Monocyte-Dependent Death of Freshly Isolated T Lymphocytes: Induction by Phorbolester and Mitogens and Differential Effects of Catalase

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Resting T cells are resistant to anti-Fas (CD95) mAb-mediated apoptosis but undergo apoptosis when triggered by anti-CD3 mAb or phorbolester PMA in the presence of PMA-activated monocytes. In this study, PMA, as well as the mitogens PHA and Con A, was found to induce death of resting T cells in the presence of autologous or allogeneic monocytes, while PWM was ineffective. Although several established monocyctic and myelocytic cell lines were potent accessory cells for the mitogen-induced expansion of T lymphocytes, they all failed to replace plastic-adherent monocytes in the induction of monocyte-dependent cell death (MDCD) by PMA or PHA. CD45RA-positive cord blood T cells were as susceptible as peripheral blood T cells from adult donors to PMA-stimulated induction of MDCD. Using optimal concentrations of phorbolester, MDCD was inhibited neither by Fas-Fc fusion protein or neutralizing anti-Fas mAb, nor by inhibitors of IL-1β-converting enzyme (ICE)-like proteases. In striking contrast, the H2O2 scavenger catalase completely prevented the PMA-stimulated T cell death, thereby revealing a potent mitogenic activity of PMA for human T cells in the presence of monocytes. Taken together, our results demonstrate that the accessory cell activity of monocytes/macrophages can be separated into “T cell death” and “T cell expansion” costimulatory functions, of which only the latter is mediated by established cell lines. Moreover, our results point to a pivotal role of reactive oxygen intermediates in the execution of MDCD triggered by PMA. The Journal of Immunology, 1998, 161: 1248–1256.

Immature thymocytes and transformed T cells undergo programmed cell death (apoptosis) in response to signaling via the CD3/TCR molecular complex. In many experimental systems, apoptosis is dependent on a functional Fas (CD95)/Fas-ligand (Fas-L)2 interaction (1). Upon CD3/TCR stimulation, Fas-L is rapidly up-regulated. Cell surface expressed Fas-L can interact with Fas on a neighboring cell or, after cleavage by metalloproteases, on the same cell, thereby triggering apoptosis (2–4). Susceptibility to programmed cell death is not limited to thymocytes and malignant T cells but can be similarly initiated in activated mature, nontransformed T lymphocytes. A variety of stimuli including anti-CD3/TCR mAb, anti-CD2 mAb, superantigens and conventional antigens can trigger apoptosis in activated T cells (5). In some of these cases, programmed cell death was shown to depend on Fas/Fas-L interaction (6). In contrast, other apoptosis pathways appear to proceed without involvement of the Fas/Fas-L system (7–9). Although resting peripheral blood T cells express low levels of Fas, they are resistant to anti-Fas mAb-induced apoptosis (10, 11). Upon in vitro activation, T cells rapidly up-regulate Fas expression. Nevertheless, they are refractory to anti-Fas mAb-triggered cell death for several days (11). Similarly, T cells need to be activated and cultured for several days before they become susceptible to superantigen- or anti-CD3/TCR mAb-triggered activation-induced cell death (AICD; Ref. 12). It appears that the initial resistance of freshly isolated T cells to CD95-dependent apoptosis is due to the lack of recruitment of the IL-1β-converting enzyme (ICE)-like protease FLICE (MACH/caspase 8) to the death-inducing signaling complex (13). In addition, the expression of FLICE inhibitory proteins (FLIPs), which interfere with the adapter protein Fas-associated death domain protein, can inhibit the recruitment and activation of the protease FLICE (14).

In striking contrast to their Fas resistance, freshly isolated human peripheral blood T cells are readily induced to undergo apoptosis when cultured for 18 h with PMA-treated monocytes and anti-CD3 mAb or PMA (15). This monocyte-dependent cell death (MDCD) was shown to require cell-cell contact between adherent blood monocytes and T cells and could be prevented by mAb against CD11a, CD18, and CD45RA (15, 16). Moreover, MDCD appeared to involve the Fas/Fas-L system, at least under conditions of suboptimal PMA concentrations during the T cell/macrophage coculture (16).

There is increasing evidence that monocytes/macrophages play an important role in the control of T cell and NK cell apoptosis. In addition to the execution of T cell death in the presence of PMA, monocytes induce apoptosis of NK cells through the release of reactive oxygen intermediates (ROI) (17). Furthermore, when cultured in the presence of M-CSF, monocytes gradually acquire the capacity to induce apoptosis in Ag-reactive T lymphocytes (18). Finally, monocytes are required for the apoptosis of CD4+ T cells induced by CD4 cross-linking, which up-regulates Fas-L expression in monocytes (19).

In this study, we have investigated the capacity of several monocyte/macrophage cell lines to substitute for peripheral blood monocytes in MDCD. We also demonstrate that, in addition to phorbolester PMA, the T cell mitogens PHA and Con A trigger MDCD of freshly isolated T cells. Finally, we show that MDCD triggered...
by optimal concentrations of PMA is completely inhibited by catalase, thus pointing to a central role of ROI in the execution of MDCD.

**Materials and Methods**

**Isolation of lymphocytes and monocytes**

PBMC were isolated from buffy coats obtained from healthy blood donors by Ficoll-Hypaque density gradient centrifugation. After washing twice in PBS, PBMC were resuspended in RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (Biochrom, Berlin, Germany). T cells were separated from PBMC by E rosette formation with neuraminidase-treated sheep erythrocytes and Ficoll-Hypaque centrifugation (20). Monocytes were prepared from nonrosetting (E−) cells by plastic adherence for 1 h at 37°C in RPMI 1640/10% FCS. Nonadherent cells were discarded, and adherent cells were recovered by scrubbing with a rubber policeman. Sheep E were lysed in NH4Cl solution. The E rosetting-forming cells (E+) were used as responder T cells. In some experiments, T cells were purified using magnetic activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). E− cells were stained with a mixture of mAb specific for CD4, CD16, CD20, CD56 (all from PharMingen, Hamburg, Germany) and HLA-DR (L243, American Type Culture Collection, Manassas, VA). After washing steps, the cells were incubated with goat-anti-mouse IgG-labeled micromagnetic particles and passage through a MACS column to deplete NK cells and activated (MHC class II positive) cells, as well as any residual B cells and monocytes/macrophages. These cell populations are referred to as purified T cells. They routinely consisted of >98% CD3+ T cells. CD4+ and CD8+ T cell subsets were isolated from E-rosetted T cells by negative-selection procedures using the MACS technology. In short, E− cells were labeled with mAb against CD16, TCRγδ, HLA-DR, and CD8 (for isolation of CD4+ cells) or mAb against CD16, TCRγδ, HLA-DR, and CD4 (for isolation of CD8+ cells), followed by incubation with goat anti-mouse IgG-labeled micromagnetic particles and passage through MACS columns (21). The resulting cell populations were >98% CD4+ or CD8+, respectively. Cord blood mononuclear cells (CBMC) were prepared fromeparinated cord blood obtained immediately after delivery of healthy newborns. CBMC were subjected to a second Ficoll-Hypaque density gradient, which has been shown to increase the purity of mononuclear cells considerably (22). Separation of T and non-T cells was done as described above.

**Cell lines**

The following monocytic or myelocytic leukemic cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany): Mono-Mac-6, THP-1, U937, HL-60, and KG-1. In addition, we used the erythroleukemic line K562, as well as Burkitt lymphoma cell lines Daudi, BJAB, Ca-46, and EBV-transformed lymphoblastoid cell lines (CLL) established from the peripheral blood of healthy adult donors.

**Cell cultures**

Cell cultures were set up in round-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) with 105 responder T cells (E+, purified T cells, CD4+ or CD8+) and 104 adherent cells (monocytes) or E− cells, or 5 x 105 cells of the various cell lines. In experiments where T cell expansion was also measured after 4 days of culture, the accessory cells were irradiated with a cesium source (2000 rad for monocytes/E− cells, 6000 rad for cell lines). The culture medium was RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated pooled human serum obtained from male donors. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

**Measurement of proliferation**

Cell proliferation was measured by [3H]Thymidine incorporation. Micromulture wells were pulsed for 6 h with 1 μCi [3H]Thymidine per well (sp. act.: 5 Ci/mmol; Amersham, Braunschweig, Germany). Thereafter, wells were harvested onto filter mats, and radioactivity was measured in a surface β-emission scanner (Trace-96, Berthold, Wildbad, Germany).

**Induction of T cell death**

The following stimuli were added at the onset of cultures: phorbol ester PMA (10 ng/ml; Sigma, Deisenhofen, Germany); PHA (PHA-P, 1 μg/ml; Wellcome, Burgwedel, Germany); Con A (8 μg/ml; Pharmacia, Freiburg, Germany); or PWM (1:100; Life Technologies, Eppelheim, Germany). In some experiments, the agonistic anti-CD95/Fas mAb Fas-1 (Becton Dickinson, Heidelberg, Germany) was added at 1 μg/ml. After 18 to 20 h incubation at 37°C, the absolute number of remaining viable T cells per microculture well was determined.

**Measurement of T cell death and T cell expansion**

The extent of T cell death was quantified by measuring the absolute number of viable T cells per microculture well with a recently developed flow cytometry method termed “standard cell dilution assay” (SCDA). The cells of interest (T cells) were stained with phycoerythrin (PE)-conjugated anti-CD3 mAb Leu4 (Becton Dickinson, Heidelberg, Germany). Shortly before flow cytometry (FCM) analysis, a known number of standard cells was added in staining buffer containing 0.2 μg/ml propidium iodide (PI; Serva, Heidelberg, Germany). The standard cells were T lymphocytes that had been labeled with FITC-conjugated anti-HLA class I mAb W6/32 and fixed in 1% paraformaldehyde; therefore, standard cells are FITC+/PI−. From the ratio of viable T cells (PE+/PI−) to standard cells (FITC+/PI−), the absolute number of viable T cells can be easily calculated (24). This method has been successfully applied to the analysis of allotypigen-induced death of human T cells (25, 26). In some experiments, CD4+ and CD8+ cells were simultaneously enumerated by SCDA. To this end, cultured cells were stained with unconjugated anti-CD4 mAb OKT4 plus FITC-conjugated anti-CD8 mAb OKT8, followed by PE-conjugated goat anti-mouse Ig. In these instances, viable CD4+ cells were identified as FITC−/PE−/PI−, whereas viable CD8+ cells were FITC+/PE−/PI+ and standard cells were FITC−/PE−/PI+. All FCM analyses were measured on a FACScan flow cytometer (Becton Dickinson) using the Lysis II software. The same method was used to measure the cellular expansion of T lymphocytes after a culture period of 4 days. Further details of the SCDA method have been published (24).

In addition, the translocation of phosphatidylserine from the inner plasma membrane to the cell surface as an early marker of apoptosis was monitored by staining with FITC-annexin V (27). To this end, the annexin V apoptosis kit (R&D Systems, Wiesbaden, Germany) was used, which combines phosphatidylserine labeling with PI staining.

**Flow cytometry**

The following mAbs were used as FITC or PE conjugates or as biotinylated (b) mAbs followed by PE-coupled streptavidin (Becton Dickinson) as a second-step reagent for phenotypic characterization of T cells, monocytes, and cell lines: Leu5b-FITC (CD2), Leu4–PE (CD3), Leu3a–PE (CD4), Leu2a–FITC (CD8), Leu5m–PE (CD11c), Leu M3–FITC (CD14), Leu11a–FITC (CD16), Leu M9–PE (CD33), Leu18–FITC (CD45RA), CD40–PE (CD50), CD45–PE (CD54), anti-BB1/B7–PE (CD80), HLA-DR–PE (MHC class II) (all from Becton Dickinson), CD13–PE (CD13) (Pharmacia, Uppsala, Sweden), CD86b–PE (CD86) (Ancell, Bayport, MN), and CD95b–CD95 (PharMingen). FITC– or PE-conjugated isotype controls were prepared from nonrosetting T cells. After two washing steps, the samples were resuspended in PBS with 1% paraformaldehyde. All analyses were measured on a FACScan (Becton Dickinson) on the basis of forward and side scatter properties and fluorescence intensity using the Lysis II software.

**Reagents**

Catatalase (EC 1.11.1.6) from bovine liver was from Sigma. Herbimycin A was purchased from Calbiochem (Bad Soden, Germany) and dissolved in DMSO. It was used at 1.5 μM. The inhibitors of ICE-like proteases Ac-YVAD-CKMK and ZVAD-FMK (Bachem, Heidelberg, Germany) were dissolved in DMSO at 100 mM. In all instances, the final concentration of DMSO in cell cultures did not exceed 0.1%. Additionally, the following compounds (all from Sigma) were used: superoxide-dismutase (2–200 U/ml), mannitol (50 μM–50 mM), deferoxamine (10–100 μM), N-acetylcycteine (1–20 mM), and aminophenylcarboxylic acid (ATA; 100 μM–1 mM). Sodium azide was obtained from Serva and was used at 10 μM–40 μM. Manilol, deferoxamine, and sodium azide were dissolved in H2O, whereas ATA was dissolved in 1 M NH4OH. Anti-Fas mAb M3 and M33 (28) and the Fas–Fe fusion protein including the extracellular domain of the Fas molecule were kindly provided by Immunex (Seattle, WA). Cocultures of E− cells and monocytes were preincubated with inhibitors for 60 min at 37°C before PMA or PHA were added. The following recombinant human cytokines were used at 1 to 10 ng/ml: IL-2 (sp. act., 3 x 107 U/mg; Eeur-0Cetus, Frankfurt, Germany), IL-4 (sp. act., 1.8 x 106 U/mg; Immunex), IL-10 (sp. act., 1 x 107 U/mg; Pharmingen), IL-12 (sp. act., 1.5 x 106 U/mg; Pharmingen), IFN-γ (sp. act., 1 x 106 U/mg; PharmBiotech, Hannover, Germany).

**Statistical analysis**

Student’s t test was used to analyze statistical significance of the results.
Results

T cell mitogens PHA and Con A trigger T cell death in freshly isolated PBMC

As reported by Wu et al. (15), freshly isolated T cells undergo apoptosis when stimulated for 18 h with PMA or anti-CD3 mAb in the presence of PMA-treated monocytes. In a first series of experiments, we asked whether T cell mitogens such as PHA, Con A, and PWM also trigger cell death in freshly isolated peripheral blood T cells. We used unfractionated PBMC as responder cells to avoid a possible impact of T cell separation procedures. Death and cellular expansion of T cells were quantified by measuring the absolute number of viable CD3+ cells by SCDA as described in Materials and Methods. We used the mitogens at previously determined optimal mitogenic concentrations (PHA: 1 μg/ml; Con A: 8 μg/ml; PWM: 1:1000), which yielded the typical time course kinetic of [3H]TdR incorporation, with PHA and Con A peaking at day 3 and PWM peaking at day 5 (Fig. 1a). In parallel, the absolute number of viable CD3+ cells was measured by SCDA after 4 days of culture (Fig. 1b). As expected, the mitogens PHA and Con A induced strong T cell expansion, while PWM was less efficient (consistent with the delayed kinetic of [3H]TdR incorporation). Surprisingly, the incubation of PBMC with PHA or Con A for 18 h induced significant T cell death, as evidenced by the reduction of viable CD3+ T cell numbers to 71 ± 18% and 74 ± 13%, respectively, of the medium control (Fig. 1c). While PMA was even more efficient in triggering T cell death (reduction of viable T cells to 55 ± 14% at day 1; n = 3), PWM was ineffective (reduction of viable T cells to 92% ± 6% of medium control; Fig. 1c). Similar experiments, without concomitant measurement of [3H]TdR incorporation, were performed with 17 additional donors, and comparable results were yielded (reduction of viable CD3+ cells in comparison to medium control, measured by SCDA at day 1: PMA, 48 ± 16%, p < 0.001; PHA, 75 ± 9%, p = 0.01; Con A, 73 ± 12%, p = 0.01; PWM, 94 ± 7%, p = 0.1).

The induction of cell death in freshly isolated T cells by PMA is monocyte dependent (15). We compared E-rosetted T cells (E+) with purified T cells (prepared from E+ cells by depletion of CD14+, CD16+, CD20+, CD56+, and HLA-DR+ cells) for their susceptibility to PMA- or PHA-induced cell death. As illustrated in Figure 2 (top), E+ and purified T cells showed negligible cell death in response to PMA treatment in the absence of monocytes. When monocytes were added at a 1:1 ratio, strong cell death was triggered in both responder cell populations (p < 0.001). In contrast, PHA induced significant death in E+ cells in the absence of monocytes (reduction of viable CD3+ cells to 68 ± 14% of the medium control; n = 10; p < 0.001), which was only moderately enhanced when monocytes were added (Fig. 2, bottom). Purified T cells were less susceptible to PHA-induced cell death (reduction of viable CD3+ cells to 82 ± 11% of the medium control; n = 10; p < 0.001), suggesting that contaminating cell populations (i.e., CD16+ or CD56+ NK cells, CD20+ B cells, or HLA-DR+ cells) contribute to the PHA-stimulated execution of CD3+ T cell death within E+ responder cells. In the presence of monocytes, cell death of purified T cells in response to PHA was further enhanced (reduction of viable CD3+ cells to 69 ± 11% of the medium control, when compared with PHA cultures in the absence of monocytes: p = 0.02, Fig. 2, bottom). Although we measured the absolute cell number, the percentage of viable T cells was determined by SCDA. Results are expressed as percentage of viable CD3+ T cells when compared with control cultures in medium only, which were set to 100% (solid bar). Mean ± SD of 10 experiments. The recovery of CD3+ T cells in medium only, which was measured after 18 h, did not result in significant cell loss (<5%) compared with the initial CD3+ T cell input (10^6 cells/well).

FIGURE 1. Mitogen-induced cell proliferation and T cell death. PBMC were cultured with optimal mitogenic concentrations of PHA (1 μg/ml; ■■■), Con A (8 μg/ml; ○○○), PWM (1:1000; □□□), or PMA (10 ng/ml; ▢▢▢). [3H]TdR incorporation was measured from day 1 to day 6 of culture (a) (mean cpm of triplicates of three experiments). In parallel cultures, the absolute number of viable CD3+ cells was measured by SCDA at day 4 (b) and day 1 after set-up of cultures (c) (mean ± SD of three experiments).

FIGURE 2. Influence of T cell purification on MDCD. E rosette-separated cells (E+) and purified T cells (isolated as described in Materials and Methods) were cocultured for 18 h with or without autologous adherent cells (APC) in the presence of PMA (narrow hash marks) or PHA (wide hash marks). The number of viable CD3+ T cells was determined by SCDA. Results are expressed as percentage of viable CD3+ T cells when compared with control cultures in medium only, which were set to 100% (solid bar). Mean ± SD of 10 experiments. The recovery of CD3+ T cells in medium only, which was measured after 18 h, did not result in significant cell loss (<5%) compared with the initial CD3+ T cell input (10^6 cells/well).
number of viable T cells as a read-out for cell death induction throughout this study, alternative methods were applied to investigate early events during the coculture of T cells with monocytes and PMA or PHA. It was observed that T cells undergoing apoptosis in these cultures could be rapidly identified by staining with FITC-conjugated annexin V. As shown in Figure 3, annexin-positive, PI-negative cells were detectable after 2 h, and more significantly, after 8 h. After 24 h, dead cells were annexin<sup>1</sup> and PI<sup>1</sup>, and few early apoptotic cells (annexin<sup>1</sup>/PI<sup>2</sup>) were left.

Monocyte/macrophage cell lines cannot substitute for peripheral blood adherent cells in MDCD

Next we investigated whether monocytes can be replaced by established monocytic/myelocytic cell lines in their accessory function in MDCD. To this end, 10<sup>5</sup> E<sup>+</sup> cells were cocultured with 10<sup>5</sup> irradiated adherent cells or 5 x 10<sup>4</sup> irradiated cell line cells, and viable CD3<sup>+</sup> T cells were enumerated after 1 day (for estimation of cell death) and 4 days (for estimation of CD3<sup>+</sup> cell expansion). We tested myelocytic cell lines (HL-60, KG-1), monocytic cell lines (Mono-Mac-6, THP-1, U937), erythroleukemic K562 cells, Burkitt’s lymphoma (Ca-46, BJAB, Daudi) and EBV-transformed LCL. As reported in Table I, these cell lines varied in their expression of CD11c, CD13, CD14, CD33, CD80, CD86, and MHC class II. CD14 and CD11c were expressed on adherent cells but were not detectable on any of the cell lines. CD54 was expressed neither on monocytes nor on cell lines (not shown). None of the tested cell lines could effectively substitute for peripheral blood adherent cells in the induction of MDCD by PMA or PHA, with the exception of THP-1 which was moderately effective with PMA (Fig. 4a). As illustrated in Figure 4b, however, all cell lines were potent accessory cells for the PHA-stimulated expansion of CD3<sup>+</sup> T cells as measured by SCDA after 4 days, suggesting that cell death-inducing and cell expansion-stimulating activities are separable functions of accessory cells.

Characterization of MDCD-susceptible T cells

CD4<sup>+</sup> and CD8<sup>+</sup> T cells purified by negative selection procedures were separately tested for their susceptibility to PMA- and PHA-triggered MDCD. As shown in Figure 5, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were equally sensitive and did not differ in their susceptibility from unseparated E<sup>+</sup> cells. Again, PMA induced more intense death than PHA in both CD4<sup>+</sup> and CD8<sup>+</sup> subsets. Similar results were obtained when the PHA-triggered MDCD was analyzed in unfiltered PBMC responder cells by simultaneous enumeration of viable CD4<sup>+</sup> and CD8<sup>+</sup> cells in individual microcultures (not shown).

Table I. Surface marker expression on peripheral blood adherent cells and cell lines<sup>a</sup>

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<sup>a</sup> A total of 10<sup>5</sup> adherent cells of three donors and various cell lines were stained with mAb against the indicated surface markers and isotype controls and measured by flow cytometry. The data are given as relative expression, based on median fluorescence intensity (− = < 50, + = 50–300, ++ = 300–1000, +++ = 1000–3000, and ++++ ≥ 3000) as measured on a 4 log scale using the Lysis II software.
Previous studies of Wu et al. indicated that CD45RA is critically involved in MDCD (16). Peripheral blood T cells of adult donors are composed of a mixture of CD45RO\(^{+}\) and CD45RA\(^{+}\) cells. We asked whether MDCD can also be triggered in naive cord blood T cells, which are exclusively CD45RA\(^{+}\). E\(^{+}\) cells were purified from CBMC and PBMC and cultured with PMA and autologous or allogeneic E\(^{+}\) cells. As shown in Figure 6 (top), cord blood T cells readily underwent MDCD when cocultured with autologous or allogeneic cord blood E\(^{+}\) cells and, to an even greater extent, with E\(^{+}\) cells from adult peripheral blood. Similarly to freshly isolated T cells from adult donors, however, cord blood T cells were resistant to anti-Fas mAb-triggered apoptosis (not shown). As illustrated in the lower part of Figure 6, cord blood E\(^{+}\) cells also served to trigger MDCD in T cells from adult donors, but less efficiently than autologous or allogeneic E\(^{+}\) cells from adult donors. Together, these results demonstrate that cord blood T cells are as susceptible to MDCD as T cells from peripheral blood of adults. Cord blood E\(^{+}\) cells, however, are less efficient in triggering MDCD than are E\(^{+}\) cells from adult donors. This is likely to be due to the lower percentage of monocytes among cord blood E\(^{+}\) cells as compared with E\(^{+}\) cells from adults (cord: 30.8 \(\pm\) 14.4% CD14\(^{+}\) cells and 53.4 \(\pm\) 13.9% CD11c\(^{+}\) cells (n = 6) vs adult:

**FIGURE 4.** Accessory cell function of established myelo-monocytic cell lines in MDCD and mitogen-triggered T cell expansion. E\(^{+}\) cells (10^5) were cultured with 10^5 adherent cells or 5 \(\times\) 10^4 cell line cells in the presence of PMA or PHA as indicated. The number of viable CD3\(^{+}\) cells was enumerated by SCDA after 1 (a) and 4 days (b). Results are expressed as percentage of viable CD3\(^{+}\) cells when compared with day 0 medium control. Mean \(\pm\) SD of three experiments (HL-60, day 4, PHA stimulation: 1 experiment).

**FIGURE 5.** CD4\(^{+}\) and CD8\(^{+}\) T cells are equally susceptible to MDCD. Purified CD4\(^{+}\) and CD8\(^{+}\) cells, or unseparated E\(^{+}\) cells, were cocultured with adherent cells and PMA or PHA as indicated. Results are expressed as percentage of viable CD3\(^{+}\) cells when compared with medium control. Mean \(\pm\) SD of five experiments.

**FIGURE 6.** Cord blood T cells are susceptible to MDCD. E\(^{+}\) cells isolated from cord blood (cord) or adult peripheral blood (adults) were cocultured with autologous or allogeneic E\(^{+}\) cells (APC) in the presence of PMA. Viable CD3\(^{+}\) cells were enumerated by SCDA after 18 h. Mean \(\pm\) SD of six (cord blood) and five experiments (adult).
52.1 ± 4.1% CD14\(^+\) cells and 73.6 ± 6.3% CD11c\(^+\) cells (n = 5)).

Inhibition of MDCD

We tested a variety of reagents and cytokines for their effects on MDCD. For these experiments, optimal conditions for the induction of MDCD were applied (i.e., 10\(^5\) E\(^1\) or purified T cells cocultured with 10\(^5\) adherent cells and 10 ng/ml PMA or 1 \(\mu\)g/ml PHA). Under these conditions, none of the tested cytokines at 1 to 10 ng/ml (IL-2, IL-4, IL-10, IL-12, and IFN-\(\gamma\)) had any effect (not shown). Similarly, MDCD was not significantly modulated by peptide inhibitors of ICE-like proteases, Ac-YVAD-CMK and ZVAD-FMK (1–100 \(\mu\)M; not shown). Moreover, MDCD was prevented neither by the antagonistic anti-Fas/CD95 mAb M3 (28) nor by the Fas-Fc fusion protein (1–15 \(\mu\)g/ml; not shown); these reagents partially inhibited the Fas/Fas-L-dependent lysis of Fas\(^+\) Jurkat cells by Fas-L-expressing T cell clones (H.-H. Oberg et al.; unpublished observations). Preincubation of T cells for 1 h with the PKC inhibitor herbimycin A reduced the PMA-stimulated MDCD in a dose-dependent manner, with optimal effects at 1.5 \(\mu\)M (Fig. 7). This effect, however, was statistically not significant. The effect of herbimycin A on the PHA-induced MDCD was even more variable (not shown). Interestingly, the PMA-triggered MDCD was completely abrogated by the \(\text{H}_2\text{O}_2\) scavenger catalase. As shown in Figure 8a, catalase prevented MDCD in the presence of PMA in a dose-dependent manner, with significant inhibition occurring at concentrations of >10 U/ml (\(p < 0.001\)). The prevention of T cell death in PMA-supplemented cultures by catalase had a dramatic effect on the mitogenic activity of PMA. In the absence of a Ca\(^{2+}\) ionophore, PMA is a poor T cell mitogen (Refs. 29 and 30; see also Fig. 1a) As illustrated in Figure 8a, the presence of catalase in cocultures of T cells with monocytes and PMA not only completely prevented MDCD at day 1, but also allowed a strong T cell expansion to take place in these cultures, as measured by SCDA after 4 days.

In contrast to the complete inhibition of PMA-induced MDCD, catalase exerted only moderate and highly variable effects on PHA-triggered MDCD (not shown). Various other inhibitors and modulators of ROI formation, including mannitol, deferoxamine, \(N\) acetylcysteine, sodium azide, superoxide dismutase (all used at concentrations as given in Materials and Methods) did not prevent the PMA-induced MDCD (not shown).

Discussion

In previous studies, Wu and coworkers established that freshly isolated peripheral blood T cells from adult donors readily undergo apoptosis when cocultured for 18 h with PMA and monocytes at a 1:1 ratio (15, 16). They also showed that CD3 cross-linking triggers MDCD in the presence of PMA-treated monocytes (15) and provided evidence for the involvement of CD11a/CD18, CD45RA, and Fas/Fas-L interactions under conditions of suboptimal PMA

![Figure 7](http://www.jimmunol.org/)  
**FIGURE 7.** Inhibition of MDCD by herbimycin A. E\(^1\) cells and adherent cells were preincubated for 60 min with the indicated concentrations of herbimycin A. Thereafter, PMA was added (10 ng/ml), and viable CD3\(^+\) cells were enumerated by SCDA after 18 h. Mean ± SD of eight experiments (\(p = 0.06\) at the concentration of 3 \(\mu\)M, and \(p = 0.05\) at the concentration of 1.5 \(\mu\)M).

![Figure 8](http://www.jimmunol.org/)  
**FIGURE 8.** Effect of catalase on MDCD and cell growth triggered by PMA. E\(^+\) cells were cocultured with adherent cells and PMA in the absence or presence of titrated concentrations (a) or a fixed concentration (1000 U/ml; b) of catalase. Viable CD3\(^+\) cells were measured by SCDA after 18 h (a) or daily over a period of 4 days (b). Mean ± SD of three representative experiments. Eleven additional experiments with E\(^+\) cells and 10 with purified T cells with only two or three concentrations of catalase revealed similar results as in a.
The execution of MDCD is primarily regulated by the number and source of monocytes. Titration experiments performed by Wu et al. (15, 31) and us (not shown) indicate that strongest MDCD occurs at a T cell:monocyte ratio of 1:1. As discussed above, E\(^+\) cells prepared from CBMC were less efficient accessory cells for the induction of MDCD than were adherent cells prepared from PBMC, possibly due to their lower content of monocytes (as defined by staining with CD14 and CD11c mAb). Further studies are required, however, to positively identify the monocyte/macrophage subset among cord blood (and peripheral blood) adherent cells that provides the most potent accessory function in MDCD. As a first approach, we have analyzed the capacity of various established myelo-monocytic cell lines to mediate MDCD. None of the tested cell lines could adequately substitute for peripheral blood adherent cells in the induction of MDCD by PMA or PHA, with the exception of the monocytic cell line THP-1 (32), which was moderately effective with PMA but not with PHA (Fig. 4a). It is interesting to note that MDCD could not be triggered by Mono-Mac-6, which is considered to represent a cell line of mature monocyte phenotype (33). The analyzed cell lines differed in the expression of CD13, CD33, CD86, and HLA-DR, indicating that none of these markers on monocytes/macrophages correlates directly with the ability to mediate MDCD. Strikingly, however, the cell lines failed to express CD11c and CD14, both of which are strongly expressed on peripheral blood adherent cells (Table I). While the cell lines failed to mediate MDCD, they all provided potent costimulation for the PHA-stimulated expansion of CD3\(^+\) T cells as measured after 4 days. Together, these results indicate that the cell death-inducing function and the costimulatory function of a given accessory cell are separable activities, with only the latter being provided by established cell lines of mono-myelocytic origin.

The effector mechanisms of MDCD of freshly isolated T cells have not been identified. A possible candidate is a Fas/Fas-L interaction. Although freshly isolated T cells are resistant to treatment with anti-Fas/CD95 mAb (and remain so for several days after in vitro activation even though Fas expression is rapidly upregulated; Ref. 11), Wu and coworkers obtained evidence for an involvement of the Fas/Fas-L system in MDCD. In their experiments, the neutralizing anti-Fas mAb DX2 and Fas-Fc fusion protein, comprising the extracellular domain of Fas linked to the Fc region of human IgG1, completely inhibited the PMA-triggered MDCD of peripheral blood T cells (16). In striking contrast, we did not observe reproducible inhibitory effects of comparable Fas-blocking reagents, i.e., neutralizing anti-Fas mAb M3 (28) and Fas-Fc fusion protein in our experiments. It should be noted, however, that these reagents only partially inhibited the cell death of Fas\(^+\) Jurkat cells triggered by Fas-L\(^+\) human T cell clones. It should be also noted that different read-outs for cell death were used in the two studies. While Wu and coworkers used a colorimetric method for the determination of metabolic activity (which correlates with the number of viable cells over a certain range), we measured the absolute number of viable CD3\(^+\) T cells following the induction of MDCD. The role of the Fas/Fas-L system in the execution of MDCD is not clear. In the experiments reported by Wu et al., Fas-L was induced in T cells but not in macrophages (16). Macrophages, however, are capable of expressing Fas-L after appropriate activation by, e.g., cross-linking of cell surface CD4 molecules (19) or infection with HIV-1 (34). Furthermore, Kiener et al. have recently shown that human monocytes contain high levels of intracellular Fas-L, which is rapidly released in an active, soluble form upon stimulation of monocytes with PHA, superantigens, or immune complexes (35). Therefore, cell surface expression of Fas-L on macrophages might provide one of several mechanisms by which macrophages can potentially kill other cells through the induction of apoptosis (36). Freshly isolated T cells, however, are resistant to Fas-dependent apoptosis and remain so for several days, even after appropriate in vitro activation (11), due to the lack of caspase 8 recruitment and the expression of caspase 8/FLICE-inhibitory proteins (13, 14). Our results are compatible with the Fas resistance of freshly isolated T cells. Moreover, there are experimental systems where MDCD can proceed clearly without involvement of Fas, e.g. the apoptosis imposed by M-CSF-differentiated macrophages on Ag-reactive T cells (18). However, recent evidence indicates that membrane Fas-L kills peripheral blood T cells (37). While our failure to inhibit MDCD by neutralizing anti-Fas mAb or Fas-Fc fusion protein suggests that the Fas system plays a minor role when using optimal PMA concentrations, we cannot exclude a role of membrane Fas-L when suboptimal PMA concentrations are used.

Cytokines, including IL-2, IL-4, IL-10, IL-12, or IFN-\(\gamma\), did not prevent MDCD in our experiments. Similarly, preincubation of T cells
cells with peptide inhibitors ZVAD-FMK and Ac-YVAD-CMK (1–100 μM) did not significantly inhibit MDCD. Although these peptides are potent inhibitors of anti-Fas mAb-induced liver cell apoptosis in vivo (38), their protective effect on T cell apoptosis is known to depend on the kind of apoptosis-inducing stimulus (39, 40).

While all the above mentioned reagents failed to prevent MDCD, we observed a strong and dose-dependent complete inhibition of T cell death during coculture with monocytes and 10 ng/ml PMA by catalase, a scavenger of H₂O₂ (41). Oxidative stress has been recognized as a potent effector mechanism of apoptosis, mediated by reactive oxygen intermediates (ROI) such as O₂⁻, OH⁻, and H₂O₂ (42). Accordingly, antioxidants prevent apoptosis in various systems (42, 43). ROI have been implicated in Fas- and p53-dependent apoptosis (44–47) and have been identified as monocyte-derived effector molecules of NK cell apoptosis (17). Kono and coworkers (48) have reported that H₂O₂ produced by tumor-derived macrophages, or LPS- or PMA-stimulated monocytes from healthy donors, can induce down-regulation of CD3ζ, CD3ε, and CD16ζ and inhibit tumor-specific CTL and NK cell activity. This inhibitory effect of activated monocytes could be prevented by catalase. Our results show that the non-thiol antioxidant catalase totally inhibits MDCD of freshly isolated T cells under conditions of optimal PMA concentrations. Thiols antioxidants, such as N-acetylcysteine, have been found to completely block the mitogen-induced death of T cell hybridomas (49) and the Fas- and CD2-dependent apoptosis of activated T cells (50). In contrast, N-acetylcysteine did not have a significant effect in our experiments. Similarly, superoxide dismutase (a scavenger of superoxide anion), hydroxyl radical scavenger such as deferoxamine and mannitol, sodium azide (a myeloperoxidase inhibitor), or the endonuclease inhibitor aurantriarcobinolic acid (ATA) did not prevent the PMA- or PHA-triggered MDCD of freshly isolated T cells (not shown). In striking contrast to the effect on PMA-induced MDCD, the PHA-mediated MDCD was not reproducibly prevented by catalase, suggesting that different mechanisms regulate the induction of MDCD by PMA or PHA. Similarly, the moderate MDCD triggered by PMA in the presence of THP-1 cells (Fig. 4a) was not inhibited by catalase. The complete prevention of PMA-induced MDCD by catalase in the presence of peripheral blood adherent cells indicates that H₂O₂ is critically involved in the execution of cell death in this situation. In agreement with the studies of Hansson et al. (17), we observed that exogenous H₂O₂, at concentrations exceeding 10 μM, induced cell death in freshly isolated T cells in the absence of monocytes. The extent of T cell death induced by H₂O₂ was further increased in the presence of PMA and was completely reversed by catalase (not shown). To investigate whether soluble factors (including H₂O₂) were involved in MDCD, we have performed experiments with supernatants of PMA- or PHA-stimulated monocytes cocultured with or without T cells for 1–16 h. Such supernatants, collected every hour for 1 to 8 h, and after 16 h, failed to induce T cell death (not shown). The lack of evidence for the involvement of soluble factor(s) in MDCD is in line with the results of Wu et al., who have shown in double chamber experiments that MDCD requires cell-cell contact or close proximity between monocytes and T cells (15, 31). It is possible that the labile H₂O₂ is effective only for a short period of time and over a short distance. This would explain why cell-cell contact is required and supernatants are ineffective. We have not identified the cellular source of H₂O₂ and are thus unable to comment on whether ROI were generated by monocytes (17) or T cells (46), or both. Interestingly, the prevention of T cell death by catalase in PMA-stimulated cocultures of T cells and monocytes had a dramatic effect on the mitogenicity of PMA (Fig. 8b). Protein kinase C activation by PMA in the absence of Ca²⁺ influx (as induced by ionomycin) is a poor mitogenic signal for human T cells (29, 30), due to the concomitant induction of apoptosis. When catalase was added to inhibit MDCD, strong T cell expansion occurred in cocultures of T cells with monocytes and PMA. These results suggest that the pleiotropic effects (growth inhibition or stimulation) that PMA exerts on different target cells may result from the balance of ROI generation and antioxidative defense mechanisms (51, 52).

To some extent, MDCD was also inhibited by protein tyrosine kinase inhibitor herbimycin A, suggesting that src-related protein tyrosine kinases are involved in the initiation of MDCD. While the precise role of src-related protein kinases in MDCD is unknown, it is interesting to note that herbimycin A inhibited apoptosis of human NK cells triggered by exposure to exogenous H₂O₂ (17). In our experiments, T cell death induced by exogenous H₂O₂ was moderately inhibited by herbimycin A (not shown). Further studies are required to define the consequence of PTK inhibition for MDCD at the molecular level.

In conclusion, our results demonstrate that induction of MDCD of freshly isolated peripheral blood and cord blood cells is a characteristic feature of certain T cell mitogens, as well as of phorbol ester PMA. In the latter situation, the complete inhibition by catalase indicates that H₂O₂ plays a pivotal role in this process.

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