Antibody-mediated Killing of Human Tumor Cells by Attached Effector Cells

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ABSTRACT

Cultured human melanoma, lung carcinoma, and colon carcinoma cells were isotope labeled and incubated with a combination of effector cells and mouse monoclonal antibodies to tumor-associated cell surface antigens. The former were derived from the peritoneal cavity of mice or from peripheral blood of healthy human subjects. Monoclonal antibodies MG-21, 96.5, and L6, which are IgG3, IgG2a, and IgG2a, respectively, were all cytolytic when added in the presence of mouse effector cells to target cells expressing the relevant antigens. MG-21 and L6 were cytolytic also with human effector cells, while monoclonal antibody 96.5 was not. The effector cells attached to plastic surfaces, stained with neutral red, were peroxidase positive and mediated their effect over a 24- to 72-h time period as compared to the 4-h generally sufficient for antibody-dependent cellular cytotoxicity by natural killer cells. In tests on human effector cells with a fluorescence-activated cell sorter, they stained with antibody LCM-3C10 to the CD14 antigen, as well as with antimonocyte antibody 61D3. The cytolytic effect of human effector cells and antitumor antibody was not abolished by incubation with antibodies FC2 or 60.3 to CD16 and CD18, respectively, known to interfere with the antibody-dependent cellular cytotoxicity activity and natural killing of natural killer cells. This suggests, together with the other findings, that the effector cells were macrophages.

INTRODUCTION

Monoclonal antibodies to tumor-associated antigens are receiving much attention as therapeutic agents, since they are used as carriers of chemotherapeutic drugs, radioisotopes, immunomodulators, etc. (1). Antibodies which can kill tumor cells in the presence of human complement (2-4), or mediate ADCC (3), MG-21 (2), and L6 (4), which are IgG2a, IgG3, and IgG2a, respectively, were all cytolytic when added in the presence of mouse effector cells to target cells expressing the relevant antigens. MG-21 and L6 were cytolytic also with human effector cells, while monoclonal antibody 96.5 was not. The effector cells attached to plastic surfaces, stained with neutral red, were peroxidase positive and mediated their effect over a 24- to 72-h time period as compared to the 4-h generally sufficient for antibody-dependent cellular cytotoxicity by natural killer cells. In tests on human effector cells with a fluorescence-activated cell sorter, they stained with antibody LCM-3C10 to the CD14 antigen, as well as with antimonocyte antibody 61D3. The cytolytic effect of human effector cells and antitumor antibody was not abolished by incubation with antibodies FC2 or 60.3 to CD16 and CD18, respectively, known to interfere with the antibody-dependent cellular cytotoxicity activity and natural killing of natural killer cells. This suggests, together with the other findings, that the effector cells were macrophages.

MATERIALS AND METHODS

Antibodies. Three mouse MAbs to tumor antigens were used, 96.5 (9), MG-21 (2), and L6 (4), which are IgG2a, IgG3, and IgG2a, respectively. The first two MAbs recognize different cell surface antigens which are expressed most strongly on melanomas, while L6 reacts with carcinomas. Our methods for obtaining and purifying the antibodies have been described, with the exception that MAb MG-21 came from hybridoma cells which were grown at Damon Biotech (Needham Heights, MA) according to an encapsulation procedure and was purified on a Protein A column. Myeloma line P1.17 was derived from American Type Culture Collection and produces an IgG2a immunoglobulin, which was purified on Protein A as previously described (4). It was used as a control. MAb TT1.1, which recognizes a human blood group antigen and is of the IgG3 isotype, was used as control for MG-21; it does not bind to the melanoma cells used. MAb FC2 (IgG2b) is specific for the CD16 Fc receptor of large granular lymphocytes and can block NK cell-mediated ADCC (10). MAb 60.3 (IgG2a) is specific for CD18, the β chain of the human LFA-1 complex and blocks NK cell-mediated ADCC (11). Mouse MAb 61D3 (to human monocytes/macrophages) and LCM-3C10 (to the CD14 antigen of human monocytes/macrophages) were obtained from Bethesda Research Laboratories, Inc., Rockville, MD.

Target Cell Cultures. Human melanoma lines M-2669 clone 13 (12) which is referred to as M-2669, M-1477, which was derived from SK MEL-28 (13), and A375-2 (obtained from the National Cancer Institute), lung carcinoma line L-2981 (4), and colon carcinoma line C-3347 (4) were used as sources of target cells. The three melanoma lines strongly react with the antimelanoma MAbs 96.5 and MG-21, and the two carcinoma lines strongly react with anticarcinoma MAb L6, while the antimelanoma antibodies bind poorly to the two carcinoma lines and MAb L6 binds poorly to cells from the three melanoma lines. Cultures were maintained in 75-cm² tissue culture flasks (Falcon Plastics, Oxnard, CA) using RPMI Medium 1640 (Gibco, Grand Island, NY) supplemented with 15% heat-inactivated FCS (HyClone, Sterile Systems, Logan, UT), at 37°C in a humidified atmosphere containing 5% CO₂; A375-2 cells were grown in Dulbecco's modified Eagle's minimal essential medium (Gibco) with 10% FCS. After the cells had grown to confluency, which took 3-4 days, they were harvested after 5-min incubation with 0.02% EDTA in phosphate-buffered saline (Baker Chemicals, Phillipsburg, NJ), and resuspended in RPMI Medium with 15% FCS. All cultures were free of mycoplasma.

Preparation and Purification of Murine Adherent Effector Cells. PC were obtained from 8-wk-old BALB/c mice (obtained from Fred Hutchinson Cancer Research Center, Seattle, WA) which 4 days prior to harvesting had received 1.0 ml thioglycollate medium (Difco Laboratories, Detroit, MI) i.p. They were harvested in RPMI Medium 1640. PC suspensions were centrifuged at 200 x g and the cells were washed, resuspended in culture medium, and plated in a 48-well tissue culture plate (Costar, Cambridge, MA) at a concentration of 1 x 10⁶ cells per well. After 1-h incubation at 37°C in a 5% CO₂ atmosphere, non-adherent cells were removed by washing twice with culture medium. The percentage of macrophages among the remaining adherent cells was assessed by neutral red uptake to be >90%.

Isolation and Culture of Human Macrophages. MNL from the peripheral blood of normal subjects were separated on a Ficoll/Lymphocyte separation medium (Litton Bionetics, Charleston, SC) incubated with monocytes/macrophages. MNL from the peripheral blood of normal subjects were separated on a Ficoll/Lymphocyte separation medium (Litton Bionetics, Charleston, SC) incubated with monocytes/macrophages. MNL from the peripheral blood of normal subjects were separated on a Ficoll/Lymphocyte separation medium (Litton Bionetics, Charleston, SC) incubated with monocytes/macrophages. MNL from the peripheral blood of normal subjects were separated on a Ficoll/Lymphocyte separation medium (Litton Bionetics, Charleston, SC) incubated with monocytes/macrophages.

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3 The abbreviations used are: ADCC, antibody-dependent cellular cytotoxicity; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; MAb, monoclonal antibody; MNL, human mononuclear lymphocytes; NK, natural killer; PC, peritoneal cells.

4 Yu, D.-S. and Yeh, M.-Y., unpublished observations.
using swing-out buckets. Cell populations layered on top of the Percoll gradient separated on the basis of their relative densities into two distinct bands. The cells from the upper band were harvested, washed twice in HBSS, and resuspended in RPMI Medium 1640 with 15% FCS. Approximately $1 \times 10^6$ monocytes were added to each well of a 48-well tissue culture plate, after which they were allowed to adhere for 1 h at 37°C in a 5% CO₂ atmosphere. After incubation, the plates were washed twice with HBSS. The presence of macrophages within the remaining adherent cell population was estimated by staining for 2 h in the dark by 1% neutral red in saline, and by staining for peroxidase according to Graham et al. (15). Tests were also performed with a Coulter EPICS-C fluorescence-activated cell sorter, measuring binding of anti-human macrophage/monocyte antibodies 61D3 and LCM-3C10. In all of these cases >90% of the adherent cells stained with neutral red contained peroxidase and bound antibodies 61D3 and LCM-3C10, indicating that they were macrophages. The plating efficiency of the adherent cells was ≥90%.

Cytotoxicity Assay. An assay was used which has been described previously (16). Briefly, tumor cells were grown to confluence and labeled for 24 h with $[^{35}S]$iodo deoxyuridine, 1.0 μCi/ml (Amersham; specific activity, 5 Ci/mg) in RPMI Medium 1640. The labeled cells were washed twice, harvested after 5-min incubation with 0.02% EDTA in PBS, and washed twice again with RPMI 1640. Subsequently, $5 \times 10^4$ cells were added to wells containing plated macrophages (see above) at an approximately 20:1 effector:target cell ratio; a control group was included with radiolabeled target cells alone. Monoclonal antibodies or control medium were then added to the appropriate wells. All groups were set up in triplicate.

Seventy-two h later (unless otherwise stated), the cultures were washed twice with HBSS and the residual adherent target cells were lysed with 0.1 ml 0.5 N NaOH and counted. The percentage of antibody-dependent cell-mediated cytotoxicity was calculated as

\[
\text{Percentage of cytotoxicity} = \frac{\text{cpm in target cells cultured} - \text{cpm in target cells cultured with test antibodies}}{\text{cpm in target cells cultured with effector cells and test antibodies}} \times 100
\]

and percentage of “spontaneous cytotoxicity” (with effector cells only) was calculated as

\[
\text{Percentage of spontaneous cytotoxicity} = \frac{\text{cpm in target cells cultured with effector cells}}{\text{cpm in target cells cultured without effector cells}} \times 100
\]

Data were calculated in the same way throughout the study. Thus, the percentage of cytotoxicity with MAb and effector cells always represents the degree of target cell killing which occurred above any cytotoxic effect observed with the effector cells alone.

We tested for inhibition of the cytolytic effect of human effector cells and antitumor MAbs (4) by adding MAb FC2 and 60.3 which inhibit the ADCC activity and natural killing of NK cells. These antibodies, which were used at a concentration of 5–25 μg/ml, were added to the wells containing effector cells, antitumor MAb, and target cells, and remained for the duration of the assay.

RESULTS

We have tested the cytolytic effects of three antitumor MAbs, MG-21, 96.5, and L6, when combined with adherent effector cells of either mouse or human origin and added to plated human tumor cells. The tumor target cells were derived from melanoma lines M-2669, M-1477, or A375-2 (all three of which can bind both MAbs 96.5 and MG-21, but only very little L6), as well as from lung carcinoma line L-2981 and colon carcinoma line C-3347 (both of which can bind MAb L6 but very little MG-21 or 96.5). The release of target cell label was measured as an indication of cytosis. Data obtained with mouse and human effector cells are presented separately.

Mouse Effector Cells. Table 1 shows experiments with mouse

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Target cells</th>
<th>% of cytotoxicity (effector cells + MAb)</th>
<th>% of SC (effector cells alone)</th>
</tr>
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<tbody>
<tr>
<td>1a</td>
<td>M-1477</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>1b</td>
<td>M-2669</td>
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<td>28</td>
</tr>
<tr>
<td>1c</td>
<td>L-2981</td>
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</tr>
<tr>
<td>2</td>
<td>M-2669</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>3a</td>
<td>M-1477</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td>3b</td>
<td>A 375-2</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>4a</td>
<td>M-1477</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4b</td>
<td>M-2669</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4c</td>
<td>L-2981</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4d</td>
<td>C-3347</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5a</td>
<td>M-1477</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>5b</td>
<td>M-2669</td>
<td>72</td>
<td>ND</td>
</tr>
<tr>
<td>6c</td>
<td>C-3347</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*SC, spontaneous cytotoxicity (effector cells alone); ND, not done.

PC as effectors. MAbs 96.5 and MG-21 (25 μg/ml) both mediated strong cytotoxicity on melanoma cells. Although there was substantial variation between individual tests, MAb 96.5 gave >20% cytotoxicity in 7 of 7 tests and ≥70% cytotoxicity in 5 of 7 tests when combined with effector cells, and the results with MG-21 were analogous. The two antimelanoma MAbs had negligible ADCC activity against cells from lung carcinoma L-2981 or colon carcinoma C-3347 (≥20% killing in 1 of 5 tests and ≥70% killing in 0 of 5 tests). Thus, the effects observed...
effector cells were used. As in the tests with mouse PC (Table 1), Fab fragments were ineffective (data not shown). Two control antibodies, P1.17 (IgG2a) and 7T1.1 (IgG3), had no effect (Table 2).

As shown in Table 2, some preparations of human effector cells were strongly "spontaneously" cytotoxic without added MAb, while others were not. To decrease the influence of this effect, the degree of ADCC as caused by a combination of MAb and effector cells, and presented as "cytotoxicity" in Tables 1–3 and Figs. 1 and 2, was always calculated by comparison with the number of counts obtained with effector cells alone. For example, the 96% cytotoxicity recorded in Experiment 7a represented a 96% decrease in cell counts from what was observed with the effector cells alone, which, by themselves, had decreased by 79% the counts from target cells observed in the absence of effector cells. As shown in Experiments 8a, 9b, and 10a, as well as in Experiment 7a, strong antibody-dependent cell-mediated cytotoxicity could be observed independent of the number of counts obtained with effector cells alone. For example, the 96% cytotoxicity recorded in Experiment 7a represented a 96% decrease in cell counts from what was observed with the effector cells alone, which, by themselves, had decreased by 79% the counts from target cells observed in the absence of effector cells. As shown in Experiments 8a, 9b, and 10a, as well as in Experiment 7a, strong antibody-dependent cell-mediated cytotoxicity could be observed independent of whether the spontaneous cytolytic activity of effector cells tested alone was high or low.

Antibody-dependent cytolytic activity was observed even at a ratio of 5 effector cells per target cell and increased with the number of effector cells. As shown in Fig. 1, the degree of ADCC as caused by a combination of MAb and effector cells, and presented as "cytotoxicity" in Tables 1–3 and Figs. 1 and 2, was always calculated by comparison with the number of counts obtained with effector cells alone. For example, the 96% cytotoxicity recorded in Experiment 7a represented a 96% decrease in cell counts from what was observed with the effector cells alone, which, by themselves, had decreased by 79% the counts from target cells observed in the absence of effector cells. As shown in Experiments 8a, 9b, and 10a, as well as in Experiment 7a, strong antibody-dependent cell-mediated cytotoxicity could be observed independent of whether the spontaneous cytolytic activity of effector cells tested alone was high or low.

An antibody-dependent cytolytic activity was observed even at a ratio of 5 effector cells per target cell and increased with the number of effector cells (Fig. 1); 20 effector cells per target cell were used as a rule (Tables 1–3). A concentration of 25 μg/ml was sufficient for maximum cytotoxicity, with >40% cytotoxicity being seen even at the lowest antibody dose tested which was 5 μg/ml. More than 25 μg MAb/ml did not increase the cytotoxicity (data not shown).

The upper and lower panels in Fig. 2 show experiments demonstrating that the degree of target cell lysis by the MAb and effector cells was negligible after 4-h incubation and was...
approximately the same after 24, 48, and 72 h. The spontaneous cytotoxicity of effector cells alone varied between 0 and 20% and did not increase appreciably over time.

Table 3 shows that the ADCC activity of MAb MG-21 and human effector cells was not significantly inhibited by MAb FC2 or 60.3, both of which can inhibit NK cell-mediated ADCC and natural killing (10, 11).

DISCUSSION

We have shown that two mouse MAbs of the IgG2a isotype, 96.5 and L6, and one IgG3 MAB, MG-21, can lysy human tumor cells expressing cell surface antigens recognized by the respective MAB when combined with effector cells of mouse origin. Two of these MAbs, L6 and MG-21, lysed tumor cells also when human effector cells were used, while MAB 96.5 did not significantly do so.

The effector cells were able to attach to plastic surfaces, were not removed by washing, stained to ≥90% with neutral red, and were peroxidase positive. They needed at least 24 h to be highly cytolytic which correlates with published work using murine macrophages (17). NK cells, on the other hand, are nonadherent and can mediate strong ADCC detectable already after a 4-h incubation (2, 4). Furthermore, >90% of the human effector cells bound MAbs LCM-3C10 and 61D3, which are specific for the CD2 antigen expressed by monocyte/macrophages, and their ADCC activity was not inhibited by antibodies to CD16 or CD18, which are known to interfere with the ADCC activity of NK cells (10, 11). Taken together our data indicate that the effector cells responsible for the activities described in this report were monocyte/macrophages.

The reactivities observed had immunological specificity, with MAbs MG-21 and 96.5 mediating ADCC against melanoma cells and MAb L6 giving ADCC against carcinoma cells. Antibodies alone were not cytotoxic, while effector cells alone sometimes lysed a substantial fraction of the target cells.

We have previously shown that MAb MG-21, but not 96.5, can inhibit the outgrowth, in nude mice, of human melanoma cells which express the antigens defined by the two respective MAbs (4). While these MAbs were approximately equally effective in lysing antigen-positive tumor cells in the present study, incubating PC of mouse origin with target cells for 3 days, only MAB MG-21 gave ADCC in a 4-h 51Cr release assay in which the effector cell had the markers of NK cells. These data suggest that the measurement of NK cell-mediated ADCC in a 4-h 51Cr release assay better correlates with antitumor activity of an antibody in nude mice than does the measurement of tumor cell killing by attached effector cells over a 3-day period. It has not been settled which of the two reactivities best correlates with antitumor activity in man.

Finally, we like to stress that a high level of tumor cell lysis could be reached by using the appropriate combination of antibodies and effector cells, e.g., MAB MG-21 and human monocytes/macrophages. A good strategy for obtaining therapeutic agents with high specific antitumor activity may, therefore, be to either select mouse MAB such as MG-21, or to manufacture "chimeric" (mouse-human) antibodies (18) of desirable specificity and a functional activity as good as that of MG-21.

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