Abstract. Background: Studies on cancer patients undergoing chemotherapy have shown inhibitory effects of anticancer drugs on certain immune activities. Materials and Methods: The in vitro effect of 5-(FU) fluorouracil was studied on both lymphocyte proliferation and natural killer (NK) cell antitumor cytotoxicity following incubation of peripheral mononuclear cells (PBMCs) from healthy donors with interleukin (IL)-2, phytohemagglutinin A (PHA) and pokeweed mitogen (PWM). Results: Activation of PBMCs with IL-2, PHA and PWM in the presence of 250 and 2500 ÌM of 5-FU caused a marked decrease in both the induction of activated natural killer (ANK) cell cytotoxic activity and DNA synthesis, while 2.5 ÌM increased DNA synthesis by 195%, 58% and 222% for IL-2, PHA and PWM, respectively, more than cells cultured without the drug. No effect of 5-FU was noted on mature ANK cells. Conclusion: 5-FU exhibits diverse effects on lymphocyte proliferation and on the generation of ANK antitumor cytotoxic activity.

5-Fluorouracil (5-FU) is a pyrimidine antimetabolite frequently used in the treatment of colorectal carcinoma and other malignancies of the digestive tract (1, 2). The target enzyme of 5-FU is thymidylate synthase, thus overexpression or mutation of thymidylate synthase (TS) is directly correlated with cancer cell resistance to 5-FU. The effect of 5-FU on tumor cells has been extensively studied (3-5) but its effect on immune responses, considering its suppressive effects on bone marrow cells (6), remains to be elucidated. Studies in mice revealed that the in vitro response of splenocytes to phytohemagglutinin (PHA) was markedly reduced in the presence of 5-FU and that this effect correlated with a decrease in the number of peripheral blood lymphocytes (7). In other animal studies, 5-FU was shown to affect natural killer (NK) cell-mediated cytotoxicity against tumor cells either directly, by increasing in vivo the number of NK cells, or indirectly, by rendering tumor target cells more susceptible to NK cell killing (8-9). Treatment of human lymphocytes with 5-FU was toxic toward both resting and PHA-IL2-activated lymphocytes (10). Similarly, administration of 5-FU to mice reduced the PHA responses of peripheral blood lymphocytes and splenocytes (7). In human studies, exposure of mononuclear cells to 1400 ÌM 5-FU reduced cell proliferation but had no effect on natural cell-mediated cytotoxicity (11). When tested in colorectal cancer patients, low dose leucovorin plus 5-FU did not alter the Th1/Th2 ratio, PHA response or the production of interleukin (IL)-6, IL-10 and soluble IL-2 receptors (12).

Activated NK cells (ANK), which are generated following incubation of human CD3+/CD16+ lymphocytes in IL-2 (13), are endowed with distinct antitumor, MHC-non-restricted activity (14). The induction of ANK cells is down-regulated by various cytokines, including transforming growth factor (TGF)-ß, prostaglandin E and IL-4 (15, 16). In addition, certain anticancer drugs, among them dacarbazine and carboplatin, substantially reduce the number of circulating CD16+ lymphocytes in the blood of melanoma patients (17). In order to reveal the possible effects of these drugs on ANK cell activity in a more controlled system, we now tested the effect of 5-FU on ANK cell generation and antitumor cytotoxic activity in vitro.

Materials and Methods

Preparation of peripheral mononuclear cells (PBMCs) and non-adherent cells. 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, Ficol, Pharmacia,
Uppsala, Sweden) and spun at 550g for 20 min. The cells that had 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 (17). For isolation of non-adherent cells, 5x10^6 PBMCs/5 ml CM were transferred to 10 mm Petri dishes and incubated at 37°C for 60 min. Non-adherent cells were collected, spun at 400g for 5 min and resuspended in CM at the desired density.

5-fluorouracil. 5-FU (1000 mg/20 ml) were purchased from Ebewe, Unterach, Austria and kept at room temperature in the dark at 50 mg/ml HBSS for up to 1 week.

Interleukin-2 (IL-2). Human recombinant IL-2 (Cetus, Emeryville, CA, USA) has a specific activity of 3x10^6 IU/mg protein and endotoxin levels below 0.5 ng/mg recombinant IL-2.

Lectins. PHA and PWM (Israel Industries, Beit-Haemek, Israel) were employed at 1:200 and 1:100 final dilution, respectively.

Proliferation assay. The proliferation assay was carried out as in (17).

PBMCs at 2x10^5/0.2 ml CM/well were incubated for 72 h in a 96-well round-bottom microplate with either 10^5 units/ml IL-2 or 1:200 PHA or 1:100 PWM, in the presence or absence of different concentrations of 5-FU, after which 2 μCi/20μl/well [3H]thymidine (Nycomed Amersham, Buckinghamshire, UK) was 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). In part of the experiments, DNA synthesis is expressed as stimulation index (SI), calculated as follows:

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SI = \frac{\text{Mean cpm of samples incubated in CM alone}}{\text{Mean cpm (3 wells) of samples incubated with mitogen with or without 5-FU}}
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Generation of ANK cells and cytotoxicity measurement. ANK cells were generated as described elsewhere (17). Briefly, 2x10^6/ml PBMCs in CM were incubated for 3 days with 1000 units/ml recombinant IL-2. The possibility of 5-FU inducing the inhibition of ANK cell generation was not related to the number of viable effector cells after incubation in IL-2 and 5-FU since no difference was seen in either the percentage of cell viability (Figure 1B) or total viable cell yield (Figure 1C) between the control culture without 5-FU and cultures with 2.5-2500 μM 5-FU.

Reduction in 5-FU-induced inhibition of ANK cell generated from non-adherent PBMCs. Macrophages had been shown to respond to anticancer drugs by delivering certain inhibitory factors, such as IL-6, transforming growth factor-β and prostaglandins (16, 18, 19). To analyze the possibility of 5-FU inducing the inhibition of ANK cell generation, both PBMCs and non-adherent cells were incubated for three days with 1000 U/ml IL-2 in the presence or absence of 5-FU at concentrations ranging from 2.5-250 μM. Incubation of PBMCs in 2.5-25 μM 5-FU had minimal effect on the generation of ANK cell cytotoxic activity (161.3 and 142.8 LU, respectively) compared to cells incubated in CM+IL-2 only (181.8 LU) (Figure 1A). Incubation of PBMCs in IL-2 together with higher concentrations of 5-FU (250 μM and 2500 μM) caused a marked reduction (of 67% and 96%, respectively) in ANK cell generation. The effect of 5-FU on the induction of ANK cells was not related to the number of viable effector cells after incubation in IL-2 and 5-FU since no difference was seen in either the percentage of cell viability (Figure 1B) or total viable cell yield (Figure 1C) between the control culture without 5-FU and cultures with 2.5-2500 μM 5-FU.

Results

Inhibition of ANK cell generation by 5-FU. PBMCs cultured in IL-2 for 3 days together with 5-FU at concentrations ranging from 2.5-2500 μM were tested for cytotoxic activity as described at the Material and Methods section. Incubation of PBMCs in 2.5-25 μM 5-FU had minimal effect on the generation of ANK cell cytotoxic activity (161.3 and 142.8 LU, respectively) compared to cells incubated in CM+IL-2 only (181.8 LU) (Figure 1A). Incubation of PBMCs in IL-2 together with higher concentrations of 5-FU (250 μM and 2500 μM) caused a marked reduction (of 67% and 96%, respectively) in ANK cell generation. The effect of 5-FU on the induction of ANK cells was not related to the number of viable effector cells after incubation in IL-2 and 5-FU since no difference was seen in either the percentage of cell viability (Figure 1B) or total viable cell yield (Figure 1C) between the control culture without 5-FU and cultures with 2.5-2500 μM 5-FU.

No effect of 5-FU on mature ANK effector cells. As shown in the previous experiments, 5-FU at 250 μM substantially inhibited ANK cell generation. PBMCs were cultured in 1000 U/ml IL-2 without 5-FU. After 3 days, the cells were collected and added to 51Cr-labeled Daudi cells in the absence (CM) or presence of different concentrations of 5-FU. As demonstrated in Figure 3, 5-FU at
concentrations of 2.5 μM and 25 μM had no effect on ANK cell cytotoxic activity (22.2 and 22.3 LU, respectively) compared to cultures lacking the drug (22.2 LU). The addition of 250 μM and of 2500 μM caused a minimal reduction of 13% and 15% in ANK activity (19.2 and 18.5 LU, respectively).

Effect of 5-FU on IL-2-induced DNA synthesis in PBMCs. We next tested the possibility that the reduction in the generation of ANK cell cytotoxic activity was due to inhibition of DNA synthesis. As shown in Figure 4, which summarizes 6 separate experiments, the addition of 5-FU increased DNA synthesis for 1670 to 2710 (62% increase) which was statistically significant (p>0.01).
Kinetics of 5-FU induced enhancement in DNA synthesis following activation with IL-2. To test the kinetics of 5-FU-induced enhancement in DNA synthesis we incubated PBMCs in IL-2 together with 2.5-2500 µM of 5-FU. As shown in Figure 5, at 24 h, DNA synthesis, expressed as mean cpm, was 172±19 in the control cultures (no 5-FU), whereas the mean cpm increased to 277±6 (p<0.01 compared to CM) and 268±49, respectively, in the presence of 2.5 µM and 25 µM 5-FU. At 250 µM and 2500 µM 5-FU, the mean cpm was 171±29 and 108±19, respectively. DNA synthesis in the control cultures was 1309±121 at 48 h, and it was 2453±181 (p<0.05 compared to CM), 1521±223 (p<0.05), 184±26 (p<0.005) and 97±40 (p<0.005) in the presence of 2.5, 25, 250 and 2500 µM 5-FU, respectively. Similarly, the mean cpm of the control cultures was 2811±240 at 72 h, and it was 5115±477 (p<0.05), 2221±208 (p<0.05), 92±29 (p<0.005) and 64±8 (p<0.005) cpm in cultures that were incubated together with 2.5, 25, 250 and 2500 µM 5-FU, respectively. These results show that 5-FU at 2.5 µM increased DNA synthesis in PBMCs by up to 182-187% following incubation in IL-2 and that this effect was retained at each time point tested.

5-FU increases DNA synthesis in PBMCs activated with PHA and PWM. Having showed that 5-FU increased DNA synthesis following incubation with IL-2, we tested the effect of other activators on 5-FU-induced proliferation. For that purpose, we incubated PBMCs for 72 h with either PHA or PWM and tested DNA synthesis as described above. As shown in Figure 6, IL-2 increased DNA synthesis in the presence of 2.5 µM of 5-FU, from 38.4 stimulation index (SI) to 74.7 SI, an increase of 95%. When the same concentration of 5-FU was employed together with either PHA or PWM, the SI index increased from 122.4 to 193.7 (58%) and from 69.2 to 153.4, (121%), respectively.

Discussion

Studies on cancer patients undergoing chemotherapy have shown inhibitory effects of anticancer drugs on various immune activities, including mitogen responses, cytokine secretion in the serum as well as on NK and ANK cell activities (17, 18).
To study the effect of anti-cancer drugs on the immune responses in a more controlled environment, we selected the in vitro effect of 5-FU on the generation and function of ANK cells following incubation of human PBMCs with IL-2. Our results demonstrated a dose-dependent effect of 5-FU on the induction of ANK cells. Whereas little or no effect was noted on ANK cell generation in the presence of 2.5 and 25 μM 5-FU, a marked reduction (of 68% and 96%, respectively) was noted at 250 and 2500 μM (Figure 1A).

Similar to our results, others have shown that 1400 μM 5-FU, a concentration comparable to serum Cmax levels in cancer patients (20), inhibited human lymphocytes response in vitro to PHA-stimulation, a response which was abrogated when combined with 500 units/ml interferon-α (7). No effect of 5-FU was noted on NK activity, however, in other in vivo (16, 21) and in vitro (9) studies. Moreover, when combined with IL-2, 5-FU augmented the generation of ANK cells that had been generated from PBMCs of advanced colorectal cancer patients (22). These opposing results for 5-FU effects on NK/ANK activities may be due to the concentrations of 5-FU employed, as was shown in our current study (Figure 1A).

The mechanism by which 5-FU exerts its inhibitory effect on ANK cell generation is not fully understood. One possibility is that 5-FU had a direct effect on DNA synthesis via its target thymidylate synthase (23). Our current results demonstrated that 250 and 2500 μM 5-FU led to a marked reduction (of up to 98%) in IL-2-induced DNA synthesis after 72 h in culture (Figure 5). Since neither the number of viable cells nor viability were altered after 3 days of incubation with 5-FU (Figure 1B-C), it is unlikely that the reduction in ANK cytotoxic activity was due to a reduced number in ANK cells. Interestingly, others have shown a significant reduction in the number of viable tumor cells following exposure to 5-FU in culture (11). The difference in sensitivity to 5-FU between ANK cells precursors and certain tumor cells (7, 9) could be due to the presence of relatively high thymidylate synthase activity in activated lymphocytes (24). The possibility that 5-FU exerted its effect in the current study by reducing cytotoxic enzymes in ANK cells following their activation by IL-2, or alternatively, by selectively affecting ANK cells precursors, is under investigation.

Another possible mechanism by which 5-FU acts on the induction of ANK cells may involve a non-direct effect. Our results support such a possibility since the effect of 5-FU on ANK cell generation was more prominent when whole PBMC populations (67% inhibition) rather than adherent cell-depleted PBMCs (36% inhibition) were employed (Figure 2). Others have shown that 5-FU activated tumoricidal activity of macrophages probably by enhancing TNF secretion (25). Since activated monocytes-macrophages were shown to secrete TGF-β together with other cytokines (16, 19) which down-regulated ANK cell activity (26-28), it is possible that under the conditions employed in our experiments activated adherent PBMC-derived monocytes partially inhibited ANK cells following exposure to 5-FU.

While 5-FU substantially inhibited the generation of LAK cells at concentrations of 250 μM and 2500 μM, there was no observable effect on mature ANK effector cell activity at concentrations ranging from 2.5-2500 μM 5-FU (Figure 3). Thus, these results imply a relatively higher sensitivity of ANK cell precursors to 5-FU than that of mature cells, similar to findings illustrated by others using bio-modulators, including TGF-β, IL-4 (29) IL-10 (30) and prostaglandins (31).

Unlike the effect of high concentrations of 5-FU on both IL-2-mediated DNA synthesis and the generation of ANK cell cytotoxic activity in PBMCs, there was a marked increase in DNA synthesis (of up to 195%) at a lower dose of the drug (2.5 μM, Figure 4), which had no effect on IL-2-induced generation of ANK cells (Figure 1). In addition, incubation of PBMCs with 5-FU at the described concentrations in the absence of IL-2 did not enhance DNA synthesis (Unpublished observation). One explanation for the increase in IL-2-induced DNA synthesis could have been that 5-FU had affected the kinetics of stimulation. This possibility was ruled out, however, by demonstrating that incubation of PBMC in IL-2 for 24-72 h at various concentrations of the drug had a similar effect on DNA synthesis at each tested time point (Figure 5). As such, the effect of 5-FU on IL-2-activated PBMCs was not due to a mere shift in DNA synthesis kinetics. Alternatively, the effect of 2.5 μM 5-FU on IL-2-induced DNA synthesis could be due to an increase in CD4+ lymphocytes secreting cytokines which synergize with IL-2 in inducing lymphocyte proliferation (32). Investigation of this possibility is currently underway.

The observed effect of 5-FU on DNA synthesis in IL-2-stimulated PBMCs was apparently not restricted to one mitogen since similar findings were obtained using PHA and PWM (known as T and B lymphocyte mitogens) instead of IL-2. Furthermore, similar to IL-2, the effect of 5-FU was most prominent in the presence of 2.5 μM of 5-FU (Figure 6).

Finally, we demonstrated opposing effects of 5-FU on both IL-2-induced DNA synthesis and the generation of anti-tumor cytotoxic activity in PBMCs of healthy individuals. Experiments designed to delineate the effect of 5-FU on PBMCs at the cellular and molecular levels from both healthy donors and cancer patients are also currently underway.

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References


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