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## EFFECT OF 5-FLUOROURACIL ON THE CELL GROWTH AND CELL CYCLE KINETICS OF A MOUSE ASCITES TUMOR GROWING IN VIVO

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### Abstract

The effect of 12, 24 and 36 mg/kg body weight doses of fluorouracil (5-FU) on the Bp8 ascites sarcoma growing in vivo was studied. From sequential studies of the total number of cells together with the composition of cells in the cell cycle, the cell cycle flow was calculated and correlated to the pharmacokinetics, which was determined by using  $^3\text{H}$ -5-FU. The dose of 12 mg/kg 5-FU affected cell growth between 24 and 72 hours, while the effect of higher doses was immediate. An early block in outflow of cells from  $G_1$  was followed by an increased outflow, indicating an early inhibition followed by an enhancement of the initiation of the DNA synthesis. This increased outflow from  $G_1$  together with the decrease in outflow from the early S-phase, i.e. decreased DNA synthesis, resulted in an accumulation of cells in the early part of the S-phase. The prolonged effects on the cell growth and the cell cycle flow despite the very fast decline in the drug concentration both in the ascites fluid and within the cells, together with a constant level of the drug in the macromolecular fraction, suggest an interaction between 5-FU and RNA/DNA at later times rather than an inhibition of the thymidylate synthetase activity.

**Key words:** Chemotherapy; 5-FU, mouse ascites tumor, cell growth, cell cycle kinetics.

Fluorouracil (5-FU) is an anticancer drug in clinical use for more than 25 years, and thus one of the most studied anticancer drugs. Many aspects of both the cellular and molecular pharmacology have been investigated, in vitro as well as in vivo. Cell kinetic studies have also been carried out, but with conflicting results (for review see 29). Thus, for in vitro growing cells, sensitivity to 5-FU was in some studies observed more or less in all cell cycle stages (2, 30), while other studies have shown an effect only on  $G_1$  and S-phase cells (17, 22). For in vitro growing cells, cell growth and cell loss were effected dose-dependently with a reduced growth rate up to 15 days, and increased cell loss up to at least 48 hours with the highest

dose (4, 11, 12, 20). A reduced number of  $G_2$  and mitotic cells was reported up to a few hours after administration of 5-FU (4, 11, 20) followed by an accumulation of  $G_1$  and S-phase cells as well as reduced cell flow from  $G_1$  to S (12, 20).

The aim of the present study was to describe the effects of 5-FU on the growth kinetics of in vivo growing Bp8 mouse ascites sarcoma cells. From changes in the cell growth and the cell cycle composition we calculated the cell flow from the different phases of the cell cycle. These results were correlated to the pharmacokinetics of the drug.

### Material and Methods

**Experimental tumor animals.** Bp8 ascites tumor cells were administered every 10th day by intraperitoneal injection into 3-month-old male NMRI mice with a body weight of 20 to 25 g. Water and standard food were given ad libitum. At the start of the experiment (day 0),  $20 \times 10^6$  cells were transplanted after appropriate dilution in 0.2 ml saline.

**Total number of cells, cell concentration and mitotic index.** The volume of ascites and the total number of tumor cells were measured indirectly by using an isotope dilution technique (21). 0.2 ml of  $^{125}\text{I}$ -labelled human albumin, specific activity: 5.6 MBq/mmol, after appropriate dilution with TRIS-NaCl-EDTA buffer (TRIS 0.1 mol/l, NaCl 0.07 mol/l, EDTA 0.005 mol/l, pH=7.5, 290 mOsm) containing 100 000 to 200 000 cpm, was injected intraperitoneally. Ten minutes after injection the animals were killed by cervical dislocation, the ascites cell suspension

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was removed and the activity in 0.025 ml was measured by a gamma spectrometer (Intertechnique CG 30). The ascites volume was calculated according to: ascites volume (ml) = ((inj. cpm-background/ascites cpm-background) × inj. volume) - inj. volume.

After estimating the cell concentration of nucleated cells by counting in a Bürker hemocytometer the total number of cells was estimated from the known ascites volume.

From DNA histograms the proportions, and the number of normal and tumor cells was estimated. For the evaluation of the mitotic index smears were prepared, fixed for 15 min in ethanol-aceton 3:1, stained with Harris hematoxylin and eosinophil solution and again fixed in ethanol. The index was estimated on 500 cells in each preparation.

**Cell cycle composition.** The cellular DNA content was measured using the rapid flow cytofluorometric method described previously (28). After correlation for background the proportion of cells in different phases of the cell cycle ( $G_1$ , early S, late S,  $G_2+M$ ) was determined from the areas of the histograms assuming a Gaussian function of the  $G_1$  and  $G_2+M$  maxima and attributing the remaining part of the DNA histograms to the cells of the S-phase, which was divided into two equal parts called early-S and late-S. Since the tumor cells contain more DNA than normal cells, the proportion of these normal cells has also been calculated from the histograms.

**Calculation of cell flow.** Details have been described and discussed earlier (26). In contrast to the well known labelled mitosis method for determination of cell cycle kinetics, the presented method can also be used under conditions of markedly disturbed cell flow. The method is based on the sequential calculation of the total number of cells together with the proportions of cells in the various parts of the cell cycle, determined by DNA measurement. In this way the outflow from the various cell cycle phases was calculated. In these calculations we assumed that no resting cells were present, as found in various ascites tumors (26). The cell outflow was related to the total number of cells in the corresponding compartments and expressed as %/h.

**Cytostatic treatment.** On day 4 after transplantation, 0.3, 0.6 and 0.9 mg of 5-FU (25 mg/ml), corresponding to about 12, 24 and 36 mg/kg body weight, was injected intraperitoneally into the mice in a volume of 0.2 ml after proper dilution with normal saline solution.

**Pharmacokinetics.**  $18.5 \times 10^4$  Bq  $^3\text{H}$ -5-FU (specific activity  $47.7 \times 10^{10}$  Bq/mmol), diluted with unlabelled 5-FU to a final 5-FU concentration of 0.6 mg/animal (final specific activity  $1.1 \times 10^{10}$  Bq/mmol) was injected intraperitoneally into mice at time 0. After 30 min, 1, 6, 12, 24, 48 and 72 hours the animals were killed by cervical dislocation and the ascites cell suspension removed. The cell suspension was centrifuged at 2000 rpm for 7 min. The ascites fluid was removed and the remaining cells were washed twice in 3.0 ml TRIS-NaCl-EDTA buffer. Twenty

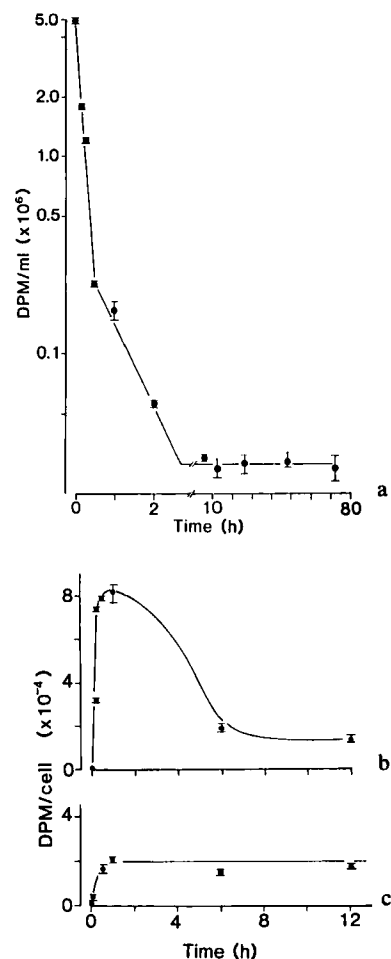


Fig. 1. Tritiated 5-FU activities in the ascites fluid (a) and in the soluble (b) and in the insoluble (c) perchloric acid fractions of the cells. A 5-FU dose of 0.6 mg/animal with a predetermined amount labelled with tritium ( $^3\text{H}$ -5-FU) was injected intraperitoneally on day 4 after transplantation. The mean values  $\pm$  SEM of 4 experiments are shown.

million cells were extracted with 2.0 ml 0.2 mol/l perchloric acid and stored for 30 min in ice water. They were then centrifuged at 10000 rpm for 5 min at  $4^\circ\text{C}$ . The acid soluble fraction was removed and the remaining precipitation was again washed in 1.0 ml 0.2 mol/l perchloric acid, centrifuged at 10000 rpm for 5 min at  $4^\circ\text{C}$ , and dissolved in 0.6 ml 0.5 mol/l NaOH at room temperature. The activity in the ascites fluid, the acid soluble and acid insoluble fractions were measured after dilution in 5 ml scintillation fluid (Packard scintillator 299). The results were expressed as dpm.

## Results

**Pharmacokinetics of 5-FU in ascites sarcoma.** In order to establish the pharmacokinetics of 5-FU,  $^3\text{H}$ -5-FU was injected intraperitoneally into mice at a final 5-FU dose corresponding to 0.6 mg/animal. The amount of  $^3\text{H}$ -5-FU

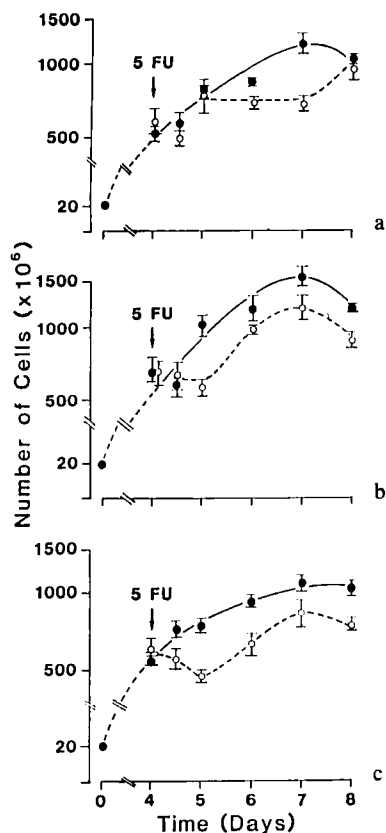


Fig. 2. Growth curves of ascites tumor, untreated (●) and treated (○) with 0.3 (a), 0.6 (b), and 0.9 (c) mg 5-FU/animal. The drug was injected intraperitoneally on day 4 after transplantation. The curves represent the mean values  $\pm$  SEM of one to 3 experiments with 3 to 5 mice at each point. The growth curves were calculated by computer according to 'best fit'.

in the ascites fluid decreased in a biphasic way with a rapid decline the first 30 min ( $T_{1/2}=7$  min) followed by a slower decline between 30 min and 2 hours ( $T_{1/2}=37$  min), and thereafter it was constant at a level of about 0.5 per cent of the amount injected to the end of the observation period of 72 hours (Fig. 1 a).

In the acid soluble and insoluble fractions of the cells the amount of  $^3\text{H}$ -5-FU reached maximum values after about 30 min.

The amount of  $^3\text{H}$ -5-FU in the insoluble fraction was unchanged for 12 hours while the amount in the soluble fraction declined to about 20 per cent of the maximum value after 6 hours and remained at this level for at least 12 hours (Fig. 1 b, c). The amount of  $^3\text{H}$ -5-FU in the insoluble fraction started to decline after 12 hours with a  $T_{1/2}$  of about 35 hours (data not shown).

**Influence of 5-FU on cell growth on ascites sarcoma cells.** The number of untreated tumor cells increased up to day 7 and then reached a plateau of growth (Fig. 2). The host animals started to die around day 15.

After intraperitoneal injection of 0.3, 0.6 and 0.9 mg 5-FU/animal, the total number of cells was determined at 0,

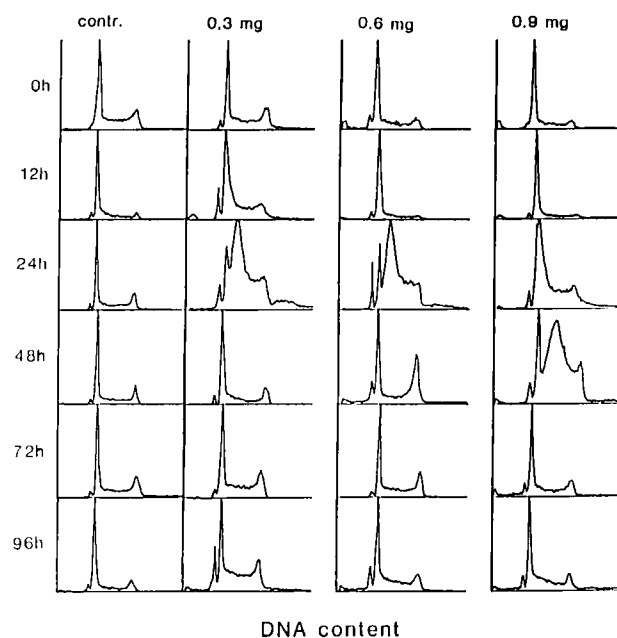


Fig. 3. Representative DNA histograms of ascites sarcoma cells untreated and treated with 0.3, 0.6 and 0.9 mg 5-FU/animal. The drug was injected intraperitoneally on day 4 after transplantation. The peak to the left corresponds to normal mouse cells. For the untreated cells the largest peak, i.e. the second peak from the left, corresponds to the  $G_1$  and the peak to the right corresponds to the  $G_2$  cells of the tumor. The S-phase is shown as the area between the  $G_1$  and the  $G_2$  peaks.

12, 24, 48, 72 and 96 hours. For the dose of 0.3 mg/animal there was no change in cell growth until after 24 hours. Between 24 and 72 hours there was no net change in total cell number, and after 72 hours the cells started to regrow and at 96 hours reached the level of the controls. A dose-dependent decrease in the growth rate was seen as early as 12 hours following administration of 0.6 and 0.9 mg/animal dosages. Regrowth started between 24 and 48 hours following treatment. The number of cells in the groups treated with 0.6 and 0.9 mg 5-FU/animal did not reach the number of cells in the controls.

**Distribution of cells in the cell cycle of ascites sarcoma cells.** Examples of DNA histograms from cell samples at different times after exposure of different concentrations of 5-FU are shown in Fig. 3. Mean values for cell cycle distribution and the proportion of mitotic cells are depicted in Fig. 4. The S-phase has been subdivided into an early and late part. The proportion of  $G_2$  cells has been calculated by subtracting the proportion of mitotic cells from the  $G_2 + \text{M}$  phase shown in the DNA histograms.

The changes in the cell cycle composition following injection of 5-FU was similar for the various doses given with an accumulation of cells in early S-phase. However, at the dose of 0.9 mg 5-FU/animal the accumulation of cells in early S-phase was more prolonged over time along with an extended decrease of the percentage of cells in the

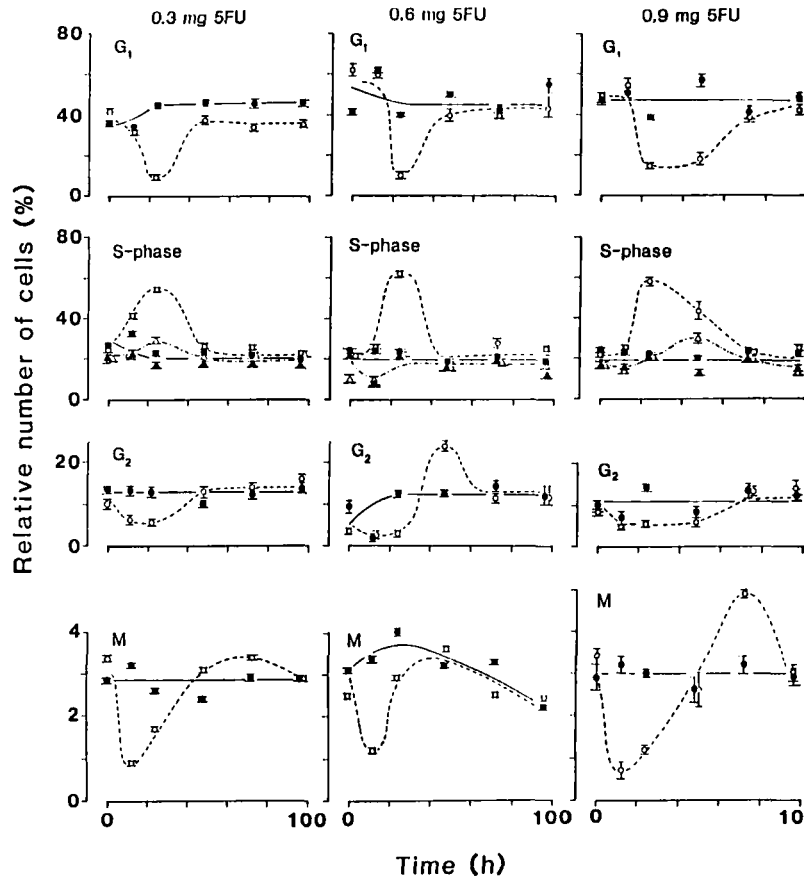


Fig. 4. Proportion of ascites cells in the various compartments on the cell cycle at different observation times after treatment with 0.3, 0.6 and 0.9 mg 5-FU/animal, treated (○) and untreated (●)

cells. The S-phase was divided into an early (○) and late (△) part. The proportion of  $G_2$  cells was calculated by subtracting the mitotic cells from the  $G_2 + M$ -phase.

$G_1$ - and  $G_2$ -phases. At the dose of 0.6 mg 5-FU/animal there was an accumulation of cells in the  $G_2$  phase not seen at that time period for cells treated with the other doses. The increased number of  $G_2$  cells at 48 hours for the dose of 0.6 mg 5-FU/animal, is due to the flow rate for cells synchronously released from the S-phase reaching the  $G_2$  phase at 48 hours. Minimum values for the mitotic cells were reached around 12 hours followed by an increase around 72 hours. This increase was not seen for the cells treated with 0.6 mg/animal. Except for the mitotic cells, the cell cycle composition was normal after about 72 hours.

*Calculated flow of cells through the cell cycle of ascites sarcoma cells after treatment of 5-FU.* Our calculations on cell flow, shown in Fig. 5, are expressed as the outflow of cells from various cell cycle compartments in relation to the number of cells in the corresponding compartments.

The cell outflow rate from the various cell cycle phases of the untreated cells declined gradually during the time interval studied (data not shown).

For cells treated with 0.3 mg 5-FU/animal the outflow

rate from  $G_1$  was already increased at 0 to 12 hours as compared with the controls, reaching a maximum outflow rate between 12 and 24 hours followed by a decrease in outflow with an outflow rate less than 50 per cent of the normal values (Fig. 5a). The outflow rates from the  $G_1$  phase of cells treated with 0.6 and 0.9 mg 5-FU/animal were similar and reduced in the beginning of the observation period followed by an increased outflow to a maximum outflow rate between 24 and 48 hours before returning back to more or less normal values (Fig. 5a).

The cell outflow from the early S-phases of cells treated with 0.3 mg 5-FU/animal was reduced by about 50 per cent between 0 and 48 hours, and more than 50 per cent between 48 and 72 hours. For the late S-phase the initial cell outflow was also inhibited by about 50 per cent but only up to 24 hours, followed by a normal outflow at 24 and 48 hours which again was decreased between 48 and 72 hours. This time to almost zero per cent (Fig. 5b, c). The outflow rate of cells treated with 0.6 mg/animal was markedly inhibited (80–100%) up to 24 hours from both the early and late S-phases. This was followed by a stimulated outflow rate, which again was followed by an ap-

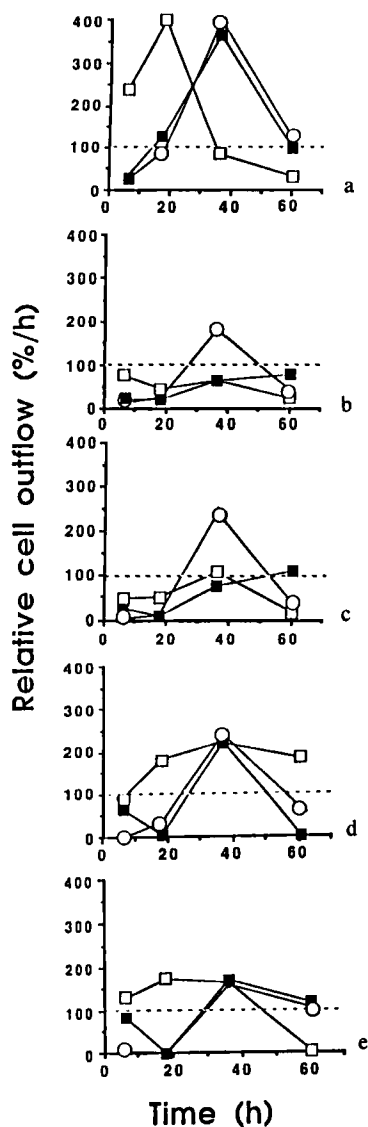


Fig. 5. Relative mean cell outflow from the various cell cycle phases of ascites tumor cells treated with 0.3 ( $\square$ ), 0.6 ( $\circ$ ) and 0.9 ( $\blacksquare$ ) mg 5-FU/animal. Values are depicted as per cent of flow of the untreated cells. The drug was injected intraperitoneally on day 4 after transplantation. The S-phase was subdivided into two parts, from the beginning to the middle of S ( $S_1$ ) and from the middle to the end of S ( $S_2$ ). The proportion of  $G_2$  cells was calculated by subtracting the mitotic cells from the  $G_2 + M$ -phase. The curves represent the mean values of one to 3 experiments with 3 to 5 mice at each point. a)  $G_1$ , b)  $S_1$ , c)  $S_2$ , d)  $G_2$  and e) M outflow.

proximately 50 per cent decrease (Fig. 5 b, c). At the dose of 0.9 mg 5-FU/animal the cells in both early and late S-phase increased their outflow rate gradually to almost normal values at the end of the observation period (Fig. 5 b, c).

From the  $G_2$  phase the outflow rate for the cells treated with 0.3 mg 5-FU/animal, was initially normal and thereafter increased for the rest of the observation period (Fig.

5 d). Cells treated with 0.6 mg/animal were almost totally depressed in the outflow from the  $G_2$  phase between 0 and 24 hours, followed by a stimulated outflow between 24 and 48 hours ending with an outflow rate of about 50 per cent compared with the controls at 48 and 72 hours (Fig. 5 d). For the highest dose, the outflow rate from the  $G_2$  phase was initially just below normal decreasing further to zero between 12 and 24 hours followed by an increased outflow between 24 and 48 hours and then decreasing back to zero between 48 and 72 hours (Fig. 5 d).

The outflow from M was stimulated between 0 and 48 hours for the lowest dose but was reduced to zero at the end of the observation (Fig. 5 e). The outflow rates from M for the higher doses were similar to those from the  $G_2$  phase except for between 48 and 72 hours where the outflow rate from M was more or less normal (Fig. 5 e).

### Discussion

In the present study we used a mouse ascites tumor which has been characterized earlier according to cell growth and cell cycle flow (7).

The effect on the cell growth following the various doses of 5-FU was similar. However, it was unexpected that the cell growth following the higher doses of 5-FU (0.6 and 0.9 mg/animal) was normalized faster than after treatment with the lower dose of 0.3 mg 5-FU/animal (Fig. 2).

The cell flow calculations describe the outflow rate from each phase between two observations. Depending on the observation intervals, some differences in the flow rates between the different doses might be explained by the stimulated or depressed outflow from the various phases of the cell cycle occurring at different times, with various durations for the different doses of 5-FU. The most interesting information from the cell flow calculation concerns the effect of 5-FU on the DNA synthesis. Thus, the outflow from  $G_1$  and S-phase gives information about the initiation of the DNA synthesis in  $G_1$  cells and the synthesis rate in S-phase cells, respectively. We found that both the initiation and the synthesis rate of DNA were inhibited immediately after adding 5-FU regardless of the dose given. However, while the synthesis rate of DNA was still partly inhibited up to 4 days, except for the middle dose between 24 and 48 hours, the initiation of DNA synthesis in  $G_1$  cells was markedly stimulated between 12 and 48 hours. The stimulated outflow from  $G_1$  and the reduced synthesis rate of DNA in early S-cells resulted in the accumulation of early S-phase cells found at 24 hours (Fig. 4). Thus, while 5-FU inhibits the synthesis rate of the DNA, the initiation of DNA synthesis in  $G_1$  is apparently stimulated.

The mechanisms of 5-FU on the molecular level have been the subject of a number of studies. There are three main theories: 1) Inactivation of thymidylate synthetase prevents de novo synthesis of dTMP resulting in inhibi-

tion of the DNA synthesis (9, 16, 18, 19, 23–35). 2) A direct effect on DNA might take place as FdUTP is likely to be synthesized intracellularly and consequently incorporated into DNA. The significance of this possible incorporation has not been well explored (5, 8, 9, 10, 13). 3) Incorporation into RNA with production of non-functional RNA and/or defect protein synthesis (3, 6, 13, 14, 27). Based on our results, it may be possible to explain the immediately depressed DNA synthesis rate by an inactivation of thymidylate synthetase. The prolonged inhibition of the DNA synthesis rate found for up to 4 days could be explained by other mechanisms since the concentration of 5-FU in the ascites fluid decreased from about 1 mmol/l to about 1  $\mu$ mol/l and the intracellular concentration from 0.2  $\mu$ mol/l to 0.02  $\mu$ mol/l within 6 hours. Even though it has been shown for rodent colon adenocarcinoma that very low concentrations of FdUMP are needed in order to inhibit thymidylate synthetase activity (1), this might not be the case in other cell lines. The amount of 5-FU incorporated into the macromolecular fraction was, however, constant for at least 12 hours, at a dose of about  $3 \times 10^{-18}$  mol/cell. The decline in the amount after 12 hours corresponded to a cell doubling time of about 30 hours. Thus, the effect on the DNA synthesis rate at these later observation times is therefore probably due to interactions between 5-FU and DNA/RNA rather than changes in the activity of the thymidylate synthetase. Furthermore, calculation of the relative cell flow from G<sub>2</sub> indicates an effect up to 24 hours on the G<sub>2</sub> cells from the higher 5-FU doses. If 5-FU acts via RNA metabolism resulting in a depressed protein synthesis rate and/or mis-coded proteins it is hard to explain the normal or stimulated outflow found after 24 hours. This is also true for the events responsible for the initiation of the DNA synthesis in G<sub>1</sub> cells. Thus, besides the mechanisms of action of 5-FU which have been discussed above, other intracellular processes important for cell growth seem also to be affected and changes in the flow rate from various parts of the cell cycle after 24 hours might be explained as the normal behaviour of an affected cell population attempting to restore balanced growth.

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