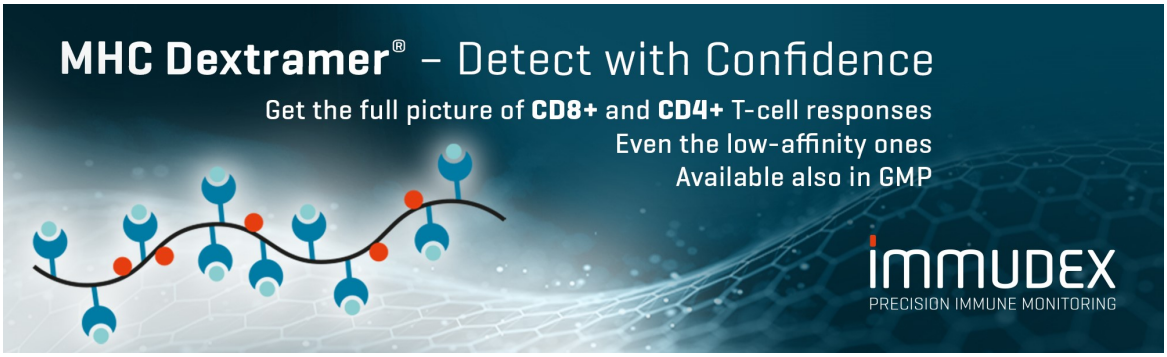

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Differential Control of Cell Cycle, Proliferation, and Survival of Primary T Lymphocytes by Purine and Pyrimidine Nucleotides[1]

Laurence Quéméneur,* Luc-Marie Gerland,† Monique Flacher,* Martine Ffrench,† Jean-Pierre Revillard,* and Laurent Genestier[2]*

Purine and pyrimidine nucleotides play critical roles in DNA and RNA synthesis as well as in membrane lipid biosynthesis and protein glycosylation. They are necessary for the development and survival of mature T lymphocytes. Activation of T lymphocytes is associated with an increase of purine and pyrimidine pools. However, the question of how purine vs pyrimidine nucleotides regulate proliferation, cell cycle, and survival of primary T lymphocytes following activation has not yet been specifically addressed. This was investigated in the present study by using well-known purine (mycophenolic acid, 6-mercaptopurine) and pyrimidine (methotrexate, 5-fluorouracil) inhibitors, which are used in neoplastic diseases or as immunosuppressive agents. The effect of these inhibitors was analyzed according to their time of addition with respect to the initiation of mitogenic activation. We showed that synthesis of both purine and pyrimidine nucleotides is required for T cell proliferation. However, purine and pyrimidine nucleotides differentially regulate the cell cycle since purines control both G_1 to S phase transition and progression through the S phase, whereas pyrimidines only control progression from early to intermediate S phase. Furthermore, inhibition of pyrimidine synthesis induces apoptosis whatever the time of inhibitor addition whereas inhibition of purine nucleotides induces apoptosis only when applied to already cycling T cells, suggesting that both purine and pyrimidine nucleotides are required for survival of cells committed into S phase. These findings reveal a hitherto unknown role of purine and pyrimidine de novo synthesis in regulating cell cycle progression and maintaining survival of activated T lymphocytes. *The Journal of Immunology,* **2003, 170: 4986–4995.**

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Triggering of the Ag-specific TCR initiates signaling cascades that induce T cell activation and proliferation. Additional costimulatory signals that are provided by other cell surface receptors are necessary for naive T cells to produce IL-2 and progress through the cell cycle (1). Failure to produce these costimulatory signals leads to Ag-specific unresponsiveness or apoptosis instead of proliferation (2). Activation of T lymphocytes with the mitogenic lectin PHA is also associated with a de novo synthesis of purine and pyrimidine which leads to a 2-fold purine and up to an 8-fold pyrimidine pool expansion, respectively, over 72 h (3). However, the precise role of these increases by de novo synthesis on T cell proliferation and survival remains elusive.

The expansion of purine and pyrimidine pools is the consequence of a marked increase in expression or activity of key enzymes involved in the de novo purine and pyrimidine synthesis pathways. In particular, two enzymes, the inosine monophosphate dehydrogenase (IMPDH)[3] which catalyzes the first step in the formation of guanine ribonucleotides from inosine monophosphate and the thymidylate synthase (TS) which catalyzes the synthesis of deoxy-thymidine 5′-monophosphate from deoxy-uridine 5′-monophosphate, both increase 10-fold 48 h after activation of T lymphocytes (4, 5). Mice heterozygous for loss of the IMPDH type II demonstrate a significant decrease of lymphocyte responsiveness to stimulation with anti-CD3 and anti-CD28 Abs, suggesting that guanine nucleotides synthesized by IMPDH type II are necessary for the proliferation of T lymphocytes (6). One may indeed speculate that clonal expansion of naive T cells depends on the activation of gene transcription involved in the stimulation of de novo purine and pyrimidine synthesis pathways (3) to provide the additional nucleotide precursors necessary for DNA synthesis. But purine and pyrimidine nucleotides also play critical roles in membrane lipid biosynthesis, protein glycosylation, RNA synthesis, and they act as the phosphate donor for phosphorylation reactions (7). These highly pleiotropic biochemical activities do not permit one to predict precisely the consequences of the de novo nucleotide synthesis pathway blockade on T cell activation, progression through the cell cycle, and survival.

Previous studies (8–10) have provided in-depth analyses of the cytostatic effect of IMPDH type II blockade, but the comparison of

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[3] Abbreviations used in this paper: IMPDH, inosine monophosphate dehydrogenase; TS, thymidylate synthase; MPA, mycophenolic acid; 6 MP, 6-mercaptopurine; MTX, methotrexate; 5FU, 5-fluorouracil; BrdU, bromodeoxyuridine; PI, propidium iodide; CDK, cyclin-dependent kinase.

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purine vs pyrimidine nucleotide depletion on primary T cell proliferation and survival has not been documented. This was investigated in the present study, by using specific nucleotide synthesis inhibitors. Because several of these inhibitors are currently used as immunosuppressive agents (11) it was anticipated that closer insight into their cytostatic/cytotoxic activities on activated primary human T cells could provide a better rationale for their clinical use. Mycophenolic acid (MPA), the active metabolite of the immunosuppressive drug mycophenolate mofetil, is a selective inhibitor of IMPDH type II which is mostly expressed in activated T and B lymphocytes (12–14). 6-Mercaptopurine (6 MP) is an inhibitor of de novo purine synthesis which depletes adenosine nucleotides to a greater extent than guanosine nucleotides in activated T lymphocytes (15). Methotrexate (MTX) is a folate antagonist that blocks dihydrofolate reductase and TS (16) but which may also block the first committed step of purine biosynthesis at low concentrations (20 nM-0.2 μM) (17). Finally, 5-fluorouracil (5FU) is converted to formation of 5-fluoro-2-deoxyuridine-5-monophosphate which forms a ternary complex with the enzyme TS and its cofactor 5,10-methylene tetrahydrofolate and as result renders this enzyme inactive (18). In the present study these inhibitors were used to achieve depletion of purine or pyrimidine pools in human PBL activated by PHA. Using this model, it was possible to investigate the effect of the inhibitors according to their time of administration with respect to the initiation of mitogenic activation. Finally, the model also allowed us to analyze discrete consequences of purine and pyrimidine nucleotide depletion according to the number of T cell divisions.

Materials and Methods

Reagents

PHA, 6 MP, MPA, and MTX were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). 5FU was obtained from Teva Pharma (Courbevoie, France). Human rIL-2 was obtained from Chiron (Suresnes, France).

Cell preparation and culture

PBL were collected from healthy donors in the presence of sodium citrate. Blood was defibrinated, then mononuclear cells were isolated by centrifugation on a layer of Histopaque (Dutcher, Brumath, France). Those suspensions contained 74.4 $\pm$ 2.0% T lymphocytes (48.1 $\pm$ 4.1% $CD4^+$ and 19 $\pm$ 2.9% $CD8^+$), 7.5 $\pm$ 1.2% B lymphocytes, 16.1 $\pm$ 1.9% NK cells, and 2.3 $\pm$ 0.4% monocytes as defined by expression of CD3, CD20, CD56, and CD14, respectively. PBL were resuspended in RPMI 1640 medium (Life Technologies, Cergy Pontoise, France) supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and cultivated in a humid atmosphere containing 5% CO_2.

Activated T lymphocytes were obtained by culture of PBL for 4 days with PHA (5 μg/ml). At this stage, dead cells were removed and viable cells (10^6/ml) were treated by the different inhibitors in the presence of IL-2 (50 U/ml).

Measurement of cell viability

Cell viability was measured by trypan blue exclusion. Viable and dead cells were counted by microscopy.

CFSE staining

To follow cell division, cells (10^7/ml) were pulsed with the fluorescent dye CFSE (1 μM) (Molecular Probes, Montluçon, France) in 2% FCS medium at 37°C. Then cells were washed and resuspended in medium at 10^6/ml. After the time of culture, cells were resuspended in PBS containing 2% BSA and 0.2% NaN_3 (PBS/BSA/azide) and fixed with 1% formaldehyde in PBS/BSA/azide buffer. CFSE staining (FL1-height) was measured by flow cytometry using a FACSCalibur and CellQuest software (BD Biosciences, Pont de Claix, France). CaliBRITE allophycocyanin beads were purchased from BD Biosciences.

Bromodeoxyuridine (BrdU) staining

Cells were labeled for 30 min at 37°C with 30 μM BrdU (Sigma-Aldrich), then washed twice with PBS. For FACS analysis, 10^6 cells were fixed with ethanol 75%. Cellular DNA was denaturated with HCl 2N, incubated with FITC-conjugated anti-BrdU Ab (BD Biosciences) and counterstained with propidium iodide (PI) containing RNase A (Sigma-Aldrich). DNA content was measured by flow cytometry using CellQuest software. Cellular debris and fixation artifacts were gated out and the G_0/G_1, S, and G_2/M fractions were quantified. Cells that were not exposed to BrdU were used as negative control. For microscopic analysis, cells were cytocentrifuged at 500 rpm for 5 min (Shandon cytospeen). Samples were fixed in absolute methanol at −20°C and DNA was denatured with formamide 95% in 20 $\times$ SSC at 70°C for 45 min. BrdU staining was performed with an anti-BrdU mAb (clone Bu20a; DAKO, Trappes, France), 30 min at room temperature, recognized by a rabbit anti-mouse Ab and an alkaline phosphatase-anti-alkaline phosphatase complex (DAKO), 30 min, with Fast Red as substrate (DAKO), 10 min.

Measurement of apoptosis

Phosphatidylserine exposure was quantified by surface binding of annexin V. Two-hundred thousands cells were resuspended in annexin V binding buffer containing FITC- or PE-conjugated annexin V for 15 min following instructions of the manufacturer (Bender MedSystems, Vienna, Austria). PI (1 μg/ml) was then added and cell suspension was immediately analyzed by flow cytometry.

ssDNA fragmentation was detected using F7-26 mAb from Alexis (Apostain; Laufelfingen, Switzerland), according to the manufacturer's instructions.

Immunoblots

Cells were washed with ice-cold PBS and collected in microcentrifuge tubes for lysis. The lysis buffer contained 50 mM HEPES, pH 7.2, 150 mM NaCl, 100 mM EDTA, 100 mM EGTA, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 300 μg/ml benzamidine, 75 μg/ml PMSF, and 10 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone. After centrifugation at 50,000 rpm for 1 h, the protein content in the supernatant was assayed by the Bradford method using Coomassie dye (Bio-Rad). Equal amounts of protein were precipitated in acetone at 4°C overnight and separated on a 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Ecquevilly, France). The membrane was blocked with 5% nonfat milk in TBST and incubated 1 h with primary Ab in blocking solution. Membranes were then washed five times with TBST and incubated 45 min with the appropriate secondary Ab. Detections were performed using the ECL system (Amersham Biosciences, Saclay, France). Anti-cyclins D3, E, A, B1, and anti-cyclin-dependent kinase (CDK) 4, 6, 1, and 2 Abs were obtained from Santa Cruz Biotechnology (Le Perray en Yvelines, France). Biotinylated secondary Abs were purchased from BD PharMingen (Pont de Claix, France). HRP-conjugated streptavidin was obtained from Amersham Biosciences. Equal amounts of loaded protein have been controlled by probing membrane with a β-actin mAb (Sigma-Aldrich).

Results

Blockade of nucleotide synthesis inhibits proliferation of primary T lymphocytes

To first assess the effect of nucleotides synthesis inhibition on proliferation of primary T lymphocytes, human PBL were activated with the mitogenic lectin PHA (5 μg/ml) in the presence or absence of 6 MP, MPA, MTX, or 5FU. The fluorescent dye CFSE was used to track cell division by FACS. After 4 days of incubation, cells cultured in medium alone remained undivided (Fig. 1*A*). Lymphocytes activated with PHA underwent one to four divisions. Addition of 6 MP, MPA, MTX, or 5FU induced an inhibition of cellular division in a dose-dependent manner. Maximal inhibitory effect was shown at 500 μM for 6 MP, 1 μM for MPA and MTX, and 10 μM for 5FU. According to these results, analysis of DNA content at 48 h showed that 97, 97.5, 95, and 91% of cells activated by PHA in the presence of 6 MP, MPA, MTX, and 5FU, respectively, were in G_0/G_1 compared with 71% of cells in G_0/G_1 in the presence of PHA alone (data not shown). In all additional experiments, nucleotide synthesis inhibitors have been used at these determined concentrations.

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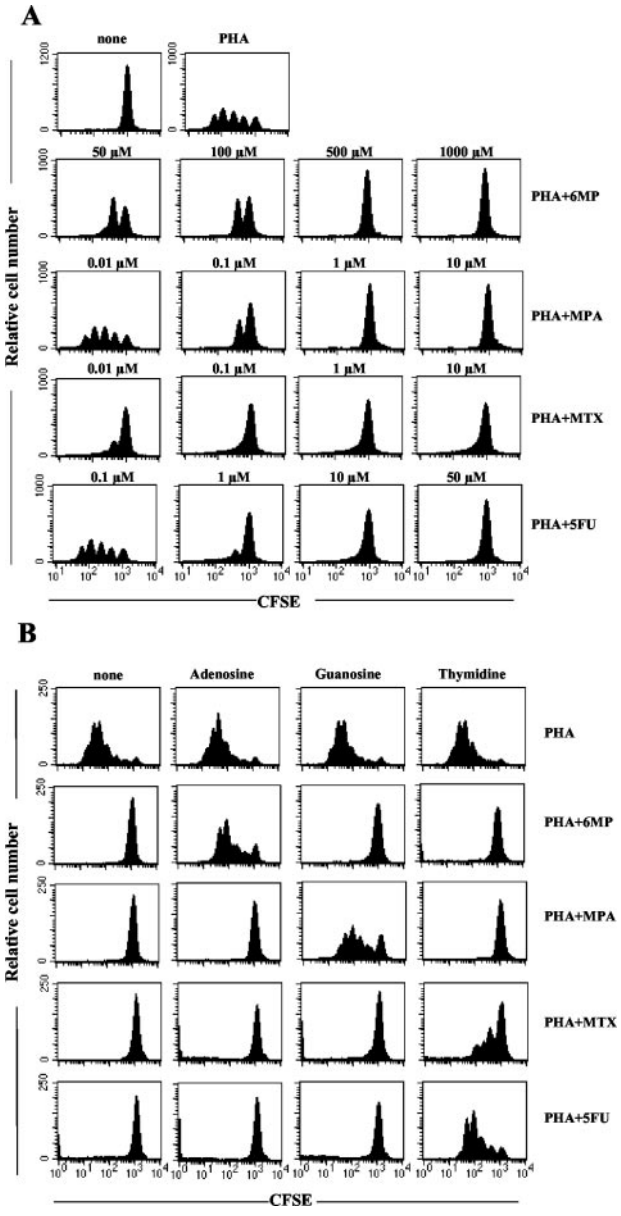

FIGURE 1. Antiproliferative effect of nucleotide synthesis inhibition in primary T lymphocytes. PBL were labeled by CFSE at the onset of culture as described in *Materials and Methods*. *A*, PBL were activated by PHA (5 μg/ml) alone or in the presence of a dose range of 6 MP, MPA, MTX, or 5FU. CFSE profiles were analyzed on a viable lymphocyte gate after 4 days of activation. *B*, Reversal effect of adenosine, guanosine, and thymidine on cellular division. PBL were activated by PHA (5 μg/ml) in the presence of 6 MP (500 μM), MPA (1 μM), MTX (1 μM), or 5FU (10 μM) and were treated by adenosine (100 μM), guanosine (10 μM), or thymidine (100 μM). Cell divisions were analyzed on viable cells after 6 days of activation. Results from one typical experiment among two showing similar results. Cell acquisitions on FACS were performed on a constant cell number in the viable lymphocyte gate.

We further investigated whether specific inhibition of purine or pyrimidine nucleotide synthesis as a consequence of blockade of the targeted enzymes could account for the antiproliferative effect of the four inhibitors. To this end, PBL were incubated with PHA and 6 MP (500 μM), MPA (1 μM), MTX (1 μM), or 5FU (10 μM) in the presence of adenosine (100 μM), guanosine (10 μM), or thymidine (100 μM) (Fig. 1*B*). Adenosine specifically reversed the inhibitory effect of 6 MP. In contrast, guanosine or thymidine did not have any effect. As expected, only guanosine prevented the inhibitory effect of MPA and only thymidine allowed cell division to proceed in 5FU-treated cells. The two major targets of MTX are dihydrofolate reductase and TS but at low concentrations (20 nM-0.2 μM); MTX was also shown to block the first committed step of purine biosynthesis (17). Therefore, we determined whether blockade of either one of the two biosynthesis pathways, purine or pyrimidine synthesis, could account for MTX-induced inhibition of cellular divisions. Thymidine partially prevented the inhibitory effect of MTX on cell division but adenosine and guanosine did not. All these results suggest that 6 MP and MPA inhibited cell division by a specific depletion of purines whereas MTX and 5FU inhibited cell division by blocking pyrimidine biosynthesis.

Inhibition of purine and pyrimidine synthesis at the onset of T cell activation accumulates PBL in G_0/G_1 and early S phase of the cell cycle, respectively

To determine whether inhibition of purine or pyrimidine biosynthesis induced a specific blockade in the cell cycle, we analyzed the cellular DNA content in parallel with a BrdU staining which reveals cells in the S phase of the cell cycle. As shown in Fig. 2*A*, 97% of cells cultured in medium alone were in G_0/G_1. After 48 h of activation with PHA, 31% of cells entered into the S phase of the cell cycle with 9, 19, and 2.6% in early, intermediate, and late S phase, respectively. In the presence of 6 MP or MPA, cells did not incorporate BrdU and accumulated in the G_0/G_1 phase (Fig. 2). By contrast, cells activated with PHA in the presence of MTX or 5FU were labeled with BrdU, with the same staining intensity as cells activated with PHA alone (Fig. 2*B*) but they accumulated in early S phase (Fig. 2*A*).

Transitional expression and activity of several cyclin-CDK complexes control progression in the different phases of the cell cycle (19–21). Cyclin D3 associated with CDK4 or CDK6 is expressed during the G_1 phase (22, 23), cyclin E/CDK2 is essential for the G_1 to S phase transition (24, 25), and cyclin A/CDK2 complexes are expressed during the S phase (26). The cyclin A/CDK1 complex regulates the transition from S to G_2 (27) whereas cyclin B/CDK1 is expressed in late G_2 (28, 29). Therefore, we assessed the expression of cyclins and CDKs after 48 h of culture (Fig. 3). As expected, cells cultured in medium alone did not express either cyclins or CDKs whereas cells activated with PHA alone expressed all cyclins and CDKs. According to results shown in Fig. 2, cells activated with PHA in the presence of 6 MP or MPA did not express any cyclin and the expression of CDKs was strongly decreased. Surprisingly, MTX or 5FU did not prevent expression of cyclins and CDKs. Of note, the accumulation of cells in early S phase induced by MTX or 5FU (Fig. 2) was not associated with an absence of cyclin A/CDK2 and cyclin B1/CDK1 expression despite the known role of cyclin A/CDK2 in the progression to the intermediate and late S phase of the cell cycle (26, 27) and that of cyclin B1/CDK1 in the G_2/M transition (28, 29).

Inhibition of pyrimidine, but not purine, synthesis induces apoptosis of PBL upon activation

Because inhibition of cell division could reflect either blockade of proliferation or induction of cell death, we analyzed whether inhibition of purine or pyrimidine synthesis could affect cell survival following T cell activation. In medium alone, cell numbers remained stable around 10^6 cells/ml during 120 h (Fig. 4*A*). After activation with PHA, counts of viable cells showed an initial decrease from 10^6 cells/ml at day 0 to 0.8×10^6 cells/ml at 48 h, then an increase to reach 2.1×10^6 cells/ml at 120 h. 6 MP or MPA did not prevent the initial decrease at 48 h and cell numbers remained stable with a slight decrease to 0.6×10^6 cells/ml after 120 h of activation. In the presence of MTX or 5FU, we also observed the

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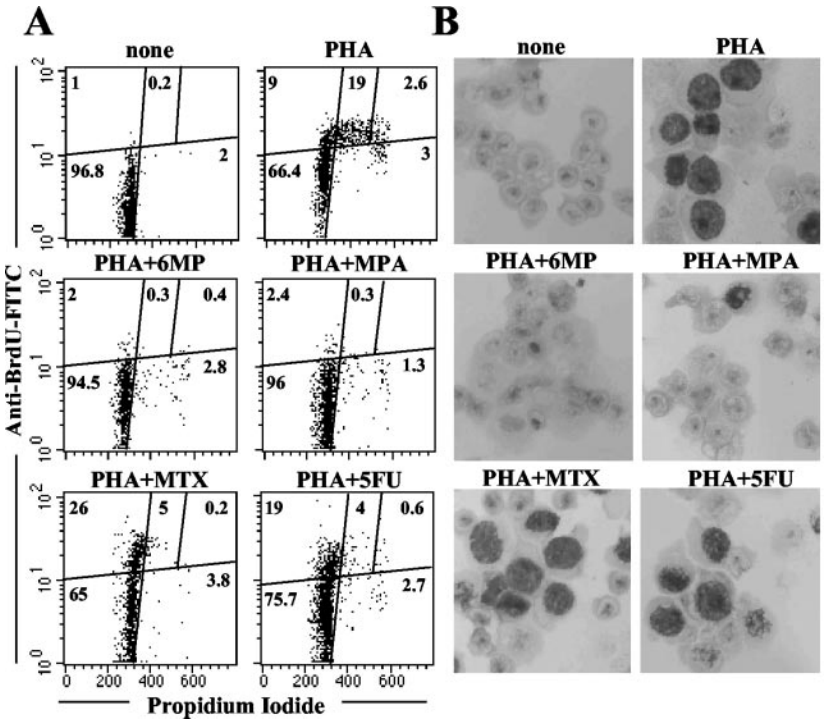

FIGURE 2. Effect of purine and pyrimidine synthesis inhibition on G_1 to S phase transition of the cell cycle. PBL were activated by PHA in the presence of 6 MP (500 μM), MPA (1 μM), MTX (1 μM), or 5FU (10 μM). After 48 h of incubation, cells were incubated with BrdU for 30 min. The fraction of cells engaged in S phase was evaluated. *A*, For FACS analysis, BrdU-labeled cells were stained with FITC-conjugated anti-BrdU Ab and PI as described in *Materials and Methods*. Percentages of cells in G_0/G_1, early S, intermediate S, late S, and G_2/M phases of the cell cycle are indicated in each dot plot. *B*, For microscopic analysis, BrdU-labeled cells were cytocentrifuged and stained as described in *Materials and Methods*. Results from one typical experiment among three showing similar results.

initial decrease, but counts of viable cells strongly dropped to 0.1×10^6 cells/ml after 120 h.

In parallel, we followed the percentage of apoptotic cells by annexin V staining (Fig. 4*B*). The percentage of apoptosis in cell suspensions cultured in medium alone was around 16% after 120 h. Activation with PHA modestly increased the percentage of apoptotic cells after 48 h and then it remained stable, around 20%. Treatment with 6 MP or MPA did not obviously affect the percentage of annexin V^+ cells until 72 h, then there was a slight increase to 34 and 41%, respectively, after 120 h of culture (Fig. 4*B*). In agreement with the drop in viable cell numbers (Fig. 4*A*), the kinetics of apoptosis showed a progressive increase as soon as 72 h to reach 70% after 120 h of activation in the presence of MTX or 5FU (Fig. 4*B*).

To more precisely assess the phenomenon of apoptosis, we also determined the percentage of cells with fragmented DNA after 120 h of culture. As shown in Fig. 4*C*, 13% of cells cultured in medium alone or activated with PHA exhibited DNA fragmentation. The percentage of DNA fragmented cells did not increase in the presence of 6 MP or MPA but raised to 46.2 and 45.6 in the presence of MTX or 5FU, respectively. Altogether these results demonstrate that inhibition of pyrimidine, but not purine, synthesis induces apoptosis of PBL upon activation.

Inhibition of purine and pyrimidine synthesis abrogates proliferation of cycling T cells

Having demonstrated the differential effect of purine and pyrimidine synthesis inhibitors on cell cycle regulation and apoptosis when added at the onset of activation, we then investigated their effect on cycling T cells. For this purpose, CFSE-labeled PBL were activated with PHA (5 μg/ml) for 4 days, then viable cells were treated with IL-2 (50 U/ml) in the presence or absence of purine and pyrimidine synthesis inhibitors. Four days after activation with PHA, lymphocytes had undergone from one to six divisions, with the highest frequency under the fourth division peak (Fig. 5*A*, *upper left panel*). After a further incubation of 48 h with IL-2, T lymphocytes had undergone up to seven divisions with the majority of cells in the fifth and sixth division peaks (Fig. 5*A*, *upper right panel*). 6 MP- or MPA-treated cells did not perform additional cell divisions and showed the same CFSE profile as compared with cells recovered after 4 days of activation with PHA (Fig. 5*A*), demonstrating that purine synthesis inhibitors completely prevent cell divisions induced by IL-2. PBL treated with MTX or 5FU in the presence of IL-2 did not undergo further divisions but disappeared from the fifth to sixth division peaks (Fig. 5*A*). Furthermore, the increase of the relative cell number under the first and second division peaks, as well as that of nondividing cells suggest that pyrimidine inhibitors not only inhibit cell division, but also trigger apoptosis of cycling T cells.

Cycling T lymphocytes accumulate in early and intermediate S phase of the cell cycle upon inhibition of purine and pyrimidine synthesis, respectively

Knowing the antiproliferative effect of purine and pyrimidine inhibitors on cycling T cells, we assessed their effects on cell cycle regulation by BrdU staining. After 24 h of culture in the presence of IL-2, 36.5% of activated PBL entered into S phase of the cell cycle with 18.4, 12.2, and 5.9% in early, intermediate, and late S phase, respectively. By contrast to their effect when added at the onset of activation, 6 MP and MPA allowed activated PBL to enter into S phase (Fig. 5*B*). However, cells can no longer pursue into G_2/M phase in the presence of these inhibitors and they accumulate into early and intermediate S phase of the cell cycle (Fig. 5*B*). In agreement with their effect when added at the onset of activation, MTX or 5FU induced an accumulation of activated PBL in early S phase.

Differential kinetics and amplitude of apoptosis of cycling T lymphocytes after inhibition of purine and pyrimidine synthesis

Because pyrimidine inhibitors profoundly modified the CFSE profiles after a 48-h culture in the presence of IL-2 (Fig. 5*A*), we investigated whether the inhibition of cell division by purine and

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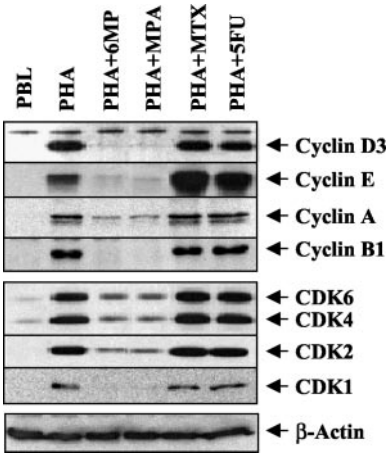

FIGURE 3. Effect of purine and pyrimidine synthesis inhibition on cyclin and CDK expression. PBL were treated for 48 h as described in Fig. 2. After a wash in PBS, cells were lyzed as described in *Materials and Methods*. A total of 100 μg of proteins were loaded and separated on 12% SDS-PAGE and expression of cyclins and CDKs was evaluated by Western blot as described in *Materials and Methods*. Amounts of loaded proteins have been controlled for homogeneity by probing membranes with an anti-β-actin mAb. Results from one typical experiment among two showing similar results.

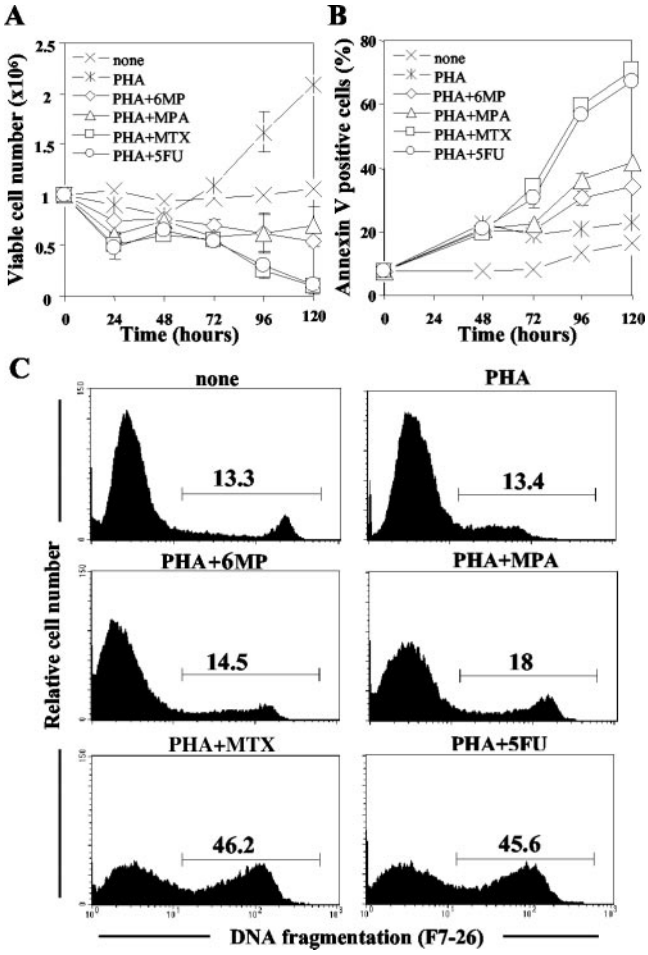

FIGURE 4. Purine and pyrimidine synthesis inhibition at the onset of culture: effect on cell survival. PBL were incubated in medium alone or stimulated with PHA (5 μg/ml) with or without 6 MP (500 μM), MPA (1 μM), MTX (1 μM), 5FU (10 μM). *A*, Effect of 6 MP, MPA, MTX, or 5FU on viable cell recovery. Viable cell number was determined by trypan blue exclusion at the indicated times. Values are the mean ± SEM of two independent experiments. *B*, Effect of 6 MP, MPA, MTX, or 5FU on apoptosis. Percent apoptotic cells were evaluated at the indicated times by annexin V binding as described in *Materials and Methods*. Results are expressed as the mean ± SEM from a triplicate measurement from a total of five showing similar results. Acquisition was performed on a constant cell number in the lymphocyte gate. *C*, After 120 h of incubation, DNA fragmentation was analyzed by using the F7-26 mAb as described in *Materials and Methods*. The percentage of cells with fragmented DNA is indicated for each histogram.

especially pyrimidine synthesis inhibitors could be the consequence of apoptosis of cycling T cells. As shown in Fig. 6*A*, activated T cells cultured in the presence of IL-2 (50 U/ml) did not undergo apoptosis, and the percentage of annexin V^+ cells remained stable around 15% even after 96 h of culture. Inhibition of pyrimidine synthesis by MTX or 5FU induced apoptosis as soon as 24 h of culture and percentage of apoptotic cells reached a maximum at 72 h. Surprisingly, inhibition of purine synthesis by 6 MP or MPA also strongly increased the percentage of apoptotic cells but only after 48 h of exposure. Moreover, the percentage of annexin V^+ cells after treatment with 6 MP or MPA was lower compared with the effect of MTX or 5FU, even after 96 h of treatment. The different kinetics of apoptosis were also evaluated by analyzing DNA fragmentation. At 24 h of culture with IL-2 alone or in the presence of 6 MP or MPA, the percentage of cells with fragmented DNA was low (10–15%) whereas in the presence of MTX or 5FU ~30% of cells exhibited DNA fragmentation (Fig. 6*B*). However at 96 h, inhibition of either purine or pyrimidine synthesis induced DNA fragmentation in 30 and 40% of cells, respectively (Fig. 6*C*), confirming the difference of kinetics and amplitude in the induction of apoptosis.

Apoptosis induced by purine or pyrimidine synthesis inhibition increases with the number of T cell divisions

Proliferation of activated T cells induced by PHA is asynchronous and after 4 days of culture, T cells have undergone from one to six divisions. We took advantage of this asynchronism to investigate whether purine or pyrimidine synthesis inhibition could differentially affect cell survival according to the number of mitoses. To this end, PBL were labeled with CFSE at the onset of activation with PHA, and after 4 days viable cells were harvested and cultured with IL-2 (50 U/ml) alone or in the presence of 6 MP, MPA, MTX, or 5FU. At the time of harvesting, cells had accomplished one to six divisions with a higher frequency under the third division peak (Fig. 7*A*). After 12 h with IL-2, a maximum number of cells was recovered under the third and fourth division peaks (Fig. 7, *A* and *B*). By contrast, in the presence of 6 MP, MPA, MTX, or 5FU, the highest frequency of cells remained within the third division peak. After 24 h with IL-2, cells underwent up to seven divisions (Fig. 7*A*). 6 MP- or MPA-treated cells did not perform additional cell divisions as compared with untreated cells and the number of viable cells for each cell division did not decrease significantly. However in the presence of MTX or 5FU, the maximum number of cells remained within the third division peak but it was associated with a strong decrease of viable cell numbers especially from the third cell division peak and beyond (Fig. 7*B*). After 48 h in the presence of IL-2, viable cell numbers increased and the maximum number of cells were within the sixth division peak. In the presence of 6 MP or MPA, repartition between division peaks remained stable (Fig. 7*A*). However the viable cell numbers decreased, especially in the fourth to sixth division peaks (Fig. 7*B*). In the presence of MTX or 5FU, the viable cell numbers strongly dropped in each peak. Of note, inhibition of de novo purine or

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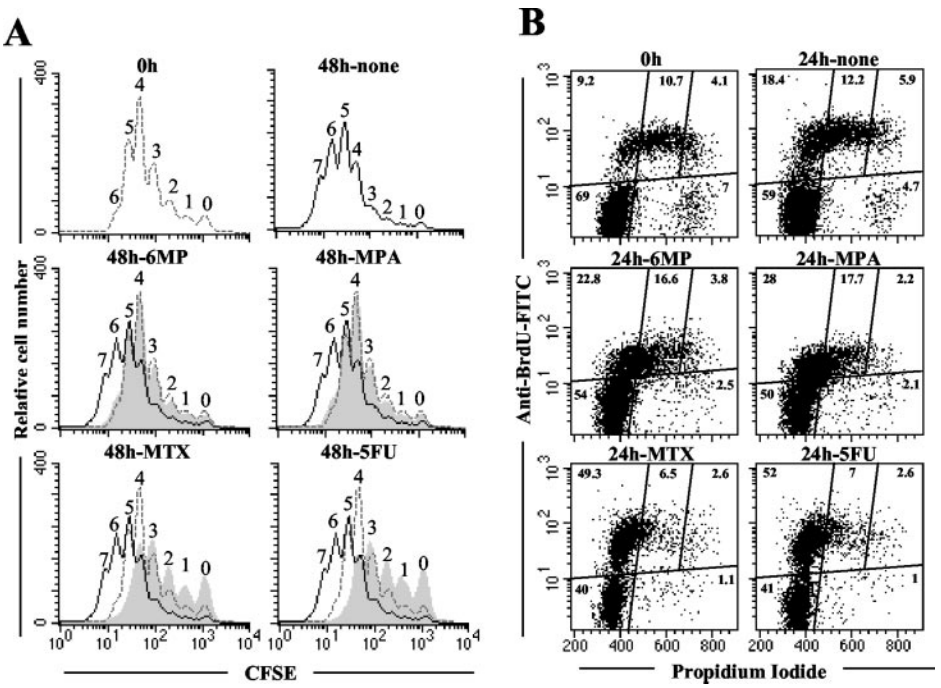

FIGURE 5. Purine and pyrimidine synthesis inhibition in cycling T cells: effect on proliferation and cell cycle. *A*, CFSE-labeled PBL were stimulated with PHA (5 μg/ml) for 4 days, then viable cells were harvested and incubated with IL-2 (50 U/ml) alone or in the presence of 6 MP (500 μM), MPA (1 μM), MTX (1 μM), or 5FU (10 μM). CFSE profiles were analyzed on a viable lymphocyte gate at the time of harvesting (dotted line) or after an additional 48 h treatment in the presence of IL-2 alone (solid line) or with purine or pyrimidine inhibitors (shaded histogram). The number of cell cycles corresponding to each peak is indicated. The constant cell number was acquired in the viable lymphocyte gate. *B*, PBL were stimulated for 4 days with PHA (5 μg/ml) (*upper left panel*), then viable cells were incubated with IL-2 (50 U/m) alone (*upper right panel*) or in the presence of 6 MP (500 μM), MPA (1 μM), MTX (1 μM), or 5FU (10 μM). After 24 h, cells were incubated with BrdU for 30 min and stained with FITC-conjugated anti-BrdU Ab and PI as in Fig. 2. Percentages of cells in G_0/G_1, early S, intermediate S, late S, and G_2/M phases of the cell cycle were indicated for each dot plot.

pyrimidine synthesis did not affect the number and viability of nondividing cells (Fig. 7, *A* and *B*).

We next determined T cell apoptosis according to the number of cell divisions. As soon as 12 h, inhibition of pyrimidine synthesis (MTX or 5FU) induced apoptosis of cells which have undergone five to six divisions (Fig. 7*C*). After 24 h, apoptosis was obvious after three divisions and the percentage of apoptosis increased with the number of cell cycles, reaching a maximum around 80% within the sixth cell division peak (Fig. 7*C*). By contrast, apoptosis induced by inhibition of purine synthesis (6 MP or MPA) was only observed at 24 and 48 h for cells that have accomplished at least five divisions. In conclusion, data in Fig. 7 suggest that susceptibility to apoptosis induced by inhibition of purine and pyrimidine synthesis on cycling T cells increases with the number of cell divisions. Furthermore, MTX and 5FU trigger apoptosis of cycling T cells more efficiently than do purine synthesis inhibitors, in agreement with the data reported in Fig. 6.

Discussion

One hallmark of the adaptative immune response is clonal expansion of appropriate effector cells needed to deal with a given pathogen. In the case of viral infections for example, the activation of $CD8^+$ cytotoxic T lymphocytes is characterized by a massive proliferation of the tetramer-specific responder T cells (30, 31). This expansion phase is necessary for complete differentiation of CD4 and CD8 effector and memory T lymphocytes. Indeed, commitment from naive CD8 T cells into fully differentiated effector and memory cells requires at least seven cell divisions (32). In agreement with these results, expression of cytokines that characterize effector lineages of CD4 Th cells is also regulated by cell division (33, 34). Fairbanks et al. (3) demonstrated a 2-fold purine and 8-fold pyrimidine pool expansion over 72 h of T cell activation. Although it was recently reported that pyrimidine-limited conditions alter the balance of Th1/Th2 cell differentiation (35), the precise role of these nucleotide increases on T cell proliferation and survival has not been systematically investigated. Our report demonstrates that inhibitors of de novo purine (6 MP and MPA) or pyrimidine (MTX and 5FU) synthesis prevent cell cycle progression and down-regulate survival of activated T lymphocytes. Our experiments using addition of exogenous nucleotides to specifically counteract the effect of these inhibitors (Fig. 1*B*) formally demonstrate the implication of key enzymes of de novo purine and pyrimidine nucleotide synthesis pathways in these different biological responses.

Previous reports had shown the importance of purine nucleotides in cell cycle regulation. In particular, depletion of guanine nucleotide pools after inhibition of IMPDH by MPA or by mizoribine potently inhibits cell cycle progression by arresting activated T cells in the G_1 phase of the cell cycle (8–10). This effect was associated with 1) inhibition of cyclin D3/CDK6 induction, and 2) down-regulation of the CDK inhibitor $p27^{Kip1}$ following PHA stimulation of PBL (8, 9). In keeping with those reports, we show in this study that MPA but also 6 MP, when added at the onset of activation, arrested T lymphocytes in the G_1 phase and completely abrogated expression of cyclins and CDKs (Fig. 3) and degradation of $p27^{Kip1}$ (data not shown). However, our results also pinpoint a second hitherto unrecognized blockade of the cell cycle induced by purine nucleotide depletion. Indeed, in contrast to their effect upon addition at the onset of activation, MPA or 6 MP when added on cycling T lymphocytes allowed activated PBL to enter into S phase but blocked their progression from early to intermediate S phase. Therefore, in addition to their critical role in the G_1

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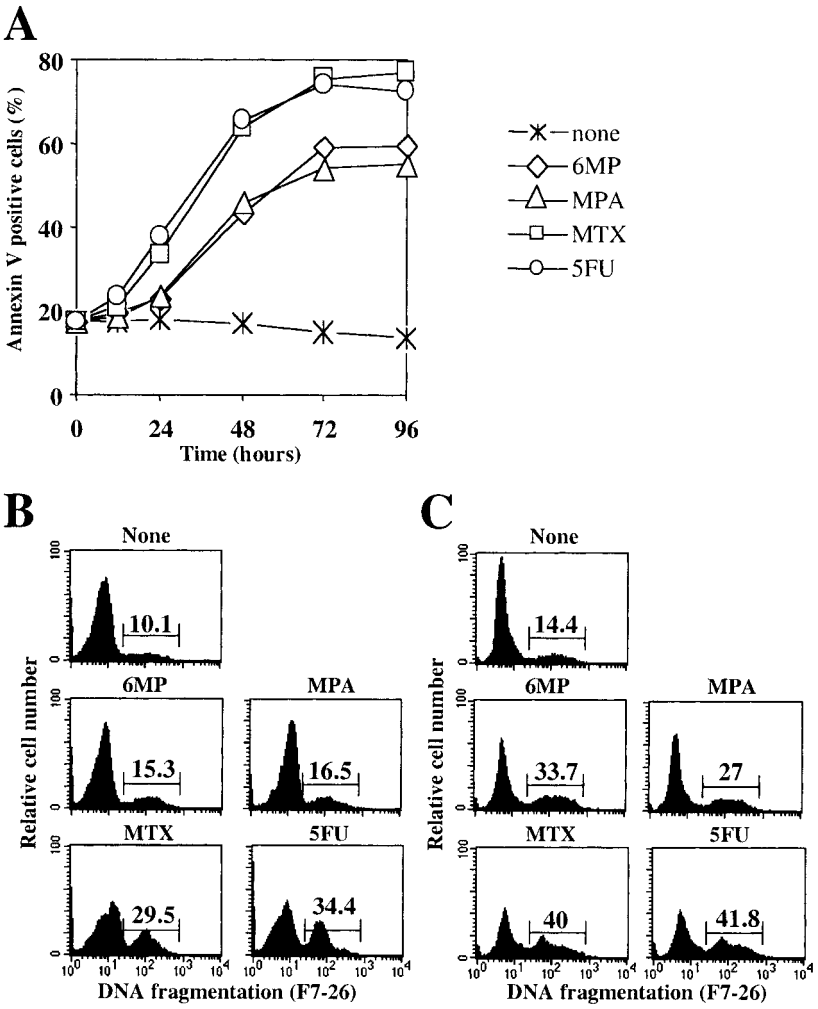

FIGURE 6. Purine and pyrimidine synthesis inhibition in cycling T cells: effect on cell survival. Four-day-activated PBL were incubated with IL-2 (50 U/ml) alone or with 6 MP (500 μM), MPA (1 μM), MTX (1 μM), or 5FU (10 μM). *A*, Percentage of apoptotic cells was evaluated at the indicated times by annexin V binding as described in *Materials and Methods*. Results are expressed as the mean ± SEM from a triplicate measurement from a total of four showing similar results. Cell acquisitions on FACS were performed on a constant cell number in the lymphocyte gate. DNA fragmentation was analyzed by using the F7-26 mAb as described in *Materials and Methods* after 24 (*B*) and 96 h (*C*) of treatment. The percentage of cells with fragmented DNA is indicated for each histogram.

to S phase transition, purine nucleotides are also required for progression throughout the S phase. In contrast, pyrimidine nucleotide inhibitors when added at the onset of T cell activation did not block the G_1 to S phase transition and did not prevent the expression of cyclin D3 associated with CDK4 or CDK6 which occurs during the G_1 phase (22, 23), that of cyclin E/CDK2 and cyclin A/CDK2 complexes expressed during the S phase (24, 25) nor that of the cyclin B1/CDK1 complex expressed at the G_2/M transition (28, 29). However, inhibition of pyrimidine nucleotide synthesis abrogated progression from early to intermediate and late S phase whatever the time of addition of the inhibitors. Therefore, in contrast to purine nucleotide depletion, pyrimidine nucleotide depletion induces only one blockade of the cell cycle. Whether this blockade and the S phase cell cycle arrest induced by purine nucleotides depend on identical or related mechanisms is not known. Although depletion of nucleotide pools is the major cause of antiproliferative effects and cell cycle arrest (Fig. 1*B* and data not shown) whatever the nucleotide inhibitor used, it is interesting to note that purine or pyrimidine depletion does not result in the same effects in term of cell cycle arrest or apoptosis.

The absence of inhibition of cyclin D3, cyclin E, cyclin A, and their respective CDKs is not surprising because cells treated with pyrimidine nucleotide inhibitors pursue their cell cycle progression until the early S phase. However, the absence of inhibition of the cyclin B1/CDK1 complex is more unlikely. Indeed, the expression of this complex should be restricted to the cells which have already completed their DNA replication, and are ready for mitosis. This kind of "unscheduled" expression of the cyclin B1/CDK1 complex has also been previously described in thymidine-treated T lymphocytes which are blocked in the G_1 phase of the cell cycle (36).

We previously demonstrated that MTX induces apoptosis of activated T lymphocytes, whatever the time of its addition (37). This effect was attributed to TS inhibition and blockade of pyrimidine nucleotide synthesis because it was fully reversible by addition of thymidine. In keeping with those results, we now demonstrate that another inhibitor of de novo pyrimidine nucleotide synthesis which directly targets TS, also induces apoptosis of T lymphocytes when introduced either at the onset of activation or on already cycling T cells. A remarkable feature of apoptosis of the activated T cell induced by inhibitors of purine or pyrimidine synthesis is that a necessary and sufficient condition for the triggering of cell death is progression to the early S phase of the cell cycle. Therefore, it may be hypothesized that among many other biological events controlled by purine and pyrimidine nucleotides, DNA synthesis is the critical step that triggers apoptotic signals. In support of this hypothesis, blockade of G_1 to S phase progression by calcineurin inhibitors, anti-IL-2R mAbs or rapamycin, prevents MTX-induced apoptosis (37). Similarly, blockade of cell cycle progression in mid-G_1 by 6 MP or MPA does not trigger T cell apoptosis (Fig. 4 and Ref. 9). However, this study also demonstrates a novel role for purine nucleotides in survival of activated T lymphocytes. Indeed, we showed that whereas purine synthesis inhibitors did not trigger apoptosis when added at the onset of activation, they profoundly

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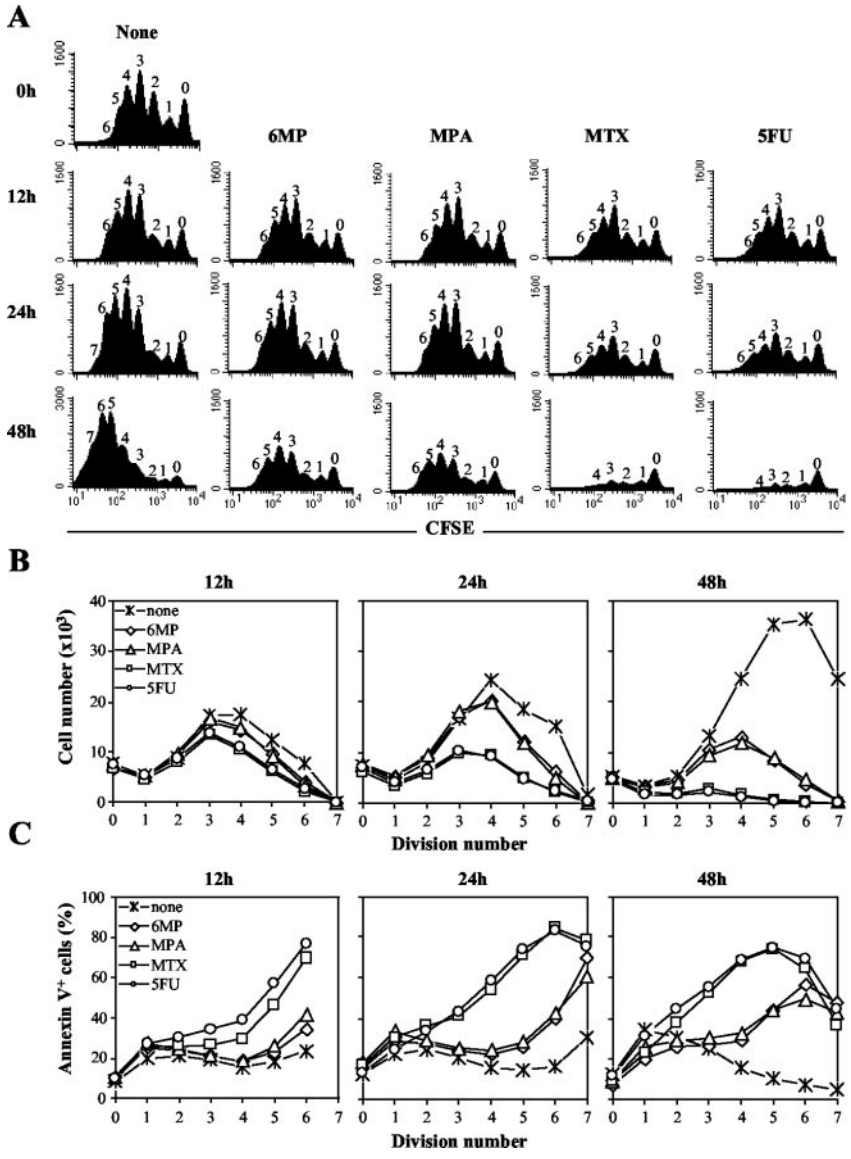

FIGURE 7. Apoptosis induced by purine or pyrimidine synthesis inhibition increases with the number of cell divisions. CFSE-labeled PBL were activated with PHA (5 μg/ml). After 4 days of stimulation, viable cells were harvested and incubated with IL-2 (50 U/ml) in the presence or absence of 6 MP (500 μM), MPA (1 μM), MTX (1 μM), or 5FU (10 μM) for the indicated times. Cell acquisition was calibrated on a constant number of allophycocyanin-labeled beads. *A*, Cells were electronically gated on viable lymphocyte population and cell divisions were analyzed by FACS. The number of the cell cycles corresponding to each peak is indicated. *B*, Cells were counted by FACS in the viable lymphocyte gate for each division. *C*, Percentage of apoptotic cells for each division was evaluated by annexin V binding in the lymphocyte gate.

affected survival of cycling T lymphocytes. The proapoptotic activity of purine nucleotide inhibitors is restricted to cycling and not to merely activated T lymphocytes. Indeed, we previously demonstrated that while blocking IL-2-stimulated proliferation, MPA preserved IL-2-driven survival of activated T lymphocytes "synchronized" in the G_1 phase of the cell cycle (9). This is in agreement with our data showing that activation of T lymphocytes in the presence of purine nucleotide inhibitors, which results in accumulation of T cells in the G_0/G_1 phase, does not trigger apoptosis. Altogether these results suggest that requirement in purine or pyrimidine nucleotides for survival of activated T lymphocytes depends on the phase of the cell cycle. In particular, different susceptibility of activated T lymphocytes to apoptosis induced by purine nucleotide inhibitors may be correlated with the two different cell cycle arrests controlled by purine nucleotides.

Our report further demonstrates that susceptibility to apoptosis induced by inhibition of purine and pyrimidine synthesis on cycling T cells increases with the number of cell divisions. As mentioned above, activation of T lymphocytes leads to a 2-fold purine and up to an 8-fold pyrimidine pool expansion over 72 h (3). This finding supported the idea that initial expansion provides the extra purine and pyrimidine nucleotides necessary for subsequent DNA replication and cell divisions. Interestingly, our results showing that addition of purine and pyrimidine synthesis inhibitors 4 days after activation did not allow further cell divisions in the presence of IL-2 (Fig. 5*A*), suggest that clonal expansion of T lymphocytes does not only require an initial purine and pyrimidine pool expansion but rather a recurrent de novo synthesis of these pools for each division to proceed. However, we assume that successive cell divisions would lead to a progressively decreasing synthesis of purine and pyrimidine nucleotides which by consequence may increase apoptotic susceptibility of cycling T cells with the number of cell divisions. One may then speculate that deletion of activated T cells at the end of an immune response could be at least partially mediated through purine and pyrimidine depletion. Indeed, the immune response is characterized by an expansion phase of specific T lymphocytes followed by a contraction phase of the responder T cells which die by apoptosis (38). Several mechanisms have been proposed to account for this phenomenon, including passive cell death or activation-induced cell death, mediated through CD95/CD178 interaction (38). However, one may also hypothesize that cell divisions associated with the expansion phase would lead to a progressively decreasing synthesis of purine and pyrimidine nucleotides with the number of cell divisions which by consequence may increase apoptotic susceptibility of cycling T cells and would finally result in the contraction phase of the immune response. An

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alternative explanation of the preferential killing of cells which have undergone a higher number of divisions could be that these cells divide faster than those undergoing a low number of divisions, and consequently enter more frequently into the S phase where they are susceptible to purine and pyrimidine nucleotide depletion.

Our results also gain information on the mechanisms regulating DNA replication in primary T lymphocytes. Indeed, we demonstrated that de novo purine or pyrimidine synthesis not only controls the cell cycle, but also regulates survival of activated T lymphocytes. Whether apoptosis is the consequence of cell cycle arrest or rather cells do not pursue their cycle because they die is still an open question. However, our cell cycle analysis demonstrated that in absence of purine or pyrimidine nucleotides, T cells accumulate in one particular phase of the cell cycle before they die. These results suggest a complex regulation of so-called DNA damage responses, in which first sensors such as ataxia telangiectasia mutated detect abnormality during DNA replication, which, owing to transducers, activates effector proteins such as p53 (39, 40). These effector proteins induce first expression of proteins such as the CDK inhibitor $p21^{Cip1/Waf1}$ that arrests the cell cycle and then, if DNA replication is altered, proapoptotic proteins such as Bax will be expressed and lead to apoptosis of activated T lymphocytes. Alternatively, apoptosis could be the consequence of the "unscheduled" cyclin B1 expression. Indeed, when cells are blocked in early or intermediate S phase and by consequence do not complete their DNA replication, they still express the cyclin B1/CDK1 complex (Figs. 2 and 3), which is the natural signal for mitosis. In this setting, the complex may trigger an apoptotic signal as was previously demonstrated (41).

Finally, these results provide a framework for the design of immunosuppressive strategies based on administration of antimetabolites. One may hypothesize that drugs which inhibit pyrimidine synthesis, as for instance MTX or leflunomide (42, 43), will primarily trigger apoptosis of activated T cells and therefore induce deletion of Ag-stimulated T cell clones. Simultaneous exposure to the drug and the nominal Ag (in allografts or autoimmune disorders) will result in clonal deletion and favor the development of peripheral tolerance. Inhibitors of de novo purine synthesis, like 6 MP, MPA, or its analogs, would prevent clonal expansion if given before and during antigenic stimulation or, alternatively, trigger apoptosis of cycling T cells if administered after the mitogenic stimulus. The cytostatic effect should be readily reversible upon drug withdrawal whereas the deletional mechanism will be more prolonged. It is noteworthy that clonal deletion is not an intrinsic property of antimetabolites that target de novo purine or pyrimidine synthesis (44, 45) but rather depends on the timing of drug administration according to that of antigenic stimulus.

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