Effects of 5-Fluorouracil on Human Mitogen-activated Peripheral Blood Lymphocytes from Healthy Individuals

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Abstract. Background: The effect of 5-fluorouracil (5-FU) on activated lymphocytes was explored. Materials and Methods: The in vitro effects of 5-FU on DNA synthesis in mitogen-activated lymphocytes from healthy volunteers were compared to those of the antimetabolites doxorubicin, cyclophosphamide and 6-mercaptopurine. These effects were assessed by alterations in the phenotypic profile and the percentage of cells in various phases of the cell cycle, as well as by the secretion of T helper (Th)1 and Th2 cytokines (ELISA). Results: Unlike 5-FU, the other antimetabolites failed to augment DNA synthesis in activated lymphocytes. The effect of 5-FU correlated with an increase in the percentage of cells in the S-phase and caused an increased in CD4+ cells, a decrease in CD56+ cells and a shift of the cytokine secretion pattern from Th2 to Th1. Conclusion: 5-FU exhibited a unique effect on DNA synthesis in activated lymphocytes which was accompanied by selective effects on various lymphocyte subpopulations.

Studies on cancer patients undergoing chemotherapy have shown the inhibitory effects of anticancer drugs on various immune activities, including mitogen responses and cytokine secretion in the serum, as well as natural killer (NK) and activated NK (ANK) cell activities (1, 2). One of the antimetabolites frequently used in the treatment of colorectal carcinoma and other malignancies of the digestive tract is the pyrimidine 5-fluorouracil (5-FU) (3-5). The target enzyme of 5-FU is thymidylate synthase (TS) and the binding of these molecules forms a stable tertiary complex which inhibits the TS conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), thus eventually inhibiting DNA synthesis and cell replication (6). The role of TS as a target for 5-FU has been further demonstrated in tumor cells overexpressing TS which renders them more resistant to 5-FU (7, 8). Other antimetabolites frequently employed in the chemotherapy of cancer patients act by inhibiting DNA synthesis through the activation of different mechanisms. Doxorubicin blocks DNA synthesis and transcription via the stabilization of topoisomerase II/DNA cleavable complexes (9). Cyclophosphamide inhibits DNA synthesis by crosslinking of DNA strands (10), and 6-mercaptopurine reduces DNA synthesis by inhibiting purine nucleotide synthesis and metabolism. Since 5-FU is widely used in cancer chemotherapy, its effects on the immune system, which mediates a variety of antitumor activities both in culture and in cancer patients (11, 12), is of substantial interest. In addition to its effect on tumor cells, 5-FU has exhibited inhibitory activities on various immune responses, including NK cell-mediated cytotoxicity (13, 14) and mitogen-induced activation of lymphocytes (15). In previous experiments on human peripheral blood lymphocytes from healthy donors (16), we demonstrated by 3H-thymidine incorporation that concentrations of 250-2500 μM of 5-FU substantially (up to 98%) reduced DNA synthesis in culture following lymphocyte activation. At a lower concentration (2.5 μM), however, 5-FU markedly increased DNA synthesis by 195%, 58% and 222% following stimulation with interleukin-2 (IL-2), phytohemagglutinin A (PHA) and pokeweed mitogen (PWM), respectively. While low concentrations of 5-FU had no effect on the generation of activated NK cells, concentrations of 250-2500 μM 5-FU caused a marked decrease in the generation of IL-2-induced NK antitumor cytotoxic activity (16). Various cytokines, such as IL-10, prostaglandins and TGF-β have also been shown to modulate both proliferation and cytokine secretion by lymphocytes (17-20).

In the present study, the effects of 5-FU on lymphocyte activation in culture was explored further by analyzing the kinetics of cell activation and percentage of cells at various cell cycle phases, by measuring the effect of other...
antimetabolites on DNA synthesis and by analyzing the effect of the 2.5 μM stimulatory concentration of 5-FU on both the phenotypic profile and the T helper (Th)1 and Th2 cytokine secretion patterns in activated lymphocytes.

Materials and Methods

Preparation of peripheral blood mononuclear cells (PBMCs). Twenty milliliters of heparinized blood obtained from healthy volunteers were diluted 1:1 in Hanks balanced salt solution (HBSS), pH 7.2. The blood was then layered on a 10-15 ml density-gradient solution (1.077 g/cm³, Ficoll; Pharmacia, Uppsala, Sweden) and spun at 550 xg for 15 min. The cells that accumulated at the interphase between the plasma and Ficoll layers were collected, washed twice in HBSS, counted in 0.2% trypan blue solution to establish the percentage of viable cells and resuspended at the desired density in enriched medium (CM) as described elsewhere (21).

Antimetabolite drugs. 5-Fluorouracil (50 mg/ml) was purchased from Endoxan-asta, Frankfurt Am Main, Germany, doxorubicin (2 mg/ml) was purchased from Pharmacia, Milan, Italy, and 6-mercaptopurine was purchased from Sigma-Aldrich, St. Louis, MO, USA (Cat No. 852678-1G-A). All the drugs were diluted in CM at the desired concentrations prior to use.

Mitogens. Human recombinant IL-2 (Cetus, Emeryville, CA, USA) had a specific activity of 3×10⁶ IU/mg protein and endotoxin levels below 0.5 ng/mg. PHA (Israel Industries, Beit-Haemek, Israel) was employed at 1:200 final dilution in CM, and PWM (Israel Industries) at 1:100 final dilution in CM.

Proliferation assay. The proliferation assay was carried out as detailed previously (21). PBMCs at 2×10⁵/0.2 ml CM/well were incubated for 24-96 h in a 96-well round-bottom microplate with either 10⁴ units (U)/ml IL-2 or 1/200 PHA or 1/100 PWM in the presence or absence of different concentrations of 5-FU, doxorubicin, cyclophosphamide or 6-mercaptopurine, after which 2 μCi/20 μl/well [³H]thymidine (Sigma-Aldrich; cat No. T 5063) were added to each well for the last 6 h of culture. The cells were then harvested and the radioactivity was determined by a liquid scintillation counter (LKB, Mt Waverley, Australia). Proliferation index was calculated as following:

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\text{Mean cpm of lymphocytes cultured with the mitogen} \times \text{Mean cpm of lymphocytes cultured in medium alone}
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\text{Mean cpm of lymphocytes cultured in medium alone}
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Cytokine measurement. The PBMCs were cultured at 10⁶ cells/ml with PHA in the presence or absence of 2.5 μM 5-FU. Control cultures were incubated without PHA in the presence or absence of 5-FU. On days 1, 2, 3 and 4, 0.5 ml of supernatant from each culture was harvested and tested for both interferon-gamma (IFN-γ) and IL-10 secretion using the ELISA Ready-SET-Go kits (eBioscience, San Diego, CA, USA) following the manufacturer’s instructions. Briefly, after coating 96-well plates with anti-IL-10 or IFN-γ antibody, 2- to 4 fold dilutions of the tested supernatant samples were applied, biotin-conjugated anti-IL-10 (clone JES3-12GB) or IFN-γ (clone 4S.B3) was added, the samples were incubated with avidin-horse radish peroxidase (HRP) and the substrate solution tetramethylbenzidine was added at the end. The results were obtained as pg or ng/10⁶ PBMC/ml.

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\text{Phenotype analysis.} \quad \text{The PBMCs were incubated for 120 h with 1000 U/ml IL-2 in the presence or absence of 2.5 μM of 5-FU.} \quad \text{The cells were then harvested, washed and incubated for 60 min with the following fluorescent mouse anti-human monoclonal antibodies (Becton Dickinson, San Jose, CA, USA): anti-CD3 (T lymphocytes), anti-CD4 (helper T lymphocytes), anti-CD8 (cytotoxic T lymphocytes), and anti-CD56 (NK lymphocytes).} \quad \text{The cells were then analyzed on a FACS Caliber (Becton Dickinson).}
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Cell cycle measurements. PBMCs incubated in 1000 U/ml IL-2 alone or in the presence of 2.5 or 250 μM 5-FU were collected after 72 h and 96 h, washed twice in HBSS and 10⁶ cells were incubated with 50 μl 1% Triton® X-100/PBS (USB Corp, Cleveland, OH, USA) followed by 50 μl of 1 mg/ml propidium iodide (Sigma-Aldrich), and analyzed using a FACSort flow cytometer (Becton Dickinson).

Statistical analysis. Statistical analysis was performed by a two-tailed Student’s t-test.

Results

Kinetics of 5-FU on mitogen-induced DNA synthesis. As shown in Figure 1A, which summarizes six different experiments, incubation of the PBMCs for 72 h with 2.5 μM 5-FU increased the response to IL-2 by 87% (from 1130±243 to 2115±363 cpm).

To analyze the kinetics of the increase in DNA synthesis induced by 2.5 μM 5-FU in the mitogen-activated cells, PBMCs were cultured with PHA for 24-96 h in the presence or absence of 2.5 μM 5-FU. As shown in Figure 1B, incubation of PBMCs with PHA in the presence of 5-FU caused an increase in DNA synthesis from 2,051 (PHA alone) to 4,464 cpm and 7,277 (PHA alone) to 13,119 cpm after 48 h and 72 h in culture, respectively, while 5-FU caused a decrease from 11,062 cpm without the drug to 6,294 cpm in the presence of 5-FU at 96 h. The mitogens IL-2 and PHA were chosen since similar results had been obtained with PHA and PWM (16).

Effects of doxorubicin, cyclophosphamide and 6-mercaptopurine on mitogen-induced DNA synthesis. To reveal the effect on activated lymphocytes of other antimetabolites PBMCs were incubated for 72 h with IL-2, PHA or PWM in the presence or absence of either 10⁻⁴ to 10⁻¹ μM doxorubicin, 10⁻¹ to 1,000 μM cyclophosphamide or 10⁻² to 1,000 μM 6-mercaptopurine.

As shown in Figure 2A, doxorubicin at 10⁻⁴ μM had no effect on DNA synthesis when any of the mitogens were used, while higher concentrations of the drug showed a gradual increase in the inhibitory effect on DNA synthesis. Similarly, cyclophosphamide (Figure 2B) substantially inhibited DNA synthesis at the highest concentrations used (1,000 μM), while lower concentrations had no effect when any of the mitogens were employed. Similarly, 6-mercaptopurine (Figure 2C) inhibited IL-2-induced DNA synthesis at 10-1,000 μM, but there was no effect when lower concentrations of the drug were tried.
None of the antimetabolites showed any increase in DNA synthesis at any of the concentrations employed.

**Effect of 5-FU on cell cycle.** As shown in Figure 3A, the presence of 2.5 μM 5-FU increased the percentage of IL-2-activated lymphocytes in the S-phase from 5.96% to 7.48% (an increase of 26%) following 72 h in culture, whereas 250 μM 5-FU caused a marked decrease in cells in the S-phase (from 5.96% to 0.62%, a reduction of 90%). After 96 h of incubation (Figure 3B), 2.5 μM 5-FU increased the percentage of cells in the S-phase from 5.94% to 7.82% (an increase of 32%) while 250 μM 5-FU reduced the cells in S-phase from 5.94% to 0.64% (a reduction of 89%). In addition, the number of cells entering the G2+M-phase increased between 72 h to 96 h from 2.3% to 3.14% (an increase of 37%) following their incubation with 2.5 μM 5-FU, whereas 250 μM 5-FU led to a decrease from 2.58% to 1.54% (a reduction of 40%) in cells entering the G2+M-phase. The number of cells undergoing apoptosis increased for both IL-2, IL-2 plus 2.5 μM 5-FU and IL-2 plus 250 μM 5-FU between 72 and 96 h of incubation and this effect was more prominent with 250 μM 5-FU (an increase of 80%) than with 2.5 μM 5-FU (an increase of 32%) when compared to cells incubated with IL-2 alone.

**Effects of 5-FU on the phenotypic profile of lymphocytes undergoing 5 days’ activation with IL-2.** As shown in a representative experiment in Figure 4, on PBMCs IL-2 in the presence of 5-FU had limited effects compared to IL-2 alone. 5-FU reduced the percentage of CD8+ cells from 16%
to 49% to 53%, while increasing the percentage of CD3+ cells from 71% to 73% and decreasing the percentage of CD56+ lymphocytes from 8% to 3% (Figure 4).

Effects of 5-FU on the secretion of IFN-γ and IL-10 in lymphocytes undergoing activation. As illustrated in a representative experiment (Figure 5A), 5-FU increased PHA-induced IL-10 secretion from 5,592 pg/10^6 cells to 7,832 pg/10^6 cells (+40%) after 48 h of incubation, whereas it reduced IL-10 secretion from 2,636 to 1,989 pg/10^6 cells (-25%), 10,281 to 7,548 pg/10^6 cells (-27%) and 8,754 to 5,624 pg/10^6 cells (-36%) at 24, 72 and 96 hours, respectively. 5-FU increased IFN-γ secretion from 17,065 to 22,269 ng/10^6 (+30%) cells after 72 h of incubation and from 27,397 to 33,393 ng/10^6 cells (+22%) after 96 h of incubation (Figure 5B).

Discussion

The substantial increase in DNA synthesis in mitogen-activated lymphocytes in the presence of a low (2.5 μM) concentration of 5-FU was prominent between 48-72 h of incubation (Figure 1B), whereas there was a decrease in DNA synthesis at 96 h, which implied that 5-FU caused a shift in the kinetics of DNA synthesis in activated lymphocytes. The stimulatory effect on DNA synthesis of low concentration 5-FU was unique to this drug. High concentrations of 5-FU as well as doxorubicin (10^-1-10^-3 μM), cyclophosphamide (1,000 μM) and 6-mercaptopurine (10-1,000 μM), substantially inhibited DNA synthesis in activated lymphocytes (Figure 2). The increase in DNA synthesis induced in activated lymphocytes by 2.5 μM 5-FU correlated with the percentage of cells in the S-phase of the cell cycle (Figure 3A-B) which increased from 5.96%
absence of 5-FU) to 7.48% (+26%) after 72 h in culture; 250 μM 5-FU caused a decrease in cells in the S-phase (from 5.96% to 0.62%, –90%). Similar results were obtained at 96 h. Others have shown an S-phase arrest after exposure of MDA435 breast cancer cells (8) and esophageal squamous cell carcinoma cells (22) to up to 1,540 μΜ 5-FU. When Yoshikawa et al. exposed SW480 and COLO320DM colon carcinoma cells to 1,000 ng/ml 5-FU, arrest in the G1/S phase was accompanied by an increase in the percentage of apoptotic cells throughout the experimental period (23), similar to the current findings (Figure 3A-B). The present results also suggested that the increase of cells in the S-phase induced by the low concentration of 2.5 μM 5-FU was not a mere accumulation of cells during this cell cycle phase, since there had been an increase of 37% in the percentage of cells (i.e. from 2.3 to 3.14%) entering the G2/M-phase between 72 to 96 h of incubation, whereas a decrease of 40% was observed during this period after treatment of cells with IL-2 in the presence of 250 μM 5-FU. The increased inhibition of cells in the S and G2/M-phases following the use of IL-2 plus 250 μM 5-FU correlated with an increase of cells undergoing apoptosis, which was most prominent at 96 h (8.28%) when compared to the cells incubated with the lower concentration (2.5 μM) of 5-FU (6.08%).

The mechanism underlying the increase in DNA synthesis induced by 2.5 μM 5-FU is still unknown. One possibility is that a low concentration of 5-FU inactivates the expression of p53 which was shown to abrogate cell cycle arrest and apoptosis induced by 5-FU in breast tumor cells (7, 24). Another mechanism might be a selective direction of DNA synthesis via the salvage pathway (22) induced by low 5-FU concentrations. Alternatively, 5-FU may increase both the activity and synthesis of TS, thus leading to an increase in thymidine and DNA synthesis. Experiments for testing this possibility are currently underway.

The mitogens which were employed in the present study activate various lymphocyte subpopulations, including T and
B lymphocytes as well as NK cells (25, 26). In this study, 2.5 μM 5-FU reduced the percentage of CD56+ cells (from 8% to 3%) and increased that of CD4+ cells (from 49% to 53%). The decrease in CD56+ cells was in agreement with our previous study in which 2.5 μM 5-FU reduced the generation of NK cell cytotoxic activity by 12% following stimulation with IL-2 (16). The increase in CD4+ lymphocytes by 2.5 μM 5-FU could apparently be attributed to an increase in Th1 cells as revealed by the increase in IFN-γ secretion after 72-96 h of incubation (Figure 5B).

Similar to other modulators, including muramyl dipeptide derivatives, cyclophosphamide and ribavirin (27-29), which affect either the Th1 or Th2 cells, 2.5 μM 5-FU could apparently be attributed to an increase in Th1 cells as revealed by the increase in IFN-γ secretion after 72-96 h of incubation (Figure 5B). Th1 cells were more sensitive to apoptosis than Th2 cells (30, 31). Alternatively, since 2.5 μM 5-FU affected DNA synthesis only in activated lymphocytes (Eisenthal, unpublished observation), the observed effect of 5-FU might have been due to a different activation pattern that had been induced in Th1 and Th2 cells by the mitogens used in the current study. Finally, the ability of 5-FU at a 2.5 μM to increase DNA synthesis and modulate the Th1/Th2 cytokine secretion pattern raises the question of the possible effects of this drug on the regulation of the activities of various lymphocytes by other biomodulators, including TGF-β, prostaglandins and free oxygen radicals. Studies in search of such effects are currently being conducted in our laboratory.

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References


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