Relationship Between Proliferation and Susceptibility to CD95- and CD2-Mediated Apoptosis in Stimulated Primary T Lymphocytes

T Cells Manifesting Proliferative Unresponsiveness Are Preferentially Susceptible to CD95-Mediated Apoptosis

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We examined the relationship between proliferation and susceptibility to Fas- and CD2-mediated apoptosis of human peripheral T lymphocytes that had been exposed in primary culture to CD3- or CD2-derived mitogenic stimuli in the presence of monocytes and exogenous IL-2. After 5 days, activated T cells were fractionated into large (F2) and small (F6) cells on Percoll density gradients and analyzed for their susceptibility to apoptosis and for their position in the cell cycle. Most F6 cells displayed a CD45RA⁺, CD25⁻, CD2R⁻ phenotype and were unable to incorporate bromodeoxyuridine (BrdUrd) during the entire culture period. However, they were activated to express Fas Ag and some cell cycle regulatory proteins specific to late G1 phase. T cells with proliferative unresponsiveness were sensitive to Fas-mediated apoptosis whether it was triggered by anti-Fas mAb or by Fas ligand, but were almost completely resistant to CD2 apoptotic signaling. In contrast, F2 cells exhibited classical activation markers (CD45RO, CD25, and CD2R), had crossed S phase at least once, and were sensitive to both Fas and CD2 apoptotic signals. In large cells harvested earlier (on day 3), the signals were operative in both BrdUrd⁺ and BrdUrd⁻ cells. Thus, S phase entry is not required for Fas- and CD2-mediated apoptosis. The profound proliferative unresponsiveness of F6 cells to CD3 and CD2 stimuli (bypassed by ionomycin plus PMA) and the CD2R⁻ conformation of their CD2 molecules suggest that they may be in vivo anergized cells whose elimination, upon restimulation, is highly dependent on the Fas death pathway.


Peripheral T cell homeostasis is in large part based on the elimination of expanded Ag-reactive cells at the end of an immunologic reaction. Apoptosis is the physiologic cell death process by which the cells are eliminated. It is characterized by extreme condensation of the chromatin and the cytoplasm, collapse of the nucleus, and, ultimately, cleavage of DNA into internucleosomal fragments (1). Apoptotic signals can be delivered to mature T cells through several membrane receptors, particularly CD3/TCR (2), Fas/Apo-1/CD95 (3, 4), the TNF receptor (5), CTLA-4 (6), and CD2 (7–9). Generally, only activated T cells develop the cellular substrates that are required for transmission of the apoptotic signals (10). Thus, freshly (resting) isolated human peripheral T cells are resistant to Fas-mediated apoptosis, although their CD45RO⁺ components express significant amounts of Fas Ag (11). They can fully up-regulate Fas expression within 24 h of stimulation through the CD3/TCR, but require several additional days of in vitro expansion to become fully susceptible to anti-Fas mAb-mediated apoptosis (12–14). Likewise, ligation of CD2 receptors with mitogenic CD2 mAb pairs causes the apoptotic death of large activated T lymphocytes generated in proliferating T cell cultures, but apparently not in nonproliferating T cells (8, 9). It has been suggested that the cellular events predisposing T lymphocytes to undergo in vitro CD3/TCR-mediated apoptosis (so-called activation-induced cell death) are linked to cell proliferation induced by growth factors (15–17). In this phenomenon, TCR occupancy results in membrane expression and release of the autocrine Fas ligand (Fas-L), which, in turn, binds to Fas receptors and triggers the suicide of activated T cells (18–20). Accordingly, the in vivo primary response of mice to Staphylococcus aureus enterotoxin B involves a strong Vβ8⁺-specific proliferative response, followed by the apoptotic death of the responding T blast cells. Only the Vβ8⁺ T cells that have proliferated are susceptible to undergo apoptosis, whereas those that have not entered the S phase (and have become anergic) escape cell death (21, 22). IL-2 seems to be particularly important not only in predisposing peripheral T cells to CD3/TCR-induced cell death (15, 17, 23), but also in creating the conditions for the execution of Fas-mediated apoptosis. This is deduced from the observation that activated T cells from IL-2-deficient mice (IL-2⁻⁻) display a Fas-resistant

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Received for publication November 21, 1996. Accepted for publication June 24, 1997.

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1 This work was supported by grants from the Association pour la Recherche sur le Cancer (ARC), Sidaaction, Etablissement Français des Greffes, and Université Paris Sud.

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Abbreviations used in this paper: Fas-L, Fas ligand; BrdUrd, bromodeoxyuridine; PE, phycoerythrin; CD6, cyclin-dependent kinase.
Materials and Methods

Cell separation

PBL were isolated from blood bank leukopheresis packs obtained from healthy volunteers of both sexes (through the courtesy of the Blood Transfusion Center from Hôpital Saint Louis, Paris, France). After Ficoll-isopaque density (density = 1.078) gradient centrifugation, adherent cells were removed by incubation on plastic dishes for 30 min at 37°C and the remaining cells were fractionated by filtration over nylon-wool columns. Monocytes were then recovered and mixed with T cells to reach a 1:10 ratio.

Monoclonal Abs and other reagents

Purified CD2 mAb were GT2 (IgG1), T11, (IgG1), and D66 (IgM) from Prof. A. Bernard (INSERM, Unit 343, Nice, France) and CD2.6 (rat IgG2a) from Dr. D. Olive (Laboratoire de Cancérologie et Thérapeutique Expérimentale, Marseille, France). Anti-Fas mAb CH-11 (IgM) and anti-CD3 mAb OKT3 (IgG2a) were purchased from Immunotech and from the American Type Culture Collection (Rockville, MD), respectively. The anti-Fas M3 and M33 mAb protein were obtained from Immunix Research and Development Corp. (Seattle, WA). In some cultures anti-CD28 mAb (248–23–2 from Dr. A. Moretta, Cancer Institute, Genova, Italy), IL-2 (from Biogen, Geneva, Switzerland), IL-4 (from Schering Plough Corp., NJ), and IL-6 (from Dr. Fiers, Laboratory of Molecular Biology, Gent, Belgium) were also added. Alternatively, cultures were performed in the presence of 5 ng/ml of PMA and 200 nM ionomycin (Sigma Chemical Co., St. Louis, MO).

Recombinant Fas-L

The yeast Pichia pastoris strain GS115 (his4) was used as the expression host and was obtained from Invitrogen (San Diego, CA). The murine IL-1R signal sequence was used to direct secretion of the extracellular portion of the molecule starting at position 103. The Fas-L gene was amplified using the 5′ primer (ATATGCTACCCTGGCTACTACAGGCACG) and cloned into pHL-D2 (Invitrogen) modified to contain the IL-1R signal sequence and the FLAG epitope (29) to the N terminus, and the antisense primer (ATATAAGCTTGGATCCTAACATAGCTAATAACACAACAATCA) which introduces a BamHI cloning site. The 5′ primer also contains an Asp7IX cloning site to allow an in-frame fusion to the IL-1R signal sequence. The PCR product was digested with Asp7I and BamHI and cloned into pHL-D2 (Invitrogen) modified to contain the IL-1R sequence under control of the AOX1 promoter. Transformation of yeast, screening for high level expressing transformants, and shaking flask inductions were conducted as previously described (30).

Culture conditions and induction of apoptosis

T lymphocytes (6 × 10⁶) were cultured in the wells of six-well flat-bottom plates (Nunc, Roskilde, Denmark) in 6 ml of RPMI 1640 medium supplemented with 10% human AB serum and antibiotics. Stimulation of the cells was performed using the mitogenic GT2 + T11; CD2 mAb pair (2.5 μg/ml) plus 100 U/ml IL-2 (Roussel UCLAF, Romainville, France) or by using 0.25 μg/ml OKT3 plus IL-2. After fractionation upon Percoll gradients (see below), 5 × 10⁵ cells were distributed into the wells of 96-well round-bottom microtiter plates and exposed for 16 h to the apoptotic stimuli. When the primary stimulation was performed with a CD2 mAb pair, the CD2 apoptotic signal came from a third CD2D (2 μg/ml). When it was performed via CD3, the CD2 apoptotic stimulus was delivered by the T11+D66 or the CD2.6+T11, CD2 mAb pair (2 μg/ml). In both cases, the cells were subjected to anti-Fas Ab (Immunotech: 1 μg/ml).

Percoll gradients

After 5 days of in vitro culture with mitogenic Abs, T cells were fractionated on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Bois d’Arcy, France). The osmolality of the Percoll was adjusted to 285 mosmol/kg H₂O. Six different concentrations of Percoll in PBS/10% FCS were used (37.5, 40, 42.5, 45, 47.5, and 60%). Fractions recovered at each interface from the top to the bottom of the gradients were numbered F1 to F6. The densities of the fractions were calibrated by density marker beads (Pharmacia Fine Chemicals) and were, respectively, 1.033, 1.038, 1.043, 1.051, 1.063, and 1.073 g/ml. After removing, cells were washed and resuspended in culture medium.

Phenotypic analysis

FITC-CD4, PE-CD8, FITC-CD45RO, PE-CD45RA, FITC-CD25, FITC-CD71, and fluorochrome-labeled, isotype-matched control Abs were purchased from Dako (Trappes, France). FITC-Fas Abs were obtained from Immunotech. Cells labeled with CD2.6 mAb were detected using FITC-labeled, affinity-purified rabbit Abs to rat IgG (Jackson ImmunoResearch Laboratories, Bar Harbor, ME). The CD2R epitope was revealed by incubating the cells first with D66, then with a rat IgG anti-mouse IgM (L2MM9 from Immunotech), and finally with a mouse anti-rat κ-chain FITC-labeled mAb (MARK, kindly provided by Dr. H. Bazin, UCL, Brussels, Belgium). Analyses were performed with a FACSscan (Becton Dickinson, Mountain View, CA).

Proliferation assays

Cells were incubated with 1 μCi/well of [3H]ThdR (Amersham, Les Ulis, France), and the amount of radioactivity incorporated was determined after a 6-h pulse.

Flow cytometric analysis of cell death

Cells were stained with propidium iodide (5 μg/ml) 10 min before examination to detect dead cells (FL3 positive) using a FACSscan or a Coulter EPICS Profile II cytometer (Coulter, Hialeah, FL).

Simultaneous measurement of RNA and DNA content

Cells were stained with the metachromatic fluorescent dye acridine orange according to the report of Terada et al. (31). Briefly, to 200 μl of culture medium containing 5 × 10⁵ cells were added 400 μl of a solution containing 0.1% Triton X-100, 0.15 M NaCl, and 0.8 M HCl. After a 1-min incubation on ice, 600 μl of a staining buffer containing 0.56 M citric acid.
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for 5 days with GT2+T11, in the presence of 10% monocytes and 100 U/ml IL-2, then fractionated on a discontinuous Percoll gradient. [3H]Tdr incorporation of 1 x 10^6 cells was assessed on day 5 by a 6-h pulse (representative of two experiments). B, T lymphocytes were stimulated in the presence of 10 μg/ml BrdUrd. On day 5, the cells were fractionated, permeabilized, and stained with anti-BrdUrd FITC mAb for cytofluorometric analysis. Values of the histograms are the mean ± SD of three independent experiments. C, Pooled F2-4 and F6 T cell fractions preactivated and isolated as described in A were restimulated in secondary cultures for 3 days with the indicated stimuli: CD3 and CD28 mAb (1 μg/ml), IL-1-β (5 U/ml), IL-2 (100 U/ml), IL-4 (10 ng/ml), IL-6 (200 U/ml), PMA (5 ng/ml), and ionomycin (200 nM). After 3 days, [3H]Tdr incorporation was assessed following a 6-h pulse. Values are the mean ± SD of triplicate cultures.

FIGURE 1. Proliferation status of large and small T cells isolated after in vitro CD2 stimulation. A, T cells were stimulated for 5 days with GT2+T11, in the presence of 10% monocytes and 100 U/ml IL-2, then fractionated on a discontinuous Percoll gradient. [3H]Tdr incorporation of 1 x 10^6 cells was assessed on day 5 by a 6-h pulse (representative of two experiments). B, T lymphocytes were stimulated in the presence of 10 μg/ml BrdUrd. On day 5, the cells were fractionated, permeabilized, and stained with anti-BrdUrd FITC mAb for cytofluorometric analysis. Values of the histograms are the mean ± SD of three independent experiments. C, Pooled F2-4 and F6 T cell fractions preactivated and isolated as described in A were restimulated in secondary cultures for 3 days with the indicated stimuli: CD3 and CD28 mAb (1 μg/ml), IL-1-β (5 U/ml), IL-2 (100 U/ml), IL-4 (10 ng/ml), IL-6 (200 U/ml), PMA (5 ng/ml), and ionomycin (200 nM). After 3 days, [3H]Tdr incorporation was assessed following a 6-h pulse. Values are the mean ± SD of triplicate cultures.

Double staining for BrdUrd incorporation and DNA content

Cells were incubated from the beginning of culture with 10 μg/ml BrdUrd. Two-color analysis of BrdUrd incorporation and DNA content was performed according to the method of Renno et al. (22). Briefly, cells were fixed in 70% ethanol for at least 30 min, and the DNA was partially denatured in 3 N HCl for 20 min, then neutralized with 0.1 M Na2B4O7, pH 8.5. The cells were subsequently stained with FITC-coupled anti-BrdUrd (Boehringer Mannheim, Meylan, France) in 50 μl of PBS/2% FCS, re-suspended in 300 μl of PBS containing 2.5% FCS, 5 μg/ml propidium iodide, 50 μg/ml RNase A (Sigma Chemical Co.), 50 mM Tris base, 50 mM NaCl, and 5 mM EDTA, pH 7.5. Cells were then incubated for 5 min at 37°C and analyzed on a Coulter EPICS profile II analyzer.

Immunoblot analysis

Detection of G1 and S phase cyclins, Cdk2, and p27kip1 inhibitor was performed using immunoblotting assays. Briefly, 5 x 10^6 cells were lysed in lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 2 mM sodium vanadate, 1 μg/ml aprotinin, 2 mM PMSF, 1 mM DTT, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A; all from Sigma Chemical Co.) on ice for 30 min. Insoluble material was removed by centrifugation at 10,000 x g for 30 min, and the supernatant was assayed for protein (micro-BCA protein assay, Pierce Chemical Co., Rockford, IL). Cell lysates proteins (50 μg) were boiled for 5 min with 2X sample buffer and resolved on 15% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose filters, and the filters were saturated for 2 h at room temperature with 5% nonfat milk in PBS containing 1% Tween-20. Filters were incubated overnight with the 2 μg/ml of mouse anti-cyclin D2 or anti-cyclin E (PharMingen, San Diego, CA), rabbit anti-cyclin D3 or mouse anti-cyclin A and B (Santa-Cruz, Le Perray en Yvelines, France), rabbit anti-Cdk2 (EUROMEDEX, Soufflyeveyrhem, France), or anti-p27kip1 (Transduction Laboratories, Lexington, KY). Blots were stained with either anti-mouse or anti-rabbit horseradish peroxidase-labeled secondary Ab (at a 1/5000 dilution; Amersham) for 1 h at room temperature. They were then developed using an enhanced chemiluminescence detection system (ECL, Amersham). Films were exposed for 1 to 15 min.

Results

A fraction of peripheral T cells manifests proliferative unresponsiveness to multiple cell surface receptor-mediated stimuli

Freshly isolated T lymphocytes were stimulated for 5 days with a CD2 mAb pair (GT2+T11) in the presence of 10% monocytes and 100 U/ml IL-2. The stimulated T cell populations were then fractionated on discontinuous Percoll density gradients to separate the cells according to size. Five different sized fractions of live cells (F2 to F6) were recovered (the low buoyant density F1 fraction was primarily constituted of dead cells). The majority of stimulated cells (-55%) sedimented in the low buoyant density F2 fraction, ~10% were in F3, ~30% of the cells were in F4, ~10% were in F5, and ~5% were in the highest buoyant density fraction F6. The rate of cell division, as measured by [3H]thymidine incorporation, decreased with cell size; F5 and F6 cells exhibited minimal DNA synthesis (Fig. 1A). To examine the possibility that some F6 cells...
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CD45RA, a feature of naive T cells, and that only ~20% of them were single positive for CD45R0, a marker of primed T cells. To gain insight into the activation status of these cells, they were also compared with resting and F2 cells markers. CD45RA/CD45RO double staining of F6 cells (Fig. 2) revealed that they were mostly (~70%) single positive for CD2R, whereas ~8% were positive for CD25 (IL-2Rα), whereas ~90% of F2 cells were positive for this marker. Unlike F2 cells, F6 cells barely expressed the CD2R epitope of CD2 molecules, a hidden epitope that is up-regulated upon T cell activation in vitro (34, 35). Accordingly, the pattern of T11, expression by F6 cells remained very similar to that of resting T cells, contrasting with the enhancement of anti-T11, staining displayed by F2 cells.

**Differential susceptibility to CD2- and Fas-mediated apoptosis of F2 and F6 T cells**

We studied two experimental models that have been previously described to detect CD2-induced apoptosis. In the first system, in vitro priming T cells with a mitogenic CD2 mAb pair (GT2+T11,) and IL-2 predisposes them to receive an apoptotic signal delivered by a third mAb (CD2R,6) directed at a different CD2 epitope (9). Transduction of the apoptotic signal probably results from a conformational change superimposed on CD2 molecules by this three-CD2 mAb setting. In the second system, CD3-activated T cells undergo apoptosis when triggered by a mitogenic CD2 mAb pair (7), namely T11, +CD2,6 mAb. Figure 3 shows that T cells isolated from the pooled F2 to F4 Percoll fractions responded to CD2 apoptotic signaling by increased cell death, whereas F6 T cells and freshly isolated resting T cells remained viable when exposed to the same stimuli. However, unlike resting T cells, a significant proportion of F6 cells generated after CD2 and CD3 stimulation was sensitive to the Fas apoptotic signal delivered by the CH-11 mAb or by a crude yeast supernatant containing recombinant human Fas-L (4). This supernatant was used at a 1/20 dilution because preliminary experiments performed with Fas” Jurkat cells indicated that at this concentration it was devoid of any nonspecific toxicity. The specificity of this assay system was assessed by using soluble anti-Fas M3 Ab, which prevents the interactions between Fas and its ligand (18). A saturating concentration (10 μg/ml) of this Ab abolished the cytotoxic effect of human Fas-L. Control anti-Fas M33, which is unable to kill...
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FIGURE 3. Comparison of the susceptibility of fractionated F2 and F6 T cells to Fas- and CD2-mediated apoptosis. Pooled F2-4 T cell fractions were isolated from T cell populations that were exposed for 5 days to CD2 mAb plus IL-2 as described in Figure 1. F6 cells were isolated from cultures exposed to either CD2 or CD3 mAb in the presence of IL-2. All these cells were subjected for 16 h to anti-CD2 or anti-Fas mAb. Values are the percentage of dead cells detected by flow cytometry after staining with propidium iodide. These results are representative of more than five experiments.

Fas-bearing cells (36) and has no antagonistic or agonistic effect on CD3-mediated apoptosis (18), did not inhibit the killing effect of human Fas-L. Note also that the Fas-L-containing supernatant was not cytotoxic to resting (Fas-negative) T cells.

Thus, cultured F6 cells were predisposed to receive the Fas, but not the CD2 death signal. As expected, most F6 cells (and hence CD45RA+ T cells) expressed the Fas Ag at a low density (Fig. 5), contrasting with resting circulating T cells, whose Fas expression is restricted to a minor CD45RO+ T cell subpopulation (11). The staining pattern of the CD2.6 epitope was identical with that of resting T cells; therefore, this epitope was available to specific mAb involved in apoptosis. The overall staining intensity was, nonetheless, lower than that of F2 cells, in agreement with the high density of CD2 molecules on large activated T cells.

F6 T cells exhibit some cell cycle regulatory proteins specific to late G1

Given that F6 cells had never entered S phase, it is clear that proliferation was not a prerequisite for Fas-mediated apoptosis of primary T cells. To investigate whether F6 cells had at least entered G1, we first measured their RNA and DNA contents simultaneously in comparison with those of resting and activated F2 to F4 T cells. After staining with acridine orange, the RNA and DNA contents of F6 cells were very similar to those of resting T cells that were sequestered in G0 (see Fig. 6A). The RNA and DNA contents of pooled F2 to F4 cells, in contrast, suggested that they were distributed among all stages of the cell cycle. We then used immunoblotting analysis of some cell cycle regulatory proteins whose expression is specifically induced in T cells during G1, namely cyclins D2 (in early G1), D3, and E (in late G1) (37, 38). For comparison, we examined the expression of cyclin A, which is preferentially induced during S and G2. Pooled F2 to F4 cells served as positive controls. As shown in Figure 6B, cyclin D2 was barely detectable in 5-day-stimulated F6 T cells, whereas cyclins D3 and E were easily detected in those cells (respectively, 2.3- and 3.7-fold over the levels detected in unstimulated cells). Neither cyclin A (Fig. 6B) nor cyclin B (not shown) was detected in F6 cells. The low level expression of cyclins D3 and E observed in unstimulated cells (resting T cells) may be due to the small proportion of CD25+ T cells (4%) present in the starting T cell preparations, which probably represents in vivo activated cells. Cdk2 is a kinase that is expressed in late G1 and binds to cyclin E/Cdk2 complexes unless it is subjected to IL-2-mediated elimination (41, 42). These cell cycle markers might, therefore, suggest that despite their low RNA content, F6 cells have passed beyond G0 and have somehow tempted to reach the late phase of G1. The finding of new proteins (and hence mRNA) that the F6 cells synthesize in primary culture, including cell cycle regulatory proteins and Fas molecules, support this idea.
Does CD2-mediated apoptosis require S phase entry?

After a 5-day stimulation period, most of the large F2 cells had entered S phase and were sensitive to CD2 apoptotic signaling, whereas F6 cells were only sensitive to the Fas signal. We, therefore, investigated whether a proliferation period was required for T cells to respond to the CD2 death signal. T cells were stimulated for 3 days only in the presence of BrdUrd, such that ~50% of F2 cells harvested at this time did not react with an anti-BrdUrd mAb (Fig. 7A). If replaced in culture for 2 more days, >80% of these cells became BrdUrd positive (Fig. 7B). This indicates that most BrdUrd-negative cells harvested on day 3 (presumably in G1, given their size) were committed to enter S phase for the first time. The susceptibilities to apoptosis of BrdUrd-negative and BrdUrd-positive F2 cells were compared. After delivery of the apoptotic signal, the cells were stained with anti-BrdUrd mAb to detect S phase passage and with propidium iodide to measure DNA content. Hypoploid cells (<2 N DNA) resulting from CD2 signaling of apoptosis were found in both the BrdUrd-positive and the BrdUrd-negative cell populations. Thus, prior S phase passage is not required for CD2-mediated apoptosis of large activated T cells (as is the case for Fas signaling of apoptosis in those cells).

Discussion

Most studies performed in vitro (15-17, 43) or in S. aureus enterotoxin B-injected mice (22) suggest that CD3/TCR-triggered activation-induced cell death of primary T cells requires a prior S phase passage. However, it is unclear whether this is true for the execution phase of apoptosis via Fas and CD2 receptors. Also, little is known about the relationship between proliferative unresponsiveness of some resting peripheral T cells that are supposedly anergic (26) and susceptibility to cell death. To address these two related issues, human peripheral T cells were activated in primary culture with mitogenic stimuli, then fractionated into large (F2) and small (F6) cells on Percoll density gradients. The susceptibility of the fractionated cells to CD2- and Fas-mediated apoptosis as well as their position in the cell cycle were investigated in this study.

We first defined F6 cells in terms of cell surface activation markers and proliferation capacity. Similar to naive resting T cells, F6 T cells displayed in majority the CD45RA+/CD25- phenotype, and their CD2 molecules were in a CD2R- conformation (34, 35). They had been unable to incorporate BrdUrd at any time during the stimulation period and hence had not entered S phase despite co-stimulation conditions adequate for the majority of surrounding T cells and the presence of exogenous IL-2. However, F6 cells had reacted to CD3 or CD2 ligation as deduced from the steady state expression of some regulatory proteins specific for the late G1 phase. Thus, like T cells synchronized in late G1 (37), F6 cells contained higher levels of cyclins D3 and E than quiescent (G0) T cells. Unlike large F2 cells that had mostly progressed into S phase, they did not contain cyclins A and B. They displayed, in contrast, enhanced expression of p27Kip1, an inhibitory protein that inactivates cyclin E/Cdk2 complexes (44) and hence prevents the cells from transiting across G1/S. The expression of Cdk2 was not higher in F6 cells than that pre-existing in G0 T cells, suggesting that these cells had not been able to respond to the progression signal that is delivered by IL-2 to "competent" cells and that usually results in de novo synthesis of this kinase (41, 45). Possibly, F6 cells had been pushed through the cell cycle to late G1 where they would have been arrested. However, cells in late G1 are generally larger and synthesize more RNA than resting T cells, which was not the case here. Alternatively, the cell cycle regulatory proteins detected in F6 cells might have been synthesized in a noncoordinated manner and represent an abortive attempt to enter the cell cycle.

F6 cells also displayed up-regulated Fas Ag expression, rendering them susceptible to Fas-mediated apoptosis, whether triggered by anti-Fas mAb or by Fas-L. This nonproliferating T cell model, therefore, indicates that prior S phase passage is not required for transmission of the Fas apoptotic signal in primary T cells activated through CD3 or CD2. Therefore, although cell proliferation is a prerequisite for CD3-triggered cell death (which we verified in our system), it is not necessary for Fas-mediated apoptosis.

F6 cells were resistant to CD2-mediated cell death. As they did not display the CD2R (restricted) epitope, we took care to treat them with CD2 mAb directed against other CD2 epitopes and that induced the death of F2 cells. Expression of these epitopes was not lower at the surface of F6 cells than that of resting T cells, and they were therefore available to CD2-specific mAb. There is evidence that the CD2R+ conformation is necessary for CD2 molecules to function (46, 47). The CD2R epitope is a conformational epitope of the CD2 ectodomain that is exposed at the cell surface after T cell activation. CD2 molecules with a CD2R+ conformation form densely packed molecular arrays that have an activation function, whereas CD2 molecules with a CD2R- conformation are isolated and have no signal transduction capacity. CD2R is spontaneously
FIGURE 5. Expression by F2 and F6 cells of Fas Ag and of the CD2.6 epitope involved in CD2 mAb killing. F2 and F6 cells labeled with anti-Fas mAb came from primary T cells incubated with a mitogenic CD2 mAb pair and IL-2. Those labeled with CD2.6 mAb came from primary T cells incubated with OKT3 and IL-2. White histograms represent negative controls, and black histograms represent staining obtained with the relevant Abs.

FIGURE 6. Comparison of simultaneous RNA/DNA staining and G1-cyclin expression in resting, F2, and F6 T cells. A, Cells were analyzed by cytofluorometry for their RNA (green fluorescence) and DNA content (red fluorescence) after staining with acridine orange. One of two similar experiments is shown. Resting T cells represent ex vivo unstimulated T cells. B, Expression of cyclins D2, D3, E, and A; Cdk2; and p27Kip1 inhibitor was examined by immunoblotting in resting cells (a), pooled F2 to F4 cells (b), and F6 cells (c). The experiment illustrated is representative of two others.
FIGURE 7. BrdUrd/propidium analysis of preactivated T cells before and after CD2 and Fas apoptotic signal. A, F2 cells were isolated from a whole T cell population that had been stimulated for 3 days by a mitogenic CD2 mAb pair in the presence of IL-2 and BrdUrd. F2 cells were subjected to CD2 or Fas apoptotic signals, then analyzed by cytofluorometry after double staining with propidium iodide (DNA content, linear scale) and anti-BrdUrd FITC-mAb (number of cells in S phase, log scale). One of three comparable experiments is reported. B, F2 cells, cultivated as described in A, were isolated on day 3, then stained with an anti-BrdUrd FITC-mAb. Part of the fractionated cells was replaced in culture for 2 additional days in the presence of BrdUrd and the initial stimuli (anti-BrdUrd staining on day 5).

induced when the total number of CD2 surface molecules reaches a critical threshold level (46). The CD2R⁺ conformation displayed by CD2 molecules of F6 cells may thus be linked to the failure of these cells to up-regulate surface CD2 copy number, resulting in the incapacity of CD2 molecules to transduce an apoptotic signal into the cells. Note that the CD2R epitope can be lost without concomitant loss of CD2 molecules from the cell surface, as shown for human alloreactive T cell clones made anergic by exposure to specific peptide/MHC ligands in the absence of CD28/B7 interactions (47). In this case, the cells are also refractory to CD2 stimulation. The reversal of specific clonal anergy by IL-2 is linked to the unmasking of CD2R (47). Thus, the absence of CD2R expression in F6 cells might reflect the failure of CD2 molecules to transduce an apoptotic signal into the cells, whereas the transduction capacity of Fas receptors is intact. Alternatively, it is possible that the effector molecules of death involved in the CD2 and Fas apoptotic cascades are different, and that F6 T cells only possess those necessary for Fas-mediated apoptosis. This possibility, which we are currently examining, is consistent with earlier work showing that protein tyrosine kinase activity is required for CD2 (but not for Fas) signaling of apoptosis (25).

In F2 cells, CD2 molecules were in a CD2R⁺ conformation and were able to transduce apoptotic signals. This allowed us to examine whether such signals could be delivered to cells that had never crossed S phase in culture and that were still in G1 (BrdUrd-negative large cells isolated on day 3). Whatever their situation in the cell cycle, the large T cell populations responded to CD2 apoptotic signals, indicating that susceptibility to CD2-mediated apoptosis could also be acquired in G1 before the first entry into S phase (as is the case for Fas-mediated apoptosis).

F6 cells resemble anergized T cells, which by definition are T cells that fail to produce IL-2 and to proliferate autonomously when optimally stimulated through the CD3/TCR and costimulatory receptors and whose proliferative block can be bypassed by phorbol ester and ionomycin (48). Like some anergic T cells, F6 cells were unable to proliferate in the presence of IL-4 (49), and
like human CD4+ T cells were rendered deeply anergic by stimulation with cross-linked CD3 mAb in the presence of IL-10, they expressed a very small amount of IL-2R, thus preventing exogenous IL-2 from reversing their putative anergic state (50). As already mentioned, the absence of CD2 expression is another feature of some anergic T cells (47). It seems unlikely that F6 cells would have acquired proliferative unresponsiveness in culture, in close proximity with neighboring T cells that proliferated vigorously under the same conditions. We favor the possibility that the unresponsive F6 cells recovered after a short stimulation period belong to a circulating pool of in vivo anergized T cells. We suggest that on encountering their specific Ag, these cells will try to enter the G1 phase of the cell cycle, display up-regulated Fas expression, and produce the biochemical substrates necessary for Fas signal transduction. They may then undergo apoptosis upon contact with Fas-L produced by other cells; purified F6 cells were indeed resistant to activation-induced cell death triggered by CD3 mAb, a phenomenon that depends on Fas-L synthesis (2.5 ± 1% cell loss relative to control vs 24.6 ± 6% in F2 cells; mean ± SD of three experiments). This, however, needs to be established, and it would be valuable to examine whether F6 cells produce membrane or soluble Fas-L, and if produced, whether Fas-L is functional, which is not always the case (51). Suda et al. have shown that a high proportion of mouse naive (CD45RA+) splenic T cells, most of which express Fas, are susceptible to Fas-L-mediated cytotoxicity in vitro and that activation through the CD3/TCR induces resistance to this factor (52). Naive human peripheral T cells seem to behave differently, because neither anti-Fas mAb nor recombinant Fas-L were cytotoxic for these cells. Although our F6 cells displayed the CD45RA + phenotype, they also showed several signs of activation. It is therefore conceivable that they did not switch from a CD45RA + to a CD45RO + phenotype during activation.

In conclusion, peripheral T cells that are unresponsive to proliferation signals appear to be sensitive to Fas cross-linking. Resistance to CD2-mediated apoptosis accompanies this functional state. Furthermore, F phase passage does not seem to be required for CD2 apoptotic signaling inactivated T cells exhibiting a CD2-sensitive phenotype.

Acknowledgments

The authors thank Arlette Verdier for her skillful technical assistance with the flow cytometric analyses, and Antoine Attiegh for helpful advice concerning anti-BrdU staining.

References