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

Kumeo Ono, Kazuaki Takahashi, Yoshio Hirabayashi, Toshihiro Itoh, Yukihiko Hiraga, and Masaru Taniguchi

Department of Immunology, School of Medicine, Chiba University, Chiba, Japan 280 [K. O., K. T., T. I., Y. H., M. T.; and Department of Biochemistry, Shizuoka College of Pharmacology, Shizuoka, Japan 422 [Y. H.]

ABSTRACT

A mouse melanoma (B16) antigen was investigated at a cellular level by three blocking experiments using monoclonal antimelanoma antibodies, soluble melanoma antigens, and enzyme-treated B16 melanoma cells as inhibitors. The activity of antime lanoma 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 (M622) reactive with the mouse melanoma-specific epitope on protein molecules. The CTL activity was also blocked by GM3 liposome 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 M622 and M2590. Antime lanoma 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 antime lanoma 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 antime lanoma IgM monoclonal antibodies M622, 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 M622 and M622 antibodies recognize different epitopes on glycoproteins with a molecular weight of 80,000 as recently demonstrated by Sakiyama et al.4 T141, an IgM monoclonal antibody without any specific binding activity, was obtained from C57BL/6 mice and used as a control for antime lanoma antibodies. Monoclonal anti-Thy-1 antibody (CMS-1) was raised in our laboratory. Monoclonal anti-Lyt-2.2 (AD4) antibody was purchased from Cederlane Laboratories Ltd., Hornby, Ontario, Canada. Anti-L3T4 monoclonal antibody (GK1.5) was originally established by Dialynas et al. (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 antigen was enriched by 50% 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 unfractionated or purified melanoma antigens was measured by enzyme-linked immunosorbent assay using the M2590-GM3 antibody or thin-layer chromatography as described previously (5).

Enzyme Treatment. B16 melanoma cells (2 x 108) 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 M622) 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 105 MMC-treated B16 melanoma cells for 3 days. The CTL activity was assayed by 12-h incubation of cultured cells with 51Cr-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-2d) spleen cells (5 x 104) for 3 days and assayed for their activity on 51Cr-labeled BALB/c-derived P815 (H-2d) mastocytoma as a target for 4 h. The specific 51Cr-release in the supernates was assayed as described (2).

Establishment of Antime lanoma 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

Received 11/4/87; revised 1/19/88; accepted 2/19/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* This work was supported by Grants-in-Aid for Cancer Research and for Scientific Research on Priority Areas from the Ministry of Education, Culture and Science, Japan, and also by the Princess Takamatsu Cancer Research Foundation and the Uehara Memorial Foundation.

To whom requests for reprints should be addressed, at Department of Immunology, Chiba University, School of Medicine, 1-8-1 Inohana, Chiba, Japan 280.

The abbreviations used are: CTL, cytotoxic T-lymphocyte; MHC, major histocompatibility complex; MNC, mitomycin C; IL-2, interleukin 2.
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 10^5 viable cells per well with RPMI 1640 supplemented with 10% fetal calf serum, 5 x 10^-4 M 2-mercaptoethanol, 200 μg/ml kanamycin, 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 10^4 irradiated syngeneic spleen cells at 8-day intervals. Seven days after the third stimulation cells from individual wells were collected for their cytototoxic activity on B16 melanoma by 51Cr release assay as described. Cell lines which showed cytototoxic 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 10^5 B16 cells and 5 x 10^4 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 10^6 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 liposomes 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 antimelanoma CTL responses, naïve C57BL/6 spleen cells (5 x 10^6/ml) were treated with monoclonal 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 antimelanoma effector CTL.

RESULTS AND DISCUSSION

In our previous studies, two types of monoclonal antimelanoma 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. Moreover, the GM3 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 antimelanoma 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 antimelanoma 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 antimelanoma CTL. The existence of this unusual antigen recognition system, quite different from that of Lyt-2-positive CTL, may be due to the fact that antimelanoma CTL without MHC restriction belong to a new CTL population. As shown in Table 1, the activity of antimelanoma CTL was abrogated by treatment with anti-Thy-1 and complement. However, anti-Lyt-2 or anti-L3T4 treatments do not affect the antimelanoma CTL activity. These results indicate that antimelanoma CTL are L3T4⁺, Lyt-2⁻ (double-negative) T-cells.

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

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

*See "Materials and Methods."

Table 5: Specific 51Cr release in the control group was 36.7 ± 1.1% in this particular experiment.

Fig. 1. Inhibitory effects of monoclonal antibodies (Mab) on antimelanoma CTL. Antimelanoma CTL induced in the primary in vitro responses were blocked by various doses of monoclonal antibodies, such as M2590 recognizing GM3(NeuAc) with cross-species melanoma epitope (A), M562 recognizing mouse melanoma-specific epitope on glycoprotein (B), M622 reacting with mouse-specific melanoma epitope similar to but different from that of M562 (C), and control T14I antibody (D). Additive inhibitory effects were also observed when M2590 and M562 antibodies were added at a 1:1 ratio (B). Specific 51Cr release in the control group was 36.7 ± 1.1% in this particular experiment.
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 GM3 or a complex of GM3 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, GM3 liposomes and B16 melanoma cells treated with an enzyme. In the first experiment, two types of syngeneic antimelanoma antibodies were used as inhibitors, because one could recognize the melanoma epitopes on GM3, 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 GM3 and M562 determinants, and the M622 epitope is not involved in the antimelanoma CTL recognition, despite the fact that both M562 and M622 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 GM3 itself is in fact involved in the CTL recognition, the second series of experiments was carried out using both GM3 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 GM3 liposomes.

The results shown in Fig. 2 demonstrated that the soluble melanoma antigen specifically inhibited the antimelanoma but not the anti-allo CTL activity in a dose-dependent manner. Similar melanoma-specific inhibitory effects were also obtained by GM3 liposomes. However, about 3,000 times more amounts of GM3 liposomes than those of the soluble antigen were required to demonstrate the same degree of inhibitory activity. Moreover, the CTL can not distinguish GM3 molecular species, for example, GM3(NeuAc) and GM3(NeuGc), while monoclonal antibodies or suppressor T-cells (Ts) do. This indicates that the antimelanoma CTL epitope and Ts/antibody epitopes are different.

Table 2. Reactivity of monoclonal antimelanoma antibodies on B16 melanoma before or after treatment with pronase.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target Antigen</th>
<th>Before Pronase</th>
<th>After Pronase</th>
</tr>
</thead>
<tbody>
<tr>
<td>M562</td>
<td>Protein</td>
<td>1214 ± 137</td>
<td>0</td>
</tr>
<tr>
<td>M2590</td>
<td>GM3</td>
<td>3117 ± 243</td>
<td>3325 ± 579</td>
</tr>
</tbody>
</table>

* B16 melanoma cells (2 x 10^6) treated with or without pronase under the conditions described in "Materials and Methods." The cells treated were reacted with ^51^Cr-labeled monoclonal antibodies and radioactivity was counted.

* Arithmetic mean cpm of three wells ± SD.

Fig. 3. Inhibition of antimelanoma CTL activity with cells treated with pronase. Untreated B16 melanoma (■), C57BL/6 derived EL-4 lymphoma (▲), and B16 melanoma treated with pronase (□) were used as inhibitors and added to the effector phase of the antimelanoma CTL response at different target/inhibitor ratios, and assayed for their CTL activity. The expression of melanoma antigens on the cell surface was investigated by cell binding assay using ^51^Cr-labeled M622 and M2590 antibodies (see Table 2).

Table 3. Blocking of antimelanoma CTL activity with monoclonal antibodies

<table>
<thead>
<tr>
<th>CTL clones</th>
<th>M622</th>
<th>M562</th>
<th>M2590</th>
<th>M622* + M590</th>
<th>B16</th>
<th>EL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Blocking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Specific Cr release</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target/Inhibitor Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/1</td>
</tr>
<tr>
<td>10/2</td>
</tr>
<tr>
<td>10/3</td>
</tr>
<tr>
<td>10/4</td>
</tr>
<tr>
<td>10/5</td>
</tr>
</tbody>
</table>

* Equal amounts (0.15 mg/well each) of two monoclonal antibodies were mixed and used in the experiment.

* Arithmetic mean cpm of three wells ± SD.

* ND, not done.
different, and that the CTL epitope is not simply GM₃ 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 GM₃ intact on the membrane, and were used as inhibitors. The B16 cells thusly treated were investigated for their expression of GM₃ 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 GM₃ 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 GM₃ liposome alone, if it is at a high dose, blocked the CTL activity. These data suggest that melanoma antigen is composed of GM₃ and protein and that some CTL recognize their combinatorial 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 GM₃ (M2590) and protein (M562), strongly indicating that CTL exist which recognize GM₃ and protein epitopes.

The idea that the melanoma antigen is not simply GM₃ itself has also been supported by the data demonstrating that antimelanoma GM₃ antibody (M2590) react with melanoma cells but not with normal target cells with GM₃ expression (4), despite the fact that the primary structure of melanoma GM₃ is the same as normal GM₃ (5). Thus, the apparent question is how the GM₃ in the melanoma antigen generates the melanoma antigenicity.

Several possible mechanisms account for the above question. First, the proteins associated with GM₃ may modify the tertiary structure of GM₃ to generate melanoma antigenicity. Second, the protein may function as molecules to assemble GM₃, resulting in an increase in the density of GM₃ epitopes and making GM₃ immunogenic. Thus, the antibody or the CTL would recognize the density of the GM₃ epitope. In fact, the M2590 antibody does react with purified normal GM₃ bound on thin-layer 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 GM₃. The third possibility is that there is an undefined melanoma anti-genic molecule with the "GM₃-like structure" other than GM₃. In such a case, T-cells and antibodies would have a high affinity to the melanoma antigen with "GM₃-like structure" but would also have binding activity to normal GM₃ with a relatively low affinity. This would be supported by recent findings that the affinity of M2590 to the soluble melanoma antigen is more than 10 times higher than that to normal GM₃ (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.

ACKNOWLEDGMENTS

We thank Chimi Saitoh for the preparation of this manuscript.

REFERENCES

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

Kumeo Ono, Kazuaki Takahashi, Yoshio Hirabayashi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/10/2730

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/48/10/2730. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.