5-Fluorouracil Treatment of a Human Colon Adenocarcinoma Implanted in the Subrenal Capsule Site of Athymic Mice

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ABSTRACT

5-Fluorouracil was used in the treatment of a recently established human adenocolon carcinoma implanted in the subrenal capsule site of athymic mice as part of a program to determine the relative effectiveness of antitumor agents in the treatment of human tumors grown as xenografts and in the clinic. 5-Fluorouracil treatments resulted in a dose-dependent inhibition of tumor growth under the two examined schedules (five daily doses and three doses every 4 days) when administered either i.v. or i.p. The various schedules and routes resulted in similar patterns of antitumor effects when the data were based on the endpoints of either final tumor weight, change in tumor weight, or relative tumor weight. Assessments of cell viability based on histological examination of tumor sections revealed little evidence of significant drug toxicity to the host.

The greatest inhibition of tumor growth, however, was accompanied by weight loss in treated mice as compared with controls. At lower doses, however, the weight loss of the mice was not extensive, which indicated that the inhibition of tumor growth was, at least in part, attributable to some selective antitumor action of the 5-FUra. The greatest inhibition of tumor growth, however, was accompanied by the most extensive animal weight loss. This correlation raised the question of the degree to which the tumor growth inhibition observed was a consequence of nontoxic drug toxicity to the host.

The current results stress the importance of detailed investigations into the nature of host-tumor parameters in order to assess the antitumor activity of candidate drugs in the treatment of human tumor xenografts.

INTRODUCTION

The athymic (nude) mouse has been well established as a suitable recipient of human tumor xenografts. Commonly, tumors are transplanted s.c., although other locations have also been used successfully (1-14). Bogden et al. and others have developed the SRC assay in athymic mice, as well as immunocompetent, normal mice, for the rapid screening, determination, and prediction of chemotherapeutic responsiveness of human tumors (15-21).

We have used the SRC assay in athymic mice in order to evaluate the effect of 5-FUra delivered by two routes of administration, under various schedules and doses, in the treatment of a transplantable human xenograft of an adenocarcinoma of the colon. Adenocarcinoma of the colon was selected for study because it is generally refractory to therapy with the available antitumor agents and thereby represents an important therapeutic challenge. 5-FUra was chosen because it has demonstrated modest levels of activity against colorectal carcinoma both clinically (22) and also in xenografted athymic animals (7).

The current study is part of a program to compare the effectiveness of therapeutic agents for treatment of human tumors grown as xenografts and in the patient. In order to assess the relationship of the SRC assay in athymic mice to the clinical experience, the following questions were addressed.

1. Could 5-FUra inhibit the growth of this human colon adenocarcinoma in the subrenal capsule site of athymic mice and could dose-response relationships be established?

2. What methods of evaluation of the tumoricidal effect of the drug were most informative? (a) Did expression of the data as “average final tumor weight,” “change in tumor weight,” or “relative tumor weight” in relation to dosage alter the dose-response curves or the interpretation of the results? (b) To what extent did initial tumor weight have an effect on final tumor weight in controls and in treated animals?

3. What was the influence of the schedules and routes of drug administration used?

4. What was the relationship of drug toxicity to the tumor and to the host? To what extent might nontoxic drug toxicity account for any of the antitumor effects observed?

5. Did the histologically determined viability of the tumors correspond to the gross observations of tumor weight and the antitumor effectiveness of the drug?

MATERIALS AND METHODS

Animals and Husbandry. Specific-pathogen-free 5-wk-old female CD-1 background nu/nu mice were used in the experiment. They were maintained (at three per cage) in ethylene oxide-sterilized microisolator units with autoclaved bedding, a pasteurized commercial diet, and autoclaved water ad libitum. The animal room was kept at 20°C, 50 ± 2% relative humidity, with a 12-h light-dark cycle. Cage cleaning was performed on a weekly basis. All cage, animal, and tumor manipulations were carried out in horizontal laminar airflow hoods using aseptic techniques, including disposable gloves and surgical masks.

Tumor and Drug. A primary sample of a human adenocarcinoma of the colon, designated CRCc2, was obtained under sterile conditions...
from a local Boston hospital in May 1982. The details of our procedures for the preparation and handling of tumor biopsies for xenografting will be presented elsewhere. Briefly, following incubation with both collagenase and DNase I in RPMI-1640 at 37°C for 1 h, the tumor cells were centrifuged and resuspended 3 times in HBSS. A viable cell count was made (6.8 x 10^6 in this case), and 10^7 cells were inoculated s.c. into 6 x 10^8 ml HBSS in the supracardinal region of five 4- to 5-wk-old female CD-1-nu/nu mice. Within 6 wk, four of five of these primary xenografts formed progressively growing tumors. Subsequent histological examination confirmed that the xenograft was morphologically similar to the original tumor sample obtained from the patient (data not shown). This tumor has shown sustained growth in the s.c. site and to date is at the 11th transplant generation since the primary inoculation. The xenografted tumor was used at passages 2 and 3 for this series of experiments. At each passage a portion of the tumor was cultured on a battery of bacterial media, and no evidence of contamination was found.

5-FUra (Adrucil; Taylor Pharmaceuticals, Decatur, IL) was supplied at a concentration of 50 mg/ml in sterile aqueous solution, pH 8.6 to 9.0, adjusted with sodium hydroxide, and stored at 20°C in aluminum foil-covered containers. It was diluted in sterile distilled water, pH 9.0, immediately prior to use.

**Table 1**

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<th>5-FUra treatment of CRC02</th>
<th>AFW</th>
<th>AITW</th>
<th>AFTW</th>
<th>ΔTW</th>
<th>RTW</th>
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**Results**

The data are presented in Tables 1 and 2 and Figs. 1–6. The experimental protocol's organization and the three methods of evaluating the antitumor effectiveness of the drug are presented in Table 1, as well as animal weight changes and initial tumor implant sizes. The three end points of AFTW, ΔTW, and RTW have been variously used for depicting antitumor effects. Final tumor weight could be thought of as an appropriate end point in its own right, but would not take into account the change in tumor weight. If the change in tumor weight were important, one could ask whether it should be examined on an absolute or a relative scale. The present analysis, using all three methods, avoids the weakness of any particular one. 3

The data were also grouped by three criteria in order to analyze the relationship between gross and histological observations and the subsequent effect of doing so on the evaluation of the drug's
antitumor effect. This analysis is illustrated for one of the end points (AFTW) in Fig. 1. Similarly shaped curves were also obtained for the other end points of ATW and RTW (data not shown). One group included all tumor observations regardless of whether the gross or histological classification was positive or negative. Note, however, that if the gross classification was negative (tumor absent), the result was included as having a value of zero. The second group included the tumors positive on gross examination, whether or not positive by histological examination. The third group included only tumors identified grossly for which there was histological confirmation.

For all of the treatment groups, there was substantial and progressive tumor inhibition as the dosage of 5-FUra was increased. This effect was evident when the results for each treatment group were expressed with respect to either AFTW, ΔTW, or RTW (Table 1; Fig. 1). While there were some anomalous data, perhaps attributable to the smaller number of animals involved in some instances or the variations in tumor weights, at sufficiently high doses, the antitumor effects of 5-FUra were substantial.

On the qdx5 schedule, animals were sacrificed at Day 11 and Day 14 in order to compare the data on tumor growth and end point evaluation. With the exception of the data for the animals treated at 19.8 mg/kg i.p. evaluated on Day 14 and the Day 11 i.v. treatment at 29.6 mg/kg, a comparison of the Day 11 and Day 14 data did not result in marked differences in interpretation of the drug’s antitumor effectiveness. Also, by evaluating the qdx5 schedule at Day 14, a direct comparison with the q4dx3 schedule was made possible. As shown in Table 1 and Fig. 1, the qdx5 schedule was more effective than the intermittent schedule by either i.p. or i.v. administration, although consistent inhibition of tumor growth was observed at higher doses on the q4dx3 schedule.

The experiments also allowed the relative effectiveness of the i.p. and i.v. routes of administration to be compared. In general, the i.p. route was a more effective method of 5-FUra administration than the i.v. method, as demonstrated by the data in the last three columns of Table 1 and the left-hand half of Fig. 1. To what extent these relative differences in route of administration represented a true pharmacological effect or simply an effect of drug deposition in proximity to the tumor (the i.p. route) remains to be evaluated.

The relationship of average final tumor weight to average initial tumor weight for animals sacrificed on Day 14 is shown in Fig. 2. The upward slanting line in each section corresponds to equality of final and initial average tumor weights. In order to judge effects on final tumor weight without regard to initial tumor weight, only the height of each point need be noted. For change in tumor weight, points above the diagonal line indicate that tumor growth occurred, and points below the line indicate that there was actual tumor regression. The ratio of final to initial average tumor weight would be identified by the slope of the line (if drawn) connecting the plotted points to the origin.

For most of the groups where the animals were sacrificed on Day 14, the points plotted in Fig. 2 (a) are lower with increasing dose of 5-FUra, (b) fall closer to the upward slanting line of equality, with the controls well above, and (c) identify lines through the origin with diminished slopes, i.e., lower ratios of final to initial weights. There was no clear relationship between the extent of tumor growth in either the controls or treated groups and the average initial tumor weight.

The relationship of final tumor weight to initial tumor weight for selected groups of individual animals is depicted in Fig. 3. Included are the data for the controls sacrificed on the 11th (Fig. 3A) and 14th (Fig. 3D) days following tumor inoculation and for the groups sacrificed on the 14th day that had been treated at 29.6 and 100 mg 5-FUra per kg on the schedules q4dx3 i.p. (Fig. 3, B and C, respectively) and q4dx3 i.v. (Fig. 3, E and F, respectively). The data are from the group that included all gross tumor observations.

There was considerable variability in initial and final tumor weights for individual animals in both the control and treated groups, which could undoubtedly have been diminished by more careful preselection for tumor size. There was, however, no clear indication that greater final tumor weights for individual animals

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4 There was only one instance in which the gross classification was negative and the histological observation positive.
depended on higher initial tumor weights. The patterns of tumor growth following drug treatment were independent of initial tumor size, as shown in Fig. 3. B and C, and E and F. The 100-mg/kg doses (Fig. 3, C and F) tended to result in data points clustered in the area of the diagonal lines, indicating that there was minimal to no tumor growth, or even a reduction in tumor size. The final tumor size was dependent on drug dose delivered to the host, not initial tumor size. In the control groups, there were a number of animals in which the final tumor weight was equal to or less than the initial tumor weight. This was more evident in the animals sacrificed on the 14th day (Fig. 3D) as compared to those sacrificed on the 11th day (Fig. 3A) following tumor implantation. To what extent this may reflect some host versus tumor graft reaction was not clear.

The data presented in Figs. 2 and 3 are germane to the question of whether, or how, to take initial tumor weights into account. From the lack of a definitive association between initial and final tumor weights for the individual tumors, final tumor weights alone, again, would have been able to serve as a basis for judging antitumor effects.

The relationship between the 5-FUra dose and the average final animal weight, the change in average animal weight, and the relative change in animal weight (final minus initial) (ΔAW) or (c) relative animal weight (average final/average initial) (RAW) is plotted in a manner to address this issue. The relative tumor weight (TWF/TWI) is plotted on a logarithmic scale on the ordinate, and the relative body weight (BWF/BWI = 1).

Lower values for TWF/TWI indicate increased inhibition of tumor growth. Lower values for BWF/BWI indicate increased inhibition of animal growth. The most desirable therapeutic result would have data points in the lower-left quadrant in each panel where there would be tumor weight reduction accompanied by a gain in animal weight. The least desirable result would be represented by points in the upper-right quadrant where the tumor gains weight despite animal weight loss. Most of the present data points fell in the upper-right quadrants of the panels, since the inhibition of tumor growth occurred at doses where there are reduced gain in animal weight as compared to controls. However, the greatest inhibition of tumor growth (Fig. 5A, qdx5 i.p. at 66.7 and 44.4 mg/kg) was accompanied by the most extensive body weight loss (lower-left quadrant). Nevertheless, there was instances in which considerable inhibition of tumor growth was obtained with only minimal retardation of animal weight gain. The latter observation suggested that there was at least a measure of specificity of the therapy, which resulted in tumor inhibition greater than that which may have occurred as the result of animal weight loss.

The relationship between the gross and histological findings was examined for animals sacrificed on Day 14 (Table 2; Fig. 6).
SRC ASSAY OF A HUMAN COLON TUMOR IN NUDE MICE

Fig. 5. The relationship of the relative body weights to relative tumor weights. Numbers beside points, dose of 5-FUra in mg/kg; C, controls. The solid vertical and horizontal lines in each panel indicate no change in body weight and no change in tumor weight, respectively. The data are for the Day 14 evaluation period. A, qdxS i.p.; B, qdx5 i.v.; C, q4dx3 i.p.; D, q4dx3 i.v.

Table 2
A comparison of gross and histological observations to tumor weight

<table>
<thead>
<tr>
<th>5-FUra dose (mg/kg)</th>
<th>No. of tumorsa</th>
<th>AFTWa</th>
<th>Av % of viabilityb</th>
<th>Mass of viable cellsb</th>
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<td>0</td>
<td>38</td>
<td>Controls</td>
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<td>13</td>
<td>7.54</td>
<td>55.7</td>
<td>4.20</td>
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</table>

a Number of evaluable tumors in data group comprised of both grossly and histologically positive tumor masses. Data are for animals sacrificed on Day 14.
b Calculated by (L x Wx2), expressed in mg.
c Determined by histological examination.
d Calculated by multiplying the AFTW by the average percentage of viability, expressed in mg.

The data are for the group in which evidence of tumor was found by both gross and histological observation. The extent of tumor cell viability was rated at 100, 75, 50, 25, or 0% (0% indicating necrotic material only with no viable cells) following histological examination of representative tumored kidney sections.

The average percentage of viable tumor cells at each dose for each of the therapy schedules is shown in Table 2. There was no clear evidence of a dose-dependent decrease in the percent-age of viability of tumor cells in the various treatment groups. As a result, correction of the tumor weights for viability lowered the values to a similar extent for both treatment and control groups. As illustrated for the qdx5 data in Fig. 6, there was a downward displacement of the curves but not an appreciable alteration in their shapes as compared to the original curves that were based on gross observations.

DISCUSSION

In order for an experimental model to be useful for the prediction of antitumor activity of drugs and treatment modalities for the clinic, it should be demonstrated that the model is amenable to quantitative studies with respect to characteristics that are pertinent to the clinic, such as the antitumor effect of various doses and treatment regimens (23). Human tumors growing in athymic mice provide an experimental counterpart to clinical neoplasia, and investigation into the nature of their responses to therapy using relevant dose-response parameters is basic to the evaluation of their predictability value for the clinic.

The SRC assay in conventional mice has been proposed as a reasonably reliable method for the evaluation of novel antineoplastic agents preclinically, and it has also demonstrated correlations with the clinical efficacy of various treatment choices (18, 21). In the current investigation using a human colon adenocarcinoma growing in the SRC of athymic mice, the dose-response curves for the fluorinated pyrimidine 5-FUra showed an increasing inhibition of tumor growth, less evident at lower dosages, that was progressive with increasing dosage. This effect was observed both for a daily treatment schedule (qdxS) and for an intermittent schedule (q4dx3) involving two routes of administration (i.p. or i.v.). The data also indicated that no appreciable differences in antitumor effectiveness were found when the tumors were evaluated at Day 11 or Day 14 postimplantation on the qdxS schedule. Further, the inhibitory activity of 5-FUra was confirmed using all three different endpointsof tumor evaluation, namely, the final tumor weight, the change in tumor weight, and the relative change in tumor weight (Table 1; Fig. 1).

The most extensive inhibition of tumor growth was obtained with the qdx5 regimen, but this was accompanied by the greatest weight loss of the animals. The qdx5 i.v. regimen appeared
to be less toxic for the host than the qd x 5 i.p. schedule, and at
two dose levels, there was evidence of tumor inhibition with
minimal to no weight loss of the animals relative to controls. The
two intermittent schedules (q4dx3 i.p. and i.v.) were similar in
their action, with evidence of retardation of tumor growth at
lower doses without extensive weight loss of the host. There
was more extensive inhibition of tumor growth at higher doses,
but this was accompanied by toxicity for the animals as reflected
in weight loss relative to controls. It would appear that the full
impact of route and schedule differences for an active drug may
not have sufficient time to become fully manifest in the type of
short-term assay used here.

Standardization of the initial tumor implant size has been
fundamental to the methodology of the SRC assay (15–19). It
was considered, however, in view of the heterogeneity of tumors,
that variation in initial tumor size with randomization of tumored
hosts could provide additional information on the influence of
initial tumor size on the extent of tumor growth. Although dis-
sected to approximately 1-mm³ in size, the tumor fragments
used in the present study showed considerable variability when
actually measured by an ocular micrometer. Obviously necrotic
areas were avoided; however, no effort was made to preselect
more carefully for initial tumor fragment size or region of the
initial tumor sample from which the tumor fragments were
prepared.

The final tumor size showed no clear dependence on the size
of the initial implant. This result was observed when the data
were presented in terms of either the average initial tumor size
versus final tumor size (Fig. 2) or by the individual animal's initial
tumor size versus final tumor size (Fig. 3). As a consequence,
the results of the therapy regimens could have been interpreted
solely on the basis of final tumor weights, without consideration
of the initial tumor weight. The many instances in which untreated
tumors showed minimal or no growth indicated, aside from
questions of viability, that only a small percentage of tumor cells
actually "took" and gave rise to progressive tumor growth within
the experimental time frames evaluated here.

Untreated and treated normal and tumored animals showed a
progressive inhibition of body weight gain with increasing dosage
of drug (Fig. 4). This inhibition was undoubtedly a reflection of a
relatively steep dose-response curve for the host toxicity of 5-
FUra and, although not measured, of accompanying reduction in
caloric intake. Goldin et al. (24) and Skipper et al. (25) have
reported a 50% reduction in the growth of sarcoma 180 and
adenocarcinoma 755, respectively, when animal weight losses
of 20 to 30% were recorded due to caloric restriction and/or
nonspecific drug toxicity. The screening program at the National
Cancer Institute recognized these findings and focused attention
on the importance of nonspecific toxicity of test antitumor
agents, with the standards for acceptance of drug activity per-
mitting only a moderate, prescribed degree of animal weight loss.
More recently, Giovannella et al. (26) reported that caloric restric-
tion inhibited the growth of human tumors heterotransplanted in
either the s.c. or SRC site in athymic mice. With increasing levels
of caloric restriction and accompanying weight loss, they ob-
served greater tumor growth inhibition. They concluded that a
weight loss greater than 20% rendered the results unreliable.

In the current study, when a comparison was made of the
change in tumor weight to change in animal weight, it was noted
that there was a 20% or greater reduction in weight gain of
treated animals as compared to that of controls at higher drug
doses. Therefore, the extensive inhibition of tumor growth ob-
served at these doses may have been attributable to the influ-
ence of nonspecific drug toxicity. At lower doses where there
was relatively little or no retardation of host weight gain, however,
there was still evidence of tumor inhibition (Table 1; Fig. 5). This
observation suggested that, at least in part, the inhibition of
tumor growth may have been the result of some specificity of 5-
FUra against the tumor cells. It is clearly important to consider
drug-induced nonspecific host weight loss in human tumor
models used for new drug evaluation and screening. Failure to
take nonspecific toxicity and accompanying weight loss into
account may lead to overprediction of antitumor activity of can-
didate drugs.

There was evidence of tumor inhibition with increasing dosage
of 5-FUra when the tumor mass was based either on gross
measurements or on the correction for the viability of tumor cells
as determined by histological examination of tumor sections
(Table 2). Although there was no evidence of a decrease in the
percentage of viability with increasing drug dosage, the tumor
weight dose-response curves, when corrected for percentage of
viability, retained their general shape but were shifted downward
(Fig. 6). Whether this absence of a dose-dependent decrease in
tumor cell percentage of viability is an inherent characteristic of
the SRC assay or is simply a reflection of the general growth
properties of this particular xenograft is an important question.
The end point chosen for the SRC assay is based on the premise
that changes in tumor size are due to the equilibrium established
between cell death, removal, and cell renewal. A closer exami-
nation of the results obtained using tumors with very high growth
fractions, which is often the case in tumors established over the
years, and those obtained with tumors more like the present one
(60% viability in controls), may provide the data required to
substantiate the use of gross and/or histological data as appro-
priate end points of the assay and thereby contribute to its
clinical utility.

In this regard, a careful study which compared tumor size
measurements with the results of a semiquantitative measure of
histopathology data involving a number of parameters may be
cited (27). In that study, a clear relationship was not evident
between gross measurements of drug effect and histological
findings. The study differed from the current one in that it used
conventional mice and utilized a short period of observation (4
and 6 days), and substantial immune response was seen by 6
days. Although the athymic mouse SRC assay is probably not
significantly affected by a host immune response, that remains
to be fully determined.

The present data showed an overall dose-dependent decrease
in tumor mass expressed either grossly or in terms of the number
of viable cells that parallels the concepts developed using murine
tumors in conventional mice; as the dosage of an effective drug
was increased, there was an increase in the percentage of kill
of tumor cells (28, 29). Goldin et al. (30) observed that increasing
numbers of leukemic cells were destroyed to give a 50% cure
rate as the dosage of methotrexate was increased. Similar
findings were reported by Skipper et al. at about the same time
(31, 32). In addition, Skipper et al. (29) formulated the "fractional
cell kill hypothesis," which states that the percentage of variously
sized leukemic cell populations killed by a specific dose of an
effective drug is reasonably constant, provided that the cell

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population is metabolically homogeneous and that all cells are exposed to the same drug concentration for the same amount of time. To what extent the tumor cell kill by 5-FUra with this solid human tumor in the SRC assay was indeed proportional to dosage with a close fit to the fractional cell kill hypothesis or, as suggested by Norton and Simon (33), was modified in its action by the heterogeneity of the tumor or other factors provides important challenges for further investigation.

ACKNOWLEDGMENTS

The authors thank Dr. W. P. Peters and Dr. E. T. Gaillard for helpful advice and histological slide evaluations, respectively. Thanks to T. F. Grace and K. J. Miller for expert technical assistance and data collation, and J. S. Foster for help in preparation of the figures. The secretarial skills of Donna Shurtleff and Beth Ouellette are gratefully acknowledged in the preparation of the manuscript.

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