Characterization of \(n\)-Butyl Alcohol Solubilized, Breast Tumor Specific Antigens Recognized by a Human Autologous Cytotoxic T-Cell Clone

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ABSTRACT

We demonstrated previously the establishment of a human cytotoxic T-cell clone, \(T_{\text{CHMC-1}}\), under culturing with recombinant interleukin that showed the specific cytotoxicity against an autologous breast tumor cell line, HMC-1-8. In the present study, the autologous tumor specific antigens that could be involved in this cytotoxicity were extracted by using \(n\)-butyl alcohol and were analyzed for their biochemical profiles.

The cytotoxicity of \(T_{\text{CHMC-1}}\) against HMC-1-8 was inhibited by adding OKT3 and OKT8 monoclonal antibodies into the cultures, or by presensitizing HMC-1-8 target cells by anti-major histocompatibility complex class I monoclonal antibodies. This suggests that T-cell antigen receptor molecule complexes T/3 on \(T_{\text{CHMC-1}}\) and corresponding specific tumor antigens on HMC-1-8 are involved in the cytotoxicity under the restriction of major histocompatibility complex class I products.

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The abbreviations used are: CTL, cytotoxic T-lymphocyte; rIL-2, recombinant interleukin 2; MHC, major histocompatibility complex; Mab, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TSTA, tumor-specific transplantation antigen; CBE, crude \(n\)-butyl alcohol extract; PBS, phosphate buffered saline; FPLC, fast protein liquid chromatography.

INTRODUCTION

Tumor cells have been demonstrated to be susceptible to cytotoxicity by various effector cells of animal hosts (1, 2). In these experiments syngeneic inbred mice or rats usually were used. However, there is no question that it is most important to use tumors and effector cells of autologous origin in order to study the host effector mechanisms against tumors, since expected differences such as minor histocompatibility antigens are possible among tumors and animals. In human cancer patients, details at the clonal level of CTL1 effector mechanisms against autologous tumors are still unknown, although a few works have demonstrated autologous human CTL against tumors (3-6).

Before the advent of rIL-2 that proliferates T-lymphocytes, CTL against tumors was assessed by using T-cells in bulk cultures; however, it was very difficult to study the diversity, proliferation, and functions of CTL at the clonal level. rIL-2 has made it possible to proliferate T-cells that retain their cytotoxic functions and to analyze antigens that bind specifically to clonotypic T-cell antigen receptor complexes T/3 (7).

We have previously demonstrated establishment of an autologous cytotoxic T-cell clone, \(T_{\text{CHMC-1}}\), that has been cultured and has retained its specific cytotoxic function against HMC-1-8 for over 1 year in the presence of rIL-2 and with continuous stimulation of target HMC-1-8 tumor antigens (8). \(T_{\text{CHMC-1}}\) and HMC-1-8 were derived from the metastatic pleural effusions of a patient with mammary carcinoma.

This specific autologous pair of CTLs and tumor cells is very useful for the investigation of the cytotoxic mechanism of human CTLS 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 blocked by addition of Mab OKT3 and OKT8, suggesting the presence of specific antigens on HMC-1-8 targets.

In this paper, we show the noncytolytic solubilization of the HMC-1-8 tumor antigens by 2.5% \(n\)-butyl alcohol (9–11). We purified partially the autologous tumor antigen molecules by several biochemical procedures. SDS-PAGE analysis indicated three separate molecules with molecular weights of 26,000, 30,000, and 32,000. Our previous study demonstrates that \(T_{\text{CHMC-1}}\) could specifically reject HMC-1-8 tumor challenge in the transplantation experiments using nude mice (8). Our present report is very important since these molecules might be a putative human homologue of antigens that are comparable with TSTAs in the animals.

MATERIALS AND METHODS

Cells. HMC-1-8 tumor cells and autologous human cytotoxic T-cell clones were reported previously (8). Briefly, both cells were derived from a malignant pleural effusion of a 35-year-old female. Propagated adherent tumor cells were cultured and passaged weekly by trypsinization (0.05% trypsin plus 0.02% EDTA). Continuous tumor cell lines were established about 1 month after initiation of the culture and were named HMC-1. T-lymphocytes were purified from pleural effusions by Percoll discontinuous density centrifugation. Approximately 1 \(\times\) \(10^6\) of these lymphocytes were stimulated with 1 \(\times\) \(10^5\) mitomycin C-treated uncloned HMC-1 autologous tumor cells for 4 days at 37°C in a 5% CO2 incubator. The cloning of these cytotoxic T-cells was carried out by a limiting dilution in 96-well microtiter plates (Costar No. 3799). The cells were continuously expanded in 0.2 ml of medium containing 20 units/ml of rIL-2, kindly provided by Dr. J. Hamuro (Ajinomoto Central Research Laboratory, Tokyo, Japan). At 2 weeks of cultivation, six clones were successfully grown in the wells, and their cytotoxic potentials against uncloned HMC-1 were assessed. Clone 1, designated \(T_{\text{CHMC-1}}\), was most cytotoxic against HMC-1 cells. Furthermore, HMC-

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1 cells were undergone to single cell cloning in order to obtain a sensitive target for the cytotoxicity of T-cell clones, because the cytotoxic potentials of T-cells against autologous uncloned HMC-1 cells were gradually decreasing during cultivation. Eight HMC-1 clones were obtained. These were used as target cell clones for the cytotoxicity assays, and the most sensitive HMC-1-8 target clone was obtained (8).

Thereafter, T$_{HMC-1}$ clone was restimulated at weekly intervals with autologous mixed tumor cell culture using mitomycin C treated HMC-1-8 cells and was expanded under the presence of rIL-2.

Allogeneic Cell Lines. Several allogeneic tumor cell lines were used as targets in the cytotoxicity assays of T$_{HMC-1}$ clone. HMC-2 and HPC-3 were derived from mammary cancer and pancreatic cancer, respectively, and were established in our laboratory (12, 13). ZR-75-1 and K562 were purchased from Flow Laboratories, Inc., Bethesda, MD.

Mabs. Mabs OKT3, OKT4, and OKT8 were obtained from Ortho Pharmaceutical. Mabs that react with determinants on human MHC class I (HH-1) and II (TC-8B1) framework structures were developed in our own laboratory (14). These Mabs were used in the cytotoxicity assays of T$_{HMC-1}$ against an HMC-1-8 target clone.

n-Butyl Alcohol Extraction. Nocyticlytic extraction of cell surface antigens from HMC-1-8 was done as reported previously (11, 15). HMC-2 cells were also treated with butanol HMC-1-8 for control of CBE to the stimulation of T$_{HMC-1}$. Briefly, 2.5% n-butyl alcohol (Nakai Chemical, Ltd., Kyoto, Japan) in PBS was mixed with 1-2 x 10$^6$ cells/ml PBS. After the solution was incubated at room temperature for 25 min with HMC-1-8 or HMC-2, it was centrifuged at 500 x g for 5 min. The supernatant was then centrifuged at 2000 x g for 10 min, dialyzed for 24 h against PBS, and concentrated partially against 50% sucrose in PBS. The supernatant was dialyzed in PBS and centrifuged at 160,000 x g for 1 h. The supernatant was referred to as CBE. All proteins for the experiments were measured by the method of Lowry and co-workers (16).

We assessed the effect of HMC-1-8 CBE on the potential of T$_{HMC-1}$ cytotoxicity against HMC-1-8 target cells. T$_{HMC-1}$ cells were cultured with 20 ml of CBE for 2 days at 37°C in 5% CO$_2$ incubator. These cells were then multiplied under the presence of 20 units/ml of rIL-2 for another 2 days at 37°C and were assessed for the cytotoxic potential against HMC-1-8. Furthermore, the effect of HMC-1-8 CBE treatment or the target specificity of T$_{HMC-1}$ cytotoxicity was studied. T$_{HMC-1}$ cells treated for 2 days with 100 mg/ml of HMC-1-8 CBE in protein content were cultured with 20 units/ml of rIL-2 for another 2 days. Then these T$_{HMC-1}$ cells were used for the cytotoxicity against allogeneic tumor lines such as HMC-2, ZR-75-1, HPC-3, and K562 at an effector/target ratio of 50 for 12 h incubation of culture. Moreover, CBE from an allogeneic mammary tumor HMC-2 line was assessed for the stimulatory effect on T$_{HMC-1}$ cytotoxicity against HMC-1-8.

To determine maximal release, 0.1 ml of 1% Nonidet P-40 (Nakai Chemical Co., Kyoto, Japan) was added to appropriate wells. A spontaneous release was assessed by incubation of target cells with medium alone, and it was usually below 15% in the experiments. All determinations were made in triplicate; the data were presented as mean ± SE.

In a separate experiment, the effects of Mabs OKT3, OKT4, OKT8, and OKT8 were assessed. Mabs OKT3, OKT4, and OKT8 were purchased from Pharmacia. Mabs that react with determinants on human MHC class I (HH-1) and II (TC-8B1) framework structures were developed in our own laboratory (14). These Mabs were used in the cytotoxicity assays against HMC-1-8.

Effect of Mabs on the Cytotoxicity of T$_{HMC-1}$ Against HMC-1-8 Targets. In order to assess the cytotoxic mechanisms of T$_{HMC-1}$ cytotoxic cell clone against HMC-1-8 cells, we studied the effects of Mab treatment on T-cytotoxic clone or target cells in the cytotoxicity assays. Table 1 showed that pretreatment of T$_{HMC-1}$ by saturated amounts of Mabs OKT3 and OKT8, but not by OKT4, resulted in inhibition of the cytotoxicity of T$_{HMC-1}$. However, the addition of concanavalin A in this assay restored T$_{HMC-1}$ cytotoxic activity, indicating that the inhibitory effect by OKT3 and OKT8 acts on the recognition phase of target cells by T$_{HMC-1}$ and that the cytotoxic potential of this clone was not lost by pretreatment of these Mabs. Table 1 also showed that the cytotoxicity of T$_{HMC-1}$ was restricted by MHC class I products, since pretreatment of HMC-1-8 target cells by anti-MHC class I Mab (HH-1) but not by anti-MHC
were enough for stimulation of \text{\textsc{T}_{\text{HMC-1}}}, cytotoxic activity. Table 2 showed the effect of HMC-1-8 CBE on DNA synthesis of \text{\textsc{T}_{\text{HMC-1}}}. The data indicate that HMC-1-8 CBE alone had no effect on DNA synthesis of \text{\textsc{T}_{\text{HMC-1}}}. There was also no enhanced or synergistic effect on DNA synthesis with CBE plus rIL-2. CBE may contain nonspecific stimulatory factors in addition to specific antigens. Furthermore, \text{n}-butyl alcohol extraction may make the conformational change of cell surface molecules that could stimulate nonspecifically to the T-lymphocytes. In the next set of experiments, we investigated whether the effects of CBE on \text{\textsc{T}_{\text{HMC-1}}}, cytotoxicity were still specific for the target cells. Fig. 2 indicates that \text{\textsc{T}_{\text{HMC-1}}}, cocultured with 100 \mu g/ml of HMC-1-8 CBE and multiplied by rIL-2 showed a specific killing effect against HMC-1-8 target, but not against other allogeneic tumor lines including breast tumor lines HMC-2 and ZR-75-1, pancreatic tumor line HPC-3, and K562. These data may indicate that the effect of CBE is the specific stimulation to \text{\textsc{T}_{\text{HMC-1}}}, rather than nonspecific. Moreover, this was confirmed by the fact that CBE from allogeneic mammary tumor cell line HMC-2 could not enhance the cytotoxic potentials of \text{\textsc{T}_{\text{HMC-1}}}, as shown in Fig. 3. \text{\textsc{T}_{\text{HMC-1}}}, cells were treated with 100 \mu g CBE from HMC-1-8, HMC-2, or PBS alone for first 2 days at 37°C in 5% CO₂ incubator and were multiplied with rIL-2. The cytotoxicity assay against HMC-1-8 target cells was performed at 4 and 11 days of cultivation of \text{\textsc{T}_{\text{HMC-1}}}, at an effector/target ratio of 50 for 12 h culture incubation. The cytotoxic potentials of \text{\textsc{T}_{\text{HMC-1}}}, cocultured with HMC-2 CBE plus rIL-2 were almost the same as those with rIL-2 alone. In contrast, the stimulation by HMC-1-8 CBE could continuously retain \text{\textsc{T}_{\text{HMC-1}}}, cytotoxic activity, although gradual loss of the cytotoxic

class II Mab (TC-8B1) inhibited \text{\textsc{T}_{\text{HMC-1}}}, cytotoxic activity. These data strongly suggested that the antigen receptor complexes T1/T3 molecules on \text{\textsc{T}_{\text{HMC-1}}}, and HMC-1-8 tumor specific antigens were involved in this cytotoxicity under the MHC class I restriction.

\text{n}-Butyl Alcohol Extraction of HMC-1-8 Antigens and Allogeneic Stimulation on \text{\textsc{T}_{\text{HMC-1}}}, Clone. The solubilization of HMC-1-8 antigens that stimulate \text{\textsc{T}_{\text{HMC-1}}}, cytotoxic activity was done with \text{n}-butyl alcohol. HMC-1-8 tumor cell surface antigens were extracted noncortyotically with treatment of HMC-1-8 by 2.5% \text{n}-butyl alcohol in PBS for 25 min at room temperature. Fig. 1 showed the effect of CBE from HMC-1-8 cells on \text{\textsc{T}_{\text{HMC-1}}}, cytotoxicity against the same cells. These \text{\textsc{T}_{\text{HMC-1}}}, cells treated without or with various CBE amounts were cultured and multiplied under 20 units of rIL-2. The cytotoxic assays were performed at an effector/target ratio of 50. It was demonstrated that crude extracted antigens with 100 \mu g/ml in protein content

<table>
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<tr>
<th>CBE (µg/ml)</th>
<th>% of cytotoxicity*</th>
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<tr>
<td>10</td>
<td>26.2 ± 1.8</td>
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<tr>
<td>25</td>
<td>44.4 ± 2.4</td>
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<td>50</td>
<td>20.1 ± 6.8</td>
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<td>100</td>
<td>48.2 ± 3.8</td>
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<td>200</td>
<td>43.6 ± 3.8</td>
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* In experiment 1, \text{T}_{\text{HMC-1}}, cells were treated with saturated amounts of Mabs at 4°C for 60 min and were used in the cytotoxicity assay against HMC-1-8 cells. In experiment 2, HMC-1-8 target cells were treated with Mabs at 4°C for 60 min and were used in the cytotoxicity assays.

The cytotoxic assays were performed at an effector/target ratio of 50 in the absence or presence of 25 µg/ml of concanavalin A for 12 h at 37°C in a 5% CO₂ incubator. For calculation of percentage of cytotoxicity, see "Materials and Methods" in the text.

Fig. 1. Effect of HMC-1-8 CBE on the potential of \text{T}_{\text{HMC-1}}, cytotoxicity against HMC-1-8 target cells. \text{T}_{\text{HMC-1}}, cells were cultured without (○) or with (●) 25, 50, 100, and 200 µg HMC-1-8 CBE/ml for 2 days at 37°C in a 5% CO₂ incubator. These cells were then cultured and multiplied with 20 units of rIL-2 for another 2 days. The cytotoxic assay against HMC-1-8 target cells was performed at an effector/target ratio of 50 for 12 h culture incubation. Symbols, mean of percent of specific cytotoxicity. Bars, SE.

Fig. 2. Effect of HMC-1-8 CBE treatment on target specificity of \text{T}_{\text{HMC-1}}, cytotoxicity. \text{T}_{\text{HMC-1}}, cells treated for 2 days at 37°C with 100 µg/ml of CBE were assessed for the cytotoxicity against allogeneic tumor lines HMC-2, ZR-75-1, HPC-3, and K562. The cytotoxicity assay was performed at an effector/target ratio of 50 for 12 h culture incubation. Columns, mean percentage of cytotoxicity. Bars, SE.

| CBE | rIL-2 | Uptake of \[^{3}H\]thymidine (cpm) of \text{T}_{\text{HMC-1}} | \hline
| -  | -   | 7,809 ± 98.2 | 7,193 ± 55.2 |
| + | +   | 11,867 ± 66.6 | 12,593 ± 89.2 |

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cytotoxic activity against HMC-1-8 target cells. Tumor cells were treated with 100 μg CBE from HMC-1-8 (•), HMC-2 (○), or PBS alone (*) for first 2 days at 37°C in a 5% CO₂ incubator and were multiplied with rIL-2. The cytotoxicity assay against HMC-1-8 target cells were performed at an effector/target ratio of 50 and 12 h culture incubation. Symbols, mean percentage of specific cytotoxicity. Bars, SE.

Fig. 3. Effect of CBE of allogeneic mammary tumor HMC-2 line on TCHMC-I cytotoxicity. CBE from allogeneic HMC-2 tumor cells was assessed for the stimulatory effect on TCHMC-I cytotoxicity against HMC-1-8 target cells. TCHMC-I cells were treated with 100 μg CBE from HMC-1-8 (●), HMC-2 (○), or PBS alone (*) for first 2 days at 37°C in a 5% CO₂ incubator and were multiplied with rIL-2. The cytotoxicity assay against HMC-1-8 target cells were performed at 4 and 11 days of cultivation of TCHMC-I clone at an effector/target ratio of 50 and 12 h culture incubation. Symbols, mean percentage of specific cytotoxicity. Bars, SE.

Fig. 4. Effect of fractions on TCHMC-I cytotoxicity and elution profile on Mono-Q FPLC of fraction 3 obtained on Sephadex G-200 column chromatography of HMC-1-8 CBE. Approximately 10 mg of HMC-1-8 were applied on the column. Antigens in fractions 1 through 9 were analyzed for stimulating potential on TCHMC-I cytotoxicity against HMC-1-8. Approximately 20 μg in protein content of antigens from each fraction were cocultured with TCHMC-I for 2 days at 37°C, and these cells were further proliferated with 20 units of rIL-2. The TCHMC-I cytotoxicity assay against HMC-1-8 was done at an effector/target ratio of 50 for 12 h culture incubation. k, thousands.

Fig. 5. Effect of fractions on TCHMC-I cytotoxicity and elution profile on Mono-Q FPLC of fraction 3 obtained on Sephadex G-200 column chromatography of HMC-1-8 CBE. Approximately 2.0 mg in protein content of fraction 3 from Sephadex G-200 chromatography were applied to Mono Q FPLC. A linear NaCl gradient from 0 to 1.0 M was applied to this column. TCHMC-I was cocultured with TCHMC-I for 2 days at 37°C. These cells were then multiplied with 20 units of rIL-2 for 2 days at 37°C and assessed for cytotoxicity against HMC-1-8 target at an effector/target ratio of 50 for 12 h culture incubation. k, percentage of specific cytotoxicity.

DISCUSSION

It has been shown that tumor cells are destroyed specifically by cytotoxic T-lymphocytes from syngeneic animals that were preimmunized with the same tumor (1, 20–23). This can lead to in vivo regression of tumor from the hosts or the inhibition of tumor growth. The antigens that are involved in this specific cytotoxicity are supposed to be TSTAs that are detected in transplantation experiments in animals (24). Although it has been very difficult to study the tumor antigens recognized by autologous human CTL, the advent of rIL-2 has made it possible to investigate the cytotoxic mechanism by CTL against autologous tumor cells.

We have previously established target tumor clone HMC-1-8 and cytotoxic T-cell clone TCHMC-I, which are specifically cytotoxic to autologous HMC-1-8 tumor cells (8). This pair of target tumor cells and cytotoxic lymphocytes was derived from metastatic ascitic fluid in a 35-year-old patient with breast carcinoma. TCHMC-I showed continuous growing and cytotoxic potentials in vitro with antigenic stimulation and under the presence of rIL-2. TCHMC-I also could inhibit specifically HMC-1-8 tumor growth in transplantation experiments using nude mice. Therefore, this pair may provide us with an opportunity to clarify a putative human homologue of TSTA molecules that were detected by CTL.

In this paper, we showed that the cytotoxicity of TCHMC-I cytotoxic clone against autologous HMC-1-8 tumor target was inhibited by pretreatment of TCHMC-I with Mab OKT3 and OKT8. Furthermore, it was also inhibited by pretreatment of target HMC-1-8 cells with Mab that reacts with MHC class I but not with MHC class II framework molecules. This fact...
The data indicated that the HMC-1-8 tumor-specific antigens were eluted into fractions containing molecules with molecular weights of approximately 200,000 on Sephadex G-200 column chromatography. The antigens were further separated into the fractions that were eluted with 0.4–0.5 M NaCl in an ionic strength on Mono Q fast protein liquid chromatography. SDS-PAGE analysis of these fractions demonstrated three separate molecular weight fractions that were eluted into fractions containing molecules with molecular weights of 26,000, 30,000, and 32,000 under reduced molecular conditions. It is not yet determined whether all or some of these molecules are components of human autologous tumors of breast tissue origin. From this point of view, it is very important to study the expression mechanism of molecules. We have currently investigated the HMC-1-8 antigens defined by Mab 3A2, which is directly involved in the cytotoxicity of TCHMC-1 against HMC-1-8. This Mab could inhibit TCHMC-1 cytotoxicity against HMC-1-8 targets when they were pretreated by this Mab. It is not yet known whether antigen molecules detected by semipurification of HMC-1-8 CBE and those by this Mab are similar, since 3A2 does not easily form immunocomplexes with solubilized membrane antigens of HMC-1-8.

Although HMC-1-8 CBE were able to enhance TCHMC-1 cytotoxic potentials, they were unsuccessful as blocking antigens in the cytotoxicity assays of the TCHMC-1 cytotoxic clone against autologous HMC-1-8 tumor cells. This might be due to the differences in affinity between HMC-1-8 CBE antigens and intact antigens expressed on live HMC-1-8 cells. It was shown that CBE could induce a conformational change in the molecules (25, 26). In fact, the stimulatory effects by MLTC to TCHMC-1 clone were greater than those by HMC-1-8 CBE or these semipurified antigens (data not shown). However, it is presently unknown why CBE did not inhibit TCHMC-1 cytotoxicity, but rather enhanced the cytotoxic potentials of this clone.

CBE from HMC-1-8 did not remarkably enhance DNA synthesis of TCHMC-1-8 clone when assayed by incorporation of [3H]thymidine, but they obviously increased the cytotoxic potentials of the clone as described above. This was true for mitomycin C treated HMC-1-8 whole cells in autologous mixed tumor cell culture or semipurified antigens separated on Sephadex G-200 column and Mono Q fast protein liquid chromatography (data not shown). The mechanism by which TCHMC-1 became more cytotoxic by these antigens is not known, but these may induce the maturation of cytotoxic granules in this clone (27, 28). It seemed that the antigen alone but not MHC class I products could increase the cytotoxic potentials of this cytotoxic clone, since molecules demonstrated on SDS-PAGE were obviously different from MHC class I products. Our previous study using the mouse colon tumor models showed that CBE had powerful immunogenic activity in tumor rejection experiments (19). The data presented in this study may suggest that the combined therapy of CBE and cytotoxic T-cells in cancer patients is more effective than immunotherapy using cytotoxic T-cell clones or CBE alone.

AUTOLOGOUS CTL CLONE AND TUMOR ANTIGEN

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