Host Interactions in the Effects of 5-Fluorouracil on Ehrlich Ascites Tumor Cells

Kevin M. Connolly, R. Douglas Armstrong, Robert B. Diasio, and Alan M. Kaplan

ABSTRACT

5-Fluorouracil (5-FUra) inoculated i.p. into Ehrlich ascites tumor (EATU)-bearing mice inhibited in vivo tumor cell proliferation and generated a population of EATU cells with half their normal content of DNA. The appearance of aberrant (approximately 2C) EATU cells from a normally aneuploid (approximately 4C) tumor cell line appeared to require a radiosensitive host cell population, since prior irradiation of animals later given injections of 5-FUra and EATU cells resulted in significantly fewer 2C tumor cells when compared with unirradiated tumor bearers. In contrast, when EATU cells were pulsed with 5-FUra and cultured in vitro, no 2C peak could be detected. Furthermore, results from adoptive transfer experiments showed that EATU cells incubated in vitro with 5-FUra gave rise to far fewer aberrant 2C EATU cells when injected into irradiated, as compared to normal, animals. Cell cycle analyses of peritoneal exudate cells from irradiated and normal 5-FUra-treated EATU bearers showed that, in addition to possessing few or no 2C tumor cells of reduced DNA content, irradiated animals possessed, within the remaining aneuploid EATU population, a high percentage of cells in the G2-M phase. Therefore, in 5-FUra-treated EATU bearers, a host cell population appeared to be necessary for the tumoricidal induction of 2C tumor cells as well as the inhibition of proliferating G2-M cells within the remaining population of aneuploid tumor cells. Further experiments are being conducted to confirm preliminary observations of macrophage-mediated host cell participation in the generation of tumor cells of reduced ploidy and in the alteration in the cell cycle distribution of the surviving tumor cells. Generation of tumor cells of reduced ploidy may be the cause or the result of a host-mediated tumoricidal mechanism.

INTRODUCTION

The mechanism(s) by which tumor cells are destroyed by Mφ* has not been well defined, although theories have been proposed linking tumor cell lysis to Mφ-secreted arginase (14), neutral proteases (1), and hydrogen peroxide (50). More recently, Alabaster et al. (2) observed that a regimen of Adriamycin and cyclophosphamide in vivo not only killed L-1210 tumor cells but generated tumor cells possessing a DNA profile showing a significant decrease in the amount of DNA per tumor cell as analyzed by FMF. Furthermore, recent observations suggest that the antitumor activity of Adriamycin may in part be related to stimulation of tumoricidal host cells (45, 61). This prompted us to evaluate the role of the host response in the induction of EATU cells of reduced ploidy after 5-FUra treatment. Our results indicate that the short-term, bulk antitumor activity of 5-FUra was not dependent upon a radiosensitive population. However, a radiosensitive host cell population was required for both the induction of tumor cells of reduced ploidy and significant alterations in the cell cycle distribution of surviving tumor cells. Future experiments will determine whether long-term remission in our model is dependent upon generation of an effector host cell population and induction of tumor cells with a reduced DNA content.

MATERIALS AND METHODS

Mice. Adult female C57BL/6J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, or Flow Laboratories, Inc., Dublin, Va.

Culture Medium and Target Cell Types. Culture medium used in in vitro cytotoxicity experiments was Eagle’s spinner medium. “Complete” medium contained 100 units penicillin per ml, 100 µg streptomycin per ml, 3 mg glutamine per ml, 10⁻⁵ M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum. The target cell used in vivo was the EATU cell line. EATU cell cultures were serially passaged by weekly inoculation of 1 x 10⁶ cells into the peritoneal cavity of C57BL/6J or C3H/HeN mice. EATU cells used in vitro were cultured in complete medium at an initial density of 5 x 10⁶ cells/ml. Cells were grown in spinner flasks incubated at 37°C in a 5% CO₂ atmosphere. When cell density reached 2 x 10⁵ cells/ml, cultures were split and diluted to a concentration of 5 x 10⁵ cells/ml.

Tumoricidal Activity of 5-FUra in Vitro. EATU cells were incubated in complete Eagle’s spinner medium with or without 10⁻⁴ M 5-FUra.
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After a 2-hr incubation at 37°, cells were washed 2 times, resuspended in 250 ml of medium, and incubated in 500-ml spinner flasks at 37°. Fifty-ml samples were removed 24, 48, 72, and 96 hr after the initial incubation. Cells were counted, and a portion was analyzed by FMF for cell cycle distribution.

PEC. All PEC were removed from mice by lavage with 7 ml of Hanks’ balanced salt solution. To obtain peritoneal exudates from 5-FUra-treated tumor bearers, 1 × 10⁸ EATU cells were injected i.p. on Day 0. 5-FUra (20 mg/kg) was injected i.p. on Days 0, 1, 2, and 3. Animals were routinely killed on Day 4, unless otherwise indicated.

Antisera. Alloantisera against H-2° were obtained following i.p. inoculation of C3H/HeN mice with 2 × 10⁷ C57BL/6J spleen and thymus cells in complete Freund’s adjuvant. After 4 biweekly inoculations, mice were bled from the heart; sera were pooled and frozen in 0.2-ml portions at −70°. C57BL/6J anti-C3H/HeN antisera was prepared in a similar manner and provided the source of alloanti-H-2° antisum. Fluorescein-conjugated Fab₂ rabbit anti-mouse IgG was obtained from Cappel Laboratories (Cochrane, Pa.). Fluoresceinated avidin and biotin-conjugated monoclonal anti-H-2° antisum were purchased from Becton, Dickinson (Rutherford, N. J.). To minimize cross-reactivity between H-2 antisera and tumor targets, all sera used for immunofluorescence were absorbed 3 times against 2 to 5 × 10⁷ EATU cells grown in either C3H/HeN or C57BL/6J mice. Anti-H-2° was absorbed with EATU cells grown in C3H/HeN, and anti-H-2° was absorbed with EATU cells grown in C57BL/6J. Cells were incubated at 4° for 30 min with 0.2 ml of sera. At the end of the third absorption, EATU cells were removed, and the sera were centrifuged for 15 min at 12,800 × g to remove cellular debris and antigen:antibody complexes.

Preparation of Cells for FMF Analysis of Cell Cycle and DNA Content. To prepare samples for FMF analyses, all cells were washed twice, fixed in 70% cold ethanol, treated with RNase A (10 µg/ml, pH 7.0) for 30 min at 37°, washed, and stained with propidium iodide (6.9 × 10⁻⁵ M in 3.8 × 10⁻² sodium citrate, pH 7.0) for 30 min at room temperature, as described previously (13).

Cells stained with propidium iodide were analyzed and sorted with a Coulter Electronics TPS-1 or EPICS IV as described previously (33). Chart 1 is a schematic diagram of the cell cycle of normal diploid host cells (G₁ = 2C = 0.9 pg DNA per cell; G₂-M = 4C) and aneuploid tumor cells (G₁ = 4C; G₂-M = 8C). The dotted line of the 2C peak represents tumor cells which have a reduced DNA content.

Detection of Host Cells by Immunofluorescence. In cell cycle experiments designed to quantitate 5-FUra-treated tumor cells of reduced ploidy, it was necessary to ascertain what percentage of the diploid (2C) peak was composed of normal host cells and how much consisted of tumor cells of reduced DNA content. Since Ehrlich ascites cells have no known host H-2 markers (10), antisera directed against host H-2 antigens was used to show the degree of host cell contamination in the total peritoneal exudate population. Whole alloantisera (or biotin-conjugated monoclonal antibody) were absorbed, diluted 1:5, and incubated in a 0.1-ml volume with 2 × 10⁶ PEC for 30 min at 4°.

The cells were washed 3 times in 0.15 M phosphate-buffered saline (pH 7.2) with 0.1% NaCl, and incubated at 4° for 30 min with fluorescein-labeled Fab₂ rabbit anti-mouse IgG or fluorescein-conjugated avidin. Cells were again washed 3 times and examined for fluorescence under a Leitz epifluorescence microscope. For calculations, all fluorescent host cells were considered to possess a 2C content of DNA, so that the number of tumor cells of reduced DNA content could only be underestimated but not overestimated. After subtracting the percentage of fluorescent cells (host cells) from the percentage of 2C population (determined by cell cycle analysis), the difference that remained could be attributed to a population of tumor cells having reduced DNA content. PEC containing a population of tumor cells of reduced DNA content are labeled as “2C” cells to distinguish them from normal host diploid 2C cells.

In experiments with anti-H-2° antisera, 2 × 10⁸ C57BL/6J thiglycollate-elicited PEC were used as a positive control. Cells used as negative controls were C3H/HeN-elicited PEC and EATU cells grown in C3H/HeN mice. In experiments with anti-H-2° antisera, 2 × 10⁸ C3H/HeN-elicited PEC were the positive control, while EATU cells and thiglycollate-elicited PEC from C57BL/6J mice were used as negative controls. Control mixing experiments where H-2° or H-2° thiglycollate-elicited PEC were added to EATU cells demonstrated that immunofluorescence with anti-H-2 sera reflected the percentage of H-2° or H-2° cells added. When positive controls were analyzed, 100 cells per field were counted under bright field illumination. In averaging 3 fields, 98 ± 1% of the cells routinely were positive for fluorescence. Thus, experimental error using indirect immunofluorescence was approximately 2%.

Irradiation Treatments. Five days before injection of 5-FUra and EATU cells, mice were lethally irradiated with 800 rads from a cobalt source. In a previous study (12), irradiation had been shown to completely abrogate the in vivo tumoricidal activity of pyran-activated Mφ.

Statistics and Analysis of Cell Cycle Data. Charts and tables describe representative experiments using 5 to 10 animals per group. In immunofluorescence work, 8 to 10 fields containing at least 100 cells were scored for host cell presence. Error bars represent S.E. A pooled Student’s t test was used in the statistical analysis of the data. Significance was defined as p < 0.05. Estimation of G₁, S, and G₂-M was done by a pattern recognition, crude stripping program, as described previously (3). Initial calibration of the 2C peak involved use of
RESULTS

5-FUra Tumoricidal Activity in Vivo and the Concomitant Appearance of EATU Cells with a Reduced Content of DNA.

The observation that 5-FUra, when injected into EATU bearers, caused the appearance of tumor cells with a reduced DNA content was made by Lindner in 1959 (43) using histological techniques available at the time. Using FMF to precisely quantitate cellular DNA and indirect immunofluorescence to distinguish H-2+ host cells from H-2- EATU cells, we showed (Table 1A) that the antitumor activity of 5-FUra was paralleled by the appearance of EATU cells with a reduced DNA content. Animals given injections of tumor cells on Day 0, followed by 4 daily injections of 5-FUra, had a 95% reduction in the number of PEC compared to the untreated tumor-bearing controls. FMF analysis revealed that approximately 35% of the total PEC population had a 2C (approximately diploid) content of DNA. Immunofluorescent antibody directed against host H-2 antigens showed that only 13 or 23% of the PEC population was of host origin. When the "percentage of host cells" was subtracted from the "percentage of 2C cells," there remained a 12 to 21% PEC population made up of aberrant 2C tumor cells containing about half their normal (almost tetraploid) content of DNA. This can be graphically seen in the histogram depicting relative DNA content of cells from EATU-bearing controls (Chart 2A) and 5-FUra-treated tumor bearers (Chart 2B). In the EATU control population, note the high, sharply defined, approximately 4C peak around Channel 40, denoting aneuploid EATU cells in G1. The broad peak around Channel 80 represents approximately 8C cells in G2-M phase. This histogram emphasizes the aneuploid nature of typical EATU cells and contrasts sharply with the DNA profile of PEC from a 5-FUra-treated tumor bearer. Here, the approximately 2C peak dominates the histogram with 12 to 21% of that peak (as shown in Table 1) comprised of tumor cells with a reduced 2C content of DNA.

Results in Table 1B represent the mean ± S.E. for 7 individual experiments and demonstrate that the 15% EATU cells in the 2C peak of 5-FUra-treated tumor bearers, when compared to the 1% in the untreated control, represent a significant increase in percentage of 2C EATU cells as calculated by a pooled Student’s t test (p < 0.025).

Lack of 2C EATU Cells in Mice Irradiated Prior to Tumor Inoculation. In Table 1B, results from the mean of 7 experiments showed that the 2C population from drug-treated tumor bearers contained a significant number of EATU cells of reduced DNA content.
duced DNA content. The corresponding 1.0% in the untreated tumor-bearing controls did not represent a significant difference as shown by Student’s t test (p > 0.25). However, one could still argue that the 1.0% represented a small but real population of 2C EATU cells and that the relative increase of 2C EATU cells in the 5-FUra-treated group may be due only to a relative resistance to 5-FUra. If the 1.0% of “2C” cells in the PEC of EATU mice was due to host cell presence that escaped detection by immunofluorescence, then reduction of host cells in the peritoneal exudate of tumor-bearing controls should reduce any putative 2C EATU cell presence. This hypothesis was tested in the 3 experiments shown in Table 2. Mice were given 800 rads of whole-body radiation 5 days before EATU cell injection, a regimen which has been shown previously to reduce the inflammatory response in the peritoneal cavity (12).

The PEC from all irradiated tumor bearers showed at least a 4-fold reduction in the percentage of host cells when compared to unirradiated tumor bearers (1.4% versus 6.4%). As predicted, a reduction in the number of contaminating host cells led to the reduction in the number of nonfluorescing 2C cells. This supported the contention that the 1.0% of nonfluorescent cells in the 2C population was not EATU cells but a small number of host cells escaping detection by immunofluorescence.

Because there was a small (2%) but real experimental error involved in determining fluorescent and nonfluorescent populations, results from all further experiments were expressed as percentages, not absolute number of cells. The difficulty in translating percentages into absolute numbers is that small variations in calculations involving the EATU group become magnified due to the large total number of cells. However, in the EATU plus 5-FUra group, there are fewer total numbers of PEC cells and therefore less magnification of any variation. For example, in Table 1B, a 2% variation in the percentage of fluorescent host cells in the EATU control represents 2.6 x 10^6 cells (0.02 times 13 x 10^6). In the 5-FUra-treated population, an equal 2% variation translates into only 6 x 10^4 cells (0.02 times 0.3 x 10^6).

**5-FUra Tumoricidal Activity in Mice Irradiated Previously.**

In earlier studies, we noted that tumoricidal activity and the induction of tumor cells with a reduced DNA content could be generated in vitro with AM6 (35, 36) or in vivo with agents which induce AM6 (i.e., pyran, C. parvum) (12, 37). We therefore wished to determine whether the tumoricidal activity of 5-FUra and/or the appearance of tumor cells of reduced ploidy was affected by the absence of an intact immune system. Test animals were lethally irradiated with 800 rads of whole-body radiation. This had been shown previously (12) to eliminate in vivo pyran induction of MΦ-mediated tumoricidal activity and the generation of tumor cells of reduced ploidy. Four days after irradiation, the mice were given injections of 1 x 10^6 EATU cells and the first of 4 daily 5-FUra (20 mg/kg) injections. When the animals were killed on Day 4, gross PEC counts alone showed no detectable abrogation of 5-FUra-induced tumoricidal activity in the irradiated animal (Table 3). Evidence from the cell count comparisons seemed to indicate that, in a short-term assay (4 days), using a large initial inoculum of tumor cells (1 x 10^6), 5-FUra needed no host cooperation to effectively kill tumor cells.

**Synergistic Role of Host Cells in 5-FUra Induction of EATU Cells with a Reduced Content of DNA.** Although short-term elimination of large numbers of tumor cells by 5-FUra was not a radiation-sensitive, cell-mediated event (Table 3), we wished to determine if there was any difference in the generation of tumor cells of reduced ploidy in irradiated or nonirradiated 5-FUra-treated EATU bearers. Table 4 shows a representative experiment comparable to results from a series of replicate experiments, all showing that the generation of tumor cells with a reduced content of DNA was significantly lower in irradiated 5-FUra-treated mice when compared to the percentage of 2C EATU cells in the peritoneal exudate of unirradiated 5-FUra-treated animals. In Table 4, a significant 21% of the PEC in the 2C peak of cells from 5-FUra-treated, normal tumor bearers were of tumor origin. In the 5-FUra-treated, irradiated tumor bearers, an insignificant 0.6% of the 2C population was composed of EATU cells.

Chart 3 shows a graphical representation of the cell cycle analysis of another experiment comparing 5-FUra-mediated generation of EATU cells of reduced ploidy in normal and irradiated mice. In this particular experiment, the 4C G0 peak of the EATU control (Chart 3C) was centered over Channel 50 due to calibration of the EPICS. This change only alters the relative fluorescence but does not change the relationship between peaks. The corresponding 8C G2-M peak was centered over Channel 100. S-phase cells were distributed between Channels 50 and 100. The 5-FUra-treated tumor-bearing group (Chart 3D) had the characteristic, dominant 2C peak centered around Channel 30. This 2C peak, comprised of host cells and aberrant EATU cells, was almost undetectable in control groups not treated with 5-FUra (Chart 3, A and C). In the 5-FUra-treated, irradiated tumor bearers (Chart 3B), it is the 8C G2-M peak (centered around Channel 100) that domi-
Cells doubled approximately once every 24 hr, reaching a final concentration of 1.1 x 10^7 cells/ml after 96 hr. The cell cycle histogram, not the much less pronounced 2C peak in irradiated animals (data not shown).

Table 4

<table>
<thead>
<tr>
<th>Host treatment</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>EATU + 5-FUra</td>
<td>2.6 ± 0.1^c</td>
<td>25.1 ± 4.1</td>
</tr>
<tr>
<td>EATU + 800 rads</td>
<td>0.23 ± 0.02</td>
<td>48 ± 4.1</td>
</tr>
<tr>
<td>EATU + 800 rads + 5-FUra</td>
<td>0.04 ± 0.01</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

^c Mice were irradiated with 800 rads on Day -5. EATU cells (1 x 10^6) were injected i.p. on Day 0. 5-FUra was injected i.p. on Days 0, 1, 2, and 3. Animals were killed on Day 4. ^d Not significant using Student's t test, p < 0.01.

The following adoptive transfer experiment (Table 6) was designed to further establish host cell participation in 2C EATU cell induction. EATU cells (6 x 10^7) were injected i.p. into irradiated or unirradiated animals following a 2-hr incubation in complete Eagle's spinner medium with or without 5-FUra (10^-4 M). When the peritoneal exudates were removed and analyzed by FMF 3 days later, the results were similar to those seen in Table 4; that is, irradiated animals, when compared to their unirradiated counterparts, possessed significantly fewer 2C EATU cells in their peritoneal exudate (2 versus 12%) (Table 6). In addition, cell cycle analysis of the PEC population showed a more pronounced G2-M shift in the aneuploid EATU cells from irradiated, as opposed to unirradiated, animals (data not shown).

Cell Cycle Analysis of 5-FUra-treated EATU Cells in Irradiated and Nonirradiated Mice. The DNA histogram from irradiated and nonirradiated 5-FUra-treated tumor bearers yielded not only differences in the size of the 2C EATU peak (Table 4; Chart 3) but also differences in the cell cycle pattern of the remaining aneuploid EATU cells (4C to 8C). Table 7 represents results from the mean of 5 experiments. Surviving aneuploid EATU cells from unirradiated, 5-FUra-treated tumor bearers did not possess a cell cycle profile significantly different from the untreated unirradiated EATU controls. That is, the total aneuploid EATU cell population was divided more or less evenly between G1 (28 or 31%), S (40 or 37%), and G2-M (27 or 26%)

Chart 3. Cell cycle analysis of PEC from irradiated and normal 5-FUra-treated EATU bearers. Animals untreated or lethally irradiated with 800 rads of whole-body radiation on Day -5. Animals were given injections i.p. of EATU cells (1 x 10^6) on Day 0. 5-FUra (20 mg/kg) was injected i.p. on Days 0, 1, 2, and 3. Animals were killed on Day 4. A, EATU + 800 rads. G1 = 27.1%, S = 28.4%, G2-M = 44.5%. B, EATU + 800 rads + 5-FUra. G1 = 7.5%, S = 5.1%, G2-M = 82.4%. C, EATU + 5-FUra. G1 = 36.4%, S = 7.4%, G2-M = 57.8%. D, EATU + 5-FUra. G1 = 25.9%, S = 34.0%, G2-M = 40.1%. In B, 7% of the cells in the peak centered around Channel 30 are of tumor origin, as determined by fluorescent microscopy. In D, 12.2% of the cells in the peak centered around Channel 30 are of tumor origin.
Cell cycle analysis of 5-FUra-treated EATU cells in normal and irradiated mice

<table>
<thead>
<tr>
<th>Host treatment</th>
<th>Treatment</th>
<th>% of host cells in PEC</th>
<th>Cell cycle analysis</th>
<th>% of 2C</th>
<th>of host cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.9% NaCl solution</td>
<td>6.6 ± 1.4*</td>
<td>6.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>5-FUra</td>
<td>5.6 ± 1.7</td>
<td>18</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>800 rads</td>
<td>5-FUra</td>
<td>1.4 ± 0.6</td>
<td>3.4</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*Four days following irradiation treatment (800 rads), C57/B1 mice were given injections i.p. with 6 x 10^6 EATU cells, as were the same number of unirradiated controls. Animals were killed 3 days after tumor cell inoculation.

Table 5

Inhibition of in vitro EATU cell growth by 5-FUra: absence of 2C peak in cell cycle

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>No. of cells (cells/ml x 10^5) at sample times (hr after initial incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>EATU</td>
<td>4.6 ± 0.2*</td>
</tr>
<tr>
<td>EATU + 5-FUra</td>
<td>6.5 ± 0.5</td>
</tr>
</tbody>
</table>

*EATU cells (1.5 x 10^5) were incubated in complete Eagle's spinner medium with or without 10^-4 M 5-FUra. After a 2-hr incubation at 37°, cells were washed twice, resuspended in 250 ml of medium, and incubated in 500-ml spinner flasks at 37°. Every 24 hr, 50 ml were removed, and the sample was counted. An aliquot of cells was stained with propidium iodide and analyzed for cell cycle distribution by FCM with an EPICS IV cell sorter. A pattern recognition, crude stripping program described in "Materials and Methods" was used to derive cell cycle. In all samples, there was no detectable 2C peak, indicating no EATU cells with a reduced DNA content.

Table 6

Injection of 5-FUra-treated EATU cells into irradiated or unirradiated mice

<table>
<thead>
<tr>
<th>Host treatment</th>
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<th>% of host cells in PEC</th>
<th>Cell cycle analysis</th>
<th>% of 2C</th>
<th>of host cells</th>
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<td>12</td>
<td></td>
</tr>
<tr>
<td>800 rads</td>
<td>5-FUra</td>
<td>1.4 ± 0.6</td>
<td>3.4</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The mechanism of 5-FUra tumoricidal activity in vitro has been explored previously in some detail (48, 56, 65). 5-FUra acts as an analog in the formation of the 45S rRNA precursor, thus producing nonfunctional rRNA (67). It is also converted into ribonucleotides which inhibit thymidylate synthetase activity, thus blocking DNA synthesis (66). While the "DNA" and the "RNA" effects are undoubtedly crucial to the tumoricidal activity of 5-FUra in vivo and in vitro, an additional phenomenon, not seen in in vitro killing, accompanies in vivo 5-FUra tumoricidal activity in the immunologically intact host, i.e., the appearance of aberrant 2C tumor cells with a reduced content of DNA (Table 1; Chart 2). These observations were made using an EATU cell line bearing no H-2 antigens and therefore distinguishable from host cells by immunofluorescence staining using anti-H-2 antibodies. This indirect method of tumor cell identification was highly reproducible (11) and confirms earlier in vitro observations of "reductive" tumor cell division seen using 2 different tumor cell targets readily distinguishable from AMφ by esterase staining and morphological differences (35, 36).

As described in "Materials and Methods," mixing experiments and analysis of positive fluorescent controls indicated that the indirect immunofluorescence assay used in this paper had a 2% experimental error. This 2% variation was not large enough to account for the 12 to 31% nonfluorescing 2C population in the PEC of the 5-FUra-treated group, but it could explain the presence of a small nonfluorescing 2C population in the PEC of the EATU control (Table 1). The 1% of EATU cells in the 2C population of the tumor-bearing control in Table 1B was not significant using Student's t test (p > 0.25). However, assuming all percentages in Table 1 were real, then in terms of absolute numbers (as opposed to shifts in percent-

(32%). However, in the irradiated, 5-FUra-treated animals, there was a very pronounced shift of cells to G2-M phase. This "G2-M shift" accounted for 53% of the aneuploid cells in mice irradiated prior to treatment with EATU plus 5-FUra, whereas the untreated irradiated control had only a 7% G2-M population. The net change of G2-M cells in the irradiated, 5-FUra-treated versus unirradiated 5-FUra-treated population was approximately +26%. Chart 3 shows an example of this G2-M shift. Chart 3A represents the irradiated tumor control, with the G2-M peak comprising 43.6% of the aneuploid population, as determined by the pattern recognition crude stripping program of the cell sorter (3, 11). Chart 3B represents irradiated tumor bearers treated with 5-FUra. The G2-M peak dominates the histogram and comprises 84.3% of the cell population, resulting in a G2-M shift of 40.7%. With the unirradiated group, the untreated tumor control in Chart 3C had a G2-M of 51.7%, while the 5-FUra-treated tumor bearer showed a G2-M of 40.1 for a net loss of 11.7%. It is interesting to note that 5-FUra caused a G2-M tumor cell shift only in those animals which had been immunologically debilitated by radiation treatment. 5-FUra treatment of tumor bearers with an intact immune response caused no G2-M block but generated EATU cells with a reduced DNA content.
can be explained by the cytotoxicity of 5-FUra which kills diploid as well as aneuploid EATU cells. Also, by virtue of their own aberrant nature, 2C cells are continually dying (35, 36), so that the percentage of 2C EATU cells may represent residual dying cells from a much larger population of 2C tumor cells. One could still argue that the relative increase of 2C EATU cells in the 5-FUra-treated group may be due only to a relative resistance to 5-FUra. However, when EATU cells were grown in vitro in the absence of host cells (Table 5) and in vivo in an irradiated animal (Table 2), 2C EATU cells were eliminated. We therefore felt that the presence of nonfluorescing 2C cells in the EATU controls was due to a small population of host cells which had escaped detection by immunofluorescence.

Whether the induction of 2C EATU cells by 5-FUra seen by us and others (43) is the result of a cytotoxic event or is an actual mechanism of tumoricidal activity has not yet been resolved. However, there are many instances where nonmitotic nuclei can be artificially induced to divide amitotically, giving rise to nuclei with less than their normal complement of DNA (34, 64). This premature nuclear division can be triggered by fusing a cell in late G2-M phase with another in G1, S, or early G2-M. The late G2-M nucleus will divide normally but, in addition, will cause the second nucleus to divide before it possesses a fully replicated complement of DNA (26, 39, 57, 62). Perhaps as a result of 5-FUra-induced in vivo tumoricidal activity, a host cell triggers premature cell division and/or amitotic nuclear cell division in tumor targets as has been described previously for AM¢ (35, 36).

In the above description of in vivo 5-FUra-mediated tumoricidal activity, the phrase “immunologically intact host” is a key one; for, in mice lethally irradiated prior to EATU and 5-FUra injections, there is little or no evidence of aberrant 2C EATU cells of reduced DNA content (Table 4). What is more, the aneuploid EATU population in 5-FUra-treated irradiated tumor bearers has a preponderance of cells in G2-M phase which is not seen in the unirradiated 5-FUra-treated tumor bearers (Table 7; Chart 3). Presumably, a host cell population may be involved in not only causing tumor cells with a reduced DNA content but also in limiting cells cycling into G2-M. In previous in vivo studies, pyran-mediated antitumor activity did not block EATU in G2-M but induced appearance of tumor cells with a reduced DNA content as well as shifting the remaining aneuploid EATU cells from G2-M to G1 (12). Krahenbuhl (41) and Kurland et al. (42) have also shown that host cells have the ability to prevent tumor target cells from entering G2-M. Moreover, Haskell (27) has shown that C. parvum-activated M¢ blocked HeLa cells in G2 phase and that Adriamycin-stimulated M¢ inhibited HeLa growth in the G2-M phase, characteristic of the free drug. He suggested that M¢ may inhibit tumor growth in 2 ways: (a) M¢ activated by biological modifiers, such as Bacillus Calmette-Guerin and C. parvum, inhibited DNA synthesis and blocked tumor cells in G1; (b) M¢ incubated with chemotherapeutic drugs like Adriamycin stored drugs in vivo and blocked tumor cells as would the free drug (in the case of Adriamycin, a G2-M block). It is unlikely that host-mediated antitumor activity in 5-FUra-treated tumor bearers was due to in vivo retention of 5-FUra by host cells, since (a) 5-FUra has a very short half-life in vivo and is almost undetectable in serum after 24 hr (9, 44); (b) injection of 5-FUra into animals given injections of thioglycollate previously induced no M¢-mediated antitumor activity in vivo or in vitro; (c) PEC from animals given injections of 5-FUra alone failed to kill tumor cells in vitro; (d) the cell cycle pattern for PEC derived from 5-FUra-treated, unirradiated, tumor-bearing mice (Chart 2) was different from that derived from the PEC of pyran-treated, tumor-bearing mice (12); that is, each possessed a dominant 2C peak of aberrant EATU cells and no shift from G1 to G2-M in the remaining aneuploid EATU cells. In contrast, the irradiated, immune-debilitated, 5-FUra-treated tumor bearers possessed PEC with the dominant G2-M peak (Chart 3; Table 7) similar to EATU cells treated in vitro with the free drug (32). We suggest that host-mediated antitumor activity in 5-FUra-treated tumor bearers is in part similar to that in pyran-treated tumor bearers. In the absence of an intact immune response, 5-FUra antitumor activity in vivo is similar to its antitumor activity in vitro.

Despite the observation that 5-FUra was equally effective in limiting the growth of 1 x 106 tumor cells in irradiated or unirradiated animals (Table 3), host-mediated immunological events may still be crucial to the ultimate effectiveness of 5-FUra. The value of host-mediated tumoricidal activity may lie in not only the short-term reduction of a larger tumor burden but also the long-term elimination of any residual cells which have survived chemotherapeutic treatment (7, 8, 31). Bremberg (6) and others (18, 19, 46, 52), in their work linking cyclophosphamide chemotherapy to host-mediated immunotherapy, noted that the major function of chemotherapeutic drugs was to decrease the tumor cell population to a certain minimal level so that the immune system could effectively destroy the residual tumor cell burden. Preliminary experiments in our laboratory have indicated that reduction of the tumor burden in the peritoneal cavity by 5-FUra results in an adherent cell population with nonspecific tumoricidal activity in vivo and in vitro. The value of an intact host immune system in chemotherapy extends beyond 5-FUra and cyclophosphamide studies (6, 20, 21, 49, 60). A number of chemotherapeutic agents, among them, Adriamycin (45, 61), 1-ß-o-arabinofuranosylcytosine (25, 30), 6-mercaptopurine (63), melphan (55), 1,3-bis(2-chloroethyl)-1-nitrosourea (47), and actinomycin D (47), act optimally only in the presence of a functioning immune system (4). This may at first appear to be paradoxical, for many of the chemotherapeutic drugs are immunosuppressive themselves as in the case with 5-FUra (5, 22, 23, 51). However, it is important to note that 5-FUra probably suppresses at the bone marrow level (22, 23). Like the immunosuppressive chemotherapeutic agents 1,3-bis(2-chloroethyl)-1-nitrosourea and cyclophosphamide (15), 5-FUra does little to inhibit the tumoricidal potential of mature host cytotoxic cells (54). Therefore, it is only necessary to strike an immunological balance, using the chemotherapeutic regimen which will lower the tumor burden without eliminating the host cell precursors (16, 17).

As to the nature of the tumoricidal host cell (24, 38, 40, 58), that is a subject under investigation by us and others (29, 29, 53). Our previous demonstration that EATU of reduced ploidy were detected after treatment of mice with agents known to activate M¢ suggests that AM¢ may play a role in induction of tumor cells of reduced ploidy after 5-FUra treatment. Prelimi-
nary data in our laboratory suggest that an adherent PEC with nonspecific tumoricidal activity can be detected after treatment of EATU with 5-FUra. Experiments are currently under way to elucidate the nature of host cell effector mechanisms in tumor-bearing mice treated with 5-FUra.

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Host Interactions in the Effects of 5-Fluorouracil on Ehrlich Ascites Tumor Cells


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