Cytochemical Effects of 5-Fluorouracil on Sensitive and Resistant Ehrlich Ascites Tumor Cells

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5-Fluorouracil was developed by Heidelberger, Duschinsky, and associates (10, 14). It was shown by Heidelberger et al. that 5-fluorouracil (5-FU) inhibits the growth of transplanted tumors in rats and mice (14). In clinical trials, regression of some human cancers after treatment with 5-FU has been reported (7). One of the biochemical effects of this series of fluorinated pyrimidines was the inhibition of deoxyribonucleic acid (DNA) synthesis by blocking the formation of the methyl group of thymine (1, 4, 8, 14, 15).

Quantitative histochemical studies of tumor cells treated with 5-FU were made to attempt a correlation of histochemical results with biochemical findings and to study the morphology of these tumor cells after such treatment. Preliminary findings were reported earlier (23).

MATERIALS AND METHODS

Tumors.—A hypotetraploid line of Ehrlich ascites tumor was used.1 At present the chromosomal mode of this tumor is 75-76, and the percentage of polyploids, 8 per cent. Its chromosomal constitution is similar to that described by Levan and Hauschka (18, 21). The growth characteristics of this hypotetraploid line were similar to those reported by Klein and Révész (17).

Animal experiment.—Groups of four female Swiss mice (Taconic Farms) bearing this tumor were given injections intraperitoneally of 5-FU, 18 mg/kg/day for 7 days, beginning 1 day after tumor inoculation. This dosage is less than the maximum effective dose, 25 mg/kg (14), but was used so that some cells would remain for examination at the end of the treatment period. For each experiment a group of control mice bearing the same tumor was injected with saline.

The mice were weighed daily during the treatment period in order to estimate the extent of tumor growth.

In addition, hypotetraploid 5-FU-resistant Ehrlich ascites tumor cells were studied. Resistance to 5-FU was produced as follows: The drug (20-35 mg/kg/day) was given for 7 days for four generations, no treatment for four generations, 25-35 mg/kg/day for six generations, then no treatment for eleven generations. The resistant mice were then divided into two groups—a control group receiving no 5-FU and the other group receiving 25 mg/kg/day for 7 days, beginning 1 day after tumor transplantation. Resistance has been carried for 122 generations in this manner.2 For the experiments with the resistant line, groups of mice bearing this tumor were treated as usual and the control and treated cells compared with each other.

Cytological technic.—At the end of the treatment periods, ascitic fluid was withdrawn from each treated and control mouse. Cell counts were made for each mouse with a hemacytometer. Smears of ascitic fluid were made and fixed while wet in Carnoy's fixative. After fixation, the smears were stained for evaluation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and basic groups of protein. The Feulgen reaction (28) was applied to demonstrate DNA. For this stain, mouse liver and kidney squash preparations were stained simultaneously with the slides of tumor cells to determine the intensity of the stain and to establish a reference system for diploid DNA values (29). For RNA, slides were stained with Azure B in a concentration of 0.25 mg/ml at pH 4.0 according to the method described by Flax and Himes (19). Since this dye stains both DNA and RNA, the DNA was extracted prior to staining with deoxyribonuclease, with a solution of 1 mg/ml at 37°C for 1 hour. For basic groups of proteins, Naphthol Yellow S (NYS) in a 1 per cent aqueous solution at pH 8.78, the method developed by Deitch (9), was used. All slides, including the mouse liver and kidney squashes, were stained together.

Chromosome counts.—Chromosomes were counted in squash preparations of control and 5-FU-treated tumor cells which were fixed and stained with aceto-orcein (21) or aceto-gentian violet solutions.3

Histological study.—Large numbers of randomly selected tumor cells were scanned for the purpose of observing differences, in the control and treated tumor cells, in nuclear and cell size, morphological changes, and number of cells per unit area of the microscopic fields.

Quantitative histochemistry.—Quantitative histochemical evaluation of DNA, RNA, and protein was carried out using photographic photometry as described by Caspersson (2, 8). For photomicroscopy, the lamp and camera were aligned on the optical axis of the microscope. The aperture of the microscope condenser was reduced to 0.4 to reduce glare. As a light source a tungsten ribbon filament lamp aligned in Köhler illumination was used. Monochromatic light was isolated by interference filters; wave-lengths of light used were as described for qualitative histochemical procedures. Kodak's Verichrome films (18) were used and the emulsions calibrated with a step wedge. Ex-

1 Carried for several years in Swiss mice in the Mc Ardle Memorial Laboratory and kindly supplied to us by Prof. Heidelberger.

2 C. Heidelberger, unpublished information.

3 The chromosome analyses were kindly done by Mr. James Hodgett and Mr. Donald Schwab.
very pale, and the DNA was arranged in irregular networks, as can be seen in Figure 1. The nuclei of control tumors ranged in diameter from 7 to 11 μm and showed a delicate and fine chromatin texture. A marked decrease in relative size of nuclei of 5-FU-treated cells, which usually appeared about the same in treated and control cells. However, with 5-FU treatment, there was an increase in RNA per cell (Figs. 5 and 6). The nucleoli were smaller in the treated group (Fig. 6). The nucleoli were markedly increased in size after 5-FU treatment.

Qualitative histochemistry.—Randomly selected control of 5-FU-treated tumor cells were scanned for differences in intensity of the Feulgen, Azure B, and NYS stains. This preliminary survey yields quick information, as the eye can judge differences in stain intensity with rather high accuracy. This procedure considerably enlarged the numbers of cells sampled.

DNA evaluation: Figures 1 and 2 demonstrate the differences in intensity of the Feulgen stain. They were photographed with a 550 mμ wavelength light. In scanning large numbers of randomly selected nuclei, those of the control tumor cells generally showed greater stain intensity than the nuclei of 5-FU-treated cells, which usually appeared larger and very pale. This was found on microscopic examination of many fields. On each slide there were mouse lymphocytes whose stain intensity served as a valuable control for possible errors due to uneven staining. It was observed that the lymphocytes from slides of control and treated tumor cells showed about the same stain intensity; therefore, it could be assumed that the staining for all slides was uniform.

RNA evaluation: Examination for RNA showed about the same Azure B stain intensity of randomly selected treated and control cells. However, since the treated cells were so much larger than the controls, it appeared that, with 5-FU treatment, there was an increase in RNA per cell (Figs. 5 and 6).

Protein evaluation: The control cells (Fig. 7) showed smaller than the 5-FU-treated cells (Fig. 8). The intensity of the Naphthol Yellow-S stain appeared about the same in treated and control cells. However, the increase in cell size suggested an increase in relative protein per cell.

Histological examination.—Feulgen-stained nuclei of the control tumors ranged in diameter from 7 to 12 μm and showed a delicate and fine chromatin network, as can be seen in Figure 1. The nuclei of treated cells as shown in Figure 2 were enlarged, very pale, and the DNA was arranged in irregular clumps and strands around vacuoles, indicating a changed arrangement of DNA within the nucleus.

Nuclei of untreated resistant tumor cells (Fig. 3) did not differ morphologically from the 5-FU-treated resistant tumor cells (Fig. 4), nor did they seem to be different from the untreated sensitive tumor cells.

Examination of Azure B-stained slides showed that the control cells (Fig. 5) were smaller than the 5-FU-treated cells (Fig. 6). The nucleoli were markedly increased in size after 5-FU treatment.

Randomly selected areas of treated tumor cells showed a marked decrease in cells per unit area (Figs. 2 and 6), as compared with areas of control cells (Figs. 1 and 5), demonstrating inhibition of tumor growth after 5-FU treatment.

RESULTS

Weights.—The mice treated with 5-FU showed less weight gain than the controls, whose increased weight can be attributed to growth of the tumor and its associated ascites; weight gains for the treated mice averaged 65 X 10^6 per tumor, while the treated mice averaged 10^6 per tumor. The mitotic index of tumor cells was reduced about the same Azure B stain intensity of randomly selected areas. However, since the treated cells were so much larger than the controls, it appeared that, with 5-FU treatment, there was an increase in RNA per cell (Figs. 5 and 6).

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indicate different DNA classes ranging from tetraploid to octaploid. Lymphocytes served as diploid markers. When comparing 50 treated nuclei with 50 control nuclei, those treated showed only about half as much relative amount of DNA per cell as the controls (Table 1). Chart 1 shows a frequency histogram of these cells. As mouse liver cells and mouse lymphocytes contain a known amount of DNA, they were taken as a reference system for relative DNA values (29, 30). The control and treated cells were grouped according to this system. The distribution of the untreated cells is similar to that described by Leuchtenberger (20) and Richards (26). Most of the treated tumor cells showed values between diploid and tetraploid, with no octaploid values and only a few intermediates between tetraploid and octaploid. From this inhibition of DNA synthesis by 5-FU is confirmed (1).

Examination of resistant treated and untreated tumor cells showed about a 6 per cent increase in the relative amounts of DNA of treated cells as compared with those of the controls. A frequency histogram (Chart 2) shows a relative decrease of tetraploid cells, together with a slight increase in octaploid cells in the treated group.

**TABLE 1**

| EHRlich ASCITES TUMOR CELLS TREATED WITH 5-FLUOROURACIL 18 MG/KG/DAY FOR 6 DAYS |
|----------------------------------|----------------------------------|
| **Data represent averages of five experiments of eight mice each, expressed in arbitrary units** |
| **Control** | **Control** |
| tumor cells | tumor cells |
| Total no. | 460x10^3 | 65x10^3 |
| DNA per cell | 16.6 (10.2–24.8)* | 7.1 (2.16–18)* |
| Total DNA | 76.2x10^8 | 4.6x10^8 |
| RNA per cell | 77.0 (40.8–146.7)* | 119.0 (53.7–342.4)* |
| Total RNA | 85.4x10^8 | 77.7x10^8 |
| Protein per cell | 72.0 (26.1–122.9)* | 108x10^8 |
| Protein per cell | 381x10^8 | 108x10^8 |
| Mitotic rate | 6.2 | 1.1 |

* Figures in parentheses represent ranges.

**Histochemical RNA quantitation.**—Figures 5 and 6 are prints of negatives used for RNA quantitation obtained by photographing Azure B-stained cells with 604 nm wavelength light. The treated tumor cells showed about twice as much RNA per cell as did the controls (Table 1). Chart 3 shows the RNA frequency histogram. In contrast to the decrease in DNA, there were many 5-FU-treated cells with higher relative RNA values.

**Histochemical protein quantitation.**—This evaluation was done on Naphthol Yellow-S-stained slides with 435 nm wavelength light. The protein per cell after 5-FU treatment was about twice that of the control cells (Table 1). Figures 7 and 8 are prints of negatives used for this protein quantitation. Chart 4 shows a frequency histogram of protein quantitation and demonstrates that many of the treated cells had higher relative protein values, in contrast to the decrease in DNA.

**DISCUSSION**

Quantitative histochemistry was developed by Caspersson, using ultraviolet light for light absorption measurements in single cells under the light microscope (2, 3). Pollister and Ris (25) developed improvements in absorption measurements with light of the visible spectrum. This method consisted of staining tissues with the Feulgen reaction (11), which was found to be specific for DNA (19, 25, 29). The Feulgen reaction can be used for histochemical quantitation of DNA, since it has been shown that light absorption of Feulgen-stained
nuclei follows Beer-Lambert’s law closely when examined under the microscope with monochromatic 550-mλ wavelength light (25, 30). According to Ris and Mirsky (27) there is agreement within 10 per cent between chemical determination of DNA and microspectrophotometric evaluation of Feulgen-stained nuclei. Flax and Himes (12) have found that, for Azure B staining for RNA, Beer-Lambert’s Law is valid.

For basic groups of protein, Deitch (9) has demonstrated that the anionie dye Napthol Yellow S can bind these groups stoichiometrically and reproducibly, thus making this reaction suitable for quantitation.

While Pollister and Ris (25) measured light absorption with “photoelectric” photometry, in our study we used “photographic” photometry, as described by Caspersson (2, 3). This technic is based on the principle that densities of exposed photographic emulsions are logarithmic functions of light absorption. The accuracy of this method depends on rigid control of the procedures of photographic densitometry (16). Photographic photometry has the particular advantage of providing a permanent record, as well as making it possible to study the morphology in detail. This method also permits the use of lower-intensity sources of radiant energy (24).

Using this method, we found diploid DNA values for lymphocytes and diploid, tetraploid, and octaploid values for mouse liver, as well as for the untreated tumor nuclei. Many nuclei of untreated cells showed some tetraploid, intermediate, and octaploid DNA values, which is consistent with Swift’s findings of DNA doubling during inter-phase (29). After 5-FU treatment many of the tumor cells showed only half as much DNA per nucleus as compared with the controls. This should probably be interpreted as owing to blocking of DNA synthesis rather than a shift to a lower ploidy. This conclusion was substantiated by chromosome analysis, which showed no change in the average number of chromosomes in treated cells as compared with untreated samples. The chromosomal mode of control and treated groups was 75-76. These findings suggest cell division without concomitant DNA synthesis having occurred. These DNA findings are confirmed by biochemical studies in which almost complete inhibition of the conversion of formate into the methyl group of thymine was found by Heidelberger et al. (14). Inhibition of DNA synthesis explains the decrease in
DNA was observed in the 5-FU-resistant strain of regularly distributed in coarse clumps and strands Ehrlich ascites tumor cells when treated with the drug and compared with untreated cells.

The RNA and protein per cell in the 5-FU-treated tumor cells was increased when compared with the untreated controls.

The morphological changes resulting from 5-FU treatment of the tumor cells are about the same as those described for early cell death (22, 31).

Cohen and Barner have described similar findings in their experiments on thymine-less cell death (3, 6), when thymidine analogs were substituted for thymine in their thymine-requiring strain of Escherichia coli. With this thymine-less cell death, mutants of E. coli bacteria enlarged or became filamentous, DNA synthesis was inhibited, while RNA and protein synthesis continued. They also stated that this “unbalanced growth” produced killing of the bacteria (cell death). It is interesting to note the similarity of the mechanism of cell death in bacteria and Ehrlich ascites tumor cells.

SUMMARY

5-Fluorouracil at 18 mg/kg/day for 7 days inhibited the growth of the Ehrlich ascites tumor at a ratio of 7:1. The tumor cells decreased from 460 million in controls to 65 million in the treated mice. The mitotic rate was decreased from about 6.2 in the controls to 1.1 in the treated cells. The quantitative histochemical analysis showed only about half as much DNA per cell in the treated tumor cells when compared with saline-treated controls. This decrease was from an average of 16 units of relative amounts of DNA per cell to 7 units. There was an increase of about 6 per cent of RNA and protein per cell in the 5-FU-resistant cells as compared with untreated resistant tumor cells. The relative amounts of RNA and protein per treated tumor cell, however, increased to about double that found in the control cells. Associated with the biochemical changes there were these distinct morphological changes:

1. After treatment with 5-FU, the cells enlarged with increasing amounts of protein and RNA.
2. DNA, as seen with the Feulgen stain, was irregularly distributed in coarse clumps and strands around vacuoles.
3. The morphological picture was that of early cell death.

In contrast to this differentiated cells such as lymphocytes and leukocytes were not affected.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1.—Feulgen-stained Ehrlich ascites tumor nuclei (untreated), measuring from 7 to 12 μ. Nuclear membranes and fine network of DNA granules and filaments are well demonstrated. t, nucleus with tetraploid DNA value; o, nucleus with octaploid DNA value; d, lymphocytes with diploid DNA values; and m, nucleus in anaphase. Photographed with 550 mμ wavelength light, ×1000.

Fig. 2.—Shows Feulgen-stained Ehrlich ascites tumor nuclei after treatment with 5-FU. These nuclei are much larger, measuring from 9 to 26 μ, than with less stain intensity than the control nuclei of Figure 1. The DNA is seen in irregular clumps and strands around vacuoles; nuclear membranes are intact. Several leukocytes (d) are present with diploid values. Note that there is a reduction in numbers of nuclei demonstrating tumor growth inhibition. Photographed with 550 mμ wavelength light. The nuclear membranes have been outlined faintly to show the borders of the nuclei more clearly. ×1000.

Fig. 3.—5-FU-resistant Ehrlich ascites tumor nuclei, untreated. Photographed with 550 mμ wavelength light. ×1000.

Fig. 4.—5-FU-resistant Ehrlich ascites tumor nuclei, treated. Photographed with 550 mμ wavelength light. ×1000.


Fig. 1.--Feulgen-stained Ehrlich ascites tumor nuclei (untreated), measuring from 7 to 12 μ. Nuclear membranes and fine network of DNA granules and filaments are well demonstrated, t, nucleus with tetraploid DNA value; o, nucleus with octaploid DNA value; d, lymphocytes with diploid DNA values; and m, nucleus in anaphase. Photographed with 550 mμ wavelength light. X 1000.

Fig. 2.--Shows Feulgen-stained Ehrlich ascites tumor nuclei after treatment with 5-FU. These nuclei are much larger, measuring from 9 to 86 μ, but with less stain intensity than the control nuclei of Figure 1. The DNA is seen in irregular clumps and strands around vacuoles; nuclear membranes are intact. Several leukocytes (d) are present with diploid values. Note that there is a reduction in numbers of nuclei demonstrating tumor growth inhibition. Photographed with 550 μ wavelength light. The nuclear membranes have been outlined faintly to show the borders of the nuclei more clearly. X 1000.

Fig. 3.--5-FU-resistant Ehrlich ascites tumor nuclei, untreated. Feulgen stain, t, nucleus with tetraploid DNA value; m, mesothelial cells; m, mitoses. Photographed with 550 μ wavelength light. X 1000.

Fig. 4.--5-FU-resistant Ehrlich ascites tumor nuclei, treated. Feulgen stain. These cells are slightly larger than those in Figure 3 and are without any degenerative changes. Photographed with 550 μ wavelength light. X 1000.
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Fig. 1.—Ehrlich ascites tumor cells, untreated, stained with Azure B for RNA. DNA was removed with deoxyribonuclease. The size of the cells ranges from 12 to 18.5 μ in diameter. There is a dark rim of cytoplasmic RNA between nuclear wall (n) and cell wall (c). Within the nucleus there are several nucleoli (nu) which contain the nucleolar RNA; these measure about 4-5 μ in diameter. Photographed with 604 μ wavelength light. ×1000.

Fig. 2.—Ehrlich ascites tumor cells stained with Azure B after treatment with 5-FU. DNA removed with deoxyribonuclease. These cells are larger than the cells in Figure 1, while the stain intensity is about the same. Cell number 6 is about 3 times larger than cell number 6 of Figure 5. Cell number 6 shows a dark rim of cytoplasmic RNA between nuclear membrane (n) and cell wall (c). Within the nucleus the dark nucleolus (nu) containing increased amounts of nucleolar RNA is about 5 times larger than the nucleoli of the control cells in Figure 5; this nucleolus measures 10 μ to 17 μ in diameter. Photographed with 604 μ wavelength light. ×1000.

Fig. 3.—Control Ehrlich ascites tumor cells stained with Naphthol Yellow S for protein. The cells measure 12-14.5 μ in diameter. Nuclear membranes and nucleoli are not visible. Photographed with 45 μ wavelength light. ×1000.

Fig. 4.—Ehrlich ascites tumor cells treated with 5-FU and stained with Naphthol Yellow S. Some of these cells are enlarged, while the intensity of the stain is about the same as that of the control cells in Figure 7. Cell number 7 is about 4 times as large as cell number 13 of Figure 7. Photographed with 45 μ wavelength light. ×1000.

Note: Cells in these figures were numbered for densitometry.


**Figure 1**.--Feulgen-stained Ehrlich ascites tumor nuclei (untreated), measuring from 7 to 12 μ. Nuclear membranes and fine network of DNA granules and filaments are well demonstrated, t, nucleus with tetraploid DNA value; o, nucleus with octaploid DNA value; d, lymphocytes with diploid DNA values; and m, nucleus in anaphase. Photographed with 550 m/ wavelength light. X 1000.

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