Mouse Melanoma Antigen Recognized by Lyt-2- and L3T4- Cytotoxic T-Lymphocytes

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ABSTRACT

A mouse melanoma (B16) antigen was investigated at a cellular level by three blocking experiments using monoclonal melanoma antibodies, soluble melanoma antigen, and enzyme-treated B16 melanoma cells as inhibitors. The activity of antimelanoma cytotoxic T-lymphocytes (CTL) was specifically reduced by addition of the mixture of two monoclonal antimelanoma antibodies, one (M2590) recognizing the cross-species melanoma epitope on Gm3(NeuAc) and the other (M562) reactive with the mouse melanoma-specific epitope on protein molecules. The CTL activity was also blocked by Gm3 liposomes as well as by the soluble antigen. However, 3,000 times more Gm3 than the soluble melanoma antigen is required to obtain a similar inhibitory effect. When pronase-treated B16 melanoma cells, which have had protein molecules removed but Gm3 left intact on the surface, were used as an inhibitor, their blocking activity was greatly reduced but was still partly observed at a high inhibitor/target ratio. These results indicate that the melanoma antigen is not Gm3 itself but is composed of the Gm3-protein complex. This finding was also supported by using an interleukin 2-dependent CTL clone whose activity was blocked by both M562 and M2590. Antimelanoma CTL were found to belong to a double-negative T-cell population with Thy-1+, Lyt-2-, L3T4- phenotypes. L3T4+ T-cells were also demonstrated to be necessary for induction of double negative antimelanoma CTL.

INTRODUCTION

Tumor cells are known to express antigens that play a decisive role in the process of recognition and rejection of tumors by the host immune system (reviewed in Ref. 1). Our previous studies have shown that melanoma cells express a cross-species melanoma antigenic epitope widely shared by various mammalian species (2-4). The cross-species epitope is demonstrated as being the sugar determinant on Gm3(NeuAc), because a syngeneic monoclonal antimelanoma antibody (M2590) with cross-species reactivity has defined the Gm3(NeuAc) carbohydrate structure (5, 6). Moreover, the primary structure of melanoma Gm3 has been found to be the same as that of normal Gm3, but indeed to have melanoma specificity (5, 6). Therefore, the immunogenic melanoma antigen is not considered as simply Gm3 itself. In fact, immunoprecipitation analysis using the syngeneic monoclonal antibody specific for Gm3 has shown that the melanoma Gm3 ganglioside is noncovalently associated with proteins under physiological conditions (3, 4). This strongly suggests that the mouse melanoma antigen is a complex of Gm3 and proteins.

The present report describes the characterization of the melanoma antigen recognized by the syngeneic CTL raised in the in vitro primary response against C57BL/6 derived B16 melanoma. The results suggest that the mouse melanoma antigen is composed of Gm3 ganglioside in association with proteins, and also that the CTL epitope is not Gm3 itself. This supports the previous biochemical findings analyzed by sequential immunoprecipitation with syngeneic monoclonal antimelanoma antibodies.

MATERIALS AND METHODS

Animals. Pathogen-free C57BL/6 mice, 8–10 weeks old, were purchased from the Experimental Animal Co., Ltd., Hamamatsu, Japan.

Monoclonal Antibodies. The antimelanoma IgM monoclonal antibodies M562, M622, and M2590, raised by syngeneic immunization of C57BL/6 mice with B16 melanoma cells, were used in this study (3, 4). M2590 recognizes Gm3(NeuAc) epitopes whereas M562 and M622 antibodies recognize different epitopes on glycoproteins with a molecular weight of 80,000 as recently demonstrated by Sakiyama et al. (7) T141, an IgM monoclonal antibody without any specific binding activity, was obtained from Cederlane Laboratories Ltd., Hornby, Ontario, Canada. Anti-L3T4 monoclonal antibody (GK1.5) was originally established by Dyaln, and others (7).

Soluble Melanoma Antigen. The soluble melanoma antigen in the spent culture medium was used. The culture medium was centrifuged at 3,000 rpm for 20 min. The antigens were enriched by 50% sodium ammonium sulfate and used as an unfractionated material. In some experiments, soluble antigens were further affinity purified on a plate coated with M2590 antibody with alkaline elution. The concentration of Gm3 in the unfractracted or purified melanoma antigens was measured by enzyme-linked immunosorbent assay using the M2590 antibody or thin-layer chromatography as described previously (5).

Enzyme Treatment. B16 melanoma cells (2 x 10⁶) were treated with 20 μg/ml of pronase (Actinase E, Kaken Seiyaku Co., Ltd., Tokyo) for 10 min at 37°C, extensively washed, and used as inhibitors for CTL. After treatment, the expression of antigens on the melanoma cell surface was assayed before use by two types of monoclonal antibodies (M2590 and M562) to determine whether the treatment was successful or not (see Table 2).

Induction of in Vitro Primary CTL and Assay System. The in vitro primary culture of C57BL/6 spleen cells for generation of melanoma specific syngeneic CTL and the assay for the CTL activity were described previously (2). In brief, 3 x 10⁶ of naive spleen cells of C57BL/6 mice were cultured with 6 x 10⁶ MMC-treated B16 melanoma cells for 3 days. The CTL activity was assayed by 12-h incubation of cultured cells with ¹⁴Cr-labeled B16 melanoma cells as a target. As a control, allo-CTL were generated in the primary culture of C57BL/6 spleen cells (2 x 10⁶) by stimulation with MMC-treated BALB/c (H-2) spleen cells (5 x 10⁴) for 4 days and assayed for their activity on ¹⁴Cr-labeled BALB/c derived P815 (H-2) mastocytoma as a target for 4 h. The specific ¹⁴Cr-release in the supernates was assayed as described (2).

Establishment of Antimelanoma CTL Clones. Syngeneic CTL clones specific for melanoma were generated by a modification of the method of Kimoto and Fathman (8). In brief, C57BL/6 mice were immunized i.p. by injecting 5 x 10⁶ MMC-treated B16 melanoma cells. Ten days...
later, spleen cells (1 x 10^6) were suspended in RPMI 1640 and cultured in a 35-mm plastic Petri dish (Falcon 3001) with 2 x 10^6 MMC-treated B16 melanoma at 37°C in 5% CO₂ in air. Three days later, cells were collected, washed twice, and cultured at 37°C in 5% CO₂ in air in 96-well flat-bottomed plates (Corning 25860) at 1 x 10^4 viable cells per well with RPMI 1640 supplemented with 10% fetal calf serum, 5 x 10⁻⁴ M 2-mercaptoethanol, 200 µg/ml kanamycin, and 2 mm glutamin in the presence of 10% concanavalin A-stimulated rat spleen culture supernatant or 0.5-1% IL-2 obtained from phorbol-myristate-acetate-stimulated EL-4 culture supernatant. The cells were stimulated with 1.5 x 10^5 MMC-treated B16 and 5 x 10^5 irradiated syngeneic spleen cells at 8-day intervals. Seven days after the third stimulation cells from individual wells were analyzed for their cytotoxic activity on B16 melanoma by ^51Cr release assay as described. Cell lines which showed cytotoxic activity were expanded in 48-well plates (Costar 3548) and were further screened for their ability to be blocked by soluble melanoma antigen. Cells were further cloned by limiting dilution in 96-well plates at 0.8 viable cells per well with 1.5 x 10^6 B16 cells and 5 x 10^5 irradiated spleen cells in IL-2-containing medium. The cloning procedures were repeated several times. After limiting dilution, cloned cell lines were selected according to their specific killing activity on melanoma. The CTL clones were stimulated with 1.5 x 10^8 B16 cells in the presence of 5 x 10^5 syngeneic feeder cells in IL-2-containing medium at 10-day intervals to propagate cells for experiments.

Inhibition of CTL Activity. The activity of CTL induced in the primary response was blocked by addition of various inhibitors in the effector phase, such as monoclonal antibodies, soluble melanoma antigens, GM3 glycoprotein complex, and enzyme-treated B16 melanoma cells. These inhibitors were incubated together with CTL and ^51Cr-labeled B16 melanoma target cells at different target/inhibitor ratios. Percentage of inhibition was calculated by the following formula:

\[
\text{% Inhibition} = \left(1 - \frac{\text{% experimental release}}{\text{% control release}}\right) \times 100
\]

Cytotoxic Treatment. In order to analyze the phenotypes of cells involved in antitumor activity, naïve C57BL/6 spleen cells (5 x 10^6/ml) were treated with monovalent antibodies at room temperature for 30 min, followed by treatment with 2-week-old rabbit complement at 1:10 dilution for 40 min at 37°C. They were then used as the responder for the CTL induction system. The same cytotoxic treatment was carried out under identical conditions on CTL that had been induced in the primary culture before ^51Cr-release assay in order to determine the phenotypes of antitumor effectors.

RESULTS AND DISCUSSION

In our previous studies, two types of monoclonal antitumor antibodies were developed by the repeated immunization of B16 melanoma cells in syngeneic C57BL/6 mice (3, 4). M2590 recognizes the cross-species epitope on melanoma cells widely shared by various mammalian species and is defined as being the GM3(NeuAc) carbohydrate structure (5, 6). M562 and M622 antibodies react with the mouse-specific melanoma epitope which is present on a glycoprotein (3). Our recent studies have demonstrated that M562 and M622 epitopes are different but expressed on the same molecule with a molecular weight of 80,000. Additionally, the GM3(NeuAc) structure seems to be associated with the glycoprotein complex, as demonstrated by sequential precipitation analysis (3, 4).

It was also found that the melanoma-specific CTL raised in the in vitro primary response recognize the cross-species melanoma epitope and act across the species barrier (2). The most reasonable explanation for this is that the melanoma CTL recognize GM3 itself and kill the target. No self molecules seem to be involved in the antigen recognition by antitumor

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1 Sakiyama et al., manuscript in preparation.

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CTL. In fact, our previous studies support this idea, showing that the conventional antisera against the MHC antigen could not block the CTL activity, whereas some antitumor monoclonal antibodies do block the CTL effector function (see Fig. 1). The results indicate that the MHC-encoded molecules are not involved in antigen recognition by the antitumor melanoma. The existence of this unusual antigen recognition system, quite different from that of Lyt-2-positive CTL, may be due to the fact that antitumor CTL without MHC restriction belong to a new CTL population. As shown in Table 1, the activity of antitumor CTL was abrogated by treatment with anti-Thy-1 and complement. However, anti-Lyt-2 or anti-L3T4 treatments do not affect the antitumor CTL activity. These results indicate that antitumor CTL are L3T4⁺, Lyt-2⁻ (double-negative) T-cells.

In the Lyt-2⁺ CTL-mediated tumor-specific immune responses, L3T4⁺ T-helper cells have been demonstrated to be required in the generation of CTL. Thus, the cell type needed for the induction of melanoma specific double-negative CTL was investigated. As shown in Table 1, treatment of naïve spleen cells with anti-Thy-1 or anti-L3T4 significantly reduced CTL generation whereas anti-Lyt-2 treatment did not affect induction of antitumor CTL. This indicates that L3T4⁺ helper T-cells are indeed responsible for the induction of double-negative CTL, as they do function in the Lyt-2⁺ CTL generation. Several investigators have recently reported the existence

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1 See "Materials and Methods."  
2 naïve C57BL/6 spleen cells (induction phase) or cells cultured in the primary CTL response system (effector phase) were treated with monoclonal antibodies and complement (see "Materials and Methods").  
3 Arithmetic mean of cpm of four wells ± SD.

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Table 1: Phenotypes of cells involved in the induction and effector phase of antitumor melanoma CTL responses

<table>
<thead>
<tr>
<th>Cells treated with</th>
<th>Effector phase</th>
<th>Induction phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B16-CTL</td>
<td>EL-4-CTL</td>
</tr>
<tr>
<td>None</td>
<td>22.8 ± 1.7</td>
<td>12.0 ± 0.2</td>
</tr>
<tr>
<td>C⁺</td>
<td>30.3 ± 3.2</td>
<td>18.1 ± 1.1</td>
</tr>
<tr>
<td>Anti-Thy-1 + C⁺</td>
<td>4.6 ± 1.6</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Anti-Lyt-2 + C⁺</td>
<td>26.3 ± 3.6</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Anti-L3T4 + C⁺</td>
<td>25.6 ± 2.3</td>
<td>16.7 ± 0.4</td>
</tr>
</tbody>
</table>

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of Thy-1+ double-negative T-cells in mouse and human, some of which were demonstrated to mediate cytotoxic function (9–12). Thus, it is clear that this cell type functions in naturally occurring tumor-specific immune responses.

To determine whether the melanoma antigen recognized by the CTL is G\textsubscript{M3} or a complex of G\textsubscript{M3} with protein molecules, four experiments were carried out in which it was attempted to block the activity of CTL with several reagents, such as monoclonal antibodies, G\textsubscript{M3} liposomes and B16 melanoma cells treated with an enzyme. In the first experiment, two types of syngeneic antimalanoma antibodies were used as inhibitors, because one could recognize the melanoma epitopes on G\textsubscript{M3}, and the other on proteins. As shown in Fig. 1, the cytotoxic activity was significantly inhibited by either of the monoclonal antibodies (M2590 or M562). Furthermore, additive effects were observed when the two antibodies were added together. The blocking effects by the monoclonal antibodies seem to be specific for melanoma antigens because the control antibody (T141) or other antimelanoma antibody (M622) recognizing the epitope different from that of M562 on the same glycoprotein molecule showed no inhibitory effects even at a high concentration. These findings strongly suggest that the epitopes recognized by the CTL are composed of the structure related to both G\textsubscript{M3} and M562 determinants, and the M622 epitope is not involved in the antimalanoma CTL recognition, despite the fact that both M562 and M662 determinants are present on the same molecule. However, it is still possible that the blocking effects are only due to steric hindrance.

In order to determine whether G\textsubscript{M3} itself is in fact involved in the CTL recognition, the second series of experiments was carried out using both G\textsubscript{M3} liposomes and the soluble form of melanoma antigens as inhibitors. The cell surface melanoma antigens detected by the two monoclonal antibodies (M2590 and M562) were also present as a soluble form in the spent culture medium of B16 melanoma cells (3, 4). We thus attempted to purify the soluble antigen with M2590, and used this as an inhibitor in order to compare its effect with that of G\textsubscript{M3} liposomes.

The results shown in Fig. 2 demonstrated that the soluble melanoma antigen specifically inhibited the antimalanoma but not the anti-allo CTL activity in a dose-dependent manner. Similar melanoma-specific inhibitory effects were also obtained by G\textsubscript{M3} liposomes. However, about 3,000 times more amounts of G\textsubscript{M3} liposomes than those of the soluble antigen were required to demonstrate the same degree of inhibitory activity. Moreover, the CTL can not distinguish G\textsubscript{M4} molecular species, such as G\textsubscript{M3}(NeuAc) and G\textsubscript{M3}(NeuGc), while monoclonal antibodies or suppressor T-cells (Ts) do. This indicates that the antimalanoma CTL epitope and Ts/antibody epitopes are different.
different, and that the CTL epitope is not simply G\(_3\)M itself.

In the third experiment, B16 melanoma cells were treated with pronase in order to remove protein molecules from the cell surface and to leave G\(_3\)M intact on the membrane, and were used as inhibitors. The B16 cells thusly treated were investigated for their expression of G\(_3\)M and proteins on the membrane by the cell binding assay with two monoclonal antibodies (M2590 and M562) before use. After the treatment, they were found to be completely negative for the protein (M562) determinant, but still intact for the G\(_3\)M expression (Table 2).

As illustrated in Fig. 3, untreated B16 melanoma cells significantly blocked the CTL activity in a dose-dependent manner, while the blocking effects were entirely abrogated by the B16 cells treated with pronase at a target/inhibitor ratio of 1:3. However, marginal inhibitory effects were still observed at the ratio of 1:9. This is consistent with the results shown in Fig. 2, demonstrating that G\(_3\)M liposome alone, if it is at a high dose, blocked the CTL activity. These data suggest that melanoma antigen is composed of G\(_3\)M and protein and that some CTL recognize their combinational epitopes.

The above results were also supported by the data shown in Table 3. In this experiment, we used antimelanoma CTL clones and attempted to block their CTL activities with two monoclonal antibodies, M562 and M2590. Some of the clones were highly susceptible to M2590. However, the CTL activities of the same clones were also largely suppressed by the M562 antibody. Moreover, the inhibition of CTL activity of the clone was greatly increased by addition of the mixture of half doses of the two antibodies recognizing G\(_3\)M (M2590) and protein (M562), strongly indicating that CTL exist which recognize G\(_3\)M and protein epitopes.

The idea that the melanoma antigen is not simply G\(_3\)M itself has also been supported by the data demonstrating that antimelanoma G\(_3\)M antibody (M2590) react with melanoma cells but not with normal target cells with G\(_3\)M expression (4), despite the fact that the primary structure of melanoma G\(_3\)M is the same as normal G\(_3\)M (5). Thus, the apparent question is how the G\(_3\)M in the melanoma antigen generates the melanoma antigenicinity.

Several possible mechanisms account for the above question. First, the proteins associated with G\(_3\)M may modify the tertiary structure of G\(_3\)M to generate melanoma antigenicity. Second, the protein may function as molecules to assemble G\(_3\)M, resulting in an increase in the density of G\(_3\)M epitopes and making G\(_3\)M immunogenic. Thus, the antibody or the CTL would recognize the density of the G\(_3\)M epitope. In fact, the M2590 antibody does react with purified normal G\(_3\)M bound on thylayer silica plates as well as melanoma cells (5). As no protein molecules are involved in this reaction, this suggests that the M2590 antibody recognizes the density of normal G\(_3\)M. The third possibility is that there is an undefined melanoma antigenic molecule with the “G\(_3\)M-like structure” other than G\(_3\)M. In such a case, T-cells and antibodies would have a high affinity to the melanoma antigen with “G\(_3\)M-like structure” but would also have binding activity to normal G\(_3\)M with a relatively low affinity. This would be supported by recent findings that the affinity of M2590 to the soluble melanoma antigen is ten times higher than that to normal G\(_3\)M (NeuAc) (13). It is not known, however, whether any one of the proposed mechanisms actually operates. Experiments to confirm these possibilities are now under way.

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