Expression of Perforin and Membrane-Bound Lymphotoxin (Tumor Necrosis Factor-β) in Virus-Specific CD4⁺ Human Cytotoxic T-Cell Clones

By Masaki Yasukawa, Yoshihiro Yakushijin, Hitoshi Hasegawa, Masazumi Miyake, Yasuo Hitsumoto, Shigeru Kimura, Nozomu Takeuchi, and Shigeru Fujita

In an attempt to clarify the mechanisms of cytotoxicity mediated by CD4⁺ cytotoxic T lymphocytes (CTL), the expression of perforin and membrane-bound lymphotoxin (LT) (tumor necrosis factor-β) in herpes simplex virus (HSV)-specific CD4⁺ human cytotoxic and noncytotoxic T-cell clones was examined. Three HSV-specific CD4⁺ human CTL clones that showed HLA-DR-restricted cytotoxicity and proliferative response were established. The cytotoxicity of these clones in 5-hour ⁵¹Cr release assays was found to be mediated by the directional target cell lysis and not by the release of cytotoxic soluble factors, i.e., “innocent bystander” killing. Northern blot analysis showed that messenger RNAs for perforin and LT, which were both expressed in HSV-specific CD4⁺ CTL and natural killer cells, were abundantly expressed in HSV-specific CD4⁺ CTL clones. Expression of perforin in the cytoplasm of CD4⁺ CTL clones was also detected by immunohistochemical staining using a monoclonal antibody against perforin. In addition, LT bound to the cell surface of CD4⁺ CTL clones was detected by flow cytometry. In contrast, little or no expression of perforin and LT was detected in three HSV-specific CD4⁺ noncytotoxic T-cell clones. Although the cytotoxicity mediated by lymphokine-activated killer cells was partly inhibited by addition of anti-LT antibody, it did not show any effect on the cytotoxicity of HSV-specific CD4⁺ CTL clones. In addition, it was found that cytotoxicity mediated by these CD4⁺ CTL clones was Ca²⁺-dependent. These data thus suggest that perforin and membrane-bound LT are both expressed in HSV-specific CD4⁺ CTL, although perforin might be the more important mediator in short-term culture.

© 1993 by The American Society of Hematology.

LYMPHOCYTE-MEDIATED cytotoxicity is extremely important physiologically, serving to protect the organism against viral and some bacterial diseases, and perhaps being involved in immune surveillance against cancer. The mechanisms of cytolysis have been studied extensively during the past decade and, consequently, various molecules, including perforin, granzymes, and tumor necrosis factor-α (TNF-α)/lymphotoxin (LT) (TNF-β) and HLA-DR antigens, have been identified as candidates for cytolytic mediators.

It has been considered that cytotoxicity is mediated mainly by CD8⁺ T cells and natural killer (NK) cells, and studies of cytolytic mechanisms have focused on these lymphocyte populations. On the other hand, CD4⁺ T cells were considered to function as helper/inducer T cells, although it was demonstrated that some CD4⁺ T cells had a capacity to lyse appropriate target cells. The mechanism of cytotoxicity mediated by CD4⁺ cytotoxic T lymphocytes (CTL) has been studied by several investigators, but the results have been controversial. Tite and Janeway demonstrated that H-2 I region-restricted, ovalbumin-specific CD4⁺ murine CTL clones mediated cytotoxicity through soluble factors released as a result of antigen-specific recognition. In contrast to such CD4⁺ T-cell-mediated “bystander killing,” CD4⁺ CTL, which directly lyse target cells in an antigen-specific and major histocompatibility complex (MHC) class II-restricted manner, have also been found in humans as well as in mice.

In the present study, we attempted to clarify the cytolytic mechanisms of virus-specific human CD4⁺ CTL, focusing on the cytolytic mediators, perforin and LT. The results demonstrated that perforin and membrane-bound LT, which were considered to be important mediators in the cytotoxicity of CD8⁺ CTL and NK cells, were both expressed abundantly in herpes simplex virus (HSV)-specific CD4⁺ human CTL clones, but not in CD4⁺ noncytotoxic T-cell clones. Of these cytolytic mediators, perforin seemed to be the more important, based on the finding that anti-LT antibody did not inhibit the cytotoxicity mediated by CD4⁺ CTL clones and that their cytotoxicity was Ca²⁺-dependent. These data thus suggest that CD4⁺ CTL, which can lyse target cells directly, may exert cytotoxicity via mechanisms basically similar to those of CD8⁺ and NK cells.

MATERIALS AND METHODS

Establishment of HSV-specific CD4⁺ T-cell clones. T-cell clones that proliferate in response to stimulation with HSV antigen were generated as described previously. Briefly, 1 x 10⁶ peripheral blood mononuclear cells (PBMC) were suspended in 20 mL RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10 mM/L HEPES buffer and 10% heat-inactivated pooled human AB serum (this medium will be referred to as “culture medium”). Thereafter, 1 x 10⁶ plaque-forming units of UV light-inactivated KOS strain of HSV-1 was added to the cells, which were then seeded in round-bottomed microwells at 1 x 10⁵ cells/well and cultured at 37°C in a 5% CO₂ incubator for 6 days. The blasts were seeded at a concentration of 1 cell/well in round-bottomed microwell plates containing 0.2 mL of culture medium with 0.5 U/mL recombinant interleukin-2 (IL-2; Takeda Pharmaceutical Industries, Osaka, Japan) and 1 x 10⁵ mitomycin C (MMC)-treated autologous PBMC. After an additional 7 to 10 days, the growing cells were transferred to 16-mm wells and expanded in culture medium containing IL-2. MMC-treated autologous PBMC and HSV-1 antigen were added to the wells once every 2 weeks.

Proliferative response. Ten thousand clone cells and 1 x 10⁶ MMC-treated autologous PBMC, as a source of antigen-presenting cells (APC) in 0.2 mL of culture medium, were seeded in round-
bottomed microtiter wells to which 0.02 mL HSV antigen was added at the optimal concentration, as determined in previous experiments. The plates were incubated at 37°C in a 5% CO₂ incubator for 72 hours. For the final 16 hours of incubation, 1 μCi of [³H]thymidine deoxyribose (Tdr; New England Nuclear, Boston, MA) was added to each well, and the cells were then harvested onto glass fiber filter paper. The incorporation of [³H]Tdr was determined by liquid scintillation counting.

Cytotoxicity assays. [³H]Cr release assays were performed as described previously. briefly, 3 × 10⁶ Epstein-Barr virus-transformed lymphoblastoid cell lines (LCL) were infected with 3 × 10⁷ plaque-forming units of HSV-I for 1 hour and cultured overnight. Various numbers of effector cells and 1 × 10⁶ [³H]Cr (Na2[³H]CrO4; New England Nuclear)-labeled target cells were incubated together in 0.2 mL of RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO) in round-bottomed microtiter wells. Target cells were also added to wells containing medium alone and to wells containing 1% Triton X-100 (WAKO Pure Chemical Industries, Ltd, Osaka, Japan) to determine the spontaneous and maximal release, respectively. To determine whether cytotoxicity is mediated by direct killing or by-stander target cell killing, in addition to [³H]Cr-labeled target cells, 1 × 10⁵ unlabelled target cells were also added to the wells. After 5 hours, 0.1 mL of supernatant was removed from each well and transferred to tubes for counting in a gamma counter. The percentage of specific [³H]Cr release was calculated as follows: (cpm experimental release – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release) × 100. The spontaneous release from target cells in 5-hour [³H]Cr release assays never exceeded 20% of the maximal release. To examine the Ca²⁺ dependency of cytotoxicity, cytotoxicity assays were performed in the presence of EGTA (Sigma, St Louis, MO) at a concentration of 2 mmol/L.

Effect of monoclonal antibody (MoAb) on the cytotoxicity and proliferative response to HSV. Blocking experiments using MoAbs were performed as described previously. Briefly, to examine the effect of anti-HLA-DR antibody on the proliferative response of cloned T cells to HSV antigen, MoAb HU-4 (anti-HLA-DR framework) was added in the culture medium at an optimal concentration (1/100), as determined previously. The incorporation of [³H]Tdr was determined as described above. To examine the ability of MoAb to block the lysis of target cells, HU-4, 626/32 (anti-HLA-A, -B, -C; American Type Culture Collection [ATCC], Rockville, MD) or OKT3 (anti-CD3; ATCC) was added to each well at the optimal dilution of 1/100, and [³H]Cr release assays were performed as detailed above.

Effect of anti-LT antibody on cytotoxicity. Polyclonal rabbit anti-LT antibody was prepared by immunizing rabbits with recombinant human LT (Kanezafuchi Chemical Co, Tokyo, Japan) emulsified in complete Freund’s adjuvant as described previously. The IgG fraction of the antisera was obtained using a protein A-conjugated Sepharose column (Pharmacia, Uppsala, Sweden). Affinity purification was then performed with LT-conjugated Fornyl-Cellulofine (Seikagaku Kogyo Co, Tokyo, Japan). The F(ab)₂ fragment of anti-LT antibody was prepared by digestion of the purified IgG with pepsin (Difco, Detroit, MI). The anti-LT antibody prepared did not cross-react with TNF-α. Effector cells were incubated with 2.5 μg of F(ab)₂, anti-LT antibody or F(ab)₂, normal rabbit IgG at 4°C for 1 hour. After being washed, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab)₂ goat antirabbit IgG at 4°C for 30 minutes. Stained cells were analyzed with an Epics profile flow cytometer (Coulter Electronics).

RESULTS

Generation of HSV-reactive CD4⁺ T-cell clones. Thirty-eight T-cell clones were generated by seeding blasts that had been activated by stimulation with HSV antigen at 1 cell/well in 576 wells. T-cell lines generated by our cloning method have been proven to be generated from a single T cell by recloning and Southern blot analysis of the T-cell receptor (TCR) gene rearrangement. Among 38 T-cell clones generated, six clones, designated MY-2.2, MY-2.13, MY-2.16, MY-2.18, MY-2.21, and MY-2.22, were selected and used for further experiments. The phenotype of all the clones was CD2+CD3+CD4+CD8⁻CD29+CD45RK. These CD4⁺ T-cell clones were maintained in culture medium containing IL-2 by periodical addition of MMC-treated autologous PBMC and HSV antigen.

HLA-DR-restricted proliferative response of CD4⁺ T-cell clones. The proliferative response of the six CD4⁺ T-cell clones of HSV to HSV antigen was measured by [³H]Thymidine incorporation into clone cells. Incorporation of [³H]Tdr into clone cells in the presence of autologous APC with or without HSV antigen and with or without anti-HLA-DR MoAb was determined during the final 16 hours of a 72-hour incubation. The values represent the mean cpm (SD) of triplicate wells.

Table 1. HLA-DR-Restricted Proliferative Response of CD4⁺ T-Cell Clones to HSV

<table>
<thead>
<tr>
<th>Clone</th>
<th>APC</th>
<th>APC + HSV</th>
<th>APC + HSV + Anti-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY-2.2</td>
<td>853 (32)</td>
<td>12,593 (1,138)</td>
<td>1,075 (131)</td>
</tr>
<tr>
<td>MY-2.13</td>
<td>516 (143)</td>
<td>8,734 (197)</td>
<td>711 (42)</td>
</tr>
<tr>
<td>MY-2.16</td>
<td>798 (101)</td>
<td>10,776 (635)</td>
<td>766 (133)</td>
</tr>
<tr>
<td>MY-2.18</td>
<td>1,092 (89)</td>
<td>9,333 (395)</td>
<td>1,251 (131)</td>
</tr>
<tr>
<td>MY-2.21</td>
<td>639 (134)</td>
<td>14,524 (978)</td>
<td>913 (184)</td>
</tr>
<tr>
<td>MY-2.22</td>
<td>416 (79)</td>
<td>11,143 (1,022)</td>
<td>785 (31)</td>
</tr>
</tbody>
</table>

Incorporation of [³H]Tdr + Anti-DR *
The proliferative response to HSV is HLA-DR. All HSV-specific CD4+ T-cell clones examined and APC in
against the HLA-DR framework and CD3, suggesting that
the cytotoxicity of these CD4+ T-cell clones was mediated
infected autologous LCL was inhibited by MoAbs directed
against HSV-infected cells. In contrast, the remaining three
clones, MY-2.13, MY-2.16, and MY-2.18, showed little cy-
toxic activity. In contrast, the remaining three
clones, MY-2.13, MY-2.16, and MY-2.18, which did not lyse the target cells spontane-
ously, was not induced in the presence of MoAb directed
against HLA and was slightly augmented by the addition of
anti-CD3 MoAb.

**Direct lysis of target cells by CD4+ T-cell clones.** It is
known that the mechanisms of cytotoxicity mediated by
CD4+ T cells are varied, involving direct lysis or bystander
killing. We next addressed the question of whether HSV-
specific CD4+ T-cell clones exhibit cytotoxicity via the release of an antigen-nonspecific soluble cytolytic factor in the culture medium, and performed "innocent bystander" experiments, in which the clone cells were incubated with 51Cr-labeled HSV-infected autologous LCL in the presence of uninfected HSV-infected autologous LCL. As shown in Table 4, no apparent 51Cr release from uninfected LCL was mediated by CD4+ T-cell clones. Similarly, no 51Cr release was observed when 51Cr-labeled HSV-infected autologous LCL and unla-
beled HSV-infected autologous LCL were incubated with
clone cells. These data thus suggest that HSV-specific CD4+
CTL clones directly lyse HSV-infected autologous cells
through antigen-specific and HLA-restricted recognition.

**Expression of perforin in CD4+ T-cell clones.** Perforin is
known to be a possible candidate for a cytotoxic mediator
of CD8+ CTL and NK cells.1,1 Accordingly, we addressed
the question of whether the ability of CD4+ T-cell clones to
lyse target cells is correlated with the expression of perforin.
Northern blot analysis of messenger RNA (mRNA) for per-

**Table 2. Cytotoxicity of CD4+ T-Cell Clones**

<table>
<thead>
<tr>
<th>Clone</th>
<th>E:T</th>
<th>Auto (HSV+)</th>
<th>Auto (-)</th>
<th>Allo (HSV+)</th>
<th>K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY-2.2</td>
<td>10:1</td>
<td>81.6</td>
<td>-5.2</td>
<td>4.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>5:1</td>
<td>70.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>53.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MY-2.13</td>
<td>10:1</td>
<td>4.6</td>
<td>-4.3</td>
<td>6.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>5:1</td>
<td>3.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>0.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MY-2.16</td>
<td>10:1</td>
<td>6.8</td>
<td>-4.0</td>
<td>1.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>5:1</td>
<td>5.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>4.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MY-2.18</td>
<td>10:1</td>
<td>8.1</td>
<td>-3.6</td>
<td>-0.1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>5:1</td>
<td>5.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>3.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MY-2.21</td>
<td>10:1</td>
<td>72.4</td>
<td>-4.6</td>
<td>4.3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>5:1</td>
<td>48.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>20.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MY-2.22</td>
<td>10:1</td>
<td>64.5</td>
<td>-3.5</td>
<td>1.4</td>
<td>-0.1</td>
</tr>
<tr>
<td></td>
<td>5:1</td>
<td>37.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>17.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* CD4+ T-cell clones generated were tested for their ability to lyse
various target cells in 5-hour 51Cr release assays.
1 Allogeneic cells used share no HLA-A, -B, -C, or -DR antigen with
MY.

**Table 3. Inhibition of Cytotoxicity by MoAb**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Auto (HSV+)</th>
<th>Auto (-)</th>
<th>Allo (HSV+)</th>
<th>Anti-HLA-A, B, C</th>
<th>Anti-HLA-DR</th>
<th>Anti-CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY-2.2</td>
<td>74.5</td>
<td>70.8</td>
<td>12.5</td>
<td>20.3</td>
<td>4.0</td>
<td>10.3</td>
</tr>
<tr>
<td>MY-2.13</td>
<td>2.9</td>
<td>2.5</td>
<td>3.0</td>
<td>4.0</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>MY-2.16</td>
<td>5.6</td>
<td>5.0</td>
<td>0.9</td>
<td>10.3</td>
<td>10.3</td>
<td>10.3</td>
</tr>
<tr>
<td>MY-2.18</td>
<td>7.0</td>
<td>6.3</td>
<td>5.0</td>
<td>13.9</td>
<td>13.9</td>
<td>13.9</td>
</tr>
<tr>
<td>MY-2.21</td>
<td>62.3</td>
<td>65.1</td>
<td>13.2</td>
<td>19.5</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>MY-2.22</td>
<td>59.8</td>
<td>60.3</td>
<td>10.3</td>
<td>12.9</td>
<td>12.9</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* Lysis of HSV-infected autologous LCL by clone cells was examined at an E:T ratio of 10:1 in the presence and absence of anti-HLA-A, -B, -C MoAb 6132; anti-HLA-DR MoAb HU-4; or anti-CD3 MoAb OKT3.

**Expression of perforin in CD4+ T-cell clones.** Perforin is
known to be a possible candidate for a cytotoxic mediator
of CD8+ CTL and NK cells.1,1 Accordingly, we addressed
the question of whether the ability of CD4+ T-cell clones to
lyse target cells is correlated with the expression of perforin.
Northern blot analysis of messenger RNA (mRNA) for per-

**Table 4. Direct Lysis of HSV-Infected Cells by CD4+ Clones**

<table>
<thead>
<tr>
<th>Clone</th>
<th>% Specific 51Cr Release From Target Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY-2.2</td>
<td>69.3</td>
</tr>
<tr>
<td>MY-2.21</td>
<td>58.8</td>
</tr>
<tr>
<td>MY-2.22</td>
<td>60.1</td>
</tr>
</tbody>
</table>

* 51Cr release assays were performed in the presence of 51Cr-labeled HSV-infected autologous LCL and unlabeled HSV-infected autologous LCL. 51Cr-labeled HSV-infected autologous LCL and unlabeled HSV-infected autologous LCL. 51Cr-labeled HSV-infected autologous LCL and unlabeled HSV-infected autologous LCL.
For perforin is shown in Fig 1. CD4+ CTL clones, MY-2.2, MY-2.21, and MY-2.22, expressed mRNA for perforin. Among these clones, MY-2.2, which showed the strongest cytotoxicity against HSV-infected autologous cells, expressed the largest amount of mRNA for perforin. On the other hand, little or no expression of mRNA for perforin was detected in the nontoxic clones, MY-2.13, MY-2.16, and MY-2.18.

Perforin is known to be stored in cytoplasmic granules of cytotoxic lymphocytes. May-Gruenwald-Giemsa staining of cytocentrifuged preparations showed that cytotoxic and noncytotoxic CD4+ T-cell clones both contained fine azurophilic granules in their cytoplasm, but the numbers of granules in nontoxic clones tended to be lower than those in cytotoxic clones (data not shown). Large amounts of perforin were detected in CD4+ CTL clones by immunohistochemical staining using anti-perforin MoAb (Fig 2A). In contrast to CTL clones, nontoxic clones exhibited no or only weak staining (Fig 2B). These data suggest that the cytotoxic reactivity of CD4+ T cells might be correlated with the expression of perforin, and that some nontoxic CD4+ T cells also express perforin, even though the level of expression is lower than that of cytotoxic clones.

Expression of membrane-bound LT in CD4+ T-cell clones. Northern blot analysis of LT, which is considered to be another important candidate for a cytotoxic mediator, in CD4+ T-cell clones is shown in Fig 3. In the same manner as for perforin, CD4+ CTL clones apparently expressed mRNA for LT, whereas nontoxic clones expressed little LT mRNA.

Recently, in addition to the secreted form, membrane-bound LT has been identified and its role in the direct lysis of target cells mediated by CTL has come under scrutiny. Because our CD4+ CTL clones appeared to lyse target cells directly, we addressed the question of whether LT is expressed on their cell surface. Results of flow cytometric analysis of membrane-bound LT are shown in Fig 4. As we expected, the cytotoxic clone, MY-2.2, expressed LT on the cell surface (Fig 4A), but no LT expression was detected on the nontoxic clone, MY-2.13 (Fig 4B). Another two CD4+ CTL clones, MY-2.21 and MY-2.22, were also found to express LT on their cell surface, even though the expression level was somewhat lower than that on MY-2.2 (data not shown). It was also found that CD4+ CTL clones produced the soluble form of LT after stimulation with HSV antigen. However, cytosis of target cells was not induced by addition of the culture supernatant of antigen-stimulated CD4+ CTL clones (data not shown).

Lack of effect of anti-LT antibody on the cytotoxicity of CD4+ T-cell clones. From the data described above, it has been found that HSV-specific CD4+ CTL clones simultaneously expressed two different kinds of cytotoxic mediator,
perforin and membrane-bound LT. To clarify which of these is more important in the cytotoxicity of CD4⁺ CTL against HSV-infected cells, we examined whether addition of anti-LT antibody to clone cells inhibited their cytotoxicity. We have found recently that LAK cells expressed membrane-bound LT and that their cytotoxicity against L929 cells in 16-hour $^{51}$Cr-release assays was partly inhibited by anti-LT antibody$^{33}$ (Table 5). In contrast to LAK cells, the addition of anti-LT antibody to assay wells had no effect on the cytotoxicity of CD4⁺ CTL clones in 5-hour $^{51}$Cr-release assays. These data therefore suggest that membrane-bound LT is not responsible for CD4⁺ CTL-mediated cytotoxicity against HSV-infected LCL, and that perforin might be more important for their cytotoxic activity in short-term culture.

**Ca²⁺-dependent cytotoxicity of CD4⁺ T-cell clones.** Because perforin-mediated cytolysis appeared to be Ca²⁺-dependent$^{34}$, cytotoxic activity of CD4⁺ CTL clones in the absence of extracellular Ca²⁺ was examined to further study the role of perforin in CD4⁺ CTL-mediated cytotoxicity. As shown in Table 6, no cytotoxicity of CD4⁺ CTL clones was observed in the presence of the Ca²⁺-chelating agent EGTA. On the other hand, the expression level of membrane-bound LT was not affected by the addition of EGTA (data not shown). These data further suggested the importance of perforin in the cytotoxicity of CD4⁺ CTL.

**DISCUSSION**

Since the discovery that some CD4⁺ T cells can exhibit cytotoxic activity, as well as CD8⁺ CTL and NK cells, the mechanisms of cytotoxicity mediated by CD4⁺ CTL have been the subject of considerable interest. The present series of experiments was undertaken to study the mechanisms of CD4⁺ CTL cytotoxicity, and a number of interesting findings
were obtained. HSV-specific CD4+ human CTL clones directly lysed HSV-infected autologous cells in an HLA class II-restricted manner, but not by “bystander killing,” i.e., through the release of antigen-nonspecific cytotoxic factors. These CD4+ CTL clones appeared to express abundantly perforin and the membrane-bound form of LT, both of which have recently been considered responsible for the cytotoxic activity of CD8+ CTL and NK cells. In contrast to the cytotoxicity of LAK cells against L929 cells, that of CD4+ CTL against HSV-infected autologous LCL in a 5-hour assay was not inhibited by the addition of anti-LT antibody. In addition, no cytotoxicity against HSV-infected autologous cells was observed in the absence of extracellular Ca2+, which appeared to be required for perforin-mediated cytolyis.34 These findings suggested that perforin is the more important mediator for the cytotoxicity of CD4+ CTL exhibited in a short period. Although the precise mechanism of cytotoxicity mediated by CD8+ CTL and NK cells is still obscure, the present data suggested that MHC class II-restricted CD4+ CTL exhibit cytotoxic activity through mechanisms basically similar to those of MHC class I-restricted CD8+ CTL and MHC-unrestricted NK cells.

The mechanism of T-cell-mediated cytotoxicity can be basically divided into two types. One is direct lysis through antigen-recognition of effector cells via CD3-TCR complex after direct contact with the target cells. The other is a mechanism termed “bystander killing,” i.e., the release of antigen-nonspecific cytotoxic substances around the effector cells after the MHC-restricted and antigen-specific recognition against appropriate APC. In a murine system, such H2I (MHC class II)-restricted CD4+ T cells exhibiting antigen-nonspecific bystander killing have been reported by several investigators.15-19 These CD4+ T cells were found to secrete TNF-like cytotoxic molecules after MHC class II-restricted antigen recognition, and it was suggested that these substances might induce the killing of bystander cells, based on the finding that anti-TNF antibody inhibited their cytotoxicity.35,36 In contrast to this type of cytotoxic CD4+ T cell, CD4+ CTL clones generated in the present study directly lysed target cells in a HLA class II-restricted manner, similar to those reported previously by us and others.20-23 Interestingly, these CD4+ CTL clones were found to express LT on their cell surface.

TNF and LT were originally identified as secretory products of macrophages and T cells.37,38 Recently, a novel type of TNF and LT, a membrane-bound form, has been identified on activated macrophages and T cells.39,43 and its role in mediating cytotoxicity during contact between effector cells and their target cells is of considerable interest. We have recently found that LT was expressed on LAK cells, and that the presence of membrane-bound LT was correlated with cytotoxic activity against LT-sensitive L929 cells.35 According to the present findings, it seemed possible that membrane-bound LT was responsible also for CD4+ CTL-mediated cytotoxicity, although the hypothesis that membrane-bound LT is essentially important for the cytotoxicity of CD4+ CTL against HSV-infected autologous cells was refuted by the following evidence: (1) whereas HSV-infected cells were found to be sensitive to recombinant human LT, a 5-hour incubation period was not enough to induce cell damage by LT, and longer-term culture (for about 16 hours) with LT was needed (data not shown); (2) addition of anti-LT antibody, which inhibited the activity of LAK cells against L929 cells, had no effect on the cytotoxicity of CD4+ CTL clones. It is thus considered that membrane-bound LT is not involved in the mechanism of CD4+ CTL-mediated cytotoxicity in the present experimental system. Similar results in a study of TNF-α have recently been reported by Liu et al.44 who found that human immunodeficiency virus-specific CD4+ T cells produced membrane-bound and secreted forms of TNF-α, although these molecules were not required for the cytolyis of target cells. Although these findings seem to suggest the independence of TNF-α and LT for CD4+ CTL-mediated cytotoxicity, the data presented here do not necessarily imply that these molecules have no significance in CD4+ T-cell-mediated cytotoxicity. It seems possible that CD4+ T cells expressing LT on their surface may lyse LT-sensitive target cells during a long incubation period. In our experimental system, the spontaneous release of 51Cr from HSV-infected LCL after a 16-hour incubation, which is usually used for TNF/LT-mediated cytotoxic assays, was too high, because of the cytopathic effect of HSV. Therefore, modification of the experimental system is needed to study further the roles of membrane-bound LT in CD4+ T-cell-mediated cytotoxicity.

Another important finding of the present study is that perforin was expressed abundantly in HSV-specific CD4+ human CTL clones. Perforin is considered to be an important candidate as an effector molecule of lymphocyte-mediated cytotoxicity.17 It has been shown that perforin is present mainly in CD3+ CD56+ NK cells,5,46 TCR-γδ T cells,47,48 and some CD8+ T cells.46 In addition, Thy-1-positive dendritic epidermal cells have also been found to express perforin in a murine system.49 Although perforin was expressed in various cytotoxic lymphocytes, previous studies showed that CD4+ T cells in peripheral blood were totally negative for perforin expression.50,46 It has also been reported that the expression of mRNA for perforin was not induced in CD4+ T cells even after stimulation with IL-2.46 In contrast with these previous reports, all HSV-specific CD4+ human CTL clones generated in the present study apparently expressed perforin. In addition, some noncytotoxic CD4+ T-cell clones also expressed a low amount of perforin mRNA. As we reported previously, some noncytotoxic CD4+ T-cell clones that proliferate in response to HSV-antigen stimulation exhibit nonspecific cytotoxicity in the presence of lectin in culture.25 This cytotoxic...
potential of CD4+ T cells without spontaneous cytotoxicity might be correlated with perforin expression.

The present study found an unexpectedly high frequency of perforin-positive CD4+ T cells. We have found that CD4+ CTL clones directed against human herpesvirus-6-infected cells also expressed a large amount of perforin after stimulation with viral antigen.50 Taken together, it is suggested that the vast majority of virus-specific CD4+ human CTL are capable of inducing perforin expression, and that activation via CD3-TCR complexes might be essential for perforin expression in CD4+ T cells. When considering the evidence that the bulk of CD4+ T cells stimulated with IL-2 are almost totally negative for perforin expression,46 it should also be considered that CD4+ T-cell clones expressing perforin might be easily selected by our cloning procedure.

Besides perforin and TNF/LT, granzymes are also considered as a candidate for cytolytic mediators of cytotoxic lymphocytes.8,11 We examined N-a-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) serine esterase activity, which reflects the enzymatic activity of granzyme A, in HSV-specific CD4+ T-cell clones. Consequently, various levels of BLT serine esterase activity were detected in CD4+ T-cell clones, but no direct correlation between this activity and cytotoxic activity was observed (data not shown). According to these data, perforin seems to be the most important cytolytic molecule in HSV-specific CD4+ CTL clones, at least in our experimental system. However, the present results do not necessarily rule out the possibility that CD4+ CTL exhibit cytotoxic activity through mechanisms other than perforin expression. Perforin-independent antigen-specific directional target cell lysis by CD4+ T cells certainly exists,51 and therefore further studies focusing on various cytotoxic mediators as well as perforin are needed to clarify the details of the cytotoxic machinery of CD4+ T cells.

In summary, we have demonstrated that virus-specific CD4+ human CTL clones simultaneously express both perforin and membrane-bound LT. Of these, perforin seems to be responsible for cytotoxicity against virus-infected autologous cells in short-term culture, whereas membrane-bound LT might also play a physiologic role in a long-term culture. Accordingly, it can be considered that CD4+ CTL possess some distinct cytotoxic mechanisms, and that they lyse various types of target cell through one or more different cytotoxic pathways.

ACKNOWLEDGMENT

We thank Prof K. Okumura (Juntendo University) for providing perforin cDNA probe and MoAb, and Dr A. Wakisaka (Hokkaido University) and Dr N. Ohta (Okayama University) for providing MoAbs.

REFERENCES

22. Kaplan DR, Griffith R, Braiciale VL, Braiciale TJ: Influenza virus-specific human cytotoxic T cell clones: Heterogeneity in anti-
genic specificity and restriction by class II MHC products. Cell Immunol 88:193, 1984
31. Yasukawa M, Zarling JM: Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. II. Bifunctional clones with cytotoxic and virus-induced proliferative activities exhibit herpes simplex virus type 1 and 2 specific or type common reactivities. J Immunol 133:2736, 1984
36. Tite JP: Evidence of a role for TNF-α in cytosis by CD4+ class II MHC-restricted cytotoxic T cells. Immunology 71:208, 1990
Expression of perforin and membrane-bound lymphotoxin (tumor necrosis factor-beta) in virus-specific CD4+ human cytotoxic T-cell clones

M Yasukawa, Y Yakushijin, H Hasegawa, M Miyake, Y Hitsumoto, S Kimura, N Takeuchi and S Fujita