioactivity with a gamma counter. The percentage of specific cytolyisis was calculated by the following formula:

\[
\text{% of specific cytolyisis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100.
\]

After 24 hr of incubation, the spontaneous cpm,
measured by incubation of target cells alone, was less than 5% of the total cpm for MH134, MM46, MM48 and MM102 tumor cells, and was approximately 20–30% for X5563 tumor cells.

Serological Winn Assay As reported elsewhere, 5 × 10^5 tumor cells were incubated with a MAb at a concentration of 90 µg per ml or with 2% FBS: RPMI 1640 medium alone at 4°C for 45 min, washed once and then resuspended in Hanks medium. One hundred thousand tumor cells treated with a MAb or medium were inoculated ip into C3H/HeN mice that had been given an ip injection of 0.5 ml of 0.9% NaCl solution or 10^4 viable units of BCG 4 days earlier. In some experiments, mice were given an additional injection of 0.5 mg of carrageenan dissolved in 0.5 ml of 0.9% NaCl solution 6 hr prior to tumor inoculation. These mice were monitored daily for mortality.

Therapeutic Experiment C3H/HeN mice were inoculated ip with 10^5 MH134 tumor cells and given an ip injection of 0.5 ml of 0.9% NaCl solution or 10^4 viable units of BCG 1 day after tumor inoculation. These mice were further given an ip injection of 100 µg of MAb or control IgG 5 days after tumor inoculation and monitored daily for mortality.

Statistical Analysis The survival rates were compared between the experimental and control groups. Statistical significance was determined by means of the Mann-Whitney U test.

RESULTS

Induction of ADCC by 11G2 and 12A2, Augmentation of the Cytotoxicity by BCG, and Effector Cell Analysis As shown in Table I, PRMφ exhibited very low cytotoxicity against MH134 tumor cells, and the cytotoxicity was only slightly elevated by coating the tumor cells with 11G2 or 12A2. On the other hand, BCG-induced PEMφ exhibited apparent cytotoxicity against MH134 tumor cells, and the cytotoxicity was significantly augmented by coating the tumor cells with the MABs. The nonadherent cells of BCG-induced PEC exhibited poor cytotoxicity to the tumor cells even in the presence of either 11G2 or 12A2. It might be possible that the cell number of the adherent cells remaining in the well is more than 2.5 × 10^5 cells per well. However, the cytotoxicity of the nonadherent cells at a cell number of more than 2.5 × 10^5 cell per well was still significantly lower than that of the adherent cells obtained from 4.2 × 10^5 BCG-induced PEC (data not shown). The addition of carrageenan resulted in significant inhibition of cytotoxicity of the adherent cells against not only medium-treated but also MAb-coated MH134 tumor cells.

Specificity of the Augmented ADCC The target specificity of the ADCC induced by these MABs was examined using BCG-induced PEMφ as effector cells, and the results are summarized in Table II. Cytotoxicity against MH134 tumor cells was significantly augmented by coating the tumor cells with 11G2 or 12A2. The augmentation of the cytotoxicity by these MABs was also seen when MM antigen-positive cells of the MM46 and MM102 tumor lines were used as targets. However, none of these MABs augmented the cytotoxicity against MM antigen-negative cells of either the MM48 or X5563.

Table I. Effector Cell Analysis of ADCC Exhibited by BCG-induced PEC against MAb-coated MH134 Tumor Cells

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Mice</th>
<th>Effector cells</th>
<th>Addition of carrageenan^a</th>
<th>% Specific cytology against MH134</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>Adherent cells</td>
<td>-</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>BCG-injected</td>
<td>Adherent cells</td>
<td>-</td>
<td>11.3</td>
</tr>
<tr>
<td>II</td>
<td>BCG-injected</td>
<td>Unfractionated cells</td>
<td>-</td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td>BCG-injected</td>
<td>Adherent cells</td>
<td>-</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonadherent cells</td>
<td>-</td>
<td>2.6</td>
</tr>
<tr>
<td>III</td>
<td>BCG-injected</td>
<td>Adherent cells</td>
<td>-</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adherent cells</td>
<td>+</td>
<td>0.6</td>
</tr>
</tbody>
</table>

^a) Carrageenan was added to a well at a final concentration of 100 µg/ml.

^b) Cytotoxicity against MH134 tumor cells pretreated with medium, 11G2 or 12A2 was assayed after 24 hr.
K. KOMUTA, ET AL.

Table II. Specificity of ADCC Induced by Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>MH134</th>
<th>MM102</th>
<th>MM46</th>
<th>MM48</th>
<th>X5563</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>18.9</td>
<td>3.8</td>
<td>5.1</td>
<td>24.1</td>
<td>41.2</td>
</tr>
<tr>
<td>7C2</td>
<td>15.4</td>
<td>2.7</td>
<td>5.7</td>
<td>ndb</td>
<td>nd</td>
</tr>
<tr>
<td>11G2</td>
<td>16.4</td>
<td>4.7</td>
<td>7.8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>12A2</td>
<td>51.4</td>
<td>23.1</td>
<td>24.1</td>
<td>29.2</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>59.4</td>
<td>26.1</td>
<td>26.0</td>
<td>24.4</td>
<td>43.1</td>
</tr>
</tbody>
</table>

a) BCG-induced PEMφ were used as effector cells.
b) Not determined.

tumor line. 7C2, a MAb of the IgM isotype, did not augment the cytotoxicity against either of these tumor lines.

Synergistic Effect of BCG and the MAbs on Prolongation of the Survival Period of Mice Bearing MH134 Ascitic Tumor (Therapeutic Experiment) Mice inoculated ip with 10^5 MH134 tumor cells were given an ip injection of BCG, the MAbs, or both. The survival periods of these mice were monitored and the results are summarized in Fig. 1. An ip injection of BCG 1 day after tumor inoculation caused a weak, though apparent, prolongation of the survival period. A single ip administration of 12A2 5 days after tumor inoculation was also found to be as effective as BCG. When mice were treated with both BCG and 12A2, they survived significantly longer than did mice given a single treatment with BCG or 12A2. Thirteen out of 20 mice given the combined treatment survived longer than 2 months without tumor growth. 11G2 also showed antitumor activity which was as effective as that of 12A2; 11 of 20 mice treated with both BCG and 11G2 were free of tumors 2 months after tumor inoculation. On the other hand, 7C2 neither showed an apparent antitumor effect when used alone, nor produced a synergistic effect with BCG such as that shown by the other two MAbs.

Abolition of the Combined Antitumor Effect by Carrageenan As shown in Table I, the ADCC of BCG-activated PEMφ was significantly suppressed by the addition of carrageenan at the effector phase of the cytotoxicity. In keeping with this observation, carrageenan blocked the development of BCG-induced augmentation of the antitumor effect of 12A2 when tested by the serological Winn assay (Fig. 2). On pretreatment of mice with
AUGMENTATION OF ADCC BY BCG

Before tumor inoculation, a weak, though apparent, prolongation of the survival period was observed. This antitumor effect of BCG was completely abolished by ip injection of carrageenan. The significant prolongation of the survival period, observed when MH134 tumor cells coated with 12A2 were inoculated ip into BCG-stimulated mice, was also significantly suppressed by the additional treatment of mice with carrageenan. When 11G2, which does not require complement for its cytotoxicity, was used as an ADCC-inducing MAb, carrageenan also blocked the development of the synergistic antitumor effect of BCG and the MAb in the serological Winn assay (data not shown).

Specificity of the Augmented ADCC in Serological Winn Assay The specificity of ADCC was also tested in the serological Winn assay, and the results are shown in Fig. 3. The pretreatment of mice with BCG before tumor inoculation exerted a weak, though apparent, antitumor effect against all of MM46, MM102, MM48 and X5563 tumors. When tumor cells coated with 12A2 were inoculated ip into BCG-stimulated mice, augmentation of the
antitumor effect was significant only against MM46 and MM102 tumors. This augmented cytotoxicity by the MAbs was not seen against either MM48 or X5563 tumor cells. 11G2 exhibited the same target specificity as that of 12A2.

**DISCUSSION**

Seto et al.\(^2\) reported that MAbs capable of inducing ADCC against cells of a murine tumor line exhibited potent antitumor effects without the help of macrophage potentiators. In their report, mice bearing the tumor as an ascitic form were treated with a single iv injection of MAbs, and a small amount of the MAbs (5 µg or less) was shown to be effective when used alone. Herlin and Koprowski\(^3\) also indicated that MAbs against human tumors, possessing the ability to induce ADCC, exhibited apparent antitumor effects against the tumors in nude mice when used alone. In their study, however, repeated injections of a large amount of MAbs (400 µg) were necessary to obtain potent effects. In contrast, the present study demonstrated that the MAbs prepared in our laboratory, showing similar activity against MH134 ascitic hepatoma, exhibited weak antitumor effects when used alone. The discrepancies among these findings may be due to differences in the susceptibility of tumor cells to the cytotoxicity, including the biochemical nature and the density of antigenic determinant(s), and/or in the affinity of the antibodies.

Augmentation of the therapeutic effectiveness of ADCC-inducing antibodies by macrophage activators has been reported using Corynebacterium parvum\(^7\) or BCG.\(^8\) In these studies, however, xenogeneic or syngeneic antisera but not MAbs was used as a source of antibodies. Therefore, the results of these studies using conventional antisera may be hampered by the complexity and heterogeneity of the antibody population. The development of monoclonal antibodies has overcome these limitations because of their defined specificity and homogeneity in their properties. The results of the present study using MAbs clearly indicate that a combination of BCG and antibodies capable of inducing ADCC against MH134 tumor cells resulted in significant augmentation of the antitumor effects.

BCG is well known to be a potent activator for macrophages.\(^9\) The results of the present study clearly indicate that a single ip injection of BCG resulted in the significant augmentation of ADCC of peritoneal cells against the MAbs-coated MH134 tumor cells, and that the effector cells of the augmented cytotoxicity were adherent and sensitive to carrageenan, which is known to be toxic for macrophages. Carrageenan also blocked the BCG-induced augmentation of ADCC in the serological Winn assay. Collectively, these results strongly suggested that activated macrophages play a major role in the synergistic antitumor effects of the combined therapy with the MAbs and BCG. However, BCG is also known to augment natural killer (NK) cell activity of peritoneal cells when injected ip.\(^10\) Furthermore, not only is carrageenan toxic to macrophages but also it is able to suppress induction of activated NK cells because it inhibits macrophages which are necessary for augmentation of NK cell activity by a variety of NK activators.\(^11\)\(^,\)\(^12\) Therefore, it might be possible that NK or K cells stimulated by BCG are also involved in the effector mechanism(s) of the combined treatment with BCG and the MAbs. In this study, however, it was shown that (1) ADCC of BCG-induced PEC was mainly mediated by the adherent cell population of the cells, (2) the effector cells required more than 16 hr to exhibit high cytotoxicity, whereas interleukin 2 (IL 2)-stimulated murine spleen cells exhibited both NK and lymphokine-activated killer (LAK) activities within 4 hr (unpublished data), (3) carrageenan inhibited the cytotoxicity when added at the effector phase of the cytotoxicity, whereas the same treatment did not inhibit either NK or LAK cytotoxicity of IL 2-stimulated spleen cells (unpublished data), and (4) carrageenan also abolished the combined antitumor effect in the serological Winn assay when administered ip 4 days after BCG injection, whereas carrageenan has been reported to block NK augmentation when administered simultaneously with an NK activator.\(^12\) Therefore, it seems unlikely that NK or K cells play a major role in the combined antitumor effects of BCG and the MAbs, even though they might be involved in part in the effector mechanism(s) of the combined therapy. In our study, BCG
AUGMENTATION OF ADCC BY BCG

administration was confined to a single injection to avoid overstimulation of macrophages. Johnson et al. showed that murine peritoneal macrophages elicited by multiple administrations of BCG exhibited significantly low ADCC against a variety of tumor cells in 24–48 hr cytotoxicity assay using antitumor MAbs, compared with the cytotoxicity exhibited by thioglycollate-induced macrophages. In this regard, they have suggested that macrophages, if fully activated, may lose their ability to mediate "slow" ADCC which is one of the important elements of antibody-mediated destruction of neoplasms in vivo.

In the serological Winn assay, however, carrageenan did not completely abolish the antitumor effect of the combined treatment with BCG and 12A2, while it completely blocked the antitumor effect of the single treatment with BCG. The weak, though apparent, antitumor effect of the combined treatment, which remained even after the treatment with carrageenan, might be attributable to the effect exhibited by 12A2 per se, because the MAb exhibited a weak antitumor effect when used alone in the therapeutic experiment. It is unlikely that this weak antitumor effect of 12A2 was mediated by CDC, because 7C2, which can induce CDC but not ADCC, exhibited no apparent antitumor effect. Conversely, 11G2, which is capable of inducing ADCC but not CDC, caused an apparent antitumor effect, as did 12A2. It seems possible that the weak effect of the MAbs alone was based on ADCC mediated by unstimulated macrophages, which might be less sensitive to carrageenan than activated macrophages. This effect might be blocked by carrageenan at a dosage of more than 0.5 mg per mouse. In a preliminary study, however, 40 to 50% of C3H/HeN mice died when 1 mg carrageenan was administered ip.

A variety of nonspecific immunopotentiators have been applied to the therapy of human cancers, and some of them have been reported to prolong the survival period in randomized controlled studies. The effector mechanism(s) of these materials has been largely ascribed to the nonspecific cytotoxicity of macrophages activated by them. However, their antitumor effects are not as potent as has been expected. The results of our study strongly suggest that combined cancer therapy consisting of biological response modifiers capable of activating macrophages and ADCC-inducing MAbs may be able to exert a potent antitumor effect.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture. (Received Oct. 27, 1986/Accepted Dec. 11, 1986)

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