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 T\textsubscript{HMC-1} 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 T\textsubscript{HMC-1} 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 colon 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\textsuperscript{1} clone, T\textsubscript{HMC-1}, 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 T\textsubscript{HMC-1} 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 T\textsubscript{HMC-1} 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 allogeneic 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. M7669, Raji, Daudi, and K562 cells were also used in our previous report (1). HMC-1-8 cells were free of murine mammary tumor virus (MuMIV) 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 x 10\textsuperscript{7} cells of HMC-1-8 two times during an interval of 14 days. Five days after the last immunization, approximately 2 x 10\textsuperscript{7} spleen cells were fused with 4 x 10\textsuperscript{7} NS-1 mouse myeloma cells by polyethyleneglycol 4000, according to the method described by Lemke 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 T\textsubscript{HMC-1} 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 x 10\textsuperscript{7} 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 serum to the cells, parallel samples were made by staining with a normal mouse serum diluted 1:5 and/or FITC-conjugated antihorse Ig alone diluted 1:40. We routinely analyzed 1 - 2 x 10\textsuperscript{7} 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 antibody selectively inhibited the cytotoxic action of T\textsubscript{HMC-1} 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 colon 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.

Received 1/17/89; revised 4/10/89; accepted 4/19/89.

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1 This work was supported by a Research Grant of the Princess Takamatsu Cancer Research Fund and a Grant-in-Aid for Special Project Research by Biotechnology.

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3 The abbreviations used are: CTL, cytotoxic T-lymphocyte; IL-2, interleukin 2; MHC, major histocompatibility complex; mab, monoclonal antibody; MuMTV, murine mammary tumor virus; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; FACS, fluorescein-activated cell sorter; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ABC, avidin-biotin complex; NK, natural killer; LAK, lymphokine-activated killer.
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 T₄HMC₁ against an Autologous HMC-1-8 Target. To investigate whether the 3A2-defined molecule could play an important role in the cytotoxicity of T₄HMC₁ 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 ¹⁸⁷⁷C 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) x 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 T₄HMC₁ 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. T₄HMC₁ 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 ¹⁸⁷⁷C 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 assessed to see 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 T₄HMC₁ 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 reacted with 3A2 mab. The data indicate that 3A2 could recognize the antigen molecule with approximately 92,000 molecular weight. This M₉, 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).

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 M₉, 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 T₄HMC₁ 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 T₄HMC₁ 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 T₄HMC₁ clone against a HMC-1-8 target. Fig. 2 indicates results of the blocking experiments by mabs of T₄HMC₁ 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,

### Table 1 Reactivity of mabs against human tumor lines

<table>
<thead>
<tr>
<th>Cells</th>
<th>HMC-1-8</th>
<th>HMC-2</th>
<th>MCF-7</th>
<th>HPC-1</th>
<th>M7609</th>
<th>Raji</th>
<th>Daudi</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A2</td>
<td>±</td>
<td></td>
<td>±</td>
<td></td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>11B4</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>3C6</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

* The scoring of data by using the FACS analyzer is mentioned in the text.
TUMOR ANTIGEN RECOGNIZED BY AUTOLOGOUS CTL

92K 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 TcHMc-I 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 TcHMc-I 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 primary autologous cancer and allogeneic cancer tissues was studied.

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 3 The effect of mabs on the cytotoxicity of TcHMc-I against HMC-1-8 target cells. In Experiment 1, HMC-1-8 target cells were treated with a saturated amount of mabs HH-1 and TC-8B1 at 4°C for 60 min, and were also included the experiments in which the effects of mabs reacting against MHC class I and II antigens on target cells as well as mabs against CD3, 4, and 8 antigens on this CTL clone were studied. As shown in Fig. 3, the cytotoxicity of TcHMc-I was blocked (1) with pretreatment of HMC-1-8 target cells by mab against MHC class I antigens and (2) with pretreatment of TcHMc-I by mabs against CD3 and 8. It was suggested that 11B4 and 3C6 could react with non-MHC cell surface antigens, since these mabs did not react with MHC class I- and II-positive...
TUMOR ANTIGEN RECOGNIZED BY AUTOLOGOUS CTL

defined antigen was assessed immunohistochemically on primary autologous mammary tumor as well as other allogeneic cancer tissues including mammary, prostatic, gastric, and colonic tumors. Deparaffinated tissue sections were reacted with mab 3A2, and were stained by using the biotin-avidin horseradish 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 Neoplastic</td>
<td>5/10</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0/10</td>
</tr>
<tr>
<td>Prostate Neoplastic</td>
<td>1/10</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0/10</td>
</tr>
<tr>
<td>Stomach Neoplastic</td>
<td>0/8</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td>0/8</td>
</tr>
<tr>
<td>Colon 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 TeHMC-1 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 TeHMC-1. These data suggest that T3/Ti complex on TeHMC-1 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 TeHMC-1, 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 TeHMC-1 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

Fig. 4. The immunohistochemical analysis of the expression of 3A2-defined antigen in autologous primary cancer and noncancerous tissues. Top, autologous primary mammary carcinomas; bottom, noncancerous mammary gland (x200).
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>HMC</sub>-1 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>HMC</sub>-1 Cytotoxicity. There seems to be less possibility of direct association between class I and antigenic 3A2-defined M, 92,000 molecule because of the large size of molecular weight of 3A2-defined antigen. However, as reported by Allen et al. (19) and Gerlier et al. (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>HMC</sub>-1 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>HMC</sub>-1 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>HMC</sub>-1 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 mammmary 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

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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