A Noncomitogenic CD2R Monoclonal Antibody Induces Apoptosis of Activated T Cells by a CD95/CD95-L-Dependent Pathway

Sylvie Fournel, Eric Robinet, Nathalie Bonnefoy-Bérard, Olga Assoussou, Monique Flacher, Herman Waldmann, Georges Bismuth and Jean-Pierre Revillard

*J Immunol* 1998; 160:4313-4321;
http://www.jimmunol.org/content/160/9/4313
A Noncomitogenic CD2R Monoclonal Antibody Induces Apoptosis of Activated T Cells by a CD95/CD95-L-Dependent Pathway

Sylvie Fournel,* Eric Robinet,* Nathalie Bonnefoy-Bérard,* Olga Assoussou,* Monique Flacher,* Herman Waldmann,† Georges Bismuth,‡ and Jean-Pierre Revillard2*

Clonal expansion of activated T and B cells is controlled by homeostatic mechanisms resulting in apoptosis of a large proportion of activated cells, mostly through interaction between CD95 (Fas or Apo-1) receptor and its ligand CD95-L. CD2, which is considered as a CD3/TCR alternative pathway of T cell activation, may trigger activation-induced cell death, but the role of CD95/CD95-L interaction in CD2-mediated apoptosis remains controversial. We show here that the CD2R mAb YTH 655.5, which does not induce comitogenic signals when associated with another CD2 mAb, triggers CD95-L expression by preactivated but not resting T cells, resulting in CD95/CD95-L-mediated apoptosis. The critical role of CD95/CD95-L interaction was supported by complete inhibition in the presence of the antagonist CD95 mAb ZB4 and by blocking CD95-L synthesis and surface expression by cycloheximide, cyclosporin A, EGTa, or cytochalasin B. YTH 655.5 was shown to stimulate p56Lck phosphorylation and enzymatic activity. However, p56Lck activation is not sufficient to trigger apoptosis, because other CD2R and CD4 mAbs that activate p56Lck do not induce apoptosis. In conclusion, CD2 can mediate nonmitogenic signals, resulting in CD95-L expression and apoptosis of CD95+ cells. The Journal of Immunology, 1998, 160: 4313–4321.

CD2 is a 50-kDa nonpolymorphic receptor of T and NK cells the ligands of which are CD58, CD59, and CD48 (1–4). Implicated in T cell activation as an adhesion and costimulatory molecule (5), CD2 is also considered as a CD3/TCR “alternative activation pathway” of T cells, because appropriate combinations of CD2 mAbs can promote IL-2 production and proliferation of human T cells in vitro (reviewed in Ref. 6). The CD2 activation pathway resembles that generated after CD3/TCR aggregation (7) and implicates early tyrosine phosphorylation. The protein tyrosine kinase (PTK) p56Lck is physically associated with CD2 (8, 9) and displays increased tyrosine kinase activity in human resting T lymphocytes exposed to mitogenic CD2 stimulation (10). Addition of a single CD2 mAb may also interfere with T cell proliferation induced by various activators (5, 11, 12).

Clonal expansion of activated T cells is controlled by homeostatic mechanisms resulting in the programmed cell death (apoptosis) of a large proportion of activated cells (13). Hence, human peripheral blood lymphocytes (PBLs) preactivated by the CD3/TCR pathway may undergo apoptosis when restimulated in vitro, a model known as “activation-induced cell death” (AICD). The major pathway of AICD is the interaction between CD95 (Fas or Apo-1) receptor, expressed by activated T and B cells, and its ligand, CD95-L (Fas-L), produced by a subset of activated T cells (14–16).

Several reports suggest that CD2 may be implicated in AICD (17–20), but the precise contribution of CD95/CD95-L interaction in CD2-mediated apoptosis of preactivated T cells remains controversial. T cells preactivated in vitro by various types of mitogens or antigens in the presence of IL-2 may undergo apoptosis when restimulated by a mitogenic pair of CD2 mAbs (20). Similarly, lamina propria T cells, which exhibit phenotypic markers of in vivo preactivated cells, undergo apoptosis when stimulated in vitro by a mitogenic pair of CD2 mAbs (19). This type of AICD depends exclusively on CD95/CD95-L interactions (19). Alternatively, Rouleau et al. (17) reported that addition of a single CD2 mAb to human PBLs that had been preactivated by a pair of mitogenic CD2 mAbs resulted in apoptosis of activated lymphocytes. However, the same CD2 mAbs did not induce apoptosis of T cells that had been activated by other mitogens, such as Con A, PMA + ionomycin (PI), and immobilized anti-CD3 mAb OKT3 (17). It was therefore hypothesized that T cell apoptosis in this model used signaling pathways distinct from those of AICD. The recent demonstration that CD95/CD95-L interaction was not involved in the CD2-mediated apoptosis supported this hypothesis (18).

Whether nonmitogenic mAbs can induce apoptosis of activated but not resting T cells is becoming an important issue for clinical applications to selective immunosuppression. Indeed, such mAbs could be used to achieve the selective deletion of in vivo-activated T cell clones in organ and bone marrow allografts or autoimmune disorders. At variance with AICD, which requires repeated activation, clonal deletion induced by such nonactivating mAbs should not be associated with the massive cytokine release triggered by activating mAbs such as OKT3 (21).
In this study, we report that a noncomitogenic CD2R mAb IgG2b mAb, YTH 655.5, which is immunosuppressive in primate models of organ transplantation (22), induces apoptosis of activated but not resting human peripheral T lymphocytes by a mechanism that involves CD95/CD95-L interaction. We show that CD95-L can be induced in preactivated but not resting T cells by this CD2R mAb. As control, we used a CD4 mAb (rat IgG2b) that activates p56lck and decreases accessory cell-dependent T cell proliferation but does not induce apoptosis of activated T cells.

Materials and Methods

mAbs and reagents

Three CD2 mAbs were used in this study: mAb YTH 655.5 (rat IgG2b) was produced by H. W. and coworkers and shown to belong to the CD2 cluster (23), the CD2 mAb D66 (mouse IgM) was a gift of P. A. Bernard (Institut National de la Santé et de la Recherche Médicale (INSERM) Unité 343, Nice, France), and the CD2 mAb X1 (mouse IgG1) was a gift of L. Boumsell (INSERM Unité 448, Créteil, France).

The CD4 mAb, Rlg2B CD4 (rat IgG2b) used as control was produced by H. W., and the CD95 mAbs CH11 (IgM) and ZB4 (IgG1) were obtained from Immunotech (Marseille, France). The anti-HLA class I mAb, mAb90, was produced as previously described (27) and purified from ascitic fluid by DEAE chromatography. The CD3 mAb, OKT3, was provided by Cilig Laboratories (Levallois-Perret, France). The anti-Ick mAb, 3AS, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-tyrosine-phosphorylated mAb, 4G10, and the anti-human lck kinase antiserum were obtained from Eurodemed (Soutelwéyrem, France). The goat anti-rat IgG-FITC-labeled mAb, YTH 655.5, which is immunosuppressive in primate models (21), was produced as previously described (24) and purified from ascitic fluid by DEAE chromatography. The CD3 mAb, OKT3, was provided by Cilig Laboratories (Levallois-Perret, France).

Cell preparation

Peripheral blood was collected from healthy donors in the presence of sodium citrate. Blood was centrifuged by gentle rotation of the flask after addition of a calcium chloride solution, and then mononuclear cells were isolated by centrifugation on a layer of FicoLite H (Dutcher, France). Cells were washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml.

Cell culture

PBLs were resuspended in RPMI 1640 (Sigma) supplemented with 10% FCS, 2 mM l-glutamine, and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml). For proliferation assay, cells (1 × 10^6/ml) were incubated for 72 h in 96-well microplates (Costar, Cambridge, MA) coated with the anti-CD3 mAb (Leu4), CD20 mAb (Leu16), and CD56 mAb (NCAM-16-2) previously shown to react with rat IgG2b (25).

The lectins PHA and Con A, the phorbol ester PMA, the calcium ionophore ionomycin, the superantigen staphylococcal enterotoxin B (SEB), and the superantigen staphylococcal enterotoxin A (SEA) were obtained from Becton Dickinson (Pont de Claix, France). The CD4 mAb, rIgG2b CD4 (rat IgG2b) used as control was produced as previously described (26) and purified from ascitic fluid by DEAE chromatography. The CD3 mAb, OKT3, was provided by Cilig Laboratories (Levallois-Perret, France). As control, we used a CD4 mAb (rat IgG2b) that activates p56lck and decreases accessory cell-dependent T cell proliferation but does not induce apoptosis of activated T cells.

Measurement of apoptosis

After 3 days of culture, activated PBLs were harvested. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml. Cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml.

Cell suspensions were incubated in 96-well microplates (Costar, Cambridge, MA) coated with the CD3 mAb (Leu4), CD20 mAb (Leu16), and CD56 mAb (NCAM-16-2) previously shown to react with rat IgG2b (25).

The lectins PHA and Con A, the phorbol ester PMA, the calcium ionophore ionomycin, the superantigen staphylococcal enterotoxin B (SEB), and the superantigen staphylococcal enterotoxin A (SEA) were obtained from Becton Dickinson (Pont de Claix, France).

The CD4 mAb, rIgG2b CD4 (rat IgG2b) used as control was produced as previously described (26) and purified from ascitic fluid by DEAE chromatography. The CD3 mAb, OKT3, was provided by Cilig Laboratories (Levallois-Perret, France) as control.

Measurement of apoptosis

After 3 days of culture, activated PBLs were harvested. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml.

Measurement of apoptosis

After 3 days of culture, activated PBLs were harvested. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml.

Measurement of apoptosis

After 3 days of culture, activated PBLs were harvested. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml.

Measurement of apoptosis

After 3 days of culture, activated PBLs were harvested. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml.

Measurement of apoptosis

After 3 days of culture, activated PBLs were harvested. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml. Dead cells were removed by centrifugation on a layer of FicoLite H (Dutcher, France) and washed three times in HBSS before culture. Those cell suspensions referred to as PBLs were shown to contain 1.8 × 10^6 cells/ml.
mN NaCl; 10% glycerol; 1% Triton X-100; 1 mM EDTA; 1 mM PMSF; 20 mM ATP and 1 mM PMSF; 20 mM MnCl₂. The noncomitogenic CD2R mAb, YTH 655.5, induces apoptosis of activated T cells. Only blast cells that express the CD2R epitope were labeled by YTH 655.5 and D66 (Fig. 3). Double labeling of cells activated by PMA, PHA, or PI, in agreement with previous results from our group (Refs. 33–35; S. Fournel et al., manuscript in preparation).

To ascertain the contribution of apoptosis to inhibition of proliferation, PBLs were activated for 3 days by PHA. Then, dead cells were eliminated; viable cells were incubated with YTH 655.5, D66, or rlgG2b CD4; and apoptosis was evaluated by different techniques. When added to PHA-activated cells, YTH 655.5 triggered decreased mitochondrial potential, phosphatidylserine surface expression, DNA fragmentation (Fig. 1), and nuclear condensation and fragmentation (Fig. 2), whereas none of these alterations were observed when resting PBLs were incubated with the same mAb. By contrast, D66, which also inhibited PHA-induced activation, did not induce apoptosis (Figs. 1 and 2). The control CD4 mAb had no apoptotic effect on activated or resting T cells (Fig. 1). Similar results were obtained when PBLs had been activated by other mitogens, such as Con A, PMA, PI, OKT3, and SEB (Fig. 2), showing that inhibition of proliferation induced by YTH 655.5, but not by D66 or CD4 mAb, was associated with apoptosis of activated cells. Of note, the percentage of specific apoptosis was not correlated with proliferation inhibition assessed by [³H]Tdr incorporation. This is likely to be accounted for by differences in the experimental protocol (the addition of Ab at day 0 or day 3).

**Kinetics and dose response of YTH 655.5-induced apoptosis**

Only blast cells that express the CD2R epitope were labeled by YTH 655.5 and D66 (Fig. 3). Double labeling of cells activated for 48 h with PHA showed that YTH 655.5 stained 70% of CD56⁺ blast cells, 90% of CD56⁺ large cells, and none of the CD2⁺ cells. Small lymphocytes were not stained by YTH 655.5. Blast cell apoptosis was induced by YTH 655.5, with a maximum at the saturating concentration (10 μg/ml). D66 was ineffective at concentrations up to 100 μg/ml (Fig. 3). Addition of YTH 655.5 at the beginning of PHA culture resulted in a decrease in the number of viable cells from 48 h to 96 h (Fig. 4A), associated with an increase of apoptotic cell number (Fig. 4B). The control CD2 mAb D66 did not induce a decrease of viable cell count (Fig. 4A). The other control mAb, rlgG2b

---

**Table I. Effect of CD2R and CD4 mAbs on [³H]Tdr incorporation**

<table>
<thead>
<tr>
<th>Activator</th>
<th>Control [³H]Tdr incorporation (cpm × 10⁻³)</th>
<th>YTH 655.5* [³H]Tdr incorporation (cpm × 10⁻³)</th>
<th>% Inhibition</th>
<th>D66 [³H]Tdr incorporation (cpm × 10⁻³)</th>
<th>% Inhibition</th>
<th>rlgG2b CD4 [³H]Tdr incorporation (cpm × 10⁻³)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.2 ± 0.5</td>
<td>0.6 ± 0.3</td>
<td></td>
<td>0.79 ± 0.0</td>
<td></td>
<td>2.1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>CD2 mAb YTH655.5 (10 μg/ml)</td>
<td>0.6 ± 0.3</td>
<td>ND</td>
<td></td>
<td>1.08 ± 0.1</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CD2 mAb X11 (1/100)</td>
<td>1.04 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td></td>
<td>19.5 ± 1.2</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PHA (5 μg/ml)</td>
<td>23.5 ± 1.6</td>
<td>16.8 ± 2.3</td>
<td>29</td>
<td>10.1 ± 0.0</td>
<td>34</td>
<td>19.6 ± 0.5</td>
<td>20</td>
</tr>
<tr>
<td>sOKT3 (100 μg/ml)</td>
<td>5.4 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>76</td>
<td>4.1 ± 0.1</td>
<td>25</td>
<td>3.8 ± 0.2</td>
<td>30</td>
</tr>
<tr>
<td>iOKT3 (5 μg/ml)</td>
<td>18.7 ± 3.0</td>
<td>3.2 ± 1.8</td>
<td>83</td>
<td>12.6 ± 0.44</td>
<td>32</td>
<td>10.3 ± 0.2</td>
<td>45</td>
</tr>
<tr>
<td>PMA (10 μg/ml)</td>
<td>6.1 ± 1.0</td>
<td>4.1 ± 0.8</td>
<td>35</td>
<td>6.1 ± 0.2</td>
<td>3</td>
<td>7.0 ± 0.0</td>
<td>0</td>
</tr>
<tr>
<td>PMA + iokt3 (100 μg/ml + 0.5 μg/ml)</td>
<td>18.6 ± 3.1</td>
<td>14.5 ± 2.13</td>
<td>22</td>
<td>20.7 ± 0.2</td>
<td>12</td>
<td>22.6 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>SEB (50 μg/ml)</td>
<td>7.9 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>81</td>
<td>7.7 ± 0.1</td>
<td>3</td>
<td>4.2 ± 0.2</td>
<td>47</td>
</tr>
<tr>
<td>SEB (10 μg/ml)</td>
<td>6.4 ± 0.8</td>
<td>1.3 ± 0.1</td>
<td>80</td>
<td>ND</td>
<td>ND</td>
<td>3.8 ± 0.1</td>
<td>41</td>
</tr>
<tr>
<td>SEB (1 μg/ml)</td>
<td>4.9 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>74</td>
<td>ND</td>
<td>ND</td>
<td>3.1 ± 0.2</td>
<td>37</td>
</tr>
<tr>
<td>MLR (Raji cells)</td>
<td>13.1 ± 3.9</td>
<td>3.0 ± 0.9</td>
<td>77</td>
<td>ND</td>
<td>ND</td>
<td>2.1 ± 0.6</td>
<td>84</td>
</tr>
</tbody>
</table>

* Proliferation was assessed by [³H]Tdr incorporation during the last 8 h of 72- or 120-h culture in the presence of mitogens or allogeneic cells, respectively. Results are expressed as cpm ± SD on two or four separate experiments.

**Results**

The noncomitogenic CD2R mAb, YTH 655.5, induces apoptosis of activated T cells

To characterize mitogenic activities of the three CD2 mAbs used in this study (YTH 655.5, D66, and X11), we cultured human PBLs in the presence of various CD2 mAb combinations. As expected, association of D66 and X11 induced a proliferative response, whereas combinations of YTH 655.5 with D66 or X11 did not (Table I), suggesting that YTH 655.5 was a noncomitogenic CD2 mAb.

We then investigated whether YTH 655.5 could inhibit T cell proliferation induced by various mitogens or allogeneic cells in MLR. As shown in Table I, YTH 655.5 strongly inhibited [³H]Tdr uptake induced by the CD3 mAb OKT3 (soluble or immobilized), the superantigen SEB, or allogeneic stimulus cells in MLR. The proliferative response to PHA, PMA, or PI was decreased by approximately 20 to 30% (Table I). D66, another CD2 mAb, decreased the proliferative response to PHA and OKT3 but to a lesser extent than YTH 655.5 and had no effect on proliferation induced by PMA, SEB, or PI. The rlgG2b CD4 decreased the proliferative responses to OKT3 or SEB, or in the MLR, but had no effect on the proliferation induced by PMA, PHA, or PI, in agreement with previous results from our group (Refs. 33–35; S. Fournel et al., manuscript in preparation).
CD4, slightly inhibited the increase of viable cell number induced by PHA activation (Fig. 4A) but did not trigger apoptosis (Fig. 4B).

Knowing that YTH 655.5 induced apoptosis after 48 h to 96 h of activation, we measured viable cell counts and percentage of apoptotic cells after addition of YTH 655.5 or control mAbs to viable PHA blasts obtained after 3 days of culture. Addition of YTH 655.5 resulted in a decreased number of viable cells starting 48 h after mAb addition, with a maximum at 96 h (Fig. 4C) associated with marked increase of apoptotic cell number at 24 h (Fig. 4D). In contrast, neither D66 nor rIgG2b CD4 had any effect on activated T cells (Fig. 4, C and D).

YTH 655.5 induces CD95-L expression by activated T cells

The major pathway of AICD results from the interaction between CD95 and CD95-L. Knowing that activated T cells express CD95 receptor (36), we investigated whether YTH 655.5 could induce CD95-L expression. To this end, PBLs or PHA-activated PBLs were treated for 6, 12, or 24 h with YTH 655.5 or PI, and CD95-L mRNA expression was measured by RT-PCR. After 6 h, CD95-L mRNA expression of PHA-activated PBLs increased by 44% in the presence of YTH 655.5 and 191% with PI (Fig. 5). CD95-L mRNA expression did not increase in unstimulated PBLs. No CD95-L mRNA was detected after 12 or 24 h of treatment. We then used the Jurkat cell line to assess the functional CD95-L cytotoxic activity. PBLs or PHA-activated PBLs were treated for 8 h with YTH 655.5 or PI and then cocultured with [3 H]TdR-labeled Jurkat cells in the presence or absence of the antagonist CD95 mAb, ZB4, for 12 h. YTH and PI did not induce lysis of Jurkat cells (data not shown). Results in Figure 6 show that the [3 H]TdR-DNA release induced by PHA-activated PBLs treated with either

![FIGURE 1. Measurement of mitochondrial transmembrane potential, phosphatidylserine expression, and DNA fragmentation after YTH 655.5 treatment. PBLs or 3-day PHA-activated PBLs (10^7 cells/ml) were incubated with or without CD2R mAbs YTH655.5 (10 μg/ml) or D66 (10 μg/ml) or with control mAb rlgG2b CD4 (10 μg/ml). After 12 h, measurement of mitochondrial transmembrane potential by flow cytometry after DiOC_6(3) staining and detection of phosphatidylserine expression by flow cytometry after addition of biotinylated annexin V were performed. After 14 h, DNA fragmentation by the TUNEL assay was measured as described in Materials and Methods.]

![FIGURE 2. The CD2R mAb, YTH655.5 induces apoptosis of activated T cells. PBLs were activated for 3 days with PHA (5 μg/ml), Con A (10 μg/ml), PMA (10 ng/ml), PI (PMA, 10 ng/ml; ionomycin, 0.5 μg/ml), SEB (50 ng/ml), soluble OKT3 (100 ng/ml), or immobilized OKT3 (5 μg/ml). After removal of dead cells, preactivated PBLs (10^7 cells/ml) were incubated with or without CD2R mAbs YTH655.5 (10 μg/ml) or D66 (10 μg/ml). The percentage of apoptotic cells was determined by microscopy after 15 h. Results are expressed as specific apoptosis, as described in Materials and Methods. Spontaneous apoptosis did not exceed 15%. Values are means ± SD of three individual experiments for YTH 655.5 (black bars) and four for D66 (numbers on the right).]

YTH 655.5 induces CD95-L expression by activated T cells

The major pathway of AICD results from the interaction between CD95 and CD95-L. Knowing that activated T cells express CD95 receptor (36), we investigated whether YTH 655.5 could induce CD95-L expression. To this end, PBLs or PHA-activated PBLs were treated for 6, 12, or 24 h with YTH 655.5 or PI, and CD95-L mRNA expression was measured by RT-PCR. After 6 h, CD95-L mRNA expression of PHA-activated PBLs increased by 44% in the presence of YTH 655.5 and 191% with PI (Fig. 5). CD95-L mRNA expression did not increase in unstimulated PBLs. No CD95-L mRNA was detected after 12 or 24 h of treatment. We then used the Jurkat cell line to assess the functional CD95-L cytotoxic activity. PBLs or PHA-activated PBLs were treated for 8 h with YTH 655.5 or PI and then cocultured with [3 H]TdR-labeled Jurkat cells in the presence or absence of the antagonist CD95 mAb, ZB4, for 12 h. YTH and PI did not induce lysis of Jurkat cells (data not shown). Results in Figure 6 show that the [3 H]TdR-DNA release induced by PHA-activated PBLs treated with either

![FIGURE 2. The CD2R mAb, YTH655.5 induces apoptosis of activated T cells. PBLs were activated for 3 days with PHA (5 μg/ml), Con A (10 μg/ml), PMA (10 ng/ml), PI (PMA, 10 ng/ml; ionomycin, 0.5 μg/ml), SEB (50 ng/ml), soluble OKT3 (100 ng/ml), or immobilized OKT3 (5 μg/ml). After removal of dead cells, preactivated PBLs (10^7 cells/ml) were incubated with or without CD2R mAbs YTH655.5 (10 μg/ml) or D66 (10 μg/ml). The percentage of apoptotic cells was determined by microscopy after 15 h. Results are expressed as specific apoptosis, as described in Materials and Methods. Spontaneous apoptosis did not exceed 15%. Values are means ± SD of three individual experiments for YTH 655.5 (black bars) and four for D66 (numbers on the right).]
YTH 655.5 or PI was markedly reduced by the antagonist CD95 mAb ZB4, indicating that YTH 655.5 induced functional CD95-L expression. As control, the anti-HLA class I mAb, mAb90, which induced apoptosis of activated PBLs in a CD95/CD95-L-independent manner (24), did not induce [3 H]TdR-DNA release from Jurkat cells (Fig. 6). The rather low DNA release activity of PI is due to the high level of cell death within 8 h in the effector cell population. These experimental conditions correspond to optimal YTH 655.5 mAb activity.

YTH 655.5-induced apoptosis depends on CD95-L expression

Knowing that YTH 655.5 triggered CD95-L expression, we studied the contribution of CD95/CD95-L interaction to YTH-induced apoptosis. The antagonist CD95 mAb ZB4 completely blocked YTH 655.5-induced apoptosis, indicating that this apoptosis was exclusively mediated by the CD95 pathway (Fig. 7A). This result was confirmed by the absence of additive effect between YTH 655.5 and the agonist CD95 mAb CH11 (Fig. 7A). That YTH 655.5-induced apoptosis requires CD95-L expression was further documented by inhibition of apoptosis in the presence of CHX, CKB (which prevents protein translocation to cell surface), CsA, and EGTA (37, 38). None of these inhibitors interfered with the apoptotic effect of the agonist CD95 mAb CH11 (Fig. 7B).

YTH 655.5 induces p56 lck activity

Knowing that CD2 is physically associated with the PTK p56 lck, which has recently been shown to control CD95-L expression in Jurkat cells (39), we studied whether binding of YTH 655.5 could activate lck. For this, PBLs and PHA-activated T cells were treated with YTH 655.5 (10 μg/ml) for 5, 15, or 30 min. Two control mAbs against surface molecules known to be associated with p56 lck were introduced in this experiment: the CD2 mAb D66 (10

FIGURE 3. Dose response of YTH 655.5 binding and induction of apoptosis. PBLs were cultivated in medium (open symbols) or in PHA (5 μg/ml) (closed symbols) for 3 days. Then, binding of YTH 655.5 at various concentrations was measured by flow cytometry (circles), and specific apoptosis (squares) was evaluated after incubation for 15 h with YTH655.5 at various concentrations, as described in Materials and Methods. Results are expressed as mean fluorescence intensity for YTH 655.5 fixation and as a percentage of specific apoptosis for YTH 655.5-induced apoptosis. Data shown are means ± SD of three independent experiments.

FIGURE 4. Kinetics of YTH 655.5-induced apoptosis. A and B, PBLs were activated with PHA (5 μg/ml) in the absence (open circles) or presence (open circles) of control mAbs rIg2a CD4 and YTH655.5 (both 10 μg/ml; closed squares) or D66 10 μg/ml (closed circles) for indicated times. C and D, 3-day PHA-activated PBLs were incubated without (open squares) or with (open circles) control mAbs rIg2a CD4 10 μg/ml, YTH655.5 10 μg/ml (closed squares), or D66 10 μg/ml (closed circles) for the indicated times. At indicated times, viable cell numbers (A and C) (determined by trypan blue exclusion) and percentage of specific apoptosis (B and D) were evaluated. Results are expressed as means ± SD of three experiments.
$\mu g/ml$ and the CD4 mAb rlgG2b (10 $\mu g/ml$). p56$\text{Lck}$ was immunoprecipitated from cell lysates, and then protein tyrosine phosphorylation and enzymatic activity of lck were evaluated. Tyrosine phosphorylation of p56$\text{Lck}$ was increased after exposure to each of the three mAbs for 5 min (Fig. 8A). A concomitant increase of lck kinase activity was detected as measured by lck autophosphorylation and by phosphorylation of enolase used as an exogenous substrate adjusted to p56$\text{Lck}$ amount (Fig. 8B, ratio B/C). The increase of lck activity was observed only after 5 min in cells exposed to YTH 655.5 and rlgG2b CD4 mAbs, and it was more sustained with the D66 mAb (Fig. 8B). No significant change in p56$\text{Lck}$ phosphorylation and no induction of enzymatic activity could be detected in fresh PBLs incubated with the three mAbs (data not shown).

Discussion

This study was undertaken to assess the contribution of CD95/CD95-L interaction in CD2-mediated apoptosis and to determine whether CD95-L expression could be triggered by CD2 in the absence of a complete mitogenic signal. The CD2 mAb YTH 655.5, which recognizes a CD2R (restricted) epitope normally “hidden” in resting T cells but exposed upon activation through TCR/CD3 or CD2 (40) provided an appropriate tool to address these questions. Indeed, we report here that YTH 655.5, unlike previous studies with other CD2 mAbs (17, 18), induces apoptosis of activated T cells by a CD95-dependent pathway. Furthermore, the same mAb activates p56$\text{Lck}$ (and other PTKs), recently implicated as a necessary and sufficient signal for CD95-dependent apoptosis (39). However, control CD2 (D66) and CD4 mAbs, which similarly activate p56$\text{Lck}$, do not trigger CD95-dependent apoptosis, so that the role of p56$\text{Lck}$ in CD2-mediated apoptosis may be reconsidered.

Repeated activation of peripheral T cells through CD3/TCR was shown to induce clonal expansion, functional inactivation (also

**FIGURE 5.** Expression of CD95-L mRNA induced by YTH 655.5. PBLs or 3-day PHA-activated cells were treated for 6 h with YTH 655.5 (10 $\mu g/ml$) or PI (PMA, 10 ng/ml; ionomycin, 0.5 $\mu g/ml$). mRNA of each sample was amplified by RT-PCR as described in Materials and Methods with primers specific for actin or CD95-L. The number of amplification cycles selected within the exponential phase of PCR was 29 for actin and 32 for CD95-L. The PCR products were separated on 2% gel agarose, and the PCR signal intensities were quantified by scanning the negative film. Results are expressed as the ratio of absorbance of CD95-L/absorbance of actin (values are means ± SD of three experiments).

**FIGURE 6.** Functional CD95-L assessed by Jurkat cell DNA fragmentation. PBLs or 3-day PHA-activated PBLs were treated for 8 h with YTH 655.5 (10 $\mu g/ml$), mAb90 (10 $\mu g/ml$), or PI (PMA, 10 $\mu g/ml$; ionomycin, 0.5 $\mu g/ml$) and then incubated with [$^{3}H$]labeled Jurkat cells (0.2 × 10^6 cells/ml) at a ratio of 1 Jurkat cell/3 PBLs, with or without the antagonist CD95 mAb ZB4 (2 $\mu g/ml$). After 12 h of culture, [$^{3}H$]DNA release induced by apoptosis of Jurkat cells was measured. Results are expressed as percentage of specific lysis as described in Materials and Methods (means ± SD of three experiments). The CD95 mAb CH11 (50 ng/ml) induced 53% of apoptosis in the Jurkat cell line.

**FIGURE 7.** Effect of ZB4, CHX, CKB, CsA, and EGTA on YTH 655.5-induced apoptosis of activated T cells. Three-day PHA-activated PBLs were incubated for 1 h with the antagonist CD95 mAb ZB4 (2 $\mu g/ml$) (A) or 3 h with CHX (0.5 $\mu g/ml$), CKB (10 $\mu M$), CsA (250 ng/ml) or EGTA (500 $\mu M$) (B). Then, YTH 655.5 (10 $\mu g/ml$) and/or agonist CD95 mAb, CH11 (1 $\mu g/ml$), were added, and the percentage of apoptotic cells was determined by microscopy after 15 h. Results are expressed as specific apoptosis, as described in Materials and Methods. Spontaneous apoptosis did not exceed 15% with all the agents tested. Values are means ± SD from three individual experiments.
referred to as anergy) and/or clonal deletion (referred to as AICD). In vivo injection of bacterial superantigens such as staphylococcal enterotoxins results in clonal expansion, anergy, and deletion of the T cell populations that express matching vβ gene products (41, 42). Intravenous, or oral administration of specific antigens in mice bearing transgenic TCRs was shown to induce deletion of specific T cell clones (43, 44). In vitro, iterative stimulation of peripheral T cells by specific antigen or by mitogenic mAbs such as CD3 or anti-TCR Abs results in AICD (44–47) primarily mediated by CD95/CD95-L interactions (16).

Iterative stimulations of the CD2 alternative activation pathway was also reported to induce AICD (46), but the mechanisms of CD2-mediated apoptosis differ from those involved in TCR/CD3-mediated T cell death. Rouleau and coworkers (17), using a model of peripheral T cell activation by mitogenic pairs of CD2 mAbs (GT2 + T11.1) in the presence of IL-2, reported that late addition of a third CD2 mAb resulted in apoptotic cell death of 40 to 60% of the cells. In this model, apoptosis was not prevented by CHX or actinomycin D, indicating that the death program was already expressed in preactivated T cells and did not require de novo CD95-L gene expression and protein synthesis. In a more recent report (18), the same group formally excluded the contribution of the CD95 pathway in their model by showing that lymphocytes from patients with a genetic defect in CD95 expression or in the CD95 signaling cascade (48) were fully susceptible to CD2-mediated apoptosis. It was suggested by the authors that the ability of CD2 to transduce an apoptotic signal may rely on certain conformation changes induced by various CD2 mAbs. For instance, the mitogenic GT2 + T11.1 CD2 mAb pair did not trigger an apoptotic signal into lymphocytes preactivated by OKT3 and IL-2, whereas the D66 + T11.1 CD2 mAb pair was highly effective.

At variance with these reports, a mitogenic pair of CD2 mAbs (T11.1 and T11.2) was recently shown to induce apoptotic death of lamina propria lymphocytes by a CD95-L pathway (19). Those lymphocytes express a phenotype of preactivated cells (CD45RO+), and their homing properties are acquired following antigenic stimulation in vivo. Considering these divergent results, it seems worth noting that repeated mitogenic activation through CD3/TCR (or CD2) may result in CD95-mediated AICD, whereas CD95-independent apoptosis was demonstrated only in culture systems involving stimulation by a pair of CD2 mAbs followed by addition of another CD2 mAb (18).

Unlike D66, which also recognizes a CD2R epitope, the YTH 655.5 mAb used in this study was devoid of comitogenic activity when associated with X11 or D66 (Table I). Yet this mAb was fully efficient in triggering apoptosis in about 30 to 40% of activated peripheral T cells. The essential role of CD95/CD95-L interaction in this effect is supported by several lines of evidence: 1) the CD95 antagonist mAb ZB4 completely inhibits apoptosis (Fig. 7A); 2) YTH 655.5 induces CD95-L mRNA expression (Fig. 5) and CD95-L functional activity measured by DNA fragmentation of the Jurkat cell line (Fig. 6); and 3) apoptosis requires protein synthesis and extracellular calcium, and it is prevented by CsA (Fig. 7B) in a way similar to that of CD95-L expression (37, 38). Further indirect evidence supporting a CD95-mediated pathway is brought by the observation that YTH 655.5 and the agonist CD95 mAb CH11 do not display additive effects when mixed together (Fig. 7A), in contrast, for instance, with anti-HLA class I mAbs, which induce CD95-independent apoptosis of a subset of activated T cells distinct from those susceptible to CD95-dependent apoptosis (24). Of note, sensitivity to CD95-dependent apoptosis is progressively acquired upon in vitro activation (36, 49) and requires an IL-2 signal (50). Furthermore, only T cells that express the CD45RO short isoform are susceptible to CD95-dependent apoptosis, even if maximal susceptibility to either pathway is not achieved at this time.

So far, CD95-dependent apoptosis has been associated with T cell-activating mAbs (e.g., CD3/TCR and CD2) (46, 20) and CD95-independent apoptosis with mAbs that do not trigger T cell activation (e.g., anti-HLA class I (24), CD30 (51), and anti-CTLA-4 (52)). However, the role of CD2 is not limited to the induction of activation or apoptosis. CD2 can also rescue T cells from apoptosis mediated by CD3 (53) or by prior ligation of CD4 by gp120 (54), by decreasing CD95 and CD95-L expression.

The role of PTKs of the src family in CD3/TCR or CD2-mediated T cell activation has been extensively documented, and CD95-L expression requires recruitment and activation of ZAP-70 (55). In a recent study, Gonzalez-Garcia et al. (39) demonstrated the critical role of p56Lck in triggering CD95-L expression. Transfection of the active form of p56Lck into normal or lck-defective cell lines was shown to be sufficient to induce expression of a functional CD95-L molecule. Our data, showing that YTH 655.5 increases p56Lck tyrosine phosphorylation and enzymatic activity (Fig. 8) and induces CD95-L expression (Fig. 5), are in keeping with these results. However, the mere activation of p56Lck is not sufficient to trigger CD95-L expression in preactivated T cells, because the CD2 mAb D66 and the CD4 mAb rlgG2b CD4, which both activate p56Lck, do not induce CD95-L expression and do not trigger apoptosis in our experiments. Activation of p56Lck is clearly not associated with induction of apoptosis, and additional signals

![Tyrosine phosphorylation pattern and p56**Lck** activity induced by YTH 655.5. PHA-activated PBLs were treated for 5, 15, or 30 min with YTH 655.5 (10 µg/ml), D66 (10 µg/ml), and rlgG2b CD4 (10 µg/ml). Then, cells were lysed and immunoprecipitated with an anti-lck mAb. A. Immunoprecipitates were separated on SDS-PAGE gel and electroblotted. The transferred tyrosine-phosphorylated proteins were identified using mAb 4G10, followed by peroxidase-labeled rabbit anti-mouse Ig and ECL detection. B. lck activity was measured as described in Materials and Methods. Products of the kinase assay were separated on SDS-PAGE gel and electroblotted, and an autoradiography of the blot was performed. C. Anti-phosphotyrosine blot was stripped, and the amount of p56Lck was revealed by the addition of the anti-lck mAb 3A5, followed by peroxidase-labeled rabbit anti-mouse Ig and ECL detection. Signal intensities of the different bands present in B and C were quantitated by scanning autoradiography using a desktop scanning densitometer. The ratio, R, corresponding to the kinase activity/amount of protein is indicated.

**FIGURE 8.** Tyrosine phosphorylation pattern and p56**Lck** activity induced by YTH 655.5. PHA-activated PBLs were treated for 5, 15, or 30 min with YTH 655.5 (10 µg/ml), D66 (10 µg/ml), and rlgG2b CD4 (10 µg/ml). Then, cells were lysed and immunoprecipitated with an anti-lck mAb. A. Immunoprecipitates were separated on SDS-PAGE gel and electroblotted. The transferred tyrosine-phosphorylated proteins were identified using mAb 4G10, followed by peroxidase-labeled rabbit anti-mouse Ig and ECL detection. B. lck activity was measured as described in Materials and Methods. Products of the kinase assay were separated on SDS-PAGE gel and electroblotted, and an autoradiography of the blot was performed. C. Anti-phosphotyrosine blot was stripped, and the amount of p56Lck was revealed by the addition of the anti-lck mAb 3A5, followed by peroxidase-labeled rabbit anti-mouse Ig and ECL detection. Signal intensities of the different bands present in B and C were quantitated by scanning autoradiography using a desktop scanning densitometer. The ratio, R, corresponding to the kinase activity/amount of protein is indicated.
References


