Pyrimethamine (2,4-Diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) Induces Apoptosis of Freshly Isolated Human T Lymphocytes, Bypassing CD95/Fas Molecule but Involving Its Intrinsic Pathway

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ABSTRACT

Pyrimethamine (2,4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine), a folic acid antagonist, may exert, in addition to antiprotozoan effects, immunomodulating activities, including induction of peripheral blood lymphocyte apoptosis. However, the molecular mechanisms underlying this proapoptotic activity remain to be elucidated. Here we show that pyrimethamine, used at a pharmacologically relevant concentration, induced per se apoptosis of activated lymphocytes via the activation of the caspase-8- and caspase-10-dependent cascade and subsequent mitochondrial depolarization. Importantly, this seems to occur independently from CD95/Fas engagement. The proapoptotic activity of pyrimethamine was further confirmed in a patient with autoimmune lymphoproliferative syndrome, an immune disorder associated with a defect of Fas-induced apoptosis. In this patient, pyrimethamine treatment resulted in a “normalization” of lymphocyte apoptosis with a significant amelioration of laboratory parameters. Altogether, these results suggest a mechanism for pyrimethamine-mediated apoptosis that seems to bypass CD95/Fas engagement but fully overlaps CD95/Fas-induced subcellular pathway. On these bases, a reappraisal of the use of pyrimethamine in immune lymphoproliferative disorders characterized by defects in CD95/Fas-mediated apoptosis should be taken into account.

The complex cascade of events triggered by apoptotic signals, including activation of specific caspases, depends on the nature of the activating stimuli (Hengartner, 2000). Two main cell death pathways have been recognized: the “mitochondrial” and the “death-receptor” pathways, involving caspase-9 and caspase-8, respectively (Schmitz et al., 1999; Fulda et al., 2001). However, both pathways converge toward specific mitochondrial activities (Fulda et al., 2001). In particular, according to the more credited theories, it would be the changes in mitochondrial membrane potential (ΔΨ) that would induce the main events of the apoptotic process, among which are the release of cytochrome c (Cyt c), the apoptosisome formation, and finally, the chromatin clumping and DNA fragmentation.

Pyrimethamine (2,4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) belongs to the group of antifolate drugs blocking the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPR) enzymes.

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ABBREVIATIONS: ΔΨ, mitochondrial membrane potential; ALPS, autoimmune lymphoproliferative syndrome; TCR, T cell receptor; FasL, Fas ligand; PBL, peripheral blood lymphocyte(s); HD, healthy donor(s); PBS, phosphate-buffered saline; PHA, phytohemagglutinin; IL, interleukin; DMSO, dimethyl sulfoxide; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; HLA-DR, human leukocyte antigen-DR; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; PMA, phorbol 12-myristate 13-acetate; IFN-γ, interferon-γ; P1, propidium iodide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; ELISA, enzyme-linked immunosorbent assay; Cyt c, cytochrome c; SEM, scanning electron microscopy; FasL, Fas ligand; Z, benzoxycarbonyl; fmk, fluoromethyl ketone; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide.
the enzyme dihydrofolate reductase essential for the synthesis of folic acid, a cofactor required for DNA synthesis. It is used in the treatment of infections caused by protozoan parasites, such as Toxoplasma gondii and Plasmodium falciparum (United States Public Health Service/Infectious Diseases Society of America, 2000; Winstanley, 2001). Accumulating evidence indicate that pyrimethamine can also modulate the immune response by suppressing the proliferation of mitogen- and antigen-stimulated human lymphocytes (Bygbjerg, 1985; Bygbjerg et al., 1986; Viora et al., 1996). In addition, a proapoptotic activity of pyrimethamine has been recently hypothesized by van der Werff ten Bosch et al. (1998, 2002) who reported reversion of autoimmune lymphoproliferative syndrome (ALPS) with pyrimethamine. ALPS is a rare immune disorder appearing in early childhood characterized by lymphadenopathy, splenomegaly, and autoimmune along with expansion of circulating T cell receptor 

Flow-Cytometry Analyses

Lymphocyte Phenotyping and BCL-2 Expression. For surface phenotyping, 500 μl of whole blood were lysed using 10 ml of lysing reagent (Ortho Diagnostics, Raritan, NJ), washed, labeled with appropriate combinations of four mAbs for 30 min at 4°C, and fixed within 1 h from blood collection. Anti-CD3 fluorescein isothiocyanate (FITC), anti-CD45RA FITC, anti-CD38 FITC, anti-CCR5 FITC, anti-CD85 FITC, anti-CCR3 FITC, anti-TCR α/β phycocyanin (PE), anti-CD62L PE, anti-CD4 peridinin chlorophyll protein (PerCP) or allophycocyanin (APC), and anti-CD8 PerCP were purchased from BD Immunocytometry Systems (San Jose, CA). Direct staining with anti-CD8 PerCP mAbs expressing cells analysis, PBL were washed once in PBS containing 2% fetal bovine serum and analyzed on a flow cytometer. Analysis of cytokine production at the single cell level was performed as previously described (Pierdominici et al., 2003). In brief, freshly isolated PBL were incubated with 16 h with 1 μg/ml ionomycin (Sigma-Aldrich) and 25 ng/ml PMA (Sigma) in the presence of 10 μg/ml brefeldin A to inhibit cytokine secretion. After a wash in PBS, cells were fixed with 4% paraformaldehyde by incubation for 5 min at room temperature, permeabilized with fluorescein-activated cell sorter-permeabilizing solution (BD Immunocytometry Systems) for 10 min, washed, and stained. The following cytokine-specific mAbs from BD Immunocytochemistry Systems were used: FITC-labeled anti-interferon-γ (IFN-γ; IgG2b), FITC-labeled anti-IL-2 (IgG1); and PE-labeled anti-IL-4 (IgG1). Surface phenotyping was performed with anti-CD4 APC and anti-CD8 PerCP. For BCL-2-expressing cells analysis, PBL were fixed and permeabilized as described above and stained with an anti-BCL-2 FITC mAb (clone 124; DakoCytomation California Inc., Carpinteria, CA) for 30 min. Surface phenotyping was performed with anti-CD4 APC and anti-CD8 PerCP mAbs.

Apoptosis. Quantitative evaluation of apoptosis was performed by a double-staining flow-cytometry method using FITC-conjugated annexin Vpropidium iodide (PI) apoptosis detection kit (Marine Biological Laboratory, Woods Hole, MA) according to the manufacturer’s protocol. FITC-conjugated annexin V-positive cells were considered as cells in the early stages of apoptosis. Cells distinguished by their ability to take up both FITC-annexin V and PI were considered as cells in the later stages of apoptosis. Live cells were those negatively stained for FITC-annexin V and PI. For selected experiments, electronically gated CD4+ and CD8+ lymphocytes were considered.

Mitochondrial Membrane Potential. ΔΨ was studied by using the lipophilic cationic probe JC-1 (Invitrogen), as described previ-
ously (Cossarizza et al., 1995). In brief, cells were incubated in complete medium for 10 min at room temperature in the dark with 10 μg/ml JC-1 probe. JC-1 was dissolved and stored according to the manufacturer’s instructions. At the end of incubation period, cells were washed twice in ice-cold PBS, resuspended in a total volume of 400 μl, and analyzed on a flow cytometer.

**Detection of Caspase-8, -10, -9, and -3 Activity.** Activation state of the caspases 8, 9, and 3 was evaluated by using the CaspGLOW fluorescent-active caspase-staining kit (Marine Biological Laboratory), whereas that of caspase-10 was evaluated by using the APO LOGIX carboxyfluorescin caspase detection cell kit (Cell Technology, Mountain View, CA). These kits provide sensitive means for detecting activated caspases in living cells and use specific caspase inhibitors (IETD-fmk for caspase-8, AEDV-fmk for caspase-10, LEHD-fmk for caspase 9, and DEVD-fmk for caspase-3) conjugated to FITC as the fluorescent marker.

These inhibitors are cell-permeant, nontoxic, and irreversibly bind to the caspase-active form. The FITC label allows detection of activated caspases in apoptotic cells directly by flow cytometry. Untreated and treated PBL were incubated with FITC-IETD-fmk, FITC-AEDV-fmk, FITC-LEHD-fmk, or FITC-DEVD-fmk for 1 h at 37°C following the manufacturer’s instruction. After this, samples were washed three times and immediately analyzed on a cytometer by using fluorescence-1 channel. Two additional experimental controls were also considered: 1) samples prepared by pretreating cells with specific caspase-8, -10, -9, or -3 inhibitors before pyrimethamine administration and 2) unlabeled PBL (negative control).

**Enzyme-Linked Immunosorbent Assays**

**Cyt c Release.** Cyt c was analyzed in total extracts of activated PBL and cultured in the presence or absence of pyrimethamine using a sensitive and specific commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). In brief, activated PBL were removed after 6, 12, 24, and 48 h of pyrimethamine exposure, washed in ice-cold PBS, lysed, and centrifuged at 1000 g for 15 min. The supernatants were then assayed according to the manufacturer’s instructions. The intensity of the light emitted was quantified by using a microplate reader at 450 nm as the primary wavelength and 490 nm as the secondary wavelength. Cyt c concentration was expressed as nanogram/milliliters. The limit of sensitivity of the assay as supplied by the manufacturer was 0.31 ng/ml.

**Soluble FasL.** Aliquots of PBL supernatants were removed 6, 12, 24, and 48 h after pyrimethamine treatment, and FasL release was analyzed by using a commercially available ELISA kit (R&D Systems) according to the manufacturer’s instructions. The intensity of the color was measured using a microplate reader with 450 nm as the primary wavelength and 570 nm as the reference wavelength. As supplied by the manufacturer, the minimum detectable dose of FasL ranged from 1.01 to 8.05 pg/ml. FasL concentration was expressed as nanogram/milliliter.

**IL-10 Determination.** Serum was removed rapidly and carefully from the red cells after clotting. Levels in serum of human IL-10 were determined using a commercially available ELISA kit (Euroclone, Wetherby, UK) according to the manufacturer’s instructions. The intensity of the color was measured using a microplate reader with 450 nm as the primary wavelength and 570 nm as the reference wavelength. The limit of sensitivity of the assay as supplied by the manufacturer was 5 pg/ml.

**Morphological Studies**

**Static Cytometry Analysis.** To visualize intracellular distribution of BCL-2 protein, PBL were collected and plated on poly-l-lysine-coated slides, fixed for 30 min at room temperature in PBS containing 3.7% paraformaldehyde, and permeabilized for 5 min with PBS containing 0.5% Triton X-100. After washings, cells were incubated at 37°C for 30 min with an anti-BCL-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then incubated with FITC-conjugated anti-rabbit IgG (whole molecule) (Alexa Fluor 488, Invitrogen). Finally, all of the samples were mounted with glycerol-PBS (2:1) and observed by intensified video microscopy by using a Nikon Microphot fluorescence microscope equipped with a color-chilled 3CCD camera (Carl Zeiss GmbH, Jena, Germany). Normalization and background subtraction were performed for each captured image. Figures were obtained by the OPTILAB software (Graftek, Mirmank, France) for image analysis.

**Scanning Electron Microscopy.** PBL were collected and plated on poly-l-lysine-coated slides and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min after different treatments as stated above. After postfixation in 1% OsO4 for 30 min, cells were dehydrated through graded ethanol, with critical point dried in CO2 and gold-coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscopy.

**Patient Clinical Features.** The in vivo proapoptotic effects of pyrimethamine were studied in a 12-year-old female with ALPS. Diagnosis of ALPS was made at the age of 10 years when immunological analysis showed 1) reduced Fas-mediated apoptosis of PHA/IL-2-stimulated T lymphocytes (30%; control values >50%), 2) increased percentage of circulating TCRβ+ CD4+ CD8+ T cells (13%; control values <1%), and 3) high IL-10 serum level (247 pg/ml; control values <10 pg/ml). Genomic DNA was prepared from PBL and screened for Fas mutations using described methods (Rieux-Laucat et al., 1995). Sequence analysis of the tumor necrosis factor receptor superfamily 6 (TNFRSF6) gene revealed no mutation. Clinical patient history showed, by 22 months of age, splenomegaly, neutropenia, thrombocytopenia, hypergammaglobulinemia with increased IgG serum levels, and recurrent urticarial rashes. Results of viral and bacterial serology were consistently negative. In the following years, this patient showed severe episodes of Combs-positive hemolytic anemia. Antinuclear (+ + + + + + + +, homogeneous pattern) as well as IgG anticardiolipin (30 GPL versus ≤10 GPL of controls) antibodies were present. Patient received many courses of steroids with transient effects. Thrombocytopenia and urticarial rash persisted after splenectomy that was performed when the patient was 9 years of age. Pyr treatment was started with an oral dose of one tablet per week.

**Data Analysis and Statistics.** Flow-cytometric analyses were performed by a four-color multiparameter flow cytometer (FACScalibur; BD Immunocytometry Systems) using the CellQuest Pro software. At least, 20,000 events were acquired. To determine marker expression on CD4+ and CD8+ cells, total lymphocytes were first identified and gated by forward and side scatter. The cells were then additionally gated for CD4 or CD8 expression. Appropriate isotypic negative controls were run in parallel. Statistical analysis was performed by Student’s t test using Stat-View program for Windows. All of the apoptosis data reported in this study are the mean ± S.D. of at least five separate experiments.

**Results**

**Pyrimethamine Induces Apoptosis of Human T Lymphocytes.** We first evaluated the dose-dependent effects of pyrimethamine on cell viability in resting or PHA/IL-2-activated PBL obtained from HD (Fig. 1, A–C). In resting lymphocytes, only pyrimethamine concentrations ≥40 μg/ml were capable of inducing significant apoptosis. Lower concentrations, corresponding to those detected in vivo at the steady state (0.4–4 μg/ml) (Weidekamm et al., 1982), did not demonstrate any proapoptotic activity in resting cells (<10%). Conversely, in activated lymphocytes, the proapoptotic effect of pyrimethamine was already remarkable at a concentration of 4 μg/ml (41 ± 5%), reaching values close to 80–90% at higher doses. Control experiments carried out with DMSO...
alone did not display any proapoptotic activity (<10%). In this set of experiments aimed at evaluating the dose-response curve of pyrimethamine, the expression of a key molecule of importance in the inhibition of apoptotic cell death pathway (i.e., the BCL-2 molecule) was also assessed. In fact, pyrimethamine-induced apoptosis was detected along with a down-regulation of BCL-2 expression, both in resting (Fig. 1D) and activated lymphocytes (Fig. 1E). Based on these results, we selected the dose of 4 µg/ml pyrimethamine as an optimal concentration for further studies on activated lymphocytes. To better evaluate pyrimethamine-induced cell death, a time-dependent analysis of apoptosis was also carried out. We cultured PHA/IL-2-activated lymphocytes in the presence of pyrimethamine at different time points (6, 12, 24, 48, and 72 h) (Fig. 2). Depending on the HD examined, the time period in which most of the T cells were annexin V single-positive was variable between 12 and 24 h (data not shown). Thus, we decided to focus our attention to 24-h pyrimethamine treatment. At this time point, lymphocytes from all HD were in fact undergoing apoptosis (annexin V/PI double-positive cells, \( p < 0.05 \) versus untreated cells). This clearly suggests a “fast” apoptotic execution phase in pyrimethamine-treated human lymphocytes. Apoptosis reached a maximum after 48 h (\( p < 0.01 \) versus untreated cells). Percentage of necrotic cells was \(<1\%\) at all of the time points analyzed. Because an active role of the CD95/CD95/FasL system in apoptosis induced by antifolate drugs was previously demonstrated [e.g., methotrexate (Debatin, 1999)], we also evaluated the expression of CD95/Fas and FasL on the cell surface. Moreover, the release of FasL molecule in the growth medium was assessed. As shown in Table 1, as compared with untreated cells, a significant up-regulation of CD95/Fas expression was detected on the surface of activated lymphocytes after 48 h of pyrimethamine treatment (\( p < 0.05 \)). Conversely, FasL expression and secretion remained unchanged at all of the time points studied, with the exception of FasL expression that showed a trend to an increase, although not significant, at 48 h of pyrimethamine treatment.

To exclude a direct involvement of CD95/Fas in pyrimethamine-induced apoptosis, PBL were treated with the ZB4 mAb; i.e., the CD95-neutralizing antibody (Fig. 3, A and B). Interestingly, the percentage of apoptosis in pyrimethamine-treated PBL preincubated with ZB4 remained substantially unchanged. As expected, control experiments carried out with activated PBL exposed to anti-CD95/Fas mAb (clone CH11) showed that ZB4 preincubation was able to significantly reduce Fas-mediated apoptosis. These results seem to suggest that the pyrimethamine-induced apoptosis does not entail the engagement of CD95/Fas molecule.

**The Role of Caspase-8 and Caspase-10.** In consideration of the well known cascade of events occurring intracellularly after apoptotic triggering, further studies were then carried out on caspase cascade; i.e., the main actors of the cell death program (Shi, 2004). Thus, to study the activation of the initiator caspases (caspases 8, 10, and 9) and the effector...
caspase-3, we incubated the activated PBL with pyrimethamine at different time points. Caspase activity was then evaluated by a fluorimetric method (Fig. 4). No significant caspase activity was found at early time points (6 and 12 h). Caspases 8, 10, and 3 were found significantly activated after 24 h of pyrimethamine treatment (Fig. 4, A, B, and D), whereas significant increase of caspase-9 was evident only after 48 h. More interestingly, at 24 h, caspase-8 activity was more pronounced compared with other caspases and statistical analyses clearly indicated a significant difference ($p < 0.01$) between the values obtained with caspase-8 with respect to all other caspases (Fig. 4A).

Next, to establish the involvement of caspase activation in pyrimethamine-mediated apoptosis, we preincubated the activated PBL with selective inhibitors of caspase-8 (Z-IETD-fmk), caspase-10 (Z-AEVD-fmk), caspase-9 (Z-LEHD-fmk), and caspase 3 (Z-DEVD-fmk). Pyrimethamine-induced apoptosis was significantly inhibited by caspase-8, -10, and -3 inhibitors (used at a concentration of 50 $\mu$M, see Materials and Methods) (Fig. 4E). Accordingly, caspase activity was significantly decreased (Fig. 4, A—D). Remarkably, 1) caspase-9 inhibitor (Z-LEHD-fmk) was unable to reduce pyrimethamine-triggered apoptosis (Fig. 4E), and 2) preincubation of activated PBL with the caspase-8 inhibitor z-IETD-fmk abrogated caspase-9 activity after pyrimethamine treatment (Fig. 4G). This suggests that pyrimethamine-induced apoptosis occurred primarily through the caspase-8- and caspase-10-dependent pathway; i.e., via extrinsic pathway.

Because altogether the above reported data were consistent with the hypothesis of a common activation pathway shared by pyrimethamine and CD95/Fas, it was mandatory to determine whether exposure to pyrimethamine could influence Fas-induced apoptosis of activated T cells. To verify this hypothesis, activated lymphocytes were treated with pyrimethamine before incubation with increasing doses of an
The Role of Mitochondria. To deeply characterize the apoptotic pathway triggered by pyrimethamine in lymphocytes, we also focused on the possible role of mitochondria, well known regulators of cell death (Kroemer et al., 1997). In particular, previous studies suggested that hallmarks of apoptosis-associated mitochondrial modification are significant decrease in ΔΨ (Ferri and Kroemer, 2001; Matarrese et al., 2003) and Cyt c release from mitochondria into the cytosol. Therefore, time-dependent changes occurring in ΔΨ and the release of Cyt c were analyzed in pyrimethamine-treated PBL. As shown in Fig. 6A, the increase of cells showing ΔΨ loss, although already detectable after 24 h of pyrimethamine treatment, reached statistical significance compared with untreated cells (p < 0.01) at 48 h. Interestingly, 2 h of pretreatment with caspase-8 and caspase-10 inhibitors was capable of significantly decreasing the percentage of cells showing ΔΨ loss in pyrimethamine-treated PBL (Fig. 6B). As a positive control, we used activated PBL treated with anti-Fas/CD95 but preincubated with caspase inhibitors (Fig. 6B). Parallel analyses carried out on cytosolic Cyt c (released from mitochondria) clearly indicated a significant release (p < 0.01) of this apoptogenic factor starting from 48 h of treatment (Table 2). The above reported results have been summarized in Fig. 7.

Clinical and Laboratory Improvement of ALPS by Pyrimethamine Treatment. To ascertain whether the pro-apoptotic effects seen in in vitro experiments could have a role in the immunoregulatory activity exerted in vivo by pyrimethamine, we studied the CD95/CD5-induced apoptosis and BCL-2 expression in T cells from a representative ALPS patient undergoing pyrimethamine administration.

No toxicity was observed during pyrimethamine treatment. Hematological analyses showed a normalization of platelet count (264,000/μl) within 1 month. IL-10 level significantly decreased but remained high (from 247 to 66.3 pg/ml); IgG level decreased from 3450 to 2030 mg/dl (normal range for age-matched HD, 640-1909 mg/dl). There was no improvement of the recurrent urticarial rashes. Five months later, the therapy was stopped when the patient developed an episode of meningitis due to Streptococcus pneumoniae. During hospitalization, patient had also an acute episode of coxo-femoralis arthritis. During the next 3 months, all treatments were stopped and the patient remained in good clinical condition. Laboratory analyses were within the normal range, with the exception of a platelet count of 41,000/μl.

Morphological scanning electron microscopy analysis of lymphocytes was conducted before and after 1 month of pyrimethamine treatment. This analysis, performed for the first time in an ALPS patient, provides useful information regarding some typical aspects of lymphocyte alteration and injury, such as modifications of microvillous structures. What we found is reported in Fig. 8; typical cell surface thin protrusions clearly visible in PHA/IL-2-activated lymphocytes from HD (A) were altered in the ALPS patient before
pyrimethamine administration (B). The same evaluation carried out after pyrimethamine therapy indicated the presence of lymphocytes with morphology similar to that detected in HD (C). Apoptotic induction by anti-CD95/Fas mAb clearly induced loss of microvillous structures and typical cell surface signs of cell death in HD (D), whereas surface structural features of lymphocytes from the ALPS patient before therapy appeared unchanged (E). Conversely, cells obtained after pyrimethamine treatment, once exposed to anti-CD95/Fas, underwent cell-surface modifications typical of cell death as in HD (F). According to these results, flow-cytometry analysis of activated T lymphocytes showed, before therapy, a significant impairment of Fas-induced apoptosis compared with HD (30 ± 2 versus 49 ± 3%, respectively, \( p < 0.001 \)) (Fig. 8G). This defect was observed in both the CD4\(^+\) (20 ± 2 versus 46 ± 5%, \( p < 0.001 \)) and, to a lesser extent, CD8\(^+\) T cell subpopulations (36 ± 2 versus 53 ± 6%, \( p < 0.01 \)). However, a “normal” CD95/Fas surface up-regulation was demonstrated on PHA/IL-2-activated lymphocytes (median fluorescence intensity, 71 ± 2 in activated cells and 38 ± 1 in untreated cells), suggesting “qualitative” but not “quantitative” defects in the transmission of the death signal delivered to the Fas molecule. Consistent with the decreased rate of anti-CD95/Fas-induced apoptosis, the percentage of activated lymphocytes with BCL-2\(^\text{low}\) expression was lower in the ALPS patient (Fig. 8H) than in HD (34 ± 2 versus 53 ± 5%, \( p < 0.01 \)). This was more evident in CD4\(^+\) (27 ± 2 versus 57 ± 7%, \( p = 0.002 \)) than in CD8\(^+\) T cell subset (33 ± 2 versus 47 ± 2%, \( p < 0.01 \)). After 1 month of pyrimethamine treatment, the percentage of Fas-mediated apoptosis increased, reaching values not significantly different from those detected in HD. Interestingly, this was associated with a normalization of BCL-2 expression (Fig. 8I), as also confirmed by intensified video microscopy analyses (Fig. 8, L and
Fig. 5. Pyrimethamine bolsters Fas-mediated apoptosis. Activated PBL were treated with pyrimethamine before incubation with increasing doses of an agonist anti-CD95/Fas mAb (clone CH11). A, reported values were obtained by considering the difference between the percentage of annexin V-FITC-positive cells found in treated cells with respect to untreated cells. Results are expressed as the means ± S.D. of five independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significance as compared with untreated control cells; + +, $p < 0.01$, significance as compared with cells treated with CH11 alone at the corresponding concentration. B, results obtained from activated PBL of a representative HD are shown. Numbers reported in the bottom and top right quadrants represent the percentages of annexin V single-positive cells and annexin V/PI double-positive cells, respectively. Pyr, pyrimethamine.

Fig. 6. Flow-cytometry analysis of $\Delta \Psi$ performed by using JC-1 probe. A, time-course analysis of the modulation of mitochondrial membrane potential by pyrimethamine. Results are expressed as mean ± S.D. of 10 independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significance as compared with untreated control cells. B, changes in $\Delta \Psi$ induced by anti-CD95/Fas mAb (CH11; 0.5 µg/ml), pyrimethamine, caspase-8 inhibitor (z-IETD-fmk), caspase-10 inhibitor (z-AEVD-fmk) alone, or in various combinations. Caspase inhibitors were used at a concentration of 50 µM. Results are expressed as mean ± S.D. of five independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significance as compared with untreated control cells. +, $p < 0.05$ and ++, $p < 0.01$, significance as compared with cells treated with pyrimethamine or CH11 alone. Pyr, pyrimethamine.
Time-course analysis of the release of Cyt c

Cyt c was analyzed in total extracts of activated PBL using a sensitive and specific commercial ELISA kit. Results are expressed as the means ± S.D. of five independent experiments. Cyt c concentration is expressed as nanogram/milliliter.

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<th>Time (h)</th>
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<tr>
<td>0</td>
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<td>3.6 ± 1</td>
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<tr>
<td>48</td>
<td>4.9 ± 0.9</td>
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**P < 0.01 compared with untreated cells.

![Diagram](Fig. 7. Scheme illustrating the possible pathways involved in pyrimethamine-induced apoptosis in activated PBL. In this pathway, pyrimethamine-induced apoptosis directly involves as initiator caspases, caspases 8 and 10, without engaging CD95/Fas. This is followed by the typical executioner cascade; i.e., mitochondrial changes and downstream events. An additional activity of pyrimethamine on mitochondria can not be ruled out. (i), a decreased expression; (ii), an inhibitory activity. Pyr, pyrimethamine.)

Discussion

In the present work, we characterized the apoptotic pathway triggered by pyrimethamine in human lymphocytes. Parallel analyses carried out in T cells from HD and a patient with ALPS depicted the same scenario; pyrimethamine was able to induce CD95/Fas downstream caspase cascade.

Activated lymphocytes are known to be vulnerable to the “physiological” signal represented by triggering of CD95/Fas with the consequent activation of caspases 8 and 10. Our data indicate that, similarly, pyrimethamine induced apoptosis of activated T cells via a mechanism bringing into play the upstream caspases 8 and 10, although it did not require CD95/Fas engagement, as demonstrated by experiments performed with a specific mAb-blocking CD95/Fas. According to this, pyrimethamine exerted its proapoptotic activity by sharing the same signaling cascade of CD95/Fas molecule and, when used in combination to a triggering anti-CD95/Fas mAb, it was able to exert a significant additive activity on Fas-mediated apoptosis.

Although our results suggest that the primary target for pyrimethamine is represented by caspase-8-driven cascade, we cannot rule out the possibility that pyrimethamine may also directly act on mitochondria, thus contributing to the powerful proapoptotic activity of the drug. Similar to other antifolate compounds, pyrimethamine led to mitochondrial membrane depolarization, a late event in the mitochondrial-driven apoptosis cascade (Kroemer et al., 1997). To this regard, it is interesting to consider that the expression of BCL-2, a regulator of mitochondrial proapoptotic activity, was down-regulated by pyrimethamine. In addition, pre-exposure to antioxidizing drugs, such as the N-acetyl-cysteine, was capable of partially protecting lymphocytes from both the loss of ΔΨ and apoptosis (unpublished observations).

Thus, our findings seem to indicate that mitochondria can not be ruled out the possibility that pyrimethamine may also directly act on mitochondria, thus contributing to the powerful proapoptotic activity of the drug. Similar to other antifolate compounds, pyrimethamine led to mitochondrial membrane depolarization, a late event in the mitochondrial-driven apoptosis cascade (Kroemer et al., 1997). To this regard, it is interesting to consider that the expression of BCL-2, a regulator of mitochondrial proapoptotic activity, was down-regulated by pyrimethamine. In addition, pre-exposure to antioxidizing drugs, such as the N-acetyl-cysteine, was capable of partially protecting lymphocytes from both the loss of ΔΨ and apoptosis (unpublished observations).

Thus, our findings seem to indicate that mitochondria can not be ruled out the possibility that pyrimethamine may also directly act on mitochondria, thus contributing to the powerful proapoptotic activity of the drug. Similar to other antifolate compounds, pyrimethamine led to mitochondrial membrane depolarization, a late event in the mitochondrial-driven apoptosis cascade (Kroemer et al., 1997). To this regard, it is interesting to consider that the expression of BCL-2, a regulator of mitochondrial proapoptotic activity, was down-regulated by pyrimethamine. In addition, pre-exposure to antioxidizing drugs, such as the N-acetyl-cysteine, was capable of partially protecting lymphocytes from both the loss of ΔΨ and apoptosis (unpublished observations).
taneously implicated. However, based on the results obtained evaluating annexin V positivity and PI permeability (i.e., a rapid execution phase with the occurrence of double-positive cells), the possibility of concomitant alternative forms of cell death (e.g., oncosis) can not be ruled out (Krysko et al., 2004; Scholz et al., 2005).

Based on these in vitro results, we also shepherded through an in vivo situation to achieve further information regarding the mechanisms responsible for the proapoptotic activity exerted by pyrimethamine. To this aim, an ALPS patient, before and after pyrimethamine treatment, was studied. This patient had no detectable Fas mutations but presented a defective Fas-induced apoptosis. A weekly dose of pyrimethamine led within 1 month of treatment to a significant increase of Fas-induced apoptosis of both CD4<sup>+</sup>- and CD8<sup>+</sup>-activated T cells in association with a normalization of BCL-2 expression. According to these data, a significant reduction in lymphocyte counts was also detected. Our results are partially at variance with those reported by van der Werff Ten Bosch et al. (2002) who observed in ALPS patients under pyrimethamine treatment a reversion of lymphoproliferative signs but no changes in Fas-induced apoptosis of T lymphocytes. This may be partially explained by the probable diverseness of genetic defects responsible for the ALPS phenotype in the patients studied. In fact, two out of seven patients described by van der Werff Ten Bosch et al. (2002) had Fas mutations, whereas the remaining five patients, as well as the ALPS patient presented in this report, had uncharacterized genetic defects in the apoptotic pathway. In addition, different experimental procedures could also be taken into account to explain such discrepancy.

Different factors may contribute to the normalization of Fas-induced apoptosis in the ALPS patient studied here. Our results are consistent with the above-described proapoptotic effects of pyrimethamine bypassing the requirement of upstream caspase-8 and caspase-10 molecules eventually defective in our ALPS patient (i.e., membrane/cytoskeleton interaction molecules or molecules involving in the death-inducing signaling complex formation). Pyrimethamine could also normalize Fas-induced apoptosis through a down-regulation in BCL-2 intracellular content. neg, FITC-conjugated isotype control.
patients as in other ALPS patients (Lopatin et al., 2001; van der Werf-Ten Bosch et al., 2002). Finally, we can not rule out the possibility that an increase in FasL expression, as detected in vitro in pyrimethamine-treated lymphocytes, may contribute to the pyrimethamine-induced apoptosis in vivo after a prolonged exposure to the drug.

From a clinical point of view, our findings elicit some noticeable considerations. The intense proapoptotic effect exerted by pyrimethamine, in addition to its well known anti-proliferative activity (Bygbjerg, 1985; Bygbjerg et al., 1986; Viora et al., 1996), might possibly result in a consistent suppression of the immune responses. Therefore, treatment with pyrimethamine should be carefully considered in patients suffering from immune defects in which a worsening of clinical conditions could be determined. To this regard, it has been previously observed that human immunodeficiency virus type 1-infected patients treated with pyrimethamine as a primary prophylaxis against toxoplasmic encephalitis showed a higher mortality rate in comparison with untreated control patients (Jacobson et al., 1994).

In contrast, many diseases sharing similar immunopathogenic mechanisms with ALPS exist in which induction of apoptosis could be beneficial. We refer in particular to autoimmune diseases as well as genetic or infectious-induced immune disorders characterized by an increased and chronic lymphocyte proliferation. In all of these conditions, the immunosuppressive effect of pyrimethamine could help in reversing the unbalanced lymphocyte homeostasis, thus leading to an amelioration of clinical status. Furthermore, on these bases, a reappraisal of the use of pyrimethamine in certain lymphoproliferative disorders can be taken into account in the long run.

References

TABLE 3
Cytofluorimetric analysis of CD4+ and CD8+ T cell subsets from an ALPS patient before and after pyrimethamine therapy
Percentage and absolute numbers in brackets of CD3+CD4+ and CD3+CD8+ T cell subsets from HD and the ALPS patient are shown. Data from HD are expressed as mean values ± S.D. ALPS patient was studied before (t0) and after 1 month (t1) of therapy. CD45RA CD62L+ were defined as naive cells, CD45RA CD62L− were identified as central memory cells, CD45RA CD62L+ were identified as effector memory cells, and CD45RA CD62L+ were identified as terminally effector memory cells.

<table>
<thead>
<tr>
<th>Cell Subsets</th>
<th>HD</th>
<th>t0</th>
<th>t1</th>
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<tbody>
<tr>
<td>CD4+</td>
<td>39 ± 6 (942 ± 140)</td>
<td>34 (1472)</td>
<td>32 (1135)</td>
</tr>
<tr>
<td>CD4+ /CD45RA CD62L+</td>
<td>64 ± 6 (588 ± 117)</td>
<td>70 (1039)</td>
<td>60 (681)</td>
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<tr>
<td>CD4+ /CD45RA CD62L−</td>
<td>29 ± 7 (270 ± 67)</td>
<td>21 (599)</td>
<td>25 (284)</td>
</tr>
<tr>
<td>CD4+ /CD45RA CD62L−</td>
<td>1 ± 0 (± 3)</td>
<td>3 (44)</td>
<td>3 (34)</td>
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<tr>
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<td>7 ± 2 (65 ± 23)</td>
<td>6 (88)</td>
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<tr>
<td>CD4+ /CD25</td>
<td>21 ± 3 (260 ± 70)</td>
<td>19 (279)</td>
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</tr>
<tr>
<td>CD4+ /CD95</td>
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<td>39 (443)</td>
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<tr>
<td>CD4+ /CCR5</td>
<td>9 ± 4 (99 ± 26)</td>
<td>10 (147)</td>
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</tr>
<tr>
<td>CD4+ /HLA-DR+</td>
<td>5 ± 2 (52 ± 10)</td>
<td>12 (177)</td>
<td>14 (159)</td>
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<td>CD4+ /IFN-γ−</td>
<td>8 ± 4 (82 ± 54)</td>
<td>9 (132)</td>
<td>5 (57)</td>
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<td>CD4+ /IL-2−</td>
<td>59 ± 14 (757 ± 271)</td>
<td>50 (736)</td>
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<td>CD8+ /CD45RA CD62L+</td>
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<td>8 (68)</td>
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<td>16 (136)</td>
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