Identification by Monoclonal Antibody of the Tumor Antigen of a Human Autologous Breast Cancer Cell That Is Involved in Cytotoxicity by a Cytotoxic T-Cell Clone

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ABSTRACT

We have already established a pair of human autologous clones, tumor-specific cytotoxic T-lymphocyte clone T9HMC1 and tumor target clone HMC-1-8, that were derived from the metastatic pleural effusion of a patient with mammary carcinoma. In this paper, we describe the target antigen that was defined by monoclonal antibody 3A2. This monoclonal antibody selectively inhibited the cytotoxic action of T9HMC1 against HMC-1-8 autologous tumor target cells, but not the cytotoxicity of lymphokine-activated killer and possibly natural killer cells against HMC-1-8 cells. Western blot analysis using the 3A2 monoclonal antibody identified a molecule with an approximate molecular weight of 92,000. This antigen was highly expressed on autologous primary cancer cells of breast carcinoma tissue, but not on the normal mammary gland in the same patient. Moreover, this antigen can be detected on approximately 50% of human allogeneic breast carcinomas, but not on other neoplastic tissues such as gastric and colonie carcinomas except for one out of 10 prostatic carcinomas. Nonneoplastic normal cells did not express this antigen. It was also suggested that the antigen is not murine mammary tumor virus-related products. These data suggest that 3A2-defined antigen could participate in the cytotoxicity by human autologous cytotoxic T-lymphocytes as the target molecule expressed on tumor cells.

INTRODUCTION

We have previously demonstrated the establishment of an autologous CTL clone T9HMC1, that has been cultured, and it has retained its specific cytotoxic function against HMC-1-8 tumor target for quite a long time in the presence of IL-2 and with continuous stimulation of HMC-1-8 tumor antigens (1). This autologous pair of T9HMC1 and HMC-1-8 was derived from the metastatic pleural effusions of a patient with mammary carcinoma. This specific pair is very useful for the investigation of the cytotoxic mechanism of human CTL at the clonal level, since the cytotoxicity of this clone is highly specific for autologous HMC-1-8 cells. This cytotoxicity was also MHC class I-restricted and was inhibited by the addition of mabs reacting against CD3 and CD8, suggesting the presence of specific target antigen molecules or some specific peptide fragments on HMC-1-8 tumor cells (2).

In this paper, we described such a target antigen molecule with a molecular weight of 92,000 that was defined by mab 3A2. This mab could specifically block the cytotoxicity of T9HMC1 against HMC-1-8 cells. Furthermore, this antigen was expressed in primary autologous breast tumor tissue, whereas it was not found in the normal mammary gland of the same patient. The data strongly suggest that 3A2-defined antigen is involved in the cytotoxicity by human autologous CTLs.

MATERIALS AND METHODS

Cells. HMC-1-8 tumor cells and autologous human cytotoxic T-lymphocyte clones were reported previously (1, 3). Several allogenic tumor cell lines were employed in the screening for hybridomas as well as in the cytotoxicity study. HMC-2 and HPC-1 cells were derived from mammary carcinoma and pancreatic carcinoma, respectively, and were established in our laboratory (4, 5). MCF-7 is a mammary carcinoma line. M7609, Raji, Daudi, and K562 cells were also used in our previous report (1). HMC-1-8 cells were free of murine mammary tumor virus (MuMTV) as determined by the immunofluorescence using guinea pig anti-MuMTV sera (Electro-Nucleonics Laboratories, Inc., Silver Spring, MD).

Monoclonal Antibodies. In order to obtain an antibody that could react specifically with HMC-1-8 membrane antigens, we developed mab. The procedure of mab development was reported elsewhere (6). Briefly, BALB/c mice were immunized i.p. with 1 × 106 cells of HMC-1-8 two times during an interval of 14 days. Five days after the last immunization, approximately 2 × 106 of spleen cells were fused with 4 × 105 NS-1 mouse myeloma cells by polyethylene glycol 4000, according to the method described by Lembke et al. (7). After cultivation for 10 days, the supernatants of hybridomas were screened for antibody activity against HMC-1-8 but not other allogeneic tumor lines by indirect immunofluorescence. The positive hybrids were cloned by limiting dilution. Thus, one hybridoma clone mab 3A2 with IgM isotype was selected and subjected to further analysis. We also obtained mabs 11B4 and 3C6. These mabs reacted with HMC-1-8 as well as other allogeneic tumor lines, and recognized non-MHC cell surface membrane antigens.

For the inhibition study of the cytotoxicity of T9HMC1 against an HMC-1-8 target, in addition to mabs described above, we used mabs reacting against CD3, 4, and 8 antigens. These were purchased from the Ortho Pharmaceutical Co. Mabs that react with determinants on human MHC class I (HH-1) and II (TC-8B1) framework structures were developed in our own laboratory (2).

FACS Analysis of Cells Stained with mabs. The cells were washed twice with PBS, and 1–2 × 106 cells in 0.2 ml PBS were incubated with a saturated amount of mab for 30 min at 4°C. Cells were washed twice with PBS and incubated with 0.1 ml of FITC-conjugated goat anti-mouse Ig diluted 1:40 in PBS for 30 min at 4°C. Cells were then washed twice with PBS and fixed in 1–2% paraformaldehyde-PBS. Samples were run on a FACS analyzer of Becton-Dickinson. For control of the nonspecific binding of mouse Ig or FITC-conjugated goat anti-mouse mouse serum diluted 1:5 and/or FITC-conjugated antimouse Ig alone diluted 1:40. We routinely analyzed 1–2 × 105 cells per sample.

Western Blot Analysis of 3A2-defined Antigen. Mab 3A2-defined antigen was analyzed by using the Western blot technique. Briefly, approximately 50 μg of 1% NP-40 solubilized antigens of HMC-1-8 were run on SDS-PAGE under a reduced condition. The antigens in a polyacrylamide gel were transferred to a nitrocellulose membrane (Immobilon PVDF transfer membrane, Nihon Millipore Kogyo K. K., Yonezawa, Japan) using a TE series transphor electrophoresis unit (HSI Co., San Francisco, CA), and were reacted with a saturated...
amount of 3A2 mab. Finally the antigen molecules were detected by Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA).

Blocking Experiment by mabs of the Cytotoxicity of T<sub>TCHMC<sub>1</sub></sub> against an Autologous HMC-1-8 Target. To investigate whether the 3A2-defined molecule could play an important role in the cytotoxicity of T<sub>TCHMC<sub>1</sub></sub> clone against HMC-1-8 autologous tumor cells, we studied the effects of mab treatment of target cells in the cytotoxicity assays. Details of the 3<sup>125</sup>C release cytotoxicity assays for the determination of T-cell cytotoxic activity were described previously (1). The percentage of lysis was determined as percentage of specific lysis = (experimental release - spontaneous release) × 100/(maximal release - spontaneous release). In this paper, the data was represented as percentage inhibition of the cytotoxicity when compared with percentage cytotoxicity of medium alone.

Although the data was already reported (2), in order to take the experimental control for blocking assays by mabs on the cytotoxicity of T<sub>TCHMC<sub>1</sub></sub> against HMC-1-8 target, we also repeated the experiments in which the effects of mabs reacting against CD3, CD4, CD8, HMC class I and class II on the cytotoxicity of this T-cytotoxic clone were studied. T<sub>TCHMC<sub>1</sub></sub> was treated with saturated amounts of anti-CD3, 4 and 8 mabs on 4°C for 60 min. The cells were washed twice with PBS and used in the cytotoxicity assays. HH-1 (anti-class I) and TC-8B1 (anti-class II) mabs were used to pretreat HMC-1-8 targets. HMC-1-8 cells treated at 4°C for 60 min with saturated amounts of these mabs were washed twice with PBS and were used in the cytotoxicity assays.

All assays were done in triplicate at 50:1 of effector/target ratio for 12-h cultivation. The spontaneous release of 3<sup>125</sup>C from target cells in the cytotoxicity experiments was less than 20% of the maximum release. The statistical analysis, i.e., standard errors, of the data in the inhibition study of the cytotoxicity was determined by comparing the mean value of percentage of inhibition of at least two separate experiments.

Preparation of NK and LAK Cells. Human peripheral blood from healthy randomly selected donors was obtained from our laboratory. Mononuclear cells were separated by Ficoll Conray centrifugation as described elsewhere (1). After washing, the cells were incubated in plastic flasks (Falcon 3024) for 3 h to remove any adherent cells, after which the nonadherent cells were run through a nylon wool column for 1 h at 37°C and collected. These cells were used as the NK cells against HMC-1-8, K562, and Daudi target cells at predetermined effector/target ratios in the cytotoxicity assays. The LAK cells were obtained by coculturing these mononuclear lymphocytes for 3 days at 37°C in the presence of 20 units of IL-2 as described above. The cells were washed with PBS and were used as LAK effector cells against HMC-1-8 target cells in the cytotoxicity assays. Mab 3A2 was also used as to whether this mab could inhibit the cytotoxicity of NK or LAK cells against HMC-1-8 target cells. The procedure for this assay was the same as that in the blocking experiment by 3A2 of the cytotoxicity of T<sub>TCHMC<sub>1</sub></sub> against HMC-1-8 autologous target cells as described above.

Immunohistochemical Staining in Primary Autologous Mammary Tumor and Allogeneic Tumor Tissues. Paraffin-embedded sections of primary autologous mammary tumor or noncancerous mammary gland, and primary allogeneic tumor tissues such as mammary, prostatic, gastric, and colonic carcinomas were obtained from freshly operated materials. These sections were deparaffinized, and were incubated with or without mab 3A2 for 1 h at room temperature. These sections were washed with PBS for 20 min and reincubated with 200x diluted serum of biotinylated goat antimouse Ig for 1 h at room temperature. After washing with PBS for another 20 min, these sections were stained by the biotin-avidin horseradish peroxidase ABC kit (Vector Laboratories, Inc.).

### RESULTS

Establishment of mab Reacting against HMC-1-8. We studied the cell surface antigen expressed specifically on HMC-1-8, since there was a possibility that the antigen expressed on the target cells could be recognized by T<sub>TCHMC<sub>1</sub></sub> CTL clone. By immunizing HMC-1-8 cells to mice, we obtained several hybridomas. The scoring system of data by using the FACS analyzer is as follows; when the median channel number was set at approximately 110, cells that showed a fluorescence of below 15%, 15-25%, and >25% were −, ±, and +, respectively. As summarized in Table 1, mab 3A2 could react specifically with HMC-1-8, although M7609 colon cancer line faintly expressed the 3A2-defined antigen on a panel of cell lines. This mab did not react with HMC-2 and MCF-7 breast cancer lines, HPC-1 pancreatic cancer line, Raji and Daudi cell lines. In contrast, mabs 11B4 and 3C6 could react with HMC-1-8 as well as other allogeneic tumor lines, and these recognized non-MHC cell surface antigens.

We assessed the determinant defined by 3A2 mab using the FACS analyzer. HMC-1-8 cells were treated with (a) 1.0 percentage of periodic acid for 60 min at 37°C, (b) 0.5 mg/ml pronase for 5 min at 37°C, and (c) 0.25 unit/ml neuraminidase for 60 min at 37°C. In this experiment, HMC-1-8 cells without treatment showed approximately 57.0% of a positive fluorescence in the FACS analysis. The cells treated with periodic acid, pronase and neuraminidase showed 13.5, 52.8, and 44.6%, respectively, suggesting that the determinant defined by 3A2 mab is composed of the carbohydrate portion.

Western Blot Analysis of 3A2-defined Antigen. Fig. 1 indicates 3A2-defined antigen analyzed by the Western blot techniques. 1% NP-40 solubilized antigens of HMC-1-8 were run on SDS-PAGE under a reduced condition. The antigens in a polyacrylamide gel were transferred to a nitrocellulose membrane, and were reacted with 3A2 mab. The data indicate that 3A2 could recognize the antigen molecule with approximately 92,000 molecular weight. This M<sub>r</sub> 92,000 component appeared to consist of a single polypeptide chain containing no disulfide bonds, as suggested by the fact that the chain ran identically in gels under both reducing and nonreducing conditions (data not shown).

Blocking by mabs of the Cytotoxicity of T<sub>TCHMC<sub>1</sub></sub> against an Autologous Target. It was demonstrated that 3A2-defined antigen could be expressed selectively on HMC-1-8. On the other hand, in our previous study T<sub>TCHMC<sub>1</sub></sub> showed the specific cytotoxicity against an autologous breast cancer line HMC-1-8. Hence, we determined whether 3A2-defined antigen could be involved in the cytotoxic mechanisms of T<sub>TCHMC<sub>1</sub></sub> clone against a HMC-1-8 target. Fig. 2 indicates results of the blocking experiments by mabs of T<sub>TCHMC<sub>1</sub></sub> cytotoxicity against a HMC-1-8 target. HMC-1-8 cells were pretreated with saturated amounts of 3A2, 11B4, and 3C6 mabs, and were used as target cells in the cytotoxicity assays. It was clearly demonstrated that 3A2 but not 11B4 and 3C6 could inhibit the cytotoxic activity of this cytotoxic lymphocyte clone. For experimental controls,
TUMOR ANTIGEN RECOGNIZED BY AUTOLOGOUS CTL

Raji cells. These results suggest that 3A2-defined antigen expressed on HMC-1-8 cells could play an important role possibly as the target molecule on the cytotoxicity of T_{HMC-1} CTL clone against an autologous-derived HMC-1-8 breast cancer line.

In the next step of the experiments, we examined whether the 3A2-defined molecule could also be involved in the mechanisms of the nonspecific cytotoxicity such as allogeneic NK and LAK cells. However, we did not have the inhibition experiment by 3A2 mab, since HMC-1-8 cells were not susceptible to the cytotoxicity of NK cells, as indicated in Table 2. In contrast, Table 2 also showed that HMC1-8 cells were highly susceptible to the cytotoxicity by LAK cells. We studied whether 3A2 could inhibit this cytotoxic activity by allogeneic LAK cells against HMC-1-8 cells. The data clearly indicated that 3A2 mab enhanced the cytotoxicity of LAK cells against HMC-1-8 target cells. These data suggest that 3A2-defined antigen molecule could not participate in the cytotoxic mechanisms of LAK cells, and possibly that this molecule may play an important role as the specific target molecule in the cytotoxicity of T_{HMC-1} CTL against an autologous breast cancer cell.

Expression of 3A2-defined Antigen on Primary Autologous Cancer and Allogeneic Cancer Tissues. The expression of 3A2-defined antigen on HMC-1-8 cells was examined by Western blot analysis. Approximately 50 μg of 1% NP40 solubilized antigens of HMC-1-8 were run on SDS-PAGE under a reduced condition. The antigens in a polyacrylamide gel were transferred to a nitrocellulose membrane, and were reacted with a saturated amount of 3A2. For details see the "Results."

**Fig. 1.** 3A2-defined antigen of HMC-1-8 cells by the Western blot analysis. Approximately 50 μg of 1% NP40 solubilized antigens of HMC-1-8 were run on SDS-PAGE under a reduced condition. The antigens in a polyacrylamide gel were transferred to a nitrocellulose membrane, and were reacted with a saturated amount of 3A2. For details see the "Results."

**Table 2.** Susceptibility of HMC-1-8 cells and the effect of 3A2 mab in the cytotoxicity of NK and LAK cells

<table>
<thead>
<tr>
<th>Target cells</th>
<th>% cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMC-1-8</td>
<td>3.7 ± 2.6</td>
</tr>
<tr>
<td>K562</td>
<td>42.8 ± 0.8</td>
</tr>
<tr>
<td>Daudi</td>
<td>19.2 ± 5.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target cells</th>
<th>3A2 treatment</th>
<th>% cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMC-1-8</td>
<td>−</td>
<td>76.2 ± 7.8</td>
</tr>
<tr>
<td>HMC-1-8</td>
<td>+</td>
<td>111.6 ± 7.2</td>
</tr>
</tbody>
</table>

* Peripheral blood nonadherent cells were run through a nylon wool column as described in "Materials and Methods." These cells were used as the NK cells against the target cells at 100 effectors/target ratio. The cultures of the cytotoxic assays were done for 12 h at 37°C in humidified 5% CO₂ incubator.

* The LAK cells were obtained by coculturing the mononuclear lymphocytes for 3 days at 37°C in the presence of 20 units of IL-2. The cells were washed with PBS and were used as LAK effector cells against HMC-1-8 target cells. HMC-1-8 target cells were also treated with a saturated amount of mab 3A2, washed with PBS and were used as target cells in the cytotoxicity assays. The effectors/target ratio was 100, and the cultures were done for 12 h.
defined antigen was assessed immunohistochemically on primary autologous mammary tumor as well as other allogeneic cancer tissues including mammary, prostatic, gastric, and colonic tumors. Deparaffinized tissue sections were reacted with mab 3A2, and were stained by using the biotin-avidin horse-radish peroxidase technique as described in “Materials and Methods.” As shown in Fig. 4, top, 3A2-defined antigen was expressed clearly on primary mammary cancer cells. It seemed that this antigen was also present in some of the lumen composed of mammary tumor cells. However, this antigen could not be detected on autologous non-cancerous mammary glands of the same patient (Fig. 4, bottom).

We studied the expression of 3A2-defined antigen on other allogeneic tumor tissues. Table 3 showed a summary of this experiment. The data indicated that five out of ten allogeneic mammary carcinomas and one out of 10 prostatic carcinomas were positive for the expression of 3A2-defined antigen. However, it was interesting to note that noncancerous glands of these six cases of mammary and prostatic carcinomas did not express this antigen. Furthermore, we examined nine cases of gastric carcinomas and five of colonic carcinomas for the expression of 3A2-defined antigen. All cases of these cancer cells as well as noncancerous cells did not express this antigen.

**Table 3 Immunohistochemical analysis of the expression of 3A2-defined antigen on allogeneic tumor and normal tissues**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Positive/cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td></td>
</tr>
<tr>
<td>Neoplastic</td>
<td>5/10</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0/10</td>
</tr>
<tr>
<td>Prostate</td>
<td></td>
</tr>
<tr>
<td>Neoplastic</td>
<td>1/10</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0/10</td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
</tr>
<tr>
<td>Neoplastic</td>
<td>0/8</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0/8</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
</tr>
<tr>
<td>Neoplastic</td>
<td>0/5</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0/5</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Although there are a number of reports that have studied CTL against tumor cells in the syngeneic animal tumor models, the effector mechanisms of autologous CTL against human tumor cells have yet to be resolved. For the past 5 years, in ours as well as other laboratories, CTL derived from tumor associated lymphocytes were successfully cloned by using a mixed lymphocyte-tumor cell culture and IL-2 (1, 8–14). CTL clones were indicated as being cytotoxic specifically against autologous human tumor targets. These experimental findings directly suggest that autologous CTL can be generated in the tumor-bearing patients. One such pair of TC_HMC1 CTL clone and HMC-1-8 tumor line was derived from the pleural effusion of the metastatic breast carcinoma (1). It was indicated that the cytotoxicity of this specific pair was MHC class I restricted. mab reacting against CD3 and CD8 molecules could inhibit the cytotoxicity of TC_HMC1. These data suggest that T3/Ti complex on TC_HMC1 clone could be involved in the cytotoxic mechanism of this specific pair of CTL and tumor cell clone. Furthermore, we considered that there could be the target antigen molecule expressed on HMC-1-8 tumor cells that is recognized by TC_HMC1, CTL alone.

In this paper, we analyzed this target antigen molecule possibly expressed on HMC-1-8 cells. We successfully obtained mab 3A2 that perhaps recognizes a target antigen, and this paper is the first demonstration of such target antigens expressed on human cancer cells that are recognized by an autologous CTL clone. The data strongly suggested that mab 3A2-defined M, 92,000 antigen could play an important role as the target molecule in the cytotoxicity of autologous TC_HMC1 CTL against HMC-1-8 mammary cancer cells. This antigen was not involved in the cytotoxicity to LAK, and 3A2 mab rather enhanced LAK cytotoxicity against HMC-1-8. It is currently uncertain as to the mechanism by which this mab could enhance LAK cytotoxicity.

The antigen was also expressed on the primary breast cancer tissue of CTL and target clone-derived patient. However, it was not detected on noncancerous normal mammary gland of the same patient. Furthermore, it was demonstrated that this antigen was expressed on allogeneic breast cancer tissues, and in the same sense with autologous tissues, it was not found in the noncancerous mammary gland of these allogeneic tissues. We could not detect this antigen on several allogeneic cancer tissues of gastric and colonic origins other than the mammary gland except for one tumor out of ten prostatic carcinomas. It is not likely that 3A2-defined antigen is a product which is antigenically related to MuMTV in terms of the molecular nature of...
antigen. Moreover, polyclonal anti-MuMTV antibody could not react with HMC-1-8 cells.

On the other hand, there has recently been a growing evidence that the MHC class I and II molecules bind small peptides derived from internal degradation and present the complexes on the cell surface for T-cell recognition (15-18). According to these reports, class I-restricted CTL could recognize peptide fragments, and the three-dimensional structure of class I molecule reveals a peptide-binding site. These fine works with respect to CTL recognition of antigen in the context of class I restriction may not precisely explain our present data. As mentioned above, the cytotoxicity of T<sub>1</sub>MC<sub>1</sub> against HMC-1-8 is clearly class I restricted, and perhaps T-cell receptor complexes may be involved, since mab reacting against CD3 blocked T<sub>1</sub>MC<sub>1</sub> Cytotoxicity. There seems to be less possibility of direct association between class I and antigen 3A2-defined M,92,000 molecule because of the large size of molecular weight of 3A2-defined antigen. However, as reported by Allen <i>et al.</i> (19) and Gerlier <i>et al.</i> (20), there are several ways for the antigen processing, indicating that some antigens could not be processed or degraded in the cells, and rather they were expressed as nonprocessed-intact molecules. It is not currently clear that the 3A2-defined molecule might be such an unprocessed antigen.

We should consider another possibility that may explain the inhibitory mechanism of T<sub>1</sub>MC<sub>1</sub> cytotoxicity by 3A2 mab. Isotype of 3A2 is IgM, and the determinants on HMC-1-8 cells recognized by mab was indicated to be a carbohydrate portion of the molecule. In this case, it is likely that 3A2 could not directly block the antigenic peptides recognized by T-cells, and this means other nonspecific mechanisms such as the steric hindrance. If 3A2-defined molecule and class I molecule could be localized very closely on the cell surface of HMC-1-8, it may explain the clear inhibition of T<sub>1</sub>MC<sub>1</sub> cytotoxicity by 3A2.

There is another possibility that the antigen may act simply as a ligand of target cells in the cell-conjugate formation between T<sub>1</sub>MC<sub>1</sub> CTL and HMC-1-8 target cells. An increasing number of recent reports (21-24) have suggested that LFA-3 and ICAM-1 could play an important role as ligands in various cell interactions including the cytotoxicity by CTL. However, it has been suggested that the expression of these molecules is not increased along with the cell transformation, but rather reduced as typically demonstrated in fibronectin (25). It is unlikely that 3A2-defined antigen is strongly associated with the neoplastic phenotype of the mammary gland. This fact suggests that this antigen is not one of the molecules which are involved in the mechanisms of cell adhesion.

Currently, we are developing new pairs of human CTL clones and autologous tumor targets. The same approaches to clarify the target antigen molecule for T-cell recognition, as demonstrated in this paper, are urgently required, since these kinds of studies directly lead not only to an understanding of the whole mechanism of the destruction of autologous tumor cells, but to clinical approaches of specific immunotherapy.

REFERENCES

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