Fas-Independent Apoptosis of Activated T Cells Induced by Antibodies to the HLA Class I α1 Domain

By Laurent Genestier, Romain Paillot, Nathalie Bonnefoy-Berard, Geneviève Meffre, Monique Flacher, David Fève, Yong Jun Liu, Philippe Le Bouteiller, Herman Waldmann, Victor H. Engelhard, Jacques Banchereau, and Jean Pierre Revillard

In addition to their major function in antigen presentation and natural killer cell activity regulation, HLA class I molecules may modulate T-cell activation and proliferation. Monoclonal antibodies (MoAbs) that recognize distinct epitopes of HLA class I molecules were reported to interfere with T-cell proliferation. We show here that two MoAbs (mouse MoAb90 and rat YTH862) that bind to an epitope of the α1 domain of HLA class I heavy chain induce apoptotic cell death of activated, but not resting, peripheral T lymphocytes. Other reference anti-HLA class I antibodies specific for distinct epitopes of the α1 (B9.12.1), α2 (W6/32), or α3 (TP25.99) domains of the heavy chain decreased T-cell proliferation but had little or no apoptotic effect. Apoptosis shown by DNA fragmentation, phosphatidylserine externalization, and decrease of mitochondrial transmembrane potential was observed whatever the type of T-cell activator. Apoptosis did not result from Fas/FasL interaction and distinct though partly overlapping populations of activated T cells were susceptible to Fas- and HLA class I-mediated apoptosis, respectively. Induction of apoptosis did not require HLA class I cross-linking inasmuch as it could be observed with monovalent Fab' fragments. The data indicate that MoAb90 and YTH862 directed against the α1 domain of HLA class I trigger apoptosis of activated T lymphocytes by a pathway which does not involve Fas-ligand.

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From the Laboratory of Immunology, INSERM U80 UCBL, Hôpital E. Herriot, Lyon, France; Schering-Plough, Laboratory for Immunological Research, Dardilly, France; Laboratory of Immune Response and Major Histocompatibility Complex, INSERM U395, Toulouse, France; Sir William Dunn School of Pathology, University of Oxford, Oxford, UK; and the Department of Microbiology, University of Virginia Center, Charlottesville.

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Address reprint requests to Jean Pierre Revillard, MD, INSERM U80, Hôpital E. Herriot, Pav. P, 5 place d’Arsonval 69437 Lyon cedex 03, France.

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MATERIALS AND METHODS

MoAbs and Reagents

MoAb90 was produced by immunization with consecutive intra-peritoneal injections of human tonsil cells (10⁶ cells) followed by the standard procedure of Köhler and Milstein. MoAb90 (IgG1) was purified from ascites fluids by protein A chromatography. YTH862 MoAb (IgG2b) was produced by immunization of rats (DA strain) with consecutive intraperitoneal injections of phytohemagglutinin (PHA)-activated human peripheral blood lymphocyte (PHL) (20 x 10⁶ cells) and fused with the Y3 hybridoma. MoAb90 bound at very high levels (>100-fold above background) to 97% to 100% of PBL from all human donors tested (n > 70). Double-immuno-fluorescent staining further showed that MoAb90 binds to 90% of human peripheral blood T cells, B cells, monocytes, and NK cells. MoAb90 binds to 30 different hematopoietic cell lines but not to Daudi (β2m deficient) and K562 that do not express HLA class I molecules. The HLA class I specificities of MoAb90 was further demonstrated by binding to HLA class I transfected cells as well as immunoprecipitation of a 45-kD HLA class I heavy chain in HLA-A, -B, -C, and -G-transfected murine fibroblasts (data not shown). The YTH862 MoAb reacts with all human cell lines tested except Daudi and precipitates class I and β2m from ³²³H-labeled PHL blast membranes. The anti-HLA class I MoAb, B9.12.1 (anti-HLA-A, -B, and -C, IgG2a), B1.23.2 (anti-HLA-B and -C, IgG2a), and the anti-β2m, B1G6 (IgG2a), were kindly provided by Dr. F. Le Bouteiller. HC10 (anti-HLA-B, IgG2a) was a gift from Prof. H. Ploegh (MIT, Cambridge, MA), TP25.99 (anti-HLA-A, -B, and -C, IgG1) was provided by Dr. R. Buelow (SangStat Medical Corp, Menlo Park, CA) and W6/32 (anti-HLA-A, -B, and -C, IgG2a) was obtained from American Type Culture Collection (ATCC; Rockville, MD). The CD3 MoAb OKT3 was from Ctlag Laboratories (Levallois-Perret, France). The agonist antihuman Fas MoAb CH11 (IgM) was from Immunotech (Marseille, France). The antagonist antihuman Fas MoAb ZB4 (IgG1) was from Kamiya Biomedical Co ( Thousand Oaks, CA). The anti-β2m MoAb BE104 (IgG2a) was prepared by C. Vincent and the anti-CD8 MoAb BL15 and the anti-CD4 MoAb BL4 by J. Broicher in our laboratory. Two irrelevant IgG1 and IgG2a MoAbs were used as isotype controls. The CD45RA (MEM-56) MoAb was from Monosan (Uden, The Netherlands) and the CD45RO (UCHL-1) MoAb was from Immunotech (Marseille, France). \* Immunofluorescence staining. Cells were washed with isotonic phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). For proliferation assay, cells (10⁷/ml) were incubated in 96-well microplates (Costar, Cambridge, MA) coated with the CD3 MoAb OKT3 (5 μg/ml) or in the presence of PHA (5 μg/ml), Con A (1 μg/ml), PWM (1/100), or PE-conjugated anti-Fas (UB2) were from Immunotech. FITC-conjugated anti-CD25, anti-CD69, anti-CD71, anti-CD4, and PE-conjugated anti-CD8 were from Becton Dickinson (Mountain View, CA). Staphylococcus aureus enterotoxin B (SEB), pokeweed mitogen (PWM), PHA, phorbol myristate acetate (PMA), ionomycin, and concanavalin A (Con A) were obtained from Sigma (St Louis, MO).

Cells and Transfectants

PBL. PB was collected from healthy donors in the presence of sodium citrate. Blood was defibrinated, then mononuclear cells were isolated by centrifugation on a layer of histopaque (Sigma). Those cell suspensions referred to as PBL contained 1.8% ± 0.4% monocytes as defined by expression of CD14. In some experiments PBMC (peripheral blood mononuclear cells) were isolated from heparinized blood by centrifugation on histopaque.

For preparation of CD4+ and CD8+ cells, PBL were depleted from CD8+ and CD4+ cells, respectively, by adherence onto plastic surfaces coated with anti-CD4 (BL4) and anti-CD8 (BL15) MoAbs. The percentages of CD8+ and CD4+ cells in those suspensions were 91.4% ± 2.4% and 95.2% ± 2.5%, respectively.

For preparation of CD45RA+ and CD45RO+ cells, PBL were depleted from CD45RO+ and CD45RA+ cells, respectively, by adherence onto plastic surfaces coated with anti-CD45RO (UCHL-1) and anti-CD45RA (MEM-56) MoAbs. The percentages of CD45RA+ cells from CD45RA+ suspension and CD45RO+ cells from CD45RO+ suspension were 93% ± 3.2% and 94.3% ± 5.1%, respectively.

Cell lines. The origin, phenotypic characteristics, and susceptibility to Fas-mediated apoptosis of Burkitt’s lymphoma cell lines (Ramos and Raji), B-lymphoblastoid cell lines (IARC970, Dakili, RPMI8866), and T-cell lines (SupT-1, CEM, Molt-4, Sud HL-1, Jurkat) are listed in a previous report. Six human T-cell lines (three CD4+ and three CD8+) were derived from human tonsils and were provided by J. Banchereau.

CIR transfecants. Transfectants of the HLA-A,-B negative human B-lymphoblastoid cell line CIR that express HLA-A2.1 (CIR-A2) or hybrid mouse/human class I molecules, DAA, ADA, AAD, and ADD were a generous gift from V.H. Engelhard and have been previously described. Briefly, interspecies hybrid class I molecules were created by reciprocal exchange of single exons encoding the three extracellular coding regions (α1, α2, and α3) between the genomic clones of HLA-A2.1 and H-2D+. The hybrid class I genes have three-letter designations, such that the first letter indicates the origin of the promoter, leader, and α1 domains; the second letter indicates the origin of the α2 domain; and the third letter indicates the origin of the α3, transmembrane, and cytoplasmic domains. An “A” represents a domain from HLA-A2.1 and “D” represents contributions from H-2D+.

Culture medium and cell proliferation. PBL were resuspended in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). For proliferation assay, cells (10⁷/ml) were incubated in 96-well microplates (Costar, Cambridge, MA) coated with the CD3 MoAb OKT3 (5 μg/ml) or in the presence of PHA (5 μg/ml), Con A (1 μg/ml), PWM (1/100), or PE-conjugated anti-Fas (UB2) were from Immunotech. FITC-conjugated anti-CD25, anti-CD69, anti-CD71, anti-CD4, and PE-conjugated anti-CD8 were from Becton Dickinson (Mountain View, CA). Staphylococcus aureus enterotoxin B (SEB), pokeweed mitogen (PWM), PHA, phorbol myristate acetate (PMA), ionomycin, and concanavalin A (Con A) were obtained from Sigma (St Louis, MO).
**RESULTS**

**MoAb90 and YTH682 Bind to the α1 Domain of HLA Class I**

Transfected C1R cells that express HLA-A2.1 (C1R-A2) or hybrid mouse/human exon-shuffled HLA-A2.1/H-2D\(^d\) genes were stained with different anti-class I MoAbs and analyzed by flow cytometry. As shown in Table 1, MoAb90, YTH682, and B9.12.1 bound to HLA-A2.1—transfected cells and to C1R-ADA, -AAD, and -ADD, that express hybrid class I major histocompatibility complex (MHC) molecules with the human α1 domain from HLA-A2.1, but not to transfected C1R-DAA cells (α1 domain from H-2D\(^d\) and α2 and α3 domain from HLA-A2.1), suggesting that they recognize a determinant that maps to the α1 domain of the HLA class I heavy chain. These experiments confirmed the binding of TP25.99 and W6/32 to the α3 and the α2 domains, respectively. Competitive cross-blocking studies confirmed these results. The binding of biotinylated MoAb90 was equally inhibited by unlabeled MoAb90 and YTH682, suggesting that both antibodies may recognize identical or overlapping epitopes. Some inhibition was observed with the two anti-β2m MoAbs BE104 and B1G6. The B9.12.1 MoAb (α1 domain) induced borderline inhibition whereas the HC10 (α1/α2 domains), W6/32 (α2), TP25.99 (α3), and B1.23.2 MoAbs did not compete with MoAb90 binding (Table 2). It was concluded that MoAb90 and YTH682 recognize a nonpolymorphic epitope of the HLA class I α1 domain that maps close to β2m and distinct from the epitopes recognized by B9.12.1 and HC10.

Several Anti-HLA Class I Antibodies Inhibit Mitogen-Induced PBL Proliferation

The functional effects of anti-HLA class I MoAbs (at 10 μg/mL) on the DNA synthesis of activated PBL were then tested. Whatever the mitogen used, MoAb90 and to a greater extent YTH682 added at the onset of culture inhibited T-cell proliferation by 46% to 99%, whereas W6/32 and TP25.99 were less efficient (Table 3). However, unlike W6/32 and TP25.99, only MoAb90 and YTH682 also inhibited the T-cell proliferation induced by PMA. The four MoAbs strongly inhibited the allogeneic MLR. Most anti-class I MoAbs known to inhibit T-cell proliferative responses were reported to interfere with early activation events. The expression of the early activation markers CD69, CD25, CD71, and CD95 (Fas/Apo-1) was therefore studied in T-cell suspensions incubated during 24 hours with PHA and various anti-class I MoAbs. W6/32 slightly but significantly inhibited the expression of G1 phase markers whereas MoAb90, YTH682, and TP25.99 did not (data not shown).

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**Table 1. Binding of Anti-HLA Class I MoAb to C1R Cells Expressing Chimeric HLA-A2 1/H-2D Molecules**

<table>
<thead>
<tr>
<th>MoAb</th>
<th>C1R-A2</th>
<th>C1R-DAA(^a)</th>
<th>C1R-ADA</th>
<th>C1R-AAD</th>
<th>C1R-ADD</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>689†</td>
<td>0</td>
<td>651</td>
<td>663</td>
<td>767</td>
</tr>
<tr>
<td>W6/32</td>
<td>319</td>
<td>57</td>
<td>0</td>
<td>286</td>
<td>2</td>
</tr>
<tr>
<td>TP25.99</td>
<td>629</td>
<td>125</td>
<td>585</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>YTH682</td>
<td>535</td>
<td>0</td>
<td>532</td>
<td>552</td>
<td>546</td>
</tr>
<tr>
<td>B9.12.1</td>
<td>448</td>
<td>0</td>
<td>385</td>
<td>382</td>
<td>466</td>
</tr>
<tr>
<td>IgG2aΔ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IgG1†</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) C1R-DAA cells expressed relatively low level of chimeric HLA-A2.1/H-2D molecules compared with other constructs.

† Results expressed as mean fluorescence intensity units after subtraction of background obtained with the untransfected C1R cells.

† IgG1 and IgG2a MoAbs were used as negative controls.

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\(\Delta\) IgG2a MoAb was used as negative control.

\(\ddagger\) Results expressed as mean fluorescence intensity units after subtraction of background obtained with the untransfected C1R cells.

\(\ddagger\) IgG1 and IgG2a MoAbs were used as negative controls.

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\(\alpha\) IgG1 and IgG2a MoAbs were used as negative controls.

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\(\alpha\) IgG1 and IgG2a MoAbs were used as negative controls.

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\(\alpha\) IgG1 and IgG2a MoAbs were used as negative controls.
Methods. Results are expressed as mean ± SD of five independent experiments.

Therefore, the latter antibodies are unlikely to interfere with early T-cell activation events.

The simultaneous addition of PHA and MoAb90 or YTH862 slightly decreased the number of viable cells at 12 hours and no more than 30% of cells seeded in culture were still viable after 96 hours (Fig 1A). In the same conditions, the addition of W6/32 or TP25.99 inhibited the proliferation induced by PHA, but did not significantly decrease viable cell recovery during the first 48 hours of culture. At 72 to 96 hours, viable cell recovery was 65% to 70% of cells seeded in culture (Fig 1A), comparable with that of nonactivated PBL in absence of MoAb.

Addition of MoAb90 or YTH862 to day 3 PHA-activated cells resulted in a rapid decrease of the viable cell recovery during the first 24 hours (Fig 1B) whereas W6/32 or TP25.99 decreased the proliferation induced by PHA but induced only a minimal cell loss (5% to 10%) that could not be detected before 96 hours of culture (Fig 1B). In the same experimental conditions, DNA synthesis was markedly inhibited by MoAb90 and YTH862, and to a lesser extent by B9.12.1 and B1.G6 (Table 2).

**MoAb90 and YTH862 Induce Apoptosis of Activated T Cells**

To study the mechanisms by which MoAb90 and YTH862 decrease viable cell recovery, several parameters of apoptotic cell death were assessed. As shown in Fig 2A, MoAb90 induces double-strand DNA breaks yielding a typical ladder only in PHA-activated but not in resting cells. No DNA fragmentation was observed in cells treated with control IgG1 and W6/32 MoAbs (Fig 2A).

PHA-activated cells treated with MoAb90 for 24 hours (Fig 2D) but not cells treated with control MoAbs (Fig 2B and C) show nuclear fragmentation as typical features of apoptotic cells. No features of apoptotic cells were observed during the first 24 hours (Fig 1B) whereas W6/32 or TP25.99 (Fig 2D) but not cells treated with control MoAbs (Fig 2B) showed nuclear fragmentation as typical features of apoptotic cells.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Specificity</th>
<th>Binding Competition</th>
<th>% Inhibition of Proliferation</th>
<th>% Specific Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>α1 (A, B, C, G)</td>
<td>66</td>
<td>43.4 ± 5.1</td>
<td>38.6 ± 3.5</td>
</tr>
<tr>
<td>YTH862</td>
<td>α1 (A, B, C, G)</td>
<td>65</td>
<td>95.6 ± 3.2</td>
<td>51.5 ± 4.6</td>
</tr>
<tr>
<td>B9.12.1</td>
<td>α1 (A, B, C)</td>
<td>15</td>
<td>21.9 ± 4.0</td>
<td>9.2 ± 2.5</td>
</tr>
<tr>
<td>W6/32</td>
<td>α2 (A, B, C)</td>
<td>0</td>
<td>4.1 ± 4.3</td>
<td>0.9 ± 1.1</td>
</tr>
<tr>
<td>TP25.99</td>
<td>α3 (A, B, C)</td>
<td>0</td>
<td>11.1 ± 3.0</td>
<td>10.9 ± 2.7</td>
</tr>
<tr>
<td>HC10</td>
<td>α1/α2 (B)</td>
<td>0</td>
<td>0.7 ± 3.8</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>B1.23.2</td>
<td>ND (B, C)</td>
<td>0</td>
<td>1.3 ± 2.9</td>
<td>0.5 ± 2.3</td>
</tr>
<tr>
<td>B1.G6</td>
<td>β2m/α1</td>
<td>37</td>
<td>21.0 ± 4.5</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>BE104</td>
<td>β2m</td>
<td>54</td>
<td>6.4 ± 1.2</td>
<td>0.2 ± 0.7</td>
</tr>
<tr>
<td>IgG2a control</td>
<td>ND</td>
<td>0</td>
<td>0.3 ± 2.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IgG1 control</td>
<td>ND</td>
<td>0</td>
<td>0.9 ± 2.5</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* Specificity represents the different domains of HLA class I molecule or β2m and the class I locus product when determined by immunoprecipitation from mouse-transfected fibroblasts.

† Results are expressed as percentage of MFI decrease by addition of biotinylated MoAb90 as described in Materials and Methods.

‡ Cells were cultured during 3 days with PHA (10 μg/mL) and then treated with different MoAbs for 24 hours. Apoptosis was assessed by Hoechst 33342 staining and inhibition of proliferation was determined as the decrease of [3H]ThdR incorporation as described in Materials and Methods. Results are expressed as mean ± SD of five independent experiments.

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**Table 2. Effect of Different Anti–Class I MoAbs on Binding of MoAb90, Proliferation, and Apoptosis of 3-Day PHA-Activated PBL**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Specificity</th>
<th>Binding</th>
<th>% Inhibition</th>
<th>% Specific Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>α1 (A, B, C, G)</td>
<td>66</td>
<td>43.4 ± 5.1</td>
<td>38.6 ± 3.5</td>
</tr>
<tr>
<td>YTH862</td>
<td>α1 (A, B, C, G)</td>
<td>65</td>
<td>95.6 ± 3.2</td>
<td>51.5 ± 4.6</td>
</tr>
<tr>
<td>B9.12.1</td>
<td>α1 (A, B, C)</td>
<td>15</td>
<td>21.9 ± 4.0</td>
<td>9.2 ± 2.5</td>
</tr>
<tr>
<td>W6/32</td>
<td>α2 (A, B, C)</td>
<td>0</td>
<td>4.1 ± 4.3</td>
<td>0.9 ± 1.1</td>
</tr>
<tr>
<td>TP25.99</td>
<td>α3 (A, B, C)</td>
<td>0</td>
<td>11.1 ± 3.0</td>
<td>10.9 ± 2.7</td>
</tr>
<tr>
<td>HC10</td>
<td>α1/α2 (B)</td>
<td>0</td>
<td>0.7 ± 3.8</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>B1.23.2</td>
<td>ND (B, C)</td>
<td>0</td>
<td>1.3 ± 2.9</td>
<td>0.5 ± 2.3</td>
</tr>
<tr>
<td>B1.G6</td>
<td>β2m/α1</td>
<td>37</td>
<td>21.0 ± 4.5</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>BE104</td>
<td>β2m</td>
<td>54</td>
<td>6.4 ± 1.2</td>
<td>0.2 ± 0.7</td>
</tr>
<tr>
<td>IgG2a control</td>
<td>ND</td>
<td>0</td>
<td>0.3 ± 2.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IgG1 control</td>
<td>ND</td>
<td>0</td>
<td>0.9 ± 2.5</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

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**Table 3. Inhibition of T-Cell Proliferation by Anti–HLA Class I MoAbs**

Proliferation was assessed by [3H]ThdR incorporation during the last 12 hours of 72 or 120 hours of culture in the presence of mitogens or allogeneic cells, respectively. Results are expressed as cpm ± SD and are representative of four separate experiments.

Abbreviation: ND, not determined.

* MoAb90, W6/32, TP25.99, and YTH862 at 10 μg/mL and activators were added simultaneously to the cells (2 × 10^5/well).
Transmission electronic microscopy further confirmed that MoAb90-treated cells present typical features of apoptosis with undamaged cytoplasmic membrane, an irregular chromatin condensation, or as a rim inside the nuclear membrane and a disruption of the nucleus into discrete fragments (Fig 2G). Cells treated with control IgG1 or W6/32 did not show nuclear injury (Fig 2E and F). It should be stressed that no necrotic cells were found on examination of a large number of MoAb90-treated cells. YTH862 MoAb that cross-reacts with MoAb90 induced a strong apoptotic effect on PHA-activated PBL (Table 2). Among the other anti-β2m and anti-α-chain MoAbs tested, only B9.12.1 and TP25.99 induced a slight apoptotic effect, which remained minimal as compared with that of MoAb90 or YTH862.

To confirm that only MoAb90 and YTH862 induced apoptosis of activated T lymphocytes, we used two other methods based on different parameters characteristic of apoptosis: externalization of phosphatidylserine and alterations in the mitochondrial transmembrane potential, measured by binding of biotinylated-annexin V and DiOC6(3) fluorescence, respectively. As shown in Fig 3, MoAb90 and YTH862 induced significant binding of annexin V and decrease of mitochondrial transmembrane potential of PHA-activated cells whereas W6/32 and TP25.99 had only minimal effect.

Knowing that MoAb90 and YTH862 induced apoptosis of activated but not resting T cells, we determined their activity on Burkitt’s lymphoma cell lines, lymphoblastoid cell lines, leukemic T-cell lines, and T-cell lines derived from tonsils stimulated with PHA and interleukin-2 (IL-2). No consistent apoptosis could be detected on any of these cell lines.

**Kinetics and Dose-Response of MoAb90-Induced Apoptosis**

The percentage of apoptotic cells measured by binding of biotinylated annexin-V increased from 8 hours to 24 hours...
of exposure to MoAb 90 (Fig 4A). Maximum apoptosis of activated T cells induced by MoAb90 was observed with 5 μg/mL (Fig 4B). Resting T cells were resistant to MoAb90 even at concentrations as high as 50 μg/mL. Interestingly, F(ab′)2 as well as monovalent Fab′ fragments also induced apoptosis of activated PBL. The AC50 (concentration required to induce 50% of maximal apoptosis) of intact IgG and F(ab′)2 was 2.1 μg/mL and that of Fab′ fragments 8.5 μg/mL (Fig 4B). As a possible contamination of Fab′ fragments by F(ab′)2, represents less than 4% (as determined by SDS-PAGE gel electrophoresis, data not shown), we conclude that HLA class I–dependent apoptosis does not require receptor cross-linking. Kinetics and dose-response studies with YTH862 MoAb showed similar results (data not shown).

**HLA Class I–Induced Apoptosis Is Not Dependent on Fas/Fas-Ligand Interaction**

Because the apoptotic activity of MoAb90 and YTH862 was effective only on activated T cells that are known to express Fas, we studied whether HLA class I–induced apoptosis was dependent on interaction of Fas with Fas-Ligand.

**Fig 3. Effect of anti–HLA class I MoAbs on phosphatidylserine externalization and mitochondrial transmembrane potential.** Three-day PHA-activated PBL were treated with control IgG1, MoAb90, YTH862, W6/32, or TP25.99 at 10 μg/mL for 24 hours. Externalization of phosphatidylserine was shown by annexin V-binding and Δψm was evaluated after staining with DiOC6(3) as described in Materials and Methods. The percentage of cells that bind annexin V and that of cells with decreased mitochondrial transmembrane potential is indicated for each histogram. In these conditions, the percentage of apoptotic cells was not modified in resting PBL.
Fig 4. Kinetics and dose-response of MoAb90-induced apoptosis. (A) Resting T cells (open symbols) or 3-day PHA-activated cells (closed symbols) were treated with control IgG1 (circles) or MoAb90 (squares) for indicated time. (B) Resting T cells (open symbols) or 3-day PHA-activated cells (closed symbols) were treated with dose range of intact MoAb90 (squares), F(ab')2 fragments (circles), and Fab' (triangles) for 24 hours. The percentage of apoptotic cells was determined by flow cytometry after staining with annexin V. Results are expressed as mean ± SD of three independent experiments.

ligand (Fas-L). To this end, PHA-activated T cells were preincubated for 1 hour with the antagonist anti-Fas MoAb ZB4 that blocks the interaction between Fas and Fas-L, before addition of either the MoAb90, YTH862, or the agonist anti-Fas MoAb CH11. Although similar levels of apoptosis and proliferation inhibition were induced by anti–HLA class I MoAbs and the agonist CH11 anti-Fas MoAb, only anti–Fas-induced apoptosis was blocked by ZB4 (Fig 5). Analysis by Northern blot further showed that MoAb90 as well as W6/32 did not increase levels of Fas-L mRNA (data not shown).

Differential Susceptibility of CD45RA+ and CD45RO+ T-Cell Subsets to Fas- and HLA Class I–Mediated Apoptosis

Because MoAb90 and YTH862 killed approximately 40% to 50% of cells, their effects on various T-cell subsets were analyzed. PHA-activated CD4+ and CD8+ enriched T-cell populations were found equally sensitive (data not shown). However, results showed that the CD45RO+ enriched T-cell subset activated with anti-CD3 was less sensitive to HLA class I–induced apoptosis (eg, ≈10% of specific apoptosis) than the CD45RA+ subset (eg, ≈70% of specific apoptosis) (Fig 6). Conversely, the CD45RO+ subset was most sensitive to Fas-mediated apoptosis (Fig 6). Further evidence that distinct subsets of activated T cells were targeted by anti-Fas and anti–HLA class I antibodies, respectively, was brought by the additive effects of the two types of MoAbs (Fig 7), and by the observation that susceptibility of T cells to HLA class I–mediated apoptosis is maximal after 2 or 3 days of activation and return to the basal level on day 6, whereas susceptibility to Fas-mediated apoptosis is maximal on day 6 (Fig 8).

DISCUSSION

In agreement with previous reports,15,22,24 we show in the present study that anti–HLA class I antibodies, including W6/32 and TP25.99, which recognize epitopes in the α2 and α3 domains, respectively, inhibited T-cell proliferation induced by various mitogens in the presence of accessory cells, but only MoAb90 and YTH862 inhibited proliferation induced by phorbol esters and triggered apoptosis of activated T cells, irrespective of the type of activation. Both MoAb90 and YTH862 bind to the α1 domain of the HLA class I and recognize closely associated or identical epitopes(s) according to competitive cross-blocking experiments. Because the epitope(s) recognized by these antibodies is(are) expressed on HLA-A, -B, -C, and -G molecules (P. Le Bouteiller, data not shown) it is(there are) quite unlikely to be associated with allele-specific determinants. Furthermore, both antibodies were found to bind to PBL and to induce apoptosis of activated T cells in 70 individuals tested.
Fig 6. Effect of MoAb90 on T-cell subpopulations. CD45RA⁺ and CD45RO⁺ T-cell subpopulations were prepared as described in Materials and Methods. After 3 days of culture in the presence of immobilized-anti-CD3 (OKT3), viable cells from PBL or CD45RA and CD45RO T-cell subsets were treated with MoAb90 (10 μg/mL), YTH862 (10 μg/mL), or anti-Fas MoAb CH11 (1 μg/mL). The percentage of CD45RA, CD45RO, and double-positive CD45RA/RO-positive cells was assessed by fluorescence-activated cell sorting (FACS) analysis, and after 24 hours of incubation with antibodies, apoptosis was evaluated by fluorescence microscopy after Hoescht 33342 staining. Results are expressed as mean ± SD of three independent experiments.

MoAb90 and YTH862 antibodies, as intact Ig but also F(ab)² and Fab' fragments, did not interfere with early activation signals but induced apoptosis of activated T cells, as shown by the initial decrease in viable cell counts, typical nuclear alterations, DNA fragmentation, externalization of phosphatidylserine, and decrease of transmembrane mitochondrial potential. Among nine anti–HLA class I or anti-β2m MoAbs tested, only these two MoAbs induced apoptosis. However, induction of apoptosis of human T cells by anti–HLA class I molecules was very recently reported by two other groups using antibodies directed against either the β2m or the α3 domain of HLA class I molecules. An important finding of the present study is that α1 domain-induced apoptosis does not require cross-linking, as shown by the fully functional effect of monovalent Fab' fragments. This excludes a possible Fc-receptor mediated antibody-dependent cell-cytotoxicity mechanism and, interestingly, suggests that the putative α1 ligand acts in a monovalent fashion. Inhibition of OKT3- or lectin-induced proliferation by...
F(ab′)2 and Fab′ fragments of anti–HLA class I MoAbs was already reported, but with regard to apoptosis, the effect of MoAb90 and YTH862 reported here differ from the effect of 5H7 MoAb and the rabbit anti-β2m antibody which induced efficient apoptosis only when cross-linked. Similarly, the RE2 MoAb, an antimouse MHC class I MoAb, but not its Fab′ fragments, induces a novel type of cell death without DNA fragmentation or swelling of mitochondria of transformed T- and B-cell lines, as well as activated T and B lymphocytes.

Unlike 5H7 MoAb and anti-β2m antibodies, which induced apoptosis of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines, normal PBL, or Jurkat cell line, MoAb90 and YTH862 induced apoptosis of peripheral T lymphocytes while resting lymphocytes, tumor, or T-cell lines are resistant. In parallel, it was shown that naive, memory, and germinal center B cells underwent apoptosis in the presence of MoAb90 or YTH862 only if activated through surface CD40. Because the epitope(s) recognized by MoAb90 and YTH862 is (are) expressed on both resting and activated T cells, it was important to assess at which stage of activation T cells became susceptible to HLA class I–mediated apoptosis. Activated T cells require IL-2 or IL-4 but not transition from G1 to S phase of the cell cycle to undergo apoptosis in the presence of these MoAbs.

The present results are at variance from data reported in the human system where the cross-linking of class I MHC molecule with 5H7 MoAb induces apoptosis in absence of T- or B-cell receptor signaling. However, the identification of early activated T cells (CD45RA+) as the main target of anti–HLA class I MoAb I bears striking similarities with the functional effects of anti–MHC class I MoAb reported in murine systems. Indeed, class I H2-MHC–reactive anti-H2 MoAbs were shown to inhibit murine T-cell proliferation in primary but not secondary allogeneic MLR, suggesting a possible resistance of preactivated memory T cells. In addition, anti-α3 domain MoAbs were shown to induce apoptosis of activated CD4+ and CD8+ precursors while mature effector T cells that had undergone extensive proliferation in vitro were resistant.

MoAb90- and YTH862-induced apoptosis should be clearly distinguished from activation-induced cell death (AICD) because MoAb90 and YTH862 do not trigger T-cell activation and neither Fas-L mRNA and protein expression nor TNF-α, which have been involved in AICD, could be detected in MoAb90- or YTH862-treated cells (data not shown). Moreover, blocking Fas/Fas-L interaction by the antagonist anti-Fas MoAb ZB4 did not interfere with HLA class I–mediated apoptosis. A mere cross-reactivity of MoAb90 and YTH862 with Fas is excluded by the fact that these MoAbs do not bind to the Daudi cell line which expresses high levels of Fas antigen. Furthermore, apoptosis is triggered by monovalent Fab′ fragments whereas Fas-mediated apoptosis requires oligomerization of surface Fas molecules and none of the anti–class I MoAbs induced apoptosis of Fas-sensitive cell lines (data not shown). Finally, the differential susceptibility among activated T cells according to the kinetics and to the expression of CD45RA or RO isoforms provides further evidence that Fas and HLA class I mediate apoptosis in partially overlapping but distinct subsets of target cells. The additive effect of anti–HLA class I antibodies fits with the hypothesis of distinct target cells according to their stage of activation, as does the observation that IL-4 is sufficient to confer susceptibility to apoptosis by anti–HLA class I antibodies but not by anti-Fas antibodies. Because of its restriction toward activated lymphocytes, the α1 domain induced apoptosis of T cells described herein may represent the basis for novel therapeutic interventions where humanized MoAb90 or YTH862, or derived fragments, may be used to induce selective deletion of antigen-specific activated T cells that appear after organ or hematopoietic cell allografts. An advantage of MoAb90 and YTH862 over MoAbs that trigger activated T-cell apoptosis, as do anti-CD3 MoAbs or association of CD2, is the lack of intrinsic T-cell–activating properties that account for severe systemic inflammatory reactions, and their capacity to delete early activated T lymphocytes that are not yet susceptible to Fas-mediated apoptosis.

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Laurent Genestier, Romain Paillot, Nathalie Bonnefoy-Berard, Geneviève Meffre, Monique Flacher, David Fèvre, Yong Jun Liu, Philippe Le Bouteiller, Herman Waldmann, Victor H. Engelhard, Jacques Banchereau and Jean Pierre Revillard