Human Purified Protein Derivative-Specific CD4+ T Cells Use Both CD95-Dependent and CD95-Independent Cytolytic Mechanisms

David M. Lewinsohn, Teresa T. Bement, Jiangchun Xu, David H. Lynch, Kenneth H. Grabstein, Steven G. Reed and Mark R. Alderson

*J Immunol* 1998; 160:2374-2379;
http://www.jimmunol.org/content/160/5/2374

**References**
This article cites 38 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/160/5/2374.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Human Purified Protein Derivative-Specific CD4+ T Cells Use Both CD95-Dependent and CD95-Independent Cytolytic Mechanisms

David M. Lewinsohn, Teresa T. Bement, Jiangchun Xu, David H. Lynch, Kenneth H. Grabstein, Steven G. Reed, and Mark R. Alderson

CTL, both CD4+ and CD8+, are essential in the eradication of intracellular pathogens. Data generated using murine T cells have suggested a critical role for CD95 (Fas, Apo-1) in CD4+ T cell-induced apoptosis of target cells. In contrast, CD8+ CTL predominantly use the perforin/granzyme lytic pathway. At present little is known about the mechanism of CD4+ CTL lytic function during intracellular infection in humans. We have used human CD4+ T cells specific for purified protein derivative (PPD) of Mycobacterium tuberculosis to explore whether CD95 is the dominant cytolytic mechanism. PPD-reactive CD4+ clones efficiently lysed Ag-pulsed autologous monocytes, adherent macrophages, and EBV-transformed B cells. Addition of an antagonistic CD95 Ab had a minimal effect on cytolyis, whereas addition of MgEGTA to block perforin/granzyme resulted in complete inhibition of killing. In contrast, lysis of activated peripheral blood B cells could be partially blocked with the antagonistic CD95 Ab. Supporting these observations, monocytes, macrophages, and EBV-transformed B cells were not lysed by an agonistic CD95 Ab. Activated B cells were readily lysed by the agonistic CD95 Ab. T cell clones triggered through the TCR with anti-CD3 were capable of lysing the CD95-sensitive Jurkat T cell line in a CD95-dependent manner, but were also able to release granzymes. We conclude that human CD4+ T cells are capable of lysing PPD-pulsed targets using both perforin/granzyme and CD95 pathways. The contribution of CD95 is strictly dependent on target cell susceptibility to CD95-mediated killing. The Journal of Immunology, 1998, 160: 2374–2379.

The mechanism by which CD4+ CTL lyse their targets has important implications for their biologic function. The use of perforin/granzyme potentially allows for lysis of all MHC class II-positive targets. In contrast, the CD95/CD95L pathway requires the target cell to both express CD95 and be susceptible to CD95-induced apoptosis. The majority of freshly isolated lymphoid and nonlymphoid cells do not undergo CD95-mediated apoptosis. As a result, CD4+ T cell dependence on the CD95/CD95L pathway would suggest a much more restricted role for these cells. Data generated in the mouse model have suggested that CD95 plays a major role in CD4+ T cell-mediated cytotoxicity (16–18). Because murine CD4+ CTL were shown to lyse only CD95-susceptible targets, these data have been used to argue that the primary role of CD4+ CTL is in the modulation of the immune response. On the other hand, recent data support a role for perforin/granzyme in CD4+ cytotoxicity. Williams and Engelhard (19) reported that short term CD4+ mixed lymphocyte cultures exhibited Ag-specific cytotoxicity that was CD95 independent and was diminished in perforin-deficient mice.

At present, little is known about the role of CD4+ CTL effector mechanisms in intracellular pathogenesis in humans. In this study we have investigated the relative contributions of the CD95 and perforin/granzyme cytolytic pathways in human PPD-specific CD4+ killing of a variety of cell targets. We have found that human CD4+ CTL efficiently lyse Ag-pulsed targets using both CD95-dependent and CD95-independent mechanisms.

Materials and Methods

Monoclonal Abs and reagents

Culture medium consisted of RPMI 1640 supplemented with 10% FBS (BioWhittaker, Walkersville, MD) and 50 μg/ml gentamicin sulfate (BioWhittaker). For the generation of primary T cell lines and clones, RPMI was supplemented with 10% human serum (Trimar Hollywood, Reseda, CA).
CA). Agonistic anti-human CD95 mAb (clone CH-11) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). CH-11 is an IgG Ab and is able to cross-link CD95 in solution and induce an apoptotic signal in susceptible cells. The agonistic CD95 Ab Fas M3 (Immunex Corp., Seattle, WA) is an IgG1 Ab that does not cross-link CD95 in solution and blocks CH-11- and CD95L-induced apoptosis (14). MgEGTA (0.1 M EGTA/0.2 M MgCl2; Sigma Chemical Co., St. Louis, MO; pH 7.4) was prepared by the method of MacLennan (20). Recombinant soluble human CD40 ligand (CD40L) was expressed and purified from the supernatant of Saccharomyces cerevisiae (21). Human IL-4 was produced at Immunex Corp.

Cell lines

The T lymphoma cell line Jurkat was obtained from American Type Culture Collection (Rockville, MD). EBV-transformed B cell lines were generated in our laboratory using supernatants from the cell line 9B5-8. Cell lines were maintained by continuous passage in RPMI culture medium supplemented with 10% FCS. Flow cytometry

Cells to be analyzed for CD95 expression were first incubated at 4°C in a blocking solution of PBS containing 2% normal rabbit serum and 2% normal goat serum to prevent nonspecific binding of mouse IgG. Cells were washed in FACS buffer (PBS containing 0.5% FBS and 0.02% sodium azide) and incubated with either the anti-CD95 Fas M3 Ab (5 μg/ml) or an IgG1 control (Becton Dickinson Immunocytometry Systems, San Jose, CA; 5 μg/ml) for 30 min at 4°C in a total volume of 50 μl. Cells were then washed and incubated in 50 μl of a 1/100 dilution of goat anti-mouse IgG-phycocerythrin (Tago, Burlingame, CA) for 30 min at 4°C. Flow cytometry was performed using a FACSCalibur (Becton Dickinson), and data were collected on 10⁴ viable cells.

Separation of target cell subsets

PBMC from healthy PPD-positive individuals (>10-mm Mantoux skin test) were isolated from heparinized blood by centrifugation over Ficoll-Hypaque (Sigma) and washed three times with culture medium. T cell-depleted targets were isolated from human PBMC by rosetting with 2-aminohexylisothiouronium bromide hydrobromide-treated sheep erythrocytes. These cells were then layered on Histopaque (Sigma), and the cell pellet was discarded. Following hypotonic lysis of sheep erythrocytes, cells were washed twice in PBS.

To generate B cell blasts, T cell-depleted PBMC were further enriched for CD19⁺ cells by positive immunomagnetic sorting (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. These cells were then incubated in the presence of CD40L (3 μg/ml) and IL-4 (30 ng/ml) for 3 days in medium containing 10% FBS.

Monocytes were prepared from fresh PBMC by countercurrent elutriation (Beckman J6-MI, Palo Alto, CA). Wright’s Giemsa-stained cytospin preparations of these cells were >90% monocytes. Monocytes were maintained in polypropylene tubes to minimize adherence.

Generation of PPD-specific T cell clones

PBMC from healthy PPD-positive individuals were isolated from heparinized blood by centrifugation over Ficoll-Hypaque and washed three times with culture medium. One million cells were cultured in the presence of PPD (Lederle, PA) at a concentration of 10 μg/ml in 16-mm wells (Costar no. 3524) in 2 ml of culture medium. Cultures were incubated at 37°C in 5% CO₂ for 5 to 7 days. At this time, FACScan analysis revealed that these cells were >85% CD4⁻. To generate a panel of CD4⁺ T cell clones reactive to PPD, a cell line was established by limiting dilution in the presence of 2 × 10⁻⁵ irradiated (3500 rad using a ¹³⁷Cs source) heterologous PBMC in the presence of anti-CD3 (10 ng/ml) and IL-2 (10 ng/ml). Cell culture media consisted of 200 μl of RPMI supplemented with 10% FBS. T cell clones were selected based upon their proliferative responses to PPD.

Cytotoxicity assay

A standard 4- to 5-h ⁵¹Cr release assay was used to assess cytolytic activity. Briefly, target cells were labeled with 50 μCi of ⁵¹Cr for 16 h at 37°C in 1 ml of culture medium in the presence of either PPD (25 μg/ml) or se-

creted proteins from Mb strain H37Rv (25 μg/ml; Colorado State University, Fort Collins, CO). T cell lines or clones to be assessed for cytolytic activity were washed twice in culture medium and serially diluted in 96-well V-bottom plates (Costar no. 3896). For anti-CD3 stimulation, the plates were coated for 16 h with anti-CD3 at 10 μg/ml and washed twice with PBS before using. Where indicated, CH11 (1 μg/ml), Fas M3 (5 μg/ml), and MgEGTA (2 μM EGTA; 4 μM MgCl₂) were added in a volume of 25 μl to achieve the final concentration indicated above. ⁵¹Cr-labeled targets (2 × 10⁴) were added immediately thereafter to make a final volume of 0.2 ml/well. After incubation for 4 h at 37°C, plates were harvested using a Skatron SCS harvesting system (Skatron, Sterling, VA). The ⁵¹Cr content of supernatants was determined using a 9600 gamma scintillation counter (Packard, Meriden, CT). The percent specific ⁵¹Cr release was calculated according to the formula: 100 × (experimental cpm) – (spontaneous cpm)/[(maximum cpm) – (spontaneous cpm)], where spontaneous cpm is the counts per minute released in the absence of effector cells, and maximum cpm is the counts per minute released in the presence of 1% Triton X-100 (Sigma).

Granzyne (N-acetylglucosaminidase) assay

Granzyne release was assessed by the modified method of Henkart (unpublished) (22). T cells (5 × 10⁵) were incubated in HBSS without phenol for 4 h at 37°C in the presence of 5% CO₂ in 16-mm culture wells previously coated with anti-CD3. Fifty microliters of supernatant was mixed with 2-aminoethylisothiouronium bromide hydrobromide-treated sheep erythrocytes by guest on July 29, 2017 http://www.jimmunol.org/ Downloaded from
Lysis of Jurkat targets by CD4
redundant.
lysis during the 4-h assay; and 3) the killing pathways were
CD95L; 2) these targets were not susceptible to CD95-mediated
susceptibilities: 1) the T cell clones were deficient in their expression of
dent manner (data not shown). We considered the following pos-
To test whether these CD4
been evaluated, all lyse Ag-pulsed macrophages in a Ca2
and all are cytolytic. Of the four clones from two donors that have
represents the mean of duplicate determinations. This experiment is rep-
experiment suggested that lysis of these targets was probably occur-
Lysis of Jurkat targets by CD4
dependent
To test whether these CD4
T cell clones were able to express
were activated with PPD at 5 µg/ml (circles) or media (squares). CD95 antagonist mAb Fas M3 (triangles) was added at 5 µg/ml at the beginning of the assay. PPD-reactive lines generated in this manner are >85% CD4⁺. Each point represents the mean of duplicate determinations. This experiment is representative of four experiments.

CD4⁺ CTL clones were capable of lysing Jurkat targets in a manner that was completely blocked by the addition of the anti-CD95 Fas M3 Ab. Supporting a primary role for CD95 in this assay, the removal of extracellular calcium with MgEGTA resulted in minimal inhibition of cytolysis (Fig. 3B). Thus, we concluded that these PPD-specific T cell clones were capable of using both the perforin-granzyme and CD95 cytolytic pathways. As a result, utilization of the CD95/CD95L pathway probably depends upon the susceptibility of the target cell to CD95-mediated apoptosis.

Expression of CD95 by a variety of targets
For a CD4⁺ CTL to kill its target via the CD95 apoptotic pathway, the target cell must both express CD95 and be susceptible to CD95-mediated apoptosis. We initially analyzed a variety of targets for CD95 expression. Flow cytometric analysis of purified cell subsets revealed the presence of CD95 on a variety of targets (Fig. 4). The Jurkat cell line, B cell blasts, elutriated monocytes, and EBV-transformed lymphoblastoid cells all exhibited high levels of CD95. Adherent macrophages expressed lower levels of CD95. The agonistic anti-CD95 Ab CH-11 was used to assess susceptibility to CD95-dependent lysis by 4-h ⁵¹Cr release or by uptake of trypan blue after overnight incubation with the CH-11 Ab (Table I). Jurkat cells and B cell blasts were both sensitive to CD95-mediated apoptosis, whereas monocytes and adherent macrophages were relatively refractory to cytolysis in these assays. Interestingly, the EBV-transformed lymphoblastoid line that expressed high levels of CD95 was relatively refractory to CH-11-mediated lysis. Similar data have been obtained using lymphoblastoid cell line (LCL) generated from four different individuals (data not shown). These data confirmed that CD95 expression was necessary but not sufficient for susceptibility to CD95-mediated lysis and predicted that among APC, only the B cell blast population would be lysed in a CD95-dependent manner.

Utilization of CD95 lytic pathway is correlated with CD95 susceptibility
To test the hypothesis that Ag-specific CD4⁺ T cell-mediated lysis could use both the perforin/granzyme and CD95 cytolytic pathways, PPD-specific T cell clones were tested for their ability to lyse a panel of target cells. As shown in Figure 5, these clones
were capable of efficient short term lysis of macrophages, elutriated monocytes, LCL, and B cell blast targets. However, significant inhibition with the CD95 Fas M3 Ab could only be demonstrated with the B cell blast targets (Table II). Addition of an isotype-matched control Ab had no effect. In addition, we assessed the ability of the PPD-specific T cell clones to lyse Mtb-infected monocytes. At an E:T cell ratio of 20:1, we observed 25% specific lysis, which was completely blocked by the addition of MgEGTA (data not shown).

CD4$^+$ T cell clones release serine esterase when stimulated

While utilization of the perforin-granzyme pathway is inferred from the dependence of lytic activity on extracellular calcium, we sought to directly demonstrate the release of granule contents of the PPD-specific T cell clones upon activation. The CD4$^+$ T cell clones were incubated in the presence of immobilized anti-CD3 for 4 h, and the supernatants were assessed for their ability to cleave the hexosaminidase substrate. As shown in Table III, PPD-specific CD4$^+$ clones released serine esterase when stimulated with anti-CD3.

CD4$^+$ T cell clones express perforin mRNA constitutively and CD95L mRNA when stimulated

Finally, to confirm the ability of CD4$^+$ T cell clones to express both CD95L and perforin, reverse transcriptase-PCR (RT-PCR) was employed. After induction with anti-CD3, both clones expressed CD95L mRNA. In contrast, both resting and stimulated clones expressed perforin mRNA (Fig. 6). Collectively, these data

**FIGURE 4.** Expression of CD95 by various cell types. Adherent macrophages, elutriated monocytes, positively selected CD19$^+$ B cells stimulated with CD40L (3 μg/ml) and IL-4 (30 ng/ml), an autologous lymphoblastoid cell line, and the Jurkat T cell line were stained with Fas M3 (5 μg/ml) or an isotype-matched control Ab followed by goat anti-mouse F(ab')2 conjugated to phycoerythrin. Flow cytometry was performed with a FACSCalibur.

**FIGURE 5.** A PPD-specific T cell clone efficiently lyses CD95-sensitive and CD95-insensitive targets. Clone 80-20 was assessed 10 days after anti-CD3 stimulation for its ability to lyse autologous adherent macrophages, elutriated monocytes, a lymphoblastoid cell line, and positively selected CD19$^+$ cells treated for 3 days with CD40L (3 μg/ml) and IL-4 (30 ng/ml). Targets were incubated overnight with secreted proteins from Mtb (20 μg/ml; circles). Before the 5-h assay, Fas M3 (5 μg/ml; triangles), MgEGTA (2 mM; diamonds), or both (open squares) were added. Control targets are shown as squares. This experiment is representative of three such experiments.
confirm the potential for CD4\(^+\) CTL to use both the CD95 and perforin/granzyme cytolytic pathways.

**Discussion**

In vitro studies with murine CD4\(^+\) T cells have suggested that the predominant mechanism for their cytolytic capacity involves CD95L interaction with CD95 on the target cell (16–18). In this study, we have found that human PPD-specific CD4\(^+\) T cell clones are capable of efficiently lysing a variety of targets using both CD95-dependent and CD95-independent mechanisms. In short term Ag-dependent cytotoxicity assays, addition of the antagonistic CD95 Ab Fas M3 had no effect on the lysis of macrophage, monocyte, and LCL targets and had a partial effect on the lysis of B cell blasts. These results are consistent with previous reports (27, 28). In contrast, B cell blasts were CD95 sensitive, a finding consistent with previous reports (27, 28).

Several experiments suggested that the short term cytosis we observed was due to activation of the perforin/granzyme system. First, we could detect the release of serine esterase following stimulation of the T cell clones and the presence of perforin mRNA by RT-PCR. Second, addition of MgEGTA resulted in complete inhibition of the CD95-insensitive targets and only partial inhibition of the B cell blast targets.

We show that the CD4\(^+\) T cell clones are capable of inducible CD95L expression by RT-PCR, and functionally in a bystander assay, as lysis of the CD95-sensitive Jurkat cell line was effectively inhibited by the antagonistic CD95 Ab, while addition of MgEGTA had little effect. Our experiments might underestimate the role of CD95 in two ways. First, chelation of extracellular calcium may inhibit T cell activation and thus the induction of CD95L expression, an obvious requirement for utilization of the CD95 lytic pathway. Second, if the kinetics of apoptosis induced by CD95 were slower than those of perforin-granzyme, our short term assays would favor detection of the former mechanism. Nonetheless, the efficient lysis of CD95-insensitive targets in a short term assay supports a dominant role for the perforin-granzyme mechanism of cytosis.

Our data also do not exclude the possibility of CD4\(^+\) T cell subsets that differentially use either the CD95 or perforin granzyme pathways. However, we have found that all our PPD-reactive T cell clones are capable of efficient short term cytosis of LCL targets. Additionally, it is possible that our CD4\(^+\) T cell clones are using an as yet undescribed, calcium-dependent mechanism that is independent of the perforin-granzyme pathway. However, experiments performed in the mouse model have suggested that the CD95 and perforin/granzyme mechanisms can account for all short term cytosis.

These observations may have important implications for the biologic role of CD4\(^+\) CTL. The ability of human CD4\(^+\) CTL clones to lyse a variety of targets using the perforin-granzyme mechanism would suggest a role for these cells in the recognition and the elimination of APC. Thus, CD4\(^+\) CTL may be important in host defense against intracellular pathogens, such as Mtb, whose host is the macrophage (29). Lysis of infected cells, particularly those in which containment has failed, might serve to release intracellular organisms into the extracellular space, allowing fresh macrophages to lyse the bacterium. Alternatively, cytotoxic T cells may play a unique role in host defense to Mtb through the release of granular constituents that promote the destruction of infected macrophages. The directed exocytosis of cytolytic granules by T cells induces apoptosis in the target cell. In this regard, it has been suggested that apoptosis will kill intracellular mycobacteria (30). However, mice deficient in the expression of perforin, granzyme, or CD95 (Fas) are still able to contain infection with Mtb (31, 32), suggesting that in the mouse model the secretion of macrophage-activating cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\) may be sufficient for protective immunity. Alternatively, other components of the cytotoxic granule may have a direct anti-mycobacterial effect (23, 33).

Since human CD4\(^+\) CTL are capable of using the CD95 lytic pathway, these cells may still have an important role in the modulation of the immune response. This role has been inferred from the development of a lymphoproliferative disorders both in the CD95-deficient \(pr\) mouse (34, 35) and in patients with the Canale-Smith syndrome (36). An immunomodulatory role for CD95 is further supported by the observation that B cells become CD95

---

**Table II. Antagonistic CD95 antibody and MgEGTA inhibit CD95 \(T\) cell lysis of B cell targets**

<table>
<thead>
<tr>
<th>Clone</th>
<th>CD95 Antagonist(^a)</th>
<th>MgEGTA(^a)</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td>80-16</td>
<td>35 ± 18(^d)</td>
<td>66 ± 37</td>
<td>87 ± 29</td>
</tr>
<tr>
<td>80-20</td>
<td>42 ± 21</td>
<td>74 ± 35</td>
<td>95 ± 20</td>
</tr>
</tbody>
</table>

\(^a\) CD19\(^+\) cells were positively selected from fresh, autologous PBMC and cultured in the presence of IL-4 and CD40L for 3 days.

\(^b\) Antagonistic anti-CD95 antibody Fas M3 was added at 5 \(\mu\)g/ml.

\(^c\) MgEGTA was added at 2 mM.

\(^d\) Results are presented as the mean ± SD of three separate experiments.

---

**Table III. CD4\(^+\) \(T\) cell clones release serine esterase when stimulated with anti-CD3\(^\alpha\)**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Spontaneous</th>
<th>CD3</th>
<th>Maximum(^b)</th>
<th>% Specific Release(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80-16</td>
<td>0.44 ± 0.04(^d)</td>
<td>0.66 ± 0.01</td>
<td>1.97 ± 0.14</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>80-20</td>
<td>0.44 ± 0.01</td>
<td>0.75 ± 0.01</td>
<td>1.65 ± 0.40</td>
<td>26 ± 9</td>
</tr>
</tbody>
</table>

\(^a\) CD4\(^+\) T cell clones were incubated in the presence of immobilized anti-CD3 for 2 h. Supernatants were assessed for the presence of serine esterase.

\(^b\) Optical density read at 450 nm with 560 nm used as a reference.

\(^d\) % Specific release = (CD3– spontaneous)/(maximum – spontaneous) × 100, where the relative esterase concentration is proportional to the OD read.

\(^d\) Maximum release was determined from T cells incubated in the presence of 0.1% Triton during the course of the assay.

\(^d\) Results are presented as the mean of triplicate determinations ± SD. Student’s t-test comparison of spontaneous and anti-CD3 stimulated release is \(p < 0.0005\) for clone 80-16 and \(p < 0.0001\) for clone 80-20.
sensitive when activated in the absence of Ag (28) and by the observation that activated CD4+ T cells lyse CD8+ T cells in a CD95-dependent manner (37). Recently, Vergelli et al. (38) have described autoreactive myelin basic protein-specific CD4+ T cell clones, a subset of which kill through CD95, a subset of which are presumed to kill through perforin, and a subset of which kill through both. Since we have not found evidence for PPD-reactive T cell clones that lyse exclusively through CD95, it is interesting to speculate that CD4+ T cell clones that are capable only of CD95-mediated lysis may play a unique immunoregulatory role.

In conclusion, we have found that human PPD-specific CD4+ CTL use both CD95-dependent and CD95-independent mechanisms. Collectively, the data suggest that CD4+ CTL may play an important role in the immune response to intracellular pathogens.

Acknowledgments

The authors thank John Belisle and colleagues at Colorado State University for providing culture filtrate proteins from Mtb. We thank Richard Armitage (Immunex Corp.) for CD40L, and Debbie Lewinsohn for her insightful discussion of the manuscript.

References